POTENTIAL ORGANOPHOSPHATE DEGRADING BACTERIA AND THEIR GENETIC VARIABILITY

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ENVIRONMENTAL SCIENCES

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CERTIFICATE

This dissertation submitted by **Ms. Arish Naseem** is accepted in its present form, by the Institute of Environmental Sciences and Engineering (IESE), School of Civil and Environmental Engineering (SCEE), National University of Sciences and Technology (NUST), Islamabad as satisfying the requirement for the degree of Masters of Environmental Sciences.

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DEDICATED….!!!

To my ever loving Parents

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Arish Naseem

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LIST OF ABBREVIATIONS

OD	Optical Density
rpm	revolutions per minute
mg/L	milligrams/litre
ml	millilitre
µL	microliter
GC	Gas Chromatography
16S rRNA	16S ribosomal ribonucleic acid analysis
LD	Lethal dose
CoA	Coenzyme A
Opd	Organophosphate degrading gene
WHO	World Health Organization
˚C	Degrees Celsius
LB	Luria Bertani
w/v	Weight by volume
EDTA	Ethylenediaminetetraacetic acid

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ABSTRACT

Profenofos an organophosphate, is a widely used pesticide for agricultural purposes which poses a great threat to entire food chain. Environmental and public health effects of profenofos due to soil, water and environmental contamination require remedial measures. Biodegradation offers the most viable solution for remediation of pesticide contaminated sites. Current study was carried out to isolate the bacterial strains capable of degrading profenofos effectively. Total fourteen pesticide degrading bacteria were isolated from agricultural soil by enrichment culture technique, two potential degrading strains were screened out. Both strains were able to grow at wide pH (6-9) range but showed growth variation at different temperatures (30, 37 and 45°C). Identification of potential strains were carried out using 16S rRNA analysis which identified the strains as *Pseudomonas aeruginosa* and *Pseudomonas putida.* Organophosphate degrading gene was found to be present in both isolates. Profenofos was utilized as second preferred carbon source by both isolates after glucose whereas lactose and methanol exhibited minimum effect on bacterial growth. Degradation studies were undertaken in bench scale reactors and percentage removal of profenofos was determined through Gas Chromatography. At profenofos concentration of 150 mg/L, degradation efficiency of individual isolates and consortium was found to be 69.9, 49.8 and 79.3% respectively within 96 hrs asserting that the efficiency enhanced when consortium of two isolates was inoculated. This study may play a significant role in onsite bioremediation of pesticide industry waste as well as contaminated fields.

Chapter 1

INTRODUCTION

Pests are the biological agents which contribute a lot in destroying the crops all over the world. The serious losses in crop production demand for a treatment system which not only kills harmful pests but also contribute to increase in yield along with fertilizers. Therefore, researchers have formulated chemical agents called Pesticides which not only control pests in the field but also reduce crop losses due to major pest attacks (Sheikh *et al.,* 2011).

As the population in the world is increasing so the need for more production which directly implies more application of fertilizers and pesticides in the field. It continued many years till the time when application of pesticides rendered no use in the field rather contributed to significant losses in production (Cerejeira *et al.,* 2003).

The use of pesticides has been considered as the most cost-effective and conventional method for pest control. Pesticides are classified into organophosphates, carbamates, organochlorine and pyethroid on the basis of their structure (USEPA, 2014). And about 38% of pesticides used all over the world are organophosphates (Cycon *et al.,* 2013).

Organophosphates are potent cholinesterase inhibitors as well as chemical warefare agents (Iyer *et al.,* 2011; Eddleston *et al.,* 2002). Cholinestrase inhibitors are the neurotoxins that inhibit acetylcholinestrase to reach its activation site and perform its action. In simple

words, the affected neuron fails to return to its resting state after activation resulting in paralysis, coma and ultimately death of insect (Aardema, 2008).

Approximately only 0.1% of pesticide is used in killing the pest and the rest ends up in the environment. The most important environmental problem caused by pesticides is their effect on soil as a function of their persistence and mobility (Walker, 2003). Pesticides not only pollute the soil and water courses but also accumulate on the fruits and enter the food chain casting drastic effects on the higher trophic level species. In addition, these also contribute to environmental pollution, losses in biodiversity and deterioration of the natural habitats (Cerejeira *et al*., 2003).

Pesticide provides a cover to farmer in terms of protection of his investment in seeds, irrigation, fertilizers and surely protection from insects and pesticides. Whereas the over anxiety of farmer to get more production and lack of proper knowledge often leads to indiscriminate use of the pesticides which causes acute and chronic effects on surrounding environment and human health (Kanekar *et al*.,2004).

Main issues regarding environmental pollution as well as effects on human health originate at the local level which is unfortunately the least literate class of the country (Kidd *et al.*, 2001). The mishandling of pesticides contribute as a threat to environmental and public health at local level but also effects the environment globally from region to continent and ultimately the global effect (Huber *et al.*, 2000).

Other than these effects there have been reported evidences of pest resurgence, resistance to the applied pesticide along with additional secondary outbreaks due to application of same group of pesticides over long periods in the field (Sattler *et al.*, 2007).

In general, organophosphates are considered safe for the crops due to their degradation properties depending upon the microbial population availability, appropriate pH, temperature, hydrolysis, photolysis, oxidation and reduction (Ragnarsdottiv, 2000). On the other hand, health effects of organophosphates include hepatic dysfunction, neurotoxic effects, carcinogenesis, oxidative stress and chronic toxicity (Hashmi *et al.*, 2004).

The pesticide selected for the study is Profenofos which belongs to organophosphates. It is used in cotton fields as well as on various vegetables like potatoes and chilli. Profenofos is a non-systematic insecticide used against mites, leafhoppers, thrips, aphids, bugs and cotton strainers. It is also found to be toxic to birds, honey bees, fish and macro-invertebrates (Akerblom, 2004). Profenofos serves as potential contaminant in aquatic and terrestrial ecosystem as well as it is harmful to human health. Moreover its residues have been found on vegetables and foods (Radwan *et al.*, 2005). Due to its harmful effects on the ecosystem and public health, it has become necessary to device a technique for degradation of profenofos.

Biodegradation offers the most viable option for pesticide degradation by employing indigenous microorganisms. The microorganisms unlike plants and animals which only detoxify the pesticides, utilize pesticide by degrading it to nutrients and energy source. This process is enzyme-specific. The fact is that organophosphates target the nervous system of insects which is absent in microorganisms so they easily utilize it as carbon and energy source. Among microorganisms, bacteria always take the lead in degradation because of a large variety of enzymes and metabolic pathways (Copley, 2009). Additionally, biodegradation is an environment friendly approach which can occur in variable environmental conditions (Latifi *et al*., 2012; Karpouzas and Walker, 2000).

1.1 Objectives

The objectives of present study were:

1. Isolation and characterization of organophosphate degrading bacteria from pesticide contaminated soil
2. Determination of genetic diversity of profenofos degrading bacteria through Polymerase Chain Reaction
3. Determination of pesticide degradation potential of isolates using Gas Chromatography

Chapter 2

LITERATURE REVIEW

Agriculture is the only sector responsible for provision of food to ever increasing population and pest attack is the major hazard in fields destroying about half of the world's food crops. This situation calls for the intensive pest control (Sheikh *et al*., 2011). On one side, pesticide application has decreased crop losses but on the other, dreadful effects in form of environmental degradation and public health are evident.

2.1 Pesticide formulations

Pesticide is composed of two components; one is active ingredient which is the actual component responsible to attack the pest and kills it. Whereas other part is inert (non-reactive), its purpose is to carry the active ingredient and make its application easier by the farmer. Majority of pesticides are available in formulations which are prepared by addition of solvents, adjuvants and fillers required as per necessity to achieve the desired composition. Pesticide formulations are majorly formed to ease the application but it tend to influence deposition on soil and plant. In addition, the formulations also determine the runoff and infiltration rate of pesticide based on its affinity with soil and water (Agrawal *et al*., 2010).

2.2 Organophosphates

Organophosphorus pesticides are most popular candidates in pesticides as these replaced the more persistent organochlorine pesticides suspected to be bio-accumulated in the food chain (Zamy *et al.*, 2004).

Organophosphates are esters or thiol derivatives of phosphoric, phosphonic and phosphoramdidic acid **(**Singh and Walker, 2006). About 38% of pesticides used all over the world are organophosphates (Cycon *et al.*, 2013). Generalised structure of organophosphate is given in Figure 2.1.

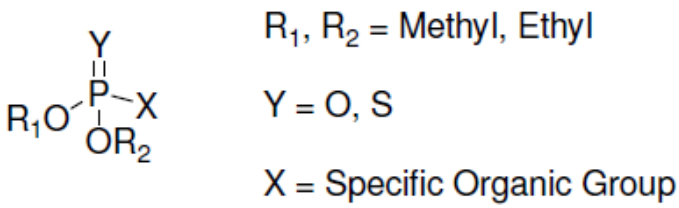


Figure 2.1 General structure of organophosphate

Generally organophosphates are used to control a range of pests including spider mites, aphids, sucking, chewing and boring insects, pests that attack fields of crops like cotton, peanuts, sugarcane, vegetables, tobacco and fruits. These have been marked as world's largest agrochemical companies (Porto *et al.*, 2011).

2.3 Mode of action of organophosphates

Organophosphate acts by inhibiting neurotransmitter acetylcholine breakdown. Acetylcholine is required for the transmission of signals from brain to muscles and other areas of body (Toole and Toole, 1995). After nerve impulse is transmitted, acetylcholine

must be hydrolysed to avoid the overstimulation of nervous system. This breakdown is catalysed by an enzyme, acetylcholinesterase. Acetylcholinesterase converts acetylcholine into choline and Acetyl CoA by forming an enzymatic complex at serine 203 which is active site of enzyme. Further, choline is released from complex and rapid reaction of acylated enzymes with water to produce acetic acid and regenerate acetylcholinesterase enzyme. It has been predicted that one acetylcholinesterase enzyme can hydrolyse 300,000 molecules of acetylcholine in a minute (Ragnarsdottiv, 2000).

Organophosphates inhibit the activity of acetylcholinesterase by covalently bonding to enzyme, hence changing the structure and function of enzyme (Singh and Walker, 2006). This phenomenon is evident in Figure 2.2.

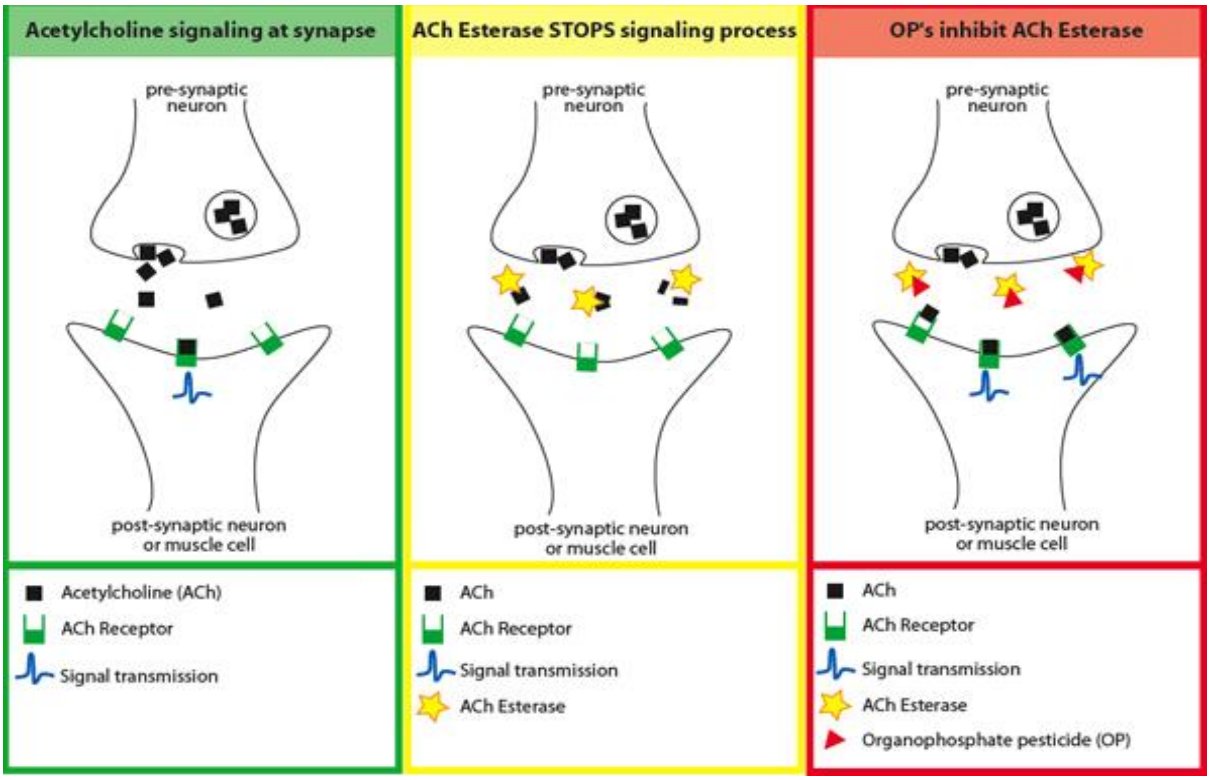


Figure 2.2 Normal transmission of nerve impulse and action of acetylcholine esterase followed by inhibition by organophosphate

Another important effect of organophosphates is that these can cause delayed neurotoxic effects which are not due to acetylcholinesterase inhibition. In the presence of organophosphates, these enzymes are inactivated. Once 80% of enzyme is inactivated, usually within four days of exposure, lethal symptoms become observable including neck muscle weakness, diarrhoea and respiratory depression in human beings (Ortiz-Hernandez *et al*., 2010).

