THE BIODEGRADATION PATHWAY OF CYPERMETHRIN

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BIODEGRADATION PATHWAY OF CYPERMETHRIN

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ABSTRACT

THE BIODEGRADATION PATHWAY OF CYPERMETHRIN

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May 2022, 130 pages

Cypermethrin (CYP) is a commonly used type Ⅱ pyrethroid for the protection of both agriculture and household, causing serious secondary environmental pollution. Environmentally friendly and effective techniques such as biodegradation can minimize or remove the contaminants and their potentially harmful metabolites from the environment. In this study, two bacterial strains isolated from agricultural soil obtained from fig farms in Adana were identified as *Enterobacter hormaechei* ZK101 and *Stenotrophomonas maltophilia* ZK102. ZK101 and ZK102 were found efficient in degrading CYP under different conditions converting CYP to 3-PBA. The maximum CYP degradation was observed at 33.9 °C, pH 6.7 with an initial CYP concentration of 5.69 mg. L^{-1} for ZK101 and at 38.1°C, pH 6.7 with an initial substrate concentration of 6.6 mg. L^{-1} CYP for ZK102. Under these conditions, 67.6% of cypermethrin for ZK101 and 81.9% of cypermethrin for ZK102 were degraded in a minimal salt medium within 5 days. While CYP was degraded accumulation of 3PBA was observed. Since the toxicity of 3-PBA is much higher than CYP, the accumulation of 3-PBA by CYP degraders would be an environmental problem. Therefore, degradation kinetics of CYP (4 mg L^{-1}) in soils inoculated with isolates ZK101 and ZK102 was established. Time-dependent removal of cypermethrin with rate constants of 0.468, 0.257 $d^{-1}$for ZK101 and ZK102 following

first-order rate kinetics was observed in soil, and time-dependent removal of 3PBA with rate constants of 0.406 and 0.278 d^{-1}, respectively. Since both strains converted the CYP to 3-PBA the first enzyme was analyzed using crude cell extracts. Enzyme assays with crude cell extracts showed an activity of equivalent of esterase. This study is one of the first studies that identified the CYP biodegradation pathway and estimated rate constants of biodegradation and 3-PBA accumulation of the isolates obtained from fig farms using the commercially available CYP. Estimating those constants helps predicting the possible negative effect that microbial activates could cause and will provide an insight of the pesticides biodegradability that could be used while potential pesticides are being applied.

Keywords: Pyrethroids, Cypermethrin, Biodegradation, *E. hormaechei*, *S. maltophilia*

ÖZ

SİPERMETRİN BİYOBOZUNMA MEKANİZMASI

Kokangül, Merve
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Sipermetrin tarımda ve evlerde koruma amaçlı yaygın kullanılan piretroid tip 2 olup ikincil çevre kirliliğine yol açmaktadır. Çevre dostu ve etkili teknik olan biyobozunma kontaminantları ve kontaminantların metabolitlerinin aza indirilmesini ya da çevreden uzaklaştırılması sağlamaktadır. Bu çalışmada Adana'da bulunan incir bahçesinden alınan toprak örneğinde iki bakteri suşu elde edildi ve bu suşlar *Enterobacter hormaechei* ZK101 and *Stenotrophomonas maltophilia* ZK102 olarak adlandırıldı. ZK101 ve ZK102 farklı koşullar altında CYP'i 3PBA'ya etkili şekilde dejenere ettiği görüldü. ZK101 suşu için maksimum CYP biyobozunması 33.9°C ve 6.7 pH aralığında olup CYP konsantrasyonu 5.69 mg. L^{-1}, ZK102 suşu için maksimum CYP biyobozunması 38.1°C ve 6.7 pH aralığında olup CYP konsantrasyonu 6.6 mg. L^{-1} dır. Bu koşullarda 5.69 mg. L^{-1} sipermetrinin %67.6'si ZK101 tarafından ve 6.6 mg. L^{-1} sipermetrinin %81.9'u ZK102 tarafından MSM'de beş günde biyobozunmaktadır. CYP bozunurken 3PBA akümüle olduğu gözlemlendi. 3PBA toksisitesinin CYP den fazla olması çevresel bir sorun oluşturabileceğini göstermektedir.

Ayrıca 4 mg L^{-1} CYP zamana bağlı bozunma hız sabitleri ZK101 için 0.468 ve ZK102 için 0.257 $gün^{-1}$ olup birinci dereceden hız sabiti olarak bulunmuştur. 3PBA için sırasıyla ZK101 ve ZK102 için 0.406 and 0.278 $gün^{-1}$ olarak bulunmuştur.

Iki bakteri suşu CYP'yi 3PBA'ya dönüştürdüğü için enzim analizi yapıldı. Ham hücre ekstrat enzim deneyleri ile biyobozunmanın esteraz eşdeğeri bir enzim gerçeklestiği tespit edildi. Bu çalışmada, ticari formda CYP kullanılan incir bahçesinden elde edilen izolatların CYP biyobozunma mekanizması ve tahmini biyolojik bozunma hız sabitleri, ve 3-PBA akümülasyonunu belirleyen ilk çalışmalardan biridir. Reaksiyon hız sabiti ve yarılanma ömürlerinin bilinmesiyle olası mikrobiyal negatif etkilerin tahmin edilebilmesine yardımcı olmaktadır.

Anahtar Kelimeler: Piretroid, Sipermetrin, Biyobozunma, *E. hormaechei*, *S. maltophilia*

To my precious family

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ABBREVIATIONS

CYP	Cypermethrin
3PBA	3-Phenoxy benzoic acid
3PBH	3-Phenoxy benzaldehyde
DCVA	3-(2 2-dichlorovinyl)-2 2-dimethylcyclopropanecarboxylic acid
HPLC	High Liquid Gas Chromatograph
PBS	Phosphate Buffer Solution
MSM	Mineral Salt Medium
OUR	Oxygen Uptake Rate
LB	Luria Bertani
LA	Luria Agar
CV-BR	Constant Volume Batch Reactor

CHAPTER 1

INTRODUCTION

All over the world, pyrethroids are commonly used to protect agricultural fields, and the excessive usage of pyrethroids causes secondary pollution in the environment. Also, it can harm aquatic animals so it is necessary to develop removal methods to degrade and eliminate contaminants and intermediates in the environment.

In the Europe, the greatest amount for only agricultural protection, approximately 42 tons, was used in the UK in 2006. France is following the UK, and 38 tons used, followed by Cyprus with 5 tons, Sweden with 2.3 tons, Estonia with 0.44 tons(Houldin, 2011)

Bioremediation, especially biodegradation, is generally considered to be a safe, environmentally friendly, and effective technique for pyrethroids elimination (Zhang et al., 2010). To date, several microorganisms show the capability of degradation to cypermethrin and their intermediate products such as Micrococcus sp. CPN1(Tallur et al., 2008), Flavobacterium haoranii sp LQY-7 (Zhang et al., 2010) Rhodopseudomonas palustris GJ-22(Zhang et al., n.d.), Serratia spp. JC1 (Zhang et al., 2010), *Azoarcus indigens* strain HZ5(Ma et al., 2013), and Serratia spp.JCN13 (Zhang et al., 2010).

To our knowledge, this is the first report about cypermethrin degrading bacteria from *Enterobacter* and *Stenotrophomonas* genus. To date, most research has focused on the cypermethrin biodegradation pathways by microorganisms (Tang et al., 2017; Tang et al., 2018). However, there are a few reports about metabolite accumulation and responsible enzymes.

1.1 Aims and Objectives

This study aims to understand the cypermethrin biodegradation mechanisms by newly isolated two strains from a heavily CYP-applied area located in Adana. The specific objectives are to:

1. Isolate pure bacterial cultures having cypermethrin degradation capacity from enrichment medium

2. Study most common intermediated, 3PBA, pattern

3. Understand how the pure bacterial colonies act on both cypermethrin and 3PBA

4. Use experimental data in Minitab to optimize concentration, temperature, and pH values for degradation

CHAPTER 2

LITERATURE REVIEW

2.1 The Overview of Pesticide Usage

Pesticides can be defined as chemicals, biological substances, or mixtures that are used to protect agriculture and homes, such as insecticides (to kill insects), fungicides (to protect from fungal infection), and herbicides (to kill unwanted plants) from unwanted organisms (Waxman, 1998). Some others, such as nematicides, acaricides, or algicides, are used against nematodes, mites, and algal growth in the water, respectively. The pesticides classify as their targets, actions, and chemistry. The most important pesticides and their characteristics were summarized in Table 2.1. These pesticides can be naturally produced or synthetically produced.

Table 2.1 Characteristics of some pesticides and their examples (Ortiz-Hernandez et al., 2013)

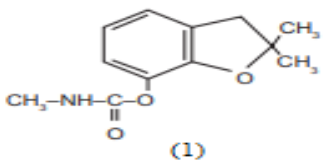
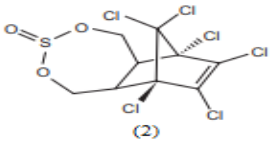
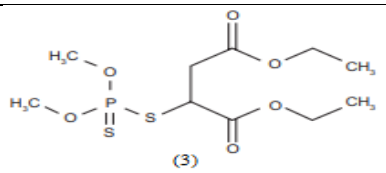
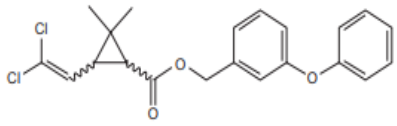
Pesticide	Characteristics	Examples
Carbamate	Kill some insects, very toxic for vertebrates, low persistence	 (1)
Organochloride	Soluble in lipids and accumulate in fatty tissue, toxic effect on animals, nonpolar and lipophilic	 (2)

Table 2.1 (cont'd)

Organophosphate	Soluble in organic solvents and water, less stable than organochlorine, stored in leaves and stems	 (3)
Pyrethroids	Less stable than others, usable in the house as household insecticides	 (4)

(1); Carbofuran, (2); Endosulfan, (3); Malathion, (4); Permethrin

The most used insecticides were organochloride (OC) and organophosphates (OPs) in the middle of the 19th century because they provided good crop protection over a long period. However, these insecticides are highly toxic to the environment because they can accumulate in the animals that belong to the end of the food chain. Even OC and OPs can affect the ion channels in the nervous system (Matthews, 2006). For that reason, some countries restricted the usage of some organochlorides and organophosphates, such as DDT and endosulfan.

Food and Agriculture Organization of the United Nations (FAO) showed that approximately 2 million tons (Mt) of pesticides were utilized worldwide in 1990, raising to 4 Mt in 2019. Worldwide consumption of pesticides between 1990 and 2019, 52.8% were in Asia, 30 % were in the USA, and 13% were in Europe (FAO, 2020a). The average use of pesticides per area of cropland increased from 1990 to 2019, as well. In 1990, Asia, America, and Europe used pesticides 2.15, 1.63, and 1.34 kg/ha, respectively. However, these amounts raised to 3.68 kg/ha for Asia, 3.7 kg/ha for America, and 1.66 kg/ha for Europe in 2019 (FAO, 2020b). According to the FAO, in Turkey, pesticide usage was 50k tons, and 14k tons of it were insecticides in 2019 (FAO, 2020a).

Pesticides are widely used to increase agricultural production. There are some other purposes to use pesticides for many reasons. For example, infectious diseases like Lyme disease can be spread by being bitten by disease-carrying ticks. According to

the EPA, some pesticides can repel this kind of disease carrier, protecting living organisms. Thus, some pyrethroids have been registered for use on pets and medical and veterinary products to control lice and ticks (EPA, 2008). Moreover, pesticides can be used to control indoor household pests, which can cause asthma and allergies. Thanks to having a broad range of functions, pesticides benefit farmers and good public health (EPA, 2008).

The pesticides have active ingredients that have pesticide activity. Some pesticides may have more than one active component, and active components are usually a mixture of inert ingredients rather than pure form. This provides easy and safe transportation and application. The mixture of active and inactive components generates pesticide formulation (Waxman, 1998). Pesticides have different active chemicals, and their characteristics are different, so different formulations are needed to optimize the activity of pesticides on the products. Several different kinds of pesticide formulations are available on the market, and these are Emulsifiable concentration (EC), granules, solution concentrates, wettable powders, and suspension concentrates. The most appropriate formulation is determined by the analysis of safety, environmental concerns, available equipment for the application, and cost (Hazra et al., 2017). The most used formulation is EC. The active components of EC formulations are insoluble in water, but they are soluble in an organic solvent (Waxman, 1998), and it is formulated by dissolving the active ingredient with emulsifying surfactant. EC formulation is easy to use, and it looks like a milky emulsion in water. However, formulation causes phytotoxicity, and dermal toxicity (Hazra et al., 2017).

2.2 Pyrethrins and Pyrethroids

The pyrethrin insecticides have been used widely since pyrethrins and pyrethroids have high insecticidal potency with low mammalian toxicity and a relatively low tendency to persist in the environment (Soderlund et al., 2002). Also, after Silent Spring was published in 1962 by Rachel Carson, big modifications on pesticide

usage were applied, so some pesticides and DDT were banned. For that reason, pyrethroids have become a commonly used and less harmful pesticide class.

The pyrethroids are synthetic derivatives of pyrethrins extracted from flowers of *Chrysanthemum cinerariaefolium* (Ortiz-Hernandez et al., 2013; Spurlock & Lee, 2008). All pyrethroids are composed of an acid moiety, a central ester bond, and an alcohol moiety. The chemical configuration and structure of pyrethroids were shown in Figure 2.1.

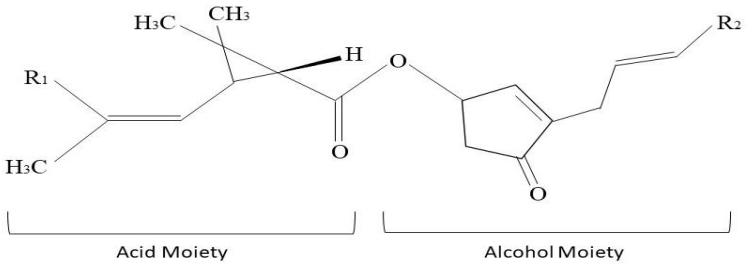


Figure 2.1 The general structure of pesticides and their functional groups (Schleier Ni & Peterson, 2011)

Pyrethrins are six insecticidally active esters, Pyrethrin I and II, Cinerin I and II, and Jasmolin I and II. They are the esters of two carboxylic acids: chrysanthemic acid (2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropane-1-carboxylic acid) and pyrethric acid. The functional groups (R_1 and R_2) of six pyrethrins and their properties were tabulated in Table 2.2. (Schleier Ni & Peterson, 2011). Most pyrethroids do not exist as single molecules and they have the same chemical formula, but their atoms have different arrangements. These kinds of molecules are called stereoisomers. The stereoisomer compounds can be diastereomer, which is not a mirror image of each other and has different physical properties, or an enantiomer, which is a non-superimposable mirror image of one another that has the same

physical properties. Both stereoisomer types have different toxicity. There are two chiral carbon atoms; the mirror-image of molecules is non-superimposable on the acid side, so pyrethroids are stereoisomeric (having trans and cis isomers). Moreover, some pyrethroids can have chiral carbon atoms on both the acid and alcohol sides, and this provides having eight different stereo enantiomers. (Costa, 2015). The stereoisomeric compound causes toxicity, and cis isomers (the functional groups lying on the same side of the carbon chain) are more toxic than trans isomers of the same pyrethroids. (Casida et al., 1983). Pyrethroids are classified into two groups according to their structure, mode of action, and symptoms. During the early 1970s, first-generation pyrethroids or type I (e.g., allethrin, imiprothrin, phenothrin, and tetramethrin) were developed without cyano groups. The second-generation pyrethroids or type II (e.g., bifenthrin, cyfluthrin, cypermethrin, deltamethrin, and permethrin) were designed with α-cyano groups on the phenoxybenzyl group by the late 1970s. Type II pyrethroids are more photostable and toxic (Weston et al., 2009; Gan et al., 2008; Kaneko, 2011).

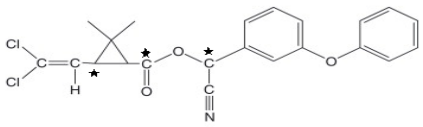
2.3 Cypermethrin

A pyrethroid insecticide, cypermethrin, is most commonly used after restricting or banning organophosphate and organochlorides (Weston et al., 2009). Cypermethrin is a carboxylic ester formed by the formal condensation between 3-(2,2-dichlorovinyl)-2,2 dimethyl cyclopropane carboxylic acid and alcoholic hydroxy group of hydroxy(3-phenoxy phenyl) acetonitrile. The UIPAC name of cypermethrin is cyano-(3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1- carboxylate, and ISO common name of cypermethrin is (RS)-a-cyano-3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate). Cypermethrin contains many functional groups like ester, halogen double, cyanide, or benzyl carbon (Huang et al., 2018).

Cypermethrin has three chiral centers, and this gives rise to eight stereoisomers (EFSA, 2011). Some of them have ISO common names: alpha-cypermethrin, beta-

cypermethrin, theta-cypermethrin, and zeta-cypermethrin. According to FAO specifications, the purity of cypermethrin should be at least 450 g/kg with 40-60% of cypermethrin's cis-isomer content (FAO, 1995). The isomers of cypermethrin are classified into four cis- and four trans-isomers and the four cis groups are more powerful insecticides than trans-isomers. Some physicochemical properties of cypermethrin were shown in Table 2.2.

Table 2.2 Some properties of cypermethrin

	Cypermethrin
Isomerism	Four cis, and four trans.
IUPAC name	(RS)-cyano(3-phenoxyphenyl) methyl (1RS)-cis-trans-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylate
CAS name	[Cyano-(3-phenoxyphenyl) methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate
CAS Number	52315-07-8
Classification	Type II class of pyrethroids
Molecular formula;	$C_{22}H_{19}Cl_2NO_3$
Molecular weight;	416.297 g. mol^{-1}
Chemical structure	
Physical state	Pale yellow liquid

"★" shows asymmetric carbon atoms

Cypermethrin uses for both protection of agricultural products and non-agricultural activity. Although using it provides many advantages, the metabolites of cypermethrin stay in the environment and agricultural products. Thus, they can affect the food chain and accumulate in living organisms (Zhang et al. 2010).

Cypermethrin can be formulated as an Emulsifiable concentrate (EC), a soluble concentrate/liquid (SC/L), and a wettable powder (WP), but in agricultural usage, EC formulation is widely used form. For example, in Turkey, a 25EC (250g. L^{-1}) formulation of cypermethrin is commonly used.

