CHAPTER II

REVIEW OF LITERATURE

Pesticides are constituents which are used to kill pests (weeds, insects, molds, rodents, etc.). There are many types of pesticides among which insecticides and rodenticides are acutely dangerous to human health. Low cost, increased productivity, simplicity in application and widespread availability has made these pesticides popular among farmers. An unavoidable part of modern agricultural practices are the pesticides. Green Revolution is the period since when the consumption of pesticides in India has elevated to several hundred times, from 154 MT in 1954 to 45,390 MT in 2013-2014 (http://www.krishijagran.com). The usage of agricultural pesticides is markedly less. In India around 0.6 kg pesticide/ha has been used against 13 kg/ha and 7 kg/ha in China and USA, respectively (OUTLOOK, 2014). In future it has high growth potential in India, according to industrial estimates (CSE, 2005). Among the 29 states of India, the maximum pesticide consumption is recorded in the states of Uttar Pradesh (9563 MT) followed by Punjab (5810 MT), Maharashtra (4639 MT), Haryana (4070 MT) and Rajasthan (3527 MT). Pesticides, highly used in India include monocrotophos (10700 MT- highest consumed), acephate (6400MT), endosulfan (5600 MT) and chlorpyrifos (5000 MT - fourth largest used) (INDIASTAT, 2015).

2.1 Classification of Pesticide

There are number of ways to classify the pesticides which provide an idea on the chemical nature of the pesticides, their mode of entry and the target organism (Zacharia, 2011). Mainly, they can be classified based on categories as depicted in Figure 2.1. The classification on the basis of target organism specifies the name of target organism on the pesticides such as for the killing of insects, insecticides are used, for weeds, weedicides are used, etc. It makes easy identification of the pesticide to be used for the particular target organism. On the basis of mode of entry, the classification is based on the means by which the insecticide enters into the target
pests. Chemical nature of the active ingredients is the basis of chemical classification of pesticides.

\[ a \]
\[ \text{Classification according to chemical nature} \]
\[ \text{Organochlorines} \]
\[ \text{Examples- DDT, Endosulfan, etc.} \]
\[ \text{Organophosphates} \]
\[ \text{Examples- Monocrotophos, Chlorpyrifos, etc.} \]
\[ \text{Carbamates} \]
\[ \text{Examples- aldicarb, Carbofuran etc.} \]
\[ \text{Pyrethroids} \]
\[ \text{Examples- Allethrin, Resmethrin, etc.} \]

\[ b \]
\[ \text{Classification on the basis of target organism} \]
\[ \text{Insecticides} \]
\[ \text{Weedicides} \]
\[ \text{Molluscicides} \]
\[ \text{Rodenticide} \]
\[ \text{Acaricides and Miticides} \]
\[ \text{Bactericides} \]
\[ \text{Fungicides} \]
\[ \text{Algicides} \]
\[ \text{Virucides} \]

\[ c \]
\[ \text{Classification on the basis of mode of entry} \]
\[ \text{Stomach Poison} \]
\[ \text{Contact Poison} \]
\[ \text{Fumigant} \]
\[ \text{Systemic Poison} \]

**Figure 2.1 Classification of pesticides**

a) Chemical nature  
b) Target organism  
c) Mode of entry
In the field of pesticides and environment, the most useful classification is the chemical classification of pesticides for researchers and for those who search for details. This method of classification provides an idea of its efficacy, chemical and physical properties. This information is very important for its mode of application and various precautionary measures required to be taken throughout its application (Buchel, 1983).

On the basis of chemical nature, pesticides can be classified as organochlorines, organophosphates, carbamates and pyrethroids. Organochlorine pesticides are the oldest discovered synthetic organic pesticides. They are placed in a larger class of compounds called chlorinated hydrocarbons as they have diverse chemical structure with chlorine molecules present in all the compounds. They contain chlorine in their structure but the arrangement differs. Figure 2.2 depicts the chemical structure of some of the commonly used organochlorines. These chlorinated hydrocarbons were used extensively during Second World War (Gupta, 2012). Till 1960s, the organochlorine pesticides have been used in agriculture and mosquito control after which their usage has been banned. Characteristic features of organochlorine pesticide include long residual effect and broad-specificity. It is responsible for opening of sodium channels in the insect's nerve cells (Vijverberg et al., 1982). They are persistent in nature which makes it very difficult for the breakdown of the pesticide. DDT, dieldrin, chlordane, mirex, kepone, toxaphene, lindane, methoxychlor, endosulfan and benzene hexachloride are some of the popularly used organochlorine pesticides. Extended usage of OCPs in huge amount coupled with its characteristics had a serious effect on the environment and health of animals and human beings. OCPs gets accumulated in mammals causing damage to them. There are reports of presence of DDT (0.510 mg/L) and BHC residues (0.195 mg/L) in breast milk (Kalra et al., 1994; Kalra and Chawla, 1981) and in bovine milk presence of DDT above 0.05 mg/kg have been reported in Punjab, India (Kalra et al., 1999). The OCPs target the CNS causing convulsions in brain and other signs of neurologic toxicity such as tremor, myoclonic jerking, ataxia, hyperreflexia and paresthesias. These were the main reasons for the ban of OCPs as pesticides. The ban on OCPs lead to the production of new synthetic pesticides.
Figure 2.2 Structure of a) DDT b) Endosulfan

Subsequent to the ban of OCPs, a new group of pesticides, the organophosphates evolved and have become the most widely used pesticides in recent times. Organophosphates are broad spectrum pesticide and hence used to control a wide range of pests. Organophosphates are nerve poisons acting as a stomach and contact poison in addition to acting as fumigant. The general chemical structure of organophosphate is shown in Figure 2.3. They are the group of pesticides which inhibits the activity of acetylcholinesterase enzyme required for degradation of the neurotransmitter acetylcholine in the synapse by phosphorylating the acetylcholine (PEHSU, 2007). Although these pesticides are biodegradable and cause less environmental pollution, they are very harmful to human health. Chlorpyrifos, monocrotophos, malathion, parathion, diazoin, dichlorvos and phosmet are some examples of organophosphate pesticides.

Figure 2.3 Chemical structure of organophosphates

Carbamates are esters of N-methyl carbamic acid which possess similar mode of action as OPs in attacking pests. Mode of action to inhibit cholinesterase is the only difference of carbamates from OPs (Fukuto et al., 1990). Figure 2.4 depicts the general chemical structure of carbamates. Carbamates are used as either dust or sprays. They get absorbed through the skin, by ingestion and inhalation. The immediate toxic effect of carbamates is very similar to that of organophosphate pesticides, but the recovery is
comparatively rapid. The symptoms of exposure includes breathing difficulties, nausea, headache, excessive sweating and salivation, general weakness and abdominal pain. They also cause muscle fasciculations (Colovic et al., 2013).

![Chemical structure of carbamates](image)

**Figure 2.4 Chemical structure of carbamates**

Pyrethroids are the last class/group of insecticides which are organic in nature and resemble the natural compound pyrethrin produced from flowers of pyrethrum (*Chrysanthemum cinerariaefolium*). They are the most commonly used household insecticide because they can be easily broken apart by sunlight within two days of its application. Thus, avoiding groundwater contamination (USEPA, 2009). There are variations in the structure of pyrethroids. Most commonly used pyrethroids are allethrin and permethrin and their structure is depicted in Figure 2.5.

![Chemical structure of Pyrethroids](image)

**Figure 2.5 Chemical structure of Pyrethroids a) Allethrin b) Permethrin**

To summarize, the pesticides were employed by the farmers to kill the pests in agricultural fields. The usage has been tremendous for various crops and the type of pesticide to be used also varies depending on the type of crops or the pest to be killed. Figure 2.6 depicts the fate of the applied pesticide in the environment. According to Pimentel, (1995), when a certain amount of pesticide is applied, only 0.1% reaches the target pest and rest of the portion goes off site (as shown in figure), such as atmosphere, uptake by plant, soil and ground water. The off sites include atmosphere, soil and ground water where they reach by volatilization, leaching and run off causing alarming situations. The pesticide remains into the soil for years and also leaches into the
groundwater. The fate of pesticide follows three stages which include degradation, adsorption and transfer. Degradation can be done by three types of methods such as photodegradation, chemical degradation and microbial degradation. Photodegradation is caused by sunlight and chemical degradation is caused by the chemicals present in the soil and the microbes (bacteria and fungi) are the one playing a role in microbial degradation.

The present study mainly focuses on chlorpyrifos pesticide, one of the most widely used organophosphate in the region. It is applied to almost all vegetables and rice crop.


**Figure 2.6 Fate of pesticide in the environment**

In Punjab, the pesticides have become the main backbone for the farmers after green revolution. It causes harm to human beings and deteriorates the environment.
2.2 Chlorpyrifos

Chlorpyrifos [O,O-diethylO-(3,5,6-trichloro-2-pyridinyl-phosphorothioate)] is a popularly used broad spectrum insecticide. It is used against insect pests of major crops which are economically important. Chlorpyrifos was first developed by Germans in the 1930s. Globally, it ranks first among the conventional pesticide active ingredient in the agricultural sector due to its consequent persistent usage. Broad-spectrum nature of chlorpyrifos makes it a favourable candidate for wide usage as insecticide leading to contamination of water and terrestrial ecosystems in abundant array (Wang et al. 2007). Chlorpyrifos was registered in 1965 for the first time in the United States by Dow Chemical Company for controlling soil-borne and foliage insect pests (NPIC, 2014). It is formulated and marketed as a liquid, gel, granular, soluble, emulsifiable and flowable concentrates, microencapsulated material, pellets, tablets, impregnated materials, baits, wettable powders, dust, and ready-to-use formulations. It is a colorless to solid white crystalline substance with a melting point of 41-42 °C. It decomposes at a temperature of 160 °C (http://www.atsdr.cdc.gov/toxprofiles/tp84-c3.pdf.). Chlorpyrifos has low water solubility (1.39 mg/L) and high soil sorption coefficient (av Koc= 8498 mL/g). In soil, the half-life of chlorpyrifos ranged from 11 to 141 days (USEPA, 1999). In Table 1.1 the physico-chemical characteristics of chlorpyrifos pesticide is provided.