2.4 Prevalence of organophosphates in Pakistan

In Pakistan, pesticide use dates back to 1960's which continued to increase over years. In Sindh and Punjab, major pesticide use is on cotton and rice followed by fruits and vegetables. Whereas in Kyber Pakhtunkhwa (KPK) and Baluchistan, pesticide use is confined to fruits and vegetables followed by maize in KPK and cotton in Baluchistan. Out of total pesticide use in Pakistan, insecticides comprises 90% followed by herbicides 7%, fungicides 3%, acaricides and fumigants 0.2% (Khan *et al.*, 2010).

During last two decades, there has been a significant increase in use of pesticides in volume as well as value. Its use has been increased by 70% (out of which 80% is used on cotton) whereas cotton yield has improved by only two folds. On the other hand, the value of pesticide exceeded over Rs. 12-14 billion which added to cost of production (Khooharo *et al*., 2008).

2.5 Environmental contamination by organophosphates

Extensive use of organophosphates has led contamination of several ecosystems worldwide (Tse *et al.,* 2004). According to Loloei *et al.,* (2014), the pesticides can pollute the ecological ecosystems by inappropriate disposal of empty pesticide containers, inadequate

use of safety equipment and spillage of pesticides residues into water canals of adjacent rivers.

2.5.1 Pesticides cycle

Major proportion of the pesticide applied remained unused and finds its way to air, soil and water by processes of air drifting, vaporization, absorption, adsorption, surface runoff and infiltration processes. By these means pesticides renders harmful effects on plants, people, pets as well as environment. Moreover, not only farmers practicing cultivation use pesticides, about more than half of pesticides are also used in urban areas in homes, gardens, schools, business areas and hospitals in form of insect repellents or insecticides. It is also reported that a larger proportion of pesticides enter in aquatic ecosystem and accumulate in fish and aquatic organisms hence enter the food chain as well as bio accumulate and bio magnify within food chain worsening the public health threat (Agrawal *et al.,* 2010). Pesticide contamination starts locally but its effects keep on adding when it travel to the air, soil and water and contaminate the region. Hence environment is degraded as whole including pollution of atmosphere and also reflected globally from region to region and continent to continent depending upon the volume and frequency of applied pesticides (Cerejeira *et al.,* 2003).

Surveys conducted by Boucard *et al.* (2004) revealed that 100% of sampled catchment areas in Scotland and 75% of sampled aquatic sites in Whales were contaminated with organophosphates used in sheep dips (insecticides and fungicides formulations used by farmers to protect their sheep from parasites). Whereas, Ahad and fellows (2000) detected dichlorvos, endosulfan, profenofos, fenitrothiom, chlorpyrifos, methyl parathion,

dimethoate, mevinphos in groundwater of Mardan Division, Khyber Pakhtunkhwa, Pakistan due to leaching and spillage into water resources.

Jabbar and his fellow researchers (1993) also have found monocrotophos, cychalothrin, dimethoate, fenvalerate, cypermethrin and profenofos in top layer soil at Samundri, a cotton growing area. Furthermore, Anwar *et al.* (2014) affirmed the presence of dichlorvos, dimethoate, methyl parathion, fenitrothion, endosulfan, mevinphos, chlorpyrifos and profenofos in soils of cotton growing areas in Bahawalpur, Punjab.

Sheikh and his co-workers (2011) reported that pesticides not only pollute soils and waters rather these persist and accumulate on fruits and vegetables as well and enter the food chain in addition to environmental pollution, biodiversity losses and natural habitat deterioration. Radwan *et al.* (2004) proclaimed the persistence of profenofos and primiphos-methyl residues on green pepper and eggplant fruits.

2.5.2 Effect on non-target organisms and resurgence

Pesticides are designed by keeping in view the general insects and pests therefore these are not species specific. However, their application methodologies can be controlled that these chemicals come in contact with only target species by avoiding the non-target ones. Talking in terms, these target species also belong to animal kingdom to which non-target species belong. They share some similar characteristics in which susceptibility to toxins is included. One chemical when proved to be toxic to one animal form then it is toxic to other organisms as well (Munkittrick, 2005).

Overuse and misuse of pesticides results in the killing of non-target organisms, development of resistance in pests against pesticide (Khan *et al*., 2010**).** There have been

reported evidences of pest resurgence; resistance to the applied pesticide along with additional secondary outbreaks due to application of same group of pesticides over long periods in the field (Sattler *et al.,* 2007).

2.6 Effects on public health

Larger doses of pesticides are lethal to human beings but smaller doses which are sprayed in fields are enough to act as endocrine disruptors. These cause disruption in sex hormones and hence reproduction defects. Thus pesticides act as xenohormones (mimicking the endogenous hormones) as well as interfere in the endocrine processes (Agrawal *et al.*, 2010).

Pesticide residues on vegetables and fruits can cause illnesses like kidney, stomach diseases, skin and eye infections as well as central nervous system related problem (Loloei *et al*., 2014). The routes of entry in human body includes ingestion, inhalation and adsorption. Among these, adsorption through the skin is most common method of pesticide entry. Pesticides are also found to be cause of children lymphoma and cancer (Flower *et al.*, 2004).

Self-poisoning in developing countries due to occupational exposures is regularly reported in agricultural areas (Eddleston, 2008). Due to self-poisoning 200,000 deaths occur worldwide in developing countries out of 3 million poisoning cases due to accidental or purposeful exposure of organophosphates (Aardema *et al.,* 2008). Imran and his colleagues (2013) has reported 145 registered organophosphate poisoning cases in Sargodha in June 2011-July 2012. 60-70% cases were of suicidal ingestion with mean age of patients to be

27 years. Similarly in Turkey, the suicidal organophosphate poisoning rate is reported to be 68% (Sungar and Guven, 2001).

Sheikh and his colleagues (2011) proclaimed that 27 different pesticides were widely used in Sothern Sindh, Pakistan on crops like corn, vegetables, potatoes and cotton. These pesticides included carbamates, nicotinoids, organophosphates and pyrethroids. Majority of pesticides were with moderately toxic or highly toxic according to WHO (2004). The findings of the research revealed that most of the farmers were not educated and applying pesticides on fields without safety measures. Moreover they were not aware of harmful effects of these pesticides on their health as they lacked appropriate knowledge of safe handling and application of these chemicals and hence were subjected to a number of respiratory diseases including asthma. The findings represent a sample of agriculture conditions on Pakistan and other developing nations where mostly farmers are not educated. There must be trainings of farmers regarding safe handling of pesticides and they should be advocated to shift towards organic farming. In addition they should not use pesticides at all or use it in minimal proportions so that effects on public health and environment could be minimized.

2.7 Possible treatments for organophosphates

In third world countries like Pakistan, the insecticides and agricultural pest management remains a major concern which calls for economical and dependable methods for organophosphate detoxification from the environment. Current methods mostly rely on chemical treatment, incineration and landfills. Chemical treatments are feasible but are problematic due to production of larger volumes of acids and alkalis which are subsequently more toxic and need to be disposed of. Incineration on the other hand

contributes a lot to environmental pollution by production of toxic fumes which in turn need to be controlled. In case of landfill which adequately serves the purpose, production of toxic leachate which contaminates groundwater calls for another treatment procedure (Sharaf *et al.*, 2007). Furthermore the suggested methods are expensive and often difficult to execute especially in extensive agricultural areas (Jain *et al*., 2005).

2.8 Biodegradation- a viable solution

Microorganisms have the ability to degrade or transform the toxic contaminants into relatively less toxic or completely non-toxic compounds (Mishra *et al*., 2001). The ability of these organisms to degrade the contaminants is a result of their long term adaptation to the environment where these chemicals exist (Porto *et al.*, 2011). One promising and most feasible method of pesticide removal is to exploit the ability of soil microorganisms to remove the contaminants from polluted sites. This alternative strategy is not only effective rather it is insignificantly hazardous, economical, adaptable as well as environment friendly (Finley *et al*., 2010).

It has been proved by a number of researchers that a fraction of soil microorganisms develops mechanism to utilize the applied pesticide as source of carbon and energy resulting in rapid degradation of applied chemical. This phenomenon known as enhanced or accelerated biodegradation (Walker and Suett, 1986). The phenomenon was first reported in 1971 (Sethunathan and Pathak, 1971) followed by extensive researches in the field in mid-1980's including isophenphos (Chapman *et al.,* 1986), fenamiphos (Stiriling *et al.,* 1992) and ethoprophos (Karpouzas *et al*., 1999).

2.8.1 Factors effecting biodegradation

Biodegradation depends on number of factors including soil properties, composition of soil micoflora, use of pesticides on the soil (soil or foliage applied), rate of use of pesticides, interval between consecutive applications and stability of soil microorganisms without existence of pesticides (Kaufman *et al*., 1985).

Houot and his co-workers (2000) proclaimed soil pH as a primary factor contributing towards the enhanced biodegradation of atrazine. This hypothesis has been supported by a number of investigations which revealed that at higher pH there is high enzymatic activity (Acosta-Martinez and Tbatabai, 2000). Sim *et al.* (2002) has also suggested that soil pH may influence the uptake of pesticide by soil microorganisms.

2.8.2 Cross-adaptation

Enhanced biodegradation is useful to the environmental health but for the farmers, it proved to be a problem. Pesticide applied to the soil field can be degraded rapidly where it has not been applied formerly but which has been exposed to a pesticide from similar chemical group (Prakash *et al*., 1996). This phenomenon is called as cross-adaptation. It has been reported for a number of pesticide groups for instance, carbamates (Morel-Chevillet *et al.*, 1996), dicaboximides (Mitchell and Cain, 1996) and isothiocyanates (Warton *et al*., 2002). On the contrary, among organophosphates, limited data is available in terms of cross adaptation (Singh *et al*., 2005).

The positive side of cross-adaptation is that microorganism able to degrade one pesticide will be able to degrade other structurally similar pesticides. Hence it can be used for bioremediation of other compounds for which no known degrading microbial system is

known. This system is well reported in case of organophosphates where parathion degrading bacteria were able to degrade a wide range of other structurally analogous compounds including warfare agents (Singh and Walker, 2006).

2.9 Microbial degradation of organophosphates

Several studies have been conducted to combat the harmful effects of pesticides in environment. A major proportion of the research focuses on microbial degradation of organophosphates.

Sharma and co-worker (2014) asserted that *Pseudomonas diminuta, Pseudomonas putida* and *Pseudomonas aeruginosa* can be utilized for efficient degradation of methyl parathion at optimised conditions of 37ºC and pH 8.5. Salinity (0.1-3.5%) and supplement (glucose 1g/L, yeast 0.5 g/L) do not cast any effect on bacterial growth so these species can be effectively utilized to treat methyl parathion contaminated soils.

Peter *et al*. (2014) affirmed that methyl parathion degrading bacteria are present in rhzizospheric soil of cabbage, guava and tomato. The isolates were identified as *Pseudomonas aeruginosa, Bacillus magaterium* and *Staphylococcus aureus*. All isolates were able to degrade methyl parathion up to 350 mg/L concentration at neutral pH and 30ºC.

Vijayalakshmi and Usha (2012) isolated fifty eight different isolates from twenty seven sampling sites having a history of chlorpyrifos use. Among these isolates ten strains were able to show better degradation efficiencies. Isolate CH532 was able to degrade 38% chlorpyrifos in un-optimized conditions and was identified as *Pseudomonas putida.* But when the conditions were optimized, the isolate was able to degrade 76% of 2%

chlorpyrifos at conditions; pH 7, temperature 35ºC, 10 ml inoculum, shaking speed of 150 rpm in presence of 200 mg/L glucose and 300 mg/L yeast within 24 hours of incubation.

Diazinon degrading *Pseudomonas peli*, *Burkholderia caryophyll* and *Brevundimonas diminuta* were isolated from soil and assessed for their organophosphate degrading ability. All these isolates were able to degrade 20 mg/L diazinon in mineral salt media as sole carbon source within 12 days of incubation. The degradation ability of isolates was enhanced when supplemented with 0.5% glucose and maximum degradation rates for these were recorded to be 4.556, 5.367 and 5.885 mg/L/day (Mahiudddin *et al*., 2014).

Chanika and fellow researchers (2011) isolated organophosphate degrading *Pseudomonas putida* and *Acinetobacter rhizopharae*. The strains have successfully hydrolysed Fenamiphos (FEN) which was further transformed by *Pseudomonas putida* solely. *Pseudomonas putida* degraded organophosphates with P-O-C linkage and also carbamaates oxamul and carbofuran proving to be the first wild type bacterial strain able to degrade both organophosphates and carbamates.

2.10 Profenofos

The pesticide selected for the study is Profenofos which is an organophosphate. It is widely used insecticide in cotton fields. It was developed for the bacterial strains which were resistant to other organophosphates. World Health organization has classified Profenofos as moderately hazardous pesticide (toxicity class II), it has moderate order of acute toxicity (WHO, 2004). It acts by inhibition of acetylcholinesterase activity leading to accumulation of acetylcholine in the pest resulting in pest death. Same phenomenon is followed by other organisms varying on dose of profenofos (Fukuto, 1990). The physical characteristics of

profenofos are illustrated in Table 2.1. and chemical structure of Profenofos (Gotoh *et al*., 2001) is given in Figure 2.3. The $logK_{ow}$ value suggests that profenofos bind strongly to the sediments (Tomlin, 1994).

