2.1 PESTICIDE:

Increasing population is a burning problem of today's world. With increasing population, demand of food and fiber has also increased. Apart from that, climate change has also affected the production of agricultural products (Shetty et al., 2008). A pesticide is a substance or mixture of substances mitigating any pest (insects, mites, nematodes, weeds, rats etc.) including insecticides, fungicides, herbicides and various other substances used to control pests (EPA, 2012). Worldwide usage of pesticides has increased upto 50 fold since 1950 and annually, 2.5 million pesticides are used (Farag et al., 2011). Worldwide approximately 9,000 species of insects and mites; 50,000 species of plant pathogens, and 8,000 species of weeds damage crops. Different pests such as insects and plants causing losses estimated in 14% and 13% respectively. Pesticides are indispensable in agricultural production. About one-third of the agricultural products are produced by using pesticides. Without pesticide application the loss of fruits, vegetables and cereals from pest injury would reach 78%, 54% and 32% respectively. Crop loss from pests declines to 35% to 42% when pesticides are used (Pimentel, 2007). According to United States Environmental Protection Agency, more than 1180 pesticides have been registered worldwide out of which, 435 are herbicides, 335 are insecticides and 410 are fungicides (Nollet and Rathore, 2009).

2.2 CLASSIFICATION OF PESTICIDES:

Pesticides are classified based on many aspects including their chemical structure, their target group, formulation etc. Mainly, pesticides can be classified into inorganic, organic and biological pesticides.

Inorganic pesticides: These pesticides do not contain carbon in their chemical structure. Inorganic pesticides mainly contain minerals like arsenic, copper, boron, mercury, sulfur, tin or zinc. They are used against plant diseases and as wood preservatives. Due to its broad range toxicity many of them have been banned or severely curtailed.

Organic pesticides: As the name suggests, these pesticides possess carbon along with hydrogen and oxygen along with nitrogen, phosphorus, sulfur or other such elements. Most of them are synthetic compounds but some of them are derived from plant or plant

extract. Due to its specificity and extreme effectiveness, most of the pesticides used today are belong to this category and thus the contamination and health related issues of pesticides are also because of these pesticides.

Biological pesticides: These pesticides are microbial pesticides which include bacteria, viruses and fungi which cause disease in pests. Such microbes are intentionally introduced to required area. They are highly specific so not harmful to other non-target organisms. Microbial products are also used sometimes as pesticides.

Classification on the basis of purpose for which they are intended into insecticides, herbicides, fungicides, algicides, fumigants, rodenticides, nimaticides and miticides.

Insecticides: This class of pesticides protect crops against insects or prevent their attack. Insecticides could be broad spectrum or could be insect specific. They work by disrupting the nervous system, damaging exoskeletons or by damaging respiratory system.

Fungicides*:* Fungicides protect crops from fungal diseases and prevent fungal infestation. Further, these fungicides are categorized in protectants or eradicants. Protectants inhibit the fungal growth and have to be applied at regular intervals while, eradicant attack the pests on application. Most of the fungicides act by damaging the fungal cell wall or by interfering in energy production within the cell.

Herbicides: Herbicides prevent the growth of herbs or specifically unwanted plants called weeds. Herbicides could be broad spectrum that could inhibit the growth of weeds along with all the plants in field like, Glufosinate ammonium which is used for vegetation control in field after cropping or for the land which is not used for cultivation. Whereas, some herbicides could be very specific that kill the unwanted plants only.

Nematicides: These pesticides are used to prevent the crops during storage. They are formulated to kill plant parasitic nematodes. These chemicals are highly volatile and promote migration through soil.

Rodenticides: These pesticides are intended to kill rodents including rats, mice, woodchunks etc. These pesticides are majorly non-specific in nature.

Apart from that, based on chemical composition, pesticides are classified as organochlorines, organophosphates, carbamates, pyrethroids, formamidines. Among which, the most commonly used pesticides fall into organochlorines, organophosphates and carbamates and all of them work by disrupting nervous system of organisms by acting on functions of particularly four targets including acetylcholine esterase, voltage-gated chloride channel, acetylcholine receptor and aminobutyric acid receptor (Kalia and Gosal, 2011). General characteristics of these commonly used pesticides are as given below: (Ortiz-Hernández, 2002; Badii and Landeros, 2007)

Organochlorines: Organochlorines are composed of carbon atoms, chlorine, hydrogen and in some cases, oxygen. They are soluble in lipid and nonpolar in nature. Thus, they can get accumulated in animal tissues. They persist for longer period.

Organophosphates: Organophosphorus pesticides consist phosphorus atom in central position. These compounds could be aliphatic, cyclic or heterocyclic. They are water as well as organic solvent soluble compounds.

Carbamates: Carbamates are generally acid derivatives. Their persistence is relatively low but highly toxic for vertebrates. Chemically, they are like plant alkaloid *Physostigma venenosum.*

Pyrethroids: Pyrethroids are generally alkaloids and are non-persistence in nature. They act by affecting the nervous system.

Biological: Biological pesticides are made from viruses, microorganisms or their metabolites. The most popular and widely used biological pesticide is derived from *Bacillus thuringiensis* (Bt).

Moreover, based on their application or work approach, pesticides are classified into systemic pesticides, foliar pesticides, soil-applied pesticides, contact pesticides,

fumigants, selective and non-selective pesticides, eradicant fungicides, protectant fungicides etc.

2.3 PESTICIDES AND INDIAN SENARIO:

In developing country like India, where, agriculture has the major share in GDP, crop production must be increased. Agricultural production is increasing rapidly thus the consumption of pesticides is also increased (FAO, 2010). On the other hand, average 45% crop loss occurs annually due to pest infestation while, 35% of crop production is lost during storage (Abhilash and Singh, 2009). Globally, India stands at 10^{th} position in pesticide consumption (Raj, 2009). Most commonly used pesticides in India are Monocrotophos (10700 Million Tonnes (MT), Acephate (6400 MT), Endosulfan (5600 MT) and Chlorpyrifos (5000 MT) (Pesticide Information, 2002). According to Section 9(3) of the insecticide act 1968, amended on 20^{th} August 2014, 246 pesticides have been registered in India for usage. Among which consumption of insecticides, herbicides, fungicides, and other pesticides is 65%, 16%, 15% and 4% respectively (www. Krishijagran.com). But, unfortunately, MRL (Maximum Residual Limit) of some pesticides have not been notified yet (Nollet and Rathore, 2009). Use of synthetic pesticides started in India at 1948 with DDT (Dichlorodiphenyltrichloroethane) for control of malaria and BHC (Benzyl Hydrocjloride) for control of locust (Gupta, 1989; Subramanian et al., 2007) but the production was started from 1952 with BHC production plant in Rishra, Kolkata and first two pesticides produced in India were BHC and DDT. Then, in 1969, Union Carbide India Limited established Union Carbide unit in Bhopal for pesticide production. Scenario of pesticide consumption in India is shown in **Figure 2.1**. WHO (World Health Organization) has been categorized each pesticide according to its toxicity. Based on their toxicity expressed in terms of LD50 value WHO categorized all the pesticides into IA: Extremely hazardous, IB: Highly hazardous, II: Moderately hazardous and III: Slightly hazardous. Toxicity of commonly used pesticide in India is as shown in **Table 2.1**. In India, the average pesticide consumption is much lesser than the developed countries, but pesticide residue problem is comparatively higher. Thus, incidences of pesticide contamination are increasing day by day. Samples of fruit, vegetables, cereals, wheat flour, oils, pulses, grains, meat, fishes, bovine milk collected

from all over India were found to be having sizable amount of pesticide residues. All the samples were found to be contaminated with four major pesticide groups, organophosphorus, carbamates, pyrethroids and organoclorine among which maximum residues were related to organophosphorus pesticides (Nollet and Rathore, 2009). In an another study, 50% vegetables were found to be contaminated by various pesticides, from which, 16% were above MRL limit (Kole et al., 2002). Highest pesticide consuming states of India in comparison to other states are Andhra Pradesh, Karnataka, Maharashtra, Gujarat and Punjab (Govt. of India, Eleventh Five-Year plan: 2008-2012).

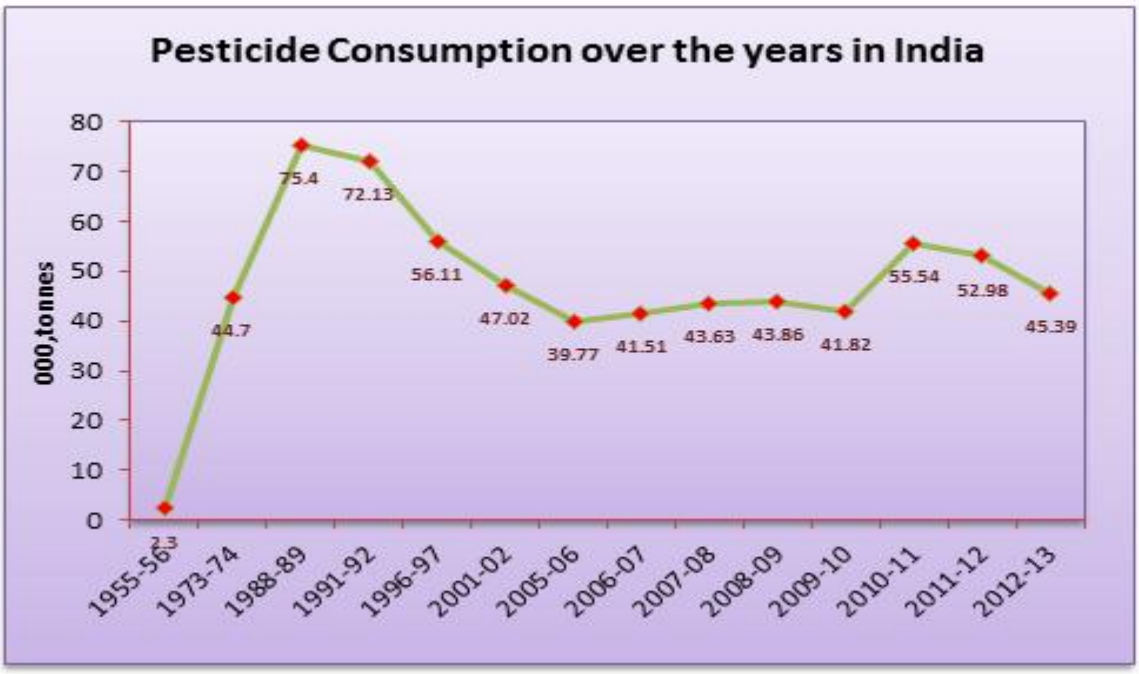


Figure 2.1: Pesticide consumption in India (www.krishijagran.com)

Table2.1: Pesticides commonly used in India (Source: Reddy G V S, 2013)

Pesticide	**Chemical family**
IA : Extremely hazardous [a]	
1. phorate	Organophosphate
Ib : Highly hazardous [a]	
2. monocrotophos	Organophosphate
3. profenofos	Organophosphate

4. cypermethrin	Organophosphate
5. carbofuran	Carbamate
II : Moderately hazardous [a]	
6. dimethanoate	Organophosphate
7. quinalphos	Organophosphate
8. endosulfan	Organochlorine
9. carbaryl	Carbamate
10. chlorpyrifos	Organophosphate
11. cyhalothrin	Pyrethroid
12. fenthion	Organophosphate
13. DDT	Organochlorine
14. Lindane	Organochlorine
III : Slightly hazardous [a]	
15. Malathion	
IV: Unlikely to cause accute hazard in normal use [a]	
16. Carbendazim	Carbamate
17. Atrazin	triazane

a: WHO classification, (2004)

2.4 PESTICIDE CONTAMINATION:

Pesticides have become unavoidable, inevitable evil needed to fulfill the ever increasing demand of food and fiber. But due to the extensive use of pesticide, land started loosing its fertility so, the demand of fertilizers has increased. First ever, Rachel Carson reported hazards of pesticide specifically DDT in 1962 in her book 'Silent Spring'. Moreover, pesticides resistant pests and insects have developed so, more toxic and broad spectrum pesticides are needed that ultimately cause almost irreversible damage to the environment and living beings. Apart from that farmers have constant pressure of social, political, economical, physiological factors and subsidies in agrochemicals, lack of alternatives and awareness, weak enforcement of laws and easy availability enhance their usage. Pesticide contamination occurs by direct application to crops and soil, accidental spills during

production and transportation and waste generated during manufacturization and treatment of cattle for ecto parasite control (EPA, 2012). Moreover, malfunctioned practices regarding production, formulation, application and disposal is major reason of pesticide contamination. Sometimes, empty pesticide containers are used for storage purpose of other materials in domestic fields which directly expose to humans and other mammals (Dalvie and London, 2001; Dalvie et al., 2006).Thus the high vulnerable or risk groups are farmers, sprayers, production workers and formulators.

Fate of pesticides in environment relies on many factors including, chemistry of pesticide, its solubility in water, adsorption-desorption of pesticide on soil particles, physico-chemical as well as biological removal of pesticides, environmental factors (Jerald 1996; Ian 2004), mode of application and amount of pesticide. Indiscriminate and frequent application of pesticide cause potential risk to water and soil. Pesticides pollute the environment by many ways like adsorption to soil particles, volatilization, soil erosion, leaching, water runoff etc. Moreover, obsolete pesticides, pesticides which are outdated, or unused or banned pesticides or which have been passed expiry date are also major source of contamination. In developing countries, due to lack of management, such unused obsolete pesticides are stockpiled which create a dangerous waste (Dasgupta et al., 2010).

2.4.1 Pesticide contamination of soil:

Soil is also get contaminated by pesticides through many ways. When the farmer spray the pesticide on crops, some particles spread through air either through or through volatilization in low troposphere when the pesticides are applied directly plant, some amount of pesticide get accumulated soil. Adsorption and persistence of pesticide in soil crucially depends on soil properties as well as chemistry of pesticide. Generally, pesticides are adsorbed strongly to clay particles and organic matter of soil which don't get leached out or evaporated easily. Soil contamination by pesticides is a threatening problem as they disrupt soil microbial activities and thus affect the nutritional quality (Handa et al., 1999). Soil environment is made up of biotic and abiotic components and that affect the fate of pesticides in soil. Thoughtless use of agrochemicals has serious negative effect on helpful, beneficial microflora of soil as the insecticidal residues remain

in soil (Ambrogioni et al., 1987) and stimulate or repress the biochemical activities carried out by them like ammonification, nitrification, respiration and nitrification that affect the fertility and productivity of soil and crop (Heinonen – Tanskj et al., 1985; Tu, 1995; Naumann, 1971; Schuster and Shroder, 1990; Jenkinson and Powlson, 1976 and Zelles et al., 1984). Insecticides can adhere to the soil particles and aerial parts of the primary producers and thus they get transported to water, air, plants, food and finally to human by means of runoff, drainage and leaching (Abraham, 2002) and thus, they are mobile within the environment and enter into the food chain and its severity increases with the possibilities of biomagnifications of these hazardous molecules in lipid bodies or tissues of organisms and mammals (Hafez and Thiemann, 2003). This problem of biomagnification has been reported in plant and animal products (Babu et al., 2003); fruits and vegetables (Waliszewaski et al., 2008) in milk and milk products (Kannan et al., 1997).

