OPTIMIZATION STUDIES ON BIODEGRADATION OF CYPERMETHRIN USING INDIGENOUS SOIL MICROORGANISM

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By

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Institute of Environmental Sciences and Engineering (IESE)
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THESIS ACCEPTANCE CERTIFICATE

Certified that the contents and form of the thesis entitled " **Optimization Studies on Biodegradation of Cypermerthin using Indigenous Soil Microorganism**" submitted by Ms. Rabia Akmal has been found satisfactory for partial fulfillment of the requirements of the degree of Master of Science in Environmental Science.

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This Thesis is dedicated to my Baba Jan.

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RABIA AKMAL

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CFU	Colony Forming Unit
SPC	Spread Plate Count
EC	Emulsified Concentration
FAO	Food and Agricultural Organization
PPSGDP	Punjab Private Sector Groundwater Development Project
beta-CP	Beta Cypermethrin
3-PBA	3-phenoxybenzoic acid
GC	Gas Chromatography
ECD	Electron Capture Detector
MSM	Mineral Salt Media
WHO	World Health Organization
mgL^{-1}	Milligram per Liter
ng/ul	Nanogram per Microliter
Ppm	Parts per Million
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
M	Specific Growth Rate
μ_{max}	Maximum Specific Growth Rate
q_{max}	Maximum Specific Degradation Rate
S	Substrate Concentration
K_s	Substrate Saturation Constant
K_i	Inhibition Constant
OD	Optical Density
DO	Dissolved Oxygen

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Degradation of cypermethrin using an isolate designated as *Bacillus safensis*, significantly enhanced degradation after acclimatization at a concentration 50 mgL^{-1} of cypermethrin in selective media. An optimum growth of isolate was exhibited at an initial concentration of 100 mgL^{-1} of cypermethrin. However, the transformation was inhibited at higher concentrations above 150 mgL^{-1}, making inhibitory effect of pesticide obvious. Based on the Gas chromatographic analysis, in semi-continuous bioreactor studies, **KY752364** was able to metabolize 88.3% of cypermethrin, with an increasing efficiency by each cycle. Moreover, the decrease in rate of removal of cypermethrin was observed i.e. 89.8% in 2 days. All the biodegradation is associated with the growth of bacteria. In present study, **KY752364** adapted quickly to the environment without any apparent lag phase and degraded cypermethrin rapidly at the start of incubation. The results indicated that *Bacillus safensis* possess a potential to be used in bioremediation of cypermethrin contaminated environment. This is very beneficial for field scale application as viable population was maintained by *Bacillus safensis* over a long period of time. For validation of experimental data various kinetic models both inhibitory and non-inhibitory were applied. The parameters of Andrew model of kinetics were estimated to be μ_{max}: 0.175 h^{-1}, K_s: 28.34 mgL^{-1} and K_i 210.06 mgL^{-1}. The high R^2 (0.9962) approaching to 1 proposes that this substrate-inhibition model is adequately fitted with the experimental data.

Key words: Cypermethrin, *Bacillus safensis, Metabolites, Degradation, Gas Chromatography, Environmental Sustainability*

INTRODUCTION

1.1 Background

Green revolution has aided to the already growing population, with UN proposing that by 2050 Pakistan's population to surpass by 300 million. Thus, the requirement for food production will increase by 70% (FAO, 2013). Consequently, the pressure of limited agricultural land, will lead to sustainable interventions by agricultural sector. Implying, that using current land for exponential yield of food. Moreover, 35% of potential yield of crop is lost before harvesting due to the pests (FAO, 2013). This loss is fulfilled through application of multiple pesticides with higher doses than recommended, at the cost of environment and health. Nevertheless, the most cost-effective and conventional method for controlling these pest is the application of pesticides. At present, besides pesticide contamination from agricultural field, the agricultural industries are also contributing relatively high quantities of toxic pesticides into the environment. Further, multiple factors are contributing toward pesticide contamination, agricultural industries is one them, adding high amounts of toxins into the environment and ecosystem.

As mentioned earlier pesticides are posing detrimental effects on health and environment. About 2.5 million tons of pesticides are applied annually worldwide and with time this share is increasing (FAO, 2013). The same drift is detected in Pakistan. In developing countries if the exposure level is 550 million people than 37,000 cancer cases are linked to the use of pesticides (WHO, 2016). Moreover, pesticide poisoning has led to 200,000 deaths each year, majority of these casualties are reported in developing nations (FAO, 2013). During last 40 years influx of scientific studies has increased towards environmental problems caused due to pesticides in Pakistan.

The transfer of sale and distribution of pesticide to the private sector increased the consumption of pesticides by many folds. These companies stirred the farmers to use higher doses of the chemicals. That leads to high accumulation of pesticides in various crops, vegetables and soil (Tariq et al., 2007). Alongside increase in imports of pesticides annually, presently more than 108 types of insecticides, 30 types of fungicides, 39 types of weedicides, 5 types of acaricides, and 6 different types of rodenticides are being used in Pakistan (Khwaja et al., 2013). The consumption of pesticide in Pakistan has amplified by 1169% in the last 20

years and each crop receives treatment of 10 various pesticides, which is a shocking scenario as far as human health is concerned (Khooharo et al., 2008).

Only 0.1% of these chemicals reach the target organism while the rest is dispersed into the environment. Due to the persistence and mobility of these chemicals, the effect on soil is supreme. They also deteriorate water courses; accumulate in foods and subsequent entry into the food chain leading to drastic effects. Additionally, contributing towards environmental pollution, deterioration of natural habitats and ultimately resulting in loss of biodiversity (Cerejeira et al., 2003).

Soil works as a filter, buffer, and degradation potential regarding storage of pollutants in terms of soil organic matter. It is a document ted fact that soil is the pathway through which pesticide are transported to water, air, food and eventually end up in humans. This transport is made feasible through runoff and subsurface drainage; interflow and leaching; and the transmission of mineral nutrients and pesticides from soils into the plants and animals. The persistent nature of pesticides and steady breakdown leads towards contamination of source. These sources are closely linked to anthropogenic activities for instance industrial, agriculture and domestic discharges. Numerous studies in Pakistan have stated detection of pesticide in soils (Tariq et al., 2007; Burauel & Baßmann, 2005).

The indigenous soil microorganisms play a vital role in transitional degradation either partial or complete degradation of pesticides Bacterial influence can start directly by mineralization, conjugation and accumulation. It may occur indirectly through secondary effect of microbial activity, wavering soil pH and redox conditions (Burauel & Baßmann, 2005).

On the basis of structure organophosphates, carbamates, organochlorine and pyrethroid are the main classes of pesticide. Among them Pyrethroids are frequently being used around the world. They are synthetically similar to a natural pyrethrin, a kind of toxin with insecticidal activity originating from the flowers of *Chrysanthemum cinerariaefolium*. Based on the toxic indicators and cyano group, they are classified under type I or type II. In insecticide market they account for more than 30%, and it's been more than 3 decades they are in use. With the removal of organophosphorus insecticide, their application is mounting (Grant & Betts, 2004).

Cypermethrin is a prime synthetic pyrethroid pesticide, immensely used on cotton, fruit and vegetable crop; home and garden pest control worldwide (Tallur et al., 2008). cypermethrin has received increasing concern not only because it is harmful to fish and invertebrates but also a threat to human health (Zhang et al., 2011). For instance, it is a possible cancer-causing agent in humans and also gives disrupting effects in nervous, immune, genetic, endocrine and reproductive system. Cypermethrin induces cell stress that alters antioxidants enzyme of human cell line (McKinlay et al., 2008; Wolansky & Harrill, 2008; Zhang et al., 2010; Wang et al., 2011). The cleavage of cypermethrin produce free radical in animal cell that are known to cause alterations in mitochondrial dehydrogenase and DNA damage Therefore, there is a critical necessity to develop an efficient method to degrade cypermethrin from the environment.

Earlier, due to their prompt activity in insects and low toxicity in mammals synthetic pyrethroid were designated safe to use (Dorman & Beasley, 1991). However, recent report shows a lot of complications in environment and health. This is because of unceasing and uncontrolled use of insecticides like cypermethrin (Cuthbertson & Murchie, 2010). It is categorized as toxic type II synthetic pyrethroid with a a-cyano group. Universally used in agricultural formulation to control a wide-ranging pests. It was persistently detected in water and soil sediments, caused by overspills from agricultural fields. Cypermethrin residues have been reported to bioaccumulate and biomagnify in the food chain, consequently manifesting its toxicity in marine life and ultimately human health (McKinlay et al., 2008).

Since its low water solubility and moderately high lipoaffinity, cypermethrin is well known for string bioconcentration in aquatic organisms (Sapiets et al., 1984; Kollman & Segawa, 1995). However, specific compounds due to their characteristic nature have resisted biodegradation or the process occurs at slower than normal, thus making biological treatment ineffective. Therefore, for a successful biological treatment process, it is essential to maintain a sustainable population of microbes that are able to degrade waste.

There are number of methods to remove cypermethrin including ozone oxidation, photo and ultrasonic degradation, incineration, fenton degradation and adsorption. All these approaches to control environmental pollution are less effective, inconvenient and more expensive than biological methods of remediation (Xiao et al., 2015). In natural environment, hydrolysis, volatilization, photolysis and aerobic degradation by microorganism can be used to degrade cypermethrin (Laskowski, 2002).

Among various treatment technologies, microbial degradation is one of the most significant process in determining the activity and outcome of cypermethrin (Chen et al., 2012a). It involves the use of living microorganisms to detoxify the hazardous substances and is considered as an efficient method to remove impurities from the environment. It is well known that specific bacterial culture is proficient to degrade the threatening organic compounds if provided with accurate environmental conditions (Khalid & Hashmi, 2016). Environmental pollutants such as pesticides, petroleum, plastic, dye, surface active agent, etc. can be metabolized through biodegradation.

Some cypermethrin degrading organisms have been reported are *Bacillus sp*., *Pseudomonas sp*., *Alcaligenes sp*., *Aspergillus sp*., *Acidomonas sp*., *Micrococcus sp*., *Sphingobium sp*., and *Klebsiella sp*. PIs. *Bacillus subtilis, Bacillus licheniformis* and *Sphingomonas sp*., *Bacillus sp*., *Micrococcus sp*. *Ochrobactrum lupine*, *Serratia sp*., *Streptomyces aureus* and fungi like *Cladosporium sp.* (Grant et al., 2002), *Micrococcus sp*. (Grant et al., 2002; Tallur et al., 2008; Zhang et al., 2010; Chen et al., 2011a).

1.2 Present Study

The study was conducted to evaluate the growth potential of microorganism to metabolize cypermethrin using bioreactor. Further growth kinetics was estimated to access the capacities of the potential isolate. The findings may also play a significant role in onsite bioremediation of cypermethrin contaminated sites as well as for water treatment facilities proving as an effective approach for environmental sustainability.

1.3 Aims and Objectives

The objectives of present study were:

1. Isolation and identification of bacteria capable of cypermethrin degradation from contaminated soil;
2. Optimization of biodegradation efficiency of isolated bacteria;
3. Validation of results through application of kinetic models.

The outcome of this research would aid environmental scientist and engineers who are working on developing means to treat compounds like cypermethrin that are hydrophobic and impervious to biological treatment system.

LITERATURE REVIEW

2.1 Pesticides

The chemical compound employed in agriculture sector includes nitrogen and phosphorus fertilizers, disinfectants, pesticides, growth regulators for plants and veterinary drugs. Among them, pesticides are the compounds that cause lethal effects to human and environment. Pesticides are substances that are primarily used to control pests (Figure 2.1). Significant amount of pesticides is used in agriculture, horticulture sector, forestry and livestock production. The presence of these chemicals and their metabolites effects entire ecosystem and food chains of target and non-target species. The persistence of pesticide in the environment pose serious health effects such as genetic mutation, caners, nervous system disorders, sterility and developmental effects. Contamination of soil, surface and ground water is mainly caused due to the improper disposal from manufacturing plants, accidental spills, leaching and surface runoff.

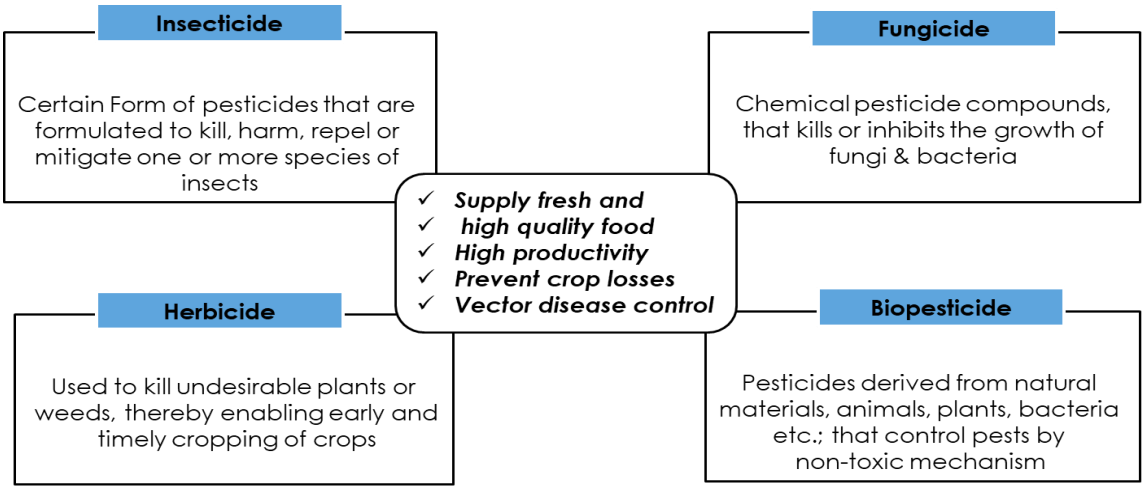


Figure 2.1: *"Agrochemicals" – Valuable to Protect the Agricultural Crop*

2.2 Pesticide Market Dynamics

Due to an increasing demand for bio-pesticides and adaptation of herbicide resistant crops, a relatively steady growth has been witnessed by the pesticide market in Pakistan. The worth of Pakistan crop protection market was projected to be USD 427.86 million in 2015. Primarily the pesticide market of Pakistan is import dependent, with about 25.30% of imports in 2016. The import worth of pesticide of USD 80.28 million was recorded in 2013. When

associated to the imports, the native production is also on the rise in Pakistan, with the production rate of USD 227.88 million in 2013. The market share of the crop protection industry in Pakistan by product type is depicted as of June 2016 (Figure 2.2).