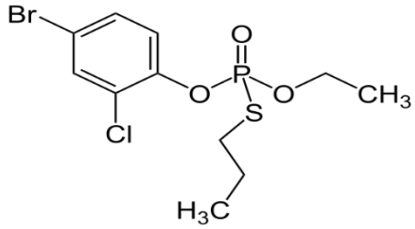


Figure 2.3 Chemical Structure of profenofos

Physical Properties of Profenofos	
Colour	Light Brown
Odour	Weak odour like cooked onion
Solubility in water at 22 ˚C	28 mg/L at pH 6.9
Molecular Formula	$C_{11}H_{15}BrClO_3PS$
Boiling Point	100^{o}C/1.80 Pa
Molecular weight	373.6 g/mol
$logK_{ow}$	4.44 (Tomlin, 1994)

Table 2.1 Physical characteristics of selected organophosphate- Profenofos

Profenofos is considered to be extremely toxic to fish, macro-invertebrates, honey bees and birds. LD_{50} of profenofos for rats is 358 mg/kg, whereas the inhalation toxicity (LC 50) is 3 mg/L. In case of rainbow trout the LC 50 is found to be 0.08 mg/L (Akerblom, 2004). Profenofos has been proved to be highly toxic to euryhaline fish, *Oreochromis mossambicus* (Rao *et al.*, 2003). First reported case of profenofos poisoning was registered in 2000 when an 88 years old women died by consuming 280 ml of 40% emulsified concentration of profenofos (Gotoh *et al.*, 2001). In vitro biotransformation of Profenofos results in desthiopropylprofenofos and hydroxyprofenofos as metabolites (Abass *et al*., 2007).

Malghani and his colleagues (2009) have isolated strain OW having profenofos degrading ability from an agricultural field having a history of pesticide use. The bacterial strain was able to survive at pH 5.5-7.2 with broad temperature profile. The degradation rate of profenofos was determined through gas chromatography. Standard physiological, biological and 16S rRNA analysis identified the strain as *Pseudomonas aeruginosa.* The isolate efficiently degraded profenofos in 96 hours of incubation. The intermediates of profenofos degradation were determined as 4-bromo-2-chlorophenol (BCP) which is hydrolysis product of profenofos Figure 2.4.

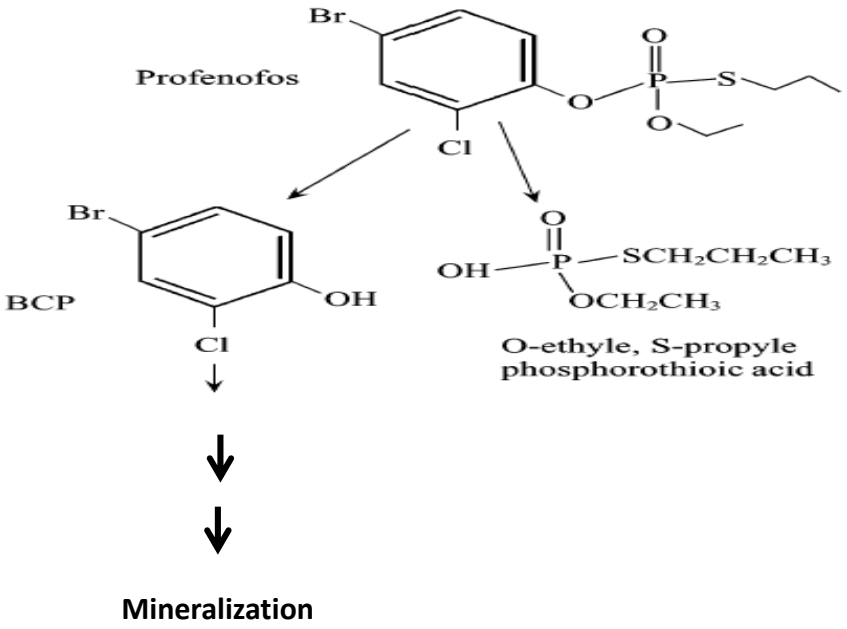


Figure 2.4 Proposed degradation pathway of profenofos (Malghani *et al*., 2009)

Tamilselvan and his colleagues (2014) isolated pesticide degrading bacteria from paddy fields of Tamilnadu, India having a history of pesticide application. The isolates were identified as *Pseudomonas aeruginosa*, *Satphylococcus aureus* and *Bacillus Subtilis.* Growth of isolates was assessed at minimal salt media containing 25 mg/L of Metribuzin and Profenofos. Their efficiency was assessed at different temperatures (25-55ºC), pH (4-8), carbon sources (Lactose, Dextrose, Fructose, Mannose and Galactose) and Nitrogen

sources including peptone, yeast extract, beef extract, malt extract and casein. Maximum bacterial activity was observed at 35ºC and pH 6. Dextrose enhanced the bacterial growth followed by fructose, galactose and mannose and minimal growth was observed in case of lactose. The isolates efficiently utilized malt extract as nitrogen source followed by peptone, yeast extract and casein. The bacterial isolates also exhibited maximum growth in profenofos in comparison to metribuzin.

2.11 Efficiency of consortium in comparison to pure cultures

With the technique of enrichment culture, from contaminated environments, consortia of microorganisms with great ability to degrade persistent and toxic compounds can be obtained (Krishna and Philip, 2009). Consortium has ability to degrade the target compounds more efficiently as competition between microbial populations and metabolites inhibition of specific microbial populations disappears (Krishna and Ligy, 2008). On several occasions, mixed bacterial cultures are isolated having the ability to degrade organophosphates but their individual components were unable to utilize the chemical as a source of energy when purified. For instance fenamiphos (Singh *et al*., 2003).

Pino and Penuela (2011) had done simultaneous degradation of methyl parathion and chlorpyrifos by consortium of *Acinetobacter sp, Pseudomonas putida, Bacillus sp., Pseudomonas aeruginosa, Citrobacter freundii, Stentrophomonas sp., Flavobacterium sp., Proteus vulgaris, Pseudomonas sp., Klebsiella sp.* and *Proteus sp.* The consortium was able to degrade 72% and 39% of 150 mg/L methyl parathion and chlorpyrifos respectively in 120 hours of incubation.

2.12 Genetic and enzymatic basis of organophosphate degradation

Organophosphate degradation is done by various enzymes which are encoded by genes either present on Plasmid or Chromosomes.

2.12.1 *Opd* gene

First organophosphate degrading gene (*opd* gene) was identified in *Pseudomonas diminuta* and was known to be present on plasmid (Serdar *et al*., 1982). Later *opd* gene found in *Flavobacterium sp.* ATCC 27551 was having analogous restriction map as *opd* gene *Pseudomonas diminuta.* The southern hybridization experiments revealed significant similarity among *opd* genes from these two bacteria. This finding of homologous genes on non-homologous plasmids of temporal and genetically different bacteria suggests that gene may be a mobile genetic element or transposon (Mulbry *et al*., 1987). Several other bacteria has also been found to have similar nucleotide sequence (Somara *et al*., 2002). It is hypothesized that the gene was present in environment long before the introduction of organophosphates in the field. The presence of *opd* gene in several bacteria that have never been exposed to organophosphates also support this argument (Richins et al., 1997). Higher number of *opd* genes are observed in higher pH soils (Singh *et al*., 2003). It is worth mentioning that Organophosphate hydrolase (OPH) has optimal activity at higher pH as *opd* gene suggesting role of *opd* gene in phosphate metabolism (Horne *et al*., 2002).

Organophosphates contain three phosphoester linkages and therefore termed as phosphotriesters. In general, the hydrolysis of any one ester linkage can contribute to significant reduction in toxicity of organophosphate. Organophosphate hydrolase can significantly hydrolyze phophoester linkage in organophosphates and reduce their toxicity

(Ningfeng *et al*., 2004). *Opd* genes coding for organophosphate hydrolase (OPH) are identified in *Pseudomonas diminuta* and *Falvobacterium sp.* (Ortiz-Hernandez and Sanchez-Salinas, 2010).

2.12.2 *OpdA* gene

A similar enzyme *OPD*A (Organophosphate deaminase) has been isolated from *A. radiobacter* and was found to be 90% similar to OPH in amino acid and secondary structure level. It is encoded by *OpdA* gene present on the chromosome of bacterium. Despite of similarities, both enzyme utilize different substrates and a major difference is that in OPDA the active site is much larger in comparison to OPH and other difference is in region of protein which is responsible to bind to phenyl ethanol which act as inhibitor for OPH (Yang *et al*., 2003). *OpdA* gene is also transposable as *opd* gene (Horne *et al*., 2003). These observations of transposable nature of *opd* and *opdA* gene supports the idea of widespread distribution of *opd* gene might be due to lateral transfer by transposition and plasmid transfer (Siddavattam *et al*., 2003).

2.12.3 *OPaA* gene

Another gene which has received attention is opaA which encodes OPAA (Organophosphorus acid anhydrolase) enzyme which was isolated and purified from *Alteromonas undina* and is composed of single polypeptide with molecular weight 53kDa (Cheng *et al*., 1997). *OpaA* gene is not functionally similar to *opd* gene as *OPAA* possesses low catalytic activity towards P-O linkage but high activity against P-F bond. Molecular and biochemical analysis of OPAA revealed that this enzyme is prolidase; a type of

dipeptase cleaving the dipeptide bond with a propyl residue at the carboxyl terminal (Cheng *et al.,* 1999).

Present study focuses on biodegradation of profenofos by indigenously isolated bacteria because there is not sufficient data in the literature in this regard. This study employs individual isolates as well as consortium in order to compare the degradation efficiency of individual strains in contrast to consortium. It also aims at detection of gene responsible for profenofos degradation in promising isolates. This research study will help in suggesting a viable consortium combination for biodegradation of organophosphates in the pesticide industry.

Chapter 3

MATERIALS AND METHODS

Biodegradation studies were carried out in bench scale bioreactor system installed in Environmental Microbiology and Biotechnology Research Lab, Institute of Environmental Sciences and Engineering, National University of Sciences and Technology.

3.1 Chemicals and materials

Commercial grade profenofos (Agro Mart) was purchased from market. The common name for profenofos is Prudent.

For analytical studies GC grade ethyl acetate and GC standard Profenofos (PESTANAL@ FLUKA, Sigma Aldrich) were purchased from Paramount Scientific, Pakistan.

3.2 Composition of the media

Sterile glassware and distilled water was used in each experiment. The sterile conditions were ensured by autoclaving the object at 121°C for 15 min. Mineral Salt media was prepared (Table 3.1) and used in degradation studies.

Sr. No.	Component	Quantity(g/L)
1.	Potassium Phosphate Dibasic (K_2HPO_4)	1.5
2.	Potassium Monobasic Phosphate (KH_2PO_4)	0.5
3.	Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	0.2
4.	Sodium Chloride (NaCl)	0.5
5.	Ammonium Nitrate (NH_4NO_3)	1.5

Table 3.1 Mineral Salt Media composition

3.3 Site description

Organophosphates contaminated agricultural fields are widespread in Pakistan. Punjab is an agricultural hub of the country therefore sampling site located in Punjab was selected. The sampling area is located in Northern Punjab where mostly vegetables and wheat are grown by local farmers to be sold in market. Sampling was done from fields located in Hassanabdal.

In sampling area, almost all seasonal vegetables are grown like cabbage, potato, tomato, chilies and spinach.

3.4 Sample collection

Soil samples were collected from three random points in the field in sterile bags from depth of 10 cm and mixed to make a composite. The sample was transported to the lab and processed within 24 hrs of collection.

3.5 Isolation and screening

The sample was sieved through 2mm sieve to remove any gravel and stones. Then 1g of the soil sample was added to 100 ml Mineral Salt Media and incubated for 2 days at 150

rpm/min at 30 ˚C. The supernatant of the Mineral Salt Media was removed and inoculated to three different concentrations (50, 100 and 150 mg/L) of MSM while 0 mg/L was kept as control.

Serial dilutions of all three concentrations were prepared and 100 µL of each dilution was spread on profenofos supplemented nutrient agar plates (50, 100 and 150 mg/L).

After 24 hrs, the plates were observed. Visually distinct bacterial colonies were selected from different dilutions and purified on profenofos supplemented nutrient agar medium by streak plate technique.

3.6 Morphological characterization

3.6.1 Colony morphology

Observing the single colony (derived from a single cell) plays a key role in description and identification of unknown microorganisms. The single colony was picked and streaked on nutrient agar repeatedly until the pure culture was obtained. Colony was observed at each step of purification in terms of color (from naked eye and under microscope), shape, margin, size, texture, elevation and pigmentation.

3.6.2 Cell morphology

Smear was prepared in a drop of distilled water by thoroughly mixing bacterial culture on a clean slide. The slide was air dried and then heat-fixed by passing through flame thrice.

After fixation, the primary stain i.e. crystal violet was applied on the smear for one minute and washed with distilled water. Then the smear was flooded with iodine solution for one

minute followed by washing with distilled water. After that, the slide was treated with decolorizing solution for twenty seconds.

The gram- positive cell wall is composed of peptidoglycan (50-90%) which is a polymer of N-acetyl Glucosamine and N-acetyl muramic acid. This cross-linkage allows the gram-positive bacteria to retain the primary stain whereas the gram-negative cell wall is composed of a thin layer of peptidoglycan (10%) which allows crystal violet to wash-off when rinsed with ethanol (Reiner, 2012).

After treating with decolorizing solution, the glass slide was air-dried and finally counter stain safranin was applied on the smear for 40-45 seconds. The gram- negative cells took Safranin and appeared pink while gram-positive retained the crystal violet and appeared purple.

3.6.3 Motility test

Hanging drop technique was used for motility test. A drop of autoclaved distilled water was placed on the cover slip, and fresh bacterial suspension was prepared with the help of sterile toothpick. Then the cover slip was carefully inverted over the depression and the drop hanged from the cover slip into the cavity slide. Subsequently, the slide was observed under the microscope at 100X to visualize the motility of bacteria.