Chlorpyrifos is highly lipid-soluble and their routes of entry inside the body include absorption through the skin, oral mucous membranes, conjunctiva, gastrointestinal and respiratory routes. People were using it for the control of mosquitoes (larvae and adults), flies, various soil pests, many foliar crop pests and household pests. Empty containers of pesticide were used by the villagers for storing most of the food items (Thakur et al., 2008). In cotton rape seeds of Punjab, 0.019 mg/kg of chlorpyrifos concentration were reported (Singh, 2004). The vegetables such as cabbage and cauliflower contains 0.08 and 1.17 mg/kg of chlorpyrifos, respectively (Department of Agriculture and Cooperation, 2009; Gill et al., 2009). Human blood sample is also reported to be contaminated with pesticides in which chlorpyrifos of 0.6662 mg/L was observed (Mathur et al., 2005). Scientists have also reported the presence of chlorpyrifos residues in the food chain (Aysal et al., 2004; Chandra et al., 2010).
2.2.1 Effect of Chlorpyrifos

Chlorpyrifos is known to cause adverse effects. The transmission of nerve impulses in the brain, skeletal muscles and in other areas require acetylcholine (Toole and Toole, 1995). Further, in order to avoid over stimulation of the nervous system after the transmission of the impulse, they must be hydrolyzed. Acetylcholine esterase is an enzyme required for the breakdown and hydrolysis of the acetylcholine. It converts acetylcholine into choline and acetyl-CoA by binding the substrate at its active site, Ser 203 to form an enzyme-substrate complex. One acetylcholine esterase enzyme can hydrolyze around 300,000 molecules of acetylcholine in one minute (Ragnarsdottir, 2000). Organophosphorus compound binds covalently to the enzyme at Ser 203, an amino acid active site of acetylcholine esterase than changes its structure and function and finally inhibits the breakdown of neurotransmitter acetylcholine (Boublík et al., 2002; Colovic et al., 2013). When the leaving group binds to the positive hydrogen of His 447 then the breakdown of phosphate occurs and the enzyme gets phosphorylated. The process of regeneration of phosphorylated acetylcholine esterase gets very slow and may take hours or days which ultimately results in the accumulation of acetylcholine at the synapses. This causes the nerves to get over stimulated and jammed (Manahan, 1992). The inhibition of AChE is irreversible causing accumulation of neurotransmitter acetylcholine and neurotoxicity occurs (Thompson and Richardson, 2004). The inhibition of acetylcholine esterase by the pesticide causes convulsion, paralysis and finally death of insects and mammals (Ragnarsdottir, 2000). Among the ill effects of chlorpyrifos poisoning such as fatality in extreme cases, birth defects, headache, nausea, muscle twitching, convulsions and disorders in the male reproductive system were reported depending on the several factors (Sai et al., 2014).

Various factors such as properties of organophosphate pesticides (physico-chemical properties, solvents and impurities), duration and routes of exposure are to be considered while studying its effect on human health. Other factors which play a key role in determining the intensity of the effect of pesticides are metabolism, sequestration, excretory processes and health status (age, gender, environmental
factors, concurrent medications, cholinergic status) of the exposed humans to the pesticides (Karalliedde et al., 2003).

Pesticide is not just the malefactor to human beings alone but also several environmental processes affecting human health. The commotion brought in biogeochemical cycles due to contamination of different constituents of the ecosystem are serious environmental alarms caused due to undue utilization of pesticides in agriculture (Singh et al., 2004). Indiscriminate usage of organophosphorus pesticides has led to the contamination of several ecosystems over the world (Singh and Walker, 2006). Several reports are there for the presence of chlorpyrifos pesticide in soil which ultimately leaches into the groundwater. Depending on the type of soil, its characteristics and temperature conditions, the time taken for leaching varies (Wang et al., 2006; Tahir et al., 2010; Zhang et al., 2011). Even after four to eight years of its application to kill termite population, chlorpyrifos residues have been identified in sixteen houses in North Carolina (Wright et al., 1991, 94). Ecological imbalance can occur as the living organisms are exposed to pesticide residues in soil and water. Beneficial insects like arthropods including bees, ladybird beetles, and parasitic wasps were also affected by chlorpyrifos toxicity. A part per million concentrations of chlorpyrifos is able to kill fish population. There are reports of abnormal cell division, delayed emergence of seedlings and fruit deformities in the plant (Cox, 1995).

Considering the harmful effects of chlorpyrifos pesticide on the environment and living beings, there is an alarming need for its degradation or removal from the environment. Scientists have reported various methods of pesticide degradation. However, no method has achieved sustained results thereby increasing the thirst of new research.

2.3 Degradation of pesticides

Researchers have made lot of attempts to find solutions for the treatment and removal of pesticide and its residues from the environment, water and soil. The degradation of pesticide is to break down the toxic chemicals into non-toxic compounds (Vargas, 1975). In general, pesticide degradation occurs by chemical or biological processes. The process of breakdown occurs in a wide range of time from hours or days to years; it totally depends on the chemical characteristics of the pesticide and the environmental
conditions. The pesticide which quickly breaks down does not persist in the environment or on the crop providing a short-term control (AGF, 2016).

The various techniques presently being studied for removal of pesticides are adsorption, biological treatment, and degradation by advanced oxidative processes (AOP) that utilize TiO$_2$/UV as a catalyst, photo-Fenton reagents (FR) (Legrini et al., 1993), and ozonation processes with O$_3$, O$_3$/UV, and O$_3$/H$_2$O$_2$ (Masten and Davies, 1994). Degradation of endosulfan was studied by Chiron et al. (1997) where photocatalyzed reactions with FeCl$_3$/H$_2$O$_2$ and TiO$_2$/H$_2$O$_2$ were performed in water samples. Removal of pesticides was effective against both the catalysts and this methodology can be applied to other situations and environmental situations. Ozonation reactions were used for the complete mineralization of methyl parathion, parathion, diazinon and cypermethrin pesticides in an accelerated degradation (Arbeli and Fuentes, 2007). The complete mineralization was assessed by intermediate products and the formation of CO$_2$.

Chemical degradation of pesticides takes place by photolysis, oxidation and reduction reactions (Sassman et al., 2004), while the action of environmental micro-organisms cause biological degradation (Ghardiri and Rose, 2001; Sassman et al., 2004).

Biological degradation of pesticides by native environmental micro-organisms is more popular due to its multiple advantages such as eco-friendly, economical, and ability to degrade a wide range of chemicals causing less harm to the environment than the commonly used methods for remediation of pollutants such as pyrolysis, land filling, incineration etc. which are responsible for the formation of more toxic intermediate compounds than the parent compound affecting the environment in a more deleterious ways (Finley et al., 2010). Also, these methods are too expensive for the cleanup (Singh and Walker, 2006). So, there is a need to understand the microbial role in the degradation of pesticide.

**2.3.1 Role of microbes in degradation**

The present study deals with chlorpyrifos pesticide degradation, hence this section will mainly focus on microbial degradation of chlorpyrifos. Chlorpyrifos can be potentially degraded by microorganisms as they possess a unique ability to completely mineralize
many aliphatic, aromatic and heterocyclic compounds, which are potentially exploited in biodegradation studies (Bhagobaty et al., 2006). Some of the microbial species reported for chlorpyrifos degradation by various researchers includes Klebsiella, Serratia, Alcaligenes, Providencia, Verticillium sp. DSP, Leuconostoc, Lactobacillus, Pseudomonas, Enterobacter, Cyanobacterium Synechocystis sp. Strain PUPCCC 64, Cladosporium cladosporioides Hu-01 (Ghanem et al., 2007; Xu et al., 2007; Surekha et al., 2008; Madhuri and Rangaswamy, 2009; Fang et al., 2008; Awad et al., 2011; Singh et al., 2011; Chen et al., 2012). Table 2.1 summarizes the microbes and their chlorpyrifos degrading potential.

In the table, different types of microbial species and their efficiency in chlorpyrifos degradation is reported. It has been observed that species reported by Yang et al. (2006) and Li et al. (2008), the Stenotrophomonas sp. YC-1 and Stenotrophomonas sp. DSP-4, respectively degraded the chlorpyrifos pesticide completely within 24 h incubation period.

Chlorpyrifos pesticide breakdown into different metabolites depending on the type of microbial enzyme and various environmental factors. Among them TCP (3,5,6-trichloropyridinol) is the primary metabolite first reported by Thiegs, (1966) using $^{36}$Cl$^-$ labelled CP in soil. Following that, in the year 1979 Bidlack identified the secondary metabolite TMP (3,5,6-trichloro-2 methoxy pyridine) in soil studies. Later Zidan et al. (1981) reported some other metabolites such as chlorpyrifos oxon, O-ethyl O-(3,5,6-trichloro-2-pyridyl) phosphate and desethyl chlorpyrifos using paper chromatography. Formation of Diethylthiophosphate (DETP) was also reported by Chen et al. (2012) and Bootharaju and Pradeep (2012). Table 2.2 enlists the chlorpyrifos metabolites. Apart from these there are reports of formation of 2, 4-bis (1, 1 dimethylethyl) phenol and 1, 2 Benzenedicarboxylic acid by Fulekar and Geetha (2008) while degradation of chlorpyrifos. Complete mineralization of chlorpyrifos with no accumulation of TCP or diethyl thiophosphate (DETP) was first reported by a bacterial strain Paracoccus sp. (Xu et al., 2008). Hydrolysis of chlorpyrifos to DETP and TCP by Entrobacter strain B-14 was observed and the strain utilized DETP for growth and energy (Singh et al., 2004).
Table 2.1 List of microorganisms involved in chlorpyrifos degradation

