ISOLATION AND MOLECULAR CHARACTERIZATION OF POTENTIAL LAMBDA CYHALOTHRIN DEGRADING BACTERIA

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CERTIFICATE

This dissertation submitted by Ms. Huma Farooq 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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It is certified that the contents and forms of the thesis entitled "**Isolation and Molecular Characterization of Potential Lambda Cyhalothrin Degrading Bacteria**" submitted by **Ms. Huma Farooq**, Registration no. 00000171945has been found satisfactory, free of errors and plagiarism for the requirements of the degree of Master of Science in Environmental Sciences.it is further certified that necessary amendments as pointed out by the GEC members of the scholar have also been incorporated in the said thesis.

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DEDICATED….!!!!

To my ever loving and supporting Parents

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Huma Farooq

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LIST OF ABBREVIATIONS

˚C	Degrees Celsius
μ	Specific Growth Rate
μ_{max}	Maximum Specific Growth Rate
16S rRNA	16S ribosomal ribonucleic acid analysis
3-PBA	3-Phenoxybenzoic acid
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
EC	Electrical Conductivity
FAO	Food and Agriculture Organization
K_i	Inhibition Constant
K_s	Half Saturation Constant
LB	Luria Bertani
LC	Lambda cyhalothrin
mg/L	Milligram per liter
MSM	Mineral Salt Medium
NB	Nutrient Broth
OD	Optical Density
PCR	Polymerase Chain Reaction
ppm	Parts per Million
S	Substrate Concentration
TDS	Total Dissolved Solids
WHO	World Health Organization

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ABSTRACT

Synthetic Pyrethroids are now extensively used for the improvement of crop production and quality in Pakistan. Along with the advantages, pyrethroids are also regarded as potentially harmful pollutants and pose a serious threat to organisms and environment. The present study is designed to evaluate the efficiency of different bacterial strains isolated from water and soil samples to degrade the pesticide Lambda Cyhalothrin (LC), a known synthetic pyrethroid. The residues of LC have been detected in runoff water, irrigation water, and soil and even on fruits and vegetables. The sampling area was selected as Rawal Lake and water samples were collected from four streams namely Nurpur, Ratahutar, Jinnah, Shahdara streams and Korang River that ultimately flow into the Rawal Lake. Soil samples contaminated with the pesticide were also collected from along streams and from agricultural fields around Rawal Lake. Physico-chemical characterization of water samples was also performed. Isolated LC degrading bacterial strains were taxonomically characterized on the basis of their morphology, gram staining, biochemical tests and phylogenetic similarity index of 16S rRNA gene sequence. A total of 20 different colonies were isolated from water and soil and purified by streak plate method. The strains were acclimatized by inoculating them into MSM (minimal salt medium) and Nutrient broth containing initial concentration of Lambda cyhalothrin (100 mg/L) as a sole source of carbon and energy. Optical cell density was also monitored with the help of UV-visible spectrophotometer at 600 nm. On the basis of screening, four strains were selected and identified as *Bacillus circulans* and *Bacillus aryabhattai* (isolated from soil) and *Pseudomonas aeruginosa* and *Proteus mirabilis* (isolated from water). Batch experiments were performed for degradation studies and percentage removal of Lambda cyhalothrin was determined through Chemical Oxygen Demand (COD). The strain, *Pseudomonas aeruginosa* has proved to be proficient bacteria by producing auspicious results in both MSM and NB media with the removal efficiency of 85 and 93.84% of Lambda cyhalothrin after 72 hours, respectively. Biodegradation kinetic model was also applied. The values of the coefficient of determination (R^2) were 0.9535 and 0.946 in MSM and NB respectively, which confirmed the applicability of the model on the experimental data. The kinetic parameters μ_{max}, K_s and K_i were also determined. Thus, *Pseudomonas aeruginosa* may tolerate lambda cyhalothrin more competently as compared to other strains and may play a vital role in the bioremediation of environmental contamination of pyrethroids.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Pests are such biological agents which are responsible for the destruction of all kinds of crops, leading to large scale losses in crop production. A proper treatment is required in order to compensate such huge losses which not only eliminate harmful pests from the system but also helps in the increment of crop yield. Considering the situation, efforts have been carried out by the researchers to formulate chemical agents called pesticides (Sheikh et al., 2011).

With increase in world's population, the use of pesticides has also largely increased since 1960s. The pesticides, on one side, have significant role in reducing crop losses and obtaining healthier and improved yield of crops such as maize, cotton, corn, and potatoes. But on the other side, their usage have also led to many environmental and human health issues. The major issue of pesticides mismanagement is getting critical locally as well as globally (Jamali et al., 2014).

These pesticides are basically an important constituent of the modern agricultural technology that has been adopted globally in order to control or prevent pests, weeds diseases and other plant pathogens, thus playing a vital role in reducing crop yield losses and sustaining high quality. Many major and minor benefits have been reported from pesticides use, among which economic benefits are the most prominent. These benefits include improvement and preservation of crop yield and quality and minimization of other costly inputs such as fuel and labor (Cooper and Dobson, 2007; Damalas, 2009).

Among the south Asian countries, Pakistan stands second in the list of most pesticide consuming countries, where 27% of the total usage of pesticides is sprayed on fruits and vegetables (Jamali et al., 2014). The use of pesticides in the country was started in 1954 and now is showing an increasing trend at the rate of 25% a year (USAID, I-LED 2006-2009).

A wide variety of pesticides are being consumed on a large scale in Pakistan in order to protect crops. An average growth of 11.6% has been observed in pesticide use during last 20 years in the country, but with no remarkable improvement in the yield and quality (Khan et al., 2015). Despite the benefits, such massive use of pesticides has also been reported to be the cause of

chronic diseases, workers' poisonings and number of fatalities in the whole country (Khan et al., 2015; Khan, 2010).

A wide range of pesticides are now available for the farmers for preventing and controlling pests and diseases such as organochlorine, carbamate, organophosphate, pyrethroid and neo-nicotinoid groups. Organophosphates are among the most broadly used groups of pesticides, contributing to more than 40% of the total pesticide market in the world. Malathion, Methyl parathion, Parathion, Dimethoate, Monocrotophos and Phorate are the organophosphorus pesticides, which are utilized extensively worldwide (Manigandan and Nelson, 2015).

Pesticides after releasing into the environment, persist in fruits and vegetables and pollute water and soils. They disturb the food chain and consequently enter in human blood via food and water. They are also responsible for deterioration of natural habitats and biodiversity losses (Cerejeira et al., 2003). There have been reported examples of pest recovery, secondary pest outbreaks, increased resistance towards pesticides and destruction of non-target species. Though the application of pesticides is well known in many other sectors, agriculture can definitely suffering the most because of the adverse effects (Sattler et al., 2007).

In order to prevent these pesticides from further exacerbate the situation, many treatment methods have been established. These methods include physical, chemical or biological processes which either detoxify or degrade the pesticides. Many conventional strategies have been implemented such as photodecomposition, ozonation, adsorption, fenton degradation, and incineration. However, these physicochemical technologies are quite expensive and at many times, they aggravate the problem, rather than eliminating it (Kumari et al., 2011). Moreover, these technologies are not eco-friendly as they release toxic substances as by-products (Chen et al., 2015).

Widespread contamination of water, soil and sediments by organophosphorus compounds (OPs) is now considered a major environmental concern. They are widely used as insecticides, pesticides and chemical warfare agents and lead to extreme toxicity of the environment. Increased environmental concern has made it imperative to adopt safe, reliable and economical approaches that are capable of alleviating these compounds. With advancements in Biotechnology, the researchers around the world are trying to investigate the degradability of the microorganisms in order to find out efficient and economical solution for the

decontamination of pesticides. The process of Bioremediation has proved to be more attractive than other conventional methods because it is more cost-effective and far less disruptive (Chapalamadugu, 1992).

Recently, bioremediation has been investigated as a potential alternative technique due to its cost-effective and eco-friendly properties in controlling pesticide residues. This approach involves the use of naturally occurring microorganisms in order to enhance the normal biological breakdown. Appropriate conditions are established in the contaminated environments which help the microorganisms to flourish and perform the metabolic activities to decontaminate the pollutants. This type of decontamination has become the method of choice since the usage of many xenobiotic compounds including pesticides by microorganisms. They determine the environmental fate of these compounds by consuming them as sources of carbon and energy for the growing and consequently mineralizing and detoxifying them. Considering the importance of microorganisms, their isolation from contaminated environment and identification has become essential. Moreover, there is also a need to investigate about the genetic determinants present on the plasmids, which are responsible for resistance (Manigandan and Nelson, 2015).

Several bacterial strains have been utilized for the degradation of a number of pesticides, whose presence is causing disruption in the environment. According to various researches, potential bacterial strains such as *Pseudomonas aeruginosa, Stenotrophomonas acidaminiphilia, Bacillus cereus, Pseudomonas fluorescens, Serratia plymuthica* and *Achromobacter spp.* were found to be able to degrade cypermethrin, bifenthrin, permethrin, deltamethrin, fastac, fenvalerate and fluvalinate (Thatheyus and Selvam, 2013).

In the present study, the focus was on the group of insecticides called pyrethroids. Their application has become extensive in agriculture, homes and gardens for the control of insect pests. Pyrethroids are basically man-made chemicals that have similar characteristics as of the natural insecticide pyrethrin. These chemicals have been widely used because of their broad spectrum, high efficacy and low pollution effect. One of the most important synthetic pyrethroid is Lambda- cyhalothrin. It was registered in 1998 by the USEPA. Lambda cyhalothrin has low water solubility due to which it has a low potential to pollute ground water, but has a high affinity for soil (Manigandan et al., 2013). This study aimed at investigating the ability and

efficiency of the isolated bacterial strains to degrade lambda-cyhalothrin in Nutrient broth and Minimal Salt Medium, using lambda-cyhalothrin as the only source of carbon. The following were, the objectives of the study:

1.2 Objectives:

The present study has the following objectives:

- Isolation and Identification of potential bacterial strains for pesticide degradation
- Biodegradability check of selected isolates through Chemical Oxygen Demand (COD)
- Determination of the kinetic parameters for the biodegradation of Lambda cyhalothrin

CHAPTER 2

LITERATURE REVIEW

2.1 Background

2.1.1 Pyrethroids

Synthetic pyrethroids (SPs) are the man made compounds having similar characteristics as pyrethrins, which are the compounds found in the floral part of *Chrysanthemum cinerariaefolium*. Although these have been indicated as active insecticidal complexes, yet Pyrethrins have not been utilized for protection of plants on larger scale because of their high degradation potential (Laskowski, 2002; Palmquist et al., 2012).

As the substitute of natural Pyrethrins, two generations of synthetic Pyrethroids have been developed by modifying the molecular structure of natural pyrethrins. The first generation was produced in 1960s which consisted of various pyrethrin derivatives including, bioresmethrin, resmethrin, bioallethrin and tetramethrin. These synthetic pesticides, although being more active then natural pyrethrum, had limited applicability due to their instability in the presence of sunlight. Second generation of SPs was developed in 1970s which included permethrin, deltamethrin and cypermethrin. Subsequently, other insecticides were also synthesized such as betacyfluthrin, fenvalerate, lambda-cyhalothrin (Kidd and James, 1991; Katsuda, 1999).

Synthetic pyrethroids act more stably in direct sunlight as compared to natural pyrethrins and work more efficiently against a wide-ranging insects. Their increased stability made them more appropriate for agricultural use (Laskowski, 2002). As the utilization of organophosphorus pesticides decreased in the beginning of 2000s, pyrethroid pesticides made a significant comeback in the market. At present, pyrethroids constitute more than 25% of the pesticide market at global scale. (Cycon and Seget, 2016).

Pyrethroids vary from the rest of pesticides in a way that they consist of one to three chiral centers; therefore, a pyrethroid compound is composed of two to eight isomers. Recent studies have demonstrated that pyrethroids' degradation also displays prominent isomer selectivity. Pyrethroids are divided into two distinct classes depending upon their physical and toxicological properties i.e. type I and type II. Type I pyrethroids comprised of d-phenothrin, permethrin, allethrin, bifenthrin, tetramethrin and resmethrin. All of these are devoid of a cyano group. On the other hand, the insecticides classified as Type II i.e. cyhalothrin, deltamethrin, cypermethrin, cyfluthrin, fenvalerate, fluvalinate, and lambda-cyhalothrin possess a cyano group in their structure (Laskowski, 2002).

Moreover, pyrethroids are extremely hydrophobic compounds high and have octanol-water partition coefficients. They are either insoluble in water or have solubility of 0.1mg/L of water (Wauchope et al., 1992; Tomlin, 2003).

2.2. Toxicity of Pyrethroids

2.2.1. Acute toxicity

Pyrethroids can result in acute toxicity through dermal exposure which results in a typical skin sensations termed as paresthesia. It occurs especially in the facial region because of the hyperactivity of sensory nerve fibers in the cutaneous region. Ingestion of pyrethroids can lead to various issues such as sore throat, vomiting, nausea, and abdominal pain. Mouth ulceration may also occur which lead to difficulty to swallow and increased secretions, termed as dysphagia (Bradberry et al., 2005).

Other symptoms associated with pyrethroid exposure include dizziness, headache and fatigue, chest tightness, palpitations, and blurred vision less frequent. There have also been anecdotal reports of flight attendants who experienced skin irritation, sore throat, mucosa, vomiting, headaches, abdominal pain, dizziness and nausea (Wei et al., 2012). According to the survey, among 3,113 cotton farmers who were observed for 72 hours after spraying pyrethroids, 26.8% were found to be suffering from dizziness, facial sensations, fatigue, headaches, nausea as well as appetite loss (Chen et al., 1991).

In spite of the fact that pyrethroid is being used worldwide, relatively few reports are available on lethal human pyrethroid poisoning. Oral toxicity studies on rats reveal that approximately

55 mg/kg of body weight is enough to kill half of the population of exposed rats (LD50) with Bifenthrin or lambda-Cyhalothrin. These two pesticides appear as the most toxic (> 100 and 632 mg/kg body weight, respectively) (WHO, 2005).

2.2.2 Chronic toxicity

Chronic toxicity usually occurs with repetitive low doses exposure to the pyrethroids. Long-term effect related to pyrethroids are unclear as maximum information is collected from animal studies while only few epidemiological studies have been performed in humans (Koureas et al., 2012).

Sub-lethal effects of pyrethroids toxicity as seen among animals include perturbations of behaviour, hormonal balance and development. In case of mammals; movement, sexual, learning, anxiety and fear behaviors may be disturbed. Certain pyrethroids modify various types of behavior like schedule controlled responses. They may also produce incoordination, fluctuate the shock response to a noise and weaken grip strength (Wolansky and Harrill, 2008).

World Health Organization published a report in 2005 which identified various neurotoxicity and developmental effects of pyrethroids upon animals. These included delayed reflex, declined learning behavior and transformed binding of neurotransmitters to brain (acetylcholinesterase in the hippocampus), as a result of exposure to Deltamethrin; enhanced open-field immobility in male offspring exposed to Fenvalerate; reduced exploratory behavior in rats as a result of in utero exposure to Cyhalothrin and in utero treatment with Bioallethrin and Deltamethrin changed brain neurotransmitter receptors (muscarinic cholinergic receptors) in neonates and adults (WHO, 2005).

Pyrethroids have been designated as endocrine disrupting compounds (EDC), which in turn may result in an increased occurrence of testicular, prostate and thyroid cancer. Long-term exposure to pesticide may result in oxidative stress and damage to DNA and may also disrupt the endocrine system, leading to cancer in rats (Meeker et al., 2009).

In case of humans, various symptoms which were observed following chronic household exposure include nausea, delayed weight and hair loss, dizziness and respiratory pain, rashes on skin, loss of muscular response, immune and memory response (Kolaczinski and Curtis, 2004). However, in 2001 it was stated that in human no clear indication of carcinogenicity has been observed so far.

2.3 Mode of Action

Nerve fibers are particularly affected by pyrethroids which bind to the protein that is responsible for regulation of voltage gated sodium channel. Due to this reason, these are also termed as axonic poisons. This gate functions in a way that it opens to cause nerve stimulation and closes for termination of nerve signal. Ions are allowed to enter the axon through channels which act as their pathways and result in their excitation. If the channels do not function properly and constantly left open, it results in the repetitive discharges from nerve cells, eventually resulting in paralysis (Shafer and Meyer 2004). This dysfunctionality is caused by pyrethroids which

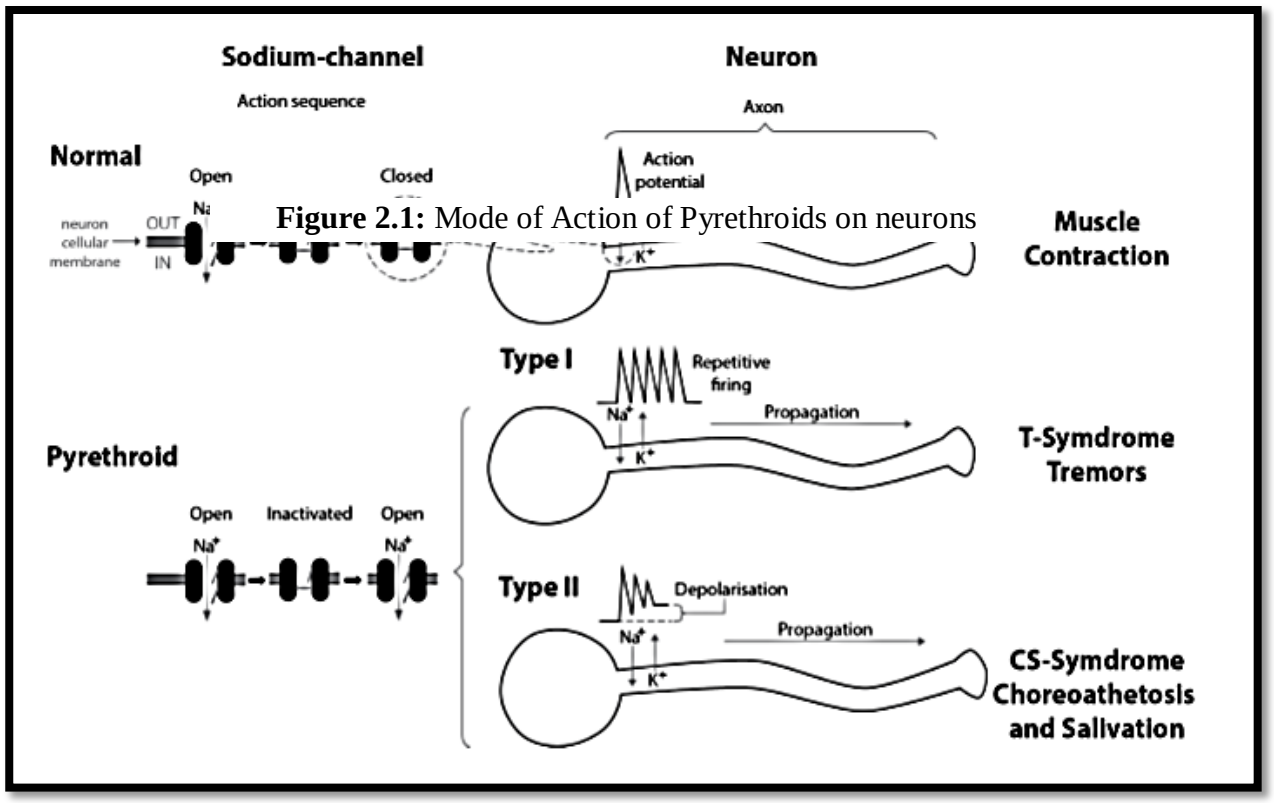


Figure 2.1: Mode of Action of Pyrethroids on neurons

bind to this gate and inhibit its proper closing, leading to tremors and constant nerve stimulation among poisoned insects. Infected organisms are unable to regulate their nervous system and fail to produce synchronized movement.