2.4.2 Pesticide contamination in water:

Pesticides applied on agricultural field migrate to surface and ground waters and contaminate them. Pesticides can contaminate surface water through runoff from treated plants or soil and ground water by leaching from the soil which takes many years to be cleaned up. Pesticides can also adsorbed on suspended particles or dissolved organic matter of water and get accumulated in sediments (Katagi, 2008; Irace-Guigand and Aaron, 2003). Apart from that, pesticide containers and tools used for application of pesticides are also source of contamination. After application, washing or disposal of such containers and tools are not well managed as they rinsed with water and such contaminated toxic wastewater is hazardous for aquatic animals, wildlife and human being (Wilson and Tisdell, 2001 and Ridgway et al., 1978). Such contaminated waste water accumulates in aquatic life through biomagnifications. Pesticides also transferred through migrating fishes and birds and get accumulated in aquatic ecosystem. Pesticide enter in food chain and by biomagnifications, it spreaded everywhere in ecosystem. Pesticide contamination of water results in failure of reproduction system of fishes (Connel, 1988; Verschueren, 1977) also cause egg shell thinning in birds like peregrine falcons, sparrow hawk and eagle owls (Lundholm, 1997). Almost all the surface water

reservoirs of India except mountain water, are highly polluted (Nollet and Rathore, 2009). Though the water is treated before supply, but these treatments are inefficient to remove pesticides and their residues. Pesticide interact with both biotic and abiotic components of environment and causes imbalance in nature. Groundwater also get contaminated by pesticide leaching out from treated fields, mixing sites, washing sites, or disposal areas (Anonymous, 2009). This way, pesticides which are water soluble get transported to ground water through leaching. Root holes and earthworm burrows in earth crust also play their role in pesticide transportation (Stagnitti et al., 1994; Magri and Haith, 2009).

2.4.3 Pesticide contamination in air:

When pesticides are applied to the crops, pesticide particles carried out by wind and form aerosols and drifts. Moreover, pesticides also contaminate the air through volatilization in which, vapor or aerosols of pesticides are formed during their application and carried to long distance with air flow from the region they have been applied and contaminate other plants (Taylor and Spencer, 1990).There are several factors like wind speed, temperature, humidity etc. that affect the rate of spreading of pesticides in air. Approximately, 80 to 90% of applied pesticides get volatilized within few days after application (Majewski and Capel, 1995). In the view Straathoff, (1986) of many esters containing herbicides get volatized from treated plant and infect the other plants nearby. Due to spray drifts, spray droplets of pesticide are transported in surroundings when the farmers are sprayed on plant and approximately 2 to 25% of the pesticides applied on crops are lost and spread over long distance from few yards to hundreds of miles (Aktar et al., 2009).

Pesticides and their residues can enter in the food chain through contaminated soil, water and air. Increasing cases of pesticide and their residue detection in food and drink products are evidence of this emerging issue. Many developing countries like India, have been started monitoring of such contamination in food and drink products and such studies have thrown light on the widespread contamination of fruits, vegetables, cereals, milk and milk products, and animal feed with pesticide residues (Singh, 2001; Kang et

al., 2000; Shah et al., 2000; Kole et al., 2002; Dubey et al., 1999; Battu et al., 2004, and Adeniyi and Oladele, 1999). Bhanti and Taneja, (2007) has reported that, vegetable samples collected from North India were analysed for pesticide residues and presence of organophosphorus pesticides like methyl parathion, chlorpyrifos, malathion were detected in the range of 2.5 to 6 ppb. Though, in most of the cases, pesticide residues are detected within the maximum residue limit (MRL), but continuous use of such food and drinks can cause accumulation of pesticide residues.

2.5 ORGANOPHOSPHORUS PESTICIDES:

Organophosphate pesticides are most common compounds fall in highly neurotoxin category used for crop, feedstock protection and warfare agents (Cho et al., 2002). Organophosphates are esters, amides or thiol derivatives of phosphoric, phosphonic, phosphothionic or phosphothioric acids (FAO, 2002). Organophosphorus compounds have three phosphoester linkages in which phosphorus is linked with either an oxygen in case of oxons (P = O) or sulfur in case of thiols (P = S) by double bond (Horne et al., 2002) as shown in **Figure 2.2**. In 1937, first organophosphorus pesticide, tetraethylpyrophosphate was developed by German chemist Gerhard Schrader (Gallo and Lawryk, 1991) and simultaneously, two warfare agents tabun and sarin were also developed (Singh and Walker, 2005) followed by further development in production of new organophosphorus compounds. Overall, organophosphorus pesticides have 27% of total sales (Agrolook International Crop Science Magazine, 2003 and Gupta, 2006) with 38% consumption of total pesticides worldwide (Singh, 2009). Approximately, 140 organophsophorus pesticides are synthesized worldwide (Ortiz-Hernandez and Salinas, 2010). According to WHO, from total 3 million pesticide poisoning cases mostly are OP pesticides related with 20,0000 deaths annually, either because of self poisoning or occupational exposure (Bird, 2008; Jeyaratnam, 1990). Pesticides and their residues were detected in sizable amount in various samples including, fruits, vegetables, cereals, wheat flour, meat, fish, oils, grains, pulses. Most commonly used oprganophosphorus pesticides are glyphosate, chlorpyrifos, parathion, methyl parathion, diazinon, coumaphos, monocrotophos, fenamiphos and phorate.

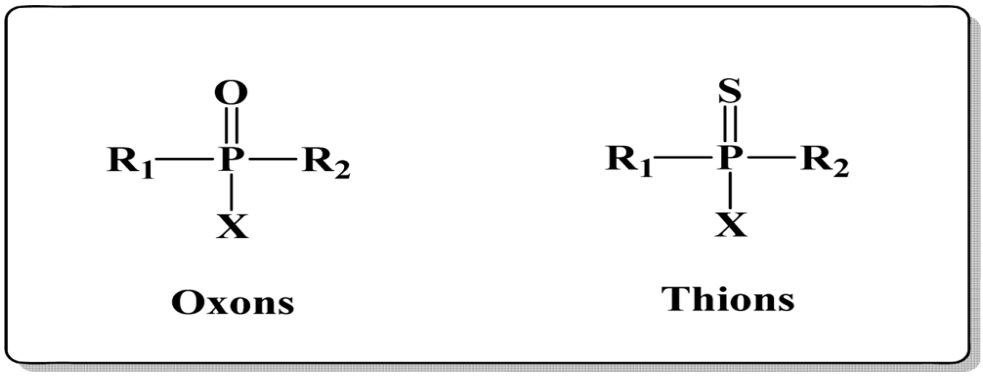
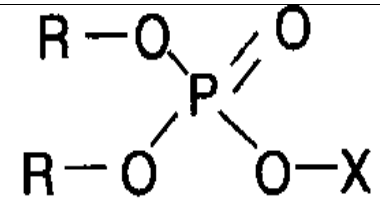
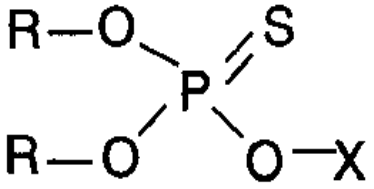
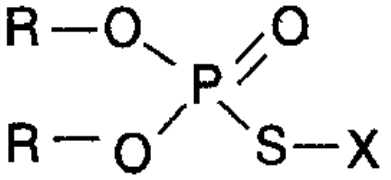
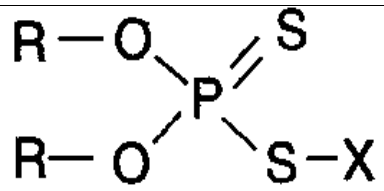
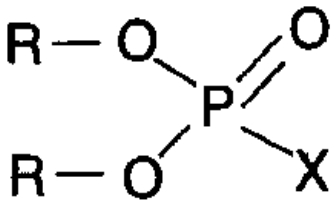
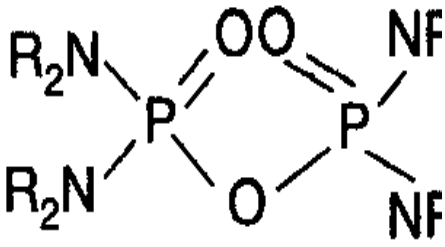


Figure 2.2. General structure of organophosphorus compounds

Here, R1 and R2 represents the aryl or alkyl groups and X represents the ‘‘leaving group’’ which get displaced during hydrolysis of OP compound. **Table 2.2** shows basic structures of oganophosphorus pesticides.

Table 2.2 Basic structures of organophosphorus pesticides (Hassal, 1999)

GROUP	**STRUCTURE**	**EXAMPLE**
Orthophosphates		Dichlorovos, mevinphos, monocrotophos, thionphosphates
Phosphorothionates		Diazinon, parathion,fenitrothion
Phosphorothiolates		Vamidothion

Dithiophosphates		Dimethoate, phorate, malathion
Phosphonates		Trichlophon, butonate
Pyrophosphoramides		Schradan

2.6 MONOCROTOPHOS:

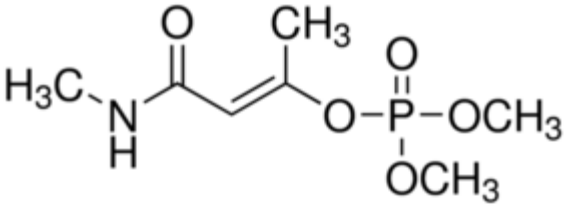


Figure 2.3: General structure of monocrotophos

Chemical formula: $C_7H_{14}NO_5P$

Molecular weight: 223.2 gm/mol

Solubility in water at 20^0C: 100 g/l

Monocrotophos [dimethyl-(E)-1-methyl,2-methylcarbamoylvinylphosphate] is an organophosphorus insecticide and acaricide which was first produced in 1965 by Cilca AG and Shell development company (Sha, 1999). Monocrotophos is amongst top 15

pesticides used in 20th century (Anonymous, 2005). It is manufactured from mono-chloro-monomethyl-acetoacetamide and trimethyl phosphate. It has P-O-C linkage and amide bond and is highly water soluble and hydrophilic by nature that is classified as highly hazardous category according to WHO, (2004). In India, it is widely used to protect crops like cotton, sugarcane, tobacco, groundnut, maize, rice, soybean and vegetables (Bhadbhade et al., 2002) against mites, stem borers, potato tuber moth, common pests of tomato, sugarcane, soybean and tobacco, sorghum midge, western flower thrips, aphides and green vegetable bugs (Vig et al., 2001 and Bhadbhade et al., 2002).

2.7 MODE OF ACTION AND TOXICOLOGY OF MONOCROTOPHOS:

Monocrotophos acts by inhibiting acetylcholine esterase which is required for transmission of nerve pulses in brain, skeletal muscles and other areas (Toole and Toole, 1995). But after transmission of impulse, acetylcholine has to be hydrolyzed to avoid overstimulation of nervous system. This hydrolysis is carried out by acetylcholine esterase that binds with active site, serine 203 of acetylcholine and form enzyme-substrate complex and convert into choline and acetyl Co-A. This normal function of acetylcholine esterase is inhibited by monocrotophos. It binds at serine 203 by covalent bond and changes its function and structure. The leaving group is then attaches to the Hiss 447 and release phosphate thus the enzyme becomes phosphorylated. This cause accumulation of acetylcholine at synapses and ultimately nerves get overstimulated and get jammed (Manahan, 1992). Loss of acetylcholine esterase cause accumulation of acetylcholine at cholinergic neuroeffector junctions made up of smooth muscles and gland cells and cause muscle contraction and secretion respectively; actylcholine accumulation at skeletal nerve-muscle junctions can results in muscle twitching, and paralysis by depolarizing the end-plate; acetylcholine accumulation in the brain can cause sensory and behavioral disturbances also results in incoordination (Waxman, 1998). There are some studies that correlate the increasing usage of organophosphorus pesticides with increasing cases of cancers like lymphoma (Nollet and Rathore, 2009).

As far as human being are concerned, these pesticides enter into bodies and cause disorders of immune system, hormonal or reproduction system. Monocrotophos acts

systemically by contact and can be absorbed through inhalation, ingestion and direct skin contact. Oral toxicity of this pesticide in case of male rate is (LD50) 23 mg/kg while in case of female rate it is 18 mg/kg. As far as direct skin exposure is concerned, its toxicity for rates is 354 mg/kg. When this pesticide is inhaled, it can affect respiratory system and cause bleeding of nose, runny nose, shortness or difficulty in breathing, chest discomfort, coughing or excessive fluid in bronchial tubes. Direct skin exposure can cause sweating, muscle contractions; while eye contact may results into tears, pupil constriction. Severe poisoning can exhibit symptoms like, highly irritation of eyes, incoordination, slurred speech, loss of reflexes, weakness, involuntary muscle contraction, irregular pulsation, increased blood pressure, blurred vision (Anonymous, 1991)and even cause paralysis and death (Ragnarsdottir, 2000). The major groups having higher risk of pesticide poisioning are workers associated with production, sprayers and farmers specially in developing countries like India, there is a lack of awareness and unavailability of the essential protective devises like, mask or respirator, goggles and proper clothing. Pesticides enter into human body and cause immune system disorders, reproductive disorders and even can damage fetus development (Nollet and Rathore, 2009). Children are more susceptible to pesticide toxicity due to their weak immunity and thus, cases of cancer specially leukemia are increasing in children (Daniel et al., 1997). There are many incidences occurred in developing countries which are directly related to monocrotophos which include, Paraguay where, monocrotophos is commonly used on cotton and found as causative agent for paralysis in children of cotton growing regions. In Egypt, farmers using monocrotophos showed symptoms of deep sensory loss and loss of reflexes in ankles and knees. Health department of Brazil also noticed 1,650 cases of monocrotophos poisoning during 1982-1991. Even in India many accidental or intentional cases of monocrotophos have been reported like in Bihar, several school children got died because of monocrotophos contaminated mid-day lunch. In Jamnagar, Gujarat total 132 poisoning cases were detected by Forensic Science team among which, 71 cases were due to insecticides and from that, 20 cases were monocrotophos related (Gupta and Vaghela, 2005). Apart from monocrotophos, poisoning cases of other pesticides have been also reported. The first pesticide poisoning case reported in India was from Kerala in 1958, where, more than 100 people were died after consuming parathion contaminated wheat

flour (Karunakaran, 1958). Nowadays, pesticides and their residues are found as cancer causing agents. Some studies reveal that, increasing incidences of non-Hodgkin's lymphoma are related with increasing usage of organophosphorus pesticides (Nollet and Rathore, 2009).

The toxicity of any hazardous compound specially, pesticides depends on the persistence and concentration of pesticide. Such pesticides contaminate the soil, air and water systems and get accumulated in plants and other living beings including, humans, birds, fishes through entering in food chain. Apart from humans, monocrotophos is extremely toxic for bees, birds and is moderately toxic for fishes and other aquatic animals. In Rachel Carson's book Silent Spring, there is a narration about death of some bird's species due to accumulation of pesticides in bird's tissues. Several fungicides can kill the earthworms which ultimately harm the population of birds that feed upon them. Like wise, pesticide polluted water bodies are also harmful to aquatic life. In water contaminated with herbicides, growth of aquatic plants get ceased that ultimately affect the oxygen amount in water and shows adverse effect on fishes and other aquatic animals. Apart from that, constant exposure to pesticides, also cause adverse effect on physiology of fishes. Pesticides and their residues remain on crops after their application are harmful to animals fed upon them.

2.8 DEGRADATION OF PESTICIDES:

2.8.1 Physico-chemical degradation of pesticides:

Removal of pesticides from the contaminated sites is essential because of their hazardous effects. Traditional methods for removal of pesticides include thermal treatment, physical treatment and chemical treatment. Principle ways in all these treatments are thermal, physical and chemical treatments. Thermal treatment includes incineration and open burning. Physical treatments involve adsorption by activated carbon and organic as well as inorganic materials, use of percolator filters, landfills, disposal pits and land cultivation. Ozonation/ UV-radiation, fenton oxidation, hydrolysis and Potassium Polyethylene Glycol Ether (KPEG) methods are considered as chemical treatment for removal of pesticides.

Incineration: Inicineration of pesticide oxidation process occurs under high temperature and pesticides get converted into inorganic gases like CO_2, volatile acids, metal oxides and ash (Felsot et al., 2003 and Alloway and Ayres, 1997). But there are some disadvantages of this process like, it needs sophisticated equipment, production of toxic gases like cyanide in the off. Apart from that, inorganic pesticides are not incinerated (Felsot et al., 2003).