3.7 Biochemical characterization

Biochemical characterization is a major step in identification of unknown bacterium through different reactions carried out by bacterium in the incubation period. In the present study, API 20E (Analytical Profile Index 20 *Enterobacteria*) kits were used for biochemical characterization of bacteria. The API 20E kits contain twenty microtubes

having dehydrated reagents. The bacterial culture is inoculated in the cupules and color change determines the positive or negative reaction.

Bacterial suspensions were prepared in sterile saline solution (0.85%). With the help of micropipettor, the microtubes were filled with the bacterial suspension. Mineral oil was added on the microtubes labeled as ADH (Arginine dihydrolase), LDC (Lysine decarboxylase), ODC (Ornithinine decarboxylase), H_2S (Hydrogen sulphide production) and URE (Urease) in order to provide the anaerobic conditions to check whether the microbe can utilize the provided material as the source of carbon and energy or not in the absence of oxygen.

After this procedure, the strips were incubated at 37° C for 24 hours and examined visually. For some tests (tryptophan deaminase, indole, voges proskauer, and nitrite production) further reagents were added for the completion of reaction before recording the final result as per manufacturer's instructions.

In case of nitrogen production, zinc dust (2-3 mg) was added in the same microtube of GLU and examined after five minutes. The changed color of microtube i.e. yellow indicated positive result in this case. In contrast, if tube turned orange-red, it is an indication of negative result. API 20E strip is shown in Figure 3.1.

Figure 3.1 API 20E strip for bacterial identification

3.7.1 Catalase test

Catalase is an enzyme produced by bacteria which decomposes the Hydrogen Peroxide into oxygen and water. Usually catalase production is characteristic of aerobes and facultative anaerobes.

The reaction taking place in bacterium by catalase production is reported as

$$2H_2O_2 + \text{Catalase} \longrightarrow 2H_2O + O_2$$

0.3% hydrogen peroxide solution was prepared. A clean glass slide was taken and 18-24 hours fresh bacterial growth was placed onto the slide with the help of sterile inoculating loop. Hydrogen peroxide solution was poured onto the growth. The immediate effervescence (Bubble Production) indicated a positive catalase test whereas no bubble production indicated negative result (Reiner, 2012).

.3.7.2 Oxidase test

Tetra-methyl-p-phenyenediamine dihydrochloride (TMPD) is used which is oxidized by cytochrome oxidase and transformed into a colored product i.e. dark blue or purple indicating presence of cytochrome oxidase also known as indophenol oxidase (Shields and Cathcart, 2012).

The TMPD solution was prepared. Then autoclaved filter paper strips were dipped in the solution and air-dried. Bacterial culture was picked with the help of inoculating loop and rubbed on dried filter paper strips. Change of bacterial culture to blue indicated positive result while no change designated the negative result represented no oxidase production.

3.8 Selection of most efficient bacteria

Out of fourteen isolates, two isolates which were able to show significant growth at highest concentration (150 mg/L) were selected for further studies.

3.9 Physiological characterization of efficient isolates

It includes the identification of optimum temperature and pH for bacterial growth.

3.9.1 Optimum pH

According to Martin and Brewer (2010), the optimum pH for bacterial growth is 7.0 but minimum and maximum pH at which bacteria may grow is 4.4 and 9.0 respectively.

Luria Bertani broth was prepared, adjusted to different pH by using 1N NaOH and1N HCl and poured into test tubes. The test tubes were properly plugged and autoclaved at 121°C for 30 minutes. After autoclaving, the broth was cooled up to room temperature.

Meanwhile the bacterial suspensions were made by suspending loopful of bacterial culture in 1 ml autoclaved distilled water. The suspension was vortexed to ensure equal distribution of bacteria in the water. 50 µl of bacterial suspension was inoculated in six test tubes of varying pH i.e. from 5 to 10 for a single strain. After inoculation the test tubes were incubated in shaking incubator at 37°C and 150 rpm for 24 hours.

The optical densities of bacterial cultures were measured at 600 nm wavelength for varying pH ranges by using UV-Visible spectrophotometer after 24 hours.

3.9.2 Optimum temperature

TLuria Bertani broth was prepared, normalized and poured into three sets of test tubes for each bacterial strain. The test tubes and controls were plugged, autoclaved and allowed to cool-down to room temperature.

Bacterial suspensions were prepared and 50 µl of these suspensions were inoculated in test tubes for each strain. Then the test tubes were incubated at different temperatures i.e. 30, 37 and 45°C for 24 hours. After the completion of incubation period, optical densities of bacterial cultures were recorded by using UV-Visible spectrophotometer at 600 nm.

3.10 Effect of co-substrate on growth of predominant isolates

Bacterial isolates tend to vary in their growth when variable carbon sources have been provided. The growth of bacterial isolates was monitored by providing glucose, lactose methanol and profenofos as carbon sources. These carbon sources were added to Mineral Salt Media at concentration of 2 g/L. The media supplemented with carbon sources and profenofos was inoculated with selected isolates and incubated at 37°C in an orbital shaker at 150 rpm for 72 hours. The growth was measured at regular intervals (0-80 hrs) in terms of optical density using be UV-Visible spectrophotometer (600 nm).

3.11 Polymerase chain reaction analysis (16S rRNA)

3.11.1 Inoculation and washing

The bacterial isolates were cultured on nutrient agar slants and incubated at 37°C for 24 hrs. After stipulated time, the slants were washed by sterile 0.85% NaCl and were stored in eppendorf tubes for DNA Extraction.

3.11.2 DNA extraction

NORGEN Biotek Corporation Soil DNA Isolation Kit (Canada) was used and DNA extraction was done as per manufacturer's instructions.

Lysate preparation

Bacterial suspension was centrifuged at 14000 rpm for thirty seconds in order to form cell pellet. The supernatant was discarded, 750 µl lysis solution was added to re-suspend cell pellet and vortexed in order to ensure the maximum contact of lysis solution with cell pellet to ensure complete breakdown of cell membranes. Then the solution was transferred to bead tube and 100 µL of Lysis additive was added. The bead tube was rubbed horizontally against a flat surface for some time and then vortexed for 5 minutes. After this, tube was centrifuged at 14000 rpm for one minute.

400 µL of supernatant was carefully removed and transferred to DNAse free micro-centrifuge tube. 100 µL of binding solution was added to tube and mixed by inverting the tube for few times. Then the tube was incubated on ice for five minutes. The lysate was spun in order to remove any debris. With the help of micropippeter, 450 µL of supernatant was collected and transferred into another DNAse free micro-centrifuge tube. Equal amount of 70% ethanol was added to lysate volume and vortexed for proper mixing.

Binding to column

Meanwhile, spin column was assembled on collection tube provided in the kit. 600µL of the above lysate and Ethanol mixture was added to the column and centrifuged at 14000 rpm for one minute. Flow through was discarded and column was reassembled.

Column washing

Afterwards, 500 µL of wash solution I was added to column and centrifuged for one minute Again flow through was discarded, wash solution II was added and centrifuged at 4000 rpm for one minute. Wash solution II was added again and centrifuged for two minutes in order to ensure that all DNA is bound with the column packing.

DNA elution

After centrifugation, column was assembled on 1.7 ml elution tube and 100 µL of elution buffer was added to column. Then column was centrifuged at 2000 rpm for two minutes followed by one minute centrifugation at 14000 rpm. The flow through containing pure DNA was stored at -20°Cfor 16S rRNA analysis.

Selection of primers

On basis of identification of API 20E, primers were selected after extensive literature review. The primers were analyzed through BLAST (Basic local alignment search tool) in order to verify the sequence. Only primers presenting 100% similarity index were selected and used for PCR.

Set of primers

Sets of primers described in Table 3.3 were used for identification of species and determination of *Opd* (Organophosphate degrading) gene.

Primer	Sequence (5'→ 3')	Target	Annealing Temp (ºC)	Product size (bp)	Reference
nirScdaF	GTGAACGTCAAGGAAACCGG	*Pseudomonas aeruginosa*	59.2	910	Srinandan *et al.*, 2011
nirScdaR	CCGACTTCGGAGTCTTGAC				
PA-SS-F	GGGGGATCTTCGGACCTCA	*Pseudomonas putida*	56.1	1662	Figuerola and Erijman, 2010
PA-SS-R	TCCTTAGAGTGCCCACCCG				
F450	CGCCACTTTCGATGCGAT	*Opd* gene	59-62	391	Iyer *et al.*, 2011
R 840	CTTCTAGACCAATCGCACTG				

Table 3.2 Primers for 16S rRNA analysis of target isolates

3.11.3 PCR amplification

For PCR amplification, mixture of 25 µl was prepared containing 20 mM $MgSO_4$ (2.5 µl), 10 mM dNTP (2 µl), 10X reaction buffer (2.5 µl), 2 µM forward and reverse primers, sample DNA template (3 µl), and 0.3 µl of Taq polymerase. Remaining volume of PCR water was added to the reaction mixture.

The reaction mixture was processed in Thermocycler (Extragene 9600). PCR denaturation was performed at 95°C followed by 40 cycles of DNA amplification, each consisting of three steps: 1 min at 95°C for DNA denaturation into single strand, 1 min for primer to hybridize at annealing temperature given in Table 3.2 and 1 min at 72°C for extension of complementary DNA strand from each primer followed by 10 min at 72°C for Taq DNA polymerase to synthesize any un-extended strand left.

The resulting PCR products were examined on 1.5% (w/v) agarose gels stained with ethidium bromide (6µl). Electrophoresis was performed at 100 V for 45 min in TBE

(Tris/Borate/EDTA) running buffer and amplified products were visualized using Geldoc system on UV transilluminator (Micro Docdeaver scientific, UK).

3.12 Degradation studies

3.12.1 Preparation of inoculum

Selected isolates (AN1 and AN3) were grown on Luria Bertani media and incubated for 24 hours at 37°C. After 24 hours the strains were washed with sterile 10 ml MSM and inoculated into the bioreactors containing 2 L of sterile MSM at different concentrations (50, 100 and 150 mg/L). The Optical density (not less than 3.2) of each isolate was measured prior to inoculation. Each set of experiment was performed in triplicates in order to validate the results.

3.12.2 Bench scale reactor setup

Bacterial isolates were maintained in MSM to evaluate profenofos biodegrading ability at the initial concentration of 50 mg/L which was increased to 100 and 150 mg/L. Bench scale reactor setup was employed for biodegradation studies at the mentioned concentrations as illustrated in Figure 3.2.

Fresh cultures were washed with sterile MSM and inoculated in the reactors. Sample collection was carried out from each reactor at regular intervals (0, 2, 4, 6, 8, 24, 48, 72 and 96 hrs) and extraction of profenofos residues for GC analysis was done using ethyl acetate.

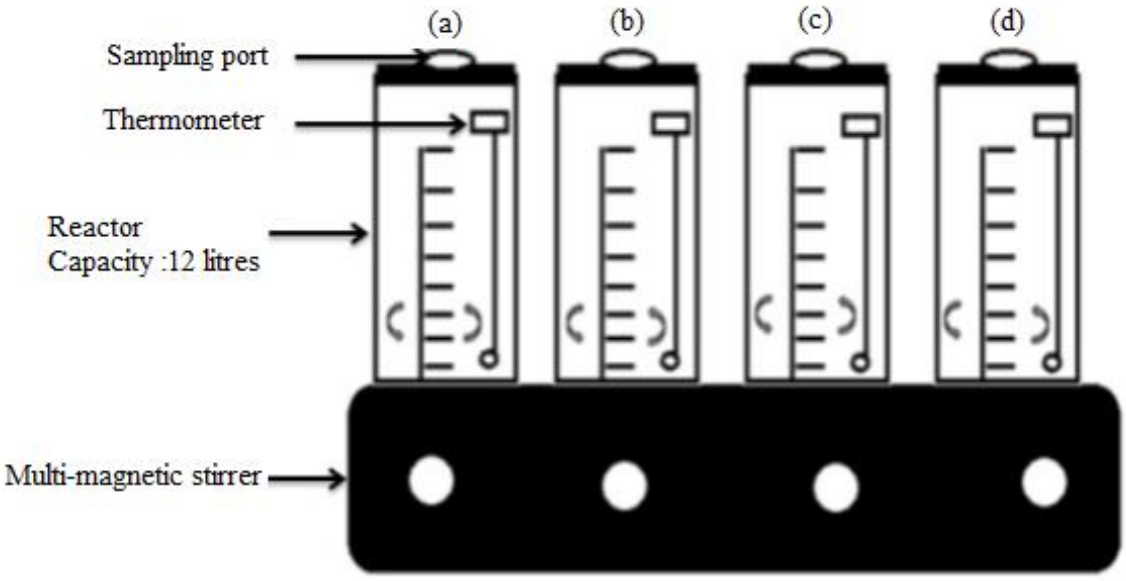


Figure 3.2. Bench scale reactor setup for pesticide degradation a) 0 mg/L, b) 50 mg/L, c) 100 mg/L and d) 150 mg/L Profenofos

3.12.3 Liquid-liquid extraction (Hunter-Nash Method, 1934)

Samples were extracted from bioreactors at regular intervals (0-96 hrs) in order to determine the rate of profenofos degradation. 5 ml of sample was taken in test tube and 1 ml GC grade ethyl acetate was added into it. These were gently shaken for few minutes and then left for some time until two distinct layers were visible. The organic layer was extracted carefully with the help of sterile glass pipette and transferred to GC vial for further analysis. The samples were stored at 4°C.

3.13 Gas chromatography

Gas chromatography is most recommended analytical technique till date to determine the pesticide residues at trace level. It is suitable technique for detection of several pesticides

in water and soil. Higher sensitivity of this instrument brings precision in the results generated (Andreu and Pico, 2004).