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the microorganism</th>
<th>Removal efficiency</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Klebsiella sp.</td>
<td>92 % in 4 days</td>
<td>Ghanem et al. (2007)</td>
</tr>
<tr>
<td>2.</td>
<td>Verticillium sp. DSP</td>
<td>50 % in 3 days</td>
<td>Fang et al. (2008)</td>
</tr>
<tr>
<td>3.</td>
<td>Phanerochaete chrysosporium</td>
<td>27.5% in 18 days</td>
<td>Bumpus et al. (1993)</td>
</tr>
<tr>
<td>4.</td>
<td>Flavobacterium sp. ATCC 27551</td>
<td>100% in 24 h</td>
<td>Mallick et al. (1999)</td>
</tr>
<tr>
<td>5.</td>
<td>Arthrobacter sp.</td>
<td>(10 mg L(^{-1})) in 48 h</td>
<td>Mallick et al. (1999)</td>
</tr>
<tr>
<td>6.</td>
<td>Hypholoma fasciculare and Coriolus versicolor</td>
<td>33 % in 42 days</td>
<td>Bending et al. (2002)</td>
</tr>
<tr>
<td>7.</td>
<td>A. faecalis DSP3</td>
<td>76.2% in 18 days</td>
<td>Yang et al. (2005)</td>
</tr>
<tr>
<td>8.</td>
<td>Trichoderma Y</td>
<td>88.53% in 7 days</td>
<td>Liu et al. (2002)</td>
</tr>
<tr>
<td>9.</td>
<td>Cyanobacterium synechocystis sp. strain PUPCCC 64</td>
<td>75% in one day</td>
<td>Singh et al. (2011)</td>
</tr>
<tr>
<td>10.</td>
<td>Serratia sp.</td>
<td>100% in 7 days</td>
<td>Xu et al. (2007)</td>
</tr>
<tr>
<td>11.</td>
<td>Bacillus and Pseudomonas</td>
<td>75% in 7 days</td>
<td>Madhuri and Rangaswamy (2009)</td>
</tr>
<tr>
<td>12.</td>
<td>Pseudomonas fluorescense, Bacillus subtilis, Bacillus cereus, Klebsiella species, Brucella melitensis, Serratia marcescens and Pseudomonas aeruginosa,</td>
<td>75-87% in 20 days</td>
<td>Lakshmi et al. (2008)</td>
</tr>
<tr>
<td>13.</td>
<td>Cladosporium cladosporioides Hu-01</td>
<td>100 % in 6 days</td>
<td>Chen et al. (2012)</td>
</tr>
<tr>
<td>14.</td>
<td>Trichoderma viride and Aspergillus niger</td>
<td>95.7% and 72.3% in 14 days</td>
<td>Mukherjee and Gopal (1996)</td>
</tr>
<tr>
<td>15.</td>
<td>Stenotrophomonas sp. DSP-4</td>
<td>100% in 24 h</td>
<td>Li et al. (2008)</td>
</tr>
<tr>
<td>16.</td>
<td>Stenotrophomonas sp. YC-1</td>
<td>100% in 24 h</td>
<td>Yang et al. (2006)</td>
</tr>
</tbody>
</table>

Four bacteria capable of growing at 1600 mg/L chlorpyrifos was isolated from the soil by Bhagobaty and Malik (2008) and through morphological and biochemical tests the bacteria was identified to be Pseudomonas sp. In liquid medium, P. aeruginosa isolated
in India could degrade 80% of chlorpyrifos (50 mg/L) after 20 days of incubation (Lakshmi et al., 2009).

### Table 2.2 List of chlorpyrifos metabolites

<table>
<thead>
<tr>
<th>S. No.</th>
<th>List of Metabolite</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3,5,6-trichloropyridinol</td>
<td>Thiegs (1966)</td>
</tr>
<tr>
<td>2.</td>
<td>TMP (3,5,6-trichloro-2 methoxy pyridine)</td>
<td>Bidlack (1979)</td>
</tr>
<tr>
<td>3.</td>
<td>Chlorpyrifos oxon</td>
<td>Zidan et al. (1981)</td>
</tr>
<tr>
<td>5.</td>
<td>Desethyl chlorpyrifos</td>
<td>Zidan et al. (1981)</td>
</tr>
<tr>
<td>7.</td>
<td>2, 4-bis (1, 1 dimethylethyl) phenol</td>
<td>Fulekar and Geetha (2008)</td>
</tr>
<tr>
<td>8.</td>
<td>1, 2 Benzenedicarboxylic acid</td>
<td>Fulekar and Geetha (2008)</td>
</tr>
</tbody>
</table>

But it is identified that in microbial degradation of pesticide it is ultimately the enzymes which catalyzes the reaction efficiently. So, researchers were exploiting microbial enzymes for degradation purpose.

#### 2.3.2 Microbial enzymes in pesticide degradation

Microbes are considered as efficient workers in pesticide degradation but enzymes makes them so much valuable causing degradation of pesticide. Though microbes take part in degradation but it is the enzymes present in them which plays a role in degradation. Hence, attempts were made to use the enzymes directly for degradation. Enzymes are responsible for the catalysis of specific biochemical reactions (Underkofer et al., 1958).

Russel et al. (1998) suggested that better bioremediation of pesticides can be achieved using enzymes as catalytic bioremediants which can be added to the environment and
the surfaces of commodities. The advantages and disadvantages of enzyme assisted bioremediation are summarized in Table 2.3.

### Table 2.3 Advantages and Disadvantages of enzymatic degradation

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Unaffected by inhibitors of microbial metabolism</td>
<td>Extraction and purification of enzyme is a time consuming process</td>
</tr>
<tr>
<td>2.</td>
<td>Operated under a wide range of environmental conditions</td>
<td>The enzymes extracted from cells may remain unstable even after immobilization on matrices</td>
</tr>
<tr>
<td>3.</td>
<td>Effective at low pesticide concentrations</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Remains active in the presence of microbial predators and toxins</td>
<td>Various enzymes requires cofactors making it difficult to apply</td>
</tr>
<tr>
<td>5.</td>
<td>Enzymes are not substrate specific</td>
<td>Diffusional constraint may hinder the interaction between enzymes and pollutants</td>
</tr>
<tr>
<td>6.</td>
<td>No specific uptake mechanism unlike microbes</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Small size of enzyme facilitates easy mobility within the soil when compared to microorganisms</td>
<td>Free enzymes can be easily degraded by microbial proteases</td>
</tr>
</tbody>
</table>

(Source: Munnecke et al., 1977)

If an effort is made to overcome the disadvantages of enzyme assisted degradation, the efficiency of pollutant degradation can be humongously evidenced. The first purified novel hydrolase from fungus *Cladosporium cladosporioides* Hu-01 whose preferred substrate was chlorpyrifos has been reported by Gao et al. (2012).

### 2.4 Enzymes involved in chlorpyrifos degradation

Those enzymes which are able to break phosphotriester bond are known as phosphotriesterase and they are involved in the breakdown process of OPs as a whole. Phosphotriesterase consists of organophosphorus hydrolase(OPH), methyl parathion
hydrolase (MPH), organophosphorus acid anhydrolase (OPAA), diisopropylfluorophosphatase (DFP), and paraoxonase 1 (PON1) (Bigley and Raushel, 2013). They share several common features but they are different in their catalytic mechanism, protein sequence and also the three-dimensional structure varies (Bigley and Raushel, 2013). Laccase, a fungal enzyme is also explored for degradation of OPs due to broad substrate specificity of the enzyme (Alcalde et al., 2006). Organophosphorus hydrolase is considered an ideal enzyme for degradation and this property is attributed to its broad substrate profile. It has a potential of hydrolysing the compounds at a rate approaching the diffusion limits (Dumas et al., 1989). In chlorpyrifos, P-O is cleaved by microbial OPH (Ang et al., 2005). Other than OPH, the laccase enzyme has also been exploited for organophosphate degradation (Alcalde et al., 2006). Previously this enzyme showed tremendous potential of degrading phenolic azo dyes (Chivukula and Renganathan, 1995). The crude enzyme isolated from strain WZ-I (Fusarium LK. ex Fx) was determined for chlorpyrifos degradation, and it has been found that the cell fragment and both intra and extracellular enzymes contributed to the degradation of chlorpyrifos which was around 60.8%, 11.3% and 48%, respectively (Xie et al., 2005). Though microbial degradation is an effective way of degradation but scientists were also trying to find out other alternatives as mentioned above regarding usage of microbial enzymes in degradation.

2.4.1 Characteristic features of enzymes involved in pesticide degradation

The different types of enzymes involved in pesticide degradation were described below.

2.4.1.1 Organophosphorus acid anhydrolase (OPAA)

It is an enzyme responsible for detoxifying organophosphorus-containing compounds for example; nerve gas used in chemical warfare. They are reported to be present in protozoa, soil bacteria and mammals. This enzyme uses an activated molecule to displace the fluoride at the phosphorus center (Dumas et al., 1989). Molecular weight of enzyme is ~58 kDa and it is composed of 517 amino acids (Cheng et al., 1995; Cheng et al., 1996). OPAA is a trimer when its structure is shown in 3D structure. Its
structure has 30% homology to E. coli amino-peptidase P and 22% amino acid homology with human prolidase (Cheng et al., 1995). This enzyme loses its activity in harsher conditions such as organic solvents, long storage and at high temperature (Hoskin et al., 1982). Figure 2.7 depicts the 3D tetramer structure of the enzyme.

![Figure 2.7 Structure of OPAA trimer](image)

2.4.1.2 Paraoxonase (PON1) (EC 3.1.8.1)

Serum PON1 is one of the subtype from the three sub types of paraoxonase reported (Li et al., 2003). Figure 2.8 illustrates the structure of paraoxonase enzyme. All mammalian species studied so far has Serum PON1 except in the serum of fish, birds, insects and reptiles. In human, molecular weight of PON1 is 43000 Daltons comprising high-density lipoprotein and it is a glycoprotein composed of 354 amino acids (Primo-Parmo et al., 1996).

![Figure 2.8 Structure of Paraoxonase](image)
Studies confirmed the degradation of oxygen analogs of chlorpyrifos and diazinon (but not parathion) in vivo by paraoxonase (Pond et al., 1995). Though it is unable to degrade paraoxon, the oxygen analogue of parathion but the name of the enzyme is generated from paraoxon analogue. The studies conducted by researchers concluded that this enzyme protects human from low doses of organophosphate pesticides. Harel et al. (2004) determined its 3D structure and they identified that in HDL binding, the unique six-bladed beta-propeller with active site lid is involved.

2.4.1.3 Diisopropylfluorophosphatase (DFPase, EC 3.1.8.2)

The enzyme acts on P-F bonds present in some OPs and it is a calcium-dependent enzyme. This enzyme is isolated from the brain and ganglion of Loligo vulgaris and known as squid-type DFPase (Latifi et al., 2015). The molecular weight is 35-kDa. The structure of enzyme has six-blades and β-propeller structure with two calcium ions. Researchers developed an interest because of its ability to catalyze warfare agent organophosphates such as tabun, sarin, soman, etc. by hydrolysing the P-F bond (Elias et al., 2013). But its intracellular production is a rate limiting step in the native strains so less interest among researchers (Yang et al., 2008). Figure 2.9 depicts the structure of DFPase enzyme. The backbone fold of DFPase was shown, containing two calcium atoms and water molecules inside the central tunnel shown as spheres. Water molecules are color-coded in blue, magenta and red.