Open burning: in this treatment, pesticide waste including pesticide containers are burnt out in piles. This method is inexpensive but, the gas emission is hazardous to workers, plants, animals and surrounding environment.

Ozonation/ UV-radiation: In presence of UV light, ozonation process produces hydrogen radicals which are very powerful oxidizing agents (Glaze, 1987). This treatment is more effective than other as it has advantages like, it is very rapid and easy process. But on the other hand it needs very high energy and the equipment cost is also very high (Kearney et al., 1986).

Fenton oxidation: In this oxidation process, hydrogen peroxide (H_2O_2) and iron salts are used and the treatment works under low pH condition (Sun and Pingnatello, 1992). Here, iron salts act as catalyst and form hydroxyl radicals which are very powerful oxidizing agents in presence of H_2O_2 by forming highly reductive hydroxyl radicals. Moreover, degradation rate is also increased by UV irradiation.

Hydrolysis: Ester linkages in pesticides like, pyrethroids, carbamates, organophosphates and acetaniledes are hydrolyzed in this method. Hydrolysis is one of the most common method for degradation which can be performed homogenously or heterogeneously under both acidic as well as alkaline conditions. In case of organophosphorus pesticides, alkaline hydrolysis is performed which involve, maintenance of alkaline condition which is very costly and at the end of the process some secondary pollutants are also produced.

Potassium polyethylene glycol ether (KPEG) method: Some pesticides are resistant to hydrolysis like, chlorinated pesticides. Such pesticides are treated with KPEG. The disadvantage of the KPEG process are 1) high clay content, acidity and high natural organic matter interferes with KPEG reaction and 2) it is not recommended for large waste volumes with concentrations above 5% for chlorinated contaminants. moreover this method is not recommended for large volume of contaminated site. Besides that, the use of chemicals like titanium dioxide is harmful to soil fertility and disrupts the ecosystem of soil (Richins et al., 1997; Sharaf et al., 2007; Jain and Gerg, 2015).

Adsorption by inorganic/organic materials: Pesticide decontamination can be done through its adsorption on anionic clays (Inacio et al., 2001). There are number of organic materials which can be used for this purpose and serve as adsorbents. Activated carbon is also used by several industries for such purpose of pesticide cleanup (Atkins, 1972 and Kobylinski et al., 1984). This method also has disadvantages like, it needs skilled person and it is costly.

Photodegradation: Photo degradation is also an attractive option for detoxification of pesticides remain on plants and soil which occur by two ways i.e. indirect photolysis and direct photolysis. Indirect photolysis occurs in presence of oxygen and form hydroxyl or phenoxy radicals while in direct photolysis, the pollutant absorb the UV light and get converted to compounds that react with environment and get detoxified by itself with time. In case of photodegaradtion, there are several reports that suggest production of toxic intermediates that persists for longer time periods (Stangroom et al. 2000; Zamy et al. 2004). Moreover, because of its composition, monocrotophos is not able to absorb the light beyond 300 nm at significant level, thus the pesticide may not get degraded by direct photolysis (Bukhard *et al*., 1975; Guth, 1994). Some studies have been conducted regarding photodegradation of monocrotophos in soil. Dureja, (1989) reported 40-50% degradation of monocrotophos within 8 hrs when it was exposed to natural sun light in Indian soil.

Besides that, other physical treatments are also applied for pesticide disposal like land cultivation, land-fills and disposal pits face problems of runoff and leaching,

susceptibility towards climate and other environmental conditions. These physical methods are inefficient and costly (Chino-Flores et al., 2012). Volatilization is also an option for pesticide degradation but it relies on many factors including vapor pressure (VP), solubility of compound, adsorption behavior and the persistence of pesticides apart from that, environmental conditions also affect the pesticide removal from natural environment (Racke, 1992).

2.8.2 Bioremediation of pesticides:

Phyto remediation:

It is an emerging approach in which plants are used to remove the pollutant from soil and water (EPA, 2005). It includes, phytoaccumulation, phytoextraction, phytotransformation and phytodegaradtion. In phytoaccumulation, contaminants from soil or water get accumulated in plant roots or leaves. While, in biotransformation, toxic compounds from environment are transformed into less toxic and more stable compound with the help of roots, rhizobia and other root or plant associated microbes. Whereas in phytodegradation, organic contaminant get metabolized by the plant enzymes. In case of phytovolatilization, contaminants get volatized as they pass through the plant leaves. In another phenomena, contaminants are immobilized and their transportation through soil get reduced which is called Phytostabilization.

Microbial degradation of pesticides:

Generally, microbial transformation of pesticides is carried out by several ways: biomineralization, biodegradation, cometabolism, biotransformation, bioaccumulation and bioremediation (Shakoori et al. 2000; Park et al., 2003; Finley et al., 2010). Bioremediation of pesticides using microorganisms in contaminated natural environment is the most efficient, cost effective and promising method (Singh and Walker, 2006; Vidali, 2001; Dua et al., 2002). Biological remediation overcomes the problem of traditional method regarding production of harmful and persistent pollutants. Pesticides generally exert toxic effect on microbial flora thus, most of the microbes could not resist the presence of pesticides but some of them get evolved with the ability to utilize the pesticide as nutrition source. Bioremediation involves the use of such microbes which

have great metabolic diversity so, they can adapt in diverse ecological niches. Besides that, they have great potential of degradation when they are exposed to xenobiotic compounds (Singh and Walker, 2006). Moreover, large variety of compounds can be degraded completely under mild conditions compared to that in other treatments. Higher multiplication rate and diversity in substrate utilization makes the use of microorganisms for pesticides removal cheaper. Microorganisms either transform pesticide into non-toxic or less toxic compounds or mineralize them into elements like, phosphorus, carbon, sulfur etc. which get released into the environment and directly involve in environmental cycles. Till the date many bacteria, fungi and algae have been isolated by researchers that could degrade pesticides by utilizing pesticides as a source of carbon and energy, or by consuming the pesticides along with other sources of food or energy. Some Algae have been observed to degrade organophosphorus pesticides by adsorption and bioaccumulation process according to Lal and Lal (1987); Laabs et al., (2007); Pablo et al., (2009). While, fungi generally degrade the pesticides by means of cometabolism (Fernando and Aust, 1994; Yadav et al., 2003). But, bacteria dominate among all because of its metabolic diversity as well as their ability to grow in diverse habitats. First ever case of biodegradation of pesticide has been reported in 1973 when Sethunathan and Yoshida, (1973) isolated *Flavobacterium* sp. having ability to degrade organophosphorus compound from diazinon-treated rice fields.

Bacterial degradation of organophosphorus pesticides:

Pesticide transformation by indigenous bacteria is an environmentally friendly way of *in situ* detoxification (Mervat, 2009). There are many reports regarding successful removal of pesticides like, chlorpyrifos, endosulfan, malathion, parathion, quinalphos, ethoprop and atrazine by the addition of bacteria (Singh et al., 2004). For biodegradation of pesticides, till the date many bacteria have been isolated from various sources like sludge samples, waste water, and soil. Many researchers have been isolated the different pesticide degrading bacteria from sludge sample collected from waste water treatment plant (Jia et al., 2006; Latifi et al., 2012; Bai et al.,2008) and pesticide manufacturing company (Wang et al., 2012 and Xiao-Hua and Hang, 2006). In present study monocrotophos degrading bacteria have been isolated from soil collected from vegetable

and cotton farm previously treated with the pesticide. As the soil is the most important site of biological interactions and few thousand of species of bacteria can be found in soil. Soil is a natural ecosystem containing approximately 3500 million bacteria per gram of soil. Soil bacteria are categorized as good natural decomposers because of their ability to produce different enzymes. Apart from that, there is a possibility that, due to pre exposure of soil bacteria to the pesticide, they might have evolved ability to tolerate or degrade it. The contaminated sites are considered as an excellent source for the isolation of the pesticide-degrading microbial community (Chishti et al., 2013). Many researchers have been used soil samples collected from different sites for the same purpose. Pesticide degrading bacteria have been isolated from farm soil having pre-exposure of pesticides (Goswamy and Singh, 2009; Rani et al., 2008; Chudhry et al., 1988; Iyer et al., 2013; Kavikarunya and Reetha, 2012; Singh et al., 2004; Gundi and Reddy, 2006; Abo-Amer, 2012; Rangaswamy and Venkateswarlu, 1992; Ramnathan and Lalithakumari, 1999; Bhadbhade et al., 2002 and Singh and Singh, 2011); from soil of pesticide manufacturing company (Wang et al., 2010; Jilani and Khan, 2004 and Qiu et al, 2006). While, Perruchon et al., (2015) used soil of waste water treatment plant; Hindumathy and Gayathri, (2012) used rhizospheric soil and Bhagobaty et al., (2007) used soil of waste water irrigated farm for isolation of pesticide degrading bacteria. During isolation a number of microbial species show different capacity to degrade pesticides. Further study in this direction is not possible in a single experiment to utilize all these isolates. Therefore to select one potent microbial species for further study among many isolated species the technique followed is screening. During screening the potent microbial species can be selected on a number of criteria like color change, its capacity to grow in higher concentration of experimental pesticide, its capacity to degrade the pesticide upto a greatest extent, etc.

Researchers have isolated pesticide degrading bacteria from soil or sludge samples using different techniques. Atrazin degrading microorganisms were enumerated by Korpraditskul et al., (1993) using most probable number (MPN) technique. Further, cultures showing positive results were transferred on medium containing atrazin by dilution plate technique and two potent isolates RK014 and RK016 were isolated having ability to degrade atrazin upto 25.5% and 35.2% within 9 days respectively. Chlorpyrifos

and monocrotophos degrading bacterial cultures were isolated by Kavikarunya and Reetha, (2012) using pour plate technique. Well growing, isolated bacterial cultures were further purified by streaking them on Nutrient agar and King's B agar plate. Some researchers isolated pesticide degrading bacteria on basis of their growth in presence of pesticide. Chlorpyrifos degrading bacteria were isolated by Sharaf et al., (2006). Chlorpyrifos contaminated soil and waste water samples were inoculated on LB medium containing 100 ppm chlorpyrifos by dilution plate method and seven bacterial strains were isolated that were able to grow in presence of 100 ppm chlorpyrifos as sole source of carbon and energy.

Enrichment culture technique is most commonly used for isolation of pesticide degrading bacteria. Many researchers have isolated monocrotophos degrading bacteria by enrichment technique including Bhadbhade et al., (2002) isolated monocrotophos degrading bacteria from soils samples collected from vegetable farms which have been exposed previously to monocrotophos. They isolated potent monocrotophos degrading bacteria which were able to utilize it as carbon source by enrichment and adaptation technique. Monocrotophos, quinalphos, cypermethrin and fenvalerate pesticide degrading bacteria were isolated by Rangaswamy and Venkateshwarlu, (1992). They enriched soil samples collected from groundnut growing farms with pesticides separately to make the final volume 50 kg/ha. Serial dilution agar plate method was used to inoculate such enriched samples and isolated four heterotrophic bacterial cultures having ability to degrade all the four pesticides. Similarly, Jia et al., (2006) isolated monocrotophos degrading bacteria by enriched sludge samples collected from waste water treatment plant of a monocrotophos manufacturing factory with 50 mg/L monocrotophos. The potent bacterial cultures were isolated by transferring on media containing monocrotophos as only carbon source.

Endosulfan degrading bacteria were also isolated using enrichment technique. For isolation of endusulfan degrading strains, mixed bacterial consortium was used by Kumar and Philip (2006). This consortium was obtained from soil samples collected from endosulfan producing factory. Three potent endosulfan degraders were isolated from this consortium by serial dilution method with increasing concentration of endosulfan and

finally three bacterial cultures having ability to tolerate 500 mg/L endosulfan were isolated. Endosulfan degrading bacterium was isolated by Narkhede et al. (2015) using enrichment technique. For isolation, they collected soil samples from cotton fields already contaminated with endosulfan. Further the bacterial culture having ability to tolerate high concentration of endosulfan was isolated by transferring the enriched sample in minimal medium containing 50-100 ppm endosulfan. Endosulfan and endosulfan sulfate degrading bacterial cultures were isolated from cotton field soil having previous exposure of pesticide by Singh and Singh, (2011). Here also soil samples were spiked with endosulfan and incubated for 5 weeks; meanwhile, concentration of endosulfan was increased from 200 ppm initially to 1000 ppm at 5^{th} week. Further, such enriched sample was inoculated in medium having 50 ppm endosulfan as the only sulfur source and bacterium having ability to degrade maximum endosulfan was isolated by transferring the enriched samples into medium with increasing concentration of endosulfan.

Isolation of chlorpyrifos degrading bacteria were also carried out using enrichment culture technique. To isolate chlorpyrifos degrading bacteria, agriculture soil was amended with 50 mg/L chlorpyrifos containing medium by Rani et al., (2008) and allowed to incubate for seven days. Soil suspensions withdrawn at regular intervals were inoculated on mineral salt medium containing 50 mg/L chlorpyrifos as the only carbon source to obtain bacterial cultures having ability to tolerate 50 mg/L chlorpyrifos. Similarly, Yang et al., (2006) also enriched sludge samples with chlorpyriphos and isolated six chlorpyrifos degrading bacteria by providing chlorpyrifos as the sole source of carbon using enrichment method.

Organophosphorus compounds contaminated sludge sample of pesticide manufacturing factory was used for the isolation of methyl parathion and p-nitrophenol methanidophos degrading bacteria by Wang et al., (2012). Such activated sludge sample was enriched with 100 mg/L methylparathion containing medium. Further culture broth from these enriched sample was transferred periodically into medium containing methyl parathion in increasing concentration from 11 to 800 mg/L and finally potent isolates having ability to withstand maximum concentration of methylparathion were obtained by

inoculating the bacterial cultures on medium plates containing 800mg/L. Likewise, another organophosphorus pesticide, mithamidofos mineralizing bacteria were isolated from soil of pesticide manufacturing company by Wang et al., (2010). Such soil sample was enriched with 50 mL mineral salt medium containing 1000 mg/L methamidofos. To isolate the bacterium having ability to tolerate maximum amount of pesticides, culture suspensions from the enriched soil sample were exposed to methamidofos with stepwise increase in concentration from 1000 to 3000 mg/L. Finally, potent degrader was obtained by inoculating cultures on mineral salt medium agar plates having 1000 mg/L methamidofos.