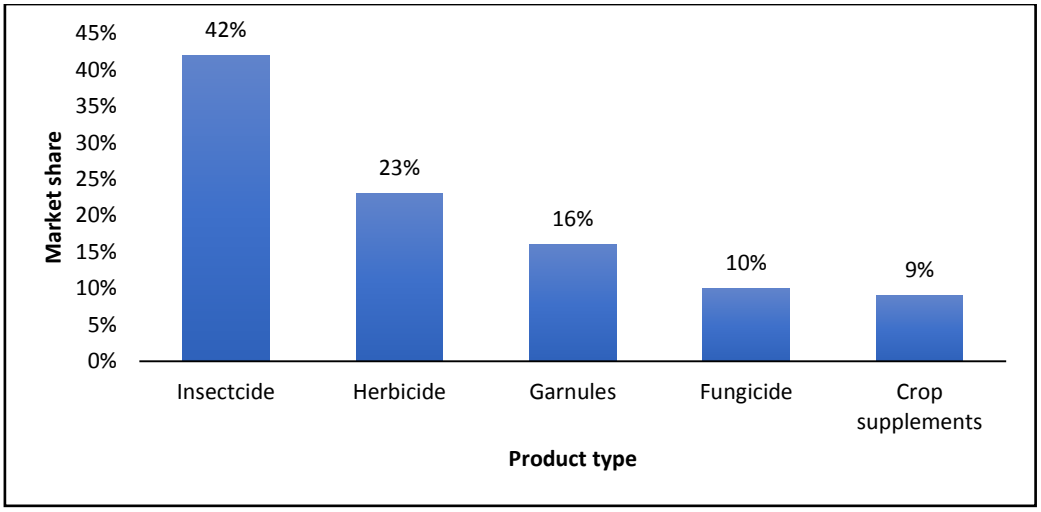


Figure 2.2: *Market share of the crop protection industry in Pakistan (2016)*

(Source: Agrochemicals and Pesticides Market in Pakistan: Business Report 2016 - Research and Markets)

In Pakistan the influences that drive the crop protection market are:

i. Rising food demand,
ii. low agricultural yield,
iii. intensification in disposable incomes,
iv. and implementation of agriculture development plan by the government of Pakistan

On the other side, the confinements limiting the market progress involve:

i. Inflation in prices of pesticides,
ii. Spread of *Bacillus thuringiensis* cotton (the highest insecticide consuming crop of the country)

With the extensive usage of pesticides in Pakistan, upcoming opportunities for market progress lie in the leaning to the adoption of novel technologies and taking up of sustainable and integrated pest management techniques in farming practices.

2.3 Key Industry Players in Pesticide Market

Some of the chief pesticide companies that control almost 75- 80% of the market share in Pakistan are:

i. Bayer Crop Science

ii. ICI Pakistan

iii. Syngenta International

iv. FMC

v. Ali Akbar

vi. Four Brothers Chemicals

Two traditional multinationals companies Dow Agro Sciences and DuPont had to finish up their operation due to massive competition and starter of generic pesticides by local companies.

2.4 Factors Leading to aggregated usage of Pesticide

2.4.1 Agricultural Yield in Pakistan

In Pakistan per hectare yield is lowest among the world. Yield in Pakistan when compared to the global average of 4 tons per hectare is at 3 tons per hectare (Figure 2.3). Developed nations like USA, France, UK and Germany are able to attain higher yield due to the advanced farming practices. This loop is often fulfilled by excessive and uncontrolled usage of pesticide.

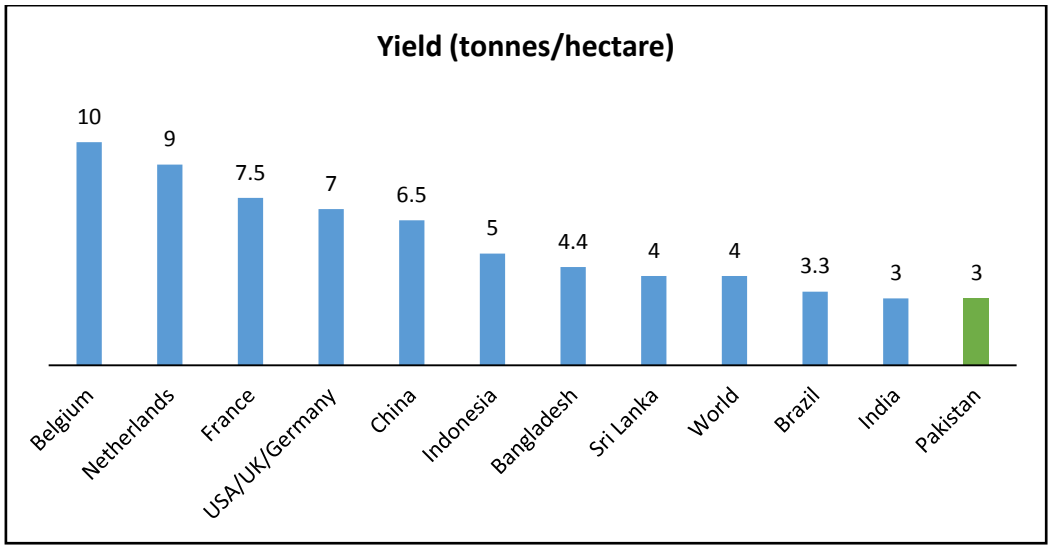


Figure 2.3: *Yield Comparison Source: Agricultural Census*

(Source: Agrochemicals and Pesticides Market in Pakistan: Business Report 2016 - Research and Markets)

2.4.2 Increasing Pest Attacks

A significant increase in total number of pest attack has been observed since 1940s. For illustration, the number of harmful pests for crops as in rice has augmented from 10 to 17 while for wheat from 2 to 19 respectively (Table 2.1). This is leading serious risk to food security and further underlines the importance of agrochemicals.

Table 2.1: *Crop wise Pests*

Crop	**1940**		**Present**	
	Number of Pests	Severe Pests	Number of Pests	Severe Pests
Wheat	20	2	100	19
Rice	35	10	240	17
Sugarcane	28	2	240	43

(Source: Agrochemicals and Pesticides Market in Pakistan: Business Report 2016 - Research and Markets)

2.5 Risk Associated with Pesticide

The presence of pesticide deposit in the soil, water and air is risky for both human and natural environment (DeLorenzo et al., 2001). It has been proved that farmers have extensively used pesticides in areas of Pakistan. Groundwater is constantly being contaminated due to insecticide use. It is clear from the biological survey that due to constant exposure farmers are at greater risk of acute and chronic health effects related to pesticides. Also, the excessive use of pesticides poses a greater risk for the pickers, field workers, and of an unacceptable residue concentration in cotton seed oil and cakes (Tariq et al., 2007).

The ultimate sink of insecticides used in agriculture is soil. The longer persistence of pesticides in soil increases adsorption of such poisonous chemicals by plants. This leads to accumulation of these compounds in plants which can be harmful and hazardous for human being as well as livestock. These pesticides in soil are gradually degraded by indigenous soil microorganisms (Vandevivere & Verstraete, 2002). Adapted microorganisms that convert these harmful pollutants into simpler non-toxic compounds enhance natural biodegradation. For environmental restoration, biodegradation and bioremediation are the most developing and growing fields (Damalas & Khan, 2007).

Contaminated environments have led to the evolution of microbial community in order to adapt to these xenobiotics (Pahm & Alexander, 1993). Consequently, for the isolation of stains proficient in degradation, these sites are most enriched ecological niches (Ortiz-Hernández et al., 2001).

2.6 Target Compound

Cypermethrin was primarily synthesized in 1974 and first marketed in 1977 as a highly active synthetic pyrethroid insecticide, efficient towards a wide range of pests in cultivation, human health, and animal husbandry. Chemically, cypermethrin is the alpha-cyano-3-phenoxy-benzyl ester of the dichloro analogue of chrysanthemic acid, 2,2-dimethyl- 3-(2,2-dichlorovinyl) cyclopropanecarboxylic acid (Figure 2.4). The molecule represents three assymetrical centres, one on the alpha cyano carbon while other two in the cyclopropane ring. cypermethrin represent mixture of eight isomers. Consequently, cypermethrin is a racemic mixture in a ratio of 50:50 if not mentioned. The physiochemical properties of cypermethrin are presented in table 2.2.

Among pyrethroid cypermethrin and Lambda-cyhalothrin are most frequently used throughout Pakistan. Cypermethrin usage alone in Hazara region accounts for 30% highest among all other chemicals used in the area (Khan et al., 2011). But due to ineffective legislation, lack of awareness and technical knowhow among the farming community, pesticide use is not accurately regulated in Pakistan. The residues of cypermethrin in irrigated soils of Pakistan are reported up to 74 mg/kg (Nafees & Jan, 2009).

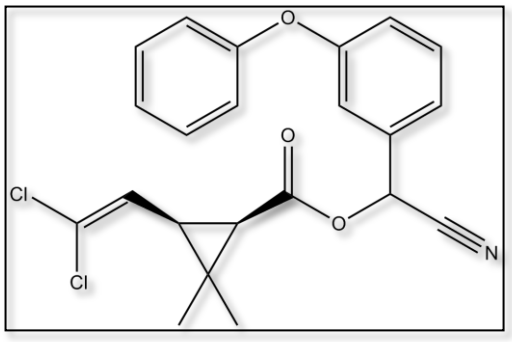


Figure 2.4: *Cypermethrin Structure*

Table 2.2: *Physiochemical Properties of Cypermethrin*

PROPERTY	DESCRIPTION
Class	Pyrethroid
Molecular Formula	$C_{22}H_{19}Cl_2NO_3$
Molecular Mass	416.3 g/mol
Color	Yellow
Odor	Odorless
Melting Point	80^oC
Boiling Point	220°C

2.7 Mode of Action of Cypermethrin

Cypermethrin, a synthetic pyrethroid insecticide is well known for its high activity in insecticides. It is reported to have low toxicity in birds and mammals, and quite stable to air and light (Cakir et al., 2008). Cypermethrin is available in forms like wettable powder or emulsifiable concentrate but in ultStrain-4ow volume. And it's also available in combined formulations with other pesticides.

Cypermethrin acts on the nervous system of vertebrates and invertebrates. It can spread poisonous by mere contact and by acting on stomach. In peripheral nervous system of the frog**,** its main action is to encourage noticeably repetitive activity and produce chains of nerve impulses by altering ion permeability of nerve membranes (Davies et al., 2007; Siegfried, 1993). These long-lasting chains generate hundreds to thousands of spontaneous nerve impulses in the sense organs. This repetitive activity is brought about by pyrethroid damage to the voltage-dependent sodium channel; as a result sodium channels stay open much longer than usual (Davies et al., 2007).

Cypermethrin has been proved to reduce ATPase enzymes involved in movement of ions against a concentration gradient regulated by active transport. This action is particularly dangerous to fish and aquatic life where ATPase enzymes provides energy necessary for active transport, and is crucial at sites of oxygen exchange. ATPase inhibit and disrupt active transport, which certainly affect movement of ion and maintaining ion balance, and disturb respiratory surfaces, thus cypermethrin is inherently more toxic to aquatic life The values of LC_{50} in fishes & EC_{50} in invertebrates are reported to be 1.5×10^{-3} mg/l and 2.1×10^{-4} mg/l respectively (Balaji et al., 2015).

2.8 Environmental Fate of Cypermethrin

2.8.1 Air

In atmosphere cypermethrin does not readily volatilize, as it has very low vapor pressure. Cypermethrin has a very low Henry's Constant value of 2.5×10^{-7} atm-m^3/mol, specifying it's no propensity to get volatilize from solutions that are aqueous in nature. As mentioned in a study conducted by Layman and coworkers, that a substance is considered almost nonvolatile if H is less than round 3×10^{-7} atm-m^3/mol (Cash, 2005).

2.8.2 Soil

Cypermethrin is a combination of both the *cis* and *trans* isomers. In a technical grade cypermethrin the ratio is 1:1. By a factor of two cis isomers are more vigorous. In soil metabolism of cypermethrin is governed by two natural phenomena of hydrolysis and photolysis. The main derivatives of hydrolysis are 3-phenoxybenzoic acid (PBA) and cyclopropanecarboxylic acid (Chen et., 2012b).

Numerous photoreaction govern degradation process in soil, the rate of such reactions depend upon the content organic matter of the soil. At favorable conditions i.e. aerobic the metabolites further degrade into CO_2, though the rate of conversion is much slower. The persistence of the metabolites is indefinite (Mollah & MacGregor, 2002).

Cypermethrin is hydrophobic as it displays very low solubility in water. It a non-polar pesticide thus showing a tendency to be absorbed onto a solid surface. Thus, making movement of cypermethrin through soil profile impossible though the products of degradation are often mobile. The metabolites PBA and DCVA are organic in nature which are mobile in soil. The chemical absorbed onto the soil greatly depends upon its organic

content. According to a report of USDA the average K_{oc} values for five different soil types was about 6.1x104 cm^3/g signifying immobility of cypermethrin in soil (Huang et al., 2018)

Microbes in soil play a substantial role in degradation. In anaerobic and waterlogged conditions, the rate of cypermethrin degradation gets affected. The degradation is also affected in sterile soils becoming slower, which demonstrates the importance of microorganisms (Chapman et al., 1981). In soils cypermethrin is relatively non-persistent. In sandy soil the typical half-life is about 2-4 weeks (Chapman & Harris, 1981). In soil with raised organic matter, high clay content, compact microbial activity and anaerobic settings, cypermethrin persistence intensified (Goswami et al., 2013).

2.8.3 Water

The solubility of cypermethrin is very low in water, 4 ppb at 200^oC (Kollman & Segawa, 1995). Cypermethrin is highly hydrophobic and instant aggregation of its particles occur in an aqueous solution. Thus, comparatively minor amounts of suspended matter in natural water bodies may remove a substantial amount of cypermethrin from the aqueous phase. The major ecological reservoirs for cypermethrin are soil and sediment for cypermethrin (Badrul Hisyam, 2012).

Cypermethrin gradually hydrolyzes in water at pH 7 and below, and hydrolyzes rapidly at pH 9. Cypermethrin is stable on hydrolysis with a half-life of >50 days, under optimum environmental temperatures and pH. It is also stable to photolysis with a half-life of >100 days. In antiseptic solution in sunlight, cypermethrin photodegrades deliberately, in 32 days, with <10% lost (Badrul Hisyam, 2012; Jillani, 2006). In the absence of sunlight, cypermethrin was fairly stable with 88.7 and 95.6% recovery after 10 days in river water and refined water, respectively.