Figure 2.9 Structure of Diisopropylfluorophosphatase from Loligo vulgaris
2.4.1.4 Methyl parathion hydrolase (EC 3.1.8.1)

It is a monomer of ~34 kDa (Chu et al., 2003). This enzyme generally catalyzes the organophosphate, methyl parathion but explored for other organophosphates also. A study conducted by Deng et al. (2015), observed that *Stenotrophomonas* sp. G1 is efficient in degrading organophosphate pesticides. They reported that the species is able to degrade 63% of chlorpyrifos concentration. Degradation occurred due to the intracellular presence of methyl parathion hydrolase. Structure of methyl parathion hydrolase (MPH) is shown in Figure 2.10.

![Figure 2.10 Structure of methyl parathion hydrolase](image)

2.4.1.5 Organophosphorus hydrolase (OPH) (EC 8.1.3.1)

This enzyme is the most widely exploited enzyme for organophosphate degradation. The molecular weight of OPH is 72 kDa representing a dimer. It consists of 336 amino acid residues in each of its two identical subunits. OPH enzyme contains two Zn$^{2+}$ or Co$^{2+}$ ions per subunit as it is a metal dependent enzyme. Catalytic activity is also exhibited by the enzyme where Zn$^{2+}$ or Co$^{2+}$ gets replaced by Cd$^{2+}$, Mn$^{2+}$ or Ni$^{2+}$. Highest catalytic activity with respect to phosphotriesters is being observed by enzyme containing Co$^{2+}$. OPH catalyzes the hydrolysis of P-O, P-F and P-S bonds. Lowest specificity was observed on P-S bond. Phosphotriesters are more prone to hydrolysis than the diesters. Inversion in the configuration of the phosphorus atom was observed when organophosphate compounds were hydrolyzed by OPH as it follows SN2 mechanism. Toxicity of organophosphates gets reduced to several magnitude when hydrolyzed using OPH (Sogorb et al., 2004). Structure of organophosphorus hydrolase was acquired from Protein Databank accession #1DPM. In Figure 2.11, the blue and
cyan color represents two identical monomer of which enzyme is composed off. Two metals are present in each monomer and they are represented as yellow/green spheres. The red space filled model represents the inhibitor diethyl 4-methylbenzylphosphonate which is the active site of the enzyme.

There is a list of microbes which are responsible for organophosphate degradation and are isolated from pollutant site containing OPH enzyme. Most widely exploited microbes for the isolation of OPH are *Pseudomonas putida*, *Flavobacterium* Sp, *Arthrobacter* Sp, etc. There are reports of chlorpyrifos degradation by the novel phosphotriesterase enzyme isolated from *Enterobacter* strain B-14 by Singh et al. (2004). In the study they have reported that enzyme is efficient in degrading chlorpyrifos concentration of 35 mg/L when supplied without any other carbon source in 24 h of incubation.

![Structure of organophosphorus hydrolase](image)

**Figure 2.11 Structure of organophosphorus hydrolase**

### 2.4.1.6 Laccase (EC 1.10.3.2)

Laccases (benzenediol: oxygen oxidoreductases) are ligninolytic enzymes with broad substrate specificity. The enzyme was first described by Yoshida in 1883. It was first extracted from the exudates of *Rhus vernicifera*, commonly called as Japanese lacquer tree (Thurston, 1994). The enzyme has gained considerable interest in industrial and environment biotechnology, due to their broad substrate specificity. It does not require any low molecular weight cofactor because their co-substrate O₂ is usually present in
the environment (Couto and Herrera, 2006). Most of the laccase enzymes are extracellular. Due to this the purification procedures for laccase enzyme is very easy. This contributes to a considerable level of stability in the extracellular environment which attributes their application in biotechnological processes.

**Source:** They are widely distributed in fungi such as *Neurospora crassa*, *Pleurotus*, *Pholiota*, *Polyporus versicolor*, *Aspergillus nidulans*, *Podospora anserina*, white-rot fungi and *Pyricularia oryzae* (Gardiol et al., 1998). They are also present in higher plants such as *Lactarius piperatus*, *Rhus vernicifera*, *Prunus persica*, *Rhus succedanea*. Few bacterial species are reported to contain laccase which are *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Streptomyces griseus* and *Bacillus subtilis* (Givaudan et al., 1993; Alexandre and Bally, 1999; Hullo et al., 2001; Solano et al., 2001; Sanchez-Amat et al., 2001; Endo et al., 2002). There are also other sources of laccase which are summarized below in Table 2.4.

### Table 2.4 Sources of laccase enzyme

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Source of laccase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Fomes annosus</em></td>
<td>Haars and Huttermann (1980)</td>
</tr>
<tr>
<td>2.</td>
<td><em>Coriolus versicolor</em></td>
<td>Archibald and Roy (1992)</td>
</tr>
<tr>
<td>3.</td>
<td><em>Armillaria mellea</em></td>
<td>Worrall et al. (1986)</td>
</tr>
<tr>
<td>4.</td>
<td><em>Azospirillum lipoferum</em> (Bacteria)</td>
<td>Givaudan et al. (1993)</td>
</tr>
<tr>
<td>7.</td>
<td><em>Phanerochaete chrysosporium</em></td>
<td>Keyser et al. (1978)</td>
</tr>
<tr>
<td>8.</td>
<td><em>Schizophyllum commune</em></td>
<td>De Vries et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Reference</td>
</tr>
<tr>
<td>---</td>
<td>---------------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>11.</td>
<td><em>Trametes versicolor</em></td>
<td>Han et al. (2005)</td>
</tr>
</tbody>
</table>

**Structure:** Laccase, representing the largest subgroup of blue multicopper oxidases, uses the distinctive redox ability of copper ions to catalyze the oxidation of a varied range of aromatic substrates, concomitantly by reducing the molecular oxygen to water (Couto and Herrera, 2006; Majeau et al., 2010). Laccases are polyphenol oxidases that catalyzes the oxidation of various aromatic compounds using molecular oxygen. Phenols (-OH) and anilines (-NH) act as electron-donating groups and oxygen as an
electron acceptor. Furthermore, laccases are ubiquitous in nature. Their structure can be monomeric, dimeric and tetrameric and each monomer consists of four atoms (Type 1, 2 and 3) of copper in its catalytic site. The oxidative property of laccase is due to Type 1 (T1) copper which provides blue color to the enzyme. Each copper center in the laccase enzyme is attached to the imidazole sidechains of histidine. The laccase structure is depicted in Figure 2.12. In the figure the copper is represented in brown colour and nitrogen in blue.

![Figure 2.12 Structure of Laccase enzyme](image)

**Mechanism:** Oxidation mechanism of laccases involves radicals where aromatic and non-aromatic hydrogen donors were oxidized using molecular oxygen. These radicals can endure additional non-enzymatic reactions, such as polymerization, hydration or hydrogen abstraction and laccase-catalyzed reactions. Formation of aryloxy radical was observed when phenolic substrates were oxidized by laccase which is an active species to convert into quinine during oxidation at the second stage. Depending on the substrate and various environmental parameters, the quinone intermediates forms soluble or insoluble colored oligomers spontaneously by reacting with one another.

Redox potential of laccase enzyme is a barrier towards its non-specificity of substrates. It ranges between 0.5 and 0.8 V (Riva, 2006). The activity of the enzyme is highly influenced by enzyme inhibitors and pH (Stoilova et al., 2010). However, non-phenolic substrates can be metabolized using redox-mediators. Like in case of pesticides the degradation can be enhanced using redox mediators viz. ABTS, HBT and veratryl alcohol (Kapich et al., 1999; Kunamneni et al., 2007). The redox-mediators are small molecule which oxidizes a non-substrate compound via a non-enzymatic reaction by
diffusing out the catalytic pocket, when it gets oxidized by the laccase. The catalytic cycle of the laccase mediator system is illustrated in Figure 2.13 (Baiocco et al., 2003). Redox mediators are of low molecular weight and have high redox potential than laccase (Farragher, 2013). In laccase mediator system (LMS) the oxidation of the mediator by laccase drive the reaction by generating a strongly oxidised intermediate which in turn oxidise the pesticides with a greater effect (Camarero et al., 2005; Morozova et al., 2007). Though this mechanism has been studied before for several years, it was not fully understood.

![Catalytic cycle of laccase mediator system](image)

**Figure 2.13** Catalytic cycle of laccase mediator system (Baiocco et al., 2003)

There are numerous industrial applications of laccase enzyme which includes decolorization of dye, biological bleaching in pulp and paper industries, detoxification of recalcitrant compounds, production of ethanol, coal solubilization and also, in food and beverage industries (Mayer and Staples, 2002; Couto and Herrera, 2006; Majeau et al., 2010).

### 2.5 Role of laccase in different industrial applications

Laccases have a broad range of substrate specificity. Due to this property, they can oxidize a varied list of pollutants found in industrial wastes (Chandra and Chowdhary, 2015). The bacterial/fungal laccases are playing important roles in the bioremediation of industrial waste because they oxidize both toxic and nontoxic substrates. Laccases are also included in the cleaning of industrial effluents, mostly from paper and pulp, textile and distillery industries. In biological method of enzymatic degradation, laccases represent an interesting group of ubiquitous oxidoreductase enzymes that show great potential for biotechnological applications. So far fungal laccases has shown wider claims, ranging from the pulp and paper industry to the pharmaceutical sector, for
reducing the kappa number. The bleaching of kraft pulp is elevated through wood pulp bleaching in the presence of mediators such as ABTS.

Moreover the decontamination of phenolic pollutants in soil could be directed by immobilizing those using laccases, which catalyzes oxidative coupling further reducing their bioavailability. In contrast, pollutants might undergo oxidative transformation (degradation) by laccases to form products that are easily taken-up by soil microflora. In paper industries, laccases are playing important roles, and one of the most studied applications is the laccase-mediated bleaching of kraft pulp; the efficiency of which has been proven in mill-scale trials.

2.5.1 Laccases and delignification

The removal of lignin from woody tissue is a process that has attracted a great deal of research, especially due to its importance in the pulp and paper industry. There are chemicals which enable the large enzyme molecule to react with fiber lignin via the small mediator molecule. In decaying of wood by white rot fungi, there is no doubt that laccase is one of the main enzymes involved in delignification. The presence of laccase, alone or together with lignin peroxidase (LiP) and manganese peroxidase (MnP), has been demonstrated in a wide variety of white rot fungi. The structure of many of these laccases has been described and surprisingly, considerable diversity was observed in molecular weight, pH optimum and other properties, despite using lignocellulose as a common substrate. The stability of some of these laccases is enhanced by the presence of phenolic substances (Mai et al., 2000). It has also been shown that the capability of laccases to break down lignocellulose is increased by certain phenolic compounds acting as mediators (Bourbonnais et al., 1995).