Cypermethrin is more permanent in soil (sixfold) by comparison within the aquatic environments. The half-life of cypermethrin in the environment depends on several parameters. Its half-life of it changes from 2 days to 165 days (Wexler, 2014). Also, biodegradation efficiency is affected by many parameters. A study was done by Gu et al. (2008), and Cycon and Piotrowska-Seget (2016) showed biodegradation rate was affected by the catabolic activity of microorganisms and soil properties like organic content. Ismail et al. (2012) studied the effect of temperature and moisture content of soil on biodegradation, and the results showed that the half-life of cypermethrin decreased when both parameters were decreased. Furthermore, the accumulation of cypermethrin in the body varies due to having different isomeric forms. For example, the rat's half-life of cypermethrin has been measured as a 3.4 for trans and 18 days for cis isomers (Wexler, 2014).

2.3.1 Degradation of Cypermethrin

Generally, pyrethroids are degraded by sunlight and soil within one to two days of exposure, but some are more durable in the environment. (Luo and Zhang, 2011)

Pyrethroids have many degradation pathways when they enter the soil or aquatic environment. They can be degraded chemically, biologically, or through adsorption. Physical degradation can happen through UV light action. Chemical degradation can be in the form of hydrolysis, oxidation, or reduction. Co-metabolism of

microorganisms, bacteria, or fungi provides biodegradation of pyrethroids (Cycon & Piotrowska-Seget, 2016).

The biodegradation of pesticides can be investigated in three sections: adsorption, cell membrane activity, and enzymatic reactions. In the first step, the target was adsorbed by the surface of the cell membrane. After adsorption, the target got inside the cell, and finally, enzymatic reactions began (Huang et al., 2018). The biodegradation of pyrethroid starts with the cleavage of the ester bonds, and it can bring about some by-products. These can be 3-phenoxybenzyl alcohol, 3-phenoxy benzaldehyde (3PBH), or 3-phenoxybenzoic acid (3-PBA) (Tallur et al., 2008, Ma et al., 2013, Xiao et al., 2015). Microorganisms can degrade cypermethrin. The studies done before showed many bacterial strains could degrade cypermethrin, and some examples of these bacterial strains were shown in Table 2.3. Moreover, most of the given bacteria degrade cypermethrin under aerobic conditions. The optimum pH and temperature for the growth of cypermethrin degraders are approximately 7 and 30°C, respectively.

Table 2.3. Some bacterial strains for cypermethrin degradation

Strain	**Source**	**Optimal conditions**	**Reference**
Acinetobacter calcoaceticus MCm5	Soil	pH 7, 30°C	Akbar et al., 2015a
Azoarcus indigens HZ5	Activated sludge	pH 7, 30°C	Ma et al., 2013
Bacillus sp. DG-02	Wastewater treatment system	pH 7.5, 30°C	Chen et al., 2012a, 2014
Bacillus licheniformis B-1	The soil in the tea garden	pH 7-7.5, 30°C	Liu et al., 2014
Brevibacillus parabrevis JCm4	Contaminated soil	pH 7, 30°C	Akbar et al., 2015b

Table 2.3 (cont'd)

Micrococcus sp. CPN 1	Contaminated soil	pH 7, 30°C	Tallur et al., 2008
Pseudomonas aeruginosa JCm8	Contaminated soil	pH 7, 30°C	Akbar et al., 2015a
Streptomyces sp. HU-S-01	Wastewater sludge	pH 7.5, 26–28◦C	Lin et al., 2011

2.3.2 Biodegradation Studies for Cypermethrin and Its Pathways

The biodegradation of cypermethrin generally starts with the activities of enzymes. The carboxylesterase or esterase is the main enzyme family responsible for the biodegradation of pyrethroids. Monooxygenase and pyrethroid hydrolase (pyrethroid catalyzing esterase) can also break down the ester bond. For example, *Streptomyces* sp can produce monooxygenase enzyme (Chen et al., 2013), and pyrethroid hydrolase enzyme can be produced by *Pseudomonas aeruginosa* GF31strains (Tang et al., 2017). Enzyme identification is made with SDS-PAGE analysis by determining molecular weight. To date, a few studies purified cypermethrin degrading enzymes. For example, esterase (66.4 kDa) from *Bacillus licheniformis* B1, aminopeptidase (53 kDa) from *Pseudomonas aeruginosa* GF31, and monooxygenase (41 kDa) from *Streptomyces* sp were purified (Chen et al., 2013; A. X. Tang et al., 2017; M. Zhang et al., 2021).

A degradation pathway of cypermethrin by *Bacillus parabrevis* BCP-09 (Tang et al., 2018) and *Pseudomonas aeruginosa* strain GF31 was found in the study (Tang et al., 2017). The bottom part of Figure 2.2 described the pathway of *Bacillus parabrevis* strain BCP-09. It converted cypermethrin to DCVA and cyano-3-phenoxybenzyl alcohol by the esterase enzyme activity. Cyano-3-phenoxybenzyl alcohol is unstable, so it was simultaneously converted to 3-PBH, and then 3-PBA was formed through the dehydrogenase enzyme activity. Phenol and benzoic acid were produced by 3-

phenoxybenzoic acid degrading enzymes via cleavage of the diphenyl ether bond. Phenol was converted to the catechol by phenol hydroxylase, and benzoic acid was converted to 3-carboxy muconic acid (Tang et al., 2018).

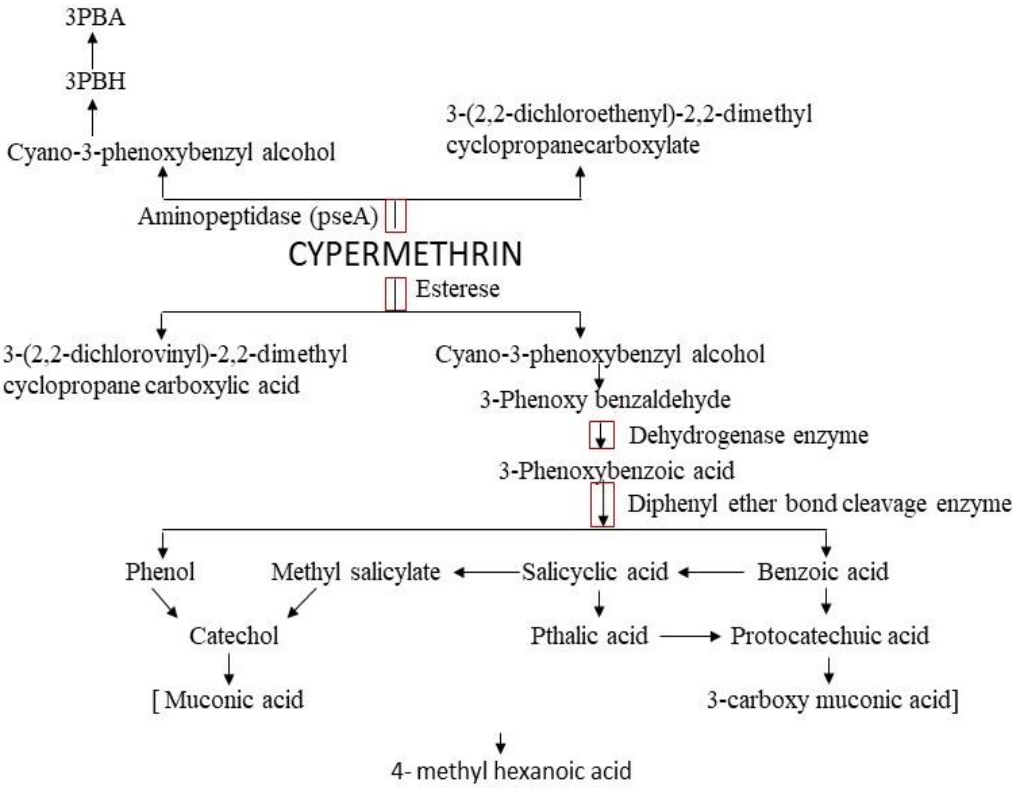


Figure 2.2 Mechanisms of cypermethrin degradation pathways (Tang et al., 2017; Tang et al., 2018) The red square shows the purified enzyme responsible for the pathway

However, it did not cover the properties of enzymes like k_m and k_{cat} constants and working mechanisms. The study said the first step was the activity of the esterase enzyme, but it did not specify the gene name. Moreover, the specific activity of

enzymes was not studied. That is important to know to analyze 3PBA accumulation in the environment. The upper part of Figure 2.2 depicted the degradation pathway of *Pseudomonas aeruginosa* GF31. The degrading enzyme in the first step was different from a monooxygenase, a peroxidase, or carboxylesterase in this study. The key enzyme which is aminopeptidase is secreted extracellularly. *P. aeurignosa* strain GF31 transformed cypermethrin to DCVA and 3PBA (Tang et al., 2017). The results of the study showed DCVA and 3PBA concentration increased first three days, and then concentration decreased. Also, the study concluded that there is no 3PBA accumulation. The above findings propose cypermethrin degrade enzyme from GF31 strain should be investigated further.

Only a few studies showed the mechanism of the exact degradation pathway and enzymes in detail, but there are several proposed pathway studies in the literature. Some examples of proposed biodegradation pathways were shown in Figure 2.3. The biodegradation metabolites of these pathways were given in Table 2.4.

For detailed physical and chemical properties of some common metabolites and parent, compounds refer to Appendix A- EPI Suite Results.

Table 2.4 Proposed pathways' intermediate compound names

Letter	Compound Name	Letter	Compound Name
A	3-(2,2-Dichloroethenyl)-2,2-dimethyl Cyclopropane carboxylate	L	Protocatechuic acid
B	α-Hydroxy-3-phenoxy-benzene acetonitrile	M	Phenol
C	3-Pheoxybezaldehyde (3PBH)	N	Phthalic acid
D	3-Pheoxybenzoic acid (3PBA)	O	2-phenoxyphenol
E	4-Hydroxybenzoate	P	Catechol
F	4-propyl benzaldehyde	R	3-carboxy-cis, cis muconoic acid
G	o-Phenoxy benzoic acid	S	Cis, cis muconic acid
H	2-hydroxy-2(3-phenoxyphenyl) acetonitrile	T	Benzoic acid 2,5-dimethyl
I	3-(2,2-Dichlorovinyl)-2,2-dimethyl-cyclopropane carboxylic acid (DCVA)	V	Benzoic acid
J	Muconic acid	Z	3,5-dimethoxy phenol
K	Cyano-3-phenoxybezylalcohol		

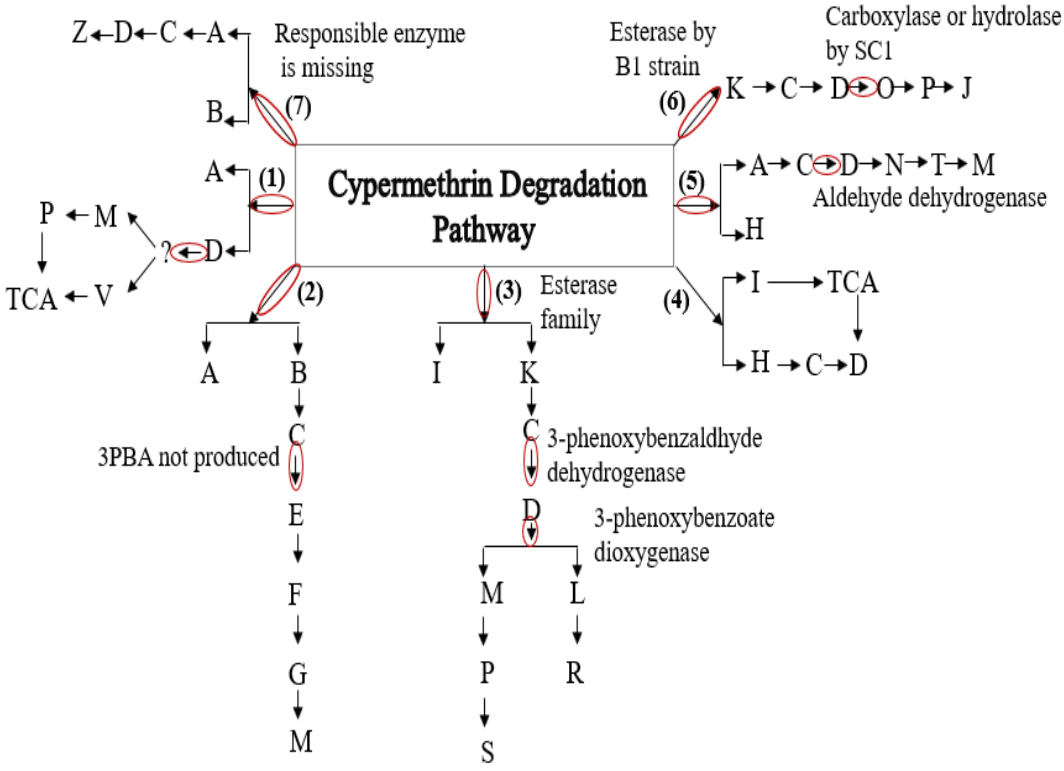


Figure 2.3 Some proposed pathways for biodegradation of cypermethrin (Pankaj et al., 2016; Tallur et al., 2008; Ma et al., 2013; Xiao et al., 2015) The first red square shows the initial enzyme in the pathways is missing

A recent study worked on genes encoding the enzymes responsible for cypermethrin and its degradation metabolites (Zhao et al., 2022). It showed that *Bacillus cereus* strain GW-01 could degrade cypermethrin and its metabolite 3PBA by α/β-hydrolase and cytochrome aa3 quinol oxidase, respectively. Cypermethrin was firstly converted to the 3-(2,2-Dichloroethenyl)-2,2-dimethyl cyclopropane carboxylate and 3PBA by α/β-hydrolase (gene id, chr_3118). Then cytochrome aa3 quinol oxidase (chr_677, 678, 679,680) enzyme transformed 3PBA into phenol and benzoic acid and further degraded to citric acid cycle intermediates. The proposed degradation mechanism was shown in the 1st pathway. Moreover, the study showed

enzyme activity of strain GW-01 was inducible rather than constitutive, unlike other enzymes (Zhao et al., 2022).

Bacillus sp. strain SG2 degradation mechanism was depicted in the 2nd pathway. However, the first enzymes responsible for degradation were not studied in the study (Pankaj et al., 2016). Unlike other findings, in the pathway, 3PBH was converted to 4-hydroxy benzoate rather than 3PBA.

In the 3rd pathway, *Micrococcus sp*. CPN1 strain degradation mechanism was depicted. It converted cypermethrin to DCVA and cyano-3-phenoxybezylalcohol by the activity of the esterase enzyme. Also, its metabolites were further degraded by the activity of 3-phenoxybenzaldehyde dehydrogenase, 3-phenoxybenzoate dioxygenase, phenol hydroxylase, protocatechuate-3,4-dioxygenase, catechol-1,2-dioxygenase, and catechol-2,3-dioxygenase. The study calculated the specific activity of these enzymes and concluded that the CPN1 strain was a good candidate for cypermethrin degradation because 3PBA did not accumulate in the environment (Tallur et al., 2008).

Azoarcus indigens strain HZ5 degradation route proposed in the 4th pathway. Cypermethrin was hydrolyzed to 3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylic acid (DCVA) and 2-hydroxy-2-(3-phenoxyphenyl) acetonitrile. Then, the 2-hydroxy-2-(3-phenoxyphenyl) acetonitrile was converted to 3PBH and 3PBA and further degraded to citric acid cycle intermediates (Ma et al., 2013).

Bacillus thuringiensis strain SG4 degradation mechanism depicted in the 5th proposed pathway. It firstly converted cypermethrin to 2-hydroxy-2-(3-phenoxyphenyl) acetonitrile and 3-(2,2-dichloroethenyl)-2,2-dimethyl cyclopropane carboxylate (does not accumulate in the human body) by the cleavage of the ester bond. Then like in the other studies, 3PBH and 3PBA were formed, and in the end, phenol was formed (Bhatt et al., 2020)

In the 6th pathway, the degradation was studied co-metabolism of *Bacillus licheniformis* B-1 and *Sphingomonas* sp. SC-1. Cypermethrin was firstly converted to cyano-3-phenoxybezylalcohol by the esterase enzyme produced by strain B-1. Cyano-3-phenoxy benzyl alcohol is unstable so it simultaneously transformed to

3PBH and then 3PBA. The B-1 strain could not degrade 3PBA, so it accumulated in the environment. However, SC-1 strain could degrade 3PBA to 2-phenoxyphenol efficiently by decarboxylase or hydrolase enzymes. The study did not specify exactly the enzyme name that is it only mentioned the enzyme family. (Liu et al., 2014)

In the 7th pathway, *Bacillus subtilis* BSF01 strain activity was shown. Cypermethrin was transformed into 3-(2,2-dichloroethenyl)-2,2-dimethyl cyclopropane carboxylate and α-hydroxy-3-phenoxy benzene acetonitrile. Compound α-hydroxy-3-phenoxy benzene acetonitrile was unstable, so it transformed to 3PBH and 3PBA, respectively. Finally, 3PBA was converted to the 3,5-Dimethylphenol (Xiao et al., 2015). The study mentioned neither the first enzyme responsible for the cleavage of the ester bond nor the activities of enzymes in the pathway.

After analyzing the result of different studies related to the degradation pathway, conclude that the esterase enzyme family is responsible to initiate cypermethrin degradation. By the activity of esterase enzyme, most formed first metabolites are 3-(2,2-Dichlorovinyl)-2,2-dimethyl-cyclopropane carboxylic acid (DCVA), 3-(2,2-Dichloroethenyl)-2,2-dimethyl cyclopropane carboxylate, cyano-3-phenoxybezylalcohol (unstable chemical), 2-hydroxy-2(3-phenoxyphenyl) acetonitrile (unstable chemical) and α-Hydroxy-3-phenoxy-benzene acetonitrile (unstable chemical). Generally, the second metabolites are 3PBH and 3PBA. However, gene encoding enzymes for cypermethrin and specific name, characteristics like a specific activity, and working principles of these enzymes remain understudied, so further enzyme studies must be carried out. It is very important to know the enzyme properties to see whether accumulation happens or not. Also, further research on a pathway should be investigated.