Similarly, isolation of othophenylphenol, cadusafos, diazinon, pyrazosulfuron-ethyl, pyridine, acetamiprid and tributyl phosphate degrading bacteria using enrichment method is also reported by Perruchon et al., (2015); Abo-Amer, (2012); Cycon et al., (2013); Xu et al., (2009); Bai et al., (2008); Xiao-Hua and Hang, (2006) and Ahire et al., (2012). An organophosphorus pesticide ortho-phenylphenol degrading bacterial strain of *Sphingomonas* has been isolated by Perruchon et al., (2015) from soil of waste water disposal site. This soil was enriched with 10 mg/kg ortho-phenylphenol three times successively at regular intervals of 15 days. Serial dilutions were prepared from the enriched culture showing more than 50% degradation and potent isolate was obtained by transferring the bacterial colonies on mineral salt medium supplemented with nitrogen and ortho-phenylphenol. To isolate the another organophosphorus pesticide cadusafos from agricultural soil, Abo-Amer, (2012) enriched the soil sample with mineral salt medium having cadusafos. Serial dilutions were prepared using the enriched cultures and inoculated on mineral salt agar medium. Isolated colonies were transferred on the similar medium and among all isolated, *Pseudomonas putida* was screened out based on its degradation ability analyzed by GLC (Gas Liquid Chromatography) analysis. For isolation of bacteria able to utilize tributylphosphate as carbon and phosphorus source, soil and water samples collected from paper and pulp industries were used by Ahire et al., (2012). Enrichment of samples was done in minimal medium with and without glucose and by providing tributylphosphate as phosphorus source. The resultant enriched cultures were transferred subsequently to respective media and further these secondary enriched cultures of both the procedures were inoculated on minimal agar medium having 5 mM

tributylphosphate to isolate potent tributylphosphate degrading culture. Activated sludge sample collected from waste water treatment plant has been enriched with 300 mg/L pyridine containing mineral salt medium and when the pyridine was disappeared from medium, culture was transferred into fresh medium followed by preparation of serial dilutions and were spreaded on mineral salt medium plates and obtained isolates were screened out by streak plate method. Finally, bacterium showing higher growth rate and degradation ability was selected as potent degrader. Acetamiprid degrading bacteria were enriched by Xiao-Hua and Hang, (2006) from sludge sample of pesticide factory. Sludge was exposed to mineral medium having 200 mg/L for more than 2 months such enriched culture was transferred to mineral agar medium having acetamiprid and bacterial strain which was able to grow in presence of acetamiprid as only carbon source was selected for further study. Similarly, Xu et al., (2009) also enriched soil sample with pyrazosulfuron-ethyl for three months and transferred such enriched culture on mineral salt agar plate having 10 mg/L pyrazosulfuron-ethyl. Well grown colonies were selected and inoculated in liquid medium and potent degrader was screened out based on its degradation ability.

Different methods have been applied by different researchers for screening of potent pesticide degraders. Monocrotophos, chlorpyrifos, fipronil, tributyl phosphate, cyhalothrin, cadusafos and methamidophos degrading bacterial strains were screened out on the basis of percentage degradation of pesticides using HPLC (High Performance Liquid Chromatography) and GLC (Gas-Liquid Chromatography) analysis by Bhadbhade et al., (2002), Yang et al., (2006), Kumar et al., (2012), Ahire et al., (2012), Chen et al., (2015), Abo-Amer, (2012) and Wang et al., (2010). Methyl parathion degrading bacteria were screened out on the basis of color change of medium containing methyl parathion or by formation of degrading haloes on medium containing methyl parathion (Qiu et al., 2006, and Wang et al., 2012). Paraxon degrading bacteria were screened out on basis of yellow color formation in pesticide containing medium (Iyer et al., (2003). While, Bai et al., (2008) inoculated activated sludge sample into pyridine containing medium and when the pyridine found to be disappeared from the medium during incubation, they transfer the culture on MSM agar plates containing pyridine. The potent pyridine degrading bacterial colonies were then purified by streak plate method. Singh and Singh, (2011); Latifi et al., (2012) and Ali et al., (2012) screened out the endosulfan, chlorpyrifos and

methyl parathion degrading bacteria on the basis of their ability to grow in presence of maximum concentration of pesticides respectively. Apart from that, Silambarasan and Abraham, (2013) screened out chlorpyrifos degrading bacteria using minimum inhibitory concentration technique. Many researchers follow the screening process based on capacity of microbial strain to degrade the pesticide at its maximum extent.

After screening of potent bacterial strain from a great volume of isolated strains the next step is its identification. Isolated potent pesticide degrading bacteria can be identified on the basis of its morphological, physiological and biochemical characteristics including *Pseudomonas* sp. A3 (Ramnathan and Lalithakumari, 1999); *Pseudomonas* strain (Bhagobaty et al., 2008); *Pseudomonas fluorescens*, *Bacillus subtilis* and *Klebsiella* sp. (Kavikarunya and Reetha, 2012); and *Pseudomonas* sp. (Jilani and Khan, 2004). Substrate utilization by chlorpyrifos degrading bacteria *Stenotrophomonas* sp.was compared with the referred strains in the BIOLOG-GN database (Yang et al., 2006). While, acetamiprid degrading bacterium was identified as a member of *Pseudomonas* sp. based on morphological characteristics, physico-chemical properties, BIOLOG GN2, 16S rDNA sequencing and phylogenetic tree analysis in the study carried out by Xiao-hua and Hang, (2015). The most recent technique of bacterial culture identification is gene sequencing (Yang et al., 2006); *Pseudomonas putida* (Abo-Amer, 2012); *Agrobacterium* sp. strain Yw12 (Wang et al., 2012); *Paracoccus* sp. strain BW001 (Bai et al., 2008); *Pseudomonas* strains (Latifi, 2012); *Achromobacter xylosoxidans* strain C8B (Singh and Singh, 2011); *Hyphomicrobium* sp. MAP-1 (Wang et al., 2010); *Ochrobactrum* sp. (Qiu et al., 2006); *Sphingomonas* strain (Perruchon et al., 2015); *Paracossus aminovorans* (Yu-bin et al., 2011); *Providencia stuartii* (Rani et al., 2008); *Pyrococcus* sp. M-1 (Jia et al., 2006); *Bordetella* sp. B9 (Goswamy and Singh, 2009); *Kocuria* sp. (Neti and Zakkula, 2012) and *Bacillus thuringiensis* strain ZS-19 (Chen et al., 2015). Molecular gene sequencing technique coupled with DNA finger printing, RTqPCR analysis enhances the value of study.

2.9 DEGRADATION OF MONOCROTOPHOS IN SYNTHETIC MEDIUM:

Monocrotophos is a widely used pesticide in the field of agriculture to protect crops from a number of pests. It is also highly toxic to mammals including human and

other non-target living beings as it acts on central nervous systems. Due to this mammalian toxicity it is necessary to remove it from contaminate sites. Bacterial degradation of monocrotophos is a well-documented technique studied on a number of pesticides using various bacterial cultures including *Paracoccus* sp. M-1 (Jia et al., 2006); *Pseudomonas fluorescens, Bacillus subtillis, Klebsiella* sp. (Kavikarunya and Reetha, 2012); *Bacillus megaterium* and *Arthrobacter atrocyabeus* (Bhadbhade et al., 2002); *Micrococcus luteus*, *Bacillus cereus* and *Pseudomonas aeruginosa* (Bhuimbar et al., 2011); *Azospirillum lipoferum* and *Bacillus* sp. (Rangaswamy and Venkateshwarlu, 1992); *Pseudomonas* and *Flavobacterium* (Nazarian and Amini, 2008); *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* ssp. *Insidiosum* SBL 11 (Singh and Singh, 2003).

Microbial degradation of pesticides is judged by a number of ways. One method is based on the ability of organism to grow in pesticide containing medium like chlorpyrifos and monocrotophos degradation by *P. fluorescens, B. subtillis* and *Klebsiella* sp. (Kavikarunya and Reetha, 2012) and methamidophos, cartap, cypermethrin, and malathion degradation by *Pseudomonas putida* (Jilani and Khan, 2004). Other methods are based on color or pH change of the medium, estimation of released degradation metabolites or by quantitative estimation of residual pesticide remained after degradation. Most of the degradation studies include the later aspect to determine the amount of pesticide in culture inoculated medium with context of incubation time.

Some researchers have also used more than one technique to confirm the degradation of pesticides by bacterial culture. Bhagobaty and Malik, (2008) isolated the chlorpyrifos degrading bacterial strains from agricultural soil. Chlorpyrifos degradation ability of these bacterial strains were confirmed by TLC (Thin Layer Chromatography) analysis and tetrazodium reduction assay. Whereas, chlorpyrifos degrading bacterium *Enterobacter* strain B-14 was isolated by Singh et al., (2004). They use radiolabled chlorpyrifos to determine the amount of radiolabeled released CO_2 and residual pesticide in medium after degradation by *Enterobacter s*p. Singh and Singh, (2011) also studied α and β endosulfan degradation by *Achromobacter xylosoxidans* using GC-ECD (Electron Capture Detector), HPLC and TLC analysis to determine both the residual pesticides and produced metabolites to confirm the degradation of pesticides. Ahire et al., (2012)

analysed tributyl phosphate degradation by fifteen bacterial isolates based on released inorganic phosphorus and bacterial growth response to the pesticide and also by estimation of residual pesticide in medium.

Some bacteria possess broad range of substrates utilization and single bacterial species degrades more than one pesticides. For ex. cadusafos, ethoprophos, fenamiphos and isazophos degrading *Pseudomonas putida* (Abo-Amer, 2012); various pyrethroids like cyhalothrin, fenpropathrin, deltamethrin, β-cypermethrin, cyfluthrin and bifenthrin degradation by *Bacillus thurengiensis*; paraxon and methylparathaion degradation by *Pseudomonas putida* (Iyer et al., 2003) is studied well. Simultaneous degradation of organophosphate pesticide parathion and organochlorine pesticide γ-HCH by a single bacterium *Sphingobium japonicum* is also reported (Cao et al., 2013).

Pesticides remain in natural bodies including soil, water, plant etc. and get degraded by biological, physical or chemical means or they may become non-persistent in the natural bodies because of their chemical nature but their metabolites remain in the contaminated sites for longer duration which might be equal or more toxic than the parent compound. Therefore it is essential to study the bacterial degradation of both, the pesticides and their metabolites. Perruchon et al., (2015) reported ortho-phenylphenol and its metabolites benzoic acid, catechol and 2,3- dihydroxybiphenyl degradation by *Sphingomonas haloaromaticamanas*. Similarly degradation of endosulfan and its metabolites endoculfan lactone and endosulfan ether by *Staphylococcus* sp. and *Bacillus circulans* (Kumar and Philip,2006); and methyl parathion and its metabolite PNP degradation using *Ochrobacterium anthropi* and *Agrobacterium* sp. has been reported by Qiu et al., (2006) and Wang et al., (2012) respectively.

2.10 OPTIMIZATION FOR PESTICIDE DEGRADATION:

The aim of microbial pesticide degradation study should be to remove the pesticide quickly and efficiently from the contaminating site. Quick removal requires maximum microbial activity. Microbial activity depends on a number of factors and is sensitive to change in pH, temperature, time, metal ions etc. Detoxification or degradation of pollutants does not dependent only upon microbial growth but wide range

of physiological and ecological parameters affect the microbial degradation. So, to enhance the degradation capacity of bacteria, it is necessary to provide favorable conditions to the degrader. Different parameters like pH, temperature, incubation time, inoculum size, inoculum age are very crucial for optimum microbial growth and ultimately effective degradation of pesticide. The cultural conditions for monocrotophos degradation by potent bacterial culture has been optimized in this study as the bacterial growth and the bacterial enzymes responsible for hydrolysis of the pollutant are pH and temperature dependent.

Considering the effect of pH on bacterial degradation of pesticide, many researchers have been worked to identify the optimum pH and variation in results was observed. Chlorpyrifos degradation by *Enterobacter* strain B14 was observed to be highest at pH 5 (Singh et al., 2004) while it was maximum at 8.4 pH by *Stenotrophomonas* sp. YC1 (Yang et al., 2006). Optimum pH for endosulfan degradation by *Achromobacter xylosoxidans* and *Pseudomonas putida* was found to be 6.8 (Singh and Singh, 2011) and 7 (Narkhede et al., 2015). Xu et al., (2008) optimized this parameter for pyrazo-sulfuron-ethyl degradation by different bacterial strains of *Pseudomonas* and *Bacillus* sp. and observed maximum degradation at pH 7 and 9, for *Pseudomonas* and *Bacillus* sp. respectively. Sahoo et al., (2011) also studied pH optima for 4- chlorophenol degradation by *Arthrobacter chlorophenolicus* A6 using response surface methodology and found 7.5 pH as optimum one. Wang et al., (2012) reported pH value of 8.4 as optimum pH for degradation of methylparathion by *Agrobacterium* sp. strain Yw12, whereas, Perruchon et al., (2015) found 5.5 and 6.5 as optimum pH values for ortho-phenylphenol degradation by *Sphingomonas haloaromaticamans.*

Temperature is also a crucial parameter which was optimized by researchers for microbial degradation of different pesticides. Clorpyrifos degradation was observed to be highest at 35^0C by *Enterobacter* strain B14 (Singh et al., 2004). Degradation of pyrazo-sulfuron-ethyl was observed to be maximum in the range of 28 – 37^0C by *Pseudomonas* and *Bacillus* sp. (Xu et al., 2008). Highest degradation of cadusafos by *Pseudomonas putida* was found at both 20 and 37^0C, where complete degradation of cadusafos was observed within 6 and 5 days respectively (Abo-Amer, 2012). Maximum endosulfan degradation was achieved at 28^0C by *Achromobacter xylosoxidans* (Singh and Singh,

2011). Sahoo et al., (2011) studied optimum temperature for 4- chlorophenol degradation by *Arthrobacter chlorophenolicus* A6 using response surface methodology and reported 29.6^0C as the optimum temperature where this pesticide degrades to its maximum amount. Wang et al., (2012) reported 30^0C as optimum temperature for degradation of methylparathion by *Agrobacterium* sp. strain Yw12. Maximum degradation of methamidophos by *Hyphomicrobium* sp. MAP-1 was obtained at 30^0C (Wang et al., 2010). Perruchon et al., (2015) found 37^0C as the optimum temperature for ortho-phenylphenol degradation by *Sphingomonas haloaromaticamans.*

Age and volume of the inoculated culture are also important parameters that affect the bacterial activity. Singh et al., (2004) studied effect of inoculum density on degradation of chlorpyrifos by *Enterobacter* strain B14 and observed that, high inoculum density of $>10^4$ cells/mL, caused complete degradation within 48 h. Sahoo et al., (2011) optimized different parameters to enhance the degradation of 4- chlorophenol by *Arthrobacter chlorophenolicus* A6 using response surface methodology.

Moreover, bacteria use the pesticide as energy and carbon source but it is essential to study the effect of any additional carbon source on efficiency of degradation. Pino and Penuela, (2011) studied the effect of additional carbon source, glucose on methyl parathion and chlorpyrifos degradation and reported enhancement in methyl parathion and chlorpyrifos degradation by bacterial consortium. On the contrary, Singh et al., (2004) studied effect of additional carbon source on degradation of chlorpyrifos by *Enterobacter* strain B14 and observed no degaradtion of chlorpyrifos during three days in presence of glucose or succinate but after three days it degraded rapidly in both cases but without addition of other carbon sources chlorpyrifos degraded completely within two days. Bai et al., (2008) also reported stimulatory effect of glucose as additional carbon source on pyridine degradation by *Paracoccus* sp. strain BW001. Slow degradation of cadusafos by *Pseudomonas putida* in presence of succinate and glucose is also reported (Abo-Amer, 2012).

Optimization of various cultural conditions can enhance the pesticide degradation ability of the bacteria which has been proved by some researchers who compared the degradation of pesticides under optimized and unoptimized conditions. Sahoo et al.,

(2011) optimized different parameters to enhance the degradation of 4-chlorophenol (4-CP) by *Arthrobacter chlorophenolicus* A6 and found that, under optimized conditions, complete degradation of 4-CP was achieved in less time compared to that under unoptimized conditions and the degradation was 23% higher with optimized conditions than under unoptimized conditions. Likewise, Wang et al., (2012) also optimized various parameters for degradation of methylparathion by *Agrobacterium* sp. strain Yw12 and pesticide as well as its metabolite PNP (p-nitrophenol) both were degraded completely sooner than that under unoptimized conditions.