An additional interesting feature is that laccase, for example from Trametes sp., can be expressed efficiently in yeast (Saccharomyces cerevisiae). The transformed yeast showed considerable resistance to phenolic compounds (Larsson et al., 2001). These results suggest that laccase from white rot fungi utilizes the products of lignin breakdown and are resistant to the phenolic compounds produced, although these might be thought as inhibitory compounds. It must be assumed that the free radicals, produced as a result of laccase action, are rapidly removed by secondary reactions.
One of the laccases produced by *Pleurotus* sp. shows activity inside the cell or in the cell wall (Palmieri *et al*., 2000). There is a little difficulty to reconcile with a role in delignification of a substrate. Although the function of laccase in delignification is qualified enough but many aspects remain unclear despite the advances made in this area. The interaction of different wood-decaying basidiomycetes has shown a highly variable pattern of laccase formation and this subject requires more detailed and reliable experiments (Iakovlev and Stenlid, 2000).

### 2.5.2 Ethanol production

Laccase enzymes are used in ethanol production. Ligninolytic enzymes such as Manganese peroxidise (MnP), Lignin peroxidise (LiP) and laccase from *Ceriporiopsis subvermispora* and *Irпex lacteus* were evaluated for ethanol production. Ninety percent ethanol production was observed after 21 days of incubation of these enzymes with wheat straw (Salvachúa *et al*., 2011).

### 2.5.3 Drug analysis

A new enzymatic method based on laccase has been developed to distinguish morphine from codeine (Bauer *et al*., 1999). An enzyme sensor was constructed by immobilizing laccase and glucose dehydrogenase in a Clark oxygen electrode. It is interesting to note that laccase oxidizes morphine consuming O₂ molecules. Subsequently, the drug is regenerated by glucose dehydrogenase. Laccase cannot oxidize codeine, so the sensor is selective for morphine. Morphine is detected between 32 nM and 100 μM concentrations. This method allows rapid and a technically simple method to identify morphine and codeine within a minute.

### 2.5.4 Wine clarification

Laccase immobilized on a copper-chelate carrier that can be regenerated was used successfully to remove phenols from white grape juice (Servili *et al*., 2000). The phenols were partially removed by the enzymatic treatment, especially epicatechin, ferulic and o-coumaric acids. Laccase was shown to form two complex compounds by disintegrating ferulic acid, i.e. trans-5-((E)-2-carboxyvinyl)-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2, 3-dihydrobenzofuran-3-carboxylic acid and (Z)-b-(4-((E)-2-carboxyvinyl)-2-methoxyphenoxy)-4-hydroxy-3-methoxy cinnamic acid (Carunchio *et
al., 2001). Minussi et al. (2002) reported that laccase enzyme is used for removal of phenolic compounds in wine and also for the stabilization of beer for long time. They are playing a key role in maintaining the color and flavor of the juices.

2.5.5 Bioremediation

Laccase enzymes isolated from fungi have been shown to possess potential to degrade numerous environmental pollutants, irrespective of their nature. The enzyme studied so far are extracellular in nature and probably involved in the degradation of wood. A laccase produced in the yeast, *Pichia pastoris*, was engineered by site-directed mutagenesis to improve the rate of electron transfer between the copper-containing active site of laccase and an electrode (Gelo-Pujic *et al.*, 1999). Thus, laccases may be usefully engineered to improve the efficiency of bioremediation processes. This study mainly focuses on the bioremediation aspects of laccase enzyme. The review will be further focused on this aspect only.

2.5.5.1 Role of laccase enzyme in pesticide degradation

Glyphosate and the mixture of pesticide degradation by laccase with different redox mediators were studied by Pizzul *et al.* (2009). They have found out that different mediators affect the degradation variably. Laccase in the presence of ABTS showed 40.9% degradation of glyphosate after 24 hours of incubation. Further, when the Mn$^{2+}$ and Tween 80 were added together with the enzyme, the degradation reached 62.8%. Later, a combination of all the three mediators was used (ABTS, Mn$^{2+}$ and Tween 80) along with laccase where 90.1% removal of glyphosate was observed. A degradation ranging from 20 to 100% was observed when the same enzyme mixture was used for the degradation of 22 other pesticides. Zhao *et al.* (2010) observed that dichlorodiphenyltrichloroethane (DDT), an insecticide has been effectively degraded by laccase showing 47-52% degradation after 25 days of enzymatic treatment. Jing *et al.* (2011) studied degradation of dimethoate, chlorpyrifos, trichlorfon and parathion-pyridazine pesticides using laccase with different inductive agents such as RB-bright blue, ABTS and o-toluidine. ABTS was the best inductive agent as the rest affected the laccase production. Pesticide degradation is always influenced by pH and temperature. The best temperature for the degradation by laccase enzyme was identified to be 25
°C. However, pH varied with pesticides. The optimal pH for degradation was 10.0 for dimethoate, chlorpyrifos and parathion-pyridazine, and 8.0 for trichlorfon. Though studies were conducted with free enzyme and they participated efficiently in degradation. But, free enzymes were associated with drawbacks of reusability etc.

2.6 Drawbacks of free enzyme

Although enzyme shows very good degradation potential but enzyme in its free form suffer from certain drawbacks such as storage instability, sensitivity at high temperatures and pH, which results in the loss of enzyme activity, reusability and recovery problems. Enzymatic degradation has a huge potential on chlorpyrifos degradation but their isolation and purification is very expensive. Due to non-reusability of free enzyme the cost associated with the usage of free enzyme is high. Due to these problems the enzymatic degradation are not widely used in industries. To overcome these problems immobilization techniques were introduced. Hence, scientists attempted for immobilizing the enzyme on to a support medium. Such support systems can help in repeated use of enzymes (Munnecke, 1977).

2.7 Immobilization

The immobilization of enzyme involves confining of enzyme molecules to a distinct region of space without affecting their catalytic properties by fixing them onto or within some suitable carrier material. Immobilization allows repeat and continuous usage of enzymes (Brena and Batista-Viera, 2006).

2.7.1 Methods used for immobilization

There are two broad categories into which immobilized enzymes can be classified: irreversible and reversible methods (Gupta and Mattiasson, 1992). Usually the force of binding is inversely related to the ease with which it can be repealed. Simultaneous implementation of objectives stability and reversibility are creating clash to deal with. The conventional approach sacrifices reversibility and are keener into the formation of bond as sturdy as feasible.

Immobilization of enzyme on a suitable carriers involves several methods which can be categorized into reversible and non-reversible of immobilization. Covalent coupling,
entrapment or micro-encapsulation, and cross-linking are the most commonly used irreversible procedure for enzyme immobilization.

2.7.1.1 Irreversible method of enzyme immobilization

The irreversible method of immobilization of biocatalysts to a solid support in such a way that it cannot be detached from the support without destroying the catalytic activity of enzyme and the material used as a support system. It involves four types of methods by which enzymes are immobilized viz. entrapment, micro-encapsulation, covalent coupling and cross-linking. Figure 2.14 depict the irreversible methods of enzyme immobilization.

2.7.1.1.1 Micro encapsulation: It is a process in which the enzymes are immobilized by entrapping them in a spherical semi-permeable membrane.

2.7.1.1.2 Entrapment: Entrapment is the incarceration of enzymes within gels or fibers using covalent or non-covalent bonds (Singh, 2009). There is no involvement of chemical bonds between the enzyme and the support material, due to which diffusion of enzyme is quite impossible. In this process of immobilization, the enzyme is not bound to surface of the material used for immobilization such as the lattice structure of a material or in polymer membranes (Subramanian et al., 1999; Klotzbach et al., 2008) but possess an ability to pass substrate and product from the material used for entrapment but enzymes remain intact inside (O'Driscoll, 1976; Costa et al., 2005). A great concern is associated with this method of enzyme immobilization involving the leakage of enzyme, but this method avoids modification of the native structure of enzyme (Sassolas et al., 2012). Sodium alginate is one such material used for entrapment purpose. Alginate-gelatin-calcium hybrid carriers provides efficient encapsulation of enzyme preventing leakage and providing an increased mechanical stability (Shen et al., 2011). Chitosan, carrageenan and alginate are also, some of the materials used for entrapment.

2.7.1.1.3 Cross-linking: It is an irreversible method. This method is different from other immobilization techniques as it does not require any support (Shi et al., 1997; Honda et al., 2006). Here, by means of multifunctional reagents, leading three dimensional cross linked aggregates forming covalent bond between the enzyme molecules. This
method is also termed as carrier-free immobilization. Glutaraldehyde is the most commonly used reagent for the process of cross-linking.

An enzyme is coupled forming covalent bond with other protein molecule using bi or multifunctional coupling agent (Sheldon, 2007; Tran and Balkus, 2011). The coupling agent cross links the enzyme with support material (Tischer and Wedekind, 1999). In this mode of immobilization, the biocatalyst formed occupies 100% weight of the protein in it, as the coupling agent has negligible molecular weight as compared to enzyme (Schoevaart et al., 2004). Structural and functional property of enzymes remain undisturbed during the process of immobilization. Cross linking agents such as bisdiazobenzidine, glutaraldehyde and hexamethylene diisocyanate are used as bi/multifunctional agents (Singh, 2009). Glutaraldehyde is the most widely used bifunctional cross-linker, because it facilitates the formation of stable inter and intra-subunit covalent bonds as it is soluble in aqueous solvents (Datta et al., 2013).

2.7.1.1.4 Covalent bonding: This method of immobilization is considered highly stable as the enzyme strongly binds to the support through covalent binding. Covalent binding is the most frequently used method for immobilization of proteins. The enzyme immobilized on matrix involving covalent binding does not release enzyme while being used in the solution due to the stability of the bonds formed (Guisan, 2006). It involves highly specific reaction so that no contaminant on the carrier material appears and also no leaking of enzyme is observed in the reaction mixture. Thermal stability and the half-life of enzyme increases when various support materials like mesoporous silica, chitosan etc. are used for covalent attachment (Hsieh et al., 2000; Ispas et al., 2009). In enzymes, the covalent attachment to support is due to amino acids like aspartic acid, arginine, histidine present in their side chain (D'Souza, 1998). Different functional groups like imidazole, indolyl, phenolic hydroxyl etc. are responsible for their degree of reactivity (Singh, 2009). Brena and Batista-Viera (2006) reported that covalent bonding alters the catalytic activity of the enzyme thereby minimizing its activity. Substrate analogs can be used for a coupling reaction to enhance the activity of the enzymes (Mattiasson and Kaul, 1991).
Figure 2.14 Enzyme immobilization irreversable methods (Brena and Batista-Viera, 2006)
Fu et al. (2011) found out that if Peptide-modified surfaces were used to immobilize enzyme linkage then higher specific activity and stability can be achieved with controlled protein orientation. To achieve enzyme immobilization by covalent bonding, functionalization followed by activation of supports using specific activating agents (such as inorganic and organic halides, carbodiimides, glutaraldehyde, etc.) is vital (Zucca and Sanjust, 2014).