Conferring to a research, the decrease in cypermethrin aqueous distillate was prompt, with about 95% lost within 24 hours after application to water and sediment contained in open drains. The reduction in concentration was chiefly due to swift sorption to sediment and suspended particles and not degradation. (Oudou & Hansen, 2002)

2.9 Degradation of Cypermethrin

Contaminated sites are rich niches for the isolation of pyrethroid degrading strains. Isolates from soils, sludge or wastewater contaminated by pyrethroid, belonging to various genera of bacteria and fungi have been isolated by using enrichment culture techniques.

(Wang et al., 2011; Cycoń et al., 2014; Akbar et al., 2015a; Chen et al., 2011a; Sundaram et al., 2013; Xiao et al., 2015; Tiwary & Dubey, 2016, Zhang et al., 2011)). It is vital to determine the potential of these isolates for the degradation under optimal conditions in liquid media, for the effective usage in the bioremediation processes.

As is exemplified in the table 2.2, microorganisms are using cypermethrin as direct source of carbon and energy and also co-metabolically (Cycoń et al., 2014; Liu et al., 2014; Akbar et al., 2015b). Moreover, the biodegradation rate in liquid media is affected by a number of factors like temperature, pH, nutrients, pesticide concentration, size of the inoculum and the properties of the strains (Zhang et al., 2010; Zhao et al., 2013).

Ochrobactrum lupine was isolated from activated sludge that metabolized beta-cypermethrin (beta-CP) and its chief metabolite 3-phenoxybenzoic acid (3-PBA) in mineral salt medium (MSM). Maximum degradation was achieved under optimum conditions i.e. at 30^{o}C and pH 7.0. Andrew's equation of kinetics was applied to explicate the inhibitory effect of cypermethrin at different initial concentrations. The estimated values of parameters q_{max}, K_s and K_i were determined to be 1.14 /d, 52.06 mgL^{-1} and 142.80 mgL^{-1}, respectively (Chen et al., 2011a).

Scientists in 2011 isolated *Pseudomonas aeruginosa* from activated sludge, that not only degraded beta-cypermethrin but also produced biosurfactant. Under optimal condition of 29.4^{o}C, pH 7.0, and inoculum of 0.15 g/L; near 90% of the beta-cypermethrin was metabolized within 12 days (Zhang et al., 2011). Similarly, in 2010, group of coworkers used two *Serratia sp*. that metabolized cypermethrin. The individual capacity of degradation for both was different. Under optimum conditions strain JC1 was able to consume 92% beta-CP with an incubation period of 10 days while strain JCN13 consumed 89% in 4 days (Zhang et al., 2010).

The growth of microorganisms in cypermethrin contaminated sites is effected at higher concentration. This fact was exemplified by a group of researchers from India who isolated, *Bacillus sp*. The strain was able to transform 86% of cypermethrin with an initial dose of 100 mgL^{-1} within 7 days. But at higher concentrations this transformation was inhibited. Likewise in 2013 a researcher used *Pseudomonas sp.* to access the potential of strain to metabolize cypermethrin. Biosimulator was used to carry biodegradation , nearly complete consumption of cypermethrin at dose of 20 mgL^{-1} was observed within 48 hours (Jillani, 2013).For the enhancement of biological treatment a new technique of degradation of

multiple pesticides by TiO_2 photocatalysis under UVA irradiation was proposed. It was suggested that for treatment of cypermethrin, chlorpyrifos and chlorothalonil pre-treatment of $UV/TiO_2/H_2O_2$ photocatalysis must be applied (Affam & Chaudhuri, 2013).

Wen-Jun and his fellow wokers in 2008, studied effect of nitrogen on metabolism of cypermethrin and its metabolites. The results indicated that the degradation of cypermethrin in soil enhanced by appropriate application of nitrogen. Maximum degradation of 80.0% was achieved after 14 days of incubation (Wen-Jun et al., 2008). Enhancement in degradation was also attained using co-cultures. This led to the reduction of cypermethrin longer half-lives. Andrew model of substrate inhibition was also applied to this study due to the inhibition of cypermethrin. The kinetic parameters were sought to be degradation rate (q_{max}) of 0.1 /h, half-saturation constant (K_s) of 31.2 mgL^{-1} and inhibition constant (K_i) of 220.5 mgL^{-1} (Chen et al., 2012b).

It is established in Table 2.3 that under optimal conditions of pH & and temperature 25-30°C, the strains that are metabolically active belong to genera *Bacillus, Pseudomonas, Brevibacillus, Sphingobium, Ochrobactrum and Serratia*. For instance, study conducted by Tiwary and Dubey reported in 2016 that *Bacillus sp*. AKD1 entirely used up cypermethrin 100 mgL^{-1}. Similar results were reported by Chen and coworkers using *Bacillus sp*. DG-02. (Tiwary & Dubey, 2016, Chen et al., 2012a). Likewise, about 90% initial dose of Cypermethrin 100 mgL−1, was utilized *Ochrobactrum anthropi* strain JCm1, *Ochrobactrum lupini* strain DG-S-0, *Ochrobactrum tritici* pyd-1, *Ochrobactrum tritici* pyd-1 and *Serratia nematodiphila* CB2 (Akbar et al., 2015b; Chen et al., 2011b; Wang et al., 2011).

For an effectual bioremediation soil that is contaminated with pyrethroids, it would be more beneficial to use multiple strains for mixture of pyrethroids. As the assembly of the compound that comes under this class are similar, helping microorganisms to metabolize multiple pyrethroid. (Cycoń et al., 2014). Several studies showed that consortium of bacterial strains lead to the boost in degradation process. For illustration Chen and coworkers in 2012 used mixed culture of *Bacillus cereus* and *S. aureus* for degradation of cypermethrin. Both the strains exhibited degradation but the proficiency decreased overtime. The efficiency of biodegradation in mixed culture was 73.1% while the individual results for alone metabolized only 37 and 23% respectively (Chen et al., 2012b). Similarly, a mutual cooperation was demonstrated by *Bacillus licheniformis* and *Sphingomonas sp.* with enhanced efficiency.

cypermethrin half-life using both strains was reduced from 71.9 to 35.7 hours (Liu et al., 2014).

Table 2.3: *Degradation Potential of Bacteria Isolated from Sites Contaminated with Cypermethrin*

BACTERIAL ISOLATES	DEGRADATION RESULTS	REFERENCES
Acinetobacter calcoaceticus MCm5	84.7% (100 mgL^{-1}) 10 days	Akbar et al., 2015a
Azoarcus indigens HZ5	70% (50 mgL^{-1}) 144 h	Ma et al., 2013
Bacillus sp. AKD1	86, 73, 67, 51, and 47% of (100, 150, 200, 400, and 500 mgL^{-1}, respectively) 7 days	Tiwary & Dubey, 2016
Bacillus sp. DG-02	89.2% (50 mgL^{-1}) 72 h	Chen et al., 2012a, 2014
Bacillus sp. ISTDS2	100% (50 mgL^{-1}) 180 h	Sundaram et al., 2013
Bacillus sp. SG2	82% (50 mgL^{-1}) 15 days	Pankaj, et al., 2016
Bacillus cereus ZH-3	78.4% (50 mgL^{-1}) 72 h	Chen et al., 2012b
*Bacillus licheniformis*B-1	50% (100 mgL^{-1}) 72 h	Liu et al., 2014
*Bacillus megaterium*JCm2	89% (100 mgL^{-1})	Akbar et al., 2015b

	10 days	
Bacillus subtilis BSF01	93.9, 89.4, and 84.7% (25, 50, and 100 mgL^{-1}, respectively) 7 days	Xiao et al., 2015
Brevibacillus parabrevis JCm4	28% (100 mgL^{-1}) 10 days	Akbar et al., 2015b
Catellibacterium sp. CC-5	90% (50, 100, and 200 mgL^{-1}) 7 days	Zhao et al., 2013
Micrococcus sp. CPN 1	90% (1000 mgL^{-1}) 8 days	Tallur et al., 2008
Ochrobactrum lupiniDG-S-01	90% (50 mgL^{-1}) 5 days	Chen et al., 2011b
Ochrobactrum haematophilum JCm7	78% (100 mgL^{-1}) 10 days	Akbar et al., 2015b
*Ochrobactrum tritici*pyd-1	100% (100 mgL^{-1}) 6 days	Wang et al., 2011
Pseudomonas aeruginosa CH7	90% (100 mgL^{-1}) 12 days	Zhang C. et al., 2011
Pseudomonas aeruginosa JCm8	46% (100 mgL^{-1}) 10 days	Akbar et al., 2015b
Pseudomonas fluorescens	37.2% (50 mgL^{-1}) 96 h	Grant et al., 2002;
Sphingomonas sp. JCm3	34% (100 mgL^{-1})	Akbar et al., 2015b

	10 days	

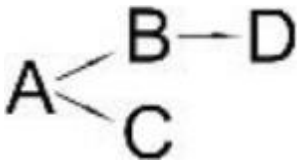
(Source: Cycoń, M., & Piotrowska-Seget, Z., 2016)

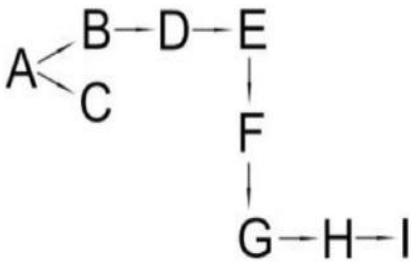
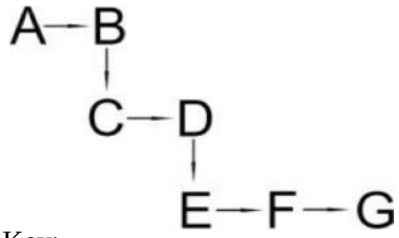
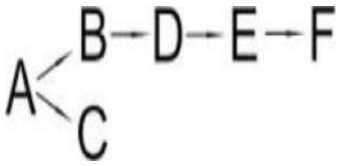
2.10 Proposed pathways for degradation of Cypermethrin

The metabolic pathway of cypermethrin was researched in single cultures belonging to the *S. aureus ,Bacillus genera, Micrococcus sp., Catellibacterium sp*. and consortium that comprised of *B. licheniformis* and *B. cereus* as well as *S. aureus* and *Sphingomonas sp*. (Tallur et al., 2008, Chen et al., 2012a, Zhao et al., 2013, Liu et al., 2014, Chen et al., 2012b).

As depicted in Table 2.4, during cypermethrin degradation, metabolites that were detected by *Bacillus sp*. SG2, *Bacillus sp*. ISTDS2 and *B. subtilis* BSF01 were α-hydroxy-3-phenoxy-benzenacetonitrile and 3-(2,2-dichloroethenyl)-2,2-dimethyl cyclopropanecarboxylate. In *Bacillus sp*. SG2 and *B. subtilis* BSF01, first product was processed into 3-phenoxybenzenaldehyde and in *Bacillus sp*. ISTDS2 into 3-phenoxybenzoic acid. The major derivative was transformed into cyano-phenoxybenzaldehyde and later converted into 3-phenoxybenzoic acid. Its breakdown produced phenol and protocatechuic. Another transitional product is 4-phenoxyphenol-2,2-dimethyl-propiophenone, in a medium injected with *B. cereus* and *S. aureus* (Chen et al., 2011a).

Table 2.4: *Degradation pathway of Cypermethrin (A) by Bacillus genera*

BACTERIAL ISOLATE	DEGRADATION PATHWAY	REFERENCES
Bacillus sp. ISTDS2	 **Key:** (B)α-Hydroxy-3-phenoxy-benzenacetonitrile (C)3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (D) 3-Phenoxybenzoic acid	Sundaram et al., 2013

Bacillus sp. SG2	 Key: B) α-Hydroxy-3-phenoxy-benzenacetonitrile (C) 3-(2,2-dichloroethenyl)-2,2-dimethyl cyclopropanecarboxylate (D)3-Phenoxybenzenaldehyde, (E)Hydroxybenzoate (F) 4-Propoyl Benzaldehyde, (G) Phenoxy benzoic acid (H) Phenol, M-tert-Butyl, (I) Phenol	Pankaj, et al., 2016
Bacillus licheniformis B-1	 Key: (B) Cyano -3-phenoxybenzyl alcohol (C)3-Phenoxybenzaldehyde (D) 3-Phenoxybenzoic acid, (E) 2-Phenoxyphenol, (F) Catechol (G) Muconic acid	Liu et al., 2014
Bacillus subtilis BSF01		Xiao et al., 2015

	Key: (B) α-Hydroxy-3-phenoxy-benzenacetonitrile, (C) 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylat (D) 3-Phenoxybenzaldehyde (E) 3-Phenoxybenzoic acid (F) 3,5-Dimetoxyphenol	

(Source: Cycoń, M., & Piotrowska-Seget, Z., 2016)

In another study reported by Pankaj and coworkers in 2016 isolated *Bacillus sp*. from pesticide-contaminated soil for cypermethrin degradation. Study of derivative compounds of biodegradation revealed that the bacteria chose a novel pathway for degradation. As the presence of 4-propylbenzoate, 4-propylbenzaldehyde, phenol M-tertbutyl and 1-dodecanol, etc. which has not been reported earlier in cypermethrin degradation (Pankaj et al., 2016).

2.11 Development of Growth Kinetic Models

A kinetic model uses a set of mathematical equations, in order to describe the behavior of cellular processes. The correlation between kinetics of microbial growth (μ) and utilization of substrate concentration (S), are represented using numerous mathematical models (Al. Malak, 2006) For an optimal design and maneuver of biological treatment plant, the information about kinetic parameters is vital (Deriase & El-Gendy, 2014).

Often in a microbial action the rate of cell growth ceases, this is due to the inhibition effect caused by the substrate or products that effects the growth of microorganism. This may be due to the accumulation of toxic byproducts or the collapse of an essential nutrient i.e. substrate. If the product of inhibition is produced, this will lead to slow growth rate and ultimate cessation. In batch studies during growth and death phase, the specific growth rate of microorganisms depends on the concentration of substrate that is being used as a source of carbon and energy. Every so often, the rate of specific growth is dependent upon the single substrate, this constituent is recognized as growth limiting substrate (Goudar et al., 2000).

Chiefly growth models are divided into two, mainly incorporating:

i. Kinetics of limiting substrate-inhibition;
ii. Only growth kinetic parameters.

These models are constructed on spatial consistency, a well-mixed environment is ensured through platform or incubator shaker. Pesticide in the media is treated as the only source of carbon and energy. Throughout the system operation, the volume and pH of the sample are anticipated to remain constant (Kim et al., 2005).

In a toxic environment specific growth rate of microbes could face two competing effects by substrate. As the substrate is increased, the specific growth rate increases, but on contrary by increasing the substrate the specific growth rate decreases. Numerous kinetic relations have been formulated that show the dual expression of specific growth on S acting as substrate and an inhibitor (Okpokwasili & Nweke, 2006).