Lambda-cyhalothrin have the ability to impact calcium and chloride channels that are vital for proper nerve functioning (Burr and Ray, 2004). Pyrethroids, with their lipophilic nature are rapidly absorbed by biological membranes and tissues. Lambda-cyhalothrin makes its way inside the insect cuticle and cause nerve conduction disruption within only minutes. This results in interruption in feeding, muscular control loss, paralysis and eventually death. This insecticide has strong repellent effect towards the insects which provides Additional protection to the crops.

2.4 Environmental Effects of Pyrethroids

2.4.1. Occurrence

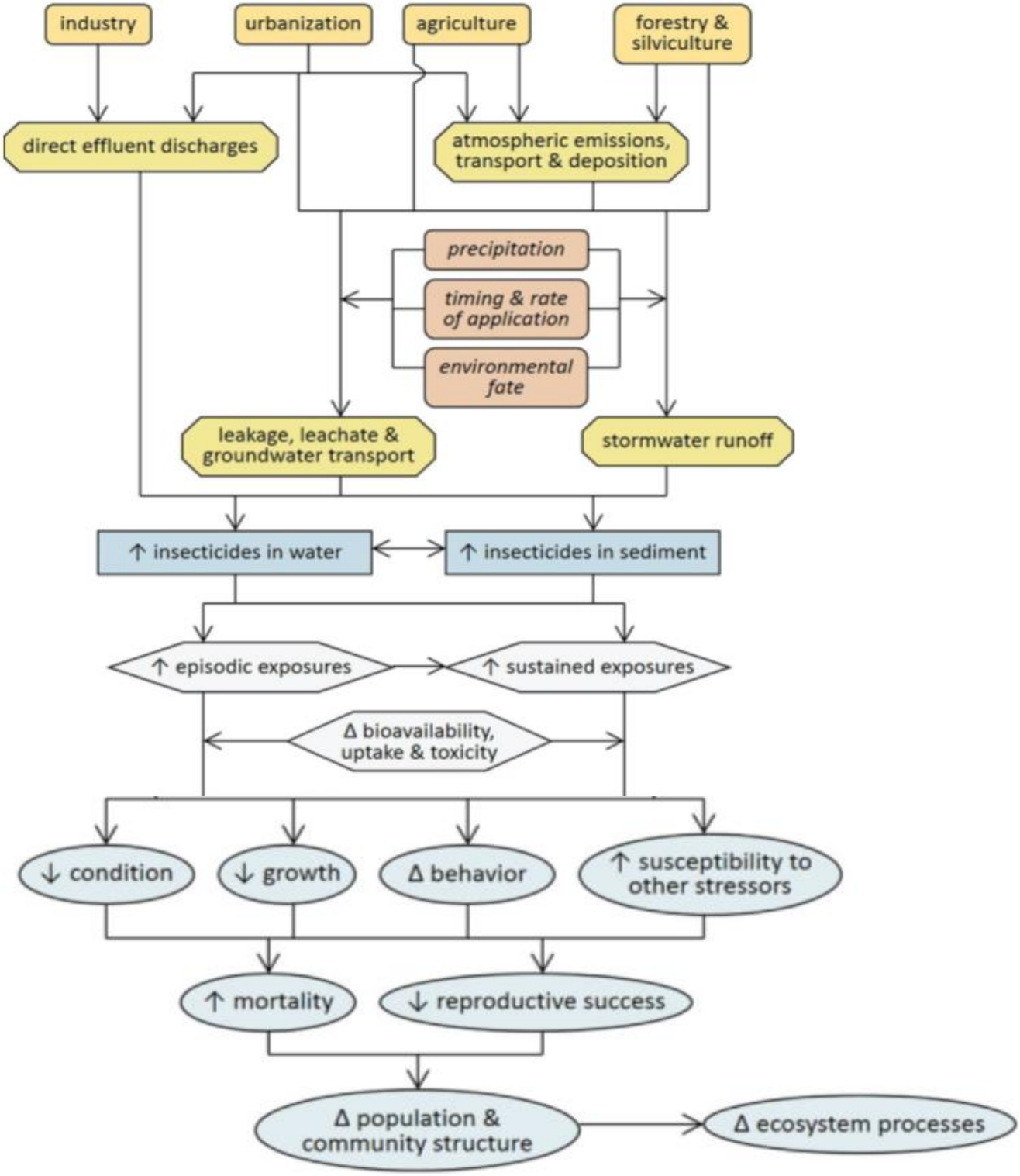
Pyrethroids move into the environment by means of drift and deposition of sprays or through leaching from agricultural and household applications. In urban areas, these pesticides are utilized for ornamentals, residential lawns, golf course turf as well as structural pest control (Kuivila et al., 2012). As pyrethroids are exceptionally harmful to aquatic animals, they are not sprayed into the water bodies directly, but can enter through leaching indirectly from treated areas and may also be released with tail water as in case of rice paddies (Oros and Werner, 2005). Even secondary treatment systems installed at municipal wastewater treatment facilities have no effect on the removal of pyrethroids (Weston and Lydy, 2010).

Pyrethroids are able to enter into the environment both through direct and indirect ways. The application of pyrethroids for in order to control pest in agriculture, silviculture and horticulture is the direct form of contamination. Mosquito larvae has been controlled by cypermethrin, deltamethrin, fenpropethrin, fenvalerate, and permethrin. Permethrin and Deltamethrin were utilized to control black fly. Permethrin was air-sprayed for spruce budworm (*Choristoneura fumiferana*) control in a spruce forest in Canada. This resulted in direct exposure of streams and ponds to the insecticide. Moreover, irrigation water in ditches, canals, and streams surrounding agricultural fields can collect direct deposits of insecticides like cypermethrin, permethrin and deltametrhin via air-spray used for cotton pests (Antwi and Reddy, 2015).

Wind, water or feed can also cause pesticides to enter in aquatic bodies indirectly. Windborne residues, also termed as pesticide drift and movement of sediment polluted with pyrethroids via wind, soil erosion and water can also transfer such chemicals to areas far away from sites of application. One of the aquatic habitats are agricultural ponds which are most likely to experience exposure from pesticides by means of surface runoff and spray drift. Permethrin and

cypermethrin have been detected in water bodies that are located nearby potatoes, cotton, vineyards and sugar beets (Crossland et al. 1982).

Figure 2.2: Mode of Entry of Pyrethroids by USEPA (2015)



Transfer of pyrethroid and their residues in runoff is largely based upon the solubility of compound in water, and the distance from the point of application to the receiving aquatic bodies. Pyrethroid residues are readily available when suspended in water as compared to when they are adsorbed onto the particulates, resulting in increased residual uptake by aquatic animals and plants from water than from food (Antwi and Reddy, 2015).

2.4.2 Effect on non-target organisms

2.4.2.1 Terrestrial organisms

Some pyrethroids inflict mammals with long-term and secondary poisoning risks. Pyrethroids cause moderately toxicity in birds (LD_{50} >1000mg/kg) however, the major risk to the birds is indirect influence upon their food supply i.e. insects. Insects are approximately 2,250 times more vulnerable to pyrethroids compared to mammals due to enhanced sodium channels senstivity, minor size and lesser temperature of body (Bradberry et al., 2005).

Bees are devoid of enzymes responsible for detoxification and may be endangered from pyrethroid insecticides (vanEngelsdorp et al., 2009). Pyrethroids contaminate bees by means of direct interaction with treated crops or nearby flowers, with spray, contact with polluted foliage as well as uptake of chemical from contaminated nectar or pollens (Goulson et al., 2008). Tau-Fluvalinate pyrethroid are able to reach apiaries indirectly when utilized as insecticides, or when used directly in hives in the form of acaricides. Deltamethrin have been detected in bees (5.9% of samples), honey, and wax and also in pollens with highest concentrations quantified (39.0 µg/kg) (Hénault-Ethier, 2015).

Earthworms, which play a vital role in regulating organic matter and serve as soil health indicators in various natural ecosystems, are also subjected to long-term risks from insecticides like Bifenthrin (Stork and Eggleton, 1992).

2.4.2.2 Aquatic organisms

Aquatic organisms are also affected negatively by pyrethroids. Young organisms (as of daphnia, copepods, and carp) are much more sensitive compared to adults, while males are more sensitive compared to females. Nutritional status also influences the probability of aquatic species to be affected by pyrethroids (Oros and Werner, 2005). Aquatic organisms may

experience reduced growth as observed in mysid shrimp and bluegill sunfish; fish suffer from transformed behavior such as equilibrium loss, quick erratic swimming, gulping respiration, lethargy, jaw spasms and darkened pigmentation; whereas water flea displays immobilization and reduced movement to stimulation.

Reproduction is also adversely affected in various aquatic species i.e. mysid shrimp, daphnia and fish. As these come in contact with the pyrethroids, their immunity level as well as the capability to combat infectious agents is challenged. There are swimming performance tests which can evaluate directly about how efficiently a fish may move and nourish itself in harsh. These tests exhibit that fish are influenced by these pesticides even at the minimum intensities that are usually considered to have negligible impacts (Hénault-Ethier, 2015).

2.5 Pyrethroids persistence and degradation

2.5.1 Factors affecting pyrethroids persistence

The impact and perseverance of pyrethroids is greatly affected by abiotic factors. For example, at low temperatures, both pyrethrins as well as synthetic pyrethroids are considered effective insecticides at low temperatures which may also affect non-target creatures (Harris and Kinoshita, 1977). Pyrethroids are able to degrade relatively quickly in the atmosphere by various biotic or abiotic routes, although these are inaccessible to degraders by adsorption into the soil and sediments.

Animal, plant and microbial degradation/photodegradation have all been established (Thatheyus and Selvam, 2013). Soil degradation rate differs for various pyrethroids and depends upon the type of soil, climate as well as the abundance and diversity of inhabiting .microorganisms. Various bacterial genus like *Pseudomonas, Erwinia*, *Enterobacter, Aeromonas, Bacillus, Stenotrophomonas, Serratia*, *Achromobacter,*and *Yersinia* have been known as degraders of different types of pyrethroids. Among these, some rely solely upon the insecticide as the source of carbon.

The processes of hydrolysis and photolysis take place relatively slowly indoors. Rapid degradation occurs within few days but is proceeded by slower degradation rates over approximately two year time period. In areas with limited sunlight and air-circulation, such as in subway tunnels or grain elevators, many of the sprayed pyrethroids e.g. D-Phenothrin retain

after almost one year.92 Cypermethrin remain present in environmental water conditions for 50 days or more while their photodegradation may need 100 days to take place (Berger et al., 1997).

Among pyrethroids, permethrin is also one of the most stable in the presence of ultraviolet light. The half-life of these compounds when adsorbed to soil particles is of 43 days. Termiticides, formulations created in order to kill termites may persist up to approx. 5 years. Pyrethroids are generally considered to possess similar physical and chemical characteristics that have impact on their environmental fate and transfer. In aerobic conditions, pyrethroids have average half-life ranges between 30 to 100 days (Oros and Werner, 2005).

2.5.2 Factors affecting degradation of pyrethroids

Pyrethroids are subjected to varying pathways as they travel through the soil environment. These include sorption-desorption, transformation/degradation, volatilization, uptake by plants, runoff into surface waters, and displacing into the groundwater. Transformation/degradation is one of the major routes controlling the fate and transfer of pyrethroids in the environment. It also covers other methods including abiotic degradation (e.g., oxidation, hydrolysis and photolysis) as well as bio-degradation. With the help of these processes, pyrethroids are completely degraded into byproducts or they are entirely mineralized. However, the important factor that determines the inherent biodegradation of pyrethroid is its molecular structure (Fenlon et al., 2011; Zhao et al., 2013; Xu et al., 2015; Zhang et al., 2016).

Ester cleavage is a major process during environmental degradation of pyrethroids that results mostly in the production of different compounds i.e. cyclopropane acid, 3-phenoxybenzyl alcohol, 3-phenoxybenzaldehyde (3-PBA) or 3-phenoxybenzoic acid. 3-Phenoxybenzyl alcohol; an intermediate and photo-catabolic product of pyrethroids, which can result in the corresponding carboxylic acid by the process of oxidation (Tyler et al., 2000; Wang et al., 2011; Xiao et al., 2015). The degradation efficiency of pyrethroids is influenced not only upon catabolic activity of soil microflora but also depends largely upon the different characteristics of soil, i.e. content of organic matter, soil texture, pH, moisture, and temperature (Cycon and Seget, 2016).

2.6 Biodegradation of pyrethroids

Microorganisms capable of degrading bacteria may be easily isolated from contaminated sites. There have been reports regarding isolation of various bacterial and fungal species belong to different genera from pyrethroid-polluted soils (Lee et al., 2016), sludge (Sundaram et al., 2013) or wastewater (Chen et al., 2014).

In order to achieve maximum benefit from microbes in the clean-up of soils contaminated with pyrethroids, it is necessary to analyze the potential of microbes for pyrethroid degradation under optimized conditions in liquid media. Numerous studies have established the fact that many of the bacterial and fungal species have the ability to degrade pyrethroids in liquid cultures.

Pyrethroids are degraded by these microorganisms either by utilizing them directly as a carbon source (Akbar et al., 2015) or co-metabolically (Lee et al., 2016). Nonetheless, the rate of the biodegradation in liquid media is based upon many factors such as temperature, pH, pyrethroid concentration, nutrients, inoculum size and the traits of bacterial or fungal strains (Chen et al., 2015).

Among all tested strains, bacteria belonging to the genera *Bacillus, Pseudomonas, Serratia, Brevibacillus, Sphingobium,* and *Ochrobactrum* have been observed as metabolically active microbes that have the ability to degrading numerous pyrethroids.

2.7 Microbial degradation of Lambda Cyhalothrin

2.7.1 Lambda Cyhalothrin

Robson and Crosby were the scientists who first introduced Lambda-cyhalothrin in 1984. ICI Agrochemicals now known as Syngenta presented the pesticide in Central America and Far East in 1985. The compound is basically a 1:1 mixture of two stereoisomers, (S)-α-cyano-3phenoxybenzyl-(Z)-(1R,3R)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethyl cyclopropane carboxylate and (R)-α-cyano-3-phenoxybenzyl-(Z) -(1S,3S) 3-(2-chloro-3, 3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate.

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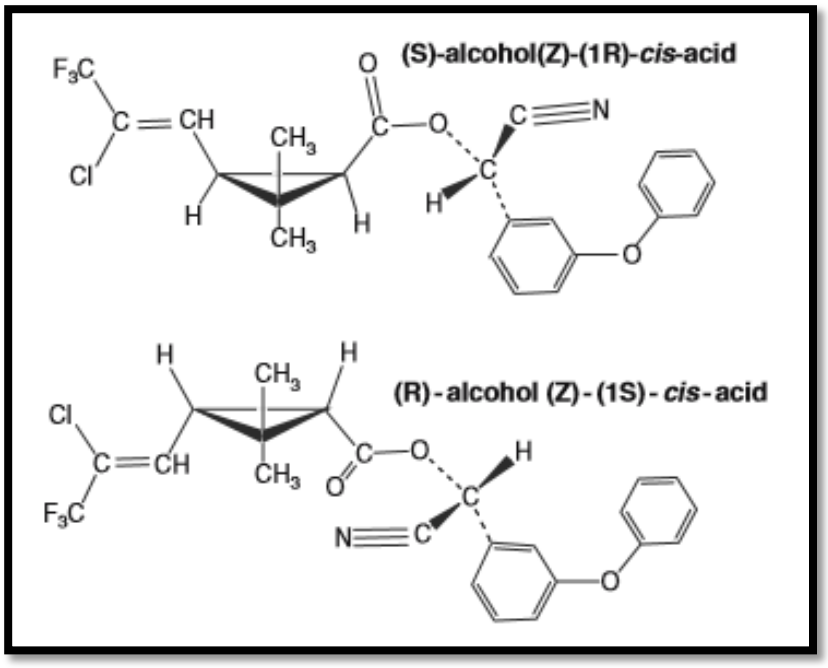
It exhibits as transparent solid at room temperature but sometimes may show yellowish color in liquid state. It cannot volatilized into atmosphere easily as it has low vapor pressure. Moreover, it has great potential to bioconcentrate because of a high octanol–water partition coefficient (K_{ow}). It also has high mean water–soil organic carbon partition coefficient (K_{oc})

Figure 2.3: The chemical structure of two isomers of Lambda cyhalothrin



Lambda cyhalothrin can easily be transported through aquatic systems as it has strong tendency to adsorb onto particulates' surfaces suspended into water column. Consequently, lambda cyhalothrin-contaminated sediments are the cause of greater risk to non-target aquatic organisms when exposed to those sediments. On the other hand, lambda cyhalothrin can alleviate its acute toxicity in water by adsorbing on bottom sediments or suspended solids as it will not be available in the water column for short period of time (Ming et al., 2008).

Table 2.1: Physical properties of Lambda cyhalothrin

Color	Colorless to Beige
Odor	Mild odor
Solubility	Very low or no solubility

Molecular Weight	449.854 g/mol
Melting Point	49.2°C
Molecular Formula	$C_{23}H_{19}ClF_3NO_3$

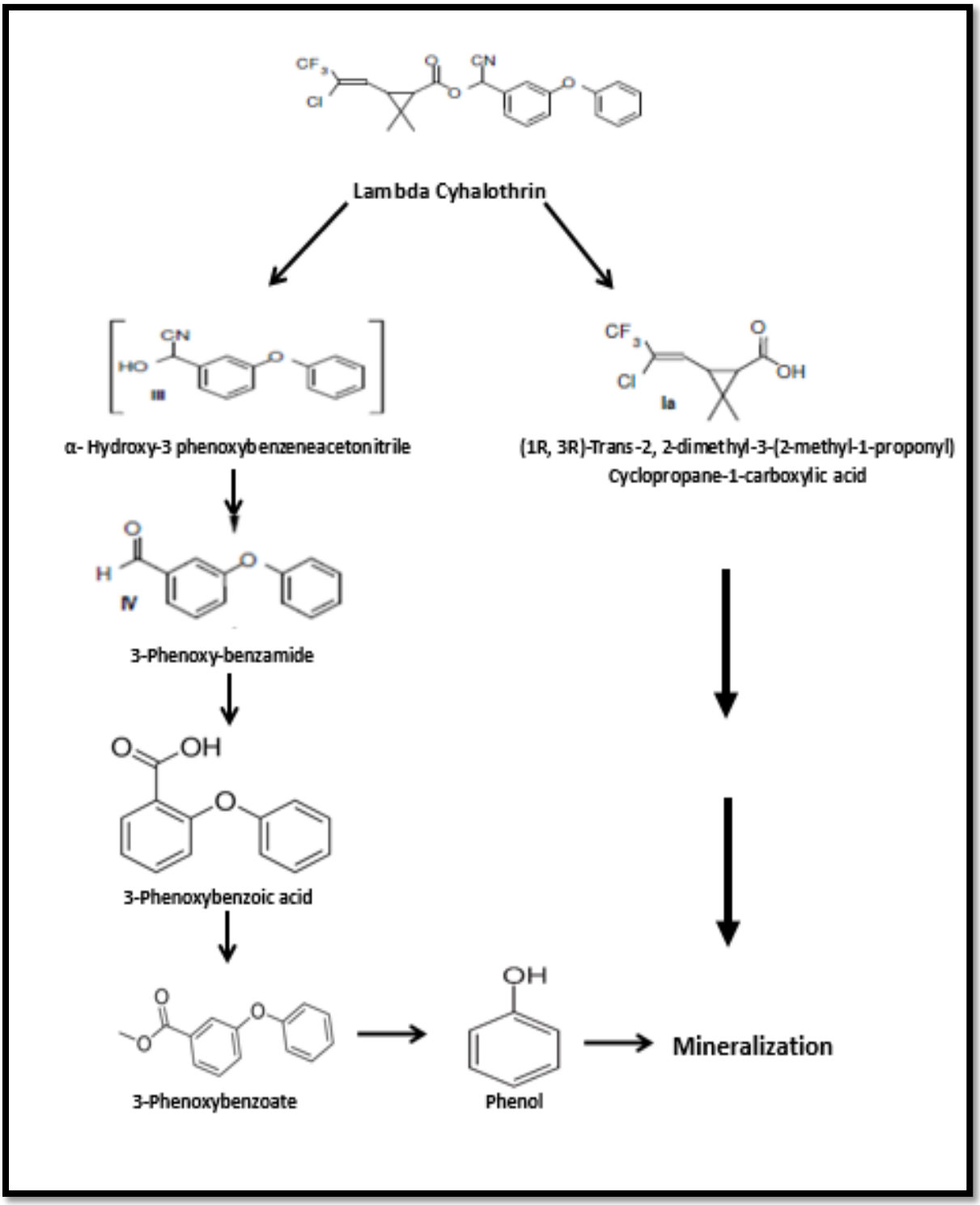
2.7.2 Biodegradation pathway of Lambda cyhalothrin

Ester-bond hydrolysis is the primary way involved in the degradation of pyrethroids by microorganisms with the help of carboxylesterases (carboxylic-ester hydrolase). Carboxylesterases are basically enzymes that facilitate the process of hydrolysis in order to degrade large number of compounds that contain ester such as organophosphates, carbamates and pyrethroids (Sogorb and Vilanova, 2002).