2.3.2.1 Cypermethrin Detoxifying Enzymes and Its Working Principle

Many pyrethroids contain ester bonds to connect the alcohol with acid moiety, and esterase enzymes have a role in detoxifying pyrethroids through the cleavage of ester bonds. There is no universal classification of esterase. For example, esterase

enzymes are categorized under the hydrolase family in IUBMB. Also, they are classified as types A, B, and C according to their interaction with OPs by Aldridge (Bhatt, et al., 2020). For enzyme purification, four steps are followed, crude enzyme preparation, ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration (Chen et al., 2013; Tang et al., 2017; Liang et al., 2005).

To date, some enzymes for the detoxification of pyrethroids have been isolated and characterized. The studies isolated carboxylesterase from *Methylobacterium* sp. A-1 (Diegelmann et al., 2015), pyrethroid hydrolase from *Aspergillus. niger* ZD11 (Liang et al., 2005), pyrethroid hydrolase from *Klebsiella* sp. ZD112 (Wu et al., 2006), PytH from *Sphingobium* sp. JZ-1(Guo et al., 2009), aminopeptidase from *Pseudomonas aeruginosa* GF31 (Tang et al., 2017), esterase from *Micrococcus* sp. strain CPN1(Tallur et al., 2008), pyrethroid-hydrolyzing carboxylesterase from *Ochrobactrum anthropic* YZ-1 (Zhai et al., 2012), esterase and monooxygenase from *Streptomyces* sp. (Chen et al., 2013). The proteins were identified by using the NCBI Blast program. Some properties of hydrolyzing enzymes were summarized in Table 2.5.

Table 2.5. Some properties of cypermethrin hydrolyzing enzymes

Accession number	Gene	Protein	Organism	Reference
C0LA90	pytH	Pyrethroid hydrolase	*Sphingobium wenxiniae*	Wang et al., 2009
FJ688006	pytH_1	Carboxylesterase	*Sphingobium wenxiniae* JZ-1	Wang et al., 2009
GU119902	pytH_2	Carboxylesterase	*Sphingobium faniae* JZ-2	Guo et al., 2009
Q52NW7	estP	Pyrethroid hydrolase	*Klebsiella* sp. ZD112	Wu et al., 2006
KT735188	pseA	Cypermethrin hydrolyzing aminopeptidase	*Pseudomonas aeruginosa* GF31	Tang et al., 2017
KP742972	MsE1	Carboxylesterase	*Methylobacterium* sp. A-1	Diegelmann et al., 2015
AEY11370	pytZ	Pyrethroid-hydrolyzing carboxylesterase	*Ochrobactrum* sp. YZ-1	Zhai et al., 2012
JQ025988	pytY	Esterase family VI	*Ochrobactrum anthropi* YZ-1	Ruan et al.,2013
JQ045333	pytZ	Esterase family VI	*Ochrobactrum anthropi* YZ-1	Zhai et al., 2012
QBK84513	EstA	Esterase	*Bacillus cereus* BCC01	Hu et al., 2019

The esterase enzyme contains a catalytic triad nucleophilic residue, an acidic residue, and a histidine residue (Montella et al., 2012; Bhatt et al., 2020). The working mechanism of the esterase enzyme (serine, histidine, and glutamate) consists of two reactions: formation and degradation of acyl-enzyme. The first step of the reaction starts via oxygen nucleophile generated by the catalytic serine, and it attacks the hydroxyl group on the carbonyl carbon of the ester bond. This attack generates a tetrahedral intermediate. To keep the reaction constant and keep pyrethroids

cypermethrin in its place, two glycine residues were added to the reaction. In the end, the process releases the alcohol metabolite (R'OH), and the enzyme is acylated because cypermethrin is a kind of carboxylic acid. In the second step of the reaction, water is added to make it acid-free and return the enzyme to its original form. Final products become alcohol (R'OH), acid (R''COOH), and enzymes with resting form (Montella et al., 2012).

Many pyrethroids are redox-active, so they can enter microorganism and causes disruption of aerobic mechanism and electron transport chain. That inactivates enzymes by the formation of reactive oxygen species such as OH or O^{2-}. Fortunately, microorganisms can develop an antioxidative defense mechanism, e.g. SOD to eliminate ROS activities (Zhao et al., 2021). Furthermore, the concentration of cypermethrin can inhibit the activity of degradation enzyme activity.

2.3.3 Microbial Aerobic Oxidation Reaction of Cypermethrin

For growth and nongrowth conditions, microorganisms get their required energy from substrate oxidation, which is reflected by the cell mass-produced, i.e., the cell yield. Most bacteria degrade cypermethrin under aerobic conditions, and aerobic degradation is faster than anaerobic degradation. The mass balance equation of aerobic microbial oxidation was shown below.

$$a\ (substrate) + b(NH_3) + c(O_2) \rightarrow d(dry\ cell\ mass) + e(CO_2) + f(H_2O)$$

As a first step, the dry cell mass molecular formula needs to be defined. According to the study done by Maier et al. (2009), the empirical formula of dry bacterial biomass can be different. The most common elements in microorganisms are C, H, O, and N, and the average empirical formula of dry bacterial biomass was found from the data given in Table 2.6.

Table 2.6 Dry cell mass empirical formula for bacterial strain

Dry Bacterial Biomass (Mass fractions are expressed as per 100g biomass)		
Name	**Formula**	**Reference**
Bacteria General	$CH_{1.666}O_{0.27}N_{0.20}$	Abbott and Clamen, 1973
Aerobacter aerogenes	$CH_{1.830}O_{0.55}N_{0.25}$	Naresh et al., 2011
Bacillus cereus	$CH_{1.490}O_{0.43}N_{0.22}$	Duboc et al., 1999

Many bacterial strains tested for cypermethrin degradation belonged to the genera *Bacillus* or *Brevibacillus* (Akbar et al., 2015a, 2015b; Bhatt et al., 2016; Chen et al., 2012b; Liu et al., 2014). For that reason, the required nitrogen amount was calculated by converting the empirical formula to molecular formula, and in this case, cell mass was assumed as $C_5H_7O_2N$ (Maier et al., 2009). The microbial aerobic oxidation reaction for cypermethrin can be written as,

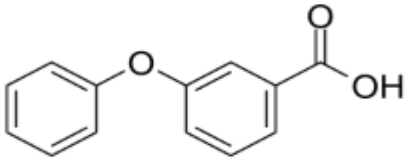
$$a\ (CYP) + b(NH_3) + c(O_2) \rightarrow d(C_5H_7NO_2) + e(CO_2) + f(H_2O)$$

The compound's coefficients (a, b, c, d, e, f) show the mole number. The mass balance reaction represents the production of a new cell mass called $C_5H_7NO_2$.

2.4 PBA (3-Phenoxy Benzoic Acid)

3-Phenoxybenzoic acid (3-PBA) is a common metabolite of many pyrethroid pesticides resulting from the degradation of parent pesticides, so it can not be used as a biomarker to detect the main pesticide (Aylward et al. 2018). The 3PBA has stronger reproductive toxicity, weaker hydrophobicity, and a longer half-life than the parent compounds so it can cause secondary pollution in the environment (White et al., 1996). Its half-life is between 120 and 180 days (Chen et al. 2011b). The physicochemical properties were shown in Table 2.7.

Table 2.7 Properties of 3-phenoxybenzoic acid

3-PBA	
IUPAC name	3-phenoxybenzoic acid
CAS name	3-phenoxybenzoic acid
CAS Number	9739-38-6
Other names	3-carboxydiphenylether; diphenylether-3-carboxylic acid; mPBAcid; 3PBA; 3PBAc; PBA
Classification	The metabolite of pyrethroids
Molecular formula;	$C_{13}H_{10}O_3$
Molecular weight;	214.22 $g.mol^{-1}$
Chemical structure	
Physical state	---
Solubility in water (20°C)	24,7 mg. L^{-1}

3PBA has weaker hydrophobicity characteristics than pyrethroids, and it joins and accumulates in the environment easily (Huang et al., 2018). 3PBA also has antibacterial activity, so it may not be degraded further to small and non-toxic intermediate like CO_2 or H_2O. That makes pyrethroids' intermediate riskier. Since the activity of enzymes in microorganisms breaks the ester bond and mostly produces 3PBA, for that reason, pyrethroid pollution can be eliminated if 3PBA is degraded efficiently. Some studies showed that *Klebsiella pneumoniae* BPBA052 (gi|1212262395 from NCBI Blast) (Tang et al.,2019), *Sphingomonas* sp. SC-1 (Tang et al., 2013), *Stenotrophomonas* sp. ZS-S-01 (Chen et al., 2011b) and *Bacillus sp.* DG-02 (Chen et al., 2012) (gi|375006100) can participate in 3PBA degradation.

The enzymatic reactions are the key element to degradation (Huang et al., 2018). After cleavage of the ester bond, 3PBA is mostly produced. The 3PBA has a diaryl ether compound that is diphenyl ether, increasing the physical, chemical, and

biological stability of 3PBA. The breaking of the diphenyl ether bond creates phenol and benzoic acid or protocatechuic acid (Tang et al., 2018; Tallur et al., 2008). The microbial degradation of 3PBA can start with the deoxygenation of diaryl ether through the Phenoxybenzoate dioxygenase enzyme produced by *Pseudomonas pseudoalcaligenes* strain POB310 (Dehmel et al., 1995). The enzyme accession number is Q52185, and the gene name is pobA from NCBI Blast. This enzyme degrades diaryl ether compound having carboxyl group 3 or 4 positions. 3PBA's carboxyl group locates in the 3-position, so Phenoxybenzoate dioxygenase can degrade 3PBA to hemiacetal, and then it is converted to phenol and protocatechuate spontaneously (Dehmel et al., 1995). The degradation enzymes must be evaluated to see the possibilities of accumulation of 3PBA. A study done before by Tallur et al. (2008) was purified esterase, 3- phenoxy benzaldehyde dehydrogenase, 3-phenoxybenzoate dioxygenase, phenol hydroxylase, protocatechuate-3,4-dioxygenase, and catechol-1,2- dioxygenase enzymes. And specific activity (U), the amount required to catalyze the formation of 1 μmol of product per minute, of these enzymes was investigated. The enzymatic activities of protocatechuate-3,4-dioxygenase and catechol-1,2-dioxygenase were higher which means 3PBA is not going to be accumulated due to the low activity of the esterase enzyme. In other words, the low specific activity of esterase produces less 3PBA, and 3PBA can be degraded further to small and less toxic metabolites (Tallur et al., 2008). According to the previous studies, Zhao et al. (2021) created some probable pathways for 3PBA degradation, as shown in Figure 2.4. Generally, monooxygenase or dioxygenase is the responsible enzyme for cleaving diphenyl ether bonds (White et al., 1996).

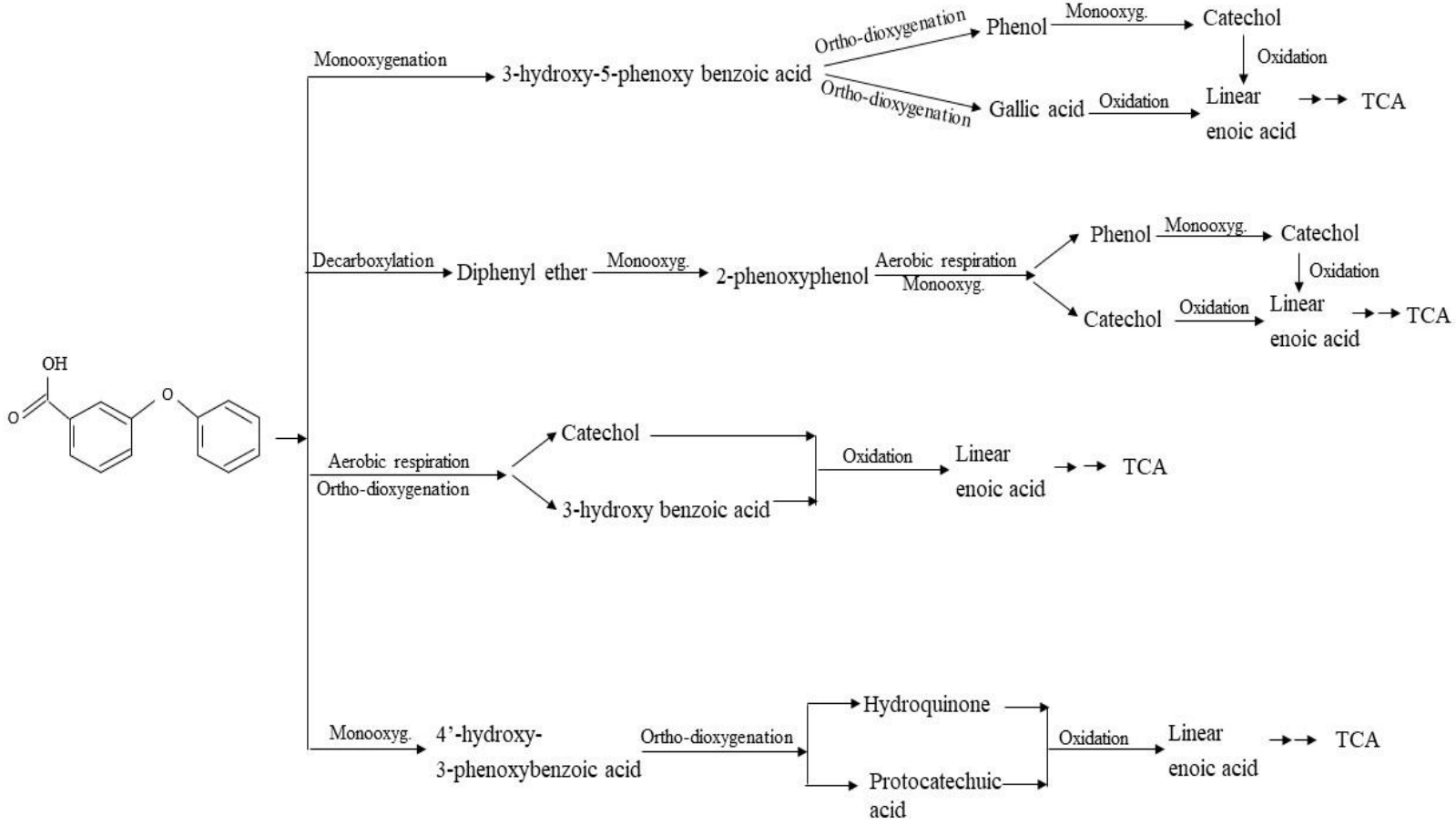


Figure 2.4 3PBA biodegradation pathways

Although 3PBA has adverse effects on the environment and living organisms' health, there are a few studies on biodegradation enzymes of 3PBA and its working mechanisms in the literature, so studies should be continued. Moreover, 3PBA degradation is higher than the parent compound, but a few studies are available in the literature.

2.5 Risk Assessment

Cypermethrin can pose a risk for aquatic organisms, non-target arthropods, and bees. The negative effect of cypermethrin application on non-target bees can vary due to different times of application. That is, the effect of cypermethrin on bees is lower when the cypermethrin applications are performed before the flowering season (Arena et al., 2018) (EFSA, 2018a). Pesticides can also cause genotoxicity, immunotoxicity, DNA damage, apoptosis, histopathological alterations, hepatotoxicity, and neurotoxicity. Genotoxicity causes the formation of nuclear abnormalities like extra DNA fragmentation. Immunotoxicity creates a low level of granulocytes and lymphocytes which are very important for the immune system by inhibiting B and T cell production. Furthermore, pesticides may enhance behavioral responses. Due to the toxic effect of pesticides dopamine active transporter activity is downregulated, and this gives rise to show irregular behaviors (Farag et al., 2021). Neurotoxicity caused by pesticides affects both PNS and CNS by changing the action potential of the sodium channel. Neurotoxicity of pyrethroids is higher against insects, unlike animals. This is because insects have more sodium channels and lower body temperature than animals. Also, they have negative effects on the ion-exchange process. Regular working sodium channels are also important for mammals since many activities such as brain activity and osmoregulation are provided by sodium channels. Exposure of cypermethrin block or decrease the antioxidant defense activities, superoxide dismutase (SOD), catalase (CAT), or glutathione-s-transferase (GST) against oxidative stress caused by enhancing reactive oxygen species like hydrogen peroxidase or hydroxyl radicals (Farag et al., 2021).

Fish are more prone to be affected by cypermethrin negatively because they are not able to produce hydrolyzing enzymes to detoxify its effects. A study done by Paravani et al (2019) showed that the DNA of gill cells is affected badly even exposure to a low concentration of cypermethrin because gill cells are directly exposed to the aqueous medium, and the lipophilic nature of cypermethrin causes absorption by gill cells. Saha and Kaviraj (2003) concluded when fish are exposed to cypermethrin, they frequently come to the surface of the water. This may happen due to having a respiratory problem. Moreover, cypermethrin can cross the blood-brain barrier and cell membrane without the need for active transport, etc. (Shi et al., 2011).

Table 2.8 The effect of cypermethrin and its metabolite on aquatic organisms (Arena et al., 2018)

Compound	Persistence in soil	Ecotoxicology
Cypermethrin	Moderate persistence Single first-order and biphasic kinetics	Pose a high risk for aquatic organisms
DCVA	Low to moderate persistence Single first-order	Low risk
3PBA	Very low persistence Single first-order and biphasic kinetics	Low risk

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The technical-grade cypermethrin, 3PBA, and chromatographic-grade acetonitrile were purchased from Sigma-Aldrich Chemical Co. The commercial-grade CYP was obtained from HEKTAŞ. Its brand is ARRIVO 25EC, and it contains 250 $g \cdot L^{-1}$ of cypermethrin. All other chemicals and solvents used were of analytical grade.

The enrichment medium contained K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$), and $Na_2EDTA.2H_2O$, $ZnSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, $NaMoO_4.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $MnSO_4.H_2O$, $MgSO_4.7H_2O$, agar (for solid media). Luria Broth was prepared with Tryptone, yeast extract, and NaCl to grow bacteria, and Luria Agar (Miller's LB) was prepared with bacteriological agar, Tryptone, yeast extract, and NaCl to isolate cypermethrin degrading bacteria. Ultrapure water, obtained from a Milli-Q water purification system, and distilled water were used. For enzyme studies, 20 mM potassium phosphate buffer (pH, 7) was prepared.

3.2 Methods

3.2.1 Sample Collection

The soil, which was used to enrich and isolate cypermethrin degrading bacteria, was obtained (0-2cm depth from the surface of the soil) from the agricultural fields of Adana, Akdeniz region (36° 56' 24" N, 35° 21' 36" E), and transferred into the plastic bags. Cypermethrin was generally used to protect the agricultural fields from the

Mediterranean fruit fly (*Ceratitis capitata*) in Adana. The satellite view of the area is shown in Figure 3.1.