2.11 GROWTH PATTERN OF PESTICIDE DEGRADING MICROORGANISM:

Bacteria able to degrade the pesticides can either tolerate the presence of it or utilize it as nutrient source. Presence of pesticide in the medium affects the physiological and reproduction characteristics and thus affects the growth pattern of bacterial cells. Growth kinetic study of bacterial cells has been done in present work to know the response of bacterial culture when monocrotophos was provided to it as sole source of carbon and energy. Many researchers have studied the bacterial growth in presence of pesticide and correlated it with the degradation of pesticide in the medium. Degradation of monocrotophos was found closely related to growth of *Paracoccus* sp. M-1 revealed by estimation of biomass and turbidity and there was no lag phase for the bacterial strain and it gradually increased from the time of inoculation and then steady growth was observed with decreasing amount of pesticide (Jia et al., 2006). Similarly, other researchers also worked on bacterial degradation of different pesticides and performed growth kinetic study in presence of pesticides using similar methods. Growth pattern of *Pseudomonas aeruginosa* strain IRLM1 and *Stenotrophomonas maltophila* strain YC1 in presence of chlorpyrifos was studied by Latif et al., (2012) and Yang et al., (2006) respectively by measuring the optical density of culture medium spectrophotometrically and found that, bacteria follow same pattern of lag, log and steady phase with decreasing concentration of pesticide. Growth of *Pseudomonas aeruginosa* strain IRLM1 was much faster in pesticide containing medium than control. Similarly, Chino-Flores et al., (2012) studied the growth pattern of *Enterobacter* strain Cons002 in presence and absence of methyl parathion by same method and observed initial lag phase in both cases but exponential growth of strain started in presence of methyl parathion which was not seen

in absence of it and it means that the bacteria requires pesticide as nutrient source and the concentration of methyl parathion decreased proportionally from media with time. Wang et al., (2012) examined growth pattern of *Agrobacterium* sp. strain Yw12 with degradation pattern of methyl parathion in mineral salt medium. Results of cell density of Yw12 and amount of released PNP (p-nitrophenol) were also related with the degradation. The bacterium was able to grow in presence of PNP. Sahoo et al., (2011) demonstrated growth kinetics of *Arthrobacter chlorophenolicus* A6 in presence of 4-chlorophenol by observing biomass and residual pesticide concentration in medium and concluded no significant repression of pesticide at its lower concentration on biomass but at higher concentration, bacterial growth followed the same pattern of lag, log and decline phase. Growth of *Paracoccus aminovorans* strain CT measured spectrophotometrically in chrysene containing medium showed that, growth pattern and concentration of pesticide were in synchronous manner (Yu-bin et al., 2011). Goswamy and Singh, (2009) found no lag phase during their study of cell biomass and cell density of *Bordetella* sp. B9 in the medium provided with endosulfan because the bacterium was collected from the soil repeatedly treated with endosulfan and the growth increased rapidly with increasing time. They also checked biomass and it showed similar pattern of increment with time in presence of pesticide. Along with pesticide, they also provided dextrose as additional carbon source and the bacterium showed exponential growth. Growth pattern of bacterial species in terms of measuring the cell dry weight and cell density in pesticide containing medium is also reported. Singh and Singh, (2011) estimated bacterial growth in terms of cell dry weight and cell density of *Achromobacter xylosoxidans* strain C8B in endosulfan containing medium. Bai et al., (2008) evaluated the growth pattern of pyridine degrading *Paracoccus* sp. BW001 and the bacterium was able to utilize the pesticide as carbon, nitrogen and energy source and thus the bacterial growth increased with increment in initial concentration of pesticide. Similarly, growth of cadusafos degrading *Pseudomonas putida* was evaluated on the basis of cell dry weight (biomass) and cell density measured spectrophotometrically by Abo-Amer, (2012).

Some researchers used different methods like total viable count or estimation of released metabolites in inoculated medium with increasing time to determine the bacterial

growth in presence of pesticide. Rani et al., (2008) recorded total viable count of *Providencia stuartii* strain MS09 in presence of chlorpyrifos and observed that bacteria had adaptation phase initially and then entered into log phase which was similar to control and then increased significantly. They also found that generation time of bacteria increased at higher concentration of chlorpyrifos. Jilani and Khan, (2004) investigated growth pattern of *Pseudomonas* sp. in presence of different concentrations of malathion, methamidophos, cartap and cypermethrin using total viable count method. Perruchon et al., (2015) evaluated the growth kinetics of *Sphingomonas* strain in mineral salt medium containing 50 mg/L orthophenyl-phenol based on enumeration of bacterial colonies using spread plate technique and reported that, the growth of bacteria was stoichiometric and was coincided with degradation of orthophenyl-phenol. When Chen et al., (2015) compared the growth pattern of cyhalothrin degrading *Bacillus thuringiensis* strain ZS19 in presence of cyhalothrin using serial dilution method for cell growth and HPLC for degradation, both bacterial growth and degradation of pesticide exhibit similar increasing trend after which bacteria followed the same log, steady and decline phase and simultaneously, pesticide got degraded gradually. While, Cook et al., (1978) measured the turbidity of the *Pseudomonas putida* inoculated, 2-aminoethylphosphonic acid containing medium, to check the bacterial growth in presence of pesticide and apart from that, they also checked protein content and released amount of ammonium and phosphate with increasing time and found that, turbidity of medium, protein and amount of metabolites increased exponentially with time and lag phase was not observed. In all these studies, pattern of pesticide concentration and bacterial biomass were found to be inversely proportional to each other. Growth pattern of the bacterial strains was similar in all cases but higher concentrations of all the pesticides inhibited the bacterial growth while, higher concentration of cypermethrin increased the lag phase of growth.

2.12 METABOLITES OF PESTICIDE DEGRADATION:

Degradation of pesticides by bacteria results in formation of metabolites which may be toxic as the parent compound or more hazardous than it. The information regarding metabolites can lead the researcher to a metabolic pathway utilized by bacteria for degradation. The metabolite identification could be done using various techniques like TLC, GC, HPLC, NMR, FTIR, LC-MS.

Monocrotophos is an organophosphorus compound and its degradation pathway has been studied by some researchers. It has been reported that, during microbial hydrolysis of monocrotophos, N-demethylation, O-demethylation, hydroxylation of N-methyl groups and cleavage of phosphate-crotanamide linkage occur which results in formation of O-desmethyl MCP, monomethyl phosphate, dimethyl phosphate, N-methyl acetoacetamide and N-methylbutyramide (Bhadbhade et al., 2002; Guth and Voss, 1970; Gundi and Reddy, 2006). Bhadbhade et al., (2002) examined the metabolites produced during monocrotophos degradation by *Arthrobacter atrocyaneus* MCM B-425 and *Bacillus megaterium* MCM B-423 using thin layer chromatograpy. They further studied for presence of ammonia by pH and color change of medium. Presence of CO_2 as end product was also determined by GC analysis of headspace and the peak showing presence of CO_2 was not detected in control. Apart from that, volatile fatty acids like, N-valeric acid and acetic acid were also found during gas chromatographic analysis. They also determined the production of phosphates as a metabolite as the amount of phosphate increased with time and this was also coincided with decreasing amount of monocrotophos with time. Gundi and Reddy, (2006) also used different analytical methods like, GC and TLC analysis to identify N-methylacetoacetamide as monocrotophos degradation product. Kanekar et al., (2004) also reported N-methylacetoacetamide, O-desmethyl-MCP and CO_2 as monocrotophos hydrolytic products.

Sometimes, metabolites produced during pesticide degradation could be more toxic than the parent compound or it could be less toxic. Metabolite production is the pathway utilized by organism for degradation and the enzymes responsible for pesticide degradation thus, it is necessary to evaluate the degradation products of pesticide degradation. Degradation products of other organophosphorus pesticides have been also studied by some researchers. Degradation products of methyl parathion by *Ochrobactrum* sp. has been determined by Qui et al., (2006). They performed HPLC and GC-MS analysis and found p-nitrophenol, 4-nitrocatechol, 1,2,4-benzenetriol and hydroquinone as end products of methylparathion degradation. Methyl parathion degradation by *Agrobacterium* sp. strain Yw12 was studied by Wang et al., (2012) and p-nitrophenol was detected as metabolite of pesticide. Chlorpyrifos degrading bacterial cultures were

isolated by Latifi et al., (2012) and 3,5,6-trichloropyridinol was identified as end product using HPLC analysis.

Goswamy and Singh, (2009) studied the α and β- endosulfan both in medium broth and soil microcosm by *Bordetella* sp. B9 and found that, endosulfan ether and endosulfan lactone as end products of endosulfan degradation by GC analysis in broth culture while, endosulfan lactone, endosulfan ether along with endosulfan sulfate were found as metabolites of endosulfan degradation in soil microcosm. Similarly, metabolites of endosulfan degradation by *Achromobacter xylosoxidans* strain C8B were determined by Singh and Singh, (2011) and found that, endosulfan sulfate is produced as an intermediate. This endosulfan sulfate was also found to be degraded by bacterium using HPLC, TLC and GC analysis and carbon dioxide was found as end product by titrimetric method. Ramanathan and Lalithakumari, (1999) used different methods like HPLC and GC-MS analysis and TLC, NMR-spectral analysis to determine metabolites of methyl parathion degradation products. They observed p-nitrophenol as one of the metabolites of methylparathion degradation. During degradation of methylparathion, nitrite was found as by-product of pesticide degradation. Ahire et al., (2012) worked on the tributyl phosphate degradation by *Providencia* sp. strain BGW4 which was organophosphate compound and observed gradual decrease in residual pesticide in medium with increasing time simultaneously, inorganic phosphorus was also produced as end product and the amount of released inorganic phosphorus increased gradually with time as the tributyl phosphate decreased from the medium. Cook et al., (1978) studied the products of degradation of 2-aminoethylphosphonic acid by *Pseudomonas putida* and observed release of ammonia and phosphate in the medium. The amount of released phosphate and ammonia was in exponential manner with decreasing amount of 2-aminoethylphosphonic and increasing growth rate. Bai et al., (2008) detected possible metabolites of pyridine hydrolysis using GC/MS analysis, standard biochemical methods and Ammonium-Testkit to identify released ammonia. Different analytical methods have been applied by different researchers to identify the metabolites of pesticides like GC-MS and HPLC analysis (Chen et al., 2015); MS/MS and GC-MS analysis (Wang et al., 2012) to identify products of bacterial degradation of chlorpyrifos, cyhalothrin and methamidophos.

2.13 DEGRADATION OF PESTICIDE IN SOIL:

Pesticides and their toxic metabolites get deposited on top soil when applied on crops and influence soil microbial activities and thus fertility as well as soil properties. Pesticide residues reach to the ground water and to other water bodies and contaminate them and ultimately, enter into food chain. Thus the bioremediation of contaminated soil is essential. Soil is the most affected system by pesticide residues thus many researchers carried out biodegradation studies in soil system. Many researchers used the bacteria found to be potent degrader of pesticide in synthetic nutrient media, for this purpose. *Pseudomonas aeruginosa* able to degrade endosulfan upto 96% within 288 hrs was studied by Narkhede et al., (2015) to degrade endosulfan in black loam soil collected from cotton growing farms. Similarly, chlorpyrifos degrading bacterium *Alcaligenes* sp. JAS1 was isolated by Silambarasan and Abraham, (2013) from agricultural soil which was able to degrade 300 mg/L chlorpyrifos within 12 hrs and found that it could degrade chlorpyrifos efficiently in soil. Soil has been categorized or classified in different orders based on its physico-chemical properties. These soil properties including organic matter, moisture content, nutrient availability, texture, pH and temperature etc. affect the biodegradation of pesticides in soil (Vischetti et al., 2002; Arias-Estevez et al., 2008).
Factors influencing the persistence and/or degradation of pesticide in soil (Arias-Estevez et al., 2008) are - pesticide (chemical nature, volatility, solubility, formulation, concentration, method of application, time period of application and frequency of application); soil (geographical location, flora and fauna, usage of other agrochemical like fertilizers and manure, cultivation, irrigation, drainage, presence of pollutants, texture, physico-chemical properties viz. minerals, pH, humus content, organic matter and cations exchange capacity); climate (wind, temperature, solar radiation, rainfall and humidity).

Pesticide degradation in soil is influenced by many factors including soil texture, organic matter, pH, moisture content etc. Rate of microbial degradation of pesticide in soil can be differ from soil to soil depending on their texture because it affects the balanced distribution of water, nutrients, oxygen etc. Mobility of pesticide also varies with various soil textures. In fine structured soil, pesticide movement could be lesser than in coarse textured soil due to the equilibrium of pesticide between soil particles or soil

aggregates and water. Apart from that, soil moisture also plays an important role. It also affects the proliferation of microbes in soil according to Daniel & Timothy, (1991). Ramdas and Gerald, (2011) suggested that, excessive water content can cause reduction in oxygen amount of soil and thus create an anaerobic condition which slows down the microbial activity and thus, biodegradation of contaminant in soil. Chlorpyrifos and fenamphifos degradation in different soil was studied by Brajesh et al, (2004) and noticed that, the degradation of pesticides was higher in soil having 40% moisture content and lower in 20% moisture containing soil. Biodegradation of pesticides depends on organic matter and clay content of soil because they affect the bioavailability of pesticides and bioavailability mainly relies on adsorption-desorption of pesticides in particular soil and there are several mechanisms by which pesticide get adsorbed with the soil particles including Van der Waal's attraction, H-bonding, hydrophobic bonding, charge transfer, ion exchange and ligand exchange. There are several types of clay minerals i.e. montmorillonite and vermiculite, which have high cation exchange capacity and high surface area. So, they higher capacity of adsorption through coulombic forces and van der Waals forces. Whereas, clay minerals like, illite, kaolinite and chlorite, have low cation exchange capacity. Moreover, they don't possess high adsorption capacity. Soil is composed of heterogeneous mixture of inorganic and organic compounds which bind with pesticides and reduce the bioavailability (Torrents & Jayasundera 1997). Clay particles and organic matter bind with the pesticide particles and reduce its availability. Organic content of soil increases the retention of pesticides. Adsorption of pesticides on organic matter of soil depends on many factors like, size of organic matter, available sorptive sites on organic matter, surface area and the particles size (Benoit et al., 2008). It is also believed that, the clay content and organic matter form complexes in soil and due to this formation, domains of organic matter responsible for adsorption get blocked or cause conformational changes in the structure of organic matter thus reduce or enhance the accessibility of pesticides (Pusino et al., 1992; Njoroge et al., 1998; Jones and Tiller, 1999; Laor et al., 1998 and Saltzman et al., 1972). Degradation of monocrotophos by *Paracoccus* sp. M1 in fluvo aquic soil and high sand soil (Jia et al., 2006) and in black vertisol soil and red alfinsol soil (Gundi and Reddy, 2006) having different physico-chemical properties was studied extensively. Cycon et al., (2013) studied the degradation

of three organophosphorus pesticides chlorpyrifos, fenitrothion and parathion by *Serratia marcescens* in three different soils and observed that degradation rate of all the three pesticides is different in all soils. Initially all the pesticides were added to soils to make final concentration of 100 mg/kg. *Serratia marcescens* degradaed 45.3%, 61.4% and 72.5% of initial concentration of chlorpyrifos, fenitrothion and parathion in sandy soil while, 61.4%, 79.7% and 64.2%, and 68.9%, 81.0% and 63.6% removal of chlorpyrifos, fenitrothion and parathion was observed in sandy loam and silty soils respectively within 42 days. Half life of fipronil was found to be varied in different soil depending upto their properties (Kumar et al., 2012); degradation of imidrcloprid and diazinon by soil microorganisms in silty loam, sandy loam and sandy soil (Hafez and Thiemann, 2003). Thus it is necessary to study the degradation of pesticide in different soil types.