A study revealed the first ever degradation pathway of cyhalothrin in which the parent compound is metabolized into six new metabolites by *Bacillus thuringiensis*. These metabolites include α-hydroxy-3-phenoxy-benzeneacetonitrile, 3-phenoxyphenyl acetonitrile, N-(2-isoproxy-phenyl)-4-phenoxybenzamide, 3-phenoxybenzaldehyde, 3-phenoxybenzoate, and phenol that ultimately mineralized into carbon dioxide.

N-(2-isoproxy-phenyl) 4-phenoxy-benzamide and 3-phenoxyphenylacetonitrile are those metabolites which were identified in the degradation of pyrethroids for the first time.

Figure 2.4: Proposed degradation pathway of Lambda cyhalothrin



Hydrolysis is the process that initially degrade the compound that results in the breakage of ester linkage. Then, further transformation was done by the cleavage of diaryl bond. Aromatic ring was then degraded followed by subsequent metabolism. Ultimately, *Bacillus thuringiensis* degraded the compound without the accumulation of any persistent product, leading to complete metabolism (Chen et al., 2015).

Many researches have been conducted in the past few years in order to minimize the harmful effects of pesticides and make the environment pollution free. Scientists are now principally focusing on the degradation potential of microorganisms for cleaning up pyrethroids from the environment.

Waheed Ahmed Ghumro and his co-workers (2017) have isolated two bacterial strains i.e. Mesorhizobium sp. Strain (S1B) and Bartonella sp. Strain (S2B) from cotton crop soil using soil enrichment technique. These strains have exhibited significant removal efficiency of Lambda cyhalothrin within 20 days. The strains S1B and S2B have exhibited 29 and 40% degradation of LC by incubating at 30°C and agitating at 200rpm under laboratory conditions. Moreover, their OD (optical density) were also measured with maximum growth absorbance of 1.19 ± 0.06 and 1.13± 0.09 respectively at 287nm, using UV-Vis Spectrophotometer.

Zhang et al. (2017) asserted that Achromobacter xylosoxidans strain may provide an efficient strategy for the degradation of lambda cyhalothrin. The strain was isolated from the activated sludge of a sewage aeration tank in a pesticide factory by enrichment acclimation and the streak plate method. The isolate was able to degrade 87.1% lambda cyhalothrin at the concentration of 500mg/L within two days.

Abdullah et al. (2016) presented eighteen bacterial strains in their study, isolated from soil samples which were collected from different agricultural sites, located in Saudi Arabia. Among the strains, the isolate DB17 was identified as *Pseudomonas putida* and was found to be much more efficient than other strains in the degradation of two pesticides i.e. profenofos and lambda cyhalothrin. Pseudomonas putida was able to degrade 99.57% of profenofos and 86.11% of lambda cyhalothrin at 30°C of incubation temperature, 100 ppm of pesticide concentration and 10^9 to 10^{11} cells / ml of bacterial inoculum concentrations for 9 days in liquid culture media. The analysis was done using GC/MS system. In this study, the genes (mpd, opd and pyty) were

also detected in the genomic DNA of Pseudomonas putida, which were responsible for the degradation of profenofos and lambda cyhalothrin pesticide.

Manigandan and Nelson (2015) isolated 52 strains from 6 soil samples collected from agricultural fields located in Tamil Nadu. Out of these strains, only three were found to be capable of exhibiting good growth in MSM media. The three strains JJC1, JJC2, and JJC3 were identified as *Stenotrophomonas maltophilia, Enterococcus faecalis* and *Pseudomonas fluorescens* based on morphology, biochemical tests and16S rRNA analysis. HPLC was used to determine the percentage of residual pesticide. It was observed that there was a decrease in residual level of pesticide with the increase in incubation period of microorganisms. A simultaneous increase in percentage degradation of lambda cyhalothrin was also noted. Thus, *Stenotrophomonas maltophilia* was found to be more efficient compared to other two strains with 85% degradation of pesticide in 8 days.

In 2015, Chen and coworkers studied the degradation of cyhalothrin. Cyhalothrin degrading bacteria was isolated from an activated sludge sample collected from a pyrethroid-contaminated area using the enrichment method. The isolate was identified as *Bacillus thuringiensis* on the basis of morphology, biochemical characterization, 16S rRNA analysis and API identification system. In minimal (MM) medium, the strain was able to completely degrade the pesticide, cyhalothrin within 72 h. While in Luria-Bertani (LB) medium, only 86% of cyhalothrin was degraded at the concentration of 100 µg/mL at 72 h post incubation and 100% degradation was achieved at 120 h by *B. thuringiensis*. Later on, when there was increase in the initial concentration of cyhalothrin to 200, 400, 600 and 800 µg/mL, significant degradation of 95.5, 87.4, 84.0, and 82.1% respectively, was observed at 72 h. Thus, the results revealed that the isolated strain exhibited great potential for the degradation of cyhalothrin.

CHAPTER 3

MATERIALS AND METHODS

Biodegradation study of lambda cyhalothrin was carried out in the 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 Lambda cyhalothrin was purchased from local Pharmacy. The common name for Lambda cyhalothrin in the market, is Nokout.

Methanol for standard stock preparation was acquired from Merck (Germany). All COD chemicals used were purchased from sigma Aldrich.

3.2 Concentrations of Lambda-cyhalothrin

In order to select the preferred media for growth of the particular strains, both Nutrient broth and Mineral salt media were used to perform comparative studies. Lambda cyhalothrin was utilized as only carbon source in Mineral salt media. Different concentrations of LC 100, 200, 300, 400, and 500 mg/L were used (Zhang et al., 2017; Ghumro et al., 2017).

3.3 Preparation of the media

All experiments were performed using sterile glassware and distilled water. Sterilization was done by autoclaving the glassware at 121°C and 15 lb/cm^2 pressure for 15 minutes. The sterilized glassware was then dried in hot air over at 150°C. Nutrient Broth and Mineral Salt media was used for degradation of pesticide. The composition of Mineral Salt media is as follows:

Table 3.1: Composition of Mineral Salt Media

Sr. No.	Component	Quantity (g/L)
1.	Diammonium Phosphate $(NH_4)_2HPO_4$	0.5
2.	Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	0.2

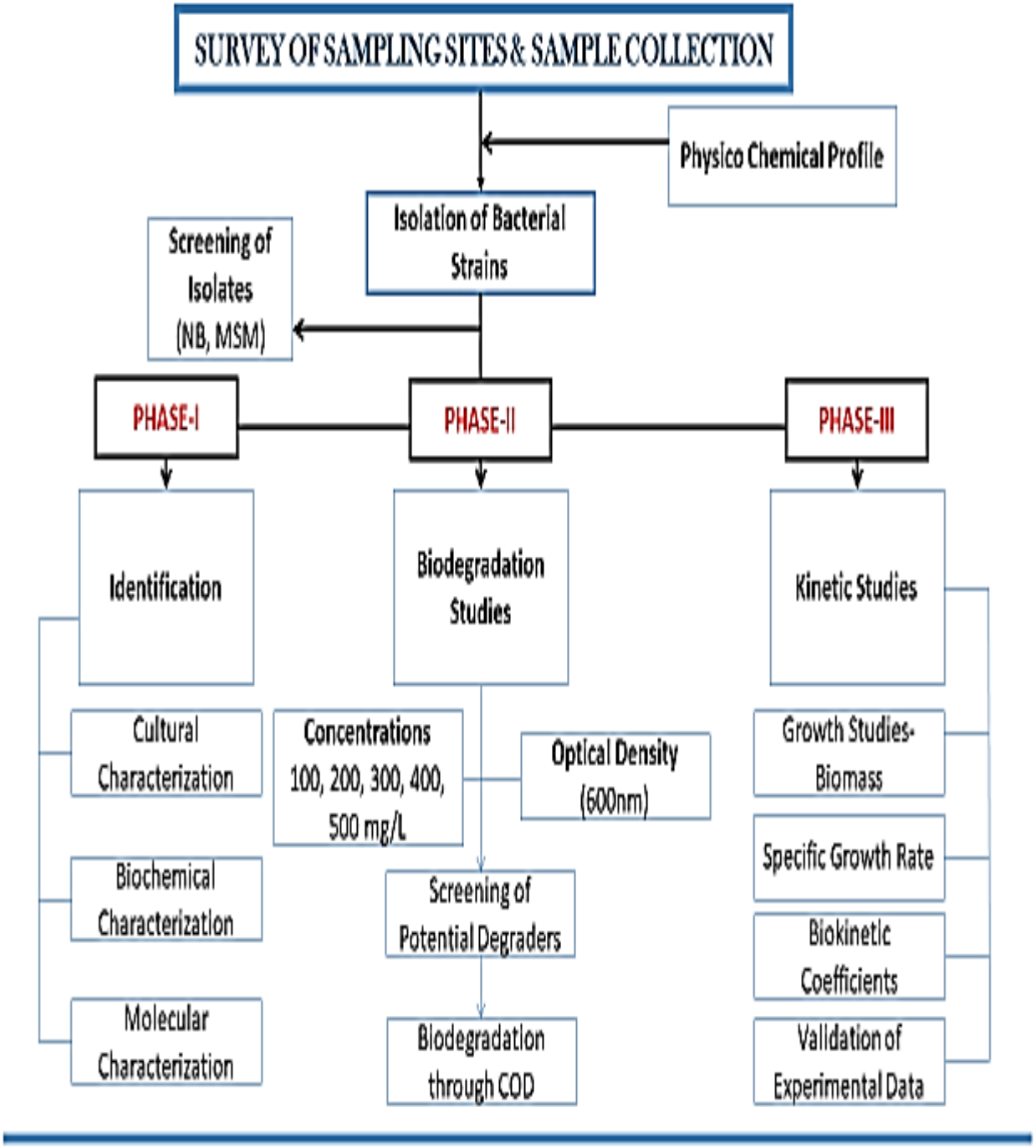
3.	Dipotassium Phosphate (K_2HPO_4)	0.1
4.	Calcium Nitrate ($Ca(NO_3)_2$)	0.01
5.	Ferrous Sulfate Heptahydrate ($FeSO_4.7H_2O$)	0.001

The above mentioned salts were added in 1L autoclaved distilled water and mixed properly (Hashmi and Kim, 2003). The pesticide Lambda Cyhalothrin (LC) was added in both media to obtain different concentrations i.e. 100, 200, 300, 400, 500 mg/L. In MSM media, LC was used as a sole source of carbon for the microorganisms.

3.3.1 Other media:

Fresh bacterial cultures were required for each experiment. For this purpose, nutrient agar plates were prepared according to standard manufactures instructions in distilled water and then it was autoclaved at 121°C and 15 lb/cm^2 pressure for 15 minutes. The nutrient agar was then cooled in water bath at 45°C and poured in autoclaved petri plates inside laminar flow hood to avoid any contamination. These plates were then placed in incubator for 24 hours at 37°C for sterility test. Similarly, nutrient broth was also prepared following standard manufactures instructions in distilled water and then autoclaving it at 121°C and 15 lb/cm^2 pressure for 15 minute. For sterility test nutrient broth was placed in incubator at 37°C for 24 hours.

Figure 3.1: Experimental Design

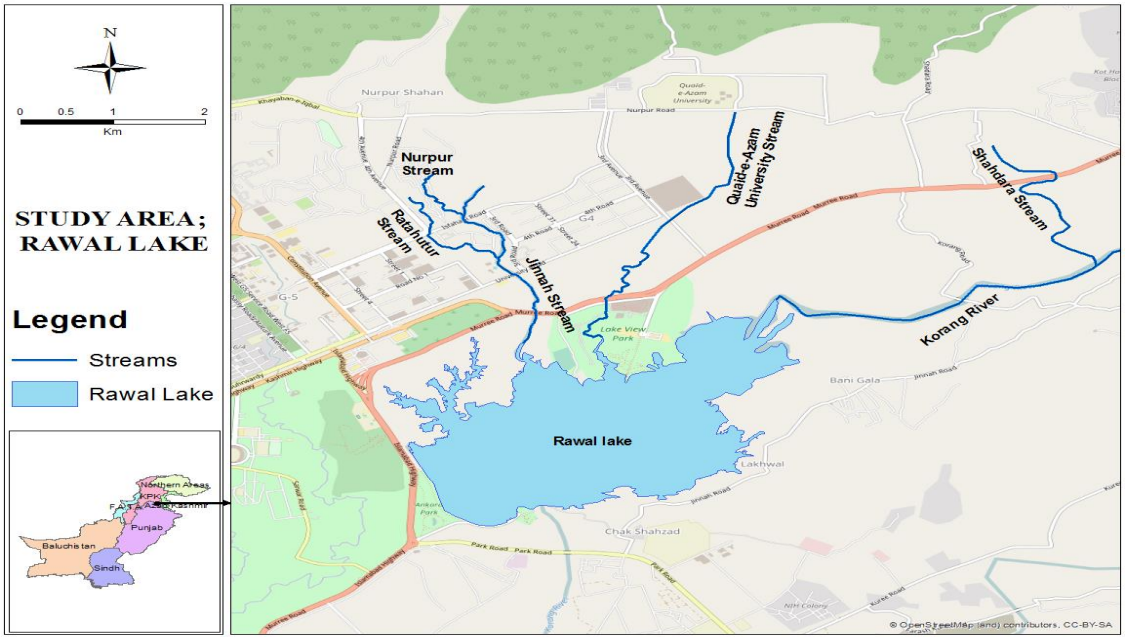


3.4 PHASE I

3.4.1 Site Description

Rawal Lake is located across the Korang River in Islamabad. The Lake serves as the main source of drinking water for the inhabitants of twin cities (Islamabad and Rawalpindi). Its neighboring streams receive water from local springs discharge and diverted untreated wastewater. The lake is exposed to a number of pollution sources from the last few decades. These include poultry waste, agricultural activities, human settlement, recreational activities, sedimentation, erosion and eutrophication in the lake (Ayaz et al., 2016).

Figure 3.2: Study Area; Rawal Lake



Upstream tributaries that congregate to form the Rawal Lake were identified and selected as sampling sites. The sampling locations along with their GPS coordinates are given in Table 3.2. Sampling was done from August to November 2016 and according to standard APHA protocols from upstream and downstream locations on the four streams and Korang River, just before they enter the Rawal Lake.

Table 3.2: Sampling locations with GPS coordinates

Sampling locations	Upstream GPS coordinates	Downstream GPS coordinates
Ratahutar stream	33°450‘06.8”N 73°050‘56.7”E	33°44’12.3”N 73°06’30.5”E
Nurpur stream	33°44’48.5”N 73°06’34.2”E	33°44’32.4”N 73°06’56.3”E
Jinnah stream	Inaccessible	33°744867”N 73°116639”E
Shahdara stream	33°746738”N 73°168261”E	33°45’35.0 ”N 73°10’25.2 ”E
Korang River	33°N 43.587; 73°E 8.416	33°N 42.707; 73°E 8.245

3.4.1.1 Sample collection

Water samples were collected in triplicates in sterile glass bottles (500mL) carefully in order to prevent surface sediments from being collected. Onsite physicochemical characterization was done using multimeter. TDS, EC and pH were checked onsite while turbidity was measured after transporting samples to the lab. The samples were stored at low temperature and transported to the lab and processed within 24 hours after collection.

Soil sample were collected from random points selected alongside streams in sterile polythene bags from depth of 10cm and then mixed to make a composite. The samples were then transported to lab and stored at 4°C before further processing.

3.4.2 Isolation of microorganisms

Serial dilutions of water samples were prepared and 100µL of each dilution was spread uniformly on nutrient agar plate using a sterilized glass spreader and was incubated for 24 hours at 37°C. NA plates were observed after 24 hour time period. Visually distinct bacterial colonies that appeared over the culture plates spread with different dilutions, were selected and further purified on NA medium using streak plate method.

In order to remove impurities i.e. gravel and stones from soil samples, these were sieved with the help of 2mm sieve. Following that, soil sample (1g) was added to distilled water (10 ml) and vortexed. After preparing serial dilutions for all the soil samples, 100 µL of each dilution was spread on NA medium under optimum conditions (24 hours, 37°C). After 24 hours, distinct bacterial colonies were chosen from different dilutions and further purified on NA medium with the help of streak plate method. In order to obtain pure and distinct colonies, each colony was subjected to 4-5 cycles of repeatedly streaking.

3.4.3 Identification of microorganisms

Isolated strains was identified with various characterization parameters including colony morphology, biochemical test and gram-staining (Collins and Lyne, 1985). Polymerase chain reaction was performed for species level identification.

3.4.3.1 Morphological Characterization

3.4.3.1.1 Colony morphology

Unknown microbes may be identified by observing the single colony which might play an important role in their characterization. In order to study colony morphology, single colony was selected and streaked on NA repeatedly so that pure culture was finally obtained. Colony was observed both from naked eye and through microscope at each step of purification in terms of different factors i.e. texture, shape, color, margin, size, elevation as well as pigmentation.

3.4.3.1.2 Cell morphology

In order to analyze cell morphology, bacterial smear was prepared by mixing bacterial culture in a drop of distilled water on the surface of a clean slide. The prepared slide was then dried by air and heat by instantly passing over the flame thrice.

As the smear was fixed over the slide, smear was treated with crystal violet i.e. the primary stain for 1 minute and then washed with the help of distilled water. Iodine solution was then flooded on the smear (for one minute) and washed again with distilled water. Subsequently, slide was treated for twenty seconds with decolorizing solution.

Cell wall of a gram- positive bacterium consists of 50-90% peptidoglycan that is a polymer of N-acetyl Glucosamine and N-acetyl muramic acid. Due to this cross-linkage, gram positive

bacteria is able to hold the primary stain i.e. crystal violet while gram-negative cell wall which is composed of only 10% peptidoglycan allows crystal violet dye to be wiped off with ethanol (Reiner, 2012).

Following treatment with decolorizing solution, glass slide was air-dried and smear was treated with counter stain i.e. safranin 40-45 seconds. The gram-negative cells appeared pink in color as they are capable to retain safranin while gram-positive bacteria showed purple color as they retained the crystal violet dye.

Figure 3.3: Gram Staining

3.4.3.2 Biochemical Characterization

3.4.3.2.1 Catalase Test

Bacteria adapted to aerobic environment produce such enzymes which are able to neutralize lethal forms of oxygen. Catalase is one such enzyme, which breaks down hydrogen peroxide into molecular oxygen and water. Bacterial colony was picked using sterilized inoculating loop and placed on clean slide. One drop of 3% hydrogen peroxide was then added to the colony on the slide. Positive result for catalase resulting in the production of bubbles.