3.13.1 Preparation of GC standards and calibration curve

GC grade standard of profenofos was purchased from Sigma Aldrich, Canada. Stock solution of 500 mg/L was prepared by addition of 3.4 µl into 10 ml ethyl acetate. Similarly, working solutions of varying concentrations (1, 25, 50, 75, 100 and 150 mg/L) were prepared from stock solution to obtain the calibration curve using GC (Model Schimadzu, 2010) equipped with Electron Capture Detector.

3.13.2 Gas chromatographic conditions for profenofos

Gas chromatograph equipped with electron capture detector (ECD) was used for analytical studies. TRB column having a length of 30 m, 0.32 mm diameter and 1 µm film thickness was used for analysis of profenofos. Gas chromatographic conditions used are presented in Table 3.4.

Conditions	
Initial temperature	50˚C
Final temperature	250˚C
Rate	15˚C/min
Run time	17.33 min
Mode	Split less
Injector temperature	240˚C
Column temperature	250˚C
Detector temperature	300˚C
Linear velocity	24.2 cm/s

Table 3.3 Operating Conditions for Gas Chromatography (Schimadzu-2010, Japan)

Chapter 4

RESULTS AND DISCUSSION

Fields sprayed with organophosphate serves as ideal source for isolation of pesticide degrading bacteria (Masahito *et al*., 2000). Soil samples from profenofos sprayed field was collected from different points and composite soil sample was prepared in order to isolate maximum profenofos degrading bacteria. Temperature and pH was determined onsite and were found to be 21ºC and 7. Overall, fourteen isolates were obtained at varying concentrations of 50, 100 and 150 mg/L (Table 4.1).

Sample	Bacterial Isolates (Assigned no.)
Soil applied with Profenofos	501,502,503,504,505,1001,1002,1003, 1004,1005, 1501,1502,1503 and1504

Table 4.1 Bacteria isolated from profenofos contaminated soil

4.1 Morphological characterization

The isolated strains were assigned codes as 501-505, 1001-1005 and 1501-1504 as mentioned in Table 4.1. Colony and cell morphology of isolates is reported in Table 4.2 and 4.3.

Majority of isolates (4/14) were observed to appear greenish in color followed by white, off-white, golden yellow and translucent in appearance. The margins of strains were entire

smooth, only a few isolates had undulate margins whereas the elevation of isolates was convex and 14% of isolates appeared to have raised elevation. In terms of texture, half of isolates were pasty, 29% isolates were creamy, and only 21% isolates were sticky. The colony size of isolates varied between <1mm-2mm.

Bacterial isolates	Visual color	Shape	Margin	Elevation	Texture	Size (mm)
501	Offwhite	Circular	Undulate	Raised	Creamy	1.5
502	White	Circular	Entire Smooth	Convex	Creamy	<1
503	White	Circular	Entire Smooth	Convex	Creamy	1.5
504	Golden yellow	Circular	Entire Smooth	Raised	Pasty	1.75
505	Offwhite	Circular	Entire Smooth	Convex	Sticky	2
1001	Greenish Yellow	Circular	Entire Smooth	Convex	Pasty	1.5
1002	Golden brown	Circular	Entire Smooth	Convex	Sticky	2
1003	Translucent	Circular	Entire Smooth	Convex	Sticky	1
1004	Translucent	Circular	Entire Smooth	Convex	Sticky	1.5
1005	Greenish Yellow	Circular	Entire Smooth	Convex	Pasty	<1
1501	Greenish	Circular	Entire Smooth	Convex	Pasty	2
1502	Greenish	Circular	Undulate	Convex	Pasty	2
1503	Offwhite	Circular	Entire Smooth	Convex	Creamy	1.5
1504	Greenish	Circular	Entire Smooth	Convex	Pasty	1.25

Table 4.2 Colony morphology of bacteria isolated from soil having a history of profenofos use

Bacterial isolates	Gram reaction	Shape	Arrangement	Motility
501	Negative	Cocci	Pairs/Small groups	-
502	Negative	Cocci	Single/Pairs/Tetrads	+
503	Negative	Small Bacilli	Single/Pairs	+
504	Negative	Bacilli	Single/Pairs	++
505	Negative	Bacilli	Single/Pairs	++
1001	Negative	Bacilli	Single/Groups	-
1002	Negative	Bacilli	Single/Pairs/Groups	+
1003	Negative	Bacilli	Pairs/Groups	++
1004	Negative	Small Bacilli	Single/Pairs	+
1005	Negative	Bacilli	Pairs	+
1501	Negative	Bacilli	Pairs	++
1502	Negative	Bacilli	Pairs	++
1503	Negative	Small Bacilli	Pairs/Groups	-
1504	Negative	Bacilli	Groups	-

++ V. fast + Fast - Static

Table 4.3 Cell morphology of the bacterial isolates obtained from profenofos contaminated soil

All of the strains turned out to be gram negative when observed under microscope after gram staining. Two isolates i.e. 501 and 502 were cocci and remaining were bacilli in shape. Strains 1005, 1501 and 1502 appeared to exist in pairs. Remaining isolates were present in more than one arrangement i.e. isolates 503, 504, 505, 1004 were present as singles and pairs while 1003, and 1503 were present as pairs and groups. Isolates 1001 and 1504 were present as single/groups and groups respectively. Bacterial strain 1002 was found to be present as single/pairs and small groups. Bacterial isolates 501, 1001, 1503 and

1504 were non-motile while remaining isolates were motile when observed under microscope at 100X.

4.2 Biochemical characterization of bacterial isolates

Biochemical characterization was performed using API20E and results were determined through analyzing the codes in API 20E software. The biochemical Characterization is listed in Table 4.4.

Table 4.4 Biochemical characterization of isolates

S. No.	Test	Bacterial Strain													
		50					100					150			
		1	2	3	4	5	1	2	3	4	5	1	2	3	4
1	*ortho*-Nitrophenyl-β-galactoside (ONPG)	-	-	-	-	-	-	-	-	-	+	-	-	-	-
2	Arginine Dihydrolase	+	+	-	+	+	+	+	+	+	+	+	+	+	+
3	Lysine Decarboxylase	-	-	-	-	-	+	-	+	-	-	+	-	-	-
4	Ornithine Decarboxylase	-	-	-	-	-	+	-	-	-	-	+	-	-	-
5	Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	H_2S production	-	-	-	-	-	-	-	-	-	+	-	-	-	-
7	Urease	-	-	-	-	-	-	+	-	-	-	+	-	-	-
8	Tryptophan Deaminase	+	+	+	+	+	+	+w	+	+	+w	+	+	-	+
9	Indole production	-	+	-	-	-	-	-	-	-	-	+	-	-	-
10	Acetoin production (Voges Proskauer)	-	+	+ l.p	-	+ v.l.p	-	-	-	-	-	-	-	-	-
11	Gelatinase	+	+	+	+	+	+	-	-	-	-	+	+	-	+

l.p = light pink v.l.p = very light pink l.g = light green p.g = pale green

g = green +w = weak

Table 4.4 Biochemical characterization results of isolates

S. No.	Test	Bacterial Strain													
		50					100					150			
		1	2	3	4	5	1	2	3	4	5	1	2	3	4
12	Oxidation/fermentation of Glucose	+	+	+ l.g	+	-	+	-	-	-	+	+	+	+	+
13	Oxidation/fermentation of Mannitol	-	-	-	-	-	-	-	-	-	+	-	-	-	-
14	Oxidation/fermentation of Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	Oxidation/fermentation of Sorbitol	-	-	-	-	-	-	-	-	-	+	-	-	-	-
16	Oxidation/fermentation of Rhamnose	-	-	-	-	-	-	-	-	-	+	-	-	-	-
17	Oxidation/fermentation of Sucrose	-	-	-	-	-	- g	-	-	-	+	-	-	-	-
18	Oxidation/fermentation of Melibiose	-	-	-	- l.g	-	-	-	-	-	+	- p.g	- p.g	-	-
19	Oxidation/fermentation of Amygdalin	-	-	-	-	-	- g	-	-	-	-	-	-	-	-
20	Oxidation/fermentation of Arabinose	+	-	- l.g	+	-	-	-	-	-	+	+	+	-	+ p.g
21	Nitrate Reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	Nitrogen Production	-	-	-	-	-	-	+	+	+	-	-	-	-	-

l.p = light pink v.l..p = very light pink l.g = light green p.g = pale green

g = green +w = weak

4.2.1 Catalase and oxidase tests

Selected bacterial isolates were capable of catalase production; only 1004 exhibited weak catalase production. Strains 502 and 503 failed to produce cytochrome oxidase. The results are mentioned in Table 4.5 along with the bacterial strains identified through API 20E.

Strains	Catalase	Oxidase	Bacterial strains identified
501	+	+	*Acinetobacter sp.*
502	+	-	*Sphingomonas sp.*
503	+	-	*Sphingomonas paucimobilis*
504	+	+	*Pseudomonas florescence*
505	+	+	*Citrobacter freundii*
1001	+	+	*Pseudomonas sp.*
1002	+	+	*Acinetobacter sp.*
1003	+	+	*Arthrobacter atrocyaneus*
1004	+	+	*Xanthromonas sp.*
1005	+	+	*Paenibacillus sp.*
1501	+	+	*Pseudomonas aeruginosa*
1502	+	+	*Pseudomonas sp.*
1503	+	+	*Pseudomonas putida*
1504	+	+	*Pseudomonas sp.*

Table 4.5 Catalase and oxidase production along with API20E identification

Results of present study coincide with numerous studies where pesticide degrading bacterial strains were isolated from pesticide contaminated site. Malghani and his co-workers (2009) have isolated profenofos degrading *Pseudomonas putida* and *Burkholderia gladioli* from soil having a history of profenofos application. Whereas Peter *et al*. (2014) have isolated *Pseudomonas aeruginosa, Bacillus megaterium* and *Staphylococcus aureus* as potential organophosphate degrading bacteria from rhizospheric soil of selected plants. *Pseudomonas fluorescence, Brucella melitenis, Bacillus subtilis, Klebsiella sp., Citrobacter freundi, Flavobacterium sp., Acinetobacter sp., Pseudomonas sp.,* and *Sphingomonas sp.* are potential organophosphate degrading bacteria isolated from

pesticide contaminated soils as reported by several researchers (Pino and Peñuela, 2011; Lakshmi *et al*., 2008).

4.3 Selection of predominant pesticide degrading isolates

On the basis of significant growth at highest concentration (150 mg/L) of profenofos in addition to preliminary degradation studies, two bacterial strains named 1501 and 1503 were selected for degradation studies. These strains were further recoded as AN1 and AN3.

4.4 Physiological characteristics of predominant isolates

Both strains showed similar growth trend in terms of pH range (6-9) whereas AN1 was able to grow at wide temperature range (30 -45˚C) as compared to AN3 which exhibited no growth after 37˚C as illustrated in Figure 4.1.

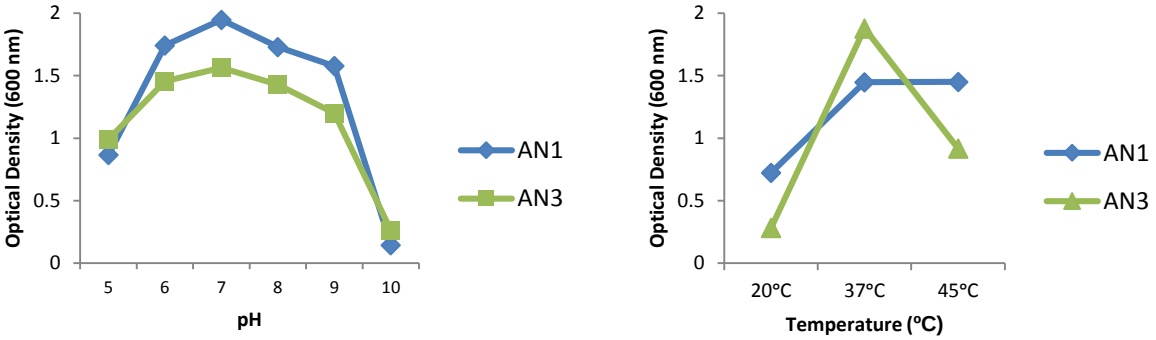


Figure 4.1 Left to right a) Growth of bacterial isolates at different pH range (6-9) b) Temperature range (30 to 45°C)

These findings are in concurrence to results produced by Tamilselvan *et al*. (2014) where optimum growth of metribuzin and profenofos degrading *Pseudomonas aeruginosa* was

observed at pH 6-8 whereas it was able to grow at wide temperature range of 25-47ºC. Vijayalakshmi and Usha (2012) have also reported optimum growth of Chlorpyrifos degrading *Pseudomonas putida* to be at pH 5-9 and temperature range of 30-40ºC. Sharma *et al*. (2014) stated that *Pseudomonas putida* fails to grow above 37ºC.