Soil pH also plays a vital role in pesticide degradation. Microbial growth and their activities are greatly affected by soil pH thus influence the biodegradation of contaminants in soil. Soil pH (Houot et al., 2000; Walker et al., 2001 and Sabadie, 1990) and moisture (Dureja, 1989) have been proved to be very influential parameters on pesticide degradation by many researchers. Ditya et al., (2012) studied chromafenozide dissipation in acidic, neutral, saline and black soil and found that, the dissipation of pesticide in soil was maximum in acidic soil followed by neutral, black and saline soil. Degradation of endosulfan isomers in soils was studied by Awasthi et al., (2000) and found that, degradation rate of pesticide differ in both the acidic and alkaline soils. Degradation was negligible in acidic soil and it increased with increasing soil pH. Moreover, moisture content was also observed as stimulating factor for degradation of endosulfan in soil. Singh et al., (2003) used soil of United Kingdom and Australia having different pH for chlorpyrifos degradation and found that degradation was low in acidic soils and increased considerably with increase in soil pH. Half life of pesticide in soil having 4.7 pH was 256 days whereas; it was 58 days in soil having 5.7 pH. Degradation was faster in neutral soil (6.7 pH) and the half life was 35 days. When the degradation of chlorpyrifos was checked in alkaline soils it was much more faster than in other soils and the half life of pesticide was 16 days. These results clearly indicate the influence of soil pH on pesticide degradation. Gold et al., (1996) reported that pH and clay content of soil

affect the persistence of bifenthrin, chlorpyrifos, cypermethrin, fenvelerate, permethrin and isopenfos.

Biodegradation of pesticide in soil is also affected by biological factors like competition with the indigenous microflora for substrate (Gupta and Gajbhiye, 2002); antagonism and predation that ultimately influence the inoculum survival and activity (Goldstein et al., 1985). Thus it is important to confirm that the degradation of pesticide is achieved either by isolated potent bacterial degrader or by indigenous microflora of soil. Thus, in present study, degradation of monocrotophos in soil was studied under three conditions including MCP containing sterile soil, MCP containing non-sterile soil which was having indigenous soil microflora and MCP containing sterile soil which was inoculated with *Bacillus subtilis* KPA-1. A number of reports suggest higher degradation of pesticide in sterilized soil inoculated with specific bacterial isolates compared to non-sterile soil containing native microflora.

Monocrotophos degrading bacterial strain *Paracoccus* strain M-1 was utilized by Jia et al., (2006) for degradation of monocrtophos in fluvo aquic soil and high sand soil. During study, they checked the role of *Paracoccus* M-1 in degradation by using both sterile and non sterile soils inoculated with *Paracoccus* strain M-1 and uninoculated with the bacterium and observed that, addition of inoculum enhanced the degradation of monocrotophos compared to uninoculated soils. Cycon et al., (2013) also compared degradation of three organophosphorus pesticides chlorpyrifos, fenitrothion and parathion in soils with and without inoculation and found that the degradation rate of all the pesticides was higher in *Serratia marcescens* inoculated nonsterile soil than uninoculated nonsetrile soils. Abo-Amer, (2012) tested the ability of *Pseudomonas putida* strain PC1 to degrade an organophosphorus pesticide cadusafos from contaminated soil by inoculating the sterile and nonsterile soils and observed that, the degradation in both sterile and nonsterile soils was equal but the rapid degradation was only observed in sterile soil. Goswamy and Singh, (2009) worked on α and β-endosulfan degradation by *Bordetella* sp.B9 in soil microcosm by application of different treatments and the degradation was found maximum in sterilized soil having endosulfan and bacterial

inoculum followed by unsterilized soil having only pesticide, unsterilized soil having both endosulfan and bacterial inoculum and control soil.

Cao et al., (2012) constructed genetically modified microorganism *Sphingobium japonicum* UT26 which could degrade both the organophosphorus and organochlorine pesticides simultaneously, and analyses the ability of strain UT26 to degrade parathion and γ-HCH in soil. They inoculated the culture in both the fumigated and nonfumigated soils, treated with parathion and γ-HCH and found that the culture was able to degrade both the parathion and γ-HCH completely within 15 days in fumigated and nonfumigated soils. Degradation rate in both cases was also similar. Yang et al., (2006) also examined degradation of chlorpyrifos by *Stenotrophomonas* sp. strain YC-1 in both fumigated and nonfumigated soils and compared it with the degradation in control, uninoculated soil and observed that, in case of nonfumigated control soil, 24% of applied pesticide was degraded in 15 days which was much slower than compared to inoculated fumigated and nonfumigated soil in which applied chlorpyrifos degraded completely within 15 days. Degradation pattern was similar in both fumigated and nonfumigated soils. *Stenotrophomonas* sp. strain YC-1 has been isolated from soil samples after degradation which indicated that, the organism was responsible for degradation.

2.14 EFFECT OF PESTICIDES ON GROWTH AND YIELD OF PLANT:

Use of pesticide is an essential evil required to fulfill the increasing food requirement of increasing population. The pesticides applied to the crop, affect the growth and yield of plant. There are contradictions in reports about the effect of pesticides on plant. So, it is necessary to evaluate the response of plant in presence of pesticide. Field study or pot experiments are conducted for this purpose. In present study, green gram (*Vigna radiate*) has been used as test crop. In India, green gram is a widely grown pulse crop which occupies 3 million hectors with 1 million tones of production (Zaidi et al., 2005; Parween et al., 2011) and 7% of total production (Singh et al., 2004). Response of *Vigna radiate* L. to foliar applications of chlorpyrifos at its different concentration was studied by Parween et al., (2011) under pot experiment. After

uprooting the seedlings at different stages like preflowering, flowering and postflowering, various parameters were analyzed and compared with that of control. During this study it was revealed that, higher dose of chlorpyrifos was detrimental for plants growth and nitrogen fixation while at lower dose, it showed stimulatory effect. During field study carried out by Kashyap and Kumar (2013), chlorpyrifos was applied to 20 days old seedlings by foliar application at the concentration of 50 to 150 ppm and the plants were uprooted at preflowering, flowering and post flowering stages and analyzed for carious growth and yield parameters. Here also lower concentration of chlorpyrifos had positive effect on all the parameters on the contrary; all the parameters were suppressed at higher concentration of pesticide. In another study, Ahemad, (2012) examined the growth and yield of four different plants chickpea, pea, greengram and lentil under the influence of quizalafop-p-ethyl at the recommended dose in pot experiment. They found during their study that, the pesticide had negative effect on symbiosis related parameters, yield parameters and nutrient uptake ability of plants. Khan et al., (2006) also determined the phytotoxicity of herbicides viz. atrazin, isoproturon, metribuzin and sulfosulfuron on greengram in pot experiment and analyzed different parameters like, length of plant, shoot, root; dry weight of plant, seed yield and number of nodules.

Development of resistance against the pesticides is the long term harmful effect of repeated and inadequate use of pesticide which will ultimately increase its usage (Ahemad et al., 2009) and the reduction in plant beneficial rhizobial microbes (Srinivas et al., 2008). Stevens et al., (2008) observed toxic effect of imidacloprid on germination and growth of rice seed. Dubey and Fulekar, (2011) also studied the effect of chlorpyrifos, cypermethrin and fenvalerate on seed germination of two grass species namely *Cenchrus setigerus* and *Pennisetumn pedicellatum.* Sauwa and Yakubu, (2013) conducted a pot experiment to check the effect of three insecticides namely dichlorvos, karate and phoskill on maize by using randomize complete block design and reported that, after eight weeks of planting, the type and rate of insecticides had nonsignificant effect on plant height and dry matter. They sprayed pesticides on plants after one month of planting and checked different parameters like, plant height, fresh and dry weight of shoot which reduced at higher dose of sevin and malathion. Wang et al., (2007) checked the effect of chlorpyrifos on wheat and oilseed rape seedlings in pot study and observed

fresh weight of wheat seedlings. Harmful effect of dimethanoate on *Glycin max* L. and *Vigna unguiculata* L. has been reported by Panduranga et al., (2005) and Mishra et al., (2008) respectively. Bashir et al., (2007) also investigated adverse effects of mancozeb on *Lens culinaris*. Effect of an organophosphorus pesticide malathion and a carbamate, sevin on carrot growth was studied by Gafar et al., (2014). All the these pesticides were applied at recommended dose as well as excessive dose and it was revealed that, pesticides at recommended dose, were beneficial to all the plant properties but higher dose negatively influenced the vegtitative growth and yield of carrot plant.

Such harmful effect of pesticides on plant properties can be eliminated by using bacterial cultures. Bacteria able to degrade the pesticide in synthetic medium and soil can be applied to reduce the toxic effect of pesticide on plant growth and yield. In present study, potent bacterium isolated from soil has been applied to reduce the pesticide effect on plant and to check the difference between response of plant to bacterial inoculation in contaminated soil and uninoculated contaminated soil. Similarly, other scientists have also focused on bioremediation of pesticides in soil and its effect on plant growth and yield. Reduction of phytotoxic effect of glyphosate, metribuzin, fluchloralin and 2,4 D on greengram by *Bradyrhizobium* sp. was determined by Zaidi et al., (2005) in pot study. In this experiment, all the pesticides were applied to the soil before sowing the seeds and the seeds were inoculated with *Bradyrhizobium* sp. Herbicide free but inoculated seeds were taken as control. Plants were uprooted at different stages of growth and were examined for various growth and yield parameters. Similarly, Khan et al., (2006) also isolated *Bradyrhizobium* from nodules of green gram and applied on the seed of green gram before sowing them. All the seeds were sown in pots and effect of 200 and 400 µg/kg atrazin, isoproturon, metribuzin and sulfosulfuron was evaluated on various plant growth and yield properties. Plants were uprooted on 45 and 70 DAS and it was observed that, each pesticide showed variable effect depending on its concentration. In general, all the pesticides did not cause significant effect at lower concentration compared to higher concentration. Ahemad and Khan, (2010a) also isolated *R. leguminosarum* strain MRP1 from nodules of pea plant which could tolerate fipronil and pyriproxyfen and conducted pot study in which, the bacterium inoculated and uninoculated pea plants were grown in sandy clay loam soil and treated with three different concentrations of both the pesticides.

Similarly, the effect of quizalafop-p-ethyl and clodinafop tolerant bacterium *Mesorhizobium* sp. on growth and yield of chickpea plant was also studied by Ahemad and Khan, (2010b). Both the herbicides were applied at different concentrations to *Mesorhizobium* sp. inoculated and uninoculated chickpea and all the concentrations proved to be toxic for uninoculated chickpea plant and reduced various plant properties like nodulation, leghaemoglobin content, nutrient uptake ability of plant, seed yield etc. but these parameters were found to be increased in inoculated plant compared to that in uninoculated herbicides treated plant.

Fox et al., (2007) also determined the effect of pesticides chrysin, methyl parathion, bisphenol A, pentachlorophenol and dichlorodiphenyltrichloroethane on alfalfa seeds inoculated with soil bacterium *S. meloloti* and observed, number of nodules, dry weight of root and shoot, number of nodules and germination of seeds in all plants treated with the pesticides compared to that of control.

2.15 EFFECT OF PESTICIDES ON PHYSICO-CHEMICAL PROPERTIES OF SOIL: Pesticides affect the soil biochemical activities and thus its physico-chemical properties. Pesticides affect the microbial communities associated with the natural biochemical cycles in soil and thus influence the availability of nutrients (Davet, 2004) which are useful for plant nutrition and microbial flora and fauna of soil. Pesticide residues remain in soil affect the macro as well as microelements of soil (Abdelelhamid, 1989) and these elements also work as cofactors of enzymes which are associated with metabolic processes (Deb et al., 2009) thus, pesticides affect the biochemical and enzymatic properties and ultimately nutritional cycles in environment. Every macro as well as micro nutrients have crucial role in ecosystem including nitrogen, phosphorus, potassium, copper, iron, zinc, cobalt, magnesium, manganese, lead etc.

There are some contradictions in reports regarding the pesticidal effect on physico-chemical properties. Some of them suggest stimulatory effect of pesticides on physico-chemical properties of soil (Das et al., 2012; Tu, 1992; Rahmansyah et al., 2009; Ghosh et al., 2014), while others suggest detrimental effect (Abul et al., 2002; Jastrzebska, 2011; Brown and Lean, 1995). The persistence of minerals or cations in soil,

similarly persistence of pesticides in soil and bioavialibility of these pesticide or their residues or microbial degradation depends on soil properties and specially cation exchange capacity of soil. Interaction between soil particles, pesticide and minerals is also very important aspect. Thus it is essential to evaluate the soil macro and micronutrients, effect of particular pesticide on them and the influence of inoculated potent bacteria on fate of harmfulness of pesticide to soil minerals. Influence of various pesticides on different soil minerals have been studied earlier. Sebiomo et al., (2012) studied the effect of recommended dose of four herbicides atrazin, primextra, paraquat and glyphosate on soil minerals like sodium, magnesium, iron and calcium for six weeks and reported that, these herbicides had ability to chelate with soil minerals and thus reduce the chances of nutrient uptake by plants. Giri et al., (2011) investigated the effect of endosulfan, herbicide 2,4-D and dithane M-45 on the microbial and biochemical properties of soil in pot study by adding these agrochemicals at their recommended doses and investigated that, endosulfan caused deleterious effect on all the properties while, dithane M-45 showed stimulatory effect in earlier stages but later on detrimental effect was observed on the contrary, herbicide 2,4-D influenced all the parameters positively. Gafar et al., (2014) worked on the effect of an organophsphate pesticide malathion and a carbamate, sevin on chemical properties of soil at recommended and higher dosage and stated that, calcium and magnesium level decreased at both dosages of pesticides due to the ionic effect of both the pesticides which affect the uptake of nutrients and their distribution in soil and plant roots.

Nitrogen is the most essential element of soil and its availability is required for plant growth and metabolism. Changes in nitrogen concentration or any effect on nitrogen assimilatory enzymes directly affect the crop production (Parween et al., 2011). Application of an organophsphate pesticide malathion and a carbamate, sevin at recommended and higher dosage reduces the total nitrogen and phosphorus contents of soil at both dosages of pesticides due to the ionic effect of both the pesticides which affect the uptake of nutrients and their distribution in soil and plant roots (Gafar et al., 2014). Sauwa and Yakubu, (2013) investigated effect of dichlorvos, karate and phoskill on soil properties at different rates under pot experiment and concluded that, when mean values of total nitrogen were compared to that of control, these chemical elements were

found to be decreased at higher rate. Significant decrease in nitrogen of loamy sand and sandy soils due to application of atrazin, pyrethrin, and a mixture of metobromuron and metachlor is also reported by Taiwo and Oso, (1997). Significant reduction in potassium content of soils is also reported by these workers in the same experiment. Likewise, information regarding influence of pesticides on other soil minerals is also very important to understand the fate of pesticides in soil. Manganese is useful for chlorophyll synthesis (Deb et al., 2009), nitrogen metabolism (Brady and Well, 2002) and also wok as integral part of respiratory enzymes. Copper is needed in nitrogen utilization by plant in form of ammonium (Marschner, 1995) and also involved in some enzyme dependent redox reactions (Deb et al., 2009). Zinc is essential for auxin synthesis, carbon metabolism and also plays role in water uptake by plant (Coyne, 2001). Paul et al., (2013) conducted a pot experiment to study the effect of 2,4 D, endosulfan and dithane M-45 on cationic micronutrients copper, zinc and manganese. During this study, they found variation in effect of pesticides and observed that, endosulfan has negative effect while, herbicide 2,4-D showed stimulatory effect on micronutrients.

2.16 EFFECT OF PESTICIDE ON ENZYMATIC ACTIVITIES OF SOIL:

Microorganisms present in soil perform their role in degradation of plant and animal residues and contribute to nutrient cycling in environment through their enzymes. Enzyme profile of soil is an index of fertility status of soil according to Hofmann and Seegerer, (1950). Pesticides affect these micro flora and fauna of soil and thus their enzymatic activities. According to Tabatabai, (1994), some pesticide can interact with the soil enzymes by binding to their active sites responsible for catalysis; whereas, some pesticides can change the balance of soil microbial communities and ultimately, the enzyme synthesis through induction or repression of their biosynthesis (Cycon et al., 2006; Tejada, 2009; Zabaloy et al., 2012 and Chishti et al., 2013). There are many reports which suggest detrimental effect of pesticides on soil enzymes (Yu et al., 2006; Jastrzebska and Kucharski, 2007; Kalam et al., 2004; Chen et al., 2001; Menon et al., 2005; Caceres et al., 2009). All the biochemical transformations occur in soil are enzyme dependent (Riah et al., 2014; Defo et al., 2011). Thus, soil enzymatic activities act as indicator of soil fertility (Antonious, 2003), disruption in biological or biochemical

imbalance due to contamination (Bending et al., 2004; Chu et al., 2003) or interference in metabolic activities involved in biogeochemical cycles (Liu et al., 2008; Hussain et al., 2009; Defo et al., 2011).