3.4.3.2.2 Simmon Citrate Test

Citrate utilization test determines the capability of bacteria to oxidize citrate into oxaloacetate. Media used in this test has citrate as the only source of carbon available for bacteria without which bacteria are unable to grow. Citrate catalyzing bacteria also break down ammonium dihydrogen phosphate to ammonium hydroxide and ammonia which results in an alkaline nature of medium. At pH 7.5 or above, bromothymol blue turns royal blue while it is green in color at neutral pH. After preparing Simmon's citrate agar plates, these were inoculated with 24 hour fresh culture and then incubated for 24 hours at 37^{o}C.

3.4.3.2.3 Oxidase Test

A single loop full of inoculum from 24 hour fresh bacterial culture was picked and placed over a piece of filter paper and then treated with a drop of 1% N, N-dimethyl-p-phenylenediamine dihydrochloride solution. Blue or purple color appearance within few seconds indicates the presence of cytochrome oxidase enzyme within the microorganism.

3.4.3.2.4 Motility test

Motility test was carried out using hanging drop technique. Fresh bacterial suspension was prepared by taking a cover slip and placing a drop of autoclaved distilled water on it and adding bacterial culture to it by using sterile toothpick. The slip was then cautiously upturned over the depression and the drop containing bacterial cell hanged from cover slip into the cavity slide. Finally, slide was observed under microscope (100X) was used to observe the slide and to check bacterial motility.

3.4.3.3 Analytical Profile Index-20E

API-20E test strip is utilized for the identification of enteric gram negative rods. The strip is equipped with 20 separate small capsules in dehydrated form, each responding to a biochemical test. Each well is rehydrated with a bacterial suspension. Inoculation was prepared with a single colony of the SM1 treated with 0.85% NaCl solution, in order to ensure homogenous and clump-free suspension. Meanwhile, LDC, ODC, ADH, H_2S, and URE capsules were half filled with remaining capacity filled with sterile mineral oil. CIT,

VP, and GEL capsule were completely filled up to the top of the wells. Strip was then labelled and positioned in an incubator at 37°C for 24 hours. Following 24 hour time period,

Figure 3.4: Interpreting API-20E Results

reagents were added into the IND, VP and TDA chambers.

The positive and negative results of API E20 were translated into numerical profiles and interpreted with the help of API web software (shown in Table 3.3).

Table 3.3: API-20E Results interpretation

Tests	**Substrate**	**Reaction Tested**	**- Results**	**+ Results**
ONPG	ONPG	Beta- galatosidase	Colorless	Yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Red/orange
LDC	Lysine	Lysine decarboxylase	Yellow	Red/orange
ODC	Ornithine	Ornithine decarboxylase	Yellow	Red/orange
CIT	Citrate	Citrate Utilization	Pale green/yellow	Blue-green/blue
H2S	Na thiosulfate	H2S production	Colorless/gray	Black deposit

URE	Urea	Urea hydrolysis	Yellow	Red/orange
TDA	Tryptophan	Deaminase	Yellow	Brown-red
IND	Tryptophan	Indole production	Yellow	Red (2 min.)
VP	Na pyruvate	Acetoin production	Colorless	Pink/red (10 min)
GEL	Charcoal gelatin	Gelatinase	No diffusion of black	Black diffuse
GLU	Glucose	Fermentation/oxidation	Blue/blue-green	Yellow
MAN	Mannitol	Fermentation/oxidation	Blue/blue-green	Yellow
INO	Inositol	Fermentation/oxidation	Blue/blue-green	Yellow
SOR	Sorbitol	Fermentation/oxidation	Blue/blue-green	Yellow
RHA	Rhamnose	Fermentation/oxidation	Blue/blue-green	Yellow
SAC	Sucrose	Fermentation/oxidation	Blue/blue-green	Yellow
MEL	Melibiose	Fermentation/oxidation	Blue/blue-green	Yellow
AMY	Amygdalin	Fermentation/oxidation	Blue/blue-green	Yellow
ARA	Arabinose	Fermentation/oxidation	Blue/blue-green	Yellow
OX	Oxidase	Oxidase	Colorless/ Yellow	Violet

3.4.4 Molecular Characterization

3.4.4.1 Polymerase Chain Reaction (PCR)

3.4.4.2 DNA Extraction

DNA from the bacterial isolates was extracted using DNA extraction kit (Norgen Biotek Corporation). 1 ml of bacterial suspension was added to eppendorf and centrifuged at

14,000 rpm for 30 sec. in order to form cell pellet. Supernatant was drained and cell pellet was treated with 250 µL of re-suspension solution. Gentle vortexing led to the resuspension of cells. 250 µL of Lysis Solution and 12 µL of Proteinase K were then added to the cell suspension, followed by thorough mixing. The mixture was then placed in incubator at 55°C for 30 minutes. Lysate was then treated with 50 µL of Binding Solution and mixed well with gentle vortexing. Mixture was subjected to spin column assembly and centrifuged in the unit for 4 min. at 8000 rpm. The flow through was discarded after centrifugation and reassembling of spin column with its collection tube was done. Wash Solution (500 µL) was applied to the column, and centrifugation was performed for 1 minute at 14,000 rpm. The spin column containing DNA bound to the resin, was assembled within a 1.7 mL Elution tube. Then, Elution Buffer (200 µL) was placed at the center of the resin bed and the elusion tube was centrifuged for 1 minute at 6,000 rpm. The assembly was again re-centrifuged at 14,000 rpm for further 2 minutes to assemble the total elution volume. The purified genomic DNA obtained was stored at 2-8°C in a refrigerator for a few days. However, -20°C temperature is recommended for longer storage.

3.4.4.3 PCR Amplification

The extracted DNA was then amplified using PCR. For this purpose, reaction mixture was prepared with total volume of 50 µl. Its composition is given in the Table 3.4.

Table 3.4: Composition of Reaction mixture

Reagents	**Volume (µl)**
Taq PCR master mix	25
DNA template	1
Primer F(10µM)	2
Primer R(10µM)	2
Nuclease free water dd H_20	20
Total volume	50

The reaction mixture was processed in Thermocycler (Extragene 9600). For the 16SrRNA gene detection , the PCR program includes 5 min at 95˚C for template denaturation, and 40 cycles for template amplification consisting of three steps: 95°C for 1 min for DNA denaturation into single strand, 61°C for 1 min for primer to anneal to their complementary sequences on either side of the target sequence, 72°C for 1 min for extension of complementary DNA strand from each primer and final elongation at 72°C for 10 min for Taq DNA polymerase to synthesize any un-extended strand left.

Figure 3.5: Thermocycler (Extragene 9600)

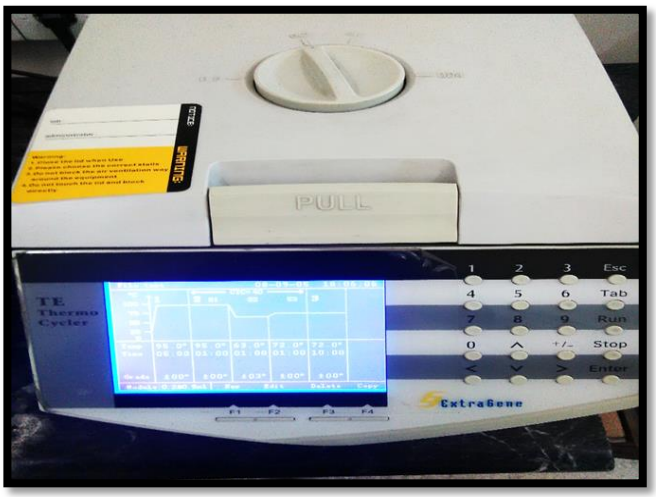
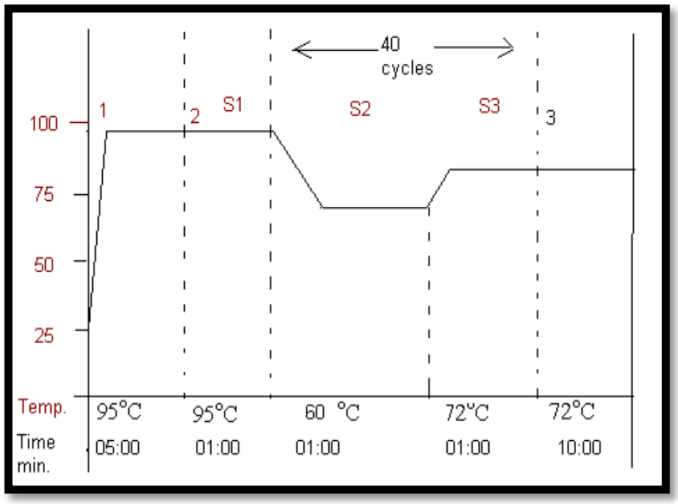


Figure 3.6: PCR Cycle



3.4.4.4 16S rRNA sequencing

Ice box was arranged in order to preserve the PCR products. These preserved isolates were then transferred to Genome analysis department Macrogen, Seoul, South Korea for 16S rRNA sequencing.

3.4.4.5 Phylogenetic analysis

The sequences were obtained from Macrogen. The junk data from sequences was removed by trimming with the helpof Bioedit software. After trimming, the sequences were analyzed through BLAST tool of National center of biotechnologcal information (NCBI). The species were then detected and identified. Their accession numbers were obtained from NCBI gene bank library. MEGA 7 software was then used in which FASTA sequences were added in order to construct the phylogenetic tree which showed linkages between the isolated strains and those at GENEBANK of NCBI.

3.5 PHASE II

3.5.1 Degradation Studies

Degradation studies were performed in order to screen out the isolates capable for bioremediation of LC and to find out the concentration at which maximum degradation was done. Experiments were conducted in triplicates. The results obtained were further validated by applying biodegradation kinetics.

3.5.2 Screening of potential LC degraders

Screening was done in both Nutrient broth and MSM media containing 100 mg/L of lambda cyhalothrin and exposing to bacterial cultures. The pesticide in the media served as the sole source of carbon and energy for the strains. The conical flasks containing the Lambda cyhalothrin and inoculum were kept in incubator at 37°C. Growth was monitored at regular intervals using single beam UV-visible spectrophotometer at 600 nm. Strains that possessed the highest degrading capability were selected for further LC degradation studies

3.5.3 Biodegradation

On the basis of the above screening step, four strains were selected which have shown the maximum growth in the presence of LC. In order to investigate the biodegradability of these selected isolates, they were inoculated into liquid media containing lambda cyhalothrin.

To prepare liquid media, specific amounts of lambda cyhalothrin were added in Nutrient broth and MSM media to attain 100, 200, 300, 400, and 500 mg/L concentrations. 24 hour fresh cultures of selected bacteria was added to the five prepared concentrations of LC in both media in equal amounts. The flasks were incubated at 37°C and the samples were monitored for 72 hours. The aliquots were drawn at regular intervals, diluted to 1000 times and analyzed for COD titration closed reflux method. According to the method, 2.5 ml of the sample was added to 1.5 ml of standard potassium dichromate digestion solution ($K_2Cr_2O_7$) and 3.5 ml of Sulfuric acid reagent in COD vials. The samples were refluxed in a thermoreactor for 2 hours at 150°C. Later on, the samples were cooled down and were transferred to Erlenmeyer flasks where 2 to 3 drops of ferroin indicator ($FeSO_4.7H_2O$) were added for further titration. Then, 0.25 M standard ferrous ammonium sulfate (FAS) was used as titrant and samples were titrated with it

and COD results were calculated. Optical Density measurements were also noted for both media at 600 nm using UV-visible spectrophotometer. Each group was run in duplicate. A control was also used which contained LC but no bacterial culture for all concentrations.

3.5.4 COD analysis

The samples were monitored for 3 days and aliquots were drawn at regular intervals i.e. 0, 4, 8, 24, 28, 48, 52, and 72 hours and analyzed for COD by standard 522 °C closed reflux titrimetric method (Standard Methods 2017). After titration, COD results were calculated using the following equation:

$$\textbf{COD as mg } \mathbf{O_2/L} = \frac{(\boldsymbol{A} - \boldsymbol{B}) \times \boldsymbol{M} \times \mathbf{8000}}{\boldsymbol{Volume\ of\ sample\ (mL)}}$$

Where:

A= Volume of FAS (mL) used for blank

B= Volume of FAS (mL) used for sample

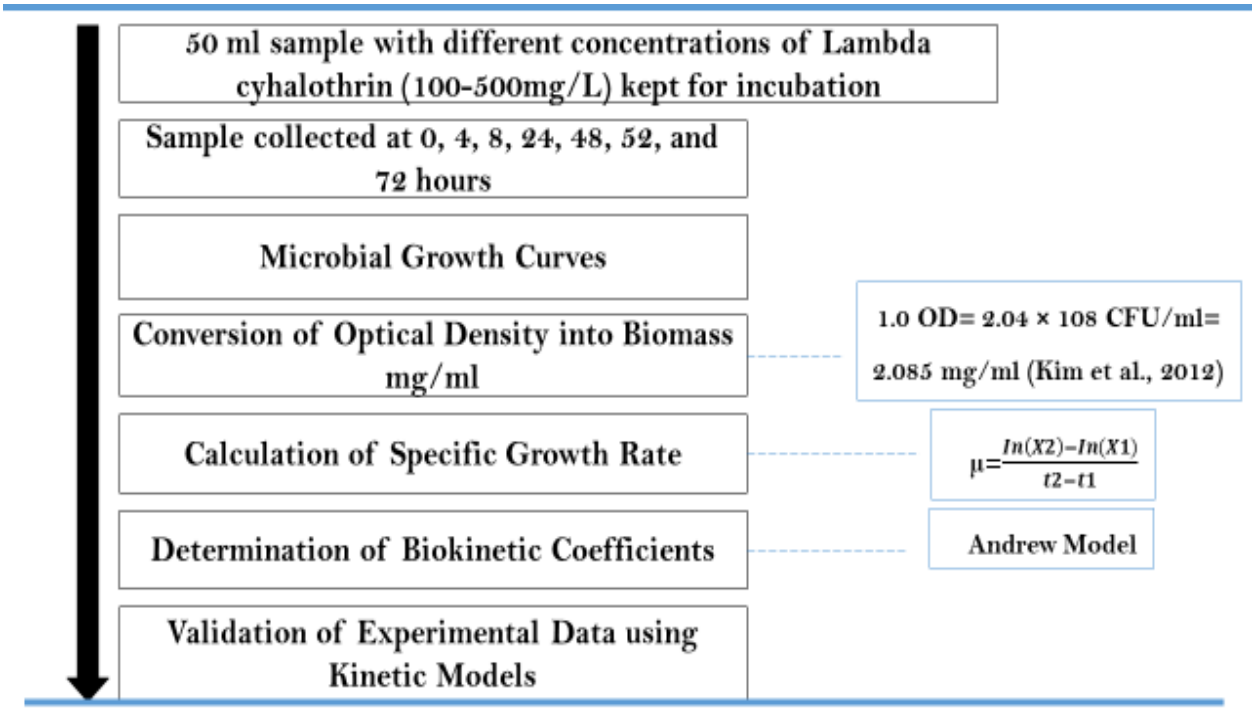
M= Molarity of FAS

8000= Milliequivalent weight of oxygen × 1000 mL/L

3.6 PHASE III

In order to evaluate the influence of initial concentration of Lambda cyhalothrin on the growth of *Pseudomonas aeruginosa,* batch experiments were conducted. Detailed steps carried out for the validation of experimental data using kinetic model are listed in the Figure 3.7.

Figure 3.7: Steps included in Kinetic Studies



3.6.1 Kinetic Modelling

Application of biochemical kinetic models is very essential as they provide assistance in understanding the behavior of microorganisms, their capacity in degradation and the processing of biological treatment system. Various kinetic models are now available in order to evaluate the data obtained in degradation. Enzymes are significant constituents of the biological reactions in which they transform the organic compounds into the cell mass and respective products. Hence, these models are primarily derived from the enzyme kinetics. Many different models are being utilized which deal with microbial growth, substrate inhibition and non-inhibition. For this study, because of its simplicity, Andrews's model has been applied and kinetic parameters have been estimated.

3.6.1.1 Andrews's model

Edward (1970) proposed various inhibitory growth kinetic models for the degradation of phenol, however, Rozich and colleagues in 1985 represented Andrews's model as the best fitted model, when they assessed the inherent variation of the growth model constants for heterogeneous populations to metabolize phenol. Due to its inclusive acceptance and mathematical simplicity, Andrews's model is one of the most widely used models. It is based on specific growth rate that describes the growth inhibition kinetics of microorganisms. The Andrews inhibitory growth kinetic equation is as follows:

$$\mu = \frac{\mu_m S}{K_S + S + S^2/K_i}$$

Where:

μ_m= maximum specific growth rate (h^{-1})

S= substrate concentration (mg/L)

K_S= half saturation constant (mg/L)

K_i= inhibition constant (mg/L)

3.6.2 Biokinetic coefficients

It is very important to have a clear concept of bio-kinetics in order to determine the appropriate growth and quantity of biomass in the system. Biokinetic coefficients involved in the process of biodegradation include specific growth rate (μ), half saturation constant (K_S) and inhibition constant (K_i).

3.6.2.1 Specific Growth Rate (μ)

It refers to the increase in the rate of cell biomass per unit of concentration of biomass. Since this increase in rate is constant during a specific period of time and is quantifiable, therefore,

specific growth rate may be measured in batch cultures. This time period exits between the lag and stationary phases. Its kinetic equation is given as follows:

$$\mu=\frac{In(X_2)-In(X_1)}{t_2-t_1}$$

Where:

X_2=Maximum biomass at time t_2

X_1=Initial biomass at time t_1

3.6.2.2 Half Saturation Constant (K_s)

The specific growth rate of microorganisms has a close linkage with the rate at which the substrate is utilized by them. Even if the substrate is in limiting condition, net growth of microbes is determined. Half Saturation Constant (K_s) is the substrate concentration at one half of the maximum specific growth rate. Its kinetic equation is given as:

$$K_S = \mu_m \frac{S}{\mu - S}$$

Where:

S= Substrate concentration (mg/L)

μ_m = Maximum specific growth rate (h^{-1})

μ= Specific growth rate (h^{-1})

3.6.2.3 Inhibition Constant (K_i)

The inhibition constant K_i indicates the inhibition tendency. It also indicates the degree of toxicity of the substrate towards the microorganisms. A lower Ki value indicates that microorganisms have a higher sensitivity to substrate inhibition.

CHAPTER 4

RESULTS AND DISCUSSION

Many researches have been conducted that focused on the biodegradation of pyrethroids, including cypermethrin, fenpropathrin and bifenthrin (Zhang et al., 2016). Though, bacteria capable of degrading lambda- cyhalothrin have been rarely stated. In the present study, lambda-cyhalothrin contaminated water and soil were used as bacterial source, in order to isolate beneficial strains with efficient degrading ability. Later on, total 20 strains were isolated through identification and characterization.

4.1 PHASE-I

4.1.1 Sampling Sites Description

Rawal Dam, situated in Islamabad, is constructed on Korang River. The river generates 84,000 acre feet of water per year with average rainfall, having a catchment area of 106 square miles. Rawal Lake and its catchment area are key sources of drinking water for Rawalpindi city and cantonment. There are four major streams, 43 small streams and Korang River contributing to its storage (Ayaz et al., 2016). These four major streams and Korang River were selected as sampling sites. Along with water samples, soil samples were also collected alongside the streams and river. The upstream and downstream locations and their coordinates are mentioned in Table 3.2. The description of sampling sites with their pictorial views are as follow:

4.1.1.1 Ratahutar Stream

The upstream location was located about 7 km away from Rawal Lake. A very few houses were built there with high mountains and lush greenery in the vicinity. The residents have their own waste discharge system instead of lobbing directly into running water. Therefore, the water was apparently clean and clear as shown in the Figure 4.1 (a). The downstream location of stream, from where the sample was collected was surrounded by slums. There were huge heaps of animal dung and other waste alongside the stream as shown in the Figure 4.1 (b). The residents of the surrounding area have dumped plastics, fabric, and feces directly into stream.