Figure 3.1. Satellite view of cypermethrin applied garden

Soil samples were obtained from the fig tree area (*Ficus carica*) in the study. The pesticide application was made using a back pump sprayer. For the 30.000m^2 agricultural area, 1L Cypermethrin (ARRIVO 25 EC) was used, and it was mixed with 1000L tap water before application. One of the soil samples was obtained one and half weeks later by applying cypermethrin (1), and the other was taken one and half months later (2). The soil samples were dried and sieved before use to prevent unwanted stones and other things.

3.2.2 Experimental Design

3.2.2.1 Medium Preparation

Three different media were used for enrichment, isolation, and cultivation of cypermethrin degrading bacteria.

A liquid mineral salt medium (MSM) supplemented with cypermethrin was prepared for enrichment studies because it is a carbon-free medium and highly selective medium that specific bacteria can be detected. Liquid MSM contained bulk medium and metal solution. The bulk medium was prepared by dissolving 2.27 g, K_2HPO_4, 0.95 g KH_2PO_4, and 0.67 g $(NH_4)_2SO_4$ in 1L ultrapure water. The metal solution was prepared by dissolving 6.37g $Na_2EDTA.2H_2O$, 1.0g $ZnSO_4.7H_2O$, 0.5g $CaCl_2.2H_2O$, 2.5g $FeSO_4.7H_2O$, 0.1g $NaMoO_4.2H_2O$, 0.1g $CuSO_4.5H_2O$, 0.2g $CoCl_2.6H_2O$, 0.52 g $MnSO_4.H_2O$, 60 g $MgSO_4.7H_2O$ in 1L ultrapure water. After autoclaving the bulk medium, 2 mL sterile metal solution was added to the medium (Hartmans et al., 1992). The ratio of chemicals used for the medium was selected to have a neutral pH.

For isolation of cypermethrin degrading bacteria, solid media were prepared. 250 mL MSM and 3.75g agar were mixed in a 500 mL flask and autoclaved at 121°C for 20min. When the medium temperature was about 45-50°C, cypermethrin (50 mg. L^{-1}) was added and mixed well. Then the medium was dispensed into disposable-sterile Petri plates near the Bunsen burner. Moreover, MSM-agar plates without cypermethrin were prepared with the same procedure.

For the cultivation of bacteria LA and LB, media were prepared in a 250 mL flask. LA medium contains 15 g bacteriological agar, 10g Tryptone, 10 g NaCl and 5 g yeast extract. The 6 g LA was dissolved in 150 mL ultrapure water. To grow bacteria LB medium was prepared like LA medium but without adding bacteriological agar. Then the media were again autoclaved at 121°C for 20 min. The solid medium was

placed in Petri plates when its temperature was about 45-50°C. All media were kept at 4°C until use.

20 mM phosphate buffer solution with neutral pH was prepared for whole-cell oxygen uptake and crude cell extract enzyme activity studies. The 0.1 M PBS was prepared by mixing through a magnetic mixer 12 g K_2HPO_4 and 7.2g KH_2PO4 in 400 ml autoclaved ultrapure water, then the solution was diluted with autoclaved ultrapure water to obtain 20 mM PBS.

3.2.2.2 HPLC Analysis

Cypermethrin content was determined using an LC-20AT HPLC instrument (Shimadzu, Kyoto, Japan) equipped with an LC-20AT pump, an Eclipse plus C18 column (100, 4.60 mm, 3.5 μm), and an SPD-M20A detector.

A series of eight solutions from the technical grade of cypermethrin was prepared to contain 15-5000 ppb cypermethrin (15, 25, 50, 100, 500, 1000, 2000, and 5000 ppb). The elution process was performed at 25°C using 85:15 (v/v) Acetonitrile: Ultrapure water and a flow rate of 0.5mL.min^{-1} with the 50 μL injection volume. The detection wavelength was 210 nm. Linear regression was carried out by plotting the cypermethrin concentrations versus the corresponding peak areas. The calibration curve equation was obtained with R^2 0.9999, and it is given in Eq. 1.

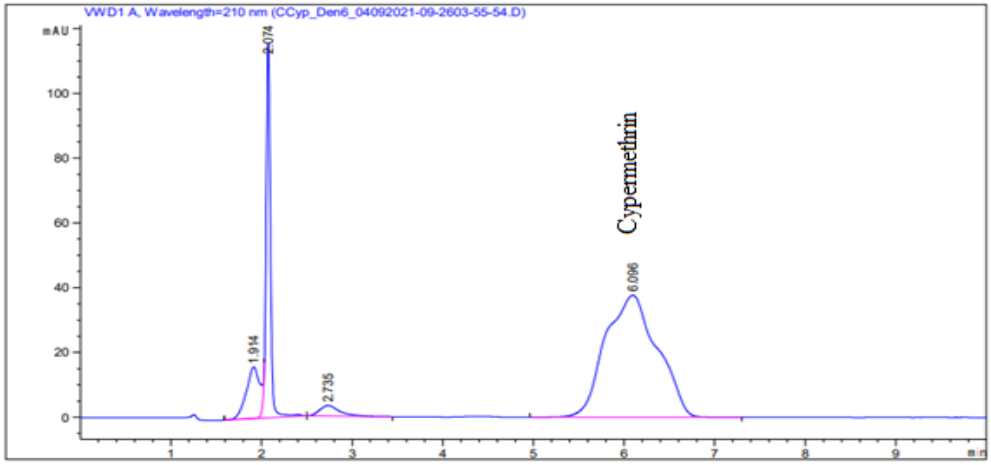
$$y = 0.762593x + 7.26033 \quad \text{Eq. 1}$$

With the same method, 3PBA was also analyzed. For the calibration curve, four different concentrations of 3PBA (5, 25, 100, and 200 ppm) from the stock solution were prepared and analyzed. The calibration curve with R^2 0.9997 was obtained and it was shown in Eq. 2. To prove that retention time belongs to 3PBA, a known amount of 3PBA from stock solution was added to the enrichment vials, and a peak was observed at the same retention time of the calibration curve.

$$y = 630.27x - 1210 \quad \text{Eq. 2}$$

HPLC results of cypermethrin (2 mg. L^{-1}) and 3PBA (3 mg. L^{-1}) were shown in Figure 3.2. The retention time for cypermethrin and 3PBA were found as 6.095 and 2.427 min, respectively.

a.



b.

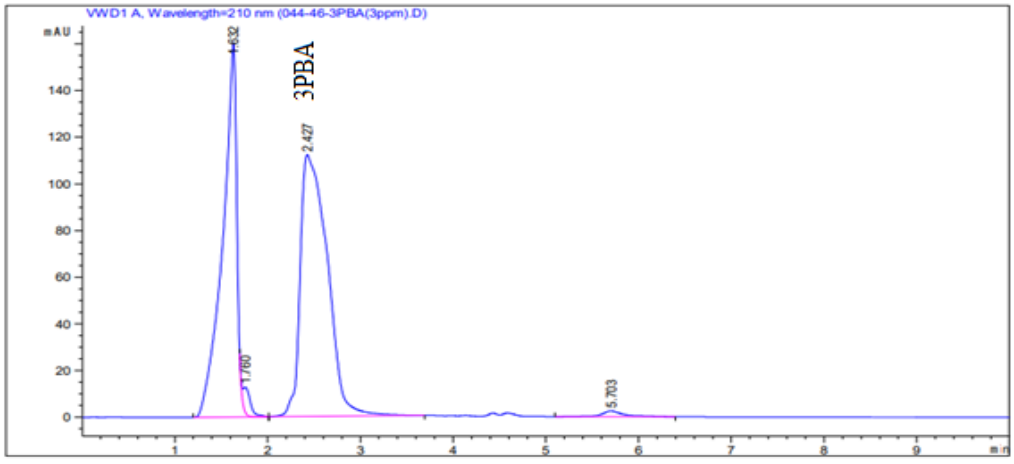


Figure 3.2 Cypermethrin and 3PBA in HPLC with Eclipse C18 column (a) cypermethrin, (b) 3PBA

For kinetic studies of cypermethrin and 3PBA, Ascentis C18 HPLC column 5µm particle size and 150 x 4.6 mm was used. The elution process was performed at 40°C using 95:5 (v/v) Acetonitrile: Ultrapure water and a flow rate of 0.5mL.min^{-1} with the 50 µL injection volume. The detection wavelength was 210 nm. According to

this method, cypermethrin was detected around 3.1min, and 3PBA was obtained around 2.7 min. The calibration curve of the second method was found and given in Eq.3 and Eq. 4, respectively.

$$y = 4849.1x - 954.57 \quad \text{Eq. 3}$$

$$y = 617.49x - 77.086 \quad \text{Eq. 4}$$

HPLC results of cypermethrin (50 mg. L^{-1}) and 3PBA with Ascentis column were shown in Figure 3.3.

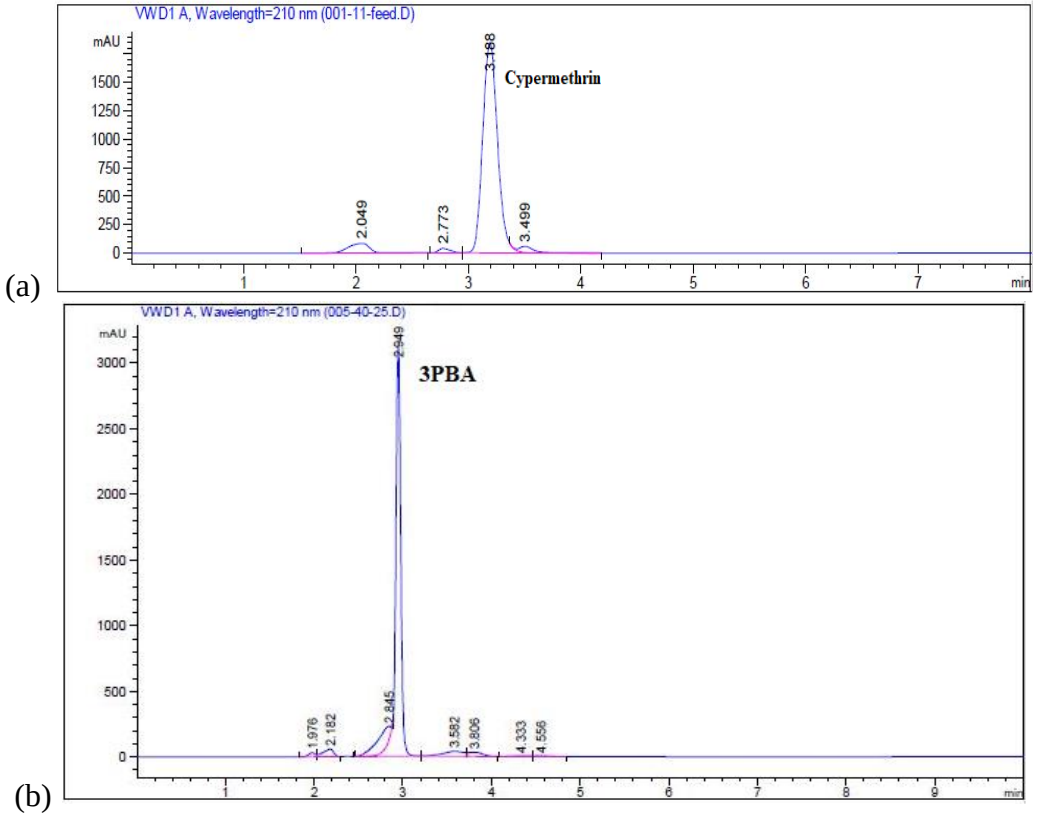


Figure 3.3. (a) cypermethrin HPLC result with Ascentis column (b) 3PBA HPLC result with Ascentis column

3.2.2.3 Enrichment Studies

In the study, there were two different enrichment sets. The first was formed using the soil sample taken recently (half a day after pesticide application). The second was created using the soil sample taken one and half weeks later pesticide application. All conditions were realized in triplicates. A total of twelve Erlenmeyer flasks were used. The autoclaved soil samples and active soil samples (2 g and 5 g) were put into a 500-mL Erlenmeyer flask containing 300 mL of MSM with 50 mg. L^{-1} CYP (60 µL). The detailed information related to the set-up is tabulated in Table 3.1 and the whole design is shown in Figure 3.4. The samples were then incubated at 30 °C on a rotary shaker at 180 rpm. Analysis was performed through HPLC.

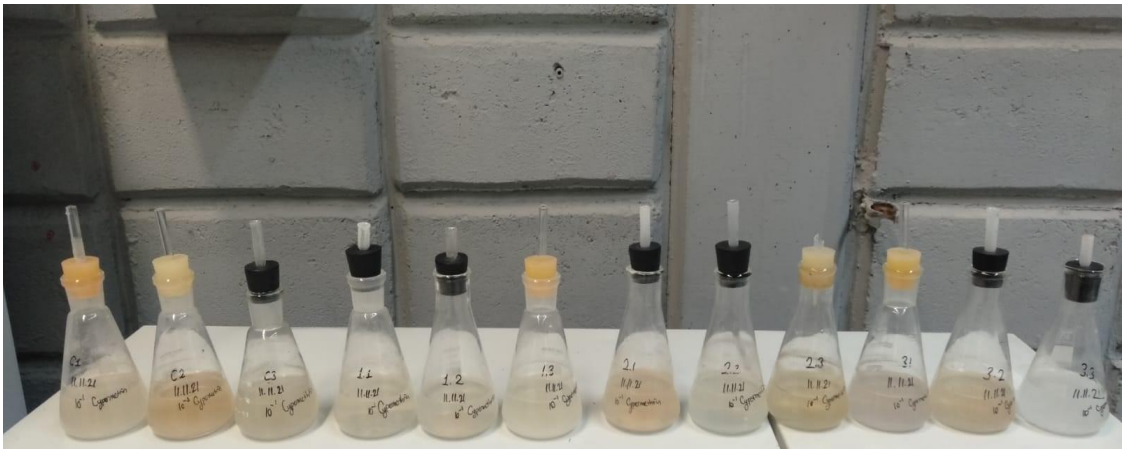


Figure 3.4. Experimental Set-up

Table 3.1. Experimental set-up for enrichment studies

Erlenmeyer flask	Medium	Sample	Substrate
C1Control Group (New soil)	MSM	2 g new autoclaved soil	50mg.L^{-1} CYP
C2 Control Group (New soil)	MSM	5 g new autoclaved soil	50mg.L^{-1} CYP
C3 Control Group (Old Soil)	MSM	5 g old, autoclaved soil	50mg.L^{-1} CYP
1.1, 1.2, 1.3 new soil sample	MSM	2 g new soil	50mg.L^{-1} CYP
2.1, 2.2, 2.3 new soil sample	MSM	5 g new soil	50mg.L^{-1} CYP
3.1, 3.2, 3.3 old soil Sample	MSM	5 g old soil	50mg.L^{-1} CYP

Due to the high adsorption capacity of the soil, control and active sets were diluted two times ten-fold that is 135 mL sterile fresh MSM supplemented with 50 mg. L^{-1} cypermethrin mixed with 15 mL active sets in 250 mL Erlenmeyer flask. Before each dilution, 100 µL samples from each culture were spread to the previously prepared MSM plates containing 50 mg. L^{-1} cypermethrin and incubated at 30°C for two days.

3.2.2.4 Isolation of bacterial strain

Isolation studies were performed for each enrichment culture. Pure colonies were picked up and transferred aseptically to the sterile liquid MSM tubes containing 50 mg. L^{-1} cypermethrin. The cultures were incubated at 30°C on a rotary shaker at 80 rpm for one week (Tang et al., 2015). The turbid tubes were transferred to the LA plates by streaking four times. After 24h incubation at 30°C, pure colonies were restreaked to the MSM with cypermethrin plates and only MSM plates for isolation. Colonies only grown on MSM with cypermethrin were chosen for further studies. Moreover, isolated cypermethrin degrading bacteria were kept at -20°C in a 20% (v/v) sterile glycerol solution for further analysis.

3.2.3 Kinetic Studies

The Michaelis–Menten equation for kinetic analysis and mass balance equation around constant-volume batch reactor for biodegradation rate calculation was used (Fogler, 1999).

For kinetic studies, one week before the experiment, 50 mg. L^{-1} stock solution was prepared with commercial-grade CYP in MSM, and 10 ml identical glass test tubes were autoclaved. In test tubes, 4.5 mL MSM was supplemented with 1, 2, and 4 mg. L^{-1} CYP and 3PBA were mixed with a 0.5 mL bacterial culture. For control groups, 5 mL 1, 2, and 4 mg. L^{-1} cypermethrin and 3PBA were added. All tubes were incubated on a rotary shaker at 35 °C and 80 rpm for 5 days.

Before kinetic studies, bacterial culture was prepared. The pure colonies grew only on LA and MSM supplemented with CYP and were transferred to the 100 mL sterile liquid MSM-CYP medium in autoclaved 250 mL flasks and incubated at 35° on a rotary shaker for three days.

For the biodegradation rate of CYP, constant-volume batch reactor mass balance (Eq 5) was combined with the reaction rate equation (Eq 6). The rate was calculated by

Eq 7 (Fogler, 1999). After finding the k values of each culture, the half-life was found by using Eq 8. For enzyme kinetics, the Lineweaver Burk plot was created by plotting C_A^{-1} vs $-r_A^{-1}$ graphs to calculate K_m and V_{max} values.

$$r_A * V = \frac{d(C_A * V)}{d_t} = \frac{V * dC_A}{d_t} \quad \text{Eq 5}$$

$$r_A = -k * C_A^n \quad \text{Eq 6}$$

$$\ln\left(\frac{C_A}{C_{A0}}\right) = k * t \quad \text{Eq 7}$$

$$\frac{ln2}{k} = t_{\frac{1}{2}} \quad \text{Eq 8}$$

3.2.4 Enzyme and Protein Analysis

The bacterial culture was inoculated with a loop to 150 ml LB medium in 250 ml Erlenmeyer flasks and incubated at 35°C on an orbital shaker at 80 rpm for seven days. Growing cultures were washed twice with MSM-CYP by centrifugation at 3200g and 4°C for five minutes. The supernatants of tubes were removed, and pellets were inoculated to MSM supplemented with 50 mg. L^{-1} CYP medium at 35°C and 80 rpm for one week. f

The one-week incubated cell cultures in cypermethrin and LB medium (used as a control) were washed with 20 mM potassium phosphate buffer solution with centrifugation under 3200 g at 4°C for 5 min. The obtained pellet was ultrasonicated at 40 kHz for 15min in the sterile Eppendorf. Then Eppendorf was centrifuged again with 15200 g at 0°C for 15min and obtained supernatants were used for crude cell extract enzyme studies (Tang et al., 2018). The experimental set-up of enzyme studies contained 8 autoclaved 15 ml glass tubes. The 5 mg. L^{-1} cypermethrin and 3PBA solution were prepared in 20 mM PBS, and both media were incubated at

30°C before the studies. Inside the tubes, 2 mL cypermethrin and 3PBA solution were added and mixed with 5µL crude cell extract. Then the tubes were incubated at 30°C. The samples were taken 15 minutes intervals and diluted one to two ratio with acetonitrile before HPLC analysis to stop the activity of the enzyme.