At high concentration cypermethrin is well known to cause inhibition effect. To describe this effect in pure and mixed culture of microbes, numerous inhibition models have been used. Due to the empirical nature of models, it's difficult to decide which models fit the study. Characteristically, hypothetical justification for the use of specific model cannot be provided, for this reason selection is purely based on the outcomes of statistical examination of model to describe set of experimental data. In spite of the empirical nature, these inhibition models are able to precisely describe experimental data on biodegradation, thus providing appropriate way of modeling biodegradation of cypermethrin (Deriase & El-Gendy, 2014).

2.11.1 Monod model

It is the most basic and an essential model for determining growth kinetics of a system. Using parameters μ_{max} and $K_{s,.}$ It correlates the growth rate of microbes to the concentration of growth-limiting substrate. When substrate is in low concentration, this model successfully describes a proportional relationship between specific growth rate and initial substrate concentration as describes in Equation 1. Usually in conditions when there is pure culture, non-inhibitory biomass growth and limited substrate concentration Monod kinetic model is used (Kumaran & Paruchuri, 1997).

$$\mu = \mu m \frac{S}{(S+Ks)} \longrightarrow \textbf{Equation 1}$$

Where

μ = specific growth rate (h^{-1}),

μ_{max} = maximum specific growth rate (h^{-1}),

S = substrate concentration (mg L^{-1}),

K_s = substrate saturation constant (mg L^{-1})

However, at higher substrate concentration the Monod model fails to account inhibition effect on the growth. In literature, several formulae are reported, in which simply Monod equation is modified to justify inhibition effect. This drawback is overcome by the models of Aiba, Haldane, Edward, Moser and Tessier (Mahanta et al., 2014).

2.11.2 Andrew Model

Among the substrate inhibition models, Andrew's model described in Equation 2 is most extensively used. It particularly explicates inhibitory effects caused by substrate at higher initial concentrations. The term inhibition coefficient, Ki quantifies the effect of toxic compound on the treatment system (Deriase & El-Gendy, 2014). Unlike Monod model Andrew model is a non-linear model. At higher values of K_i, it diminishes to Monod's equation (Dutta et al., 2015).

$$\varepsilon = \frac{(\varepsilon \max \check{I}\, S)}{(Ks+S+S^2/Ki)} \longrightarrow \textbf{Equation 2}$$

Where

μ_{max} = maximum specific growth rate (h^{-1}),

K_i = inhibition constant (mg L^{-1}),

K_s = half-saturation constant (mg L^{-1}),

S = substrate concentration (mg L^{-1}).

MATERIALS AND METHODS

Semi-continuous biodegradation studies were carried out in Environmental Microbiology Teaching Lab, Environmental Toxicology and Biotechnology Research Labs, of Institute of Environmental Sciences and Engineering, National University of Sciences and Technology, Islamabad.

3.1 Chemicals and Pesticide

Commercial grade cypermethrin (RB Avari Enterprises, 10% EC) was purchased from local market. Analytical grade cypermethrin (Pestanal @ FLUKA, Sigma Aldrich) was purchased from Science Centre, to carry out gas chromatographic analysis. Stock solution was diluted in GC grade Acetonitrile (solvent) to make various dilution (5, 1, 0.5, 0.1 & 0.05 mgL^{-1}) in order to form a calibration curve. Later, for analytical work samples were extracted through Liquid- Liquid extraction, using GC grade Ethyl acetate (solvent).

3.2 Concentrations of Cypermethrin

Nutrient broth and Mineral salt media were used to perform growth studies of the selected strain. Cypermethrin was used as a sole carbon source in Mineral Salt Media. Various initial concentrations of 50, 100, 200, 300, 400 and 500 mgL^{-1} were used (Chen et al., 2011a; Chen et al., 2012a).

3.3 Preparation of Media

3.3.1 Mineral Salt Media

To ensure sterility, sterile glass ware and autoclaved distilled water was used throughout degradation studies. Mineral salt media was prepared for the isolation and degradation of cypermethrin. (Source) The composition of mineral salt media is shown in Table 3.1.

Table 3.1: *Composition of Mineral Salt Media*

SALTS	QUANTITY (gL^{-1})
NaCl	0.5
$MgSO_4$	0.2
KH_2PO_4	0.5
K_2HPO_4	1.5
NH_4NO_3	1.0

(Source: Khalid & Hashmi, 2016)

3.3.2 Other Media

Nutrient agar slants and plates were prepared to revive the culture and bacterial inoculum. For this 20 g of nutrient agar was added in 1-liter distilled water and then it was autoclaved at 121^oC and 15 lb./cm^2 pressure for 15 minutes. Then nutrient agar was poured in autoclaved petri plates and test tubes. In order to confirm sterility, petri plates and test tubes were placed in incubator at 37^oC for 24 hours.

NB (OXOID) preparation for degradation studies was done by adding 8 g of media in 1L distilled water followed by autoclaving at 121^oC and 15 lb. /cm^2 pressure for 15 minutes. Sterility test was carried by placing NB in incubator at 37^oC for 24 hours.

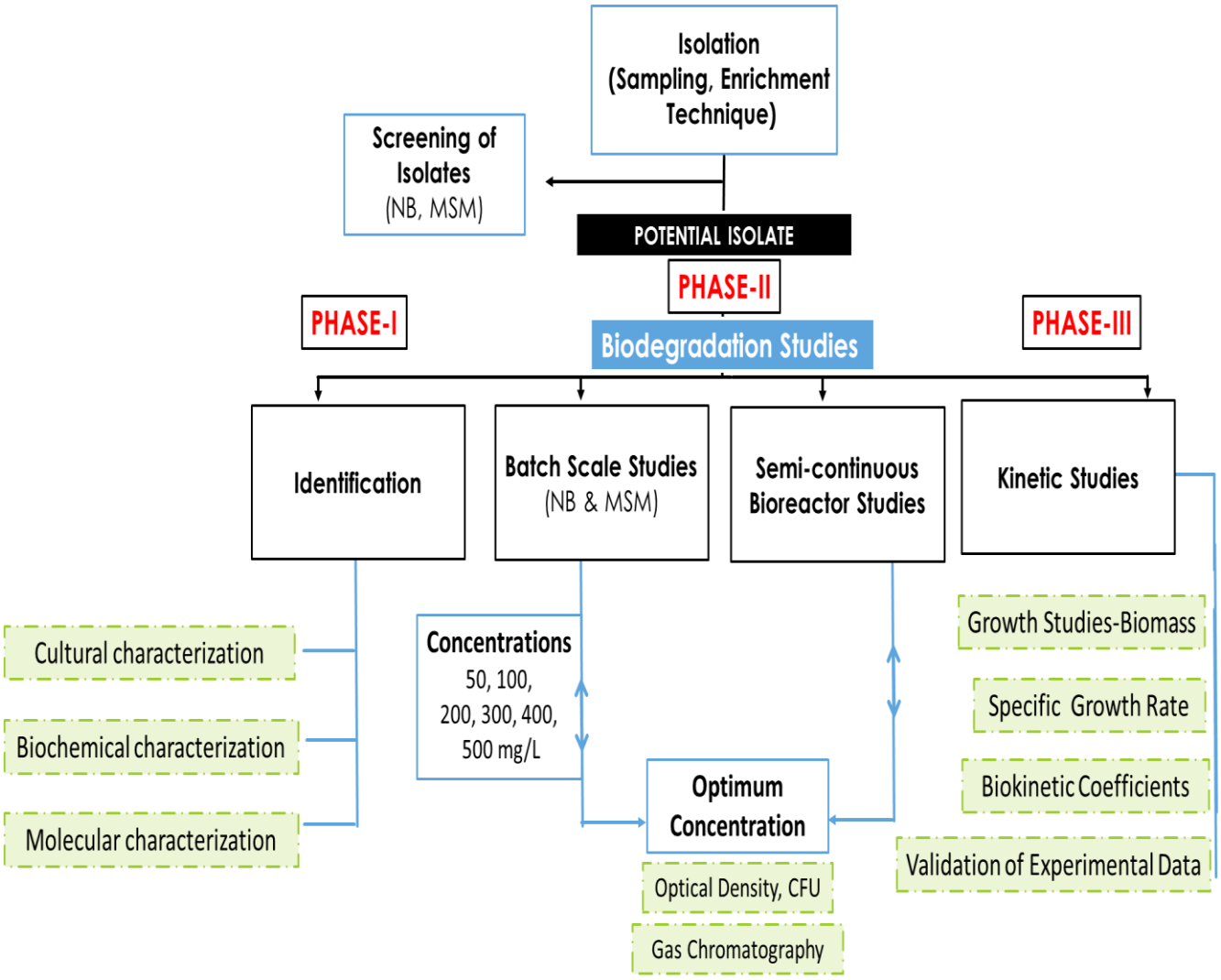


Figure 3.1: *Experimental Design*

3.4 PHASE-I

3.4.1 Isolation

3.4.1.1 Soil Sampling

Soil samples were collected from wheat field of Ahmadpur, East Punjab on September 5th, 2016. The soil has received periodic treatment of cypermethrin for years. Topsoil was collected from first 15 cm depth from various points to make composite sample. Later, it was transported in air tight bags within 24 hours in Environmental Microbiology and Teaching laboratory, IESE, SCEE NUST, Islamabad.

3.4.1.2 Soil Preparation

The soil sample was oven dried up to 60^{o}C for moisture removal. The soil was sieved through 2mm sieve and then kept in 4^{o}C in sterile polythene bags for further laboratory analysis. Soil pH was measured using portable pH meter (Aldén et al., 2001)

3.4.3 Soil Enrichment

Microorganisms capable of utilizing cypermethrin as sole carbon were isolated using enrichment technique. For isolation 10 g of prepared soil sample was added to MSM containing cypermethrin (50 mgL^{-1}) as a sole carbon and energy source and incubated at 37^{o}C. After 7 days, 10 ml of the suspension was added to fresh MSM containing cypermethrin (50 mgL^{-1}). With three successive transfers 1 ml of sample was serially diluted and spread on nutrient agar plates (Chen et al., 2011b). Isolates that showed notable growth were further purified through streaking and selected for further analysis. The selected stains for biodegradation studies were initially named as Strain-1, Strain-2, Strain-3, Strain-4.

3.4.2 Identification

3.4.2.1 Morphological Characterization

The procedure outlined in Bergey's Manual of Determinative Bacteriology was used to determine the biological and physiological characteristic of the isolated bacterial strains (Vos et al., 2011).

3.4.2.2 Colony Morphology

The foremost important step in description and identification of unknown isolate is observation of single colony by naked eye and microscope. The colony was purified by successive streaking until pure culture was attained. Several characteristics like shape, size, margin, elevation and texture pigmentation of colony were observed (Chatterjee et al., 2007).

3.4.2.3 Cell Morphology

The bacteria's that are Gram positive have peptidoglycan (50-90%) in their cell wall allowing them to retain primary dyes. On the other hand, the ratio of peptidoglycan in Gram-negative cell wall's is round 10% making primary dyes rinse away upon washing with ethanol (Reiner, 2012).

For gram staining a smear of bacterial culture was prepared onto a clean slide. The slide was set to air dry and then heat fixed. Later, a drop of crystal violet and iodine solution were applied separately followed by successive washing with distilled water. After that, it was washed with decolorizing agent for 15 seconds. Than a counter stain of safranin was applied with a final wash with distilled water. The prepared slides were mounted on a light microscope and were observed under oil immersion under 100X resolution (Fawole & Oso, 2004). The Gram- negative bacteria's acquired safranin dye appearing pink while Gram-positive retained the crystal violet dye appearing purple.

3.4.3 Biochemical Characterization

3.4.3.1 Catalase Test

A clean glass slide was taken and 18-24 hours fresh bacterial culture was placed onto the slide with the help of sterile inoculating loop. Hydrogen peroxide solution was poured onto the culture. In aerobic environments bacteria continue living by neutralizing toxic forms of oxygen by production of various enzymes. One of the enzyme is catalase, which breakdowns hydrogen peroxide into oxygen and water. With the help of sterilized inoculating loop a fresh colony was placed on clean slide. Than a drop of 3% Hydrogen peroxide solution was added onto the slide. Instant

effervescence shows a positive catalase test while no bubble creation shows negative result for catalase enzyme.

3.4.3.2 Oxidase Test

A 24-hour fresh culture was placed on filter paper via inoculating loop. Later a drop of 1% N, N-dimethyl-p-phenylenediamine dihydrochloride solution was added and air dried. Immediate coloration into blue indicated oxidase positive while no change in color specified negative results for oxidase production.

3.4.3.3 MacConkey Agar Test

MacConkey agar is a basically differential medium for Gram negative bacteria. The basis of differentiation is lactose fermentation. Fresh cultures were made to streak on already prepared plates of MacConkey agar. Later, the plates were incubated for 24 hours. Appearance of pink colonies shows lactose fermenters.

3.4.4 Molecular Characterization

3.4.4.1 16S rRNA Sequencing

For 16S rRNA sequencing, preserved isolates were sent to Genome analysis department Macrogen, Seoul, South Korea**.**

3.4.4.2 Phylogenetic Analysis

On the basis of genetic characteristics phylogentic analysis shows ancestral relationship among biological beings (Tamura et al., 2013). The sequence obtained was trimmed using bioedit software tool. Later, they were scrutinized using BLAST tool of National center of biotechnologcal information NCBI. A comaprison of seuqnce was made with seuquence reported in GENEBANK of NCBI. MEGA 7 software was run to get phylogentic analyis.

3.5 PHASE-II

3.5.1 Degradation Studies

3.5.1.1 Batch Scale Studies

Batch scale studies were conducted for the screening of potential isolate for bioremediation at optimum concentration of cypermethrin. Experiments were

conducted in triplicates. The strain potential was further enhanced in semi-continuous bioreactor system.

3.5.1.2 Preparation of Inoculum

The inoculum for all of the experiments was prepared by growing bacteria in 10 ml of NB overnight at 37°C in an incubator. After 24 hours the optical density of the inoculum was measured using spectrophotometer. For all experiments, OD: 0.8 were used and inoculated into successive samples in equal amount of 5 ml (Qiu et al., 2007).

3.5.1.3 Screening of Potential Isolate

Screening for potential isolate was done in NB and MSM containing cypermethrin 50 mgL^{-1} (sole carbon and energy source) incubated at $37^{o}C$ at 100 rpm in an incubator shaker (Table 3.2). Single beam spectrophotometer at 600 nm was used to monitor growth at regular intervals. One strain labeled as Strain-4, which possessed the highest degrading capacity, was selected for further cypermethrin degrading studies.