Figure 4.1: Ratahutar Stream (a) upstream (b) downstream

(a)

(b)

4.1.1.2 Nurpur Stream

The upstream Nurpur was passing through a village, with huge heaps of waste beside the stream. There was direct discharge of different kinds of wastes from houses including, plastic bags, bottles, and animal feces. The water was apparently murky and odorous due to increased algal growth. Large amount of wastes including plastic bags, food, feces can be seen in the Figure 4.2 (b), dumped by the locality of the surrounding area. The stream was observed to be highly polluted and water was extremely unfit for domestic usage. Massive algal growth in water was also reported.

Figure 4.2: Nurpur Stream (a) upstream (b) downstream

(a)

(b)

4.1.1.3 Jinnah Stream

Upstream location was inaccessible. The downstream location was situated near Bari Imam, with huge heavy traffic passing by. Massive growth of wild plants may be seen in the Figures 4.3 (a) and (b), but dumping of construction waste and garbage from nearby houses was also reported. The stream was appeared to be eutrophic due to enormous algal growth.

Figure 4.3: Jinnah Stream (a, b) downstream

(a) **(b)**

4.1.1.4 Shahdara Stream

The upstream shahdara (Figure 4.4 a) was a rocky area and the water flowing was clean and clear apparently. Few houses were also built but majority of the area was occupied by huge mountains. The downstream shahdara was also a rocky area with mountains and wild plants growth in the surroundings. The stream water has become odorous because of dumped garbage and contained enormous growth of algae, as shown in the Figure 4.4 (b).

Figure 4.4: Shahdara Stream (a) upstream (b) downstream

(a)

(b)

4.1.1.5 Korang River

The Figure 4.5 (a) is showing the upstream site of Korang River. The flow of water was static and it has become muddy and murky because of the presence of brick and tile factories located nearby. The residents of the area were using water for washing vehicles.

The Figure 4.5 (b) is showing the downstream site of Korang River. The discharge from nearby Brick and tile factories has made water muddy and murky. Cattle were also observed nearby. Water was used for washing cars.

Figure 4.5: Korang River (a) upstream (b) downstream

(a)

(b)

4.1.2 Soil Characteristics:

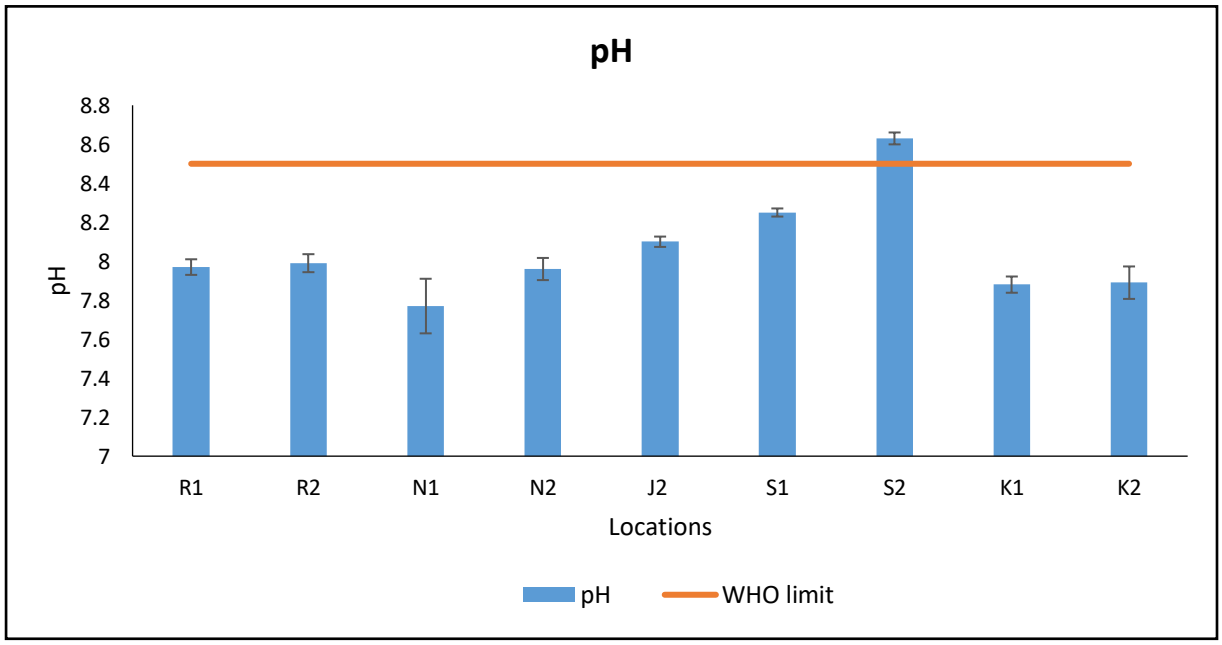
Soil that had already received treatment of various pesticides including lambda cyhalothrin was used for the isolation of bacterial strains. The pH soil was measured as it is the critical factor that influences the composition and behavior of soil bacterial communities. The pH was observed to be 7.3.

4.1.3 Physicochemical Analysis:

Physicochemical parameters of water samples including pH, Electrical Conductivity, Total Dissolved Solids and turbidity were recorded and compared with the standards given by WHO.

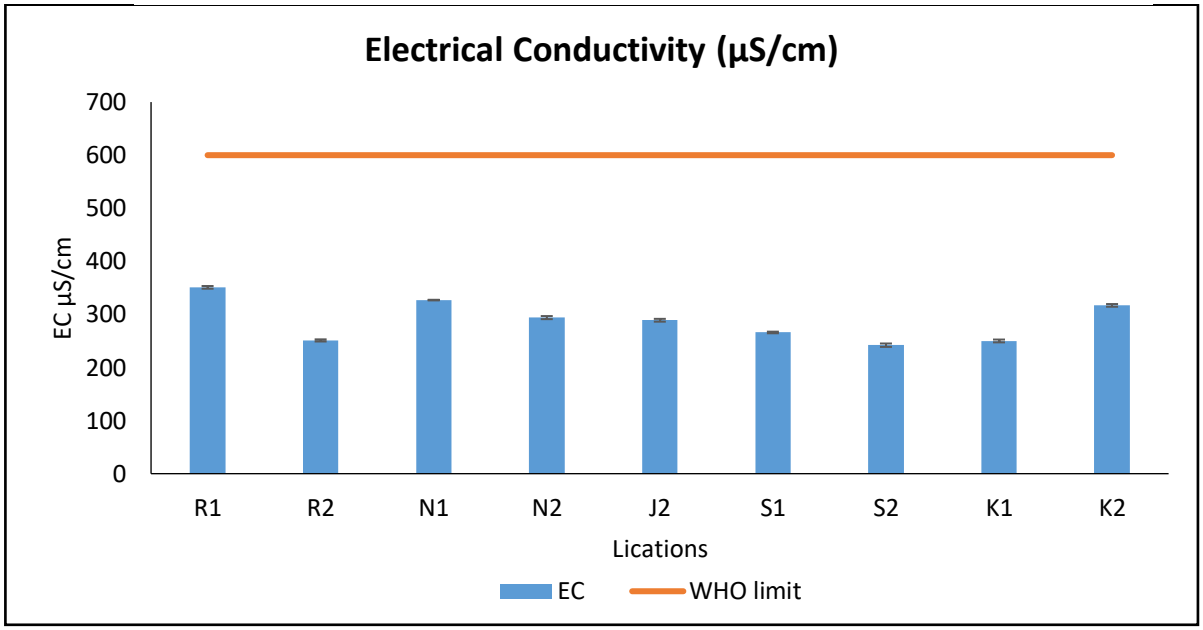
4.1.3.1 pH: It is one of the most important physicochemical parameters. pH of the water samples collected from different sites is shown in the Figure 4.6. Recorded pH of water samples was observed to be in the range of 7.77-8.63. The normal range of pH for water samples is 6.5-8.5, according to WHO. It is concluded that pH of all water samples was found to be within the normal range given by WHO, except for S2 water sample, whose pH is slightly more basic.

Figure 4.6: pH profile of water samples



4.1.3.2 Electrical Conductivity (EC): This parameter is strongly influenced by the suspended impurities in water and also depends upon the ion concentration in water. The recorded EC values for water samples can be seen in the Figure 4.7. The normal range stated by WHO for electrical conductivity of water is 400-600 µS/cm. It is observed that electrical conductivity of water collected from all sites was below the normal range except for sites R1, N1 and K2. The samples collected from these three specific sites may be considered more contaminated as they contained increased concentrations of dissolved ions in the water (IUCN, 2015).

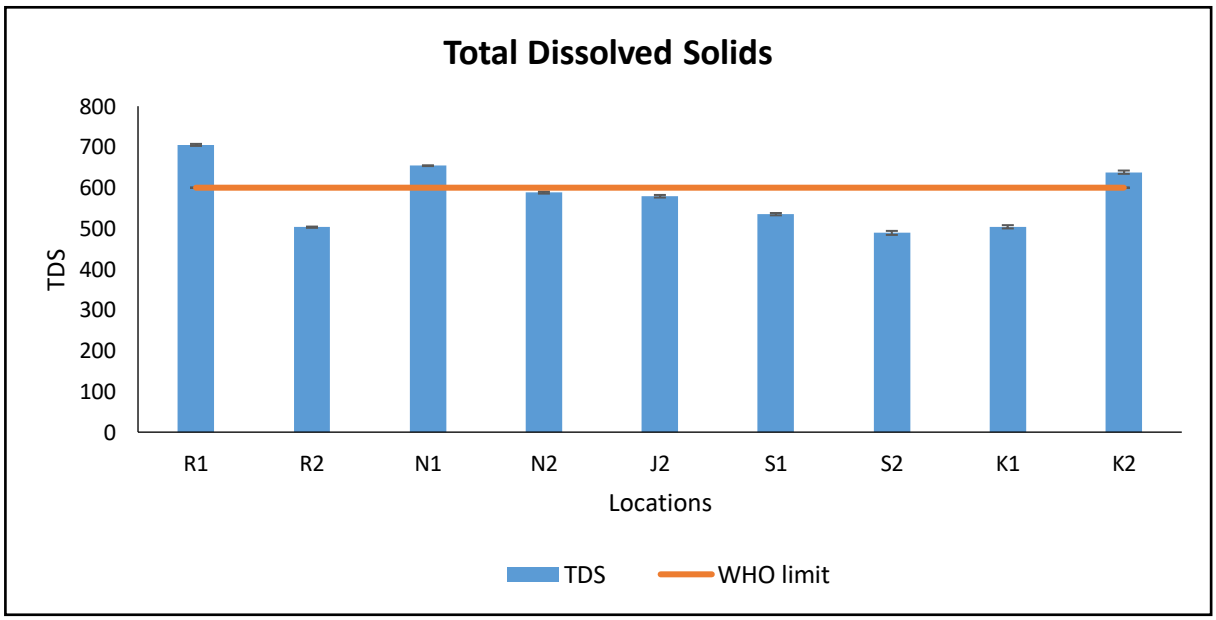
Figure 4.7: Electrical conductivity (EC) profile of water samples



4.1.3.3 Total Dissolved Solids (TDS):

Values of TDS are given in Figure 4.8. The normal value of TDS for water specified by WHO is 600 mg/L. Values of TDS for all water samples were measured. It is observed that all sites have the values of TDS lie within normal range except for R1, N1 and K2 sites where TDS exceed the normal range given by WHO, as shown in the figure 4.8. The measure of TDS is

Figure 4.8: TDS profile of water samples



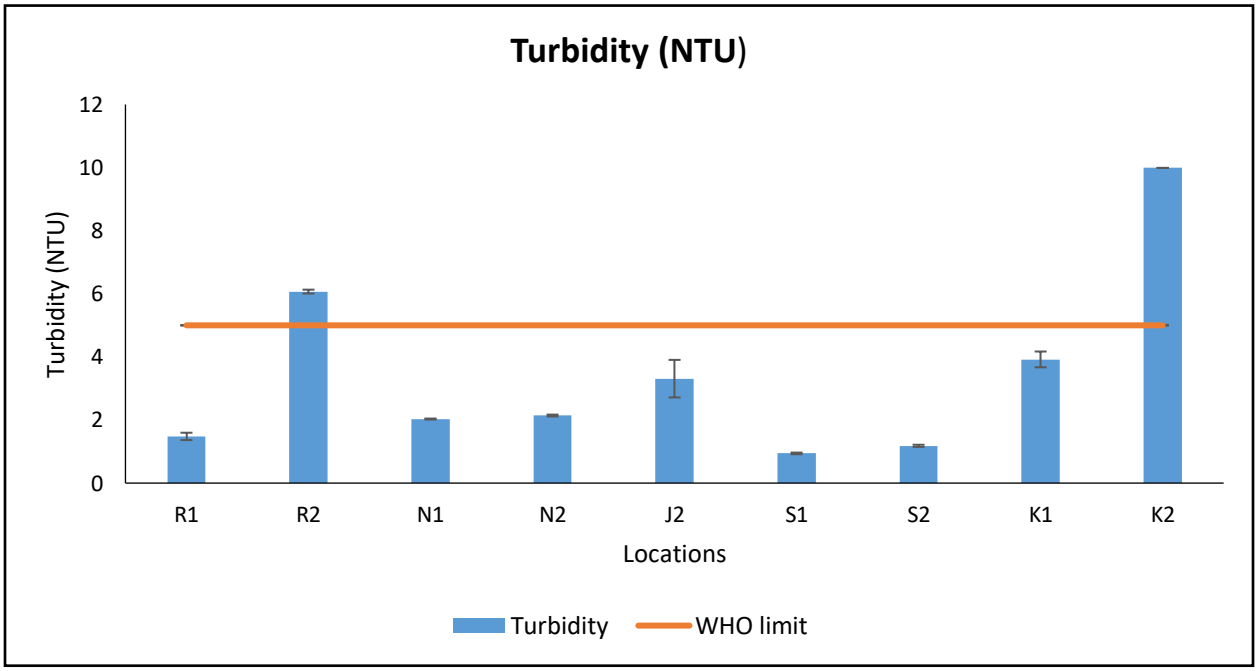
directly proportional to the extent of pollution in water (Masood et al. (2015). The high values

correlate with the addition of solids in runoff water from nearby factories, domestic and commercial areas.

4.1.3.4 Turbidity:

Values of turbidity are shown in Figure 4.9. The parameter of turbidity is mainly related to the concentration of suspended particles in water. The more total suspended solids in the water, the more it seems murkier and the higher the turbidity (IUCN, 2015). It is suggested by WHO that turbidity should not exceed 5 NTU and should be less than 1 NTU ideally. Turbidity of water samples was measured with maximum value of 9.99 NTU at K2 site, which is above the normal range and minimum value of 0.94 at S1 site. High turbidity at K2 and R2 sites may be because of sediment erosion, algal growth and waste discharge from nearby factories.

Figure 4.9: Turbidity profile of water samples



4.1.4 Isolation and Identification

A total of 20 strains were isolated from both water and soil. All the strains were given proper labels, shown in the Table 4.1. Screening was performed later on in order to obtain the potential strains for biodegradation.

Table 4.1: Isolated bacterial strains with their assigned labels

Strains isolated from water	Strains isolated from soil
W-1	S-1
W-2	S-2
W-3	S-3
W-4	S-4
W-5	S-5
W-6	S-6
W-7	S-7
W-8	S-8
W-9	S-9
W-10	S-10

4.1.5 Morphological Characterization

4.1.5.1 Colony Morphology

The colony characteristics of the isolated strains are given in the Table 4.2. Most of the strains appeared to be circular in shape, followed by entire margin and smooth in texture. Some of the strains also have shiny, slimy texture. 50% of the isolated strains have convex elevation, 30% have flat and 20% have raised elevation. In terms of color, 40% cream, 20% white, and 20% yellow colonies were observed. Some colonies also have brown and blueish-green color. Size varied from small to large.

Table 4.2: Colony morphology of bacteria isolated from LC contaminated soil and water

Bacterial ID	Color	Shape	Margin	Elevation	Size	Texture
S1	cream	circular	entire	flat	small	smooth
S2	white	circular	entire	convex	small	smooth

S3	yellow	circular	entire	convex	small	smooth
S4	yellow	circular	undulated	flat	large	rough
S5	white	circular	entire	convex	small	smooth
S6	white	irregular	undulated	convex	large	dry
S7	brown	circular	entire	convex	large	smooth
S8	translucent	circular	entire	convex	small	Shiny smooth
S9	yellow to green	irregular	entire	convex	large	mucoid
S10	translucent	circular	undulated	raised	small	Shiny smooth
W1	white	circular	curled	flat	small	smooth
W2	cream	circular	undulated	flat	large	rough
W3	cream	circular	entire	convex	small	mucoid
W4	grey	circular	undulated	raised	small	smooth
W5	Off-white	punctiform	undulated	raised	small	Smooth moist
W6	Blueish-green	circular	entire	flat	small	mucoid
W7	cream	circular	entire	Convex	small	smooth
W8	cream	circular	entire	flat	small	mucoid
W9	cream	irregular	undulated	convex	large	dry
W10	cream	circular	entire	raised	small	smooth

4.1.5.2 Cell Morphology

Most of the colonies were gram-negative and only five turned out to be gram-positive, observed under microscope. All were rods in shape. 80% of the strains appeared to exist single or in pairs. While other four were found in the form of groups, clusters and chains. Cell morphology of the strains is mentioned in the Table 4.3.

Table 4.3: Cell morphology of bacteria isolated from LC contaminated soil and water

Bacterial ID	Gram Reaction	Shape	Arrangement
S1	-	rods	single
S2	-	rods	Single, pairs
S3	-	rods	groups

S4	**+**	rods	Single, pairs
S5	**-**	rods	Single, pairs
S6	**+**	rods	Single, pairs
S7	**-**	rods	Single
S8	**-**	rods	Single, pairs
S9	**-**	rods	Single
S10	**-**	rods	Single
W1	**-**	rods	Single, pairs
W2	**+**	rods	Single, pairs
W3	**-**	rods	Single, pairs
W4	**-**	rods	Short chains
W5	**-**	rods	Single, pairs
W6	**-**	rods	Short chains
W7	+	rods	Single
W8	**-**	rods	Single, pairs
W9	**+**	rods	Single, pairs
W10	**-**	rods	Clusters

4.1.6 Biochemical Characterization

Biochemical characterization was performed using API20E kits and results were determined through analyzing the codes in API 20E software. Bacterial ID with their species names are listed in Table 4.4.

Table 4.4: Identified bacteria with their IDs and species names

Bacterial ID	Specie Identified
S1	*Pseudomonas fluorescens*
S2	*Salmonella sp*
S3	*Sphingomonas paucimobilis*
S4	*Bacillus aryabhattai*
S5	*Salmonella sp*

S6	*Bacillus circulans*
S7	*Aerm. salmonicida*
S8	*Flav. meningosepticum*
S9	*Vibrio sp*
S10	*Escherichia sp*
W1	*Alcaifaciens*
W2	*Bacillus sp*
W3	*K. Pemuno sp*
W4	*Proteus sp*
W5	*Escherichia sp*
W6	*Pseudomonas sp*
W7	*Flav. multivorum*
W8	*Pseudomonas sp*
W9	*Bacillus sp*
W10	*Ser. liqueficans*

4.1.7 Catalase, Oxidase and Simmon Citrate Test

85% of the colonies were catalase positive, 45% were oxidase positive, 60% were citrate positive and 85% were motile, as mentioned in the Table 4.5.

Table 4.5: Results for catalase, oxidase, citrate and motility tests for isolated strains

Bacterial ID	Catalase	Oxidase	Citrate	Motility
S1	+	+	-	+
S2	+	-	-	+
S3	+	+	-	+
S4	+	-	+	+
S5	+	-	-	+
S6	+	+	-	+
S7	+	+	+	-
S8	+	+	+	-

S9	+	+	+	+
S10	-	-	+	+
W1	+	-	+	+
W2	+	-	+	+
W3	+	-	+	-
W4	+	-	+	+
W5	-	-	+	+
W6	+	+	-	+
W7	-	-	+	+
W8	+	+	-	+
W9	+	+	-	+
W10	+	-	+	+

4.1.8 Molecular Characterization

4.1.9 PCR Amplification

Identification of bacteria was done advanced molecular biology technique. After extracting the DNA of the bacterial isolates using DNA spin kit, they were visualized in UV-transilluminator and the products are illustrated in the Figures 4.10 and 4.11.