Isolated bacterial culture was incubated with commercial-grade CYP (CYP-induced cell) and cultures were incubated on LB medium (non-induced) for two weeks. Growing cultures were washed with 20µM PBS, and then ultrasonication (40kHz, 30 mins) was applied. Then Eppendorf was centrifuged again with maximum speed at 0°C for 30 min and obtained supernatants were used to find cellular protein expression. Protein expressions were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12 % polyacrylamide gels.

One-week-old cultures in LB (control group) and MSM with commercial-grade CYP were centrifuged at 3200g, and pellets were ultrasonicated at 40kHz for 20 minutes. Then, cultures were centrifuged at 0 °C with 15200g for 30 mins. Supernatants were used to find the size of the enzyme with the SDS-PAGE method. As a first step, ammonium sulfate precipitation was applied with crude cell extracts.

The crude cell extracts were saturated with ammonium sulfate. Firstly, 55% saturation was achieved, and then the cloudy suspension was centrifuged at 15200×*g* for 15 min to obtain supernatant. Secondly, the supernatant was saturated to 60% with ammonium sulfate, centrifugation was applied, and the obtained pellet was dissolved in PBS, and concentrated at 2 ml. After ammonium sulfate saturation, DEAE-Sepharose ion-exchange chromatography was applied. The concentrated enzyme was loaded onto the DEAE-Sepharose Fast Flow anion-exchange column. Then gel filtration with Sephacryl™ S-100 column was used to further purification.

3.2.5 Oxygen Uptake

For the study, bacterial culture was incubated at 35°C and 80 rpm for one week.
For experimental, 8 balloons were autoclaved and 20 mM PBS was prepared.

Firstly, the oxygen uptake rate of 25 mL PBS was measured with Oxygraph, and then cypermethrin (5 mg. L^{-1}) was added and OUR was measured. Finally, a 2.5 mL bacterial culture was added to the balloon, and OUR was calculated. For noninduced culture and 3PBA, all steps were repeated (Tallur et al., 2008).

3.2.6 16s Bacterial Identification Analysis

EurX GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit (Poland) was used to isolate total DNA from bacterial strains. After isolation of DNA, spectrophotometric measurement in Thermo Scientific Nanodrop 2000 (USA) was applied to check DNA purification and the amount of DNA. 16S rDNA gene was amplified using the universal primers, 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3').

A typical reaction mixture in each PCR tube for 35 µL of the total volume contained 3 µL of the appropriate DNA template, 1X PCR reaction buffer, 2U of Taq DNA polymerase, and 0.2 mM of dNTP mix, 1.5 mM of $MgCl_2$, and 0.3 µM of F and R primer. The PCR cycling conditions consisted of initial denaturation at 95 °C for 5 min, then 40 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 60 s with the last cycle followed by a 5 min extension at 72 °C. The identification of two species was done by the analysis of the 16S rDNA sequence, compared with the National Center for Biotechnology Information (NCBI) database using the online BLAST search (http://www.ncbi. nlm.nih.gov/BLAST).

3.2.7 Defining Optimum Conditions for Cypermethrin Degradation

Response surface methodology (RSM) based on the three variables Box–Behnken experimental design was used to investigate the effects of the concentration of cypermethrin, temperature, and pH. The design consists of 15 experimental runs at the center point. The degradation rates were determined after 5 days of cultivation.

The quadratic polynomial equation was given in Eq. 9, where Y is the degradation rate, X_i and X_j are variables, β_0 is the constant, β_i is the linear coefficient, β_{ij} is the interaction coefficient, and β_{ii} is the quadratic coefficient (Myers and Montgomery, 2002). The quadratic equation was found by using Minitab software. Table 3.2 show the symbols and code level of independent variables. After five days of incubation, degradation rates would be found.

$$Y = \beta_0 \sum_{i=1}^{k} \beta_i X_i + \sum_{i+1}^{j-1} \sum_{j=1}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2 \qquad \text{Eq 9}$$

Table 3.2 The symbols and levels of independent variables in RSM

Symbol	Code Level of Variables		
	-1	0	+1
X_1 (mg. L^{-1})	2	4	6
X_2	6	7	8
X_3 (°C)	30	35	40

The procedure for optimum concentration, temperature, and pH calculation was followed as described. The 5 mL glass test tubes and MSM medium were autoclaved, and to adjust the concentration, stock solution with 50 mg. L^{-1} concentration was prepared in a 40 mL autoclaved balloon. The pH of the MSM was adjusted with 65% (v/v) HNO_3 and 64.7% (w/v) NaOH solutions, then the required amount of stock solution was added into autoclaved test tubes. 1.8 mL pH adjusted MSM medium was transferred to the test tube, and a 0.2 mL bacterial culture was added. In this experiment amount of bacterial culture, shaker rpm, and incubation time were kept constant.

CHAPTER 4

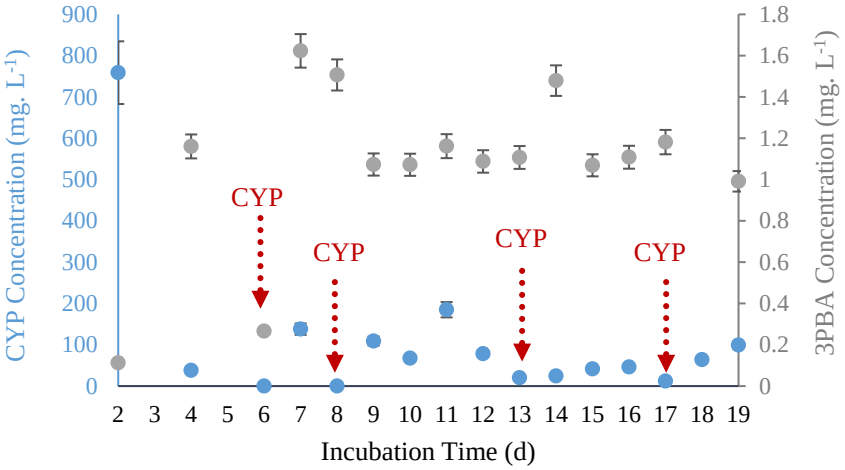
RESULTS AND DISCUSSIONS

This chapter summarizes the results obtained through the studies. In summary, enrichment studies, isolation, and identification of the strain, kinetic studies of the whole cells and enzymes, and oxygen uptake of whole cells are illustrated in this chapter.

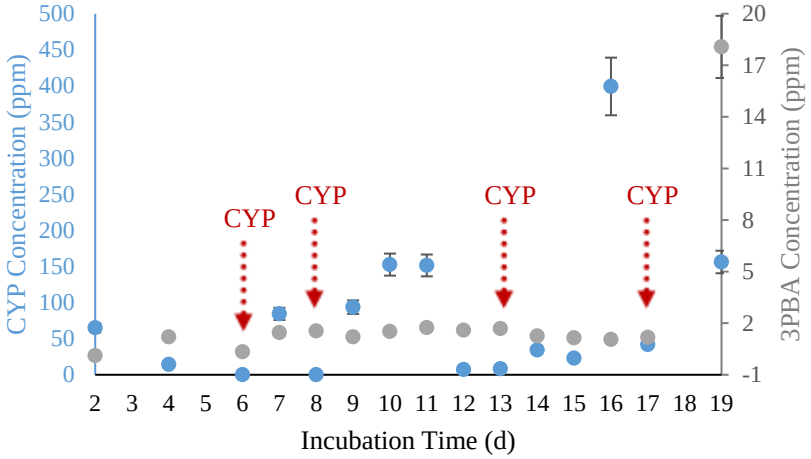
4.1 Enrichment and Isolation Results

Enrichment using soil samples from agricultural areas was performed to obtain mixed culture capable of degrading commercially applied CYP.

CYP degradation and 3PBA formation rates from the first enrichment culture were calculated. For three weeks, on the sixth, eighth, thirteenth, and seventeenth-day commercial-grade CYP was added to each flask. The CYP and 3PBA concentration changing with time were shown in Figure 4.1.



a.



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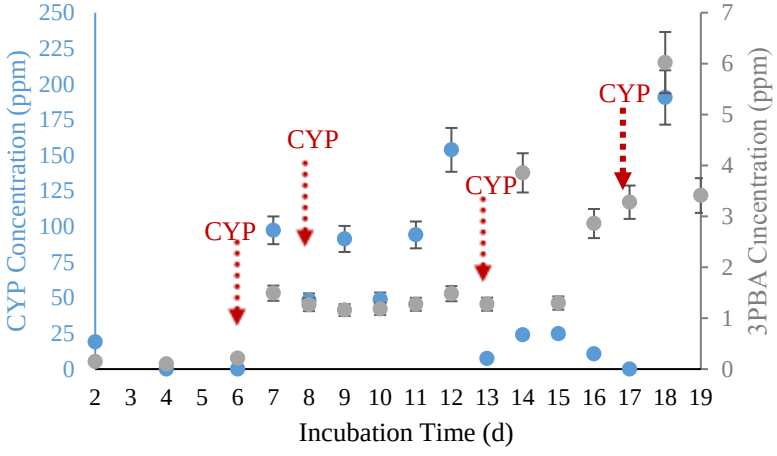


c.

Figure 4.1 3PBA and CYP concentration in the first enrichment culture measured in triplicates. The red arrow shows the commercial CYP addition day

After the two days of incubation, CYP concentration dramatically decreased as more than 90% of degradation for the cultures illustrated in Fig 4.1 a, b, c respectively. The overall biodegradation rate of the soil samples was calculated to be 9.641, 1.482, and 0.641 mg of CYP per g of soil for the enrichments in Figures 4.1 a, b, and c. Those rates were similar to previously published CYP degradation rates (Akbar et al., 2015b). The difference in degradation could be explained as the difference in soil adsorption capacities.

While CYP degradation was detected in the flasks a common byproduct of the previously established CYP degradation pathway 3PBA was also detected in the cultures. A study done by Tallur et al (2008) found 3PBA formation while CYP degradation. Fortunately, the study showed that 3PBA did not accumulate in the environment by the specific activities of the enzymes.

The first 3PBA production rates were 0.259, 0.109, and 0.003 mg. L^{-1}. d^{-1}. g^{-1} soil for flasks containing 2g first soil sample, 5g first soil sample, and 5g second soil sample, respectively. The CYP biodegradation rates were 72.094, 5.082, and 1.903 mg. L^{-1}. d^{-1}. g^{-1} soil, respectively. That means 3PBA formation rates decrease while CYP biodegradation rates decrease. After commercial-grade CYP feeding (sixth day of incubation), the 3PBA formation rate in the flasks containing 2 g soil and 5 g soil obtained one and a half weeks after application, and 5 g soil obtained one and a half months after application increased to 0.679, 0.225, and 0.256 mg. L^{-1}. d^{-1}. g^{-1} soil, respectively. Even though an enriched culture capable of CYP degradation was forming in the enrichments, 3PBA formation rates have been observed to increase. After the addition of CYP on the 16^{th} day, 3PBA formation was calculated to increase to 10.3, 3.4, and 0.548 mg. L^{-1}. d^{-1}. g^{-1} soil. This also could be explained as a lacking ingredient to further degrade 3PBA or some biological cultures that might be preventing 3PBA degradation. The study found that 3PBA accumulates in the enrichment medium which would represent the situation in the field where CYP would be applied constantly. Up to now, some studies found that bacterial strains could degrade both CYP and 3PBA at the same time with isolated cultures such as

Actinomycetes HU-S-01(Lin et al., 2011a). Also, the cooperation of two different strains enhanced the degradation of CYP and 3PBA like *B. licheniformis* B-1 and *Sphingomonas* sp. SC-1 (Liu et al., 2014). Moreover, the study done by Deng et al. (2015) showed that the removal of 3PBA from the medium was lower and 3PBA resisted degradation when the medium was acidic. However, our results showed that a mixed culture present in the agricultural fields mixed could degrade CYP while accumulating a more toxic by-product such as 3PBA.

3-Phenoxybenzoic acid (3-PBA) is a common degradation intermediate of many synthetic pyrethroid pesticides (SPs) so, 3-PBA evaluation after pyrethroid application is required. The studies reported that 3-PBA has stronger reproductive toxicity, weaker hydrophobicity, and a longer half-life than the parent compounds. For that reason, this intermediate is more likely to accumulate in the environment, causing secondary pollution of agricultural products (White et al. 1996).

4.2 Isolation and Identification of cultures

Isolation was performed by spreading 100 µL of each culture on MSM-agar plates containing commercial CYP (approximately 50 mg L^{-1}) and incubating at 30°C for two days (Figure 4.2). The single colonies were picked aseptically on plates and were inoculated to the liquid MSM containing CYP medium and incubated on a rotary shaker at 80 rpm and 35°C for two weeks (Figure 4.3).

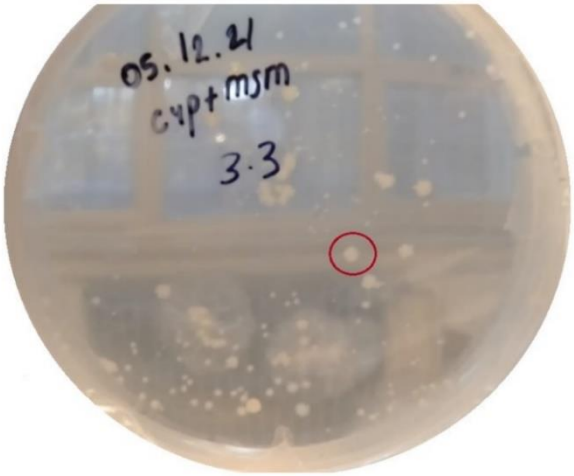


Figure 4.2. Growth on MSM-CYP plate by spread method

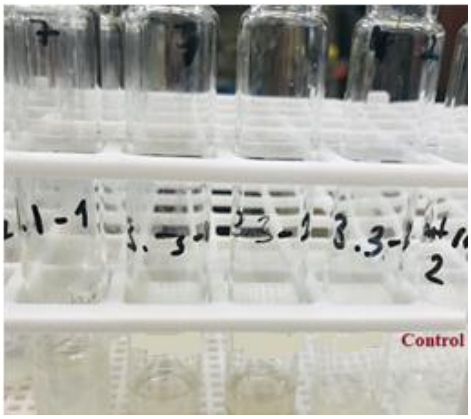


Figure 4.3 Turbidity of the growth in liquid MSM-CYP medium with the isolates. All tubes from 5 g soil taken one and a half months after the application

The cultures from turbid tubes were streaked to LA plates and incubated at 30°C for one day to verify that they were not contaminated. Then purified colonies were re-streaked to MSM-CYP and MSM-agar plates to isolate bacteria using CYP as a carbon source. As a result, two isolates called ZK101 and ZK102 were selected from enrichment cultures for CYP degradation studies. The illustration of the isolates is

shown in Figure 4.4. For preserving the cultures 20% (v/v) sterile glycerol solution was prepared to inoculate cultures, and cultures were stored at -20°C.

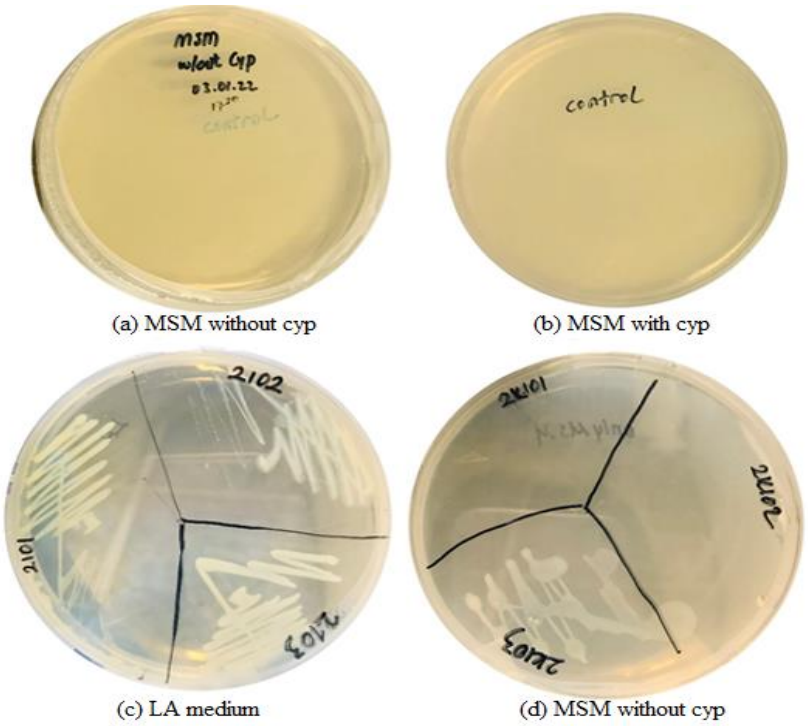


Figure 4.4 Isolation results of enrichment studies. (a) Control for only MSM plate (b) Control for MSM with CYP plate (c) LA medium (d) Growth on MSM without CYP plate

CYP degrading culture was isolated from only a 5 g soil sample obtained one and a half months after application. This illustrated that bacteria acclimated to CYP in a month. Moreover, the moisture contents, external temperatures, and porosity of the second soil sample (taken one and a half months later of the CYP application) were higher than those of the first soil sample (taken one and a half weeks later of the

application), and the pH of the first sample was higher than the second soil sample (Table 4.1). The second soil sample's properties were more like previous studies.

Table 4.1 Soil Samples Physicochemical properties

Sample	pH	Moisture Content (%)	External Temperature	Porosity (%)
(1)	8.28	5.2	28 C°	39
(2)	8.08	7.0	35 C°	42.9

According to previous studies, CYP degrading bacteria grow optimally around 30-35°C and have a pH of 7-7.5 (Akbar et al., 2015a; Liu et al., 2014). The temperature and pH of the Enrichment prepared with the second soil sample were similar to these results.