There are several important soil enzymes which play crucial role in soil fertility, plant growth and ecological cycles. Thus, the effect of pesticides on such enzymes should be studied very well. Urease is an important soil enzyme which covert nitrogen from organic to inorganic form (Nasreen et al., 2012) by hydrolyzing urea to carbon dioxide and ammonia and play an important role in nitrogen cycle (Riah et al., 2014). Effect of various pesticides on urease enzyme has been studied by many researchers. Xia et al., (2011) studied the effect of five pesticides viz. zineb, copforce, carbendazim, mancozeb and hymexazol on soil enzymatic activities and found that , urease activity was suppressed by each of these pesticides. Effect of an organophosphorus insecticide, fenamiphos on urease activity of two different soils was studied by Caceres et al., (2009) at different concentrations and did not observe any significant detrimental effect on urease activity. Toxic effect of chlorpyrifos on soil urease activity during 90 days experiment was also noticed by Tejada, (2009). All the reports regarding the effect of pesticides on soil enzymes, shows variation in results thus it is necessary to check the effect of monocrotophos on soil urease. Nasreen et al., (2012) studied the effect of an organophosphate pesticide, profenophos and an organochlorine pesticide, endosulfan on urease activity in black clay soil at both the recommended dose and higher than recommended dose. They did not show any significant influence of pesticides on urrease activity. Adverse effect of two and three continuous applications of buprofezin and acephate on urease activity of soil at different concentrations, were determined by Raju and Venkateswarlu, (2013).

Phosphatases are also important group of enzymes (Dick, 1994; Jordan et al., 1995) which play critical role in phosphorus cycle by mineralizing the organic phosphorus substrates (Schneider et al., 2001; Appiah and Thompson, 1974). Inhibitory effect of Monocrotophos and quinalphos on soil phosphatase at 7.5kg/ha concentration was noticed by (Rangaswamy and Venkateswarlu, 1996) in four different experimental soils. Many researchers have been studied the effect of different pesticides like mefenoxam (Monkiedje et al., 2002), pentachlorophenol (Cristina et al., 2006),

acetamiprid (Punitha et al., 2012), endosulfan (Defo et al., 2011), and quinalphos (Mayanglambam et al., 2005) on soil phosphatases. Defo et al., (2011) reported effect of endosulfan at recommended dose on enzyme activities of two different soils namely mandong soil which was a medium loam soil and Minkoa-Meyos which was clay loam soil under microcosm study. This study showed inhibitory effect of pesticide on acid and alkaline phosphatase activities. Ghosh et al., (2014) studied the influence of endosulfan, 2,4 dichlorophenoxy acetic acid (2,4-D) and dithane-M-45 on acid and alkaline phosphatase activities of soil under pot experiment and observed positive influence of all the pesticides on phosphatase activities at recommended doses. Cycon et al., (2010) evaluated the effect of two fungicides mancozeb and dimethomorph. Both the fungicides were applied at different concentrations of 15, 75 and 1500 mg/kg in loamy sand and sandy loam soils and observed that, the fungicides negatively affected acid and alkaline phosphatases. Chen et al., (2001) reported the inhibitory effect of two fungicides captan and benomyl on phosphatase activity in soils.

2.17 EFFECT OF PESTICIDE DEGRADATION ON MICROBIAL PARAMETERS OF SOIL:

Soil is a dynamic ecosystem having large diversity of microflora and fauna (Defo et al., 2011). Soil microbes play key role in maintaining nutritional cycles like carbon, phosphorus and of other important elements. Soil bacteria are found in range of 10^9 to 10^{15} in one gram of soil (Alexander, 1977) because of their diverse metabolism. Pesticide may cause suppression or promotion of soil microbial growth (Boldt and Jacobson, 1998; Haney et al., 2000) which depends on many factors. May be the pesticide application can not cause significant effect on soil microbial number or their heterogenicity but can alter the active soil bacterial community structure and may cause imbalance (Martina et al., 2004). Balance between different soil microbial communities like, autotrophs, heterotrophs, N_2-fixers, cellulose degraders, thermophiles, psychrophiles etc., can be disturbed by pesticide application. Adverse effect of pesticide application on soil microbial population whether it is foliar or direct has been reported by many researchers (Singh and Singh, 2005; Omar and Abdel-Sater, 2001; Sarnaik et al., 2006) which are

responsible for various biochemical cycles (Paul et al., 2013) and soil fertility (Wichern et al., 2006; Kalia and Gosal, 2011).

Inhibitory effect of pesticides on soil microflora has been reported by many researchers (Ingram et al., 2005; Littlefield-Wyer et al., 2008; Zhou et al., 2006 and Pampulha and Oliveira, 2006; Araujo et al., 1999). Certain organophosphates like monocrotophos, quinalphos and fenamiphos, phosphamidon were toxic to algae and cyanobacteria (Ramakrishnan *et al*., 2010). Monocrotophos (Rajendran *et al*., 2007) and fenitrothion (Lal, 1987) inhibited biological processes - photosynthesis and nitrogen fixation in *Anabena* sp., *Aulosira fertilissuma* and *Tolypothrix scytonemoides* at low concentration - 10 µg /mL in media. Effect of chlorpyrifos on soil microbial population was studied by Tejada et al., (2009) and total bacteria as well as fungal population were observed to be decreased significantly. Some microbes use pesticides as nutrient source while others cannot tolerate them thus, sometimes, microbial diversity of soil may get reduced but some microbial community may flourish (Wang et al., 2006) viz. urea hydrolyzers, nitrifiers, phosphate solubilizers etc. There are number of ways to evaluate microbial population of soil. Effect of pesticides on microflora can be studied by various ways like observing soil microbial biomass, enumeration of microbial populations, estimation of functional microbial enzymes (Topp et al., 1997), soil respiration, by estimation of specific respiration of biomass, qCO_2, ratio of basal soil respiration to substrate induced respiration (Qr), FDA hydrolyzing activity (Pal et al., 2006; Chowdhury et al. 2008) etc. Effect of cypermethrin on microbial biomass and its activity in an alluvial soil at field recommended rate (FR) and 10 times higher than field recommended rate (10 FR) was investigated by Goswamy et al., (2012). They observed that, the soil microbial biomass carbon decreased gradually at FR and 10 FR dose of cypermethrin compared to the control. While the basal soil respiration and substrate induced soil respiration follow the same pattern and declined for FR and 10FR dose. They estimated the total microbial activity by Fluorescein Diacetate Hydrolyzing Activity (FDHA) and found that, the FDHA was significantly reduced upto at FR and 10 FR dose of cypermethrin applied to the soil.

Sarnaik et al., (2006) studied the effect of application of various pesticides to soyabean on microbial population of soil by applying the pesticides in three ways i.e.

seed application, soil application and foliar spray. Carbosulphan 25DS, thiamethoxam 70WS, imidacloprid 70WS, chlorpyrifos 20EC were used for seed application. Phorate 10G and carbofuran 3G were used for soil application while chlorpyrifos 20EC, thiamethoxam 25WG and imidacloprid 200EC were used for foliar spray application and observed increment in rhizobial count with application of carbofuran 3G, thiamethoxam 70WS and chlorpyrifos 20E. while, application of Phorate 10G, carbosulphan 25DS, thiamethoxam 25WG and imidacloprid 200EC caused reduction in rhizobial count.

Xiaoqiang et al., (2008) did a laboratory experiment to evaluate the inhibitory effect of chlorpyrifos alone and together with chlorothalonil at different concentrations on bacterial, fungal and actinomycetes population of soil and it was observed that, combination of both the pesticides reduced bacterial, fungal and actinomycetes population significantly and hiher concentrations of chlorpyrifos was also detrimental to microbial community of soil. Taiwo and Oso, (1997) treated loamy sand and sandy soil with atrazin, pyrethrin and a mixture of metobromurona and metolachor for eight weeks and observed its effect on bacterial, fungal and actinomycetes count and the results revealed that, the microbial count was reduced significantly in presence of all the pesticides. Dubey and Fulekar, (2011) studied the effect of cypermethrin, chlorpyrifos and fenvalerate on microbial biomass of rhizosphere zone and bulk soil using two grass species *Canchrus setigerus* and *Pennisetum pedicellatum* and reported inhibitory effect of pesticides on microbial population of soil. Gilani et al., (2010) did a soil incubation study to see the influence of chlorpyrifos at 100 and 1000 ppm concentrations on soil microbial parameters. They observed that, when the soil was fortified with 100 ppm chlorpyrifos, no significant effect was seen but at 1000 ppm, growth of some microbial population was found to be suppressed.

Considering the influence of pesticides on fungal population of soil, Paul et al., (2013) determined the effect of 2,4-D, endosulfan and dithane M-45 on fungal population of soil. On the contrary, Ghosh et al., (2014) estimated both the microbial biomass carbon and phosphate solibilizing bacterial count of soil to check the effect of endosulfan, dithane M-45 and 2,4-D and reported stimulation in these microbial parameters under the influence of all the pesticides. Hindumathy and Gayathri, (2013) studied the effect of

chlorpyrifos on bacterial population of rhizospheric and non-rhizospheric soil microflora of two plants marigold and canna. They found that, in chlorpyrifos contaminated soil, bacterial population was reduced while fungal population was increased incase of nonrhizospheric soil. Whereas, in rhizosperic soil of canna, detrimental effect of chlorpyrifos on bacterial population was observed but bacterial population of rhizosphereic soil of marigold did not affected by pesticides. Cycon et al., (2010) also observed in plate count study that, after application of fungisides, mancozeb and dimethomorph in sandy loam and loamy sand soils, total number of culturable bacteria was increased in both the soils but, nitrogen fixing bacteria were reduced in loamy sand and sandy loam soils at higher concentrations. As per the views of Johnsen et al., (2001), recommended dose of pesticide also cause slight effect on microbial population and their activities within soil, so continuous and long term application of pesticides may alter the biochemical balance of soil and ultimately the local metabolism.

2.18 ROLE OF ENZYMES IN PESTICIDE DEGRADATION:

Pesticide degradation through enzymes is an innovative and recent technique for removal of pesticides from contaminated environment (Verma et al., 2014). Usage of purified enzyme extracted from bacteria for the pesticide detoxification has many advantages like, bioremediation is not dependent on bacterial growth thus, the degradation process could be quicker, detoxification process relies on the catalytic properties and concentration of the enzymes (Scott et al., 2008). Metabolism of pesticides may occur in three phases according to Ortiz-Hernandez et al., (2013). In first phase, parent compound get transformed to less toxic and water soluble compound by hydrolysis or oxidation-reduction reactions. In second phase, pesticide metabolite get associated with sugar or amino acids and transformed to less toxic and more water soluble compound while in third phase, metabolites of phase two are converted to non-toxic compounds. Microbial enzyme are needed to perform every step of this pesticide metabolism.

For pesticides degradation, there are mainly enzyme systems involved: hydrolases, esterases (also hydrolases), the mixed function oxidases (MFO), these enzyme function in the first metabolism stage, and the glutathione S-transferases (GST) act in the second phase (Li et al., 2007). Several enzymes catalyze metabolic reactions

including hydrolysis, oxidation, addition of an oxygen to a double bound, oxidation of an amino group (NH_2) to a nitro group, addition of a hydroxyl group to a benzene ring, dehalogenation, reduction of a nitro group (NO_2) to an amino group, replacement of a sulfur with an oxygen, metabolism of side chains and ring cleavage. Enzymatic degradation of organophosphorus pesticides was found to be faster than chemical hydrolysis according to Munnecke, (1976) and the enzymes involved in this could be phosphatases, estearses and hydrolases (Kanekar et al., 2004).

Esterase enzymes catalyze the hydrolysis of carboxylic esters amides and phosphate esters (Bansal, 2002) and produce alcohol and acids as given below:

$$R = O - OCH_3 + H_2O \longleftrightarrow R = O - OH + CH_3OH$$

The most important step of bacterial degradation of an organophosphorus pesticide is hydrolysis of P-O alkyl and aryl bonds and esterase is one of the required enzyme for that (Kumar et al., 1996; Bhadbhade et al., 2002; Srinivas et al., 2004). Apart from that, methyl amine is produced as metabolite by monocrotophos degradation which may be due to esterase enzyme. Thus the ability of potent bacterium to produce esterase enzyme has to be checked. Production, characterization and purification of esterase by *Bacillus* sp. is well documented (Eggert et al., 2000; Kaiser et al., 2006; Maqbool et al., 2002).

Like other enzymes, estearses are also classified as extracellular, intracellular and membrane-bound based on location of its production. *Bacillus* sp. DVL2 was found to produce all the extracellular, intracellular as well as membrane-bound esterase enzyme according to Kumar et al., (2012). Bhadbhade et al., (2002) studied the role of esterase enzyme produced by *Arthrobacter atrocyaneus* MCM B-425 and *Bacillus megaterium* MCM B-423 in biomineralization of monocrotophos. Role of esterase enzyme in degradation of other organophosphorus pesticides have been also reported. Organophosphorus pesticide degradation by monoesterase enzyme was studied by Thengodkar and Sivakami, (2010). Carbofuran degradation by esterase producing strains of *Pseudomonas* and *Alcaligens* and methomyl degaradtion by *Flavobacterium* and *Alcaligens* srains was observed by Omolo et al., (2012). Zeinat et al., (2008) worked on malathion degradation by *Bacillus thuringiensis* Mos-5. Metabolites produced by

degradation of malathion were malathion mono-acid and di-acid which suggested the role of esterase enzyme in degradation. Production of esterase enzyme by monocrotophos degrading strains of *Arthrobacter atrocyaneus* MCM B-425 and *Bacillus megaterium* MCM B-423 was confirmed on the basis of zone of clearance produced by both bacteria on monocrotophos agar medium containing 1% (v/v) tributirin (Bhadbhade et al., 2002). Similarly, Faiz et al., (2007) isolated esterase producing, thermophilic bacterium *Anoxybacillus gonensis* A4 which was able to give a zone of clearance on tributyrene agar plate. While, other researchers observed esterase production by using different p-nitrophenyl esters as substrate, Kim et al., (2013) used p -nitrophenyl butyrate as substrate to observe the esterase producing ability of *Lactobacillus brevis* NJ13. Soliman et al., (2007) performed cloning of esterase gene of *Geobacillus thermoleovorans* YN and expressed it in *E. coli*. using p-nitrophenyl laurate as substrate.

Organophosphate pesticide consists of phosphate-chrotanamide linkage which can be break down by phosphatase enzyme (Rosenberg and Alexander, 1979). Organohospates are phosphate esters which could be hydrolyzed by phosphatase. Brajesh et al., (2006) worked on microbial degradation of organophosphorus pesticides. They described that, degradation of these pesticides reduce mammalian toxicity by several orders of magnitudes and its degradation occurs through hydrolysis of P-O alkyl and P-O aryl bonds which is the most important step in detoxification and the final enzyme in degradation pathway is alkaline phosphatase. It was also suggested by Bookstein et al., (1990) that, *Bacillus* sp. produce alkaline phosphatase enzyme during the time when phosphate acts as growth limiting factor or at the time of sporulation. Location of alkaline phosphatase production in bacterial cell may differ in different bacterial genus or species. Based on its location, the enzyme cloud be intracellular, extracellular or membrane bounded. Bacterial intracellular as well as extracellular alkaline phosphatase production by *Bacillus licheniformis* (Hulett et al., 1991), *Bacillus subtilis* (Yamane and Maruo, 1978) and *Bacillus cereus* (Kostadinova and Marhova, 2010) have been reported earlier. Degradation of organophosphorus pesticides by bacterial alkaline phosphatase is well documented in number of studies carried out by Theriot and Grunden, (2010); Gilbert et al., (1991) and Kim et al., (2005).