Figure 4.10: Detection of isolated bacteria from soil, through 16S rRNA gene analysis, S1-S10 represent PCR amplification products of isolated bacteria, Right side: 100 bp DNA Ladder (Genedire), Left side: 1KB DNA Ladder (Genedire)

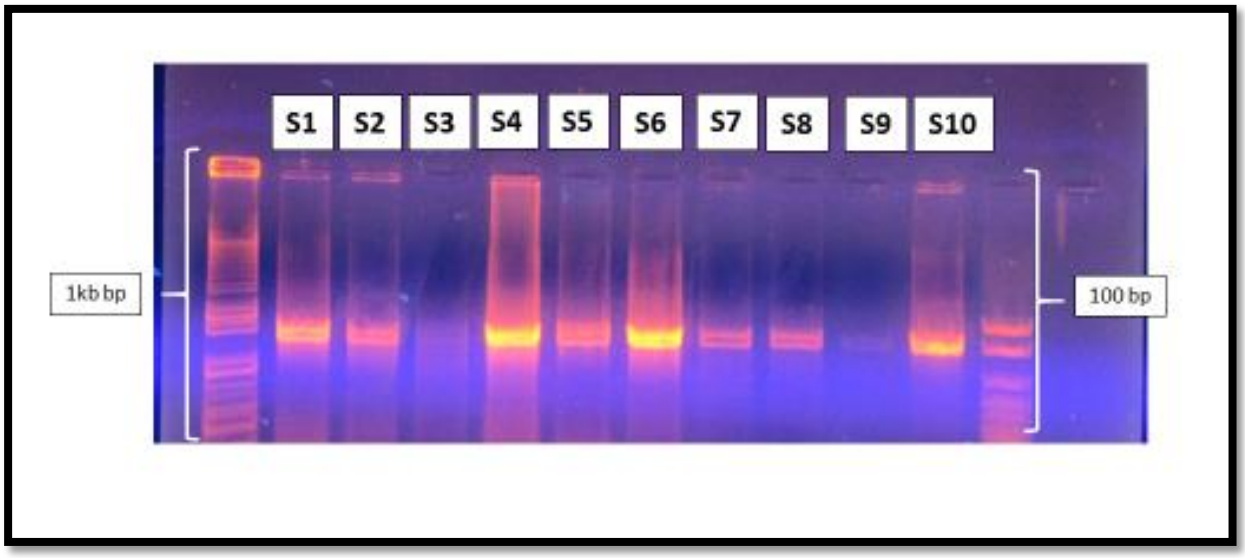
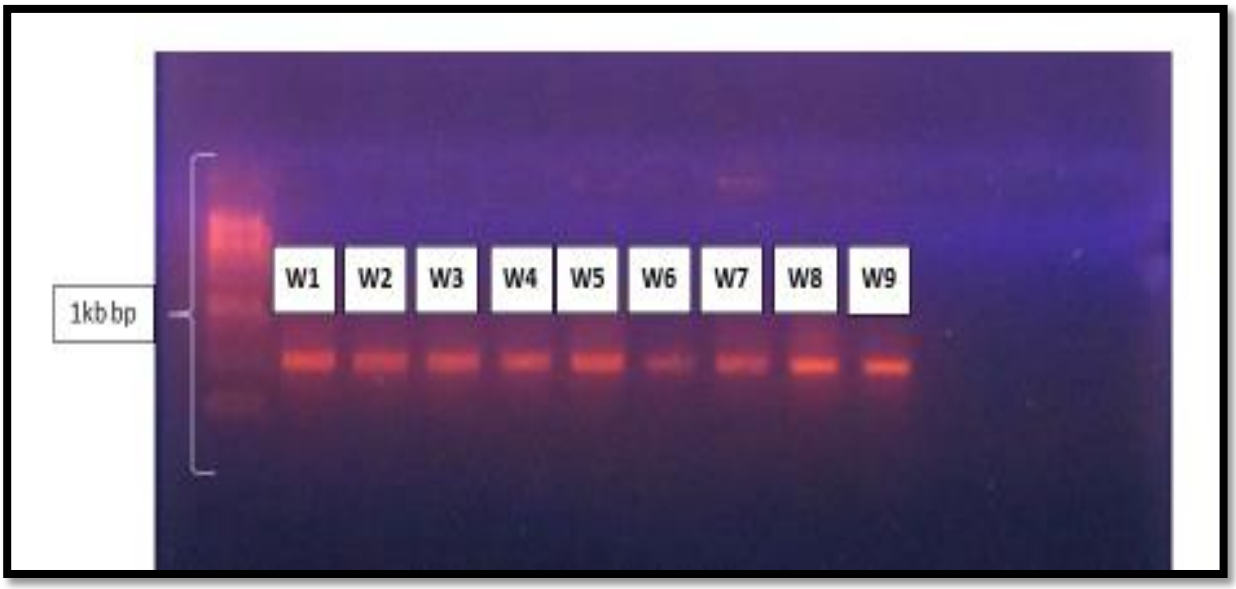


Figure 4.11: Detection of isolated bacteria from water, through 16S rRNA gene analysis, W1-W9 represent PCR amplification products of isolated bacteria, Left side: 1KB DNA Ladder

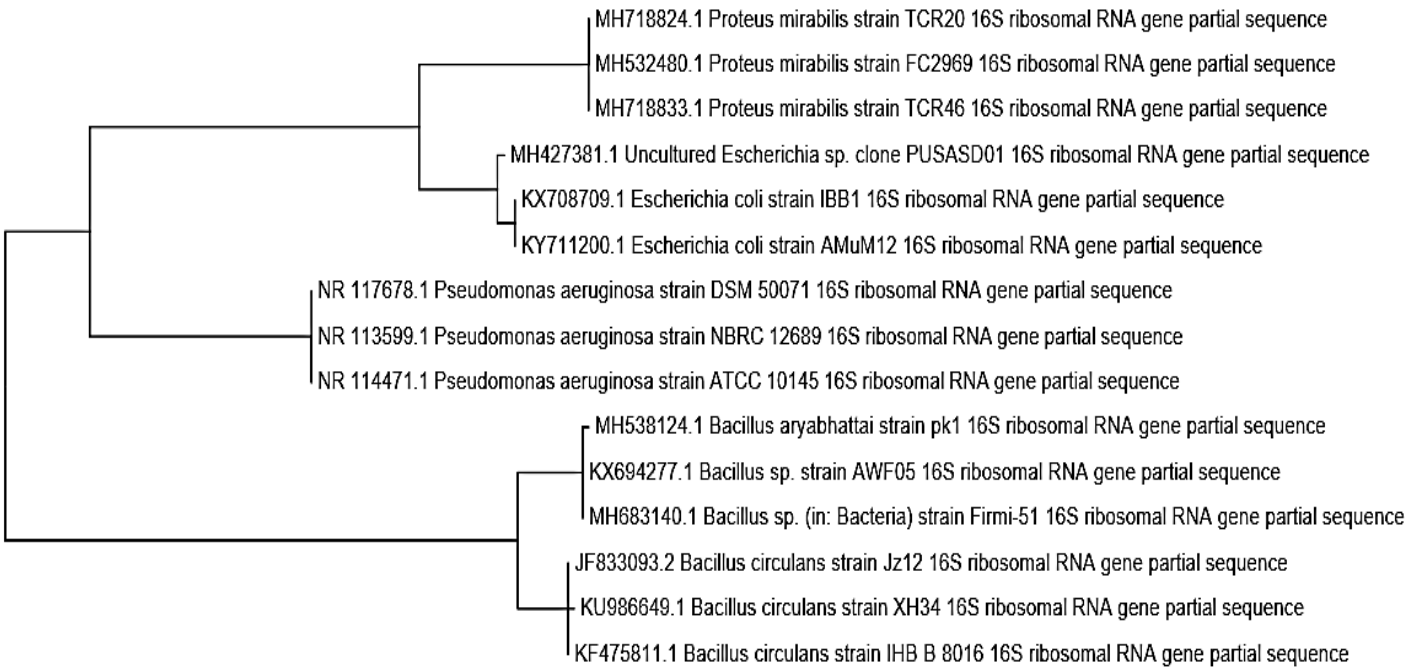


(Genedire)

4.1.1.0 Phylogenetic analysis

The sequences obtained from Macrogen were analyzed through BLAST tool and further processed by MEGA 7 software in order to construct the phylogenetic tree which showed linkages between the isolated strains and those at GENEBANK of NCBI. The Phylogenetic tree is presented in the Figure 4.12. According to various studies on lambda-cyhalohrin, bacteria from genera *Bacillus* and *Pseudomonas* are recognized as highly proficient microrganisms, playing vital role in the degradation of variety of pesticides.

Figure 4.12: Phylogenetic tree demonstrating relatedness and linkage to different bacterial strains



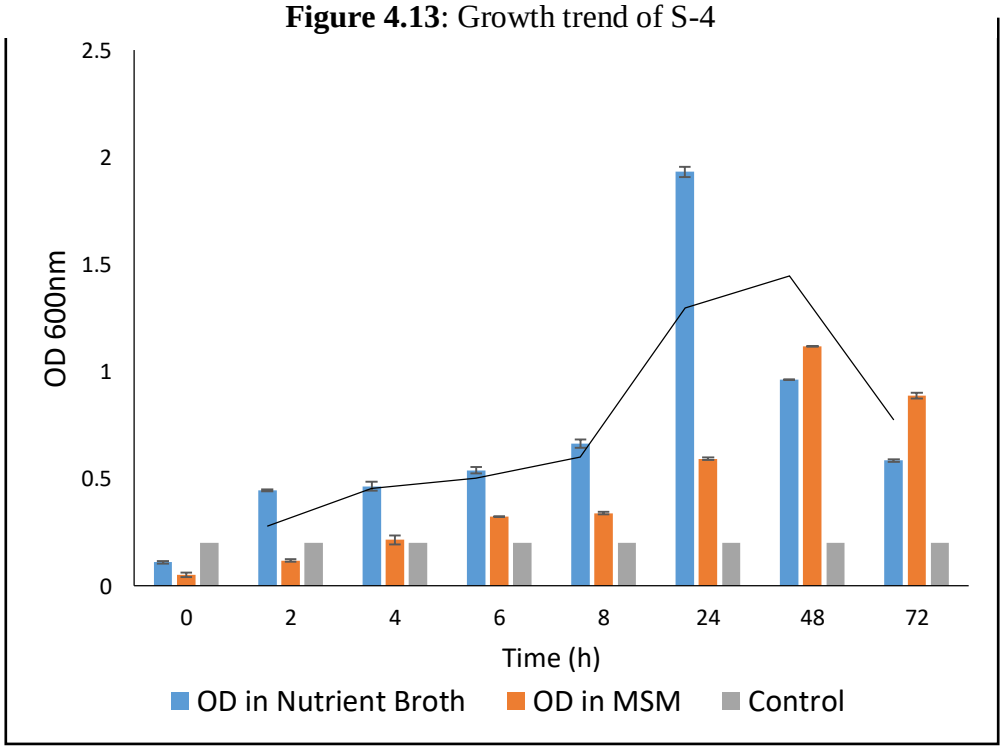
4.2 PHASE II

4.2.1 Degradation Studies

4.2.2 Screening of potential isolates

Screening was performed in order to determine the effect of initial concentration of Lambda cyhalothrin on the growth of isolated strains. On the basis of screening, strains that exhibited the maximum growth were selected for further LC degradation. The effect of initial LC concentration of 100 mg/L on the growth of best selected isolates in both Nutrient broth and MSM is presented in the Figures 4.13, 4.14, 4.15 and 4.16. It can be observed that strains are readily utilizing LC and other nutrients for their growth, particularly in Nutrient broth. Whereas growth in MSM is depicting that bacterial strains are consuming LC as sole source of carbon and energy. All strains have shown their maximum possible growth in Nutrient broth as compared to MSM due to availability of more nutrients in Nutrient broth. The growth increased gradually on increasing the incubation time but decrease trend on prolonged incubation correlates to nutrient depletion. Abdullah and co-workers also used enrichment culture

Figure 4.13: Growth trend of S-4



technique by supplementing media with initial concentration of lambda-cyhalothrin as 100 mg/L (Abdullah et al., 2016).

Figure 4.14: Growth trend of S-6

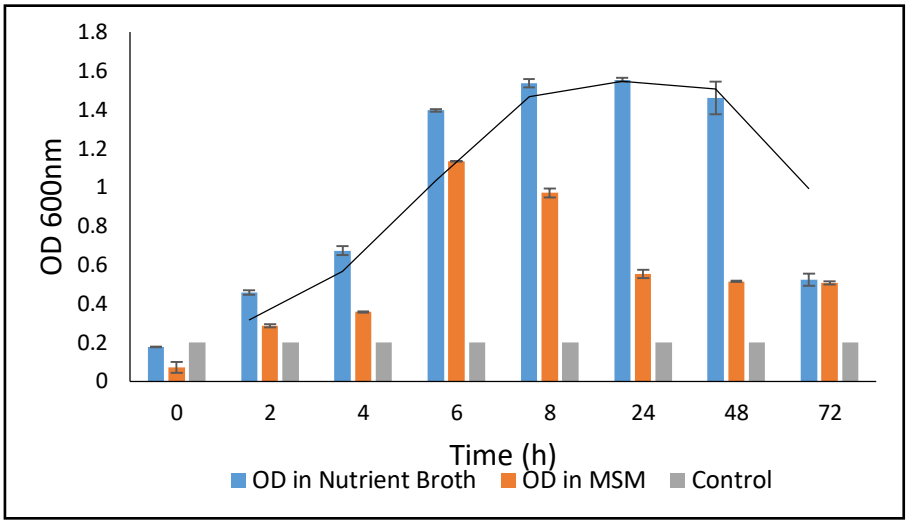


Figure 4.15: Growth trend of W-4

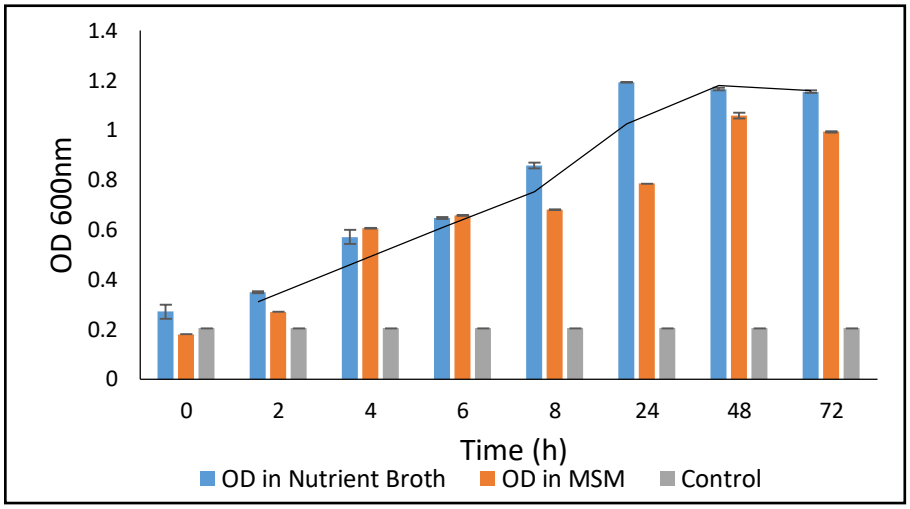
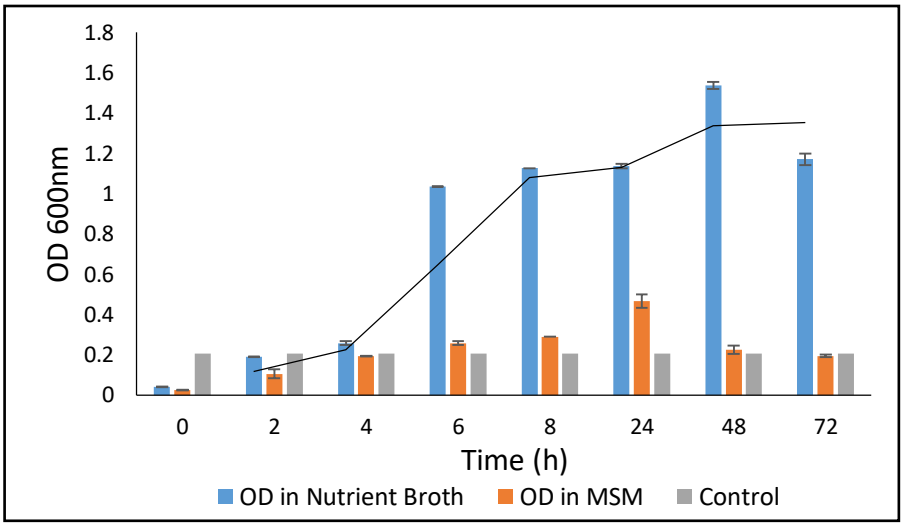


Figure 4.16: Growth trend of W-6

4.2.3 Degradation of Lambda-cyhalothrin by *Bacillus aryabhattai*

The degradation studies were carried out in Nutrient broth and Mineral salt media. Degradation was observed and results were obtained by analyzing samples at regular intervals (0-72 hrs) through Chemical Oxygen Demand (COD). *Bacillus aryabhattai* has shown significant removal in COD, when inoculated in MSM and NB media containing lambda-cyhalothrin, as illustrated in Figures 4.17 and 4.18. The strain was capable to metabolize lambda cyhalothrin with maximum COD removal of 55% at the concentration of 100 mg/L of pesticide in MSM, with maximum growth possible. *Bacillus aryabhattai* exhibited to be a slow degrader when the



pesticide concentration increases. This might be due to reason that higher concentrations of Lambda cyhalothrin exhibited inhibitory effect on growth of isolate, given in Figure 4.17. Chen et al. (2015) reported that *Bacillus thuringiensis* degraded 86% cyhalothrin at the concentration of 100 mg/L at 72 h post inoculation (Chen et al., 2015).

Bacillus aryabhattai when inoculated into nutrient broth at different pesticide concentrations, gave the maximum COD removal at 200 mg/L i.e. 41% degradation of LC. The strain again showed a slow degradation trend on increasing pesticide concentrations. Degradation rates of 35.8, 40.6, 37.5, and 36% were observed at 100, 300, 400, and 500 mg/L, as illustrated in the Figure 4.18. Zheng et al. (2012) have reported the similar findings when they inoculated *Citrobacter braakii* in nutrient broth containing 200 mg/L of lambda cyhalothrin and obtained a degradation rate of 50% after 72 h (Zheng et al., 2012).