16s rDNA results of the isolated strains showed that ZK101 and ZK102 were 99.9 and 100% identical to *Enterobacter hormaechei* (ON329342) and *Stenotrophomonas maltophilia* (ON329343), respectively. The nucleotide sequences of the two strains were given in Appendix II. In this study, two new bacterial strains that efficiently degrade CYP were isolated, *Enterobacter hormaechei* ZK101 (ZK101) and *Stenotrophomonas maltophilia* ZK102 (ZK102).

The phylogenetic tree was created based on previously finding CYP degrading strains with their 16s rDNA sequences. The accession numbers of bacteria used in the tree (Figure 4.5) and % the degradation of CYP were given in Table 4.2. This is the first report of CYP degrading bacteria isolated from Enterobacter and Stenotrophomonas genus yet.

Table 4.2 CYP degrader strains and degradation percentage

Bacteria	CYP Concentration (mg. L^{-1})	% Degradation	Accession Number	Reference
Enterobacter hormaechei ZK101	5.69	67.7	ON329342	
Stenotrophomonas maltophilia ZK102	6.62	81.9	ON329343	
Brevibacillus parabrevis strain FCm9	100	99	KJ009250	Akbar et al.,2015a
Acinetobacter calcoaceucus MCm5	100	84.7	KJ009251	Akbar et al.,2015a
Sphingomonas sp. RCm6	100	91.8	KJ009252	Akbar et al.,2015a
Serratia spp. JC1	100	92	FJ009445	Zhang et al., 2010
Serratia spp. JCN13	100	89	FJ009447	Zhang et al., 2010
Azoarcus indigens HZ5	50	71.6	GU592532	Ma et al., 2013
Rhodopseudomonas palustris GJ22	50	83.5	FJ824030	Yin et al., 2012
Bacillus subtilis 1D	100	95	MG948470	Gangola et al., 2018
Bacillus subtilis BSF01	50	89.4	JF06263	Xiao et al.,2015

Table 4.2 cont'd

Bacillus thuringiensis SG4	50	80	KT186610	Bhatt et al., 2020
Bacillus cereus BCC01	100	99.6	MH588686	Hu et al., 2019
Bacillus parabrevis BCP09	30	75.87	JX556220	Tang et al., 2018
Flavobacterium haoranii LQY-7	---	---	GQ988780	Zhang et al., 2010
Pseudomonas aeruginosa GF31	---	---	KT735188	Tang et al., 2017

The common properties of ZK101 and ZK102 strains were gram-negative, and both strains are α-proteobacteria and pathogenic. In the tree, except for *Bacillus* species, they are all gram-negative and most of them are aerobic (*Brevibacillus, Pseudomonas, Acinetobacter, Sphingomonas, and Rhodopseudomonas*).

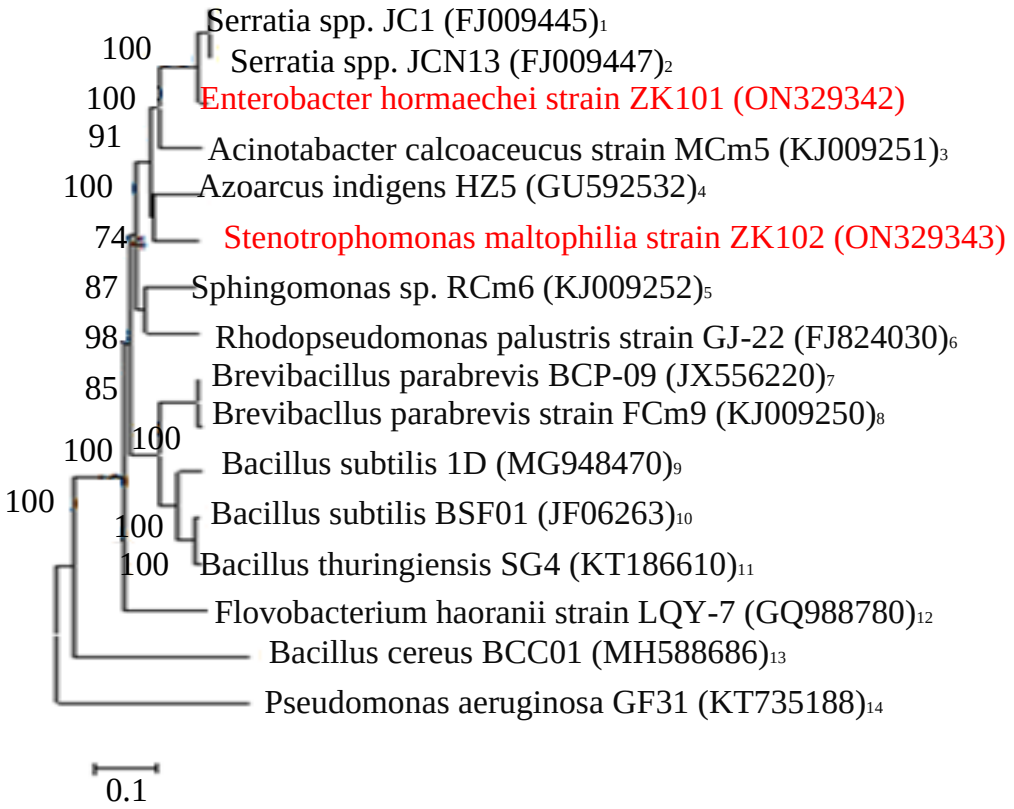


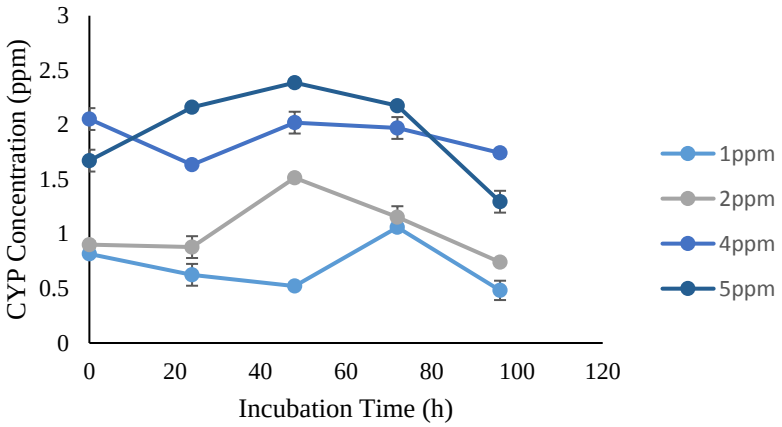
Figure 4.5 Phylogenetic tree based on the 16S rDNA sequences of strains ZK101 and ZK102, and related strains. The numbers in parentheses are the accession number in GenBank for strains. Numbers at the nodes indicate bootstrap values from the neighborhood-joining analysis of 1000 resampled data sets

4.3 Kinetic Studies of CYP and 3PBA by ZK101 and ZK102

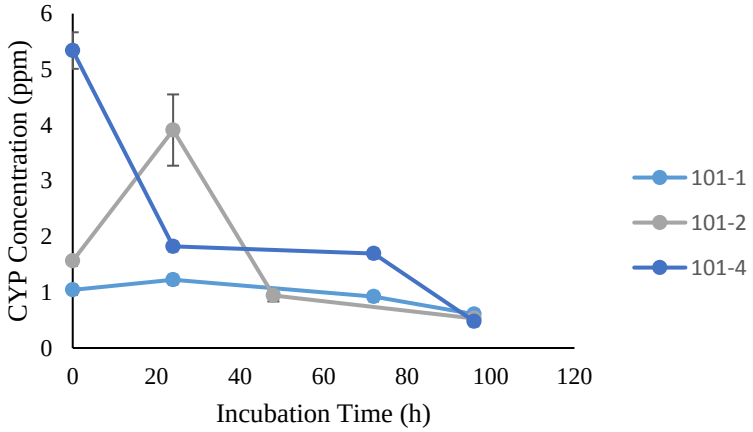
The purpose of kinetic studies is to see whether isolated bacteria are effective in CYP and its most common metabolite, 3PBA, biodegradation or not.

Due to the low solubility of CYP, 50 mg. L^{-1} stock solution was prepared. CYP with 1, 2, 4, and 5 mg. L^{-1} for kinetic studies was prepared from stock solution, and the concentration of CYP was measured in 0, 24, 48, 72, 96, and 120 h with the second

method. The concentration changing with the time by the control and whole-cell of ZK101 and ZK102 were given in Figure 4.6.



a.



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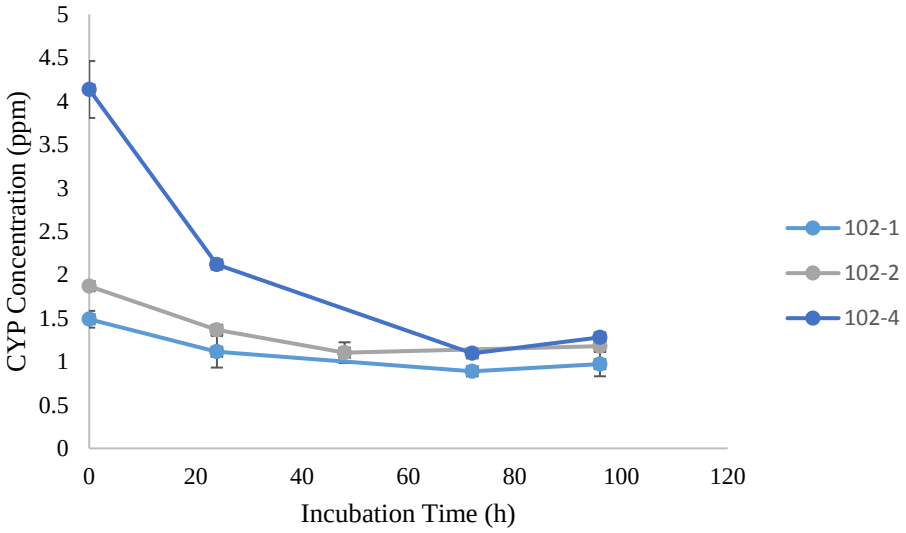


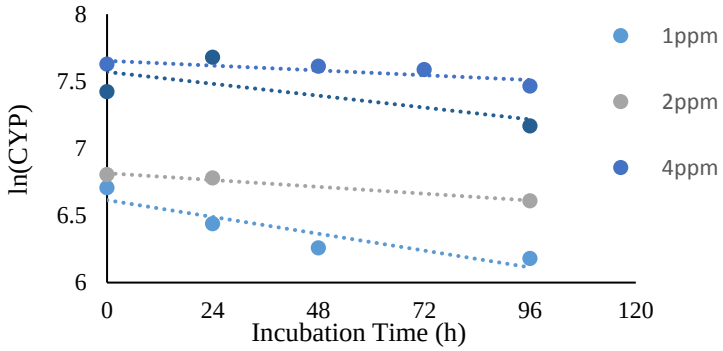
c.

Figure 4.6 CYP concentration changing by control, and whole cells of ZK101 and ZK102 with time (a) control (b) ZK101 (c) ZK102

In the control groups, initial CYP concentration did not change significantly. The percentage of abiotic degradation in the control groups supplemented with 1, 2, and 4 mg. L^{-1} CYP showed that biodegradation is more efficient than abiotic degradation. Only 40.9 % of 1 ppm, 17.8% of 2 ppm, and 15.1% of 4 ppm CYP were degraded after 4 days of incubation under abiotic conditions. In the control group photodegradation of CYP could be observed in the presence of $Cu^{2+}$and $Fe^{2+\cdot}$, but enhanced Fe levels increased the photo and microbial degradation of CYP. Moreover, the MSM medium was prepared with a trace amount of metal solution in the study, so in the control, groups photodegradation could occur.

However, with ZK101, 57.2% of 1 ppm, 65.4% of 2 ppm, and 91.5% of 4 ppm CYP were degraded, and ZK102 degraded 1, 2, and 4 ppm CYP with 63.7, 67.9, and 79.7%, respectively. The 22.5% of 5 ppm CYP was degraded in control, and 36% of 5 mg.L^{-1} was degraded by ZK101. The semi-ln graphs were plotted with time to find rate equations, rate constants, and of the cultures and control groups and are shown in Figure 4.7.

a.



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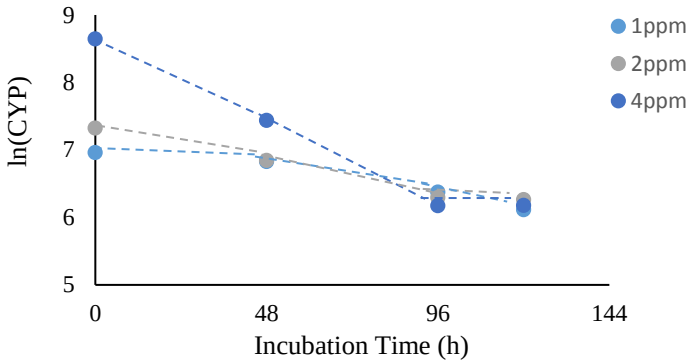

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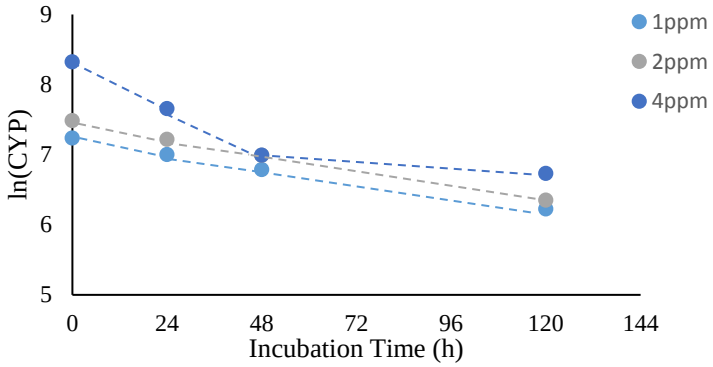


d.

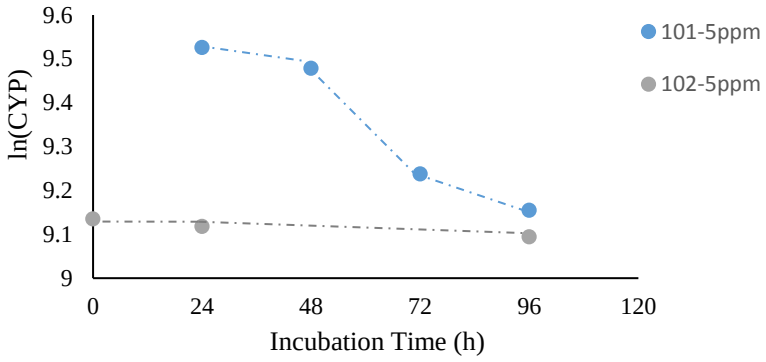


Figure 4.7 Rate constant and half-life calculation graphics for kinetic studies (a) control groups (b) ZK101(c) ZK102 (d) 5 ppm substrate curves for ZK101 and ZK102

The first-order biodegradation reaction occurs in a constant volume batch reactor at constant temperature and constant shaking speed, so rate calculations were performed accordingly. The biodegradation rate equation of CYP was calculated and summarized in Table 4.3.

Table 4.3 First order kinetic parameters of CYP degradation by control and cultures

Control	**Equation**	**k (d^{-1})**	**$t_{1/2}$ (d)**	**R^2**
1ppm	$y = -0.0052x + 6.643$	0.125	6	0.8379
2ppm	$y = -0.0021x + 6.815$	0.050	14	0.9841
4ppm	$y = -0.0015x + 7.652$	0.036	19	0.6768
E. hormaechei	**Regression Equation**	**k (d^{-1})**	**$t_{1/2}$ (d)**	**R^2**
1ppm	$C_A = 7.0412 * e^{-0.0072t}$	0.0480	14.4	0.9384
2ppm	$C_A = 7.2977 * e^{-0.0094t}$	0.0175	3.96	0.9748
4ppm	$C_A = 8.5479 * e^{-0.0218t}$	0.468	1.5	0.9636
S. maltophilia	**Regression Equation**	**k (d^{-1})**	**$t_{1/2}$ (d)**	**R^2**
1ppm	$C_A = 7.2136 * e^{-0.0083t}$	0.0740	9.4	0.9973
2ppm	$C_A = 7.46 * e^{-0.0093t}$	0.0173	4	0.9978
4ppm	$C_A = 8.014 * e^{-0.0122t}$	0.257	2.7	0.7887

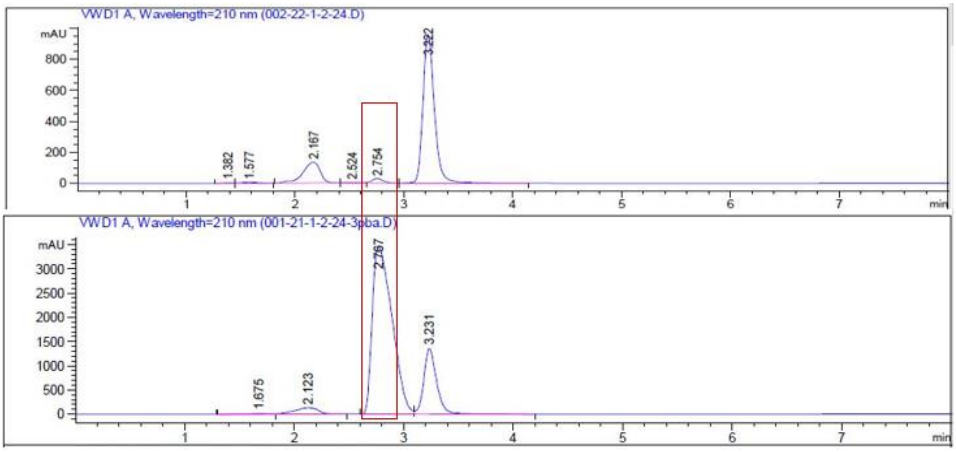
The kinetic study result of CYP concluded that CYP treatment with ZK101 and ZK102 is more efficient than abiotic treatment. The degradation percentage is higher, and its half-life of it is lower. Studies done by Akbar et al. (2015a) and Bhatt et al. (2020) also supported biotic degradation of CYP and 3PBA was more efficient than

abiotic degradation. The half-life of (100 mg L^{-1}) CYP by *Acinetobacter calcoaceticus* MCm5, *Brevibacillus parabrevis* FCm9, and *Sphingomonas* sp. RCm6 were 17.07, 9.6, and 14.35 days for 10 days of incubation, respectively. CYP was degraded biotically by strains MCm5, FCm9, and RCm6 because the ester bond was broken through carboxylesterase produced by these strains. However, without inoculation (100 mg L^{-1}) CYP half-life was calculated as 48.47 days after 10 days of incubation (Akbar et al., 2015a). Moreover, 50 mg L^{-1} of CYP was added to MSM (abiotic) and MSM with *Bacillus thuringiensis* strain SG4 (biotic) medium. After 15 days of incubation, results indicated the half-life of CYP became 165 and 6.7 days for abiotic and biotic cultures, respectively (Bhatt et al., 2020). In addition, *Ochrobactrumanthropi* JCm1, *Bacillus megaterium* JCm2, and *Rhodococcus* sp JCm5 degraded 90.5%, 86.6%, and 78% of 100 mg L^{-1} CYP after 10 days of incubation, respectively. The biodegradation rate constants of JCm1, JCm2 and JCm5 were 0.0516, 0.0425 and 0.0807 d^{-1}, respectively. (Akbar et al., 2015b).