2.7.1.2 Reversible method of enzyme immobilization

In this method of enzyme immobilization, under gentle conditions immobilized enzymes can be detached easily from the support system depending on the type of the enzyme-support binding used. Decay or loss in the enzymatic activity, requirement of support regeneration and reloading of fresh enzyme makes reversible methods of immobilization a highly attractive and economical option. While developing an immobilized catalyst, various cost factors needs to be considered. The expenditure on the support material is often considered as a prime factor determining the overall cost of the immobilized catalyst. In case of bioanalytical applications, the reversible method of enzyme immobilization has a wide application (Gupta and Mattiasson, 1992). The reversible method of enzyme immobilization involves 5 types of process. Figure 2.15 depicts the schematic view of reversible methods of enzyme immobilization.

2.7.1.2.1 Adsorption: This process of enzyme immobilization is based on the principle of weak non-covalent interactions such as hydrogen bond, Van Der Waal’s interaction and hydrophobic interaction of the enzyme to the surface of the carrier (Flickinger and Drew, 1999; Jegannathan et al., 2008). Aminoacylase adsorbed to a DEAE-Sephadex is the first procedure in industry which involved immobilized enzyme for continuous resolution of amino acids.

2.7.1.2.2 Chelation: This process is also known as metal binding. Here, without prior derivatization of activated support, the transition metal compounds such as titanium and zirconium (Kennedy and Cabral, 1985) were used to activate the surface of support. Chelate formation takes place and direct coupling of enzyme occurs (Cabral et al., 1986).
Figure 2.15 Enzyme immobilization-reversible methods (Brena and Batista-Viera, 2006)
It is the most commonly used reversible method of enzyme immobilization in chromatographic method. It involves safety issues and is reasonably expensive, so this method is not popular in industries.

2.7.1.2.3 Disulphide bond: Involvement of disulfide (–S–S–) bond formation with the support is done in this method of enzyme immobilization. The exposed non-essential thiol groups (SH) present in the enzyme can be immobilized onto thiol-reactive supports endowed with reactive disulfides or disulfide oxides under placid circumstances (Ovsejevi et al., 2013). It has an advantage that the bonds formed among the activated support and the enzyme containing exposed non-essential thiol can be reversed using an excess of a low-molecular-weight thiol (e.g., dithiothreitol [DTT]) (Carlsson et al., 1998).

2.7.1.2.4 Ionic bonding: It involves the binding of enzymes to the support material by salt linkages. In chromatography, the protein ligand interaction principles were the basis of this process of enzyme immobilization. This method is the reversible immobilization and in ion exchangers it was used for the first time (Sharp et al., 1969).

2.7.1.2.5 Affinity binding: This technique is based on high affinity interaction between biomolecules. The matrix to be used as a carrier for enzyme immobilization is synthesized only for a single type of enzyme containing antibodies against specific epitopes on the antigen. There are two ways by which affinity binding can be achieved between enzyme and support. One way is that, for the target enzyme the support needs to be pre-coupled to an affinity ligand or an entity having affinity for support should be conjugated to the enzyme (Sardar et al., 2000; Datta et al., 2013).

2.8 Carriers for immobilization

Carriers are support system required for holding the enzyme on its surface during the process of immobilization. The selection of a suitable carrier is very important for immobilization. There are different types of support system chosen depending on the type of activity to be performed. A good support system will always improve the enzyme immobilization efficiency.

The vital features in determining the performance of the immobilized enzyme system are matrix characteristics. A good carrier should possess properties such as physical
resistance to compression, ability to resist microbial attack, hydrophilicity, easy retrieval of enzyme, compatibility with biological material and economical (Treven, 1980; Buchholz and Klein, 1987). According to their chemical composition the support system can be classified into inorganic and organic (Figure 2.16). Classification of organic support includes natural and synthetic polymers (Cabral and Kennedy, 1991). Table 2.5 summarizes the various properties of organic and inorganic carriers used for immobilization.

Table 2.5 Properties of inorganic and organic support system

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Inorganic support system (ISS)</th>
<th>Organic Support system (OSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Non-porous metal and its oxides has less binding surfaces</td>
<td>Natural OSS carbohydrate is cost effective and widely available</td>
</tr>
<tr>
<td>2.</td>
<td>Defined porous material like silica are expensive and unstable in alkaline conditions</td>
<td>Synthetic OSS are compatible with almost all enzymatic processes</td>
</tr>
<tr>
<td>3.</td>
<td>Thermally stable</td>
<td>The gel structures are less stable</td>
</tr>
<tr>
<td>4.</td>
<td>Highly resistant against microbial attack</td>
<td>Inertness against microbial attack</td>
</tr>
<tr>
<td>5.</td>
<td>Can be possibly regenerated by single pyrolysis process</td>
<td>In chemical binding or adsorption of cells, OSS can bind more cells than ISS</td>
</tr>
</tbody>
</table>

The success of immobilized system depends upon physical characteristics of the support system such as bulging behavior, mean particle diameter mechanical strength and compression behavior (Guisan, 2006). The capacity for binding of enzymes to the support material gets critically affected by the pore parameters and particle size determining the total surface area. Though nonporous support materials are associated with less diffusional limitations but they suffers low loading capacity. Due to this, the porous support materials are generally preferred over them as they have high surface area allowing more enzyme loading (Wang et al., 2008). Although inorganic carrier has numerous benefits such as high stability against physical, chemical and microbial degradation, but usually the industrial applications are executed using organic matrices
only because they have more binding surfaces. The level of immobilized enzyme activity depends on the hydrophilic character of the carrier (Gemeiner, 1992).

2.8.1 Classification of carriers

The materials used for the immobilization of enzyme can be divided depending on the origin of material and also on the basis of their mode of interaction with enzyme. On the basis of their origin they can be classified as inorganic materials and organic polymer. Organic is further divided into natural polymer and synthetic polymer. Figure 2.16 depicts the classification of support material based on their origin.

2.8.1.1 Organic materials

Organic material can be classified into natural and synthetic polymer based on their origin of generation.

2.8.1.1.1 Natural Polymer

The polymers which are not synthesized in the laboratory conditions rather originated in nature.

Agarose: Agarose is the most extensively used natural organic polymer. It is an exceptional matrix used for immobilization (Porath and Axén, 1976). High porosity behavior leads to a higher percentage of protein addition to matrix. Other advantages include hydrophilic character, enzymes can be easily derived, absence of charged groups (nonspecific adsorption of products and substrate is prevented) and it is commercially accessible (Taylor, 1991). However, the expensive behavior of agarose and other porous material such as agar makes their usage limited. Utilizing reversible methods that permit matrix revival and re-use can thwart this crisis. Interactions ranging from reversible physical adsorption and ionic linkages to secure covalent bonds can be employed for the attachment of the enzymes to the support (Gupta and Mattiasson, 1992).

Chitosan and chitin: Chitin and chitosan are the natural polymers which are used as support material for immobilization of enzyme (Vaillant et al., 2000). The binding of enzyme with chitosan molecule, the protein or carbohydrate moiety of enzyme participates actively (Hsieh et al., 2000).
2.8.1.1.2 Synthetic polymers: The synthetic organic polymers which are used for enzyme immobilization includes amberlite, polyvinylalcohol hydrogel (Grosová et al., 2008), polyacrylamide (Deshpande et al., 1987), zeolites, silica, mesoporous silica, alumina and DEAE (Diethylaminoethyl) cellulose. For the trapping of enzyme on a porous surface, the ion exchange resins/polymer are used, which acts as an insoluble support material (Kumari and Kayastha, 2011). They studied the immobilization of α-amylase on renewable matrices with large surface area such as amberlite and DEAE cellulose. Polyacrylamide gel was used for the immobilization of alkaline phosphatase enzyme using entrapment method (Gonzalez-Saiz and Pizzaro, 2001). Acid phosphatase enzyme extracted from potato was immobilized using physical adsorption process on the commercial 13X (Alfani et al., 1994). In the year 2012, Petkova and his colleagues studied the immobilization of alcohol dehydrogenase obtained from horse liver on silica particles. They reported changes in the conformational structure of the enzyme.

2.8.1.2 Inorganic materials

2.8.1.2.1 Silica: Silica is the most commonly used inorganic material for enzyme immobilization. To improve the cleaning performance of detergents, silica nanoparticles
were used to immobilize α-Amylase enzyme. Silica nanoparticles are associated with numerous advantages such as high surface area, high stability to chemical and mechanical forces and ordered arrangement (Soleimani et al., 2012).

**2.8.1.2.2 Titania:** The TiO₂ nanoparticles are the second popularly used inorganic nanoparticles in enzyme immobilization due to its multiple advantages such as low toxicity, high mechanical strength, biocompatible, cheaper, chemical and physical stability. Covalent bonding occurs for enzyme immobilization on Titania particles as its surface is highly reactive. Commercial TiO₂ nanoparticles were used for biosensor preparation by several researchers. Numerous enzymes such as lyophilized horseradish, hemoglobin, glucose oxidase, tyrosinase, horseradish peroxidase and laccase have been immobilized on TiO₂ nanoparticles (Zhou et al., 2005; Tang et al., 2010; Zhou et al., 2006; Meizler et al., 2011; Hou et al., 2014).

**2.8.1.2.3 Magnetic supports:** Magnetite is a well known magnetic support material for enzyme immobilization. Covalent immobilization is the popularly used method of immobilization on magnetic supports (Zucca and Sanjust, 2014). Cobalt and nickel ferrites showing properties such as hardness, magnetization, and chemical inertness are other magnetic support material which are being used for enzyme immobilization. Some of the few enzymes immobilized on magnetic supports are lipase enzyme on Fe₃O₄ treated with (3-aminopropyl) triethoxysilane, glucose oxidase on cobalt based silica core/ shell and laccase enzyme on Fe₃O₄/SiO₂ nanoparticles (Xie and Ma, 2009; Lee et al., 2012; Wang et al., 2013).