Table 3.2: *Technical Data of Incubator Shaker*

SPECIFICATIONS	VALUES
Speed	150 rpm
Operating Temperature	$37^{o}C$
Volumetric Load	250 ml
Inoculum size	OD : 0.78 (600_{nm})
Sample Collection	0, 4, 6, 12, 24 hours
Speed	150 rpm
Operating Temperature	$37^{o}C$

3.5.1.4 Screening of Optimum Concentration

Batch scale studies were conducted to find out the optimum concentration of cypermethrin for bioremediation. For this various concentration of cypermethrin in

NB & MSM from 100 to 500 mgL^{-1} were added to conical flask with both medias. Overnight grown bacterial culture was added to each flask in equal amounts. Afterwards, the flask was incubated at 37^{o}C and 150 rpm. Each of the group was made to run in triplicates. The control was used without bacterial culture for all concentrations. Optical density at several time intervals was measured by means of UV- spectrophotometer at 600 nm (Munoz et al., 2011).

3.5.2 Semi-Continuous Bioreactor Studies

Bioreactor used in this study consisted of two main units: influent reservoir and reactor unit. Dimensions for the bioreactor were 40 cm length, 24 cm internal diameter, 14 L of the total volume and 4L of working volume (Table: 3.3). Magnetic stirrer and air pumps helped to maintain a uniform level of biomass and cypermethrin. Inlet and outlet ports supported feeding and sample collection during operation of bioreactor. Autoclaved cotton plugs were used to avoid contamination. The reactor was made to operate at ambient temperature.

Table 3.3: *Operating Conditions of Bioreactor*

PARAMETERS	VALUES
Co-Substrate	Minimal Media
Initial pH	5.5
Dissolved Oxygen	8.38 mgL^{-1}
Pesticide	Cypermethrin (10% EC)
Pesticide Concentration	100 mgL^{-1}
Bacterial Species	*Bacillus safensis*
Bacterial Inoculum	OD: 0.9 (600_{nm})

3.5.2.1 Bioreactor Operation

Synthetic wastewater was prepared by adding K_2HPO_4 and KCL 0.5 and 0.25 g/L, respectively in autoclaved water and cypermethrin was added at a concentration of 100 mgL^{-1}. Biomass developed from *Bacillus safensis* was used as seeding

inoculum for the reactor. Strain potential for cypermethrin removal in synthetic wastewater was evaluated at regular intervals. The lab scale setup of bioreactor used is attached in Anexxure 1. In a reactor, when concentration was reduced to 8-10 mgL^{-1}, cypermethrin was replenished and its removal efficiency was monitored for remaining cycles. Parameters like pH, DO, OD and removal efficiency were monitored at regular intervals (Khalid & Hashmi, 2015).

3.5.2.2 Bioreactor Functioning

The operation of bioreactor was based on fill & draw principle maintaining a total volume of 4 L. During fill phase reactor received feed from influent reservoir. Than it was allowed to react in the presence of live inoculum. Aeration was ensured during first two stages. During off time of diffusers biomass was allowed to settle at the bottom, followed by decanting stage used to draw 2 L treated water (Figure 3.2) For successive cycles the operation was repeated (Khalid & Hashmi, 2015).

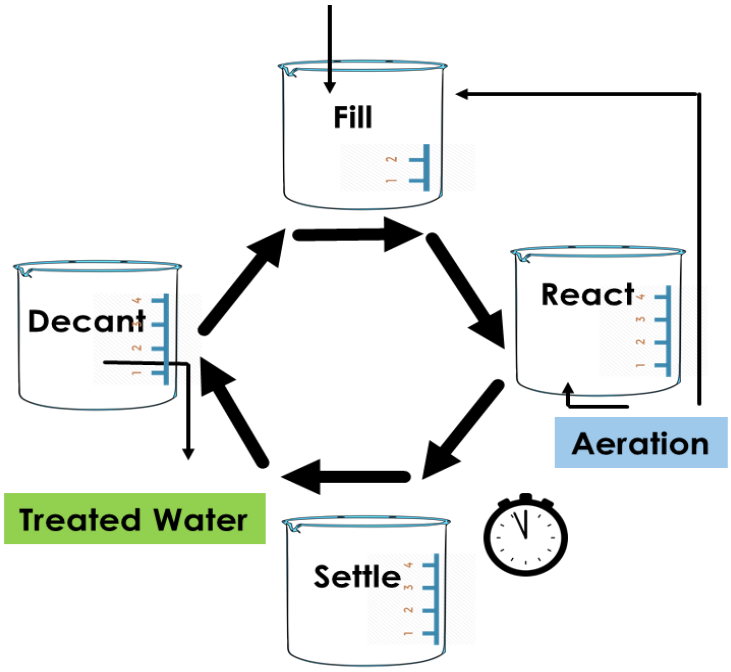


Figure 3.2 *Operation of Bioreactor*

3.5.3 Analytical Procedures

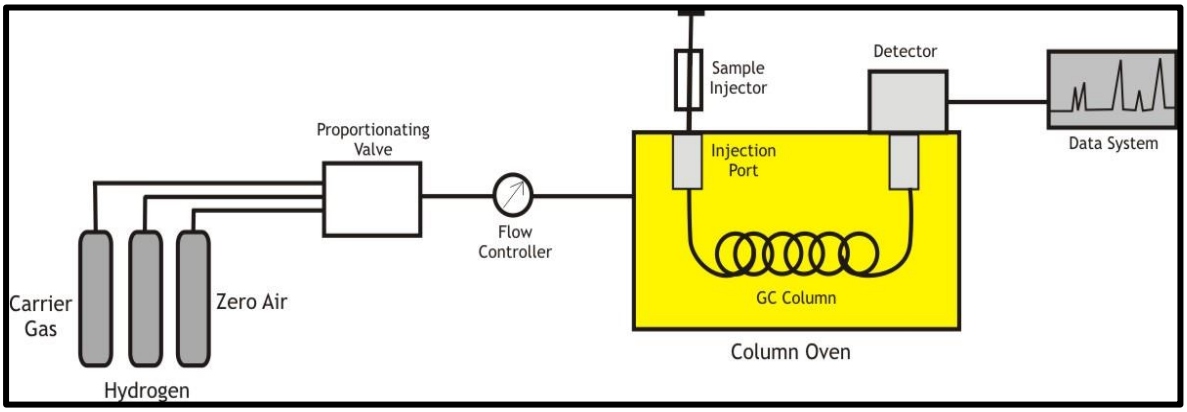
Analytical methods employed to monitor the biodegradation potential of the isolated strain for cypermethrin was determined using UV-spectrophotometer, and gas chromatography (GC).

3.5.3.1 Spectrophotometric Analysis

The sample was withdrawn at regular intervals to determine optical density using UV-Visible spectrophotometer. The wavelength used for analysis was 600 nm which is bacteria specific (Sundaram et al., 2013).

3.5.3.2 Gas Chromatographic Analysis

Pesticide residues at trace level were determined using the most recommended technique of Gas chromatography. To carry out analysis Shimadzu GC 2010 with Electron Capture Detector (ECD) was used. The separation column used was merged with silica capillary with 30 m length. Liquid samples were swiftly vaporized and transferred through column by mobile phase (Figure 3.3 & 3.4).



(Source: retrieved from: http://lab-training.com/2013/04/12/gas-chromatography-diagram/)

Figure 3.3 *GC Schematic Diagram*

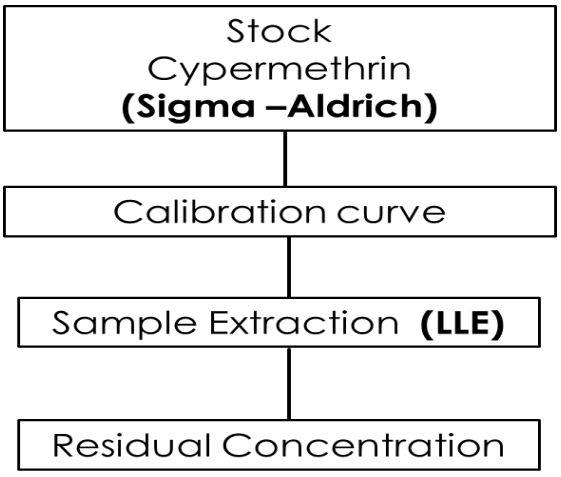


Figure 3.4 *Various Steps in GC analysis*

3.5.4 Optimization of GC conditions

Before analyzing sample for traces of cypermethrin, the instrument was optimized. The parameters such as injector temperature, detector temperature, column temperature, split ratio and flow rate were adjusted to get optimized conditions (Table 3.4).

Table 3.4 *Operating Conditions for Gas Chromatography (Schimadzu-2010, Japan)*

PARAMETERS	CONDITIONS
Injection port	
Temperature	220°C
Injection mode	Split
Column	
Initial temperature	50 °C
Final temperature	250 °C
Column length	25 m
Electron Capture Detector (ECD)	
Temperature	280 °C
Carrier gas	Helium
Makeup gas	Nitrogen
Makeup flow	15 mL/min

3.5.4.1 GC Standards and Calibration Curve preparation

Working solution of 5, 1, 0.5, 0.1, 0.05 ppm were prepared in GC grade Acetonitrile from a stock of 100 ng/ul FLUKA cypermethrin PESTANAL® (Sigma – Aldrich). Each of the working solution was injected in GC, to obtain the retention time of insecticide. It was further used to prepare calibration curve and line equation. The unknown concentration in the sample was determined using the line equation mentioned in Figure 4.4.

3.5.4.2 Sample Preparation

In order to estimate rate of cypermethrin degradation, extraction of sample from bioreactor was done at regular intervals. For the extraction of sample liquid-liquid extraction technique was used. For this, 20 ml sample was collected in a test tube and 5 ml of organic solvent ethyl acetate was added into it. The sample was than vigorously shaken for few second using a vortex. Later it was sonicated for 2 minutes and left uninterrupted for 20 minutes. This led to the formation of two distinct organic and inorganic layers. The upper organic layer was collected using microscopic pipette and transferred to GC vials. The process was repeated twice. The sample was kept in refrigerator under 4^{o}C for later chromatographic analysis.

3.6 PHASE-III

Batch experiments were conducted to evaluate the effect of initial concentration of cypermethrin on the growth kinetics of *Bacillus safensis*. In Figure 3.3 the detailed steps carried out in validation of experimental data of biodegradation study are listed.

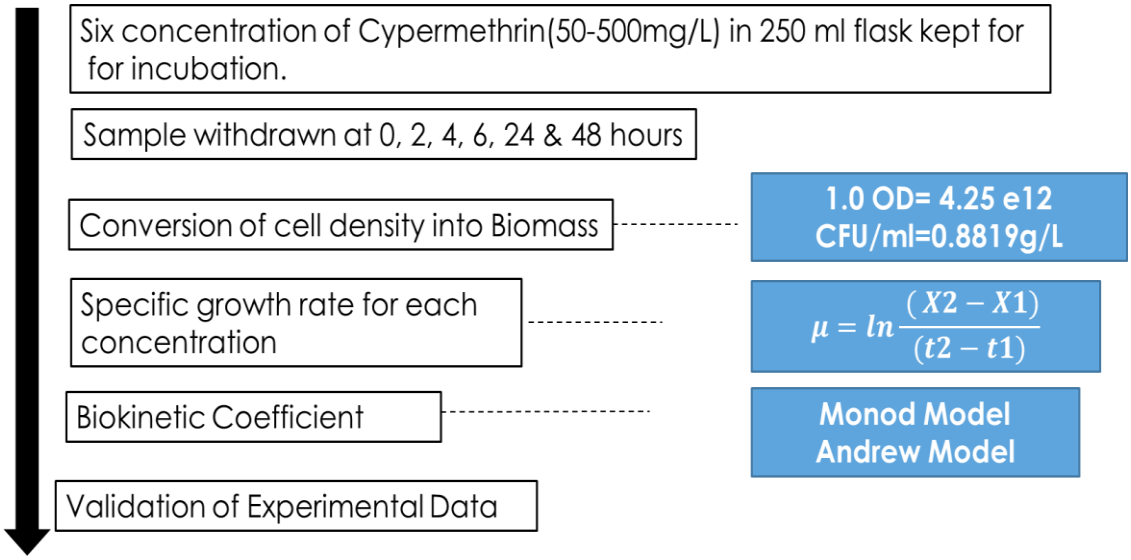


Figure 3.5 *Various Steps in Kinetic Studies*

3.6.1 Growth Kinetics of Single Culture

Based on the growth patterns of *Bacillus safensis*, individual specific growth rate was evaluated for each initial substrate concentrations (50, 100, 200, 300, 400 and 500 mgL^{-1}) using the following relation:

$$\mu = ln \frac{(X_2 - X_1)}{(t_2 - t_1)} \longrightarrow \textbf{Equation 3}$$

Where during exponential phase:

X_2 = Biomass concentration at maximum growth time t_2

X_1 = Biomass concentration at an initial growth time t_1,

The experimental and fitted relation between specific growth rate and substrate is plotted in for Monod and Andrew models, respectively (Kim *et al.,* 2005).

3.6.2 Parameter Estimation Method

Estimation of biokinetic parameters has its own implications and necessary for understanding the degradation potential of microorganisms along with corrects operation of bioreactor systems. After calculation of specific growth rate, biokinetic parameters were estimated through linear and non-linear regression analysis. Some of the definitions of biokinetic parameters are as under.

3.6.2.1 Specific Growth Rate (μ)

In bioreactor and batch studies, the rate of change of biomass, dX/dt is directly proportional to the concentration of biomass X present in reactor, and this proportionality factor is termed as specific growth rate μ. The kinetic equation describing this relation is mentioned below:

$$Rg = \frac{dX}{dt} = \mu X$$

$$\mu = \frac{(dX/dt)}{X} \longrightarrow \textbf{Equation 4}$$

Where

μ= specific growth rate

X= concentration of biomass

dX/dt= rate of change of biomass

R_g= growth rate of biomass

3.6.2.2 Half Velocity Constant (K_s)

The specific growth rate of microbes depends upon the rate of substrate utilization. Even when the substrate is in limiting conditions net growth is observed. Half velocity constant (K_s) is the concentration of substrate at one half of the maximum specific growth rate. The kinetic equation describing half velocity constant

$$Ks = \mu m \frac{S}{(\mu - S)} \longrightarrow \textbf{Equation 5}$$

Where

S= substrate concentration

μ_m= maximum specific growth rate

μ= specific growth rate

RESULTS AND DISCUSSION

4.1 PHASE-I

4.1.1 Soil Characteristics

Agriculture soil that had already received treatment of various pesticides including cypermethrin was collected form Ahmadpur, East Punjab (Table 4.1). The pH of the soil sample was 7.1. A critical factor that impacts the compositions of soil bacterial communities is the pH of soil. Neutral soils are more diverse in bacterial niches (Liu et al., 2014).