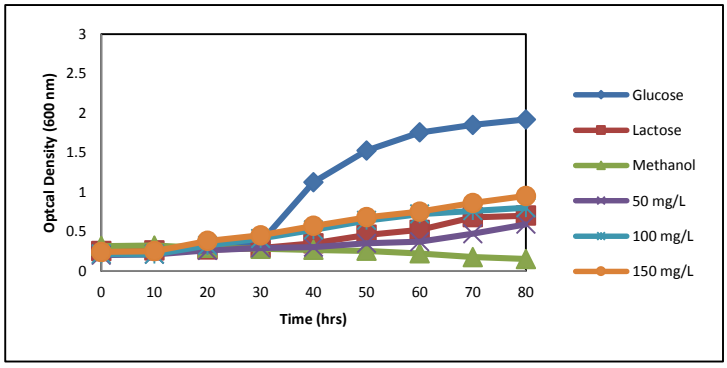
4.5 Effect of co-substrate on bacterial growth

Growth of both isolates was determined using four different carbon sources including glucose, profenofos, lactose and methanol (2 g/L). The effect of substrates was examined individually as well as in combination with different concentrations of profenofos. AN1 showed highest growth at glucose followed by 150 mg/L profenofos, 100 mg/L profenofos, Lactose, 50 mg/L profenofos and methanol as presented in Figure 4.2. (a). In case of profenofos varying concentrations {Figure 4.2 (b, c and d)}, the isolate utilized glucose as primary carbon source even at higher concentrations of profenofos. These findings suggest that isolate utilizes profenofos as second most utilized carbon source after glucose. Survival of isolate was observed when combination (profenofos and methanol) was administered. A very interesting feature in terms of isolate survival in the presence of methanol along with profenofos was observed which could be due to the fact that methanol did not show any toxic effect in the presence of co-substrate (profenofos).

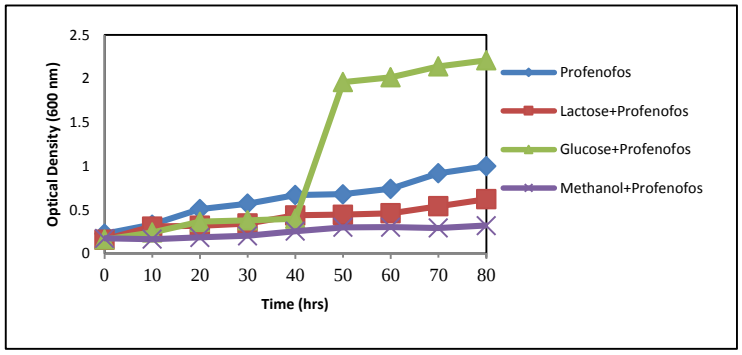
Malghani *et al.* (2009) affirmed the same pattern of carbon source utilization in organophosphate degrading *Pseudomonas aeruginosa.* In case of AN3, higher concentrations of profenofos did not favored the efficient growth of the isolate. 100 mg/L profenofos proved to be the most favorable concentration for the isolate's growth as it is evident from Figure 4.3 (a). The isolate was able to utilize glucose along with Profenofos

at approximately the same rate suggesting that profenofos efficiently serves as a source of carbon and energy to the isolate. In case of AN3, lactose and methanol exhibited minimum effect on growth of isolate as presented in Figure 4.3 (b, c and d). Our findings are in agreement with researches where microorganisms utilize organophosphates as their sole carbon and phosphorus source (Subhas and Dileep, 2003). Tamilselvan *et al*. (2014) also found that lactose showed minimum effect on bacteria able to degrade metribuzin and profenofos.

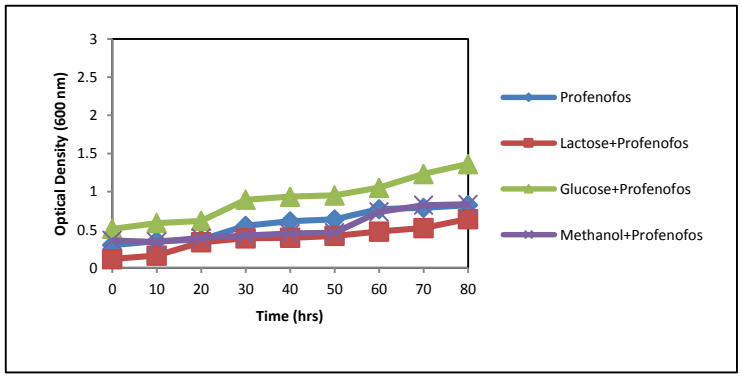
(a)



(b)



(c)



(d)

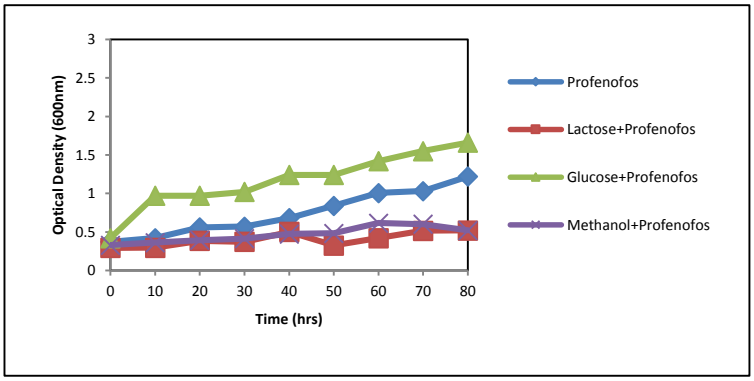
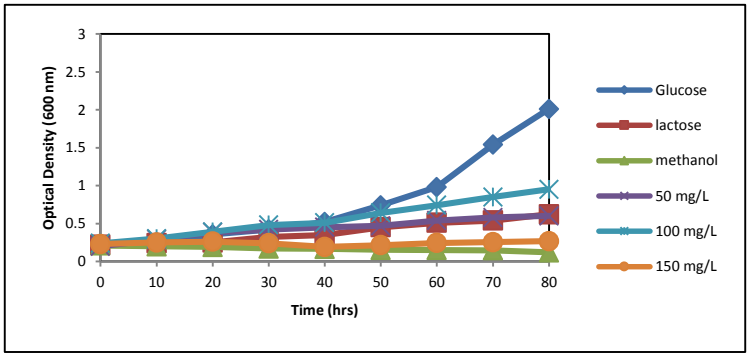
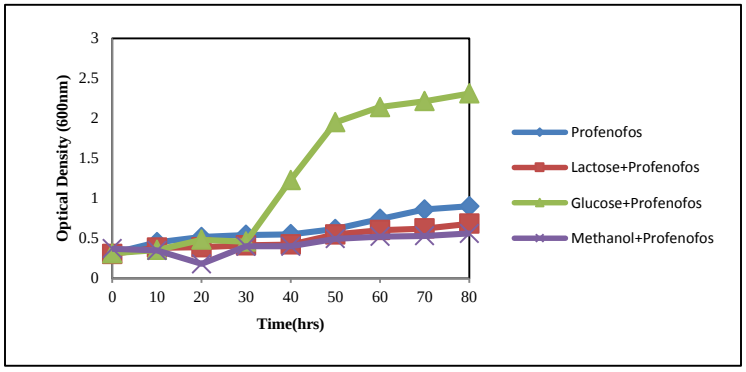


Figure 4.2. Growth characteristics of AN1 at (a) Different carbon sources (b) 50 mg/L (c) 100 mg/L and (d) 150 mg/L profenofos in Mineral Salt Media

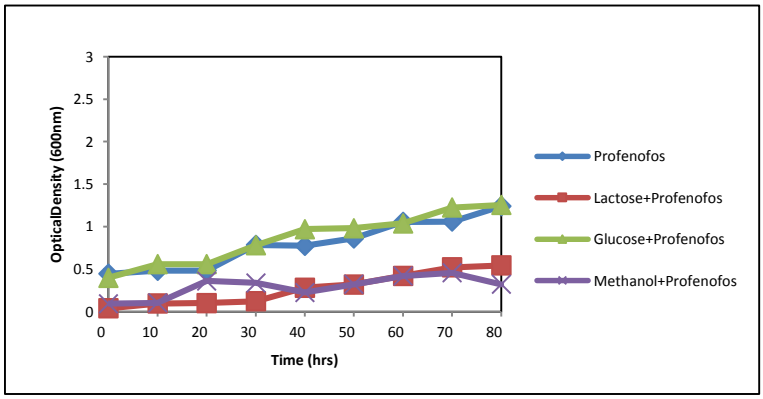
(a)



(b)



(c)



(d)

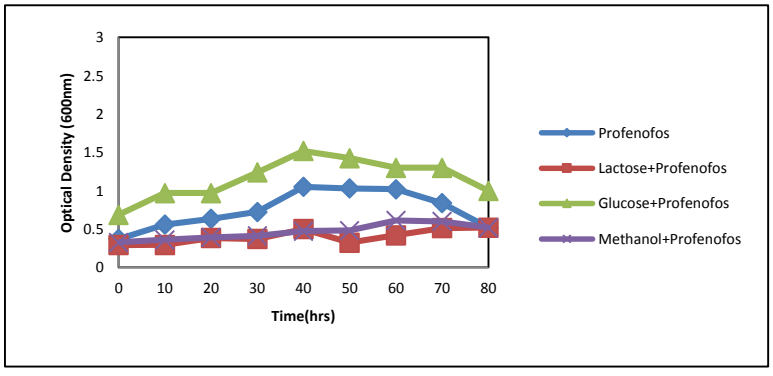


Figure 4.3 Growth characteristics of AN3 at (a) Different carbon sources (b) 50 mg/L (c) 100 mg/L and (d) 150 mg/L profenofos in Mineral Salt Media

4.6 PCR amplification

4.6.1 Determination of Genetic Variability

Bacterial identification was carried out by advanced molecular biology technique. DNA of bacterial isolates was extracted using DNA spin Kit (Norgen, Canada). The bacterial DNA was visualized in UV-transilluminator and the products are shown in Figure 4.4.

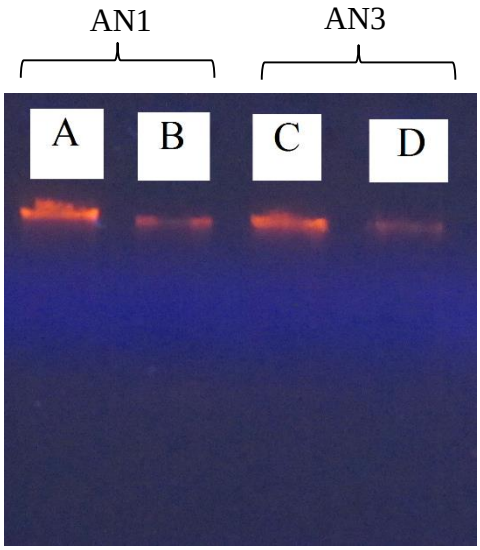


Figure 4.4 Lane A & B are DNA isolated from AN1 isolate whereas Lane C & D are DNA isolated from AN3

Products were amplified by optimizing thermocycling conditions mentioned earlier. For *Pseudomonas aeruginosa,* the annealing temperature was found to be 59.3˚C (Figure 4.5) using given set of primers whereas for *Pseudomonas putida,* the annealing temperature was observed at 56.1˚C (Figure 4.6). Same species were previously detected by Sharma and co-workers (2014) as potential methyl parathion degrading strains which supports the observation that *Pseudomonas sp.* have promising potential to utilize pesticides as their carbon and energy source.

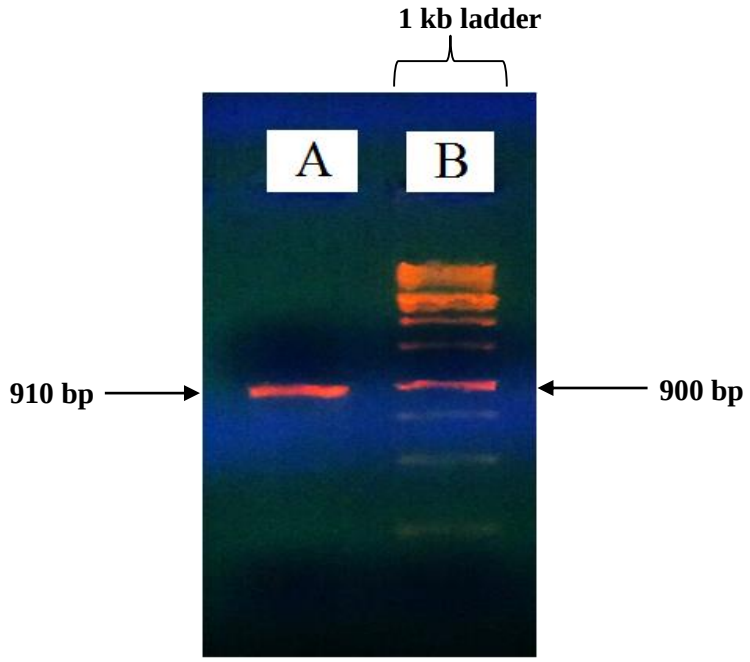


Figure 4.5 Detection of *Pseudomonas aeruginosa* through 16S rRNA gene analysis A) Lane 1 represents the PCR amplification product at 910 bp B) 1 KB DNA Ladder (Genedire)

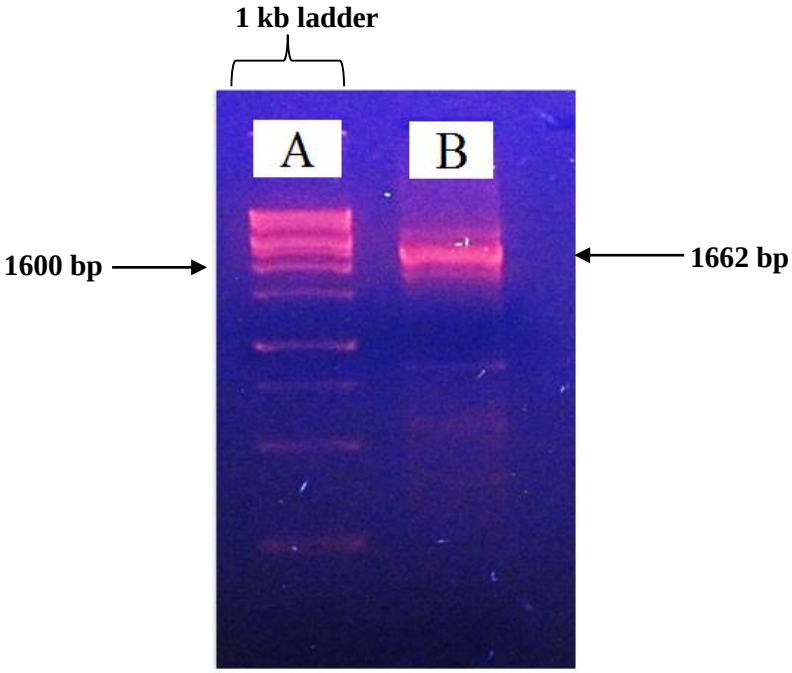


Figure 4.6 Detection of *Pseudomonas putida* through 16S rRNA gene analysis A) 1 KB DNA Ladder (Genedire) B) PCR amplification product at 1662 bp

4.6.2 Determination of organophosphate degrading gene (*Opd* gene)

The thermocycling conditions mentioned in Table 3.2 were followed and annealing temperature for *Opd* was optimized within range i.e. 59-62ºC. DNA template from sludge served as positive control whereas *Escherichia coli* was selected as negative control for determination of *opd* gene in the profenofos degrading bacteria (AN1 and AN3). The products were visible at 391 kb as shown in Figure 4.7.