**2.9 Role of immobilized enzymes and whole cell in degradation**

Researchers have studied the role of immobilized enzyme and whole cell on degradation of pollutants. Immobilized cells were more efficient than the free cells because of their potential of reusability. Further their slower contact with the substrate (pesticide) facilitates degradation process (Vijayalakshmi and Usha, 2012). It was reported that immobilized cells of recombinant strain of *Escherichia coli* possessed detoxification rate approximately twice that of freely suspended cells for organophosphate coumaphos pesticide. The immobilized cells retained the catalytic
activity and mechanical stability for over a period of 4-month. A fluorescent 
Pseudomonas species immobilized in sodium alginate showed 83.2% degradation of 
catechol when compared to free cells (82.2%) (Tewari and Malviya, 2002). In the study, 
more efficiency was observed with an advantage of reusability associated with 
immobilized cells. In another study, immobilized cells of Arthrobacter citreus showed 
100% mineralization when used for the degradation of phenol. When the alginate and 
agar immobilized cells were used for phenol metabolism, than within a period of eight 
days in batch fermentation process complete mineralization was observed (Karigar et al., 2006). Pradeep and Subbaiah (2015) studied the role of Ca-alginate immobilized 
cells of Pseudomonas putida in chlorpyrifos degradation. They observed that at the end 
of the 50th cycle, immobilized cells showed 65% chlorpyrifos degradation. In the same 
study, 100% degradation was observed for chlorpyrifos concentration of 2 mg/L while 
in case of 10 mg/L of pesticide concentration, 98% degradation was achieved. 
Xie et al. (2010) studied the chlorpyrifos degradation by immobilizing an enzyme 
isolated from Fusarium fungus WZ-I and found that the immobilized enzyme can 
withstand harsher conditions than the free extract of enzyme. The immobilized enzyme 
showed a loss in its activity retaining 69.4% of its activity after three repeated use. Liu 
et al. (2016) reported that laccase immobilized on the surface of engineered cells of 
Pseudomonas putida has efficiently degraded the total chlorpyrifos concentration. 
Complete detoxification of the medium was confirmed by performing bioassays using 
Caenorhabditis elegans as an indicator organism. In soil slurry, chlorpyrifos 
degradation using laccase immobilized in sodium alginate was performed by Wang et 
el. (2016) at 25 °C and shaking speed of 120 rpm. Chlorpyrifos degradation achieved 
after 48 h was 70%. When the chlorpyrifos concentration was 100 mg/mL, maximum 
degradation rate was achieved but it significantly reduced when concentration reached 
200 mg/mL. High chlorpyrifos concentration might have inhibited the catalytic function 
of laccase.

Carriers used so far for immobilization suffered from various issues such as large size, 
low strength, weight issues and dearth of thermal and electrical property. Though 
immobilization was achieved using different types of carrier material, still the technology
could not be successfully transferred to the field due to certain problems and lacunas. With the objective to fill the lacunas, researchers began to explore more efficient support systems. Nanoparticles came into framework of research due to its several advantages such as high surface area to volume ratio, small size and various other properties. Further review will focus on nanomaterials as potential carrier for enzyme immobilization.

2.10 Nanomaterials as carriers

Nanotechnology is an upcoming field with a potential of providing ample solution to combat the environmental problems at the nano-scale (Zhang, 2003; Zhang and Elliott, 2006). Nanoparticles display exclusive properties due to its large surface to volume ratio. The unique properties of these petite particles have increased the space for its application in various fields like biomedicine, pharmaceuticals, cosmetics, and environment. Figure 2.17 describes the merit of nanoparticles. Nanoparticles were considered as a perfect support material for enzyme immobilization due to high surface area per unit mass, high enzyme loading capability, less diffusional limitations and mass transfer resistance (McHenry and Laughlin, 2000; Asuri et al., 2006; Afkhami et al., 2010; Lei and Ju, 2012; Saifuddin et al., 2012; Hwang and Gu, 2013). Nanoparticles can easily diffuse through macromolecular substrates where the diffusion problem is more relevant making it an ideal carrier. When enzyme immobilized nanoparticles were dispersed in aqueous solutions, it follows brownian movement proving the better efficiency of immobilized enzyme than the free enzyme. Moreover, immobilization of enzymes onto the nanoparticles reduces the protein unfolding and improves the performance and stability of immobilized enzyme.

Garcia-Galan et al. (2011) reported that the purity of the enzyme impairs the volumetric activity, when non-porous nanoparticle supports were used for immobilization. In addition to all the above mentioned advantages of nanomaterials as a carrier for enzyme immobilization they are also associated with the benefits of maintaining the regulation of the proteins/enzymes orientation onto it. They are devoid of external diffusion problems associated with porous supports. This behaviour of nanoparticles makes them an efficient choice for their usage in solid-liquid systems at industry level on a large-scale process (Colombié et al., 2001).
There are several reports of enzyme immobilization onto different types of nanoparticles (polymeric nanoparticles, porous nanoparticles, magnetic nanoparticles, metal nanoparticles and metal oxide nanoparticles). Enzymatic immobilization have been performed with enzymes like lysozyme, glucose oxidase, amino-peptidase and alcohol dehydrogenase on Au and Ag nanoparticles. Catalytic efficiencies were remarkably high when fumed silica nanoparticles were used for the immobilization of enzyme from *Candida antarctica* lipase B (CALB) and S. *Carlsberg* for its application on non-aqueous media (Cruz *et al.*, 2009). For paraoxon sensing, the acetylcholinesterase enzyme was immobilized onto magnetic glasses based on iron oxide/silica (Won *et al.*, 2010). Later the same enzyme, acetylcholinesterase was tried on nickel nanoparticles which showed a high detection sensitivity for organophosphate pesticide (Ganesana *et al.*, 2011). The amino-functionalized Fe$_3$O$_4$@SiO$_2$ nanoparticles were used to construct glucose biosensor where ferrocene monocarboxylic acid is covalently bonded to nanoparticles as the building block of biosensor (Qiu *et al.*, 2007). When glucose is added to the medium the steady state current of the biosensor reached to 95% within 10s. According to Ahmad and Sardar (2015), the enzyme immobilized on TiO$_2$ nanoparticles showed accelerated activity than the free counterpart. It was also reported that the enzyme in its soluble form showed reduced stability at higher temperature than the immobilized counterpart.
Numerous nanoparticles were prepared using different elements such as silver, gold, copper, silica, carbon and iron. But iron nanoparticles possess an additional advantage of easy separation and low cost to be used as a support carrier.

2.10.1 Iron nanoparticles

Iron nanoparticles are the new generation impetus for the remediation of environmental pollutants providing a cost-effective solution (Masciaglioli and Zhang, 2003). Low cost, eco-friendly, non-toxic nature, durable and magnetic strength have made it popular among all other nanomaterials. Magnetic nature provides an easy separation of the material from the treatment site. Iron oxide nanoparticles have been widely used in many fields due to its magnetic property. Few to be mentioned are magnetically assisted drug delivery (Riedinger et al., 2013), separation of biochemical products (Majewski et al., 2013) and enzyme immobilization (Chang and Tang, 2014). Iron nanoparticles can be easily modified with functional groups or inorganic compounds (Ansari and Hussain, 2012). Henceforth, they play a promising role in enzyme immobilization as a magnetic carrier. Bio-desulfurization activity has been observed by coating magnetite nanoparticles on dibenzothiophene-desulfurizing bacterial strain *R. erythropolis* LSSE8-1 and *P. delafiedelii* (Li et al., 2009). Coating with ammonium oleate modified magnetite nanoparticles showed repeated bio-desulfurization activity. The collection of these magnetite nanoparticles is very easy and done by using an external magnet.

The iron nanoparticles are prepared by varied types of methods such as water-in-oil microemulsion, polyol, co-precipitation, sol-gel, sonolysis and hydrothermal methods. Every method possesses specific procedure and conditions that results in nanoparticles of different properties (shape, average size, size distribution, crystallinity, magnetic properties, dispersibility, etc). Among these methods, the co-precipitation method is the commonly used methods due to numerous advantages associated with this method. Advantages of co-precipitation over other methods include cost effectiveness, good homogeneity, high purity of product and no requirement of heat treatment and organic solvents. Recently, co-precipitation method has been developed
for preparation of magnetite nanoparticles using metal-organic precursors (Nazari et al., 2014). Other methods are associated with drawbacks such as water in oil microemulsion technique is very expensive as it involves compressed CO₂. Further the method also suffers from low production yield (Zielińska-Jurek et al., 2012). The sol-gel method generates 3D oxide networks among nanoparticles so it has a limitation of forming independent and disconnected nanoparticles (Hasany et al., 2012). The hydrothermal process suffers from complexity in process control and is non-reliable involving high reaction temperature and pressure (Umer et al., 2012). Co-precipitation method suffers from the problem of agglomeration because of its small size, more surface area and high energy. Property also gets affected by the reaction temperature, alkali used and the type of emulsifier used (Zhao et al., 2008). To overcome the problem of agglomeration capping agents were used such as oleic acid and particles were also coated using chitosan. Chitosan is the choice of material for coating as it provides functional groups for enzyme immobilization, environment friendly and its wide availability. Chitosan coating prevents the agglomeration of particles. Larger surface area for contaminant degradation is the main reason for oxidation of nanoparticles. Oxidation caused a high rate of aggregation and ultimately nanoparticles get stuck on the pores of the material. So, researchers were trying to use various suitable stabilizers like chitosan, carrageenan (Reddy et al., 2012), guar gum (Tiraferri et al., 2008), polyacrylate (Schrick et al., 2004) etc. to coat or stabilize the nanoparticles to prevent the oxidation (Reddy et al., 2012).

2.11 Magnetic iron nanoparticles in pollutant degradation/removal

Magnetic iron nanoparticles (MNPs) are widely explored in pollutant removal and degradation. Some of the areas where MNPs were widely explored are discussed below.