Figure 4.17: Removal efficiency of *Bacillus aryabhattai* in MSM

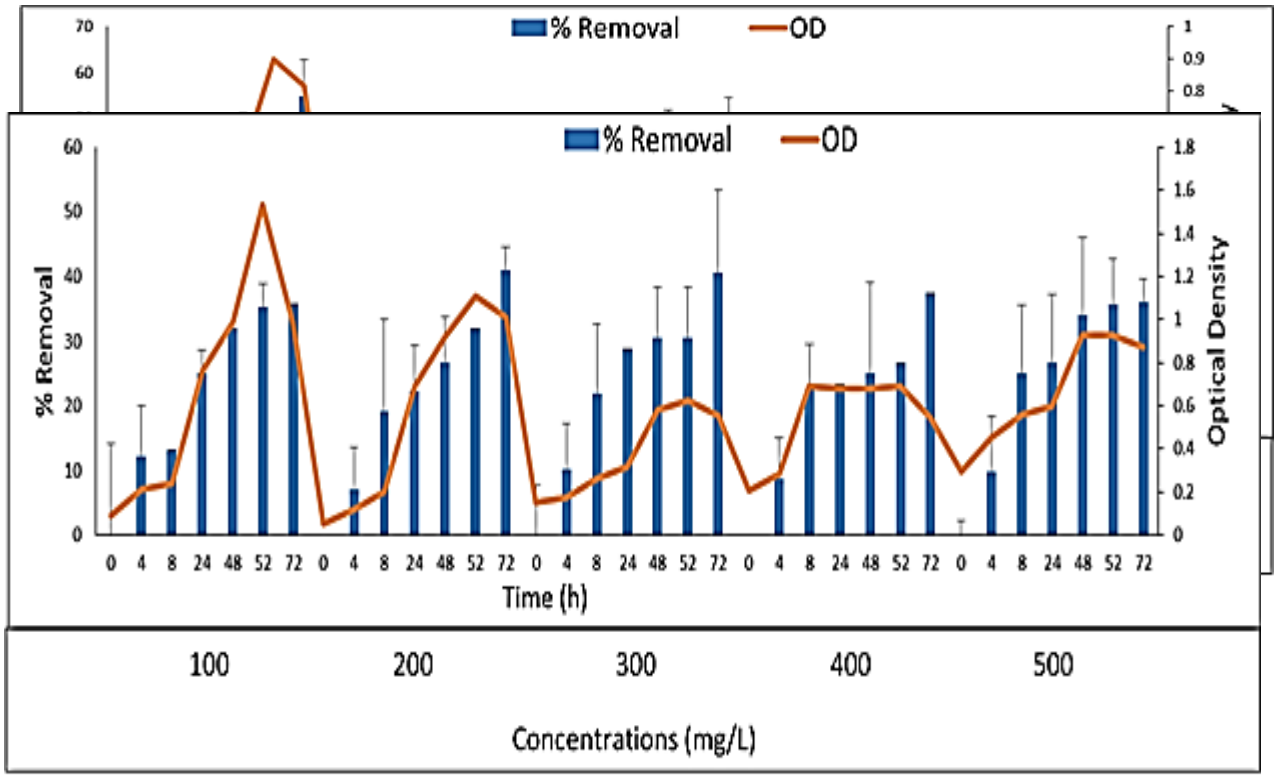


Figure 4.18: Removal efficiency of *Bacillus aryabhattai* in NB

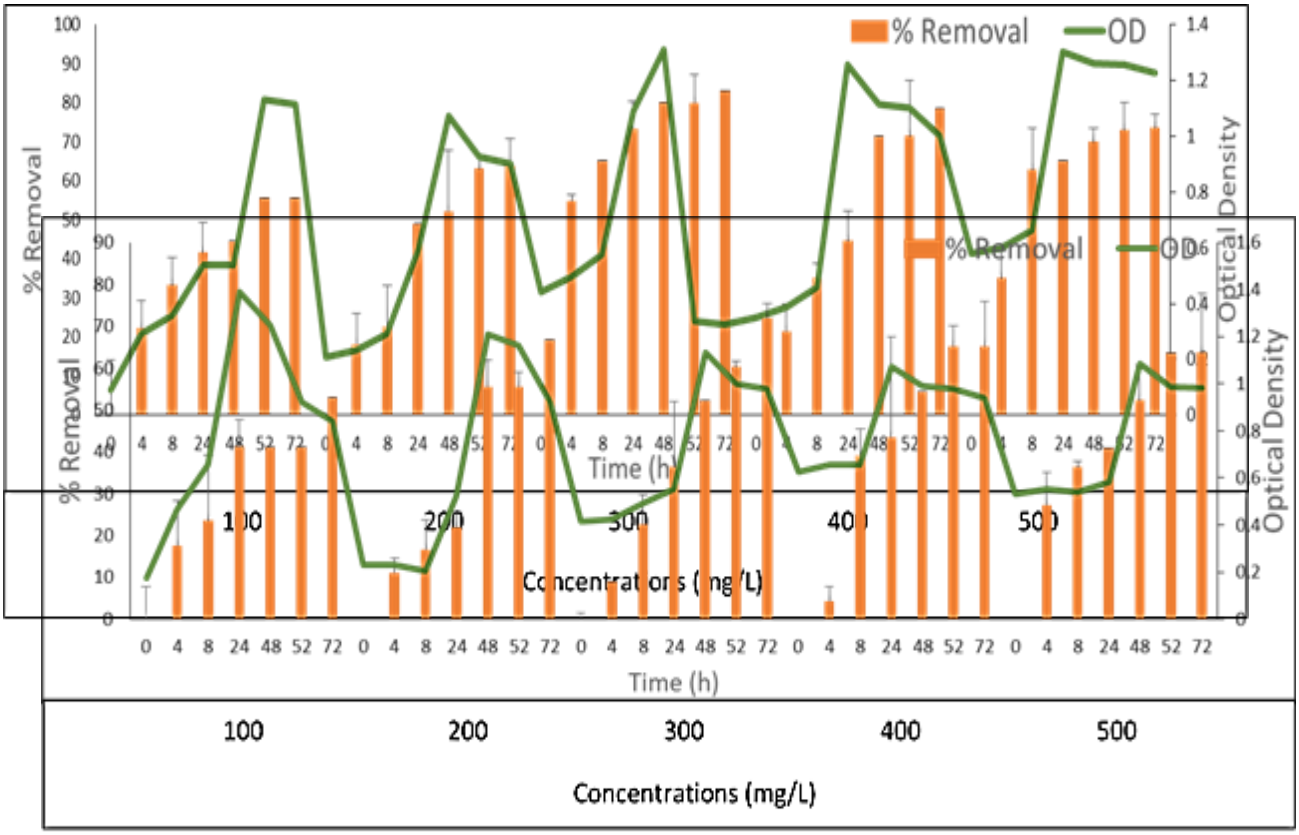
Bacillus sp. are involved in the degradation of a number of organic compound including pesticides, herbicides and dyes and proved to be proficient strains in the elimination of these compounds from the environment. A study reported the use of *Bacillius licheniformis* in the degradation of other pyrethroids such as deltamethrin, cypermethrin and fenvalerate with

significant removal rates of 41.2, 61.7 and 53.8% respectively, at 30 °C for 5 days (Ding et al., 2003).

4.2.4 Degradation of Lambda-cyhalothrin by *Bacillus circulans*

Bacillus circulans proved to be an efficient strain by producing remarkable results in the degradation of lambda-cyhalothrin. Figure 4.19 is showing COD removal rates of LC at different concentrations ranging from 100 to 500 mg/L in MSM. The degradation of lambda cyhalothrin was recorded to be 55.45, 63.64, 83, 73.56 and 71.43% at 100, 200, 300, 400, and 500 mg/L concentrations, respectively. *Bacillus circulans* can tolerate higher concentrations of pesticide by showing maximum percentage removal at concentration of 300 mg/L concentration after 72 hrs. Similar observations were made in the nutrient broth, where *Bacillus circulans* degraded 72% lambda cyhalothrin at higher concentration of 300mg/L, as shown in the Figure 4.20. These findings are in correlation to those produced by Zhang et al. (2016), in which they concluded that 87.1% degradation of lambda cyhalothrin was observed at 500 mg/L concentration in the culture within two days (Zhang et al., 2016).

Figure 4.19: Removal efficiency of *Bacillus circulans* in MSM



Bacillus sp. play a responsive role in the decontamination of environment by eliminating pesticides as well as herbicides. COD reduction rates depend on differences in bacteria present in the media, which show different results. Research was conducted in which *Bacillus simplex* and *Bacillus muralis* showed different COD reduction efficiencies of 94 and 78% in the media containing chlorsulfuron (Erguven and Yildirim, 2016).

4.2.5 Degradation of Lambda-cyhalothrin by *Proteus mirabilis*

Proteus mirabilis turned out to be capable of degrading lambda-cyhalothrin at all concentrations (100-500 mg/L) in MSM, as shown in the Figure 4.21. The degradation of lambda cyhalothrin

Figure 4.20: Removal efficiency of *Bacillus circulans* in NB

was recorded to be 41.25, 48.32, 48.21, 40 and 34.78% at 100 to 500 mg/L respectively. *Proteus mirabilis* showed maximum percentage removal of 48.32% at 200 mg/L concentration in MSM after 72 hrs. These findings correlate with the results produced by Ghumro and his co-workers (2017), where maximum degradation of lambda-cyhalothrin was observed to be 40% at 250mg/L concentration (Ghumro et al., 2017). A similar trend was obtained when *Proteus mirabilis* was inoculated in Nutrient broth. LC percentage removal of 40.61, 31.85, 37.51, and 25.58% was reported at 100, 300, 400, and 500 mg/L concentrations with maximum removal observed at 200 mg/L i.e. 55.54%, as depicted in the Figure 4.22. *Proteus mirabilis* was able to degrade more Lambda cyhalothrin in Nutrient broth than MSM as more nutrients and other energy sources along with the pesticide, were available for the strain.

Figure 4.21: Removal efficiency of *Proteus mirabilis* in MSM

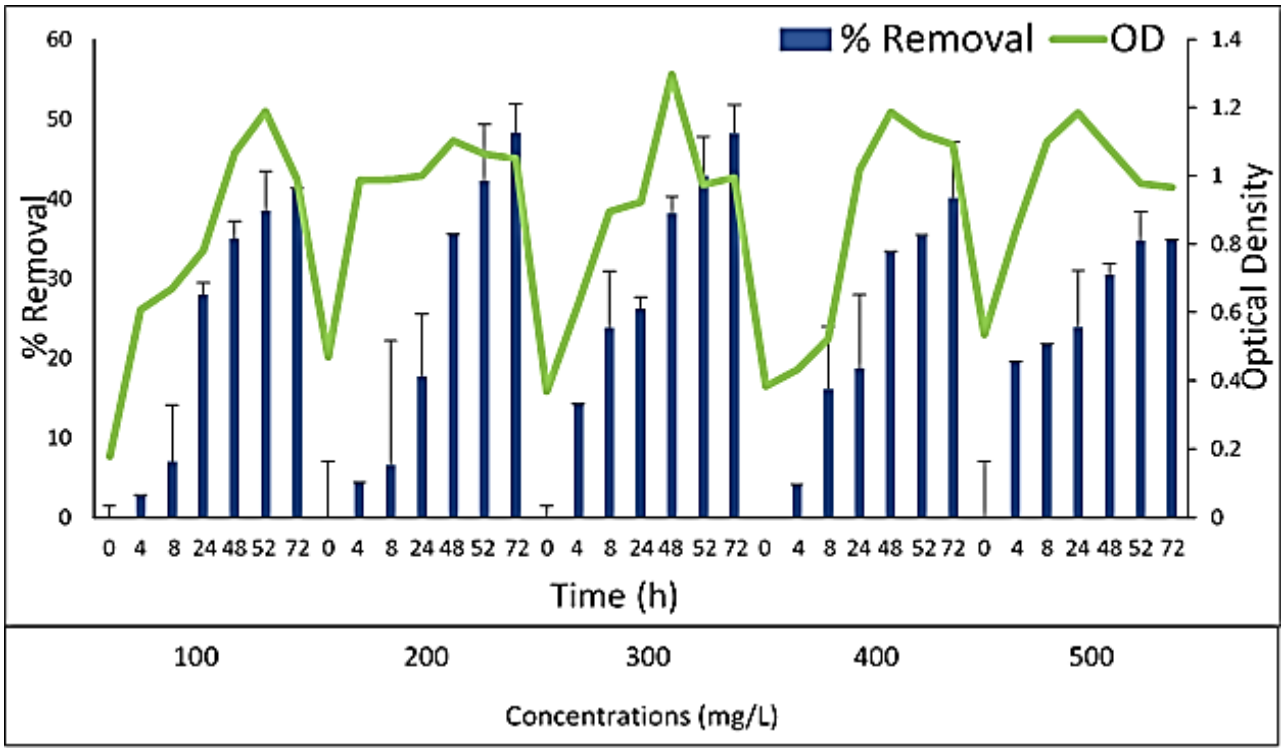
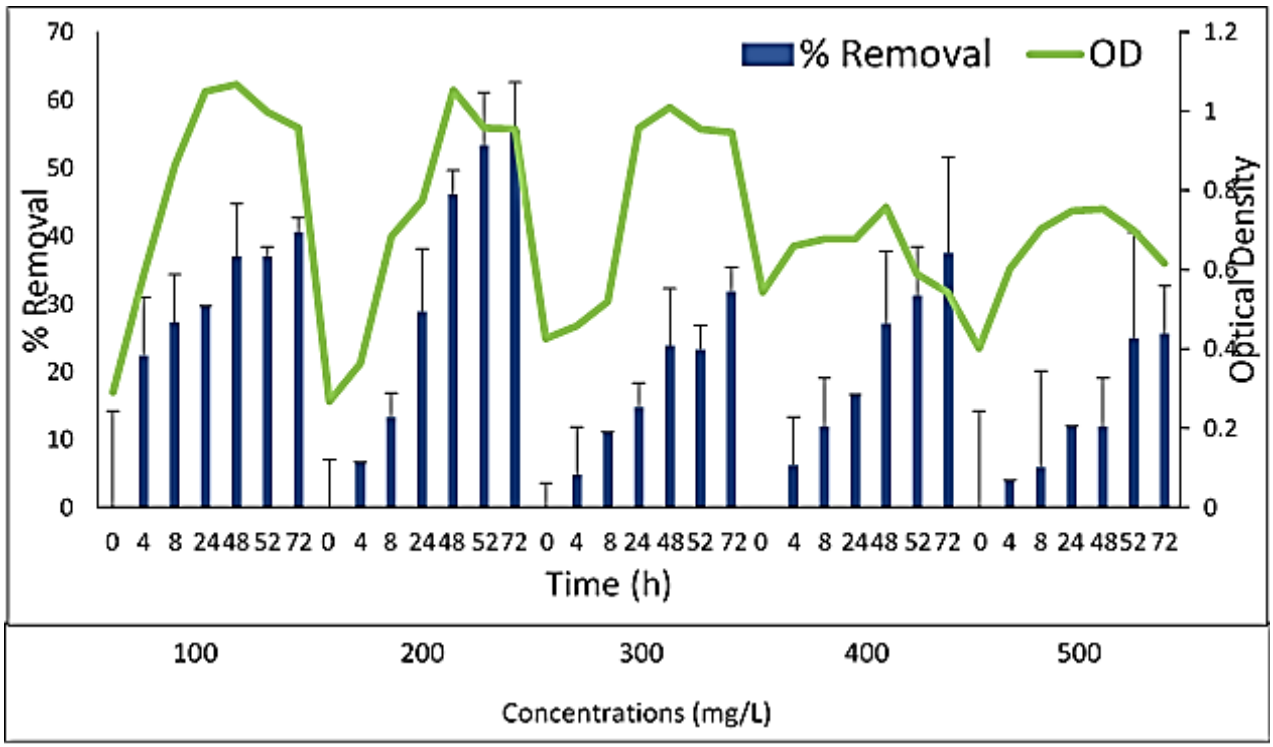


Figure 4.22: Removal efficiency of *Proteus mirabilis* in NB

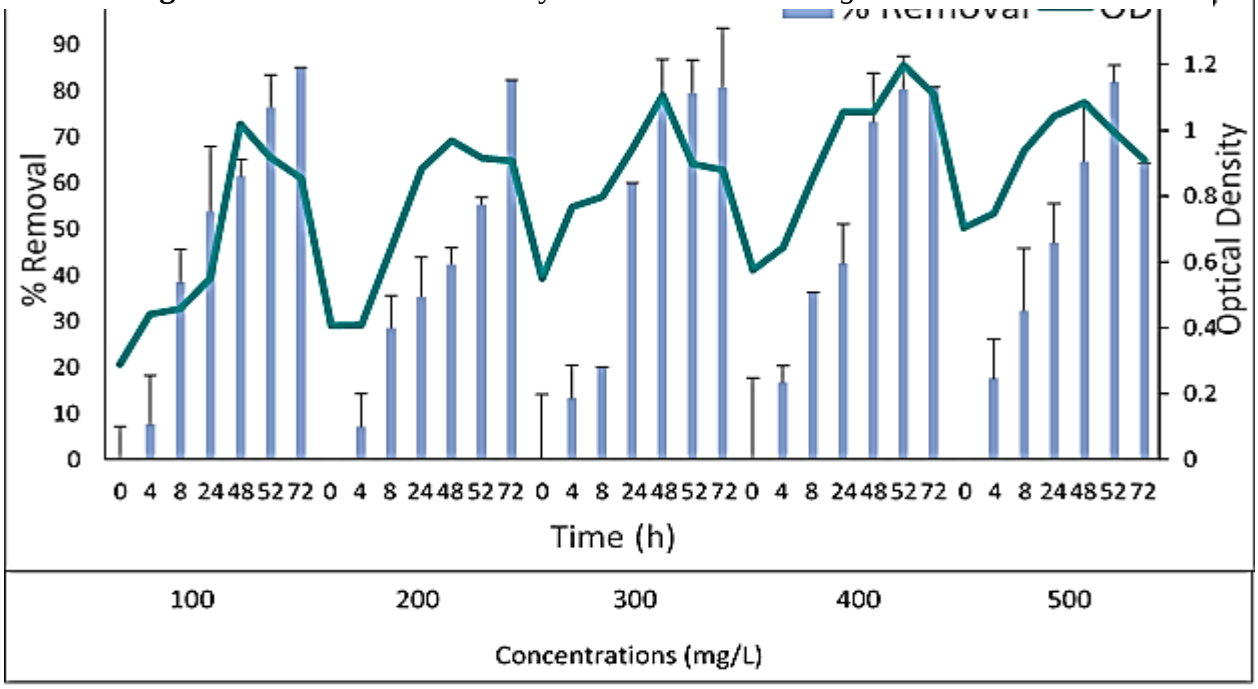


4.2.6 Degradation of Lambda-cyhalothrin by *Pseudomonas aeruginosa*

The growth of *Pseudomonas aeruginosa* with lambda cyhalothrin as the growth substrate in MSM medium has been shown in the Figure 4.23. At all concentrations, the bacterial cells and lambda cyhalothrin degradation revealed a rapid increasing trend in the initial phase of cultivation (0-24 h). After 24 h, the rate of degradation continue to increase gradually while a decrease in the number of bacterial cells has been observed. *Pseudomonas aeruginosa* metabolized 84% of lambda cyhalothrin at the concentration of 100 mg/L within 72 h. In contrast, when *Pseudomonas aeruginosa* was inoculated in nutrient broth, the strain showed maximum degradation of 93.84% at LC concentration of 100 mg/L after 72, as observed in the Figure 4.24.