During degradation of Monocrotophos by *Bacillus megaterium* and *Arthrobacter atrocyaneous* Bhadbhade et al., (2002) observed release of phosphates in the medium broth which didn't consist phosphates initially which indicated production of phosphatase enzyme by bacteria and both the bacteria were found to produce 71 and 46 U/mL phosphatase respectively. Omolo et al., (2012) isolated carbofuran degrading strains of *Pseudomonas* and *Alkaligens* and methomyl degrading strains of *Flavobacterium* and *Alkaligens*. Estimation of phosphatase enzyme and its role in pesticide degradation was studied by using p-nitrophenolate as standard. Production of alkaline phosphatase by all the bacteria was observed when grown in low phosphates containing medium. Likewise, Zhang et al., (2014) reported the correlation between degradation of five organophosphorus pesticides chlorpyrifos, diazinon, fenitrothion, malathion and methyl parathion and phosphatase production in skimmed milk by lactic acid bacteria. The pesticide containing milk was inoculated with bacteria and estimated for phosphatase production and the results revealed that, *Lactobacillus brevis* showed phosphatase activity. It was found by the Spearman's correlation coefficient analysis that, there was a positive correlation between the degradation rate of pesticides and phosphatase production which suggested the role of phosphatase in organophosphorus pesticide degradation. Harishankar et al., (2013) isolated three intestinal bacteria *Lactobacillus fermentum, Lactobacillus plantarum* and *Escherichia coli* which were able to degrade an organophosphorus pesticide, chlorpyrifos and they found that, all the three bacteria showed intracellular and extracellular phosphatase activity using p-nitrophenolphosphate as substrate.

2.18.1 Process optimization:

Process optimizations help to improve the productivity and also help to understand the effects of various media components and other physicochemical parameters on fermentation. Process optimizations which includes the media composition, culture conditions like temperature, pH, oxygen tension, aeration, agitation and type of cultivation etc plays a major role in enhancing enzyme yield. The need to achieve higher titers of enzymes, faster production rates and improved control over culture conditions among several other requirements for large scale production makes this process inevitable in industries. In the conventional method for the optimization of

enzyme production, the “one variable at a time” approach is used, which involves changing one parameter at a time while keeping the other entire parameters constant (Greasham and Inamine, 1986; Duan *et al.*, 1994). But this process is cost, labor and time intensive, and also does not consider the interaction between different variables. An alternative and more efficient approach is the use of statistical methods. Several statistical methods ranging from two factorial to multi factorial designs are available (Berenson *et al.*, 2011). Plackett & Burman design (Plackett and Burman, 1946) and Response Surface Methodologies (Myers *et al.*, 2009) are employed for identifying the important parameters in the fermentation process and optimizing these for obtaining maximum enzyme production. Optimization of cultural parameters to enhance the enzyme production by bacteria is essential. Important parameters like temperature, pH, incubation time, inoculum size and age, additional carbon source, nitrogen source etc. are optimized to improve the ability of bacteria to produce the enzyme. Optimization of parameters can be done in wet laboratory experiments as well as statistical methods. Traditional method to optimize the parameters is also called OFAT (One Factor At a Time) in which only one factor or parameter is optimized keeping other parameters constant at a time. Nowadays, many researchers use advance statistical techniques to optimize the significant factors (Abdet-Fattah, 2012; Pandey and Banik, 2010; Ren et al, 2006; Patel and Sharma, 2014). Response Surface Methodology (RSM) has advantage over conventional methods available that, it is suitable for multiple factor experiments and searches the common relationship between various factors and find out the most suitable condition for maximum enzyme production (Kaur and Satyanarayana, 2005). Conventional optimization experiments are not only tedious but, also can lead to misinterpretation of results, especially because in conventional method, interaction between different factors is overlooked. Response Surface Methodology (RSM) is widely used statistical technique for multiple regression analysis by using experimental data obtained from experiments designed using Central Composite Design (CCD). In present study, parameters including pH, temperature, incubation time, inoculum size and age, carbon source and nitrogen source were optimized by conventional techniques in wet lab experiments.

Optimum pH for esterase production was determined to enhance the esterase production by many researchers using various bacterial strains including *Lactobacillus brevis* NJ13 (Kim et al., 2013); *Anoxybacillus gonensis* A4 (Faiz et al., 2007); *Salimicrobium* sp. LY19 (Xin and Hui-Ying, 2013); *Streptomyces coelicolor* A3(2) (Brault et al., 2012) and *Bacillus subtillis* RRL 1789 (Kaiser et al., 2006). Likewise, temperature provided during incubation was also optimized to achieve maximum esterase production from *Lactobacillus brevis* NJ13 (Kim et al., 2013), *Anoxybacillus gonensis* A4 (Faiz et al., 2007), *Hallobacillus* sp. strain LY5 (Li et al., 2012), *Salimicrobium* sp. LY19 (Xin and Hui-Ying, 2013) and *Bacillus subtillis* RRL 1789 (Kaiser et al., 2006). Similarly, cultural conditions for maximum phosphatase production were also optimized for better degradation. Enzyme produced by different bacterial cultures may have different optimum conditions thus many researchers have done optimization study. Alkaline phosphatase production by *Bacillus flexus* FPB 17 (Patel and Sharma, 2012); *Bacillus licheniformis* MTCC1483 (Pandey and Banik, 2012); and *Bacillus* sp. (Omran and Quaddoori, 2015) have been optimized in earlier studies.

2.18.2 Purification and characterization of enzyme:

Purification of crude enzyme extracted from bacterial culture medium is most important aspect for application of enzyme which enhances the specific activity of enzyme. For purification of esterase enzyme, different procedures have been applied by different researchers. Faiz et al., (2007) partially purified esterase enzyme produced by thermophilic bacterium *Anoxybacillus gonensis* A4 using ammonium sulfate precipitation method at 30-60% saturation. Apart from ammonium sulfate precipitation at 60% saturation, Xin and Hui-Ying, (2013) used DEAE-Sepharose ion exchange chromatography and Sephacryl S-100 gel filtration chromatography to purify the esterase enzyme produced by *Salimicrobium* sp. LY19. Similarly, Li et al., (2012) also performed purification of esterase enzyme produced by *Halobacillus* sp. strain LY5 stepwise by 80% ammonium sulfate precipitation, DEAE- cellulose ion exchange and sephacryl S-100 gel filtration chromatography. Kaiser et al., (2006) reported purification of esterase enzyme of *Bacillus subtillis* RRL 1789 by 70-95% ammonium sulfate precipitation

having three fold increment in specific activity which further purified by FPLC using phenyl sepharose column and ultra filtration. Soliman et al., (2007) performed cloning of esterase gene of *Geobacillus thermoleovorans* YN and expressed it in *E. coli* and purified it. For purification, they initially used one-step IMAC (Immobilized Metal Affinity Chromatography) purification procedure and obtained homogenous protein which was further purified by gel filtration.

Likewise, there are various methods to purify the crude phosphatase. Kostadinova and Marhova, (2015) purified the enzyme by precipitation with 2-propanol, APSE (2-(4- aminophenylsulphonyl)-ethyl) cellulose cheomatography and sephadex G-200 gel filtration. Asencio, (2012) partially purified the enzyme produced by *Arthrospira platensis* using triton X-114. Purification of alkaline phosphatase produced from *Streptomyces* sp. JS-20 was done by Kumar et al., (2012) using ammonium sulfate precipitation method at 20-50% saturation followed by dialysis of precipitates and then applied to Sephadex G-75 column. The protein content in the fractions was measured by UV absorbance at 280 nm. After purification molecular weight estimation of the purified enzyme is also an essential step. The molecular weight of extracellular and membrane-bound phosphatase was determined by SDS-PAGE analysis. The SDS-PAGE electrophoresis was performed to know the molecular weight of partially purified enzyme. Characterization and partial purification of alkaline phosphatase was done by Mahesh et al., (2010) using *Bacillus spp.* They partially purified the crude enzyme by DEAE cellulose ion exchange choromatography and also performed SDS-PAGE electrophoresis to determine the molecular weight of enzyme. Whereas, alkaline phosphatase produced by *Geobacillus caldoxylosilyticus* TK4 was purified using nickel affinity chromatography (Col et al., 2010).

After purification of both the enzyme, molecular weight of enzymes was determined by SDS- PAGE electrophoresis. Molecular weight of esterase enzymes produced by *Streptomyces coelicolor* A3(2) (Brault et al., 2012); *Geobacillus thermoleovorans* YN (Soliman et al., 2007); *Halobacillus* sp. strain LY5 (Li et al., 2012); *Bacillus subtillis* RRL 1789 (Kaiser et al., 2006); *Anoxybacillus gonensis* A4 (Faiz et al., 2007); *Salimicrobium* sp. LY19 (Xin and Hui-Ying, 2013) was determined using SDS- PAGE electrophoresis and found that each enzyme was having different

molecular weight ranging from 43 to 96 kDa depending upto its source of production. Molecular weight of phosphatase enzymes produced from *Pyrococcus abyssi*, *Streptomyces* sp. Js-20, *Bacillus spp*., *Geobacillus Caldoxylosilyticus* TK4 and *Bacillus cereus* were also determined by Zappa et al., (2001); Kumar et al., (2012) Mahesh et al., (2010); Col et al, (2010); Kostadinova and Marhova, (2015) respectively. molecular weight of phosphatases from each bacteria were different ranging from 42 to 84 kDa.

In enzymatic study, it is very important to determine the effect of inhibitors on enzyme because it helps to evaluate the specificity of an enzyme, the physical as well as chemical structure of enzyme active site and the kinetics of enzymeatic reaction. Inhibitors are chemicals those substances which decrease the velocity of enzyme-substrate reaction. During characterization study of esterase produced from *Lactobacillus brevis* NJ13, Kim et al., (2013) studied the effect of different organic solvents viz. DMFA (Dimethylformamide), DMSO (Dimethyl sulfoxide), acetone, acetonitrile, dichloromethane, tert butanol, isopropanol, ethanol and methanol as well as detergents (Tween 80, Tween 60, triton X 100 and SDS) and metal ions viz. Ca, Mg, Mn, and EDTA (Ethylene Diamine Tetra Aceticacid) on esterase production. Experiment regarding the effect of various metal ions, organic solvents like glycerol, DMSO, benzene, acetonitrile, ethanol, acetone, n-hexane and chemical reagents on esterase enzyme produced from *Salimicrobium* sp. LY19 was also performed by Xin and Hui-Ying, (2013). Likewise, influence of various organic solvents on esterase enzyme of *Streptomyces coelicolor* A3(2) was observed by Brault et al., (2012). Brod et al., (2010) constructed a recombinant esterase from *Lactobacillus plantarum* and checked the effect of metal chelators EDTA, EGTA (Ethylene Glycol Tetraacetic Acid) and PMSF (Phenylmethanesulfonylfluride); metal ions, chemical reagents like triton X 100 (0.1% v/v) and DMSO (15%) and SDS (Sodium dodecyl sulfate) on esterase activity. Soliman et al., (2007) performed cloning of esterase gene of *Geobacillus thermoleovorans* YN and expressed it in *E. coli* and investigated the influence of solvent and metal ions on it. Faiz et al., (2007) isolated esterase producing, thermophilic bacterium *Anoxybacillus gonensis* A4 and studied the effect of divalent and trivalent ions using chloride salts of different metals having 1mM final concentration as well as influence of organic solvents on enzyme activity. Li et al., (2012) performed purification and characterization of esterase

enzyme produced by *Halobacillus* sp. strain LY5. During characterization study, effect of temperature, pH was studied and observed that, the enzyme was highly active under broad range of pH and temperature but the maximum activity was obtained at 50^0C and 10 pH. Brod et al., (2010) constructed a recombinant esterase from *Lactobacillus plantarum* and expressed it in *E.coli* and further the esterase enzyme produced was characterized for favorable temperature and pH. Soliman et al., (2007) performed cloning of esterase gene of *Geobacillus thermoleovorans* YN and expressed it in *E. coli* and the esterase enzyme was characterized further for optimum temperature and pH.

Characterization study of purified or partially purified phosphatase enzyme was also performed by many researchers. Thengodkar and Sivakami, (2010) characterized alkaline phosphatase produced from cyanobacterium *Spirulina platensis* and effect of pH, incubation temperature and enzyme volume was examined during the study. Alkaline phosphatase produced from Bacillus sp. was characterized by Mahesh et al., (2010). During this study, effect of pH, temperature, reaction time, substrate concentration was evaluated. Apart from that, influence of various enzyme activators and inhibitors was also studied. Alkaline phosphatases are classified as metaloenzymes (Trowsdale et al., 1990; Hulett et al., 1991; Wojciechowski et al., 2002; Mori et al., 1999). Effect of metal ions on phosphatase was studied by many researchers. (Kostadinova and Marhova, 2015; Asencio, 2012; Mahesh et al., 2010; Col et al., 2010). Effect of detergents, organic solvents and other chemical agents have also observed by Kostadinova and Marhova, (2010); Asencio, (2012); Mahesh et al., (2010); Thengodkar and Sivakami, (2010) and Col et al., (2010).

2.19 GENETIC BASIS OF PESTICIDE DEGRADATION:

The microbial degradation of pesticide involves various types of enzyme. The enzymes responsible for organophosphorus compounds are belong to organophosphous hydrolases which could be encoded by *opd, mpd, opdA* etc (Ali et al., 2012). Such genes have been identified from the organisms which are having ability to utilize pesticides as nutrient source specifically carbon and energy source as shown in **Table 2.3**. Pesticide degrading genes found to be located on chromosomes, plasmids and transoposons

(Kanekar et al., 2004). All these enzymes can act upon broad range of substrates having P-O, P-CN, P-F and P-S bonds (Cheng and DeFrank, 2000; Lai et al., 1995). These microbial genes might be differing in their location depending upto the organism. *Pseudomonas diminuta* strain MG, this enzyme encoded by a gene called opd (organophosphate-degrading) and shows a highly catalytic activity towards organophosphate pesticides. The organophosphorus degrading gene *opd* has been first identified from *Flavobacterium s*p. ATCC27551 (Mulbry and Karns, 1989 and Kawahara et al., 2010). Later on other *opd* homologus genes have been identified from different bacteria like, *opdA* which was identified from *Agrobacterium radiobacter* P230 having 88% similarity with the nucleotide sequence of *opd* genes identified from *Brevundiomonas diminuta* GM and *Flavobacterium* sp. ATCC27551(Horne et al., 2002a). Another gene *mpd* was identified from *Pseudomonas* WBC-3, *Stenotrophomonas* sp. YC-1 and *Plesiomonas* M6 which was responsible for methylparathion hydrolase production and was homologus to *opd* gene (Zongli et al., 2001; Yang et al., 2005 and Yang et al., 2006). Study was also carried out to identify the monocrotophos degrading genes and it was found that *Pseudomonas mendocina* MCM B-424 was having plasmids harbouring monocrotophos degrading genes (Kanekar et al., 2004).

Table 2.3 Genes responsible from organophosphorus degrading bacteria

Gene	**Bacteria**
Opd	*Pseudomonas diminuta*
opaA	*Alteromonas spp.*
opdA	*A. Radiobacter*
ophB	*Burkholderia* sp. JBA3.
OpdB	*Lactobacillus brevis*
Oph	*Arthrobacter* sp
opdE	*Enterobacter* sp.