2.11.1 Dye degradation

Many industrial effluents contain dyes whose degradation products are stated as mutagenic and extremely carcinogenic. They cause food chain contamination, resulting
in adverse effects on human and animal health due to their long half-life in water reservoirs (Kaur et al., 2014). Previously, scientists have mostly focused and reported successful role of MNPs for the removal of dyes and phenolics from waste water. Qadri and his coworkers (2009) reported that within the first 20 min of contact time γ-Fe$_2$O$_3$ was able to remove 98% of the Acidine orange dye from the solution. Weng et al. (2010) reported that new coccine (acidic dye) was removed efficiently from a solution using Fe$_3$O$_4$ nanoparticles. The adsorption capacity of new coccine decreased to 35% of its original capacity after 5 regeneration cycles in the test of regeneration. The removal efficiency of Procion red using Fe$_3$O$_4$ nanoparticles was 24.4 % and adsorption capacity was 30.5 mg/g within 30 minutes contact time under room temperature (Hariani et al., 2013). Subsequently, Fe$_3$O$_4$ nanoparticles were also reported to remove dyes viz. Congo red [CR (azo dye)], Coomassie Brilliant Blue R-250 [CBB (anionic dye)] and Acidine orange [AO (cationic dye)] from the aqueous solution by Chaudhary et al. (2013). The maximum adsorption capacity was 0.056, 0.082 and 0.078 mg/g for CR, CBB and AO, respectively. Further studies conducted by Mahmoodi et al. (2014) used Ferrite MNPs for the removal of dye viz. direct red 31 and observed $q_{\text{max}}$ of 0.271 mg/g.

Modified iron nanoparticles were also used for the removal of dyes. The experimental results using Prussian-blue (PB)-modified γ-Fe$_2$O$_3$ magnetic nanoparticles (PBMNPs) showed efficient removal of the target compound from the solution over a wide range of pH from 3 to 10. In the presence of PBMNPs, peroxidase acts as a catalyst and H$_2$O$_2$ as an oxidant. Under optimal conditions, after 120 min of reaction at 298 K complete removal of Methylene Blue (MB) dye was observed. Around 53.6% and 35% chemical oxygen demand (COD) and the total organic carbon elimination efficiency, respectively were observed during the process (Wang and Huang, 2011). Iram et al. (2010) stated that Fe$_3$O$_4$ hollow nanospheres are an efficient sorbent showing highest adsorption capacity of 90 mg/g for red dye.

2.11.2 Role in wastewater treatment

Due to unique properties of iron oxide nanomaterials, they received severe attention from the scientific community for their role in environmental remediation. Researchers
have proposed several clean-up methods for the treatment of wastewater where iron oxide nanomaterials were practically used as nanosorbents and photocatalysts. In addition, immobilization technology for enhanced removal efficiency using iron oxide nanomaterials tends to be a novel investigative point (Huang et al., 2003; Sulek et al., 2010).

Hu et al. (2005) evaluated the role of maghemite nanoparticles for chromium recovery from the wastewater. The maghemite particles could remove chromium at an optimum pH 2.5. In the following year Hu et al. (2006) reported that maghemite nanoparticles prepared using sol-gel method can efficiently remove heavy metal from the wastewater generated in electroplating industry. The industrial wastewater contained Cr(VI), Cu(II) and Ni(II.) In the year 2009, Afkhami and Norooz-Asl investigated maghemite (γ-Fe₂O₃) nanoparticles for the removal of Mo(VI) from water and wastewater. Maximum adsorption of Mo(VI) was observed within the pH range 4 to 6. Nassar et al. (2014) reported in their study that γ-Fe₂O₃ nanoparticles were successful and effective in dephenolization, decolourization and COD reduction from synthetic and diluted real olive mill wastewater (OMW). The equilibrium of the solution was achieved within 10 minutes for phenol and 30 minutes for COD removal. The adsorption rate was fast enough. Another study conducted by Nassar et al. (2015) evaluated the role of magnetic iron nanoparticles (γ-Fe₂O₃) for adsorption of dye from synthetic and real textile water. Equilibrium was achieved in less than 125 min and adsorption was fast for dyes (crystal violet and bromocresol green) in real textile wastewater.

2.11.3 Role in pesticide degradation

Fang et al. (2012) have reported the successful degradation of pesticides like 2,4 Dichlorophenoxyacetic acid (2, 4-D) using magnetic nanoparticles. Remediation of surface contaminants like petrochemical compounds and even sub-surface contaminants like pesticides, organic solvents, fertilizers and heavy metals can be done using iron nanoparticles. Due to their efficient role in environmental remediation the iron nanoparticles were further evaluated for their role as a carrier for enzyme immobilization. Singhal et al. (2012) studied the role of zero valent iron nanoparticles in the remediation of soil contaminated with malathion. They reported that 0.1 g of zero
valent nanoparticles are efficient to degrade one kg of soil containing 10 µg/g of malathion. In the year 2009, Elliott and his colleagues evaluated lindane degradation using zerovalent iron nanoparticles and observed that within 24 h of incubation, 0.015-0.39 g/L lindane was removed efficiently from the aqueous solution. In the same year zerovalent iron nanoparticles were explored for remediation of water contaminated with alachlor and atrazine by Bezbaruah et al. (2009). In their study, they observed that atrazine could not be degraded while within 72 h incubation, 96% alachlor was removed with an initial concentration of 40 mg/L. Zerovalent iron nanoparticles were also explored for remediation of chlorpyrifos contaminated soil (Reddy et al., 2012). They observed 90% remediation when the chlorpyrifos contaminated soil was incubated with zerovalent iron nanoparticles for 10 days while micro zero-valent iron nanoparticles achieved only 32% degradation.

2.12 Role of enzyme immobilized on iron nanomaterial in pollutant degradation

An attempt was made to immobilize α-amylase enzyme on magnetic poly (2-hydroxyethyl methacrylate-N-methacryloyl-( I)-phenylalanine) nanoparticles (Uygun et al., 2012). The particles showed increased substrate affinity and high specific activity of 85% even after 10 times of reuse. In another study, Khoshnevisan et al. (2011) obtained very less activity of the immobilized cellulase on magnetic nanoparticles. Temperature influences the activity of enzymes. Hence, when the temperature was increased to 80°C the activity of the enzyme also increased to 0.1 unit (µmol/min mL).

Laccase enzyme immobilized Fe₃O₄/SiO₂ nanoparticles were reported to remove more than 80% color of the dyes (Procion Red MX-5B and azophloxine) within one hour of incubation time (Wang et al., 2013). At pH 5.0 and temperature 55 °C, 95% (0.02 mmol L⁻¹) of Rhodamine B (RhB) was removed within 15 min due to catalysis of reaction by the ultrasound assisted breakdown of H₂O₂ using Fe₃O₄ MNPs. Around 6.5 and 37.6 folds increased activity was achieved by catalytic H₂O₂-Fe₃O₄ system and ultrasonic US-H₂O₂ systems, respectively (Wang et al., 2010). A study conducted by Sadighi and Faramarzi (2013) reported immobilization of chitosan nanoparticles (CS-NPs) is being facilitated on the surface of activated glass beads. Direct attachment of nanoparticulated immobilized laccase enzyme on the surface of the glass beads
resulted in the decolorization of a harmful industrial dye, congo red. Even after 25 successive cycles, 98% of initial decolorization activity of the nanoparticulated immobilized enzyme was sustained. Degradation of phenolic azo dyes were accomplished by laccase immobilized on Fe$_3$O$_4$/SiO$_2$ nanoparticles with particle size below 30 nm through glutaraldehyde coupling. After immobilization of laccase enzyme onto Fe$_3$O$_4$/SiO$_2$ nanoparticles, the operational stability for phenolic azo dye decolorization (Procion Red MX-5B and azophloxine) using laccase improved remarkably. For each dye, around 80% removal of colour was observed within 1 h (Wang et al., 2013). Jofenek, and Zajoncová, (2015) also reported decolorization of direct blue 78, acid blue 225, reactive red 195, acid blue 74 and phenol red dyes using laccase immobilized magnetic iron nanoparticles.

In the same year, degradation of 2,4-dinitrophenol with laccase immobilized on nanoporous silica beads was studied. Around 90% of 2,4-DNP was degraded within 12 h of treatment with immobilized laccase. The stability of the immobilized enzyme was maintained more than 85% of initial activity even after 30 days of storage (Dehghanifard et al., 2013). Likewise, degradation rate of phenol in coking waste water was 2-fold higher for immobilized laccase. Even after 10 successive batch treatments, 71.3% of its initial degradation ability was retained by the immobilized laccase (Wang et al., 2012). Other enzymes such as manganese peroxidase (MnP) is being used for the degradation of polycyclic aromatic hydrocarbons by immobilizing the enzyme on nanoclay. The immobilized enzyme degraded pyrene (>86%), anthracene (>65%), fluoranthene (<15.2%), phenanthrene (<8.6%) individually and in mixture. An increased stability to high temperature, pH, storage period and PAHs degradation was achieved in soil treated with immobilized MnP (Acevedo et al., 2010). Enzyme gycerophosphodiesterase (GpdQ) isolated from Enterobacter aerogenes was evaluated for its role in organophosphate degradation (Daumann et al., 2014). They reported that the enzyme showed a specific activity of 3.55 μmol/mg/min immediately after immobilization and 3.39 μmol mg$^{-1}$ min$^{-1}$ when incubated with the substrate bis(4-nitrophenyl)phosphate (BPNPP) for a period of one week. The specific activity
remained as 3.36 μmol mg⁻¹ min⁻¹ even after 120 days of use indicating the sustained activity of the GpdQ immobilized enzyme after multiple cycles and long storage period.

2.13 Summary: The insight attained from the literature review indicated that pesticide usage since green revolution has created a havoc in the environment, ultimately affecting the living beings. Organophosphates are most commonly used pesticide after the ban on organochlorines pesticides. Chlorpyrifos is the most commonly used pesticide among organophosphate pesticides because they are applied on numerous vegetables and crops for the purpose of pest control. Microbial remediation, an economical and eco-friendly method was considered to be a most suitable method for chlorpyrifos degradation. Enzymes which are the key players in microbial degradation can facilitate faster and effective degradation of chlorpyrifos. Among the several enzymes being exploited for pollutant degradation, laccase was most preferred by researchers due to its easy availability and broad substrate specificity. However, unlike other enzymes laccase also suffered problems of reusability, stability, high cost for purification. Immobilization on suitable carriers could solve the problems of reusability but selection of suitable and successful carrier is again a huge problem. Nanomaterials having a wide spread application in various field can provide a solution to problems associated with carriers.

Low cost, easy availability, easy separation with limited mechanical stress on enzymes and reusability made magnetic iron nanoparticles an attractive carrier for enzyme immobilization. Previous studies showed that the magnetic iron nanoparticles can serve as a catalyst for pollutant remediation and carrier for enzyme immobilization with an application in various fields. To summarize, nanoparticles can contribute significantly and take the degradation to the next level with laccase enzyme. Thus, the present study will evaluate the potential of laccase enzyme immobilized on magnetic iron nanoparticles for chlorpyrifos degradation.