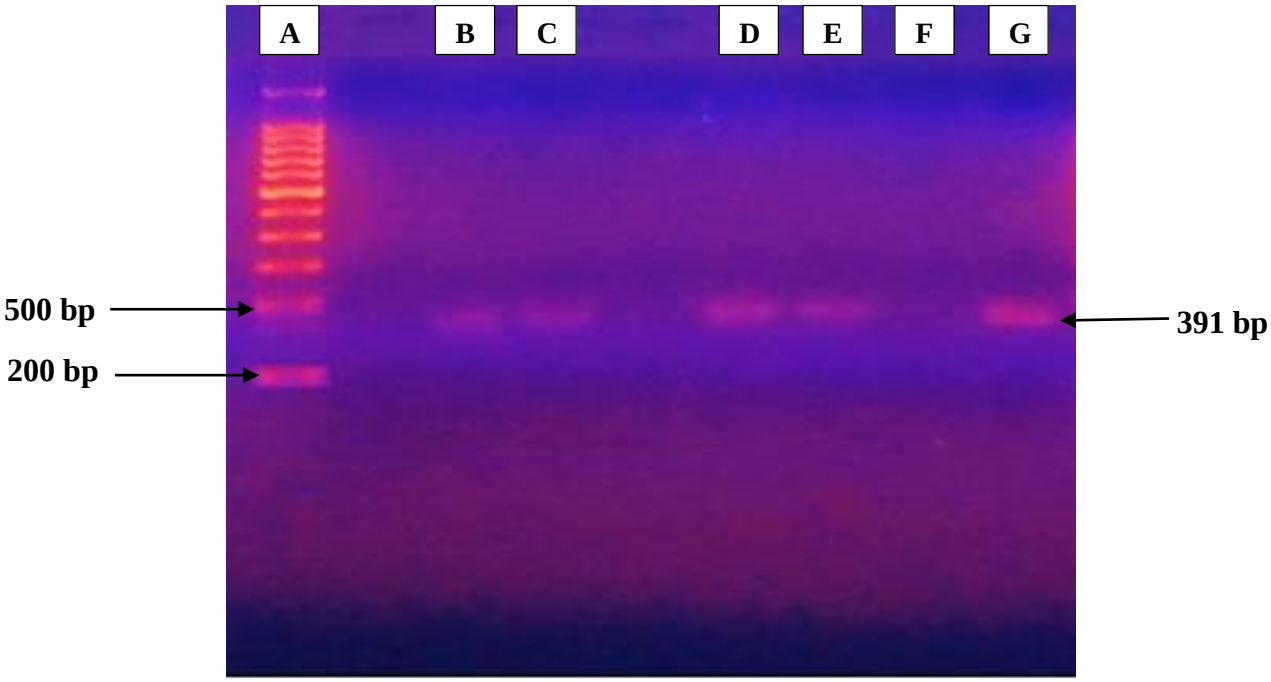


Figure 4.7 Left to right A) 1 Kb ladder B & C) *Pseudomonas aeruginosa opd* gene D & E) *Pseudomonas putida opd* gene F) Negative Control (*E.coli*), G) Positive Control (Sludge) J) 1 KB ladder

Both promising profenofos degrading strains contained *opd* gene which encodes enzymes for organophosphate degradation. These observations are similar to results produced by Iyer and co-worker (2011) who also detected organophosphate degrading gene in paraoxon metabolizing *Pseudomonas* species. The PCR products were visualized at 391 bp same as

reported by Iyer and colleagues (2011). This supports our finding that the bands observed were actually of *Opd* gene. Iyer and Iken (2013) also found *opd* gene in water-borne *Aeromonas, Stenotrophomonas* and *Exiguobacterium* supporting the fact that *opd* degradation is not stressed derived trait in bacteria and that it is transposable because of its presence on plasmid.

Malghani *et al*. (2009) affirmed that profenofos is degraded into bicholorophenol and O-ethyl, S, propyl phosphotrioic acid by action of *Pseudomonas aeruginosa.* Bicholorphenol is hydrolytic product of profenofos hence verifying the fact that organophosphate hydrolase acts upon the C-O-P bond in profenofos ultimately degrading it. *Opd* gene codes for OPH indicating that both of the strains isolated in the present study act by hydrolyzing profenofos through enzyme mediated process (Ningfeng *et al*., 2004).

4.7 Degradation efficiency of individual isolates and their consortium

The calibration curve is presented in Figure 4.8. The degradation studies were carried out in mineral salt media in bench scale setup at optimized pH (7) and temperature (30°C) at 150 rpm. Degradation was observed and results were obtained by analyzing extracted samples at regular intervals (0-96 hrs) in GC. Gas chromatogram of profenofos standard presenting retention time of ethyl acetate and profenofos is presented in Figure 4.9.

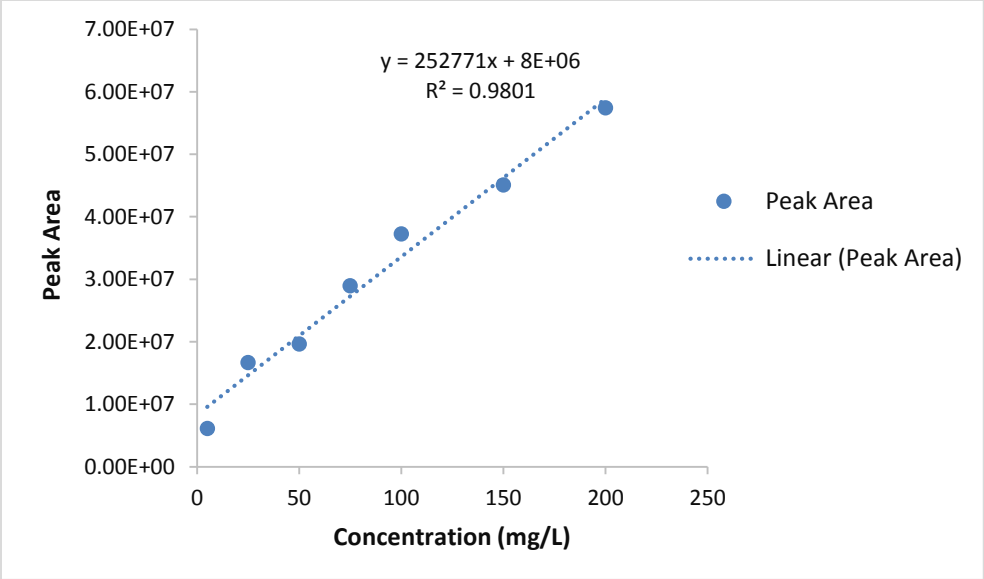


Figure 4.8 Calibration curve of different concentrations of profenofos

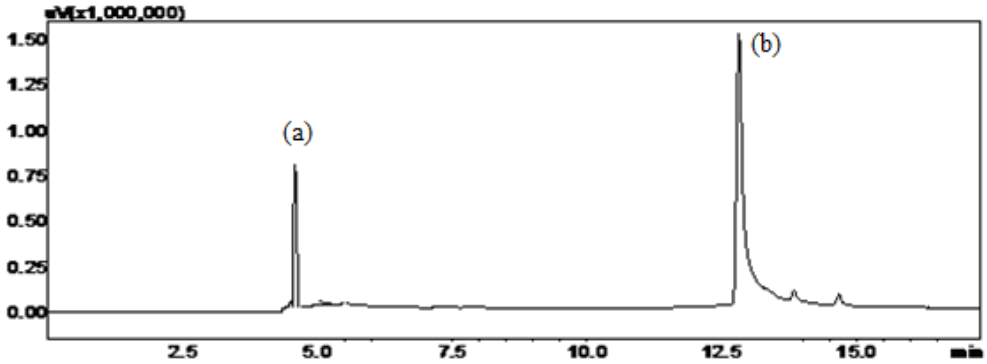


Figure 4.9 GC Chromatograph of a) Ethyl acetate (Retention Time 4.9 min) and b) Profenofos (Retention Time 12.78 min)

Pseudomonas aeruginosa turned out to be efficient in degrading Profenofos at all concentrations as compared to *Pseudomonas putida*. At 50 mg/L concentration, *Pseudomonas aeruginosa* metabolized profenofos completely in 96 hrs. Whereas degradation of profenofos was recorded to be 75.5 and 65% at 100 and 150 mg/L

respectively after 96 hrs (Figure 4.10). The degradation chromatograms are given in Annexure II. These findings coincides with the results produced by Malghani and his coworkers (2009) where rapid degradation of profenofos was observed within 48 hrs of incubation followed by slow degradation over longer incubation times. Peter *et al.* (2014) also reported *Pseudomonas aeruginosa* as a proficient strain which degraded 350 mg/L methyl parathion in 96 hrs.

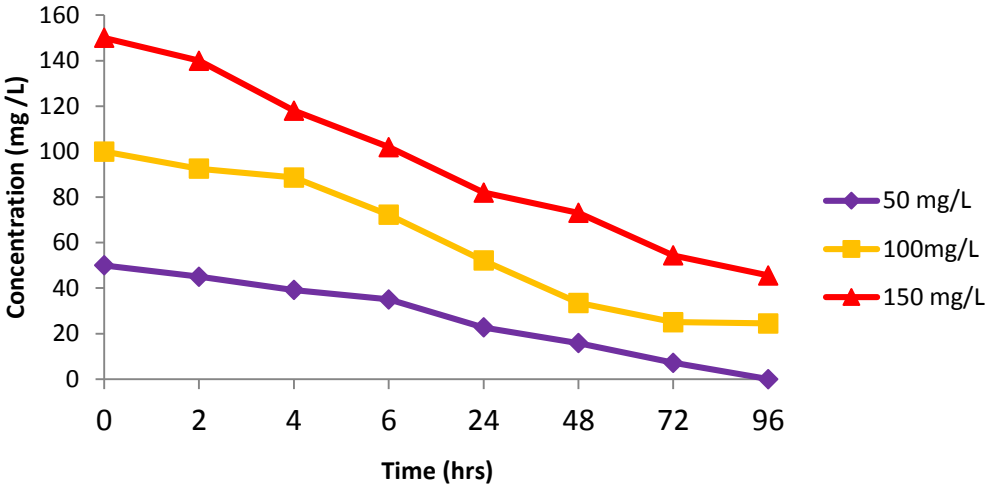


Figure 4.10 Rate of degradation of profenofos by *Pseudomonas aeruginosa*

Chanika and fellow researchers (2011) exclaimed that *Pseudomonas putida* is able to degrade both organophosphates and carbamates. However in the present study, an inverse relationship between concentration of pesticide and growth rate was observed while keeping the conditions optimized. AN3 exhibited to be slow degrader in comparison to AN1 and degradation of profenofos was recorded to be 80, 57.1 and 49.8% at 50, 100 and 150 mg/L (Figure 4.11). This might be due to reason that higher concentration of profenofos (150 mg/L) exhibited inhibitory effect on growth of isolate (Annexure III).

Vijayalakshmi and Usha (2012) reported that *Pseudomonas putida* efficiently degraded 76% of chlorpyrifos at operating conditions of neutral pH at 35°C.

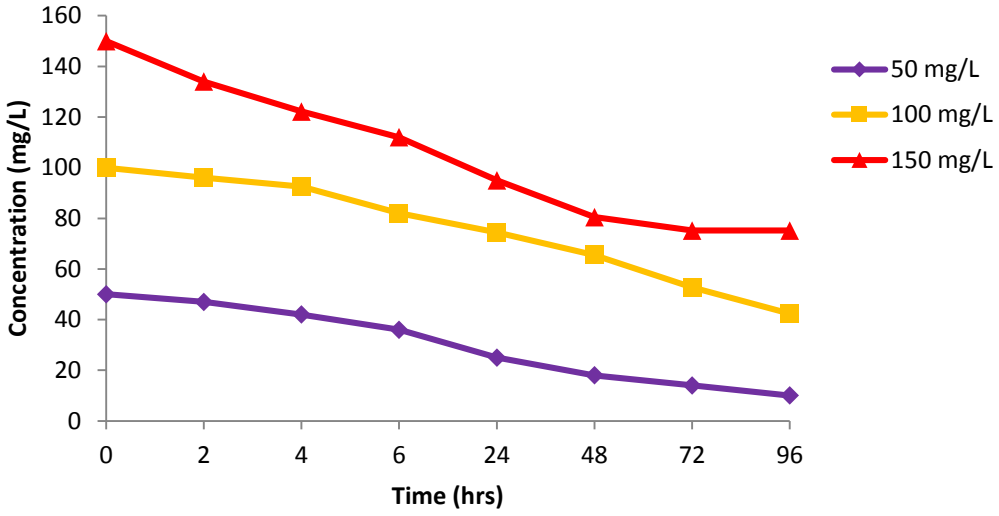


Figure 4.11 Rate of degradation of profenofos by *Pseudomonas putida*

Promising results were obtained when consortium of AN1 and AN3 was utilized for profenofos degradation (Annexure IV). Lower concentration of profenofos (50 mg/L) degraded completely within 48 hrs of incubation while about 90% degradation at 100 mg/L and 79.3% degradation at 150 mg/L (Figure 4.12) was recorded suggesting that the consortium is most effective in biodegradation of profenofos. Several studies have reported individual isolate's degradation capability but it has been proved that consortium possess highest potential in biodegradation of pesticide as well as reclamation of pesticide contaminated sites. Kumar and Philip (2006) also reported the efficiency of microbial consortium as it inhibits the accumulation of toxic materials in the environment as well as in controlled experiments.