Commercial CYP (4 mg L^{-1}) degradation rate constants by ZK101 and ZK102 degradation were calculated as 0.468 and 0.257 d^{-1}, respectively. That is higher than other studies. Moreover, commercial-grade CYP half-life was decreased from 19 days to 1.5 days with ZK101 and 2.7 days with ZK102. In summary, ZK101 and ZK102 acted like other cypermethrin degrading isolates, they formed 3PBA as a metabolite and decreased the half-life of CYP compared to control groups.

3PBA is the most formed metabolite in CYP degradation, and the study detected 3PBA formation in the Enrichment so to see the ability of the isolated strain ZK101 and ZK102 on 3PBA degradation, kinetic studies with 3PBA were applied. Also, this experiment helped to find the biodegradation pathway of commercial-grade CYP. Cultures were mixed with 1, 2, 4, and 5 mg. L^{-1} 3PBA supplemented MSM medium, and concentrations of 3PBA were found in the HPLC. In the study, 3PBA was detected around 2.8 min, and to prove that 3PBA from stock solution was added to 2 and 5 ppm CYP sample vials (Figure 4.8).

a.



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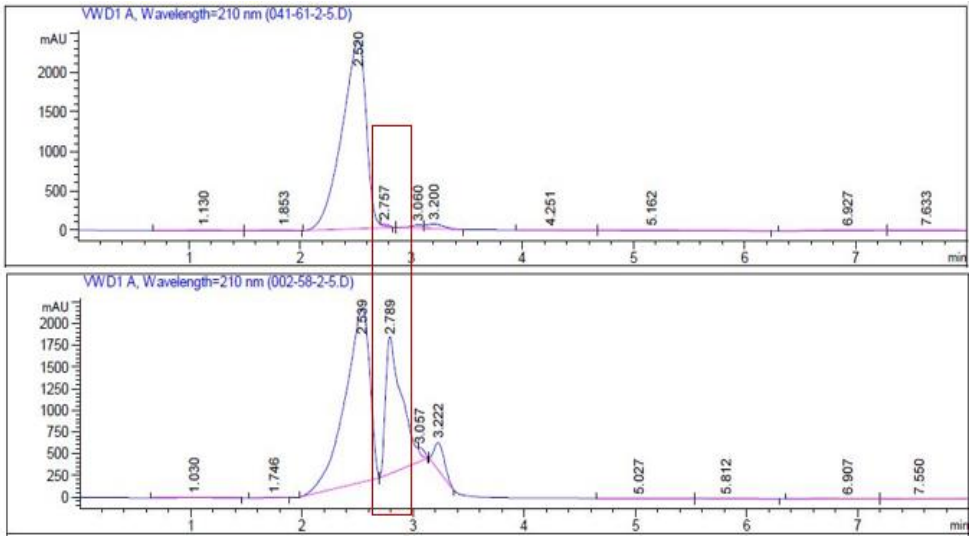
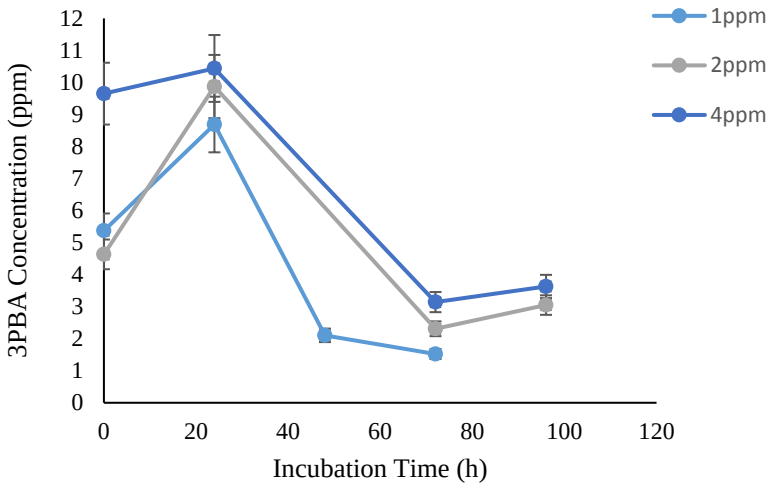
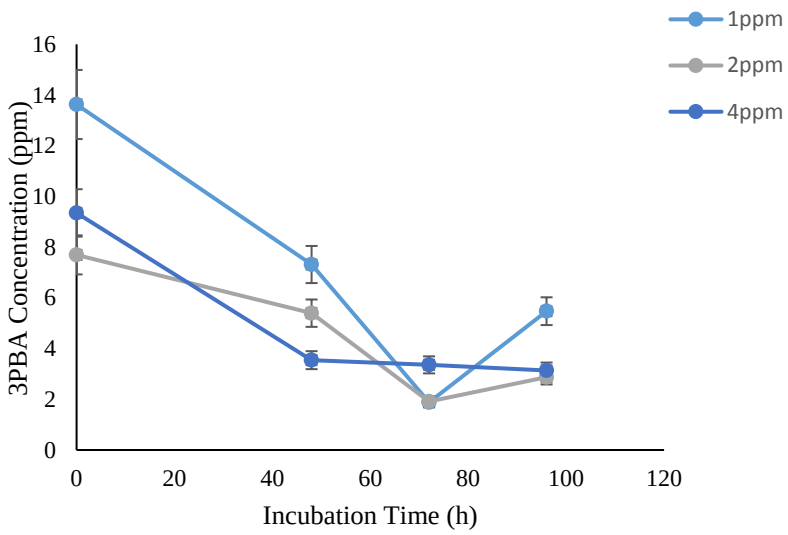


Figure 4.8 3PBA analysis through Ascentis column method (a) 2ppm CYP sample (b) 5ppm CYP sample

After proving 3PBA, concentration change with time, graphs were created (Figure 4.9), and the rate equations, rate constants, and half-life of the cultures were found by plotting semi-ln graphs shown in Figure 4.10, and they are tabulated in Table 4.4. When CYP and 3PBA half-life were compared in Tables 4.3 and 4.4, the half-life of 3PBA was lower than CYP.

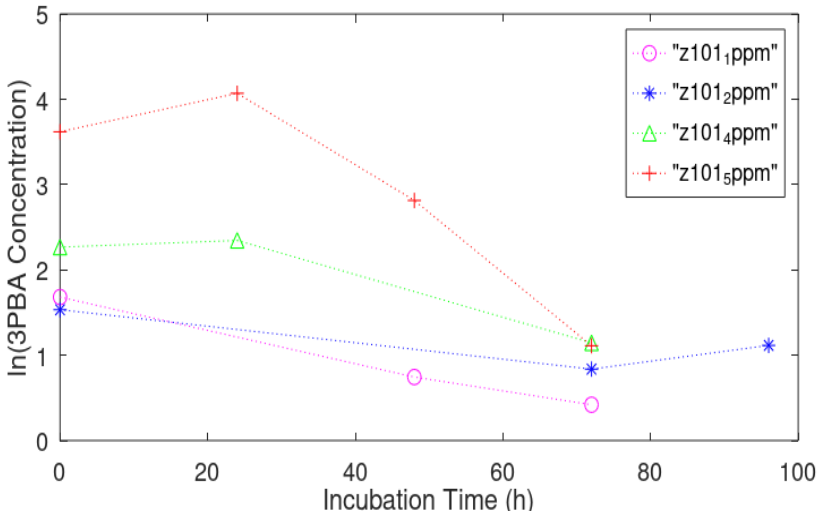


a.

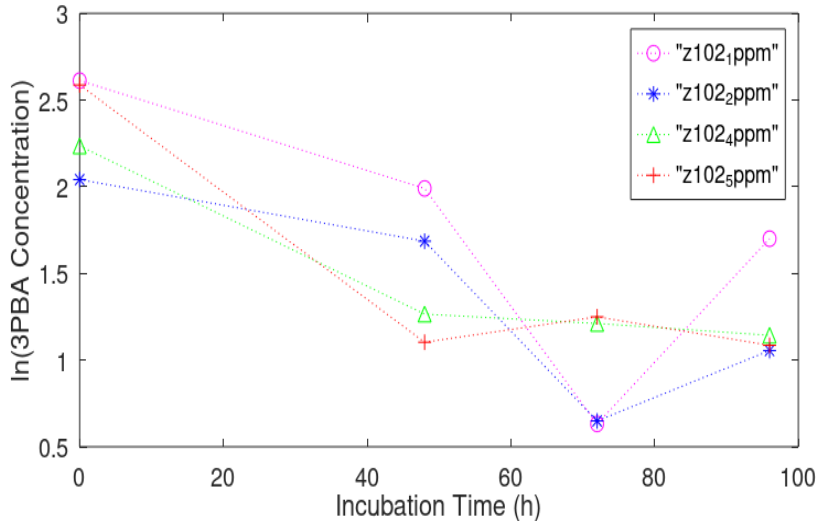


b.

Figure 4.9 The concentration change of 3PBA with time (a) ZK101 (b) ZK102



a.



b.

Figure 4.10 Rate constant and half-life calculation graphics for kinetic studies (a) ZK101 (b) ZK102

Table 4.4 First order kinetic parameters of 3PBA degradation by ZK101 and ZK102

E. hormaechei	**Equation**	**k (d^{-1})**	**$t_{1/2}$ (d)**	**R^2**
1ppm	$y = -0.0178x + 1.661$	0.427	1.6	0.9931
2ppm	$y = -0.0056x + 1.474$	0.134	5.2	0.6334
4ppm	$y = -0.0169x + 2.461$	0.406	1.7	0.8541
5ppm	$y = -0.0616x + 5.624$	1.386	0.5	0.9924
S. maltophilia	**Equation**	**k (d^{-1})**	**$t_{1/2}$ (d)**	**R^2**
1ppm	$y = -0.0095x + 2.557$	0.228	3	0.9576
2ppm	$y = -0.0128x + 2.047$	0.307	2.3	0.7027
4ppm	$y = -0.0116x + 2.090$	0.278	2.5	0.8471
5ppm	$y = -0.0155x + 2.345$	0.372	1.9	0.7719

3PBA was formed by the cleavage of the ester bond of almost all pyrethroids, and studies investigated both mother pyrethroids and their metabolite 3PBA. Many strains could degrade 3PBA efficiently. Bacillus sp. DG-02 degraded 95.6% of 50 mg L^{-1} 3PBA within 72h with 0.2014 d^{-1} rates constant, and the half-life of 3PBA decreased from 101.9 to 3.4 days with DG-02 strain(Chen et al., 2014). Actinomycetes HU-S-01 strain was isolated, and the study found that the CYP degradation rate was higher than 3PBA with the same concentration (Lin et al., 2011). *Stenotrophomonas* sp. Strain ZS-S-01 showed the degradation capability of CYP, 3PBA, and other types II pyrethroids. MSM supplemented with 50 mg L^{-1} 3PBA was inoculated with strain ZS-S-01 and without culture and incubated for 9 days. The rate constants for 3PBA control, 3PBA with culture, and CYP were found as 0.0079, 0.1811, and 0.3633d^{-1}, respectively (Chen et al., 2011). Moreover, the half-life of 3PBA was decreased from 87.9 to 3.8 days

3PBA (4 mg L^{-1}) degradation rate constants by ZK101 and ZK102 were calculated as 0.427 and 0.228 d^{-1}, respectively which is higher than other studies' calculations.

In the previously done studies, the half-life of the same concentration of CYP and 3PBA showed that 3PBA can accumulate in the environment because 3PBA has a higher half-life and lower degradation constant (Chen et al., 2011; Tang et al., 2008). In the Enrichment culture, 3PBA accumulated because of constant CYP feeding, and in the mixed culture, some bacteria could prevent further 3PBA degradation. However, the kinetic studies showed that 3PBA did not accumulate. To prove that specific activities of an enzyme are needed to be calculated.

4.4 Optimization of Biodegradation by Experimental Design 2^3 with Central Point in Triplicate for RSM

The effect of substrate concentration, temperature, and pH on the biodegradation of CYP was examined using Minitab software. The experimental setup was designed as an initial CYP concentration between 2 and 6 mg L^{-1}, an incubation temperature of 30–40°C, and a pH between 6 and 8. During the experiment, the shaking speed was 80 rpm, 10% of cultures were incubated in identical 10 ml glass tubes, and incubation time was five days in the MSM-CYP medium. The result of the study was tabulated in Table 4.5

Table 4.5 Results of the optimization of different initial concentrations, temperature, and pH

RUN	X_1 (ppm)	X_2 (°C)	X_3	Biodegradation by *Enterobacter hormaechei* (%)	Biodegradation by *Stenotrophomonas maltophilia* (%)
1	4	40	6	65.2	64.4
2	4	30	6	46.6	36.2
3	2	35	6	74.0	78.9
4	2	35	6	49.3	60.1

Table 4.5 (cont'd)

5	4	40	8	75.8	75.7
6	4	30	8	71.0	56.6
7	6	35	8	87.0	85.5
8	2	35	8	56.0	53.2
9	2	40	7	30.6	28.5
10	6	30	7	69.8	39.0
11	6	40	7	44.7	83.5
12	2	30	7	24.5	10.6
13	4	35	7	64.1	58.6
14	4	35	7	64.0	64.7
15	4	35	7	60.0	76.6

The RSM-based Box–Behnken design was applied to find the optimum substrate concentration (X_1), incubation temperature (X_2), and pH (X_3) (Table 4.5). Quadratic polynomial equations (Eq. 1, 2) were found using Minitab. In Eq. (1 and 2), Y is the predicted biodegradation rate of CYP by ZK101 and ZK102. Both models' summary is shown in Table 4.6.

$$y = -215 + 47.4X_1 + 39.2X_2 - 155X_3 - 0.78X_1X_2 + 0.79X_1X_3 - 0.690X_2X_3 - 2.297X_1^2 - 0.445X_2^2 + 13.06X_3^2 \quad \text{Eq. (1)}$$

$$y = -504 - 11.6X_1 + 55.7X_2 - 131.2X_3 + 0.665X_1X_2 + 1.69X_1X_3 - 0.455X_2X_3 - 1.88X_1^2 - 0.749X_2^2 + 10.31X_3^2 \quad \text{Eq. (2)}$$

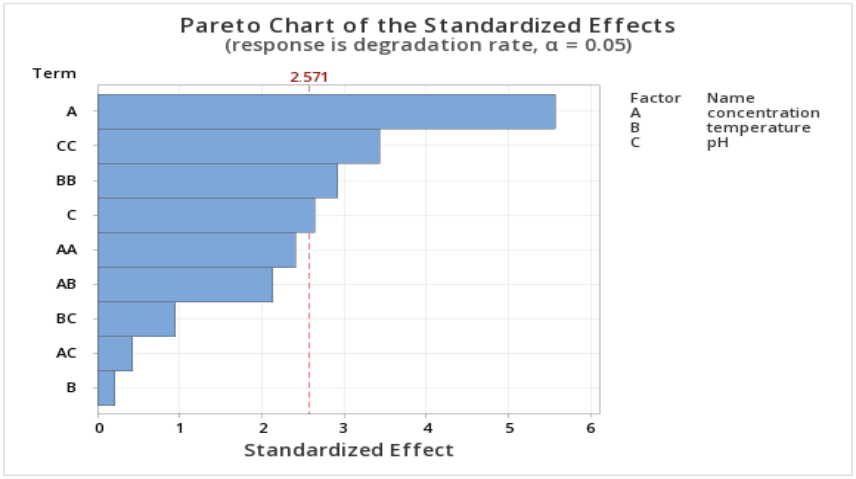
Table 4.6 Model Summary

Equation	S	R-sq	R-sq(adj)	R-sq(pred)
(1)	7.30250	93.50%	81.79%	0.00%
(2)	9.53435	93.08%	80.63%	24.40%

The first-order partial derivatives of each variable of the quadratic polynomial equations would give optimum parameters. The optimum biodegradation rate of CYP by ZK101 was 67.7%, with an initial CYP concentration of 5.69 mg. L^{-1}, incubation temperature 33.9°C, and pH 6.66. The optimum biodegradation conditions for ZK102 were incubation at 38.1 °C, with an initial CYP concentration of 6.62 mg. l^{-1} and pH 6.66 81.9% of CYP was degraded under these conditions. Generally, optimization for concentration was studied between 50 and 150 mg. L^{-1} CYP for almost two weeks of incubation, but low concentrations were supplied in this study, and incubation time was kept shorter than in other studies. The optimum incubation temperature and pH were reported to be around 35°C and neutral pH range using Minitab software (Tallur et al.,2008; Akbar et al., 2015a).