Table 4.1: *Physical characteristics of soil*

SOIL CHARACTERISTICS	DESCRIPTION
Texture	Silt Loamy
pH	7.1

4.1.2 Isolation and Identification

4.1.2.1 Colony Characterization

The colony characteristics of isolates are reported in Table 4.2. All of isolates (4/4) were observed to appear circular, followed by an entire margin and glistening in appearance. The elevation of isolates was convex and only one was flat. In terms of texture, half of isolates were smooth, one isolate was creamy, and one was slimy. Size varied from moderate to punctiform. Non-pigmented colonies dominated, only one appeared pigmented.

Table 4.2: *Colony Morphology of Isolated strains*

SHAPE	MARGIN	ELEVATION	SIZE	TEXTURE	PIGMENTATION
Strain-1					
Circular	Entire	Convex	Punctiform	Smooth	Pigmented
Strain-2					
Circular	Entire	Convex	Moderate	Slimy	Non-pigmented
Strain-3					
Circular	Entire	Flat	Punctiform	Smooth	Non-pigmented
Strain-4					
Circular	Entire	Convex	Small	Creamy	Non-pigmented

4.1.2.2 Cell Morphology

Colonies of the strains turned out to be Gram negative and one Gram positive when observed under microscope. One of the strain was cocci and remaining were bacilli in shape. One appeared to exist in pairs and small groups while other two appeared in the form of long chains. Table 4.3 shows the cell morphology for all the four strains.

Table 4.3: *Cell Morphology of Isolated strains*

BACTERIAL ID	SHAPE
Strain-1	Cocci
Strain-2	Bacilli
Strain-3	Bacilli
Strain-4	Bacilli

4.1.2.3 Catalase, Oxidase test and MacConkey Test

75% of the strains were catalase and oxidase positive and were lactose fermenter as reported in Table 4.4.

Table 4.4: *Catalase, Oxidase and Lactose production of Isolated strains*

BACTERIAL ID	CATALASE	OXIDASE	MACCONKEY
Strain-1	+ive	-ive	-ive
Strain-2	-ive	-ive	Lactose fermenter
Strain-3	-+ive	-+ive	Lactose fermenter
Strain-4	+ive	-ive	Lactose fermenter

4.1.2.4 Molecular Identification

Maximum cypermethrin degrading potential was exhibited by isolate Strain-4. Based on the biochemical characterization and 16SrRNA gene analysis the isolate was identified as *Bacillus safensis*. The phylogenetic tree is presented in Figure 4.1. According to various studies on cypermethrin, bacteria from genera *Pseudomonas* and *Bacillus*, are recognized as metabolically active microorganisms, capable of metabolizing variety of pesticides (Grant et al., 2002; Chen et al., 2011b).

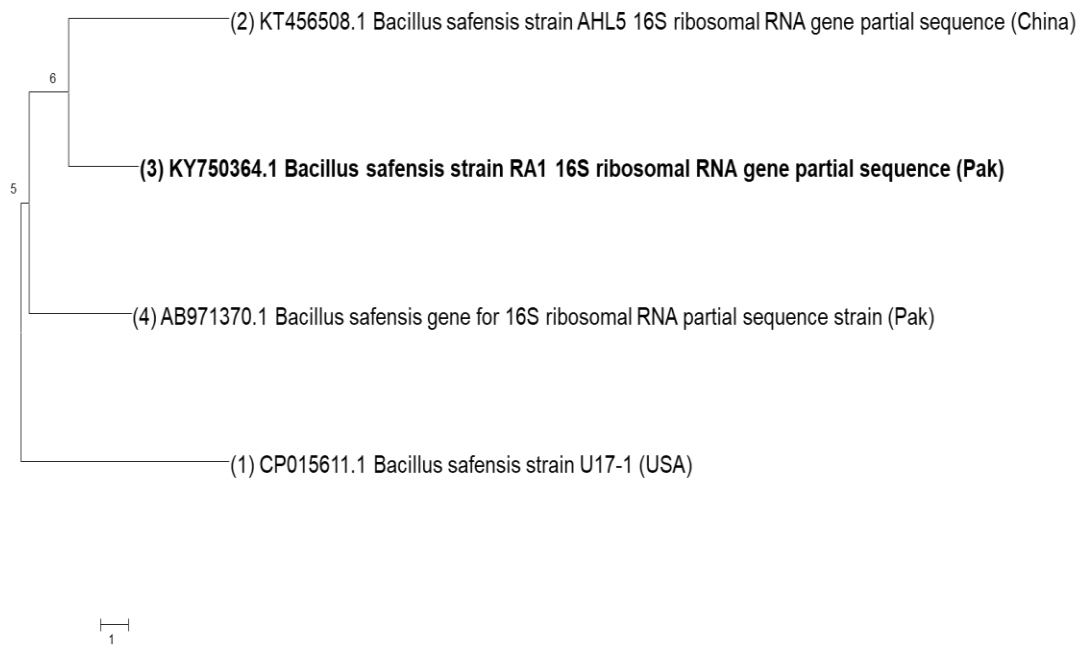


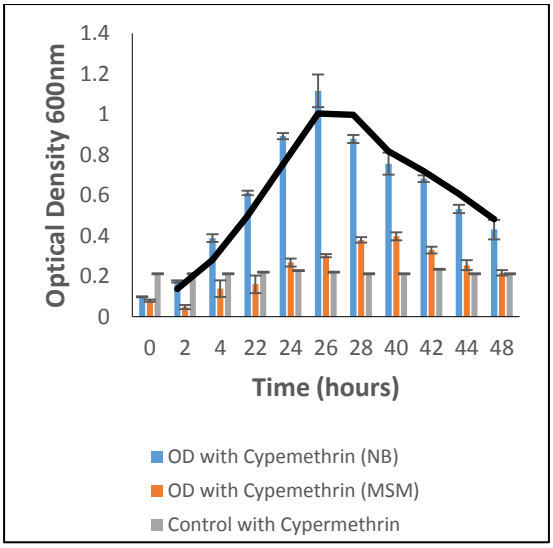
Figure 4.1: *Phylogenetic Tree Demonstrating Relatedness and Linkage to Different Bacterial Strains*

4.2 PHASE-II

4.2.1 Degradation Studies

4.2.1.1 Screening of Best Isolate

Effect of initial cypermethrin concentration of 50 mgL^{-1} on Strain-1, 2, 3 & 4 were studied in both NB and MSM as presented from Figure 4.2(a) to 4.2(d). The bacterial growth in Figure 4.2(c) and 4.2(d) suggests that Strain-3 and Strain-4 has the potency to grow on cypermethrin as the sole carbon source. Chen and coworkers also used enrichment culture technique by supplementing media with a final concentration of cypermethrin as 50 mgL^{-1} (Chen et al., 2012a).



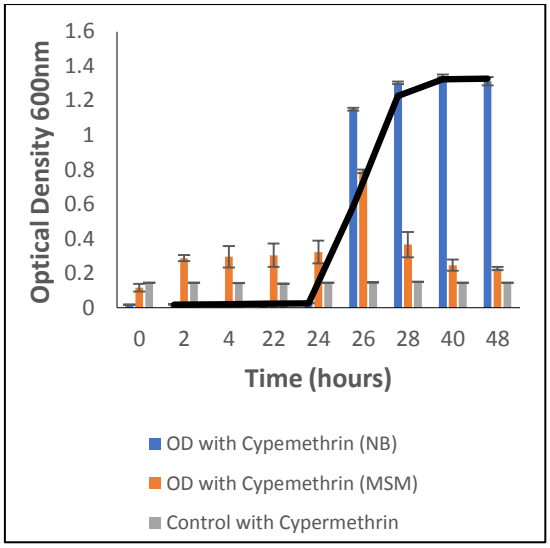
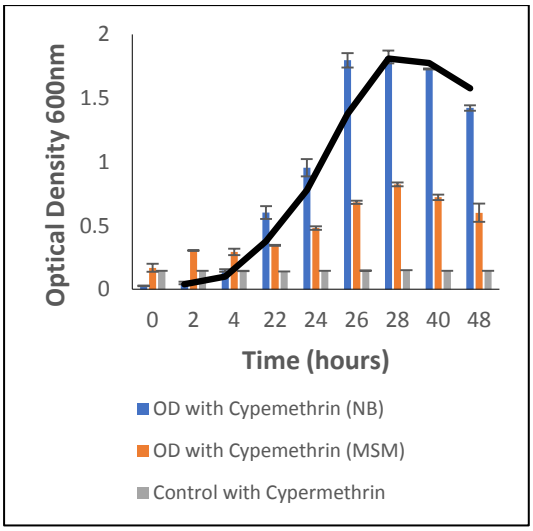


Figure 4.2 (a) *Growth Trend of Colony Strain-1*, **Figure 4.2** (b) *Growth Trend of Colony Strain-2*



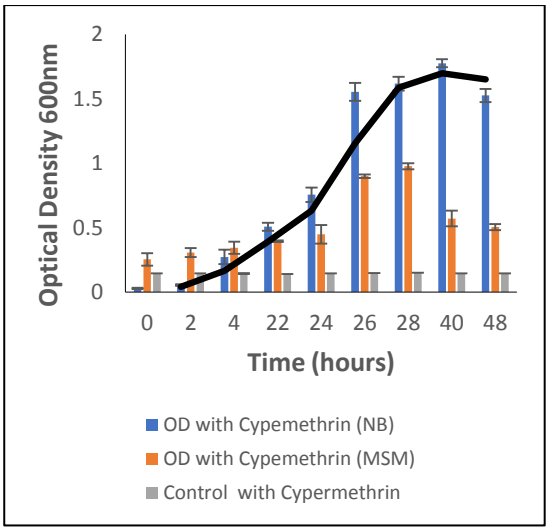


Figure 4.2 (c) *Growth Trend of Colony Strain-3*, **Figure 4.2** (d) *Growth Trend of Colony Strain-4*

4.2.2 Screening of Optimum Concentration

The effect of pesticide concentration on cypermethrin biodegradation was observed by adding different concentration of cypermethrin in NB and MSM which varied from 50-500 mgL^{-1}. Cypermethrin biodegradation is affected by a number of environmental factors. One of the significant factors is the initial concentration of cypermethrin that affects biodegradation in environmental samples. As presented in Figure 4.3(a) and 4.3(b), at higher initial concentrations 300, 400 and 500 *Bacillus*

safensis growth showed considerable decline. Therefore, optimum dose of cypermethrin was determined to be 100 mgL^{-1}.

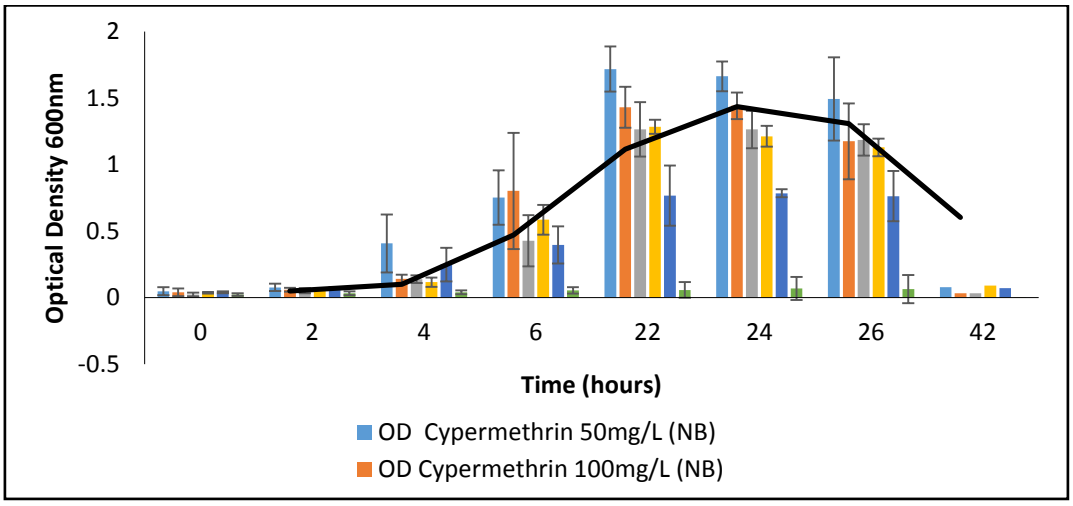


Figure 4.3: (a) *Effect of Initial Concentration of Cypermethrin (NB)*

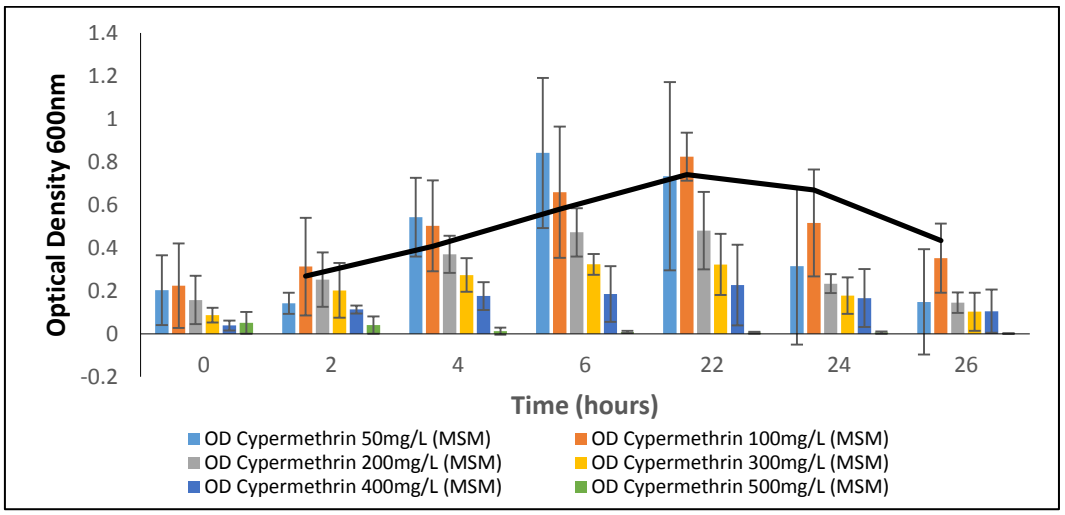


Figure 4.3: (b) *Effect of Initial Concentration of Cypermethrin (MSM)*

Chen and his fellow colleagues reported in 2012 that cypermethrin degradation showed considerable decline at higher initial concentration (Chen et al., 2012a). This decline is due to the inhibitory effect of pesticide at higher concentration on the growth of the organism or on its enzymes. Moreover, with the increasing cypermethrin concentration biomass slightly decreased. This might be due to the fact that microbial growth requires an acclimation period before an accelerated biodegradation happens at higher concentrations.