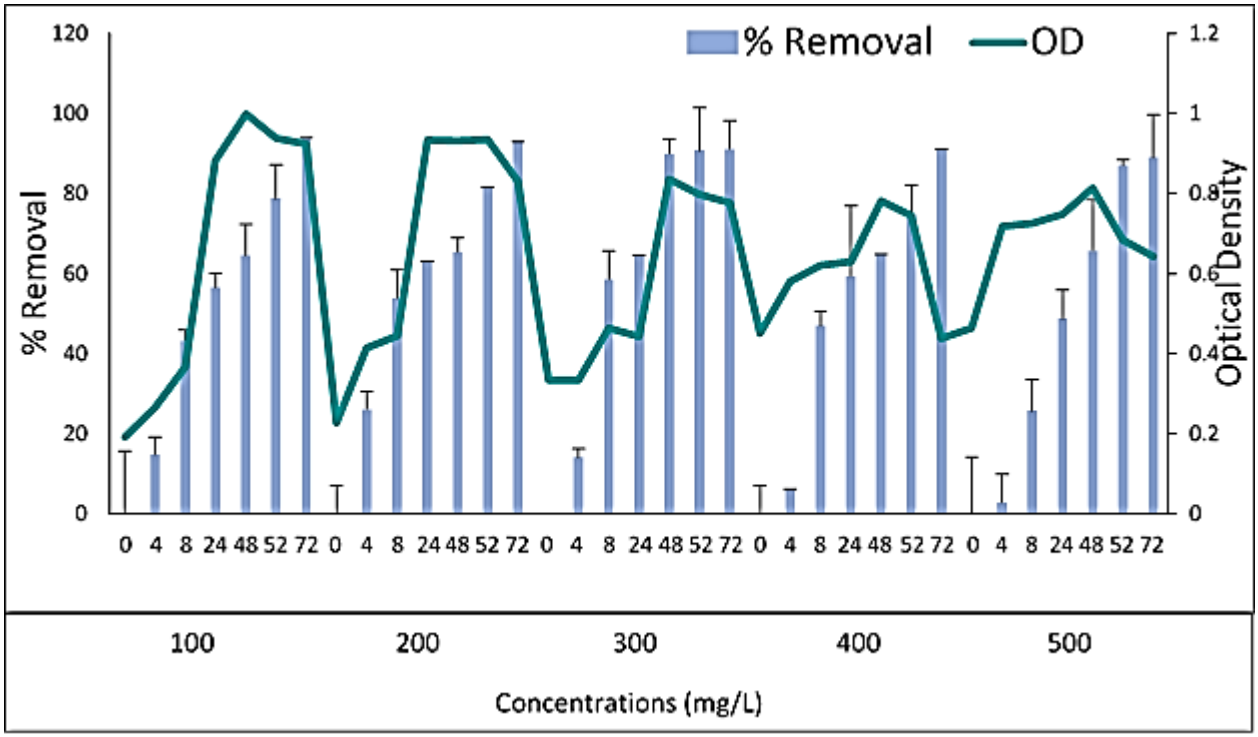
Abdullah and his co-workers (2016) have publicized similar results when they inoculated *Pseudomonas putida* in liquid media and obtained 86.11% degradation for lambda cyhalothrin at the end of the experiment. *Pseudomonas* species have been found to be efficient in the degradation of a number of pyrethroid insecticides. Manaswi Gurjar and his co-worker (2018) have utilized the specie, *Pseudomonas aeruginosa* for the degradation of cypermethrin, a

Figure 4.23: Removal efficiency of *Pseudomonas aeruginosa* in MSM



synthetic pyrethroid pesticide. They supplemented minimal medium with cypermethrin (150 mg/L) and observed 77.47% reduction in COD after 14 days.

Figure 4.24: Removal efficiency of *Pseudomonas aeruginosa* in NB



Various studies revealed degradation rates of 55.64, 52.19, and 44.56% of bifenthrin, cypermethrin and fenpropathrin, respectively by *Pseudomonas sp.* (Wang 2005). Peter et al. (2014) also reported complete degradation of methyl parathion at the concentration of 350 mg/L in 96 h, by a proficient strain of *Pseudomonas aeruginosa.*

According to the results mentioned above, the strains have generated different removal efficiencies in nutrient broth and MSM media.In MSM media, *Pseudomonas aeruginosa* was able to degrade maximum amount of the pesticide followed by *Bacillus circulans*, *Bacillus aryabhattai*, and *Proteus mirabilis*. While in Nutrient broth, *Pseudomonas aeruginosa* has proved to be an efficient LC degrading strain by showing remarkable results, followed by *Bacillus circulans*, *Proteus mirabilis* and *Bacillus aryabhattai*.

Moreover, it is also observed that *Bacillus circulans* and *Bacillus aryabhattai* (isolated from soil) have shown their maximum removal efficiency in MSM media. Whereas, *Pseudomonas*

aeruginosa and *Proteus mirabilis* (isolated from water) were able to degrade maximum lambda cyhalothrin in nutrient broth. These strains utilized the pesticide as a nutrient source for their growth and degraded them by using additional nutrients in the broth. *Bacillus circulans* and *Bacillus aryabhattai* from soil contaminated with lambda cyhalothrin, degraded the pesticide by utilizing it as carbon and energy source without the accumulation of any other compound (Manigandan et al., 2013).

In the current study, the percentage removal efficiency was observed to be higher in the case of *Pseudomonas aeruginosa.* Manaswi Gurjar and Venkat Hamde (2018) have utilized the same strain, *Pseudomonas aeruginosa* for the degradation of another pyrethroid i.e. cypermethrin and found that the strain has a remarkable capacity of growing at high concentrations of cypermethrin. Thus, the strain may tolerate lambda cyhalothrin more competently as compared to other strains and may play a vital role in the bioremediation of environmental contamination of pyrethroids.

4.3 PHASE III

4.3.1 Conversion of Cell density to Biomass (mg/ml)

The optical density (OD), measured at different time intervals during batch experiments, was converted into biomass by the relation given by Kim et al., 2012. The conversion of OD into biomass is presented in the table below:

1.0 OD= 2.04 × 108 CFU/ml= 2.085 mg/ml (Kim et al., 2012)

Table 4.6: Conversion of optical density to biomass

Time (hours)	Optical Density	Biomass (mg/ml)
0	0.289	0.602
4	0.442	0.921
8	0.458	0.954
24	1.05	2.18
48	1.02	2.12
52	0.918	1.914
72	0.554	1.155

The data obtained from lab scale experiments and the computed data were used in order to evaluate the Biokinetic parameters of Andrews's model (Annexures I and II).

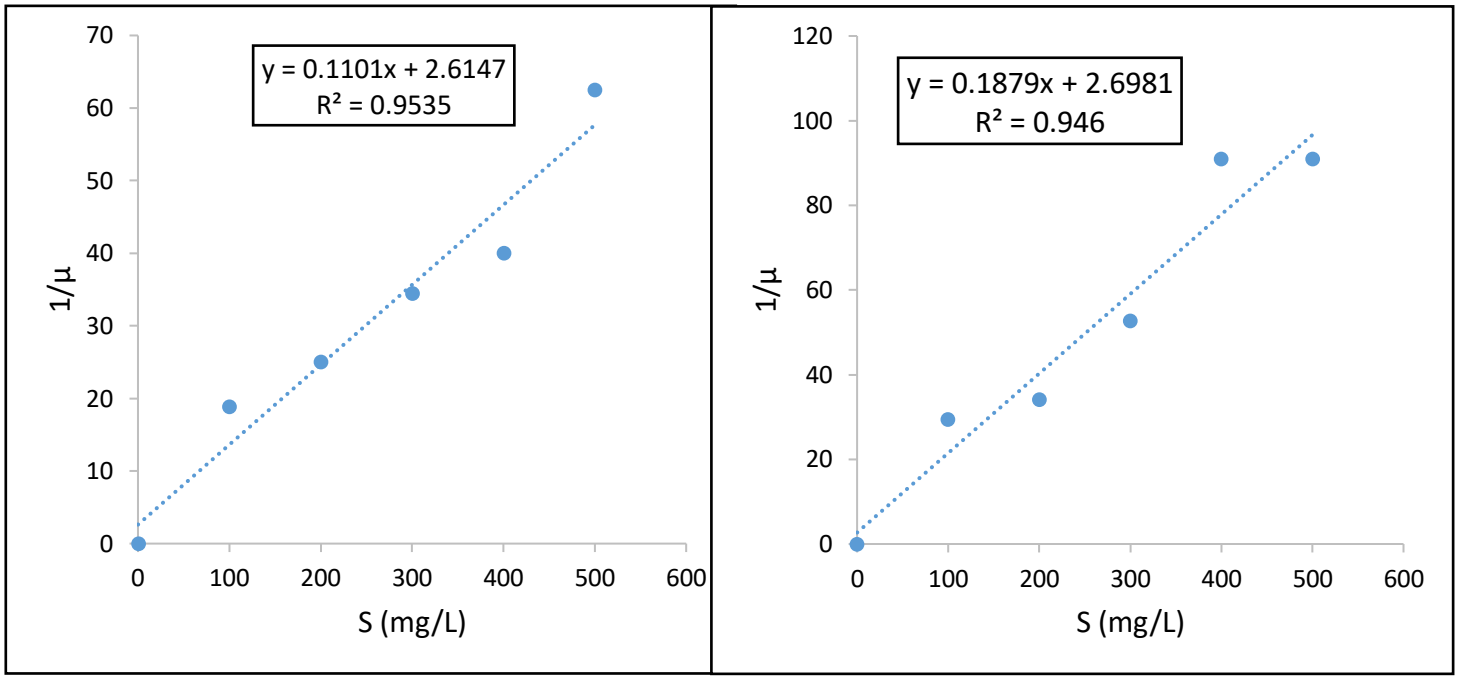
4.3.2 Estimation of Kinetic Parameters

Experimental data attained from batch experiments performed in lab were validated and justified by applying inhibition kinetic model i.e. Andrews's model. The relationship between specific growth rate and initial concentration of Lambda cyhalothrin in both media may be illustrated by the Figures 4.26 and 4.27. The Biokinetic parameters such as μ_{max}, K_s and K_i of the Andrews's model were assessed by adopting a graphical method, where the coefficient of determination (R^2) exhibited how well the experimental data are replicated by the model, as shown in the Figure 4.25 a and b. For this present study, the values of Biokinetic parameters are given in the Table 4.7.

Table 4.7: Andrews's kinetic parameters for experimental data attained from Lambda cyhalothrin degradation by *Pseudomonas aeruginosa*

Biokinetic Model	**μ_{max} (h^{-1})**	**K_s (mg/L)**	**K_i (mg/L)**
Andrews (MSM)	0.382	350	23.74
Andrews (NB)	0.370	320	14.35

Table 4.25: Andrews's kinetic parameters for experimental data attained from Lambda cyhalothrin degradation by *Pseudomonas aeruginosa* (a) MSM (b) NB



(a) (b)

The values of R^2 were 0.9535 and 0.946 in MSM and NB respectively, which confirmed the applicability of the model on the experimental data. The relationship between specific growth rate and initial concentration of Lambda cyhalothrin in both media as depicted in the Figures 4.26 and 4.27, concluded that maximum specific growth rate was observed at lower initial concentration of Lambda cyhalothrin i.e. 100 mg/L. At higher concentrations, specific growth rate tends to decrease with the significant increase in the inhibition by Lambda cyhalothrin.

The value of μ_{max} indicates the utilization level of pesticide (substrate) by the microorganism. In the present study, the substrate Lambda cyhalothrin is readily utilized by the isolated bacteria for its growth. The high value of Half Saturation Constant (K_s) exhibited strong affinity between the substrate and biomass. The value of K_i indicates the level of resistance show by the microorganisms against the toxic effects of substrate. Lower values of K_i revealed much higher sensitivity of microorganisms to substrate inhibition.

Researchers in 2015 determined the degradation kinetics of 3-BPA (3-Phenoxybenzoic acid), a metabolite of Lambda cyhalothrin by using Andrews equation. The kinetic parameters q_{max}, K_s and K_i were found to be 0.1270 h^{-1}, 64.0577 mg/L 24.36 mg/L, respectively. In another study,

Andrews model was used to evaluate the degradation kinetics of another pyrethroid i.e. beta-cypermethrin. The kinetic parameters q_{max}, K_s and K_i were determined to be 2.19 h^{-1}, 76.37 mg/L, and 54.14 mg/L, respectively (Chen et al., 2015; Xiao et al., 2015).

Figure 4.26: Experimental data versus model fit using Andrews's model for *Pseudomonas aeruginosa* in MSM

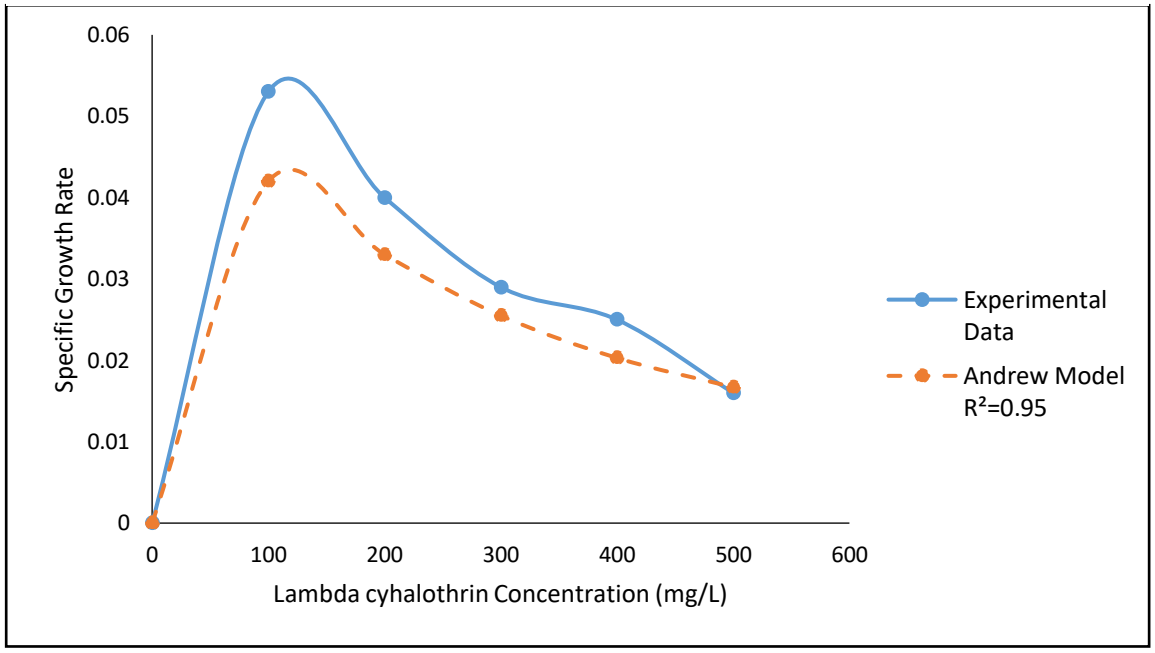
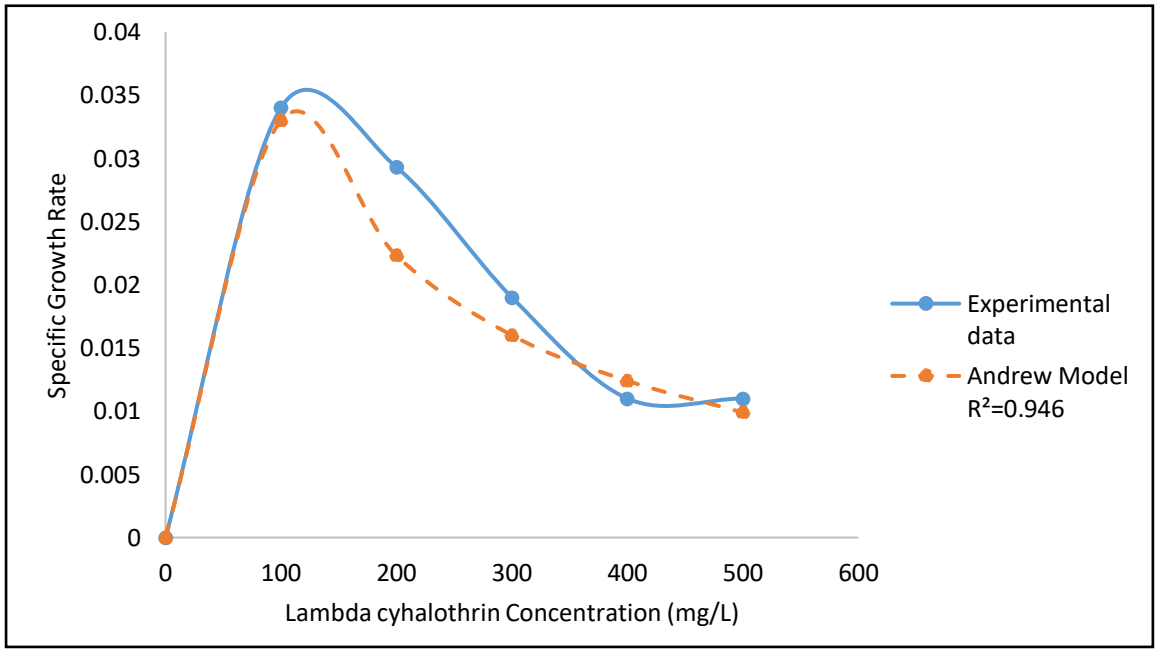


Figure 4.27: Experimental data versus model fit using Andrews's model for *Pseudomonas aeruginosa* in NB



CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Following outcomes are derived from the current study:

- A total of twenty bacterial strains were isolated from Lambda cyhalothrin contaminated water and soil. They were purified and identified as the members of *Pseudomonas sp., Salmonella sp., Sphingomonas sp., Bacillus sp., Serratia sp., and Flavobacterium sp.*
- On the basis of screening, four potential strains were selected and utilized for Lambda cyhalothrin degradation. Biochemical characterization, API 20E analysis and 16S rRNA analysis revealed that the four potential bacterial strains were identified as *Bacillus circulans* and *Bacillus aryabhattai* (isolated from soil) and *Pseudomonas aeruginosa* and *Proteus mirabilis* (isolated from water).
- Among these strains, *Pseudomonas aeruginosa* has produced auspicious results in both MSM and NB media with the removal efficiency of 84 and 93.84% of Lambda cyhalothrin, respectively.
- At higher concentrations, the effect of inhibition by Lambda cyhalothrin became prominent. For this purpose, Andrews kinetic model was applied and the values of its parameters μ_{max}, K_s and K_i were determined to be 0.382 h^{-1}, 350 mg/L and 23.74 mg/L in Mineral Salt Medium and 0.370 h^{-1}, 320 mg/L and 14.35 mg/L in Nutrient broth, respectively.
- The values of the coefficient of determination R^2 were 0.9535 and 0.946 in MSM and NB respectively, which confirmed the applicability of the model on the experimental data.
- Thus, *Pseudomonas aeruginosa* has proved itself to be potential bacterium that may tolerate lambda cyhalothrin more competently as compared to other strains and may play a vital role in the bioremediation of environmental contamination of pyrethroids.

5.2 Recommendations

- Bacterial consortium and mixture of pesticides may be used in bench scale degradation studies.
- Pesticide degradation with respect to enzymatic involvement should be undertaken.
- Quantitative studies on toxicity of pesticide and its metabolites should be commenced.
- Detailed study should be performed taking account the adverse effects of pesticides and its metabolites on the environment.

CHAPTER 6

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ANNEXURE-I

Calculation for kinetic parameters μ, μ_{max}, K_s and K_i for MSM

Data Analysis for Figure 4.25 (a)

From the Figure 4.25 (a)

From the intercept at y-axis

μ_{max} = 2.6147 (From the intercept at y-axis)

=1/2.6147

=0.384 h^{-1}

- K_i = 0.1101 (slope)

  = 1/0.1101

  =9.082×2.6147 (From the intercept at y-axis)

=23.74 mg/L

- K_s = Half Saturation Constant (K_S) is the substrate concentration at one half of the maximum specific growth rate (From Figure 4.26).
- The value of specific growth rate was found out by Andrews equation:

$$\mu = \frac{\mu_m S}{K_S + S + \frac{S^2}{K_i}}$$

By using the above Andrews equation, the values of specific growth rate at each substrate concentration are given as follows:

Substrate Concentration (mg/L)	Specific Growth Rate (h^{-1})
100	0.0420
200	0.033
300	0.0255
400	0.0203
500	0.0167

ANNEXURE-II

Calculation for kinetic parameters μ, μ_{max}, K_s and K_i for NB

Data Analysis for Figure 4.25 (b)

From the Figure 4.25 (b)

From the intercept at y-axis

- μ_{max} = 2.6981(From the intercept at y-axis)
  =1/2.981
  =0.370 h^{-1}
- K_i = 0.1879 (slope)
  = 1/0.1879
  =5.321×2.6981 (From the intercept at y-axis)

=14.35 mg/L

- K_s = Half Saturation Constant (K_S) is the substrate concentration at one half of the maximum specific growth rate (From Figure 4.27)
- The value of specific growth rate was found out by Andrews equation:

$$\mu = \frac{\mu_m S}{K_S + S + \frac{S^2}{K_i}}$$

By using the above Andrews equation, the values of specific growth rate at each substrate concentration are given as follows:

Substrate Concentration (mg/L)	**Specific Growth Rate (h^{-1})**
100	0.033
200	0.0223
300	0.0160
400	0.0124
500	0.0099