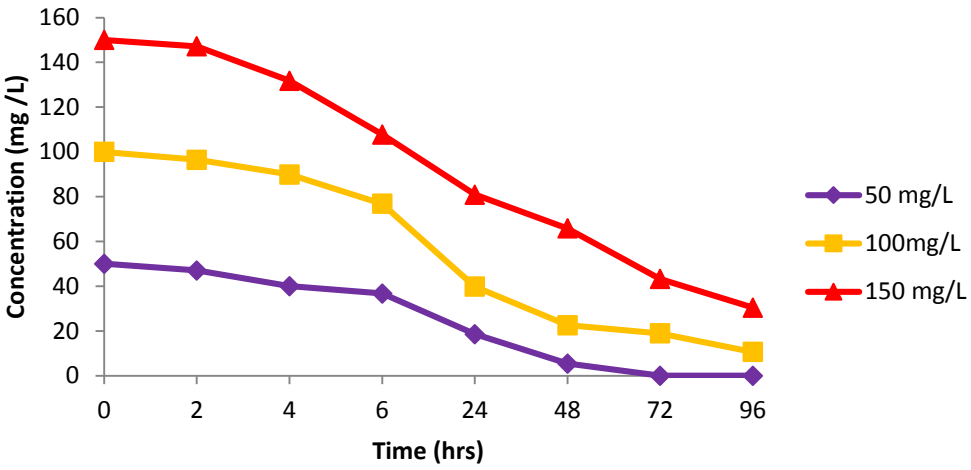


Figure 4.12 Rate of degradation of profenofos by consortium of *Pseudomonas aeruginosa* and *Pseudomonas putida*

Maximum degradation efficiency by consortium may be attributed to the fact that microorganisms are able to work co-metabolically thus facilitating each other to utilize the contaminant actively (Horne *et al*., 2002). When consortium was inoculated for degradation of profenofos, it increased the degradation rate enormously. As it was observed in Figure 4.3 that *Pseudomonas putida* was not able to survive effectively at high concentrations of profenofos while when applied in combination to *Pseudomonas aeruginosa*, it performed efficiently and increased the degradation rate of profenofos even at 150 mg/L. The results affirm that metabolically cooperative colonies may act synergistically and degrade the organophosphate at faster rate. Pino and Peñuela (2011) also degraded organophosphates actively using the bacterial consortium containing *Pseudomonas aeruginosa* and *Pseudomonas putida* along with other species.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Following are the conclusions drawn from the present study:

- A total of fourteen bacterial strains were isolated from profenofos contaminated soil and were identified to be members of *Pseudomonas sp*., *Shingomonas sp*., *Arthrobacter sp.* , *Citrobacter sp*., *Acinetobacter sp*., *Paenibacillus sp.* and *Xanthromonas sp.*
- Two strains produced promising results in terms of profenofos degradation and were later identified as *Pseudomonas aeruginosa* and *Pseudomonas putida* by 16S rRNA analysis. Both strains were found to contain *Opd* (organophosphate degrading) gene.
- Individual degradation rates of *Pseudomonas aeruginosa* and *Pseudomonas putida* at 150 mg/L profenofos concentration were recorded to be 65.3 and 49.8% however in consortium, the rate of degradation of profenofos increased and 79.3% degradation was achieved in 96 hours.

5.2 Recommendations

- Bench scale degradation system to be used for simultaneous degradation of mixture of organophosphates
- Bio kinetics studies of pesticide degradation process should be investigated
- Enzymatic studies on pesticide degradation should be undertaken

Chapter 6

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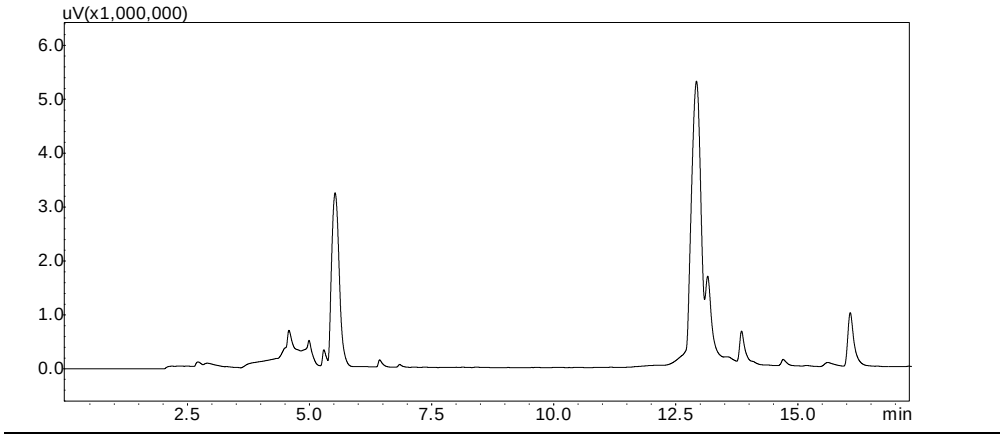
ANNEXURE I

Catalase negative (Left), Catalase positive (Right)

Oxidase positive (left), Oxidase negative (Right)

ANNEXURE II

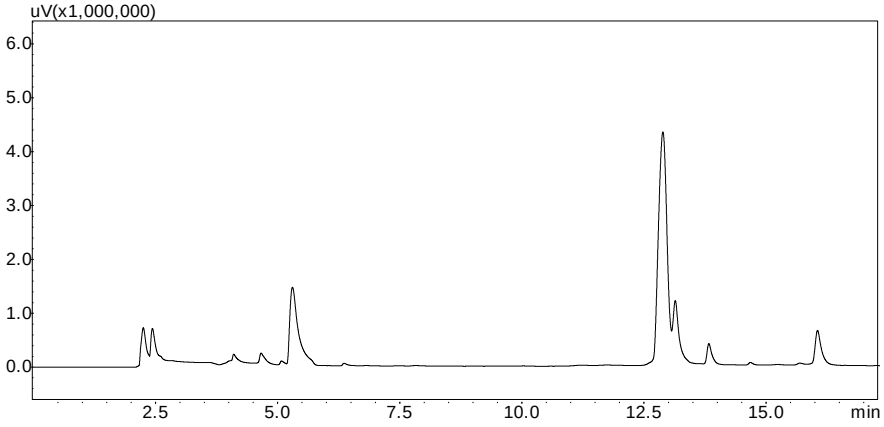
Degradation by *Pseudomonas aeruginosa*



150mg/L- 0 hrs

Retention time **12.78**

Peak area **130657639.6**



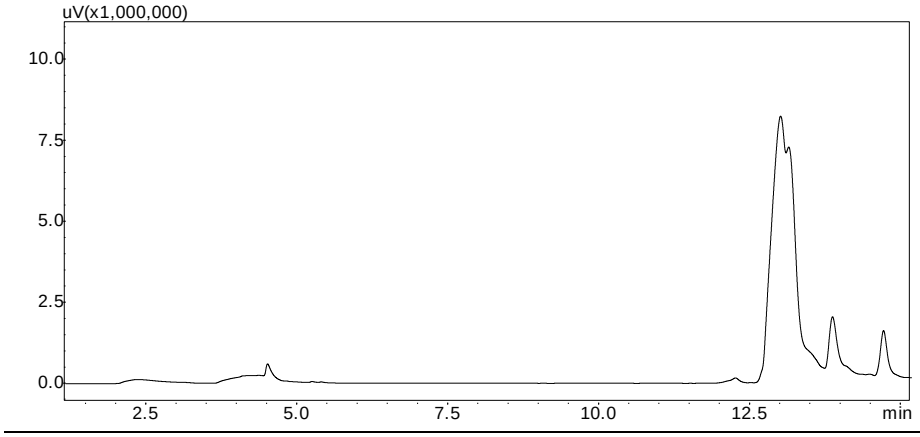
150mg/L-96 hrs

Retention time **12.78**

Peak area **73111383.0**

ANNEXURE III

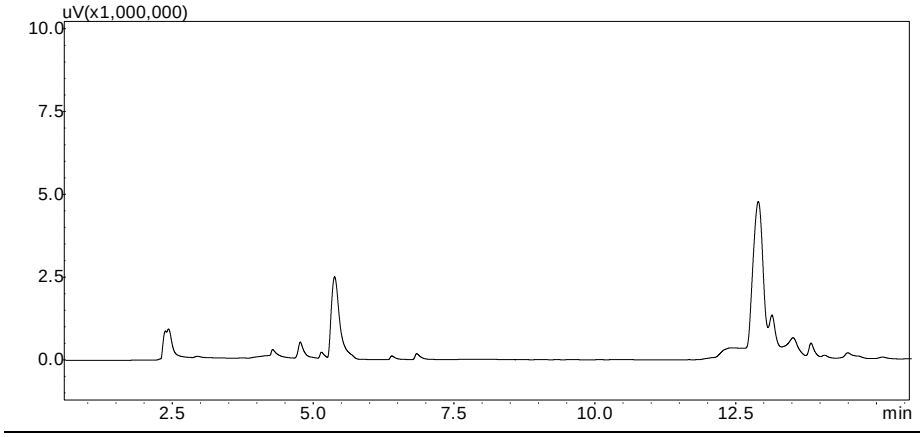
Degradation by *Pseudomonas putida*



150mg/L- 0 hrs

Retention time **12.8**

Peak area **128493587.4**



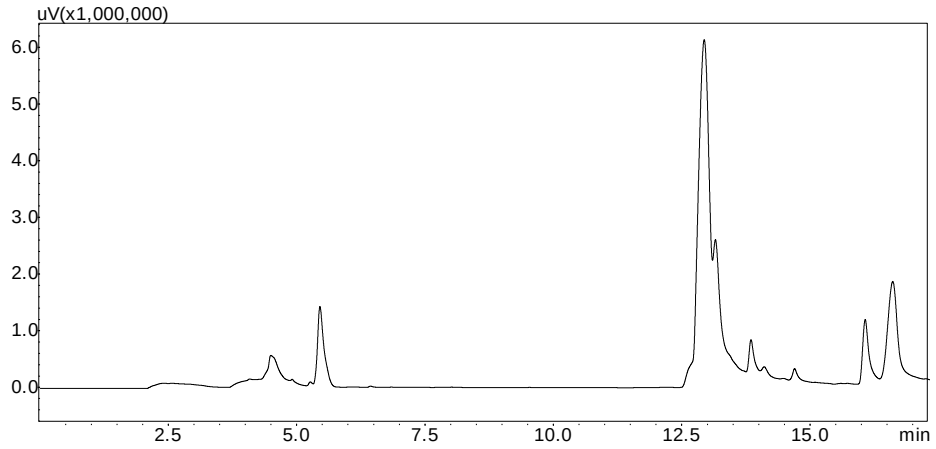
150mg/L-96 hrs

Retention time **12.8**

Peak area **82880094.5**

ANNEXURE IV

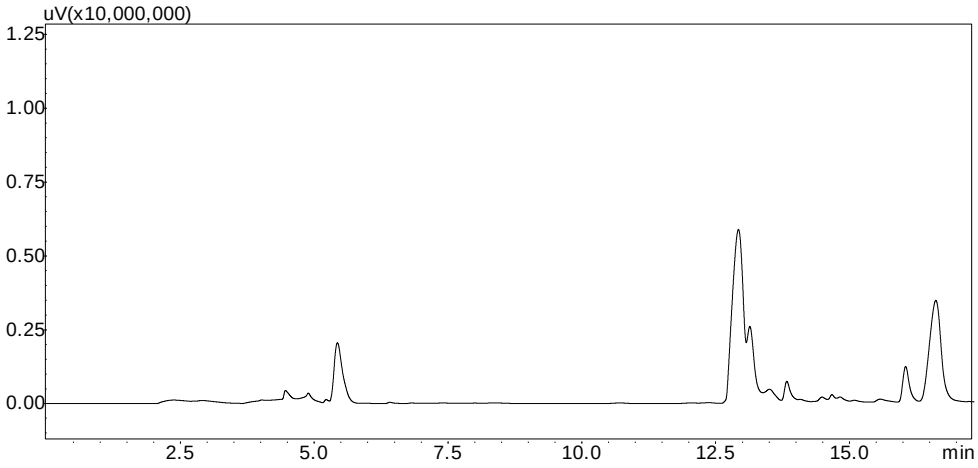
Degradation by Consortium (*P.aeruginosa+P.putida*)



150mg/L - 0 hrs

Retention time **12.78**

Peak area **143883284.1**



150mg/L-96 hrs

Retention time **12.78**

Peak area **5182002.5**