4.2.3 Semi-Continuous Bioreactor Studies

The Laboratory scale setup of a semi-continuous bioreactor system is attached as Annexure I.

4.2.3.1 Standard Calibration Curve

From standard of cypermethrin working solution was prepared in Acetonitrile (organic solvent). Various dilutions were prepared from the stock in acetonitrile (5, 1, 0.5, 0.1, 0.05 ppm). 1 µl of each dilution was made to inject into the injection port and signals were observed. The interpreted calibration curve along with the line equation is presented in Figure 4.4.

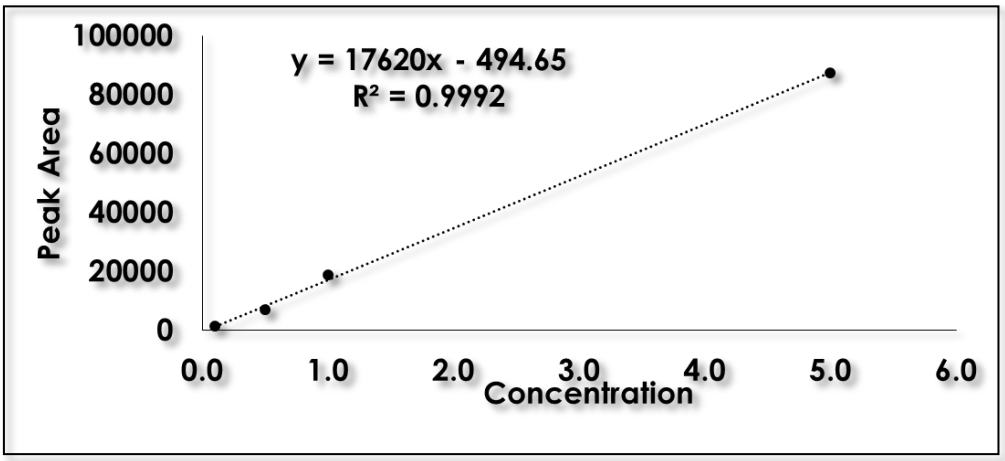


Figure 4.4: *Standard Calibration Curve and Line Equation of Cypermethrin*

4.2.3.2 Retention Time

The average retention time was calculated by injecting working dilutions at least three times. Two different solvents were used; acetonitrile for making working solutions from stock and ethyl acetate as extraction solvent. The retention time for the solvent Ethyl-acetate, acetonitrile and cypermethrin is presented below (Table 4.5). The residual concentration of cypermethrin in sample was calculated using chromatograms (Annexure II).

Table 4.5: *Properties and Retention Time of Analytes*

ANALYTE	MOLAR MASS(g/Mol)	BOILING POINT (°C)	RETENTION TIME (min)	(R^2)
CYPERMETHRIN	416.3	220.0	12.8	0.99
ETHYL ACETATE	88.11	77.1	2.6	0.99

4.2.3.3 Utilization of Cypermethrin by *Bacillus safensis*

Later in batch scale studies a comparison was made to investigate the utilization potential of cyermethrin by *Bacillus safensis*. In NB 73% of cypermethrin was degraded within 2 days, while, in MSM 61.5% cypermethrin was degraded in 2 days. However, after a short period, apparent lag phase may be observed in both medias Figure 4.5 (a) & (b) (Jillani, 2013). Therefore to maximize potential of this strain experiments were conducted in bioreactor with minimal media.

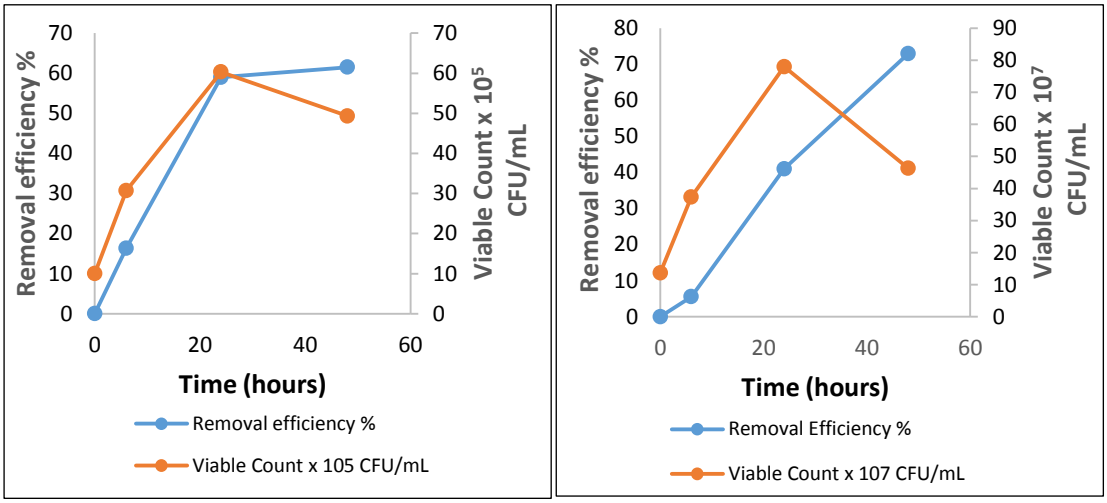


Figure 4.5 (a) *Removal Efficiency of Bacillus safensis (NB)* **(b)** *Removal Efficiency of Bacillus safensis (MSM)*

An aerobic environment was provided to *Bacillus safensis* in bioreactor system. At various intervals quantitative analysis of the trace concentration of cypermethrin was done. The sample was regularly harvested and after extraction was injected into the injection port. The *Bacillus safensis* utilized cypermethrin as sole carbon and energy source in MSM. After 24 hours the growth was most optimum, as shown in Figure 4.6(a)-4.6(c).

As presented in Figure, in all three cycles with the growth of *Bacillus safensis* the removal efficiency is increasing. From first cycle a gradual increase in cypermethrin removal efficiency was observed up to 3rd cycle i.e. 92%. Probably, because bacterial cells tolerance to cypermethrin with each cycle has amplified. In previous studies conducted on chlorpyrifos degradation successfully used *Pseudomonas kilonensis* in batch reactor for 6 cycles. (Khalid & Hashmi, 2016) Similarly for atrazine metabolism, Swissa and coworkers used *Raoultella planticola* for 10 cycles (Swissa et al., 2014).

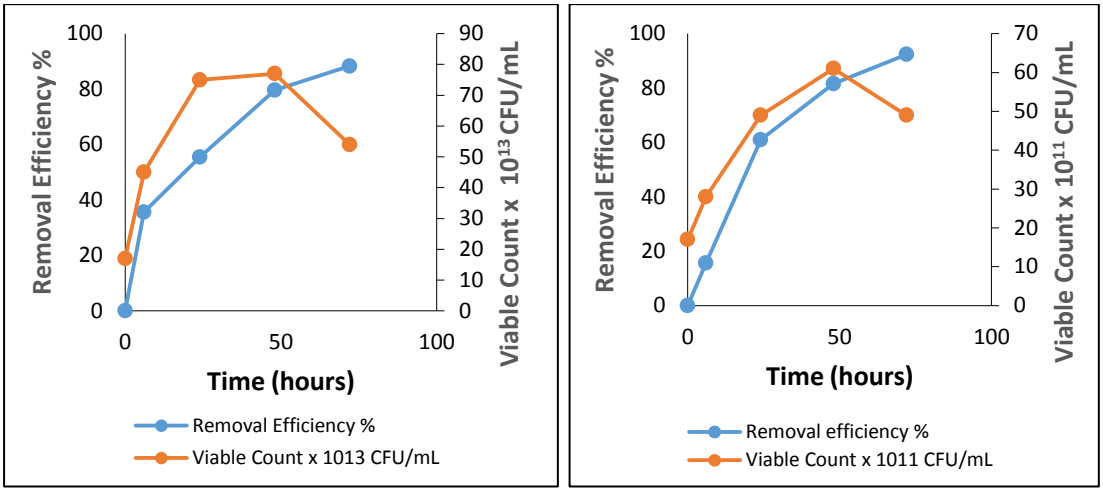


Figure 4.6: (a) *Removal Efficiency of Bacillus safensis during Cycle 1.* (b) *Removal Efficiency of Bacillus safensis during Cycle 2.*

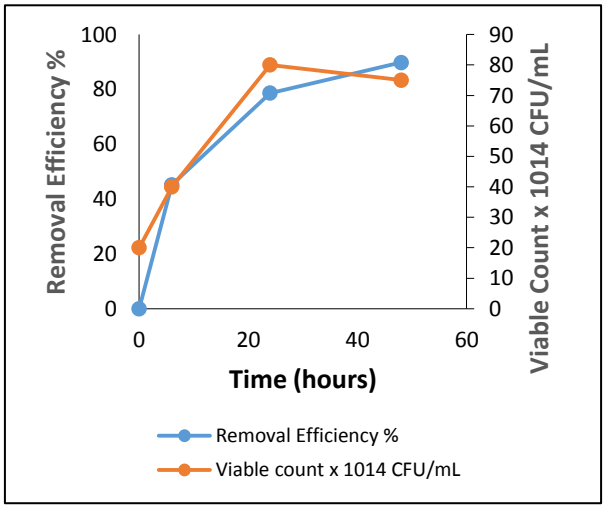


Figure 4.6: (c) *Removal Efficiency of Bacillus safensis during Cycle 3.*

Moreover, in the present study, the rate of removal efficiency of cypermethrin by *Bacillus safensis* increased with each cycle i.e. 2-3 days as shown in Figure 5.3. Zhang and fellow workers reported that Serratia sp. degraded 92% beta-cypermethrin

within 10 days (Zhang et al., 2010). Similarly, Chen and coworkers, reported 90% of beta-cypermethrin degradation by Ochrobactrum lupini within 5 days (Chen et al., 2011a). Likewise, in 2016, Tiwary and Dubey isolated *Bacillus sp*. and transformed 86% of cypermethrin in 7 days. In contrast to this present study *Bacillus safensis* was able to degrade 92% of cypermethrin in 2 days.

4.2.3.4 Concentration vs. Growth

After each cycle slight increase in tendency of *Bacillus safensis* to metabolize cypermethrin was observed. The concentration reduced to 11.4 and 8.3 mgL^{-1} in 72 hours respectively during first two cycles. While in third cycle within 48 hour the concentration reduced to 12.2 mgL^{-1} (Figure 4.7). Under the optimal conditions, *Bacillus safensis* metabolized cypermethrin rapidly reaching up to 88 - 92% of initial concentration of 100 mgL^{-1} during a period of 3 cycles.

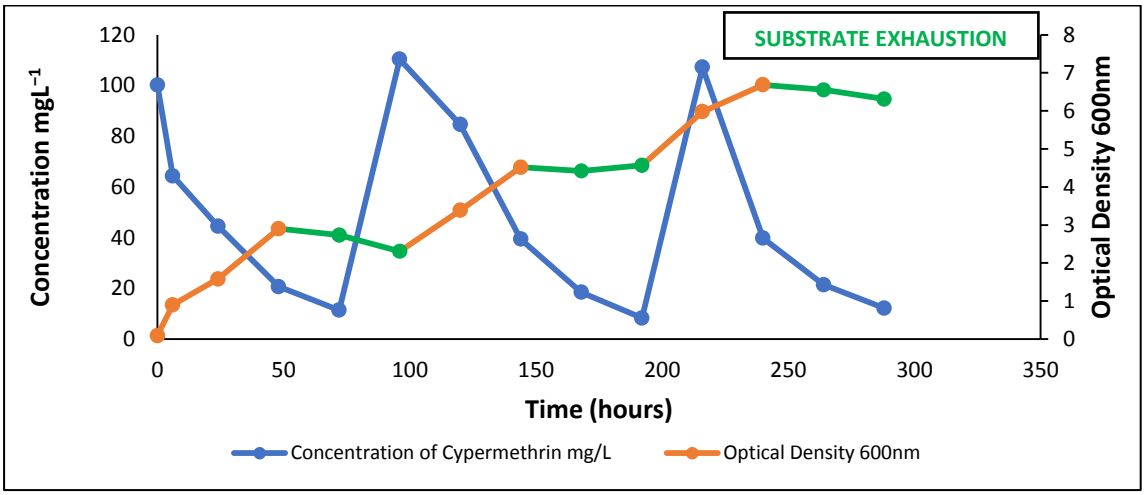


Figure 4.7: *Relationship between Bacillus safensis growth and Cypermethrin concentration*

The phenomenon of biodegradation is associated with the growth of bacteria. It was reported by group of researchers in 2009 that a prominent lag phase is observed by addition of pesticide as the sole carbon sources followed by enhanced biodegradation (Anwar et al., 2009). In present study, however, *Bacillus safensis* adapted quickly to the environment without any apparent lag phase (Figure 4.7). There are some stationary phases detected during the study, they represent substrate exhaustion that cypermethrin becomes completely used up.

The results indicated that *Bacillus safensis* possess a potential to be used in bioremediation of cypermethrin contaminated environment. This is very beneficial for

field scale application as sustainable population was maintained by *Bacillus safensis* over a long period of time.

4.3 PHASE-III

The data derived from lab scale experiments and computed data (Annexure II) was used to estimate biokinetic parameters of Monod and Andrew model.

4.3.1.1 Estimation of Kinetic Parameters

Experimental results obtained from batch studies have been subjected to justification by various mathematical models. The relationship between specific growth rate (μ) and initial cypermethrin concentration is shown in Figure 4.9. The parameters μ_{max}, K_s and K_i of Monod and Andrew were computed using linear and non-linear regression analysis of experimental data (Figure 4.8). The values of biokinetic parameters obtained from this study are given in Table 4.7. The lower values of K_s, indicate that this strain cannot grow well at higher cypermethrin concentration. These results are supported by the work of Chen and Zhang who modeled the growth rate kinetic using the Andrew model for the degradation studies for cypermethrin (Zhang et al., 2010; Chen et al., 2011b)

Table 4.7 *Estimated values of Parameters for Various Kinetic Models for the Fitting of Experimental Data Obtained during Cypermethrin degradation using Bacillus safensis*

REFERENCES	BIOKINETIC MODEL	μ_{max} (h^{-1})	K_s (mgL^{-1})	K_i (mgL^{-1})
Present Study	Monod	0.23	36.15	-
	Andrew	0.17	28.34	210.06
Chen et al., 2011b	Andrew	q_{max} 1.14/d	50.06	142.80
Zhan et al., 2011	Andrew	q_{max} 0.11/h	31.23	220.57

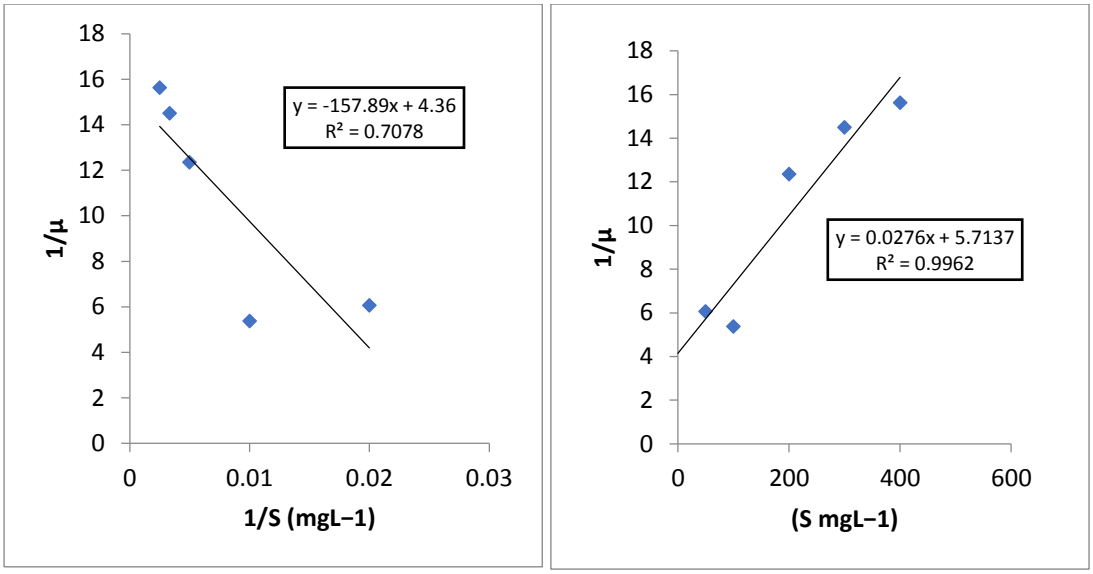


Figure 4.8: *Determination of Kinetic Constants* (a) K_s *and* μ_{max} *Monod Model* (b) K_i *and* $\mu_{max.}$ *Andrew Model*

The value of R^2 was 0.9962 indicating that the experimental data was well associated with Andrew's equation. Treatment of experimental data with various model equations confirm that Monod's model has limitations when used with inhibitory compounds. As depicted in Figure 4.9, at lower initial concentration of cypermethrin specific growth rate gradually increased. At higher concentrations above 100 mgL^{-1}, specific growth significantly decreased making the effect of inhibition evident.

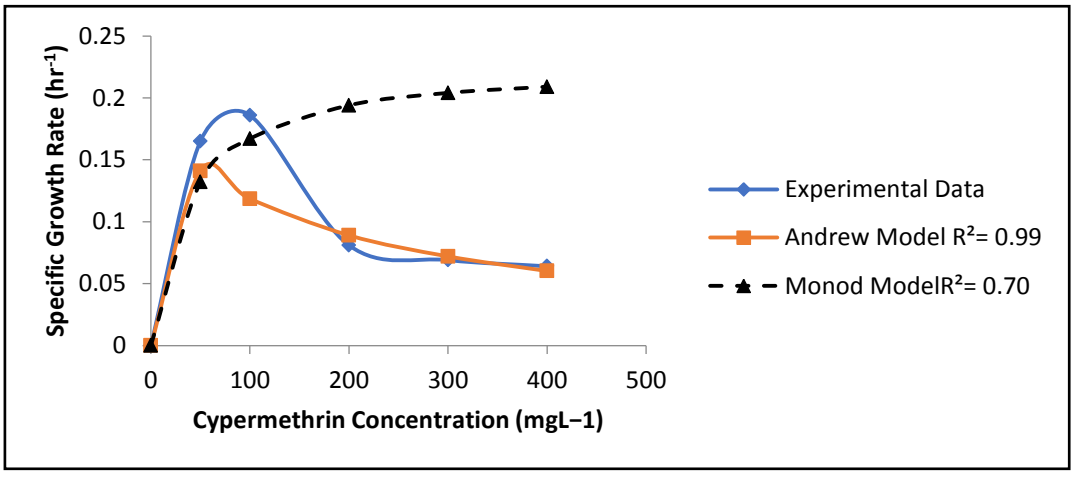


Figure: 4.9 *Experimental Data of Specific Growth Rate Versus Substrate Concentration and Model Fit using Andrew model (inhibitory model) and Monod model (non-inhibitory model) for Bacillus safensis*

A group of researchers in 2011 used Andrews equation to determine degradation kinetics for cypermethrin degradation at different initial concentrations. The kinetic

parameters for degradation q_{max}, K_s and K_i were estimated to be 1.14 /day, 52.06 and 142.80 mgL^{-1}, respectively. Similarly, in another study for cypermethrin degradation q_{max}, K_s, and K_i of 0.1051/h, 31.23 and 220.57 mgL^{-1}, respectively. These research findings confirm the inhibitory nature of cypermethrin at higher concentrations (Chen et al., 2011; Chen et al., 2012b).

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The outcomes drawn from the present study are listed below:

1. On the basis of 16SrRNA sequencing, the bacteria isolated from contaminated soil for cypermethrin biodegradation was identified as *Bacillus safensis.*
2. The optimized dose for the biodegradation of cypermethrin was determined to be 100 mgL^{-1}.
3. However, the growth of *Bacillus safensis* was inhibited at higher concentrations above 150 mgL^{-1}, making inhibitory effect of pesticide obvious.
4. In semi-continuous bioreactor system, gas chromatographic analysis showed removal efficiency to be 88.26, 92.34 and 89.8% during three consecutive cycles.
5. Due to the inhibitory effect of cypermethrin at higher concentration, Andrew model of kinetics was applied. The parameters of Andrew model of kinetics were, μ_{max}: 0.175 h^{-1}, K_s: 28.34 mgL^{-1} and K_i 210.06 mgL^{-1}.
6. The high R^2 (0.9905) approaching to 1 proposes that this substrate-inhibition model is adequately fitted with the experimental data.
7. With the addition of cypermethrin at start of each cycle, *Bacillus safensis* adapted quickly to the environment without any apparent lag phase and degraded cypermethrin rapidly at the start of incubation.
8. Thus, *Bacillus safensis* makes itself a promising bacterium for the biodegradation of cypermethrin as it is able to maintain population over a long period of time.

5.2 Recommendations

1. Evaluating biodegradation potential through application of *Bacillus Safensis* on the soil sample.
2. Bioreactor system optimization by using the given biokinetic coefficients.
3. Pilot scale studies using consortium and mixture of pesticides
4. Enzymatic studies on pesticide degradation should be undertaken.
5. Quantitative studies on toxicity of pesticide and its metabolites.
6. Interaction of pesticide and its metabolites with environment should be studied in depth.

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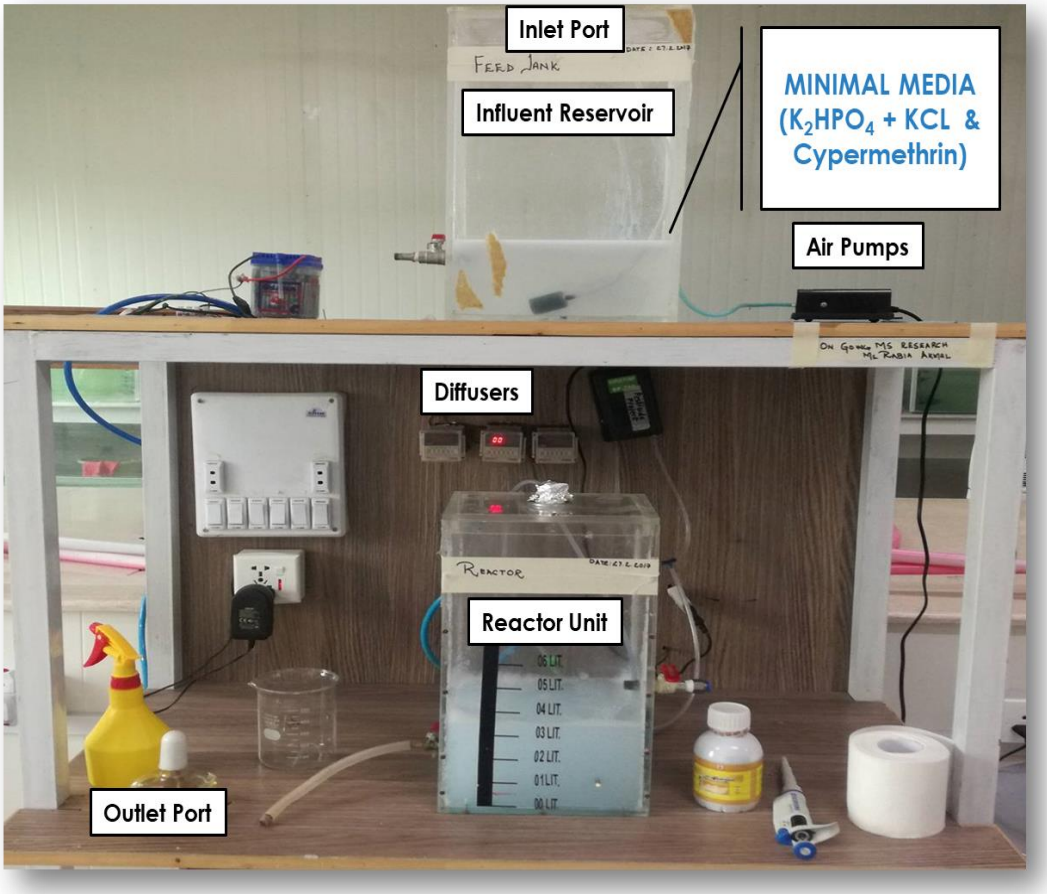
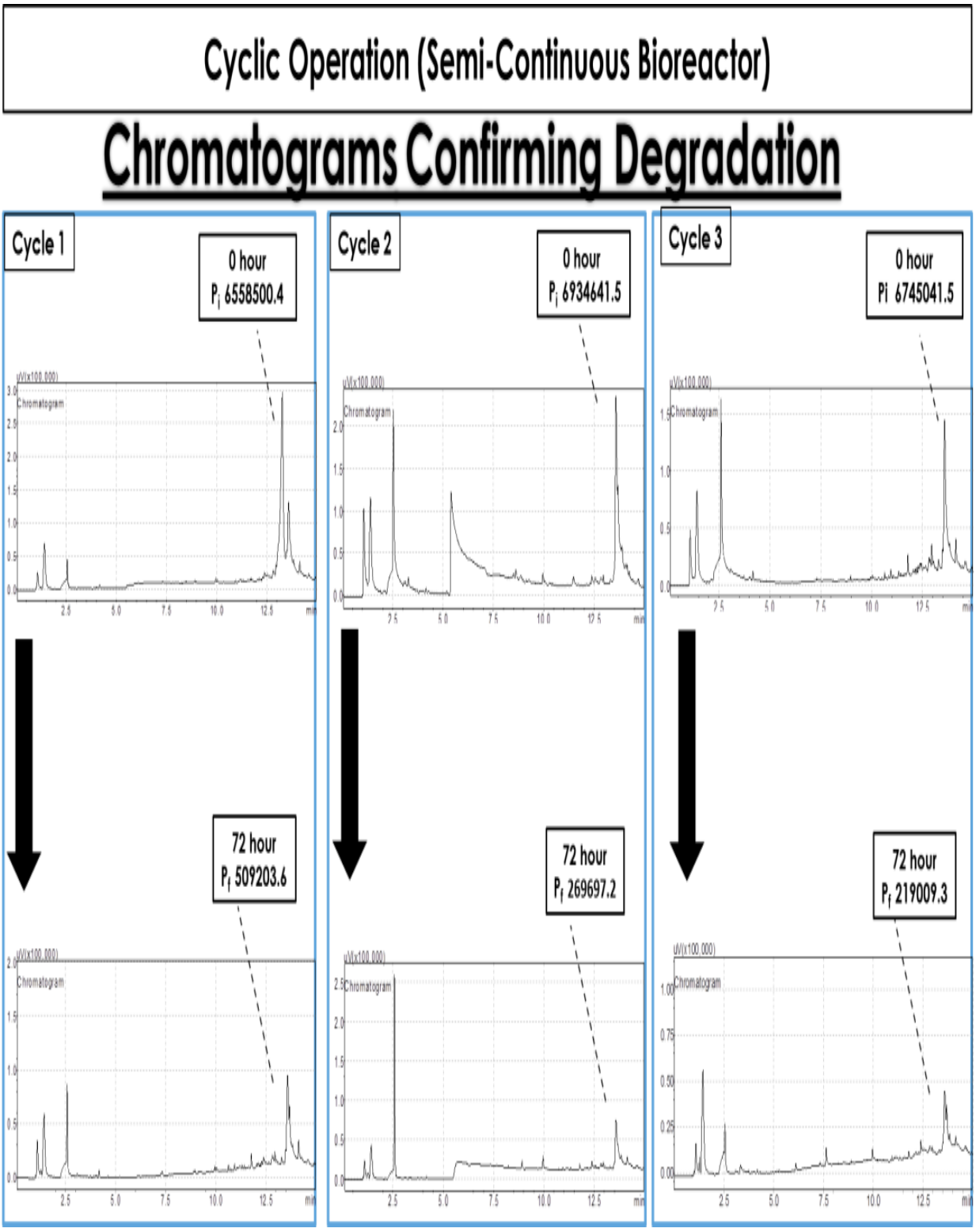
Annexure I

Semi-Continuous Bioreactor System



Annexure II


Cyclic Operation (Semi-Continuous Bioreactor)
Chromatograms Confirming Degradation
Cycle 1
0 hour
P_i 6558500.4
72 hour
P_f 509203.6
Cycle 2
0 hour
P_i 6934641.5
72 hour
P_f 269697.2
Cycle 3
0 hour
Pi 6745041.5
72 hour
P_f 219009.3

Annexure III

X_1	X_2	X_2-X_1	Experimental Data	1/S	1/μ	Andrew Model	Monod Model
0.124	0.7419	0.59	0.165	0.02	6.060606	0.141	0.132
0.257	0.726	0.451	0.186	0.01	5.376344	0.11849	0.167
0.22	0.423	0.203	0.081	0.005	12.34568	0.089	0.194
0.178	0.284	0.106	0.069	0.003333	14.49275	0.072	0.204
0.099	0.199	0.1	0.064	0.0025	15.625	0.0602	0.209