Moreover, Pareto charts were created to see the significance of independent variables. In the obtained Pareto chart (Figure 4.11), it was observed that the most significant factor for ZK101 was the initial CYP concentration (X_1), and the pH of the medium (X_3). That is higher initial concentrations of CYP and medium pH promoted increased biodegradation of CYP under the evaluated conditions. The independent factor temperature was nonsignificant in the range of 30−40 °C for biodegradation.

a)



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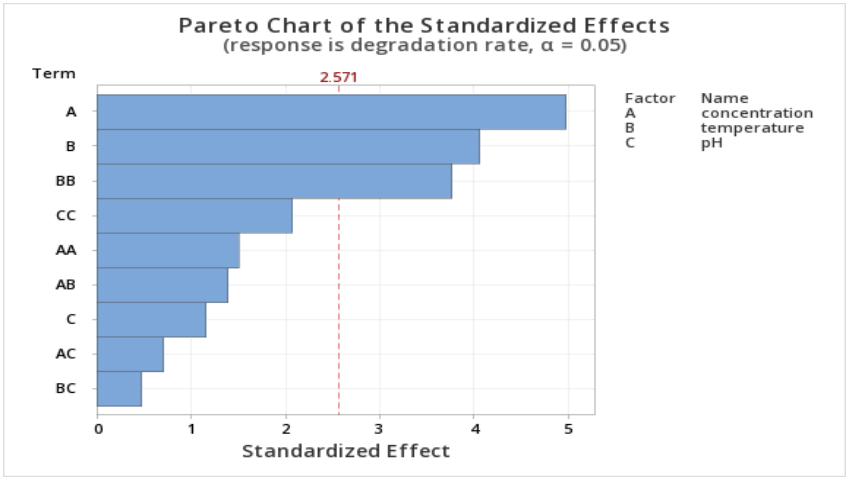
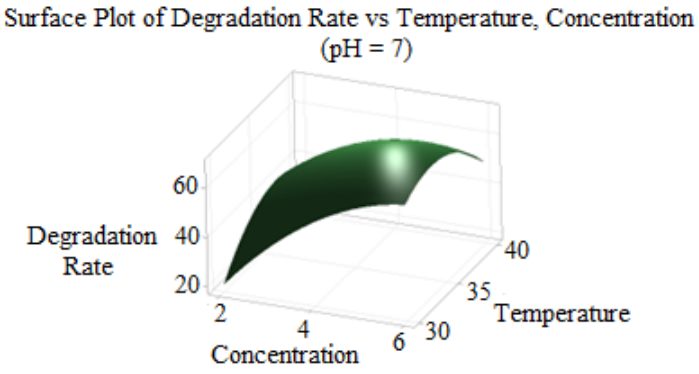
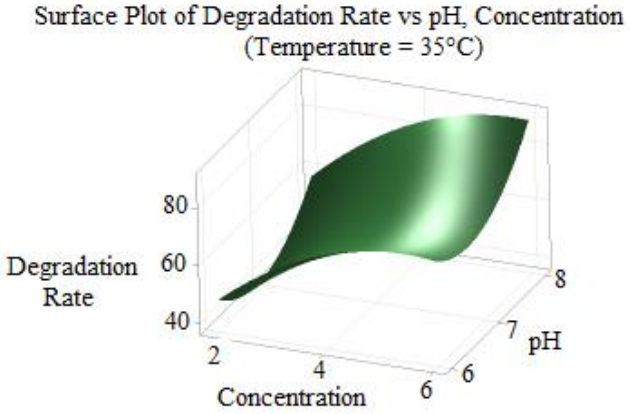


Figure 4.11 Pareto charts for significant effects on biodegradation with ZK101 and ZK102 (a) ZK101 and (b) ZK102.

In addition, it was seen that the most significant factor for ZK102 was the CYP concentration (X_1), and the incubation temperature of the medium (X_2), so higher initial concentrations of CYP and medium temperature increased the biodegradation of CYP under the evaluated conditions. The independent factor pH was nonsignificant in the 6-8 range for biodegradation. Surface plots are also created to evaluate all three independent variables' effects on biodegradation. The surface plots of concentration, temperature, and pH effect on CYP biodegradation by both cultures are shown in Figures 4.12 and 4.13.



a.



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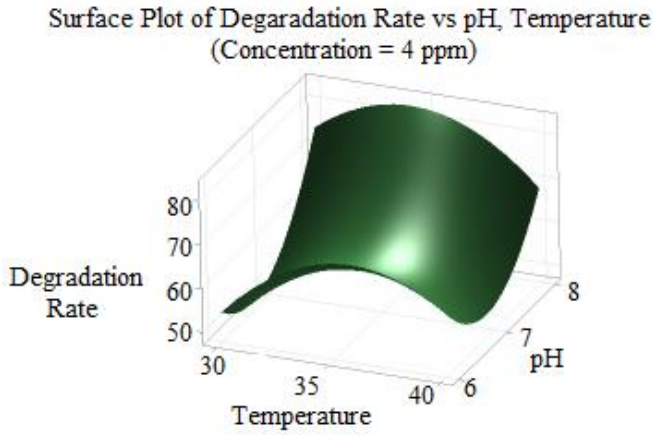
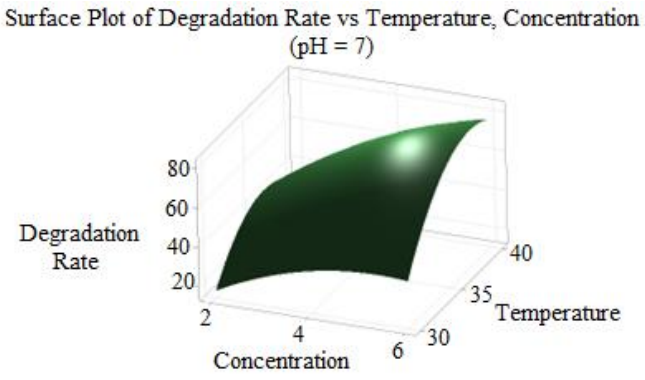


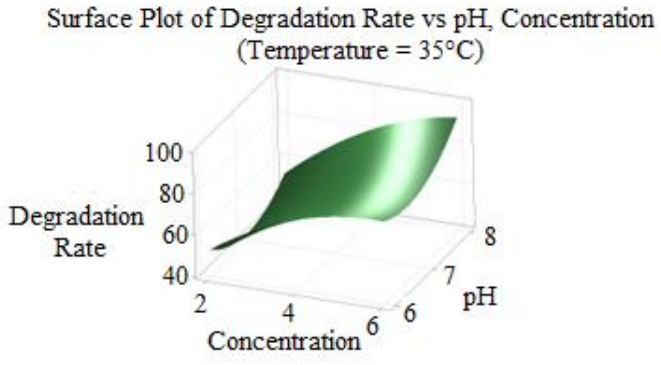
c.

Figure 4.12 Surface plots of ZK101 for biodegradation under different conditions

The response surface plots (Figures 4.12 and 4.13) are curved because the model contains quadratic terms that are statistically significant. The Pareto chart (Figure 4.11a) showed that concentration and pH were significant for ZK101. The highest percentage of biodegradation is in the upper right corner of the surface plot (Figure 4.12b), which corresponds with high CYP concentration and pH value values. The lowest degradation percentage is in the lower-left corner of the plot, which corresponds with low values of both concentration and pH. The third independent variable, temperature, is not shown in the plot. Minitab software keeps the temperature value constant at 35°C when calculating the fitted response values of the degradation percentage of CYP.



a.



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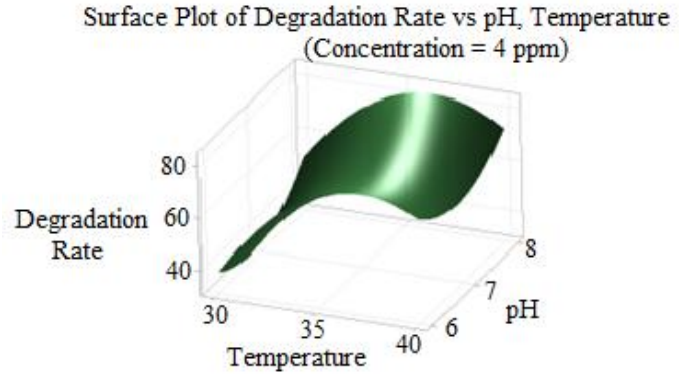


c.

Figure 4.13 Surface plots of ZK102 for biodegradation under different conditions

The Pareto chart (Figure 4.11b) showed that concentration and temperature were significant for ZK102. The highest values of the percentage of CYP biodegradation are in the upper right corner of the surface plot (Figure 4.13a), which corresponds with high values of both initial CYP concentrations and incubation temperature. The lowest degradation percentage is in the lower-left corner of the plot, which corresponds with low values of both concentration and temperature. The third independent variable, pH, is not shown in the plot. Minitab software keeps the pH value constant at 7 when calculating the fitted response values of the degradation percentage of CYP.

According to previous studies, the optimum temperature and pH for CYP degradation were 30°C and 7 (Ma et al., 2013; Tallur et al., 2008; Lin et al., 2011) the initial CYP concentration was 100 mg. L^{-1}. However, in this study, ZK101 and ZK102 degraded CYP efficiently at 33.9 and 38.1 °C higher than in other studies, respectively. These isolates also degraded CYP around a neutral pH range like other strains. This could be explained by the fact that both strains have been related to pathogenic activities and could grow better at higher temperatures than environmental strains.

Box-Behnken experimental design results show that ZK101 and ZK102 degraded CYP efficiently and converted it to the 3PBA. With this experiment, optimum incubation conditions with optimum concentration of commercial-grade CYP were estimated.

4.5 Enzyme and Protein Study Result

Microbial cleavage and detoxification of CYP occur because of the hydrolysis of the carboxyl ester linkage of the CYP by carboxylesterase, aminopeptidase, or esterase. Another breakdown mechanism involving dichlorination, oxidation, or hydrolysis has also been reported in other studies. This study clearly showed that the isolated ZK101 and ZK102 broke down the CYP carboxyl ester linkage and so used commercial-CYP as a carbon source.

ZK101 and ZK102 produced an enzyme to degrade CYP by cleavage of the ester bond. To date, a few studies purified CYP degrading enzymes and found K_m and V_{max} constants. For example, esterase (66.4 kDa) from *Bacillus licheniformis* B1, aminopeptidase (53 kDa) from *Pseudomonas aeruginosa* GF31, and monooxygenase (41 kDa) from *Streptomyces* sp, Pyrethroid hydrolase *PytZ* (25KDa) from *O. anthropi* YZ-1, *PytH* (31KDa) from *S.* JZ-1 and *S.* JZ-2, Pyrethroid hydrolyzing esterase *pye3* (31kDa), *EstP* (73 kDa) from *K.* sp. ZD112 Aminopeptidase 53 kDa, Esterase (26 kDa) from *B. subtilis* 1D, and Phenol hydroxylase (41KDa) were purified (Chen et al., 2013; Tang et al., 2017; M. Zhang et al., 2021). With monooxygenase enzyme, CYP was converted to the 2-(4-hydroxy phenoxy) benzoic methyl ester then phenol and benzoic acid derivatives were produced, and 3PBA was not formed. However, with both esterase and aminopeptidase, CYP was firstly converted to cyano-3-phenoxybenzyl alcohol and 3PBA. Figure 4.14 depicted some biodegradation pathways of CYP with initial enzymes.

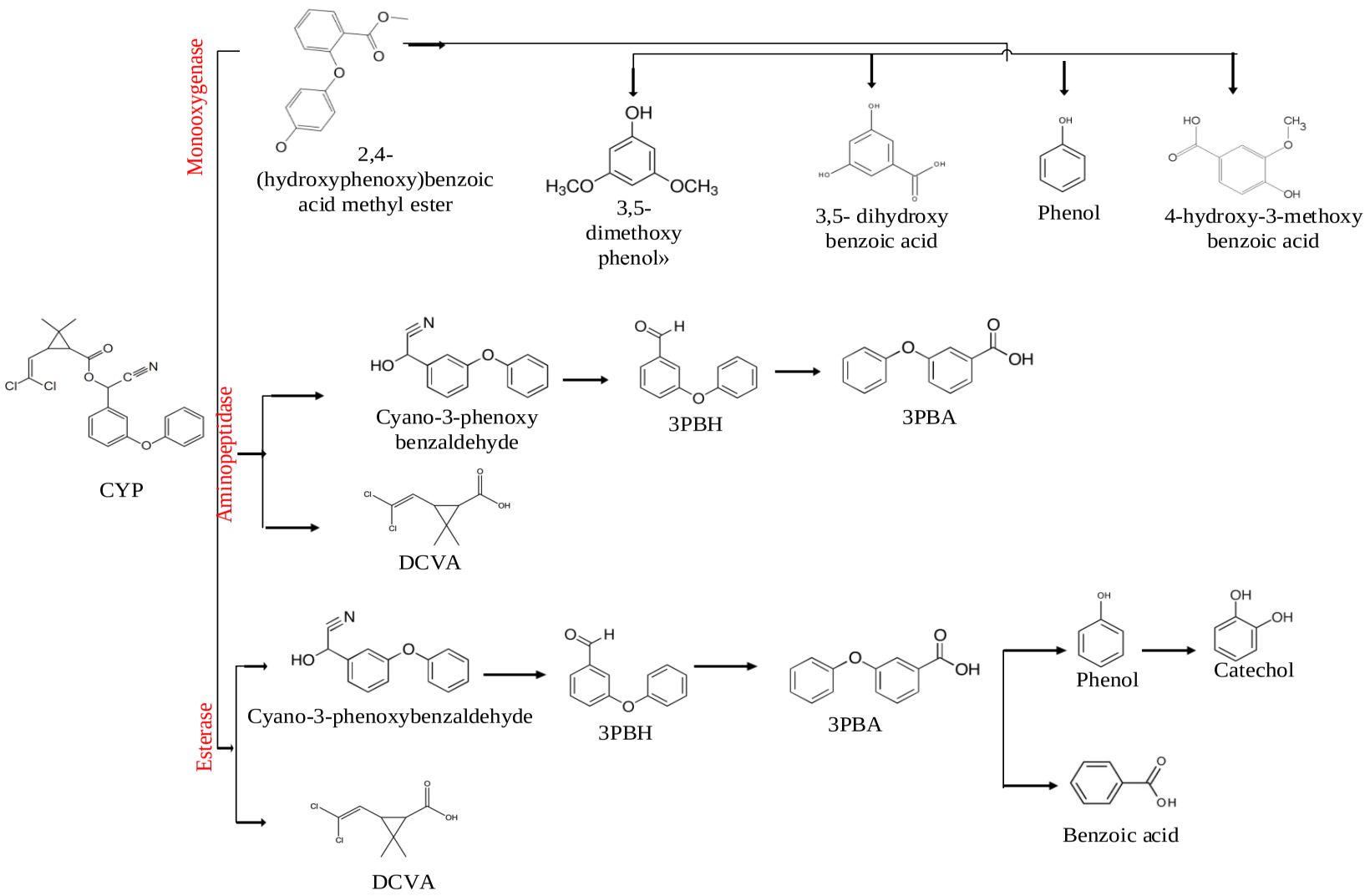
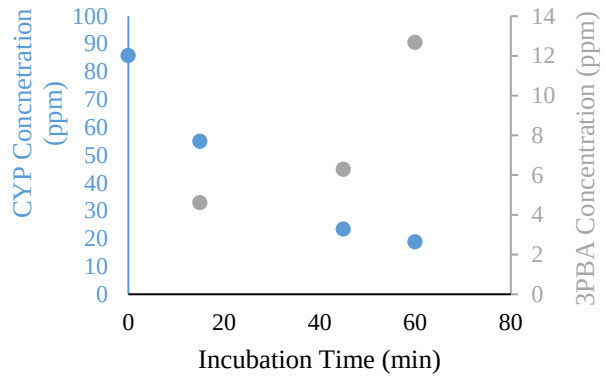


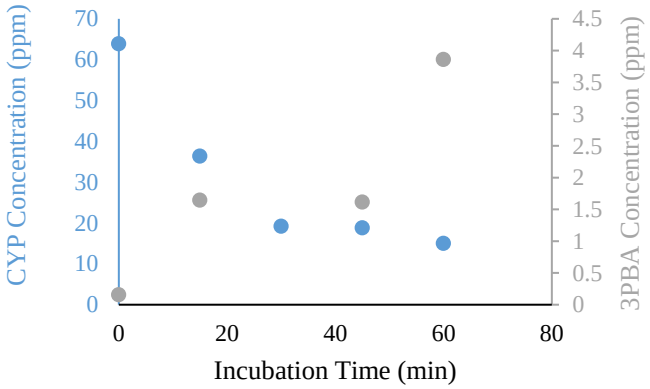
Figure 4.14 CYP degradation pathways by monooxygenase, aminopeptidase and esterase

In the study, enzyme kinetics need to be investigated to understand the enzyme mechanisms produced by ZK101 and ZK102.

Enzyme kinetics of ZK101 and ZK102 were studied in both CYP and 3PBA medium in PBS, and analyses were performed in a one-hour incubation time on an incubator at 30C°. HPLC analysis showed that CYP concentration was decreasing while 3PBA formation was increasing. Figure 4.15 shows both CYP and 3PBA concentration changes over time.



a.



b.

Figure 4.15 CYP degradation and 3PBA production with crude cell extracts' enzymes. (a) ZK101, (b) ZK102
(● ; CYP, ● ; 3PBA)

For enzyme kinetics, Lineweaver Burk plots (Figure 4.16) were created. K_m and V_{max} were calculated for ZK101 and ZK102, 8.364 μM, 4.26 min, and 19.579 μM, 21.48 min, respectively. That means, ZK102 can degrade commercial-grade CYP more effectively than ZK101 due to having higher K_m and V_{max} constants. Also, Lin et al. (2011) found K_m and V_{max} for both CYP and 3PBA 6.418 μmol.ml^{-1} and 1.263 min and 5.186 μmol.ml^{-1} and 0.831 min, respectively. The study concluded that CYP was degraded more efficiently than 3PBA. Protein assay was applied with the Lowry method, and it was found as 0.062 and 4.956 μg. ml^{-1} for ZK101 and ZK102 induced cells. For non-induced ZK101 and ZK102, it was measured as 2.559 and 2.562 μg. ml^{-1}, respectively.

Acinetobacter calcoaceticus MCm5, *Brevibacillus parabrevis* FCm9, and *Sphingomonas* sp. RCm6 induced total protein amounts were 178.2, 211.4, and 195.5 μg. ml^{-1}. The carboxylesterase and 3PBA dioxygenase were purified from these strains. The specific activities of carboxylesterase were 0.89, 0.911, and 0.879 units per mg protein, and the specific activities of 3PBA dioxygenase were 2.012, 2.220, and 1.930 units per mg protein, for MCm5, FCm9, and RCm6, respectively (Akbar et al., 2015b). The higher units. mg protein^{-1} showed 3PBA did not accumulate by these strains. Moreover, the specific activity of 3PBA dioxygenase (0.341) was higher than esterase (0.100) for Micrococcus sp CPN1, so the strain is a good candidate for removal of CYP and 3PBA (Tallur et al., 2008). The specific activities of esterase and 3PBA dioxygenase produced by strain ZK101 and ZK102 were 1.613 and 20.968 for ZK101 and 0.010 and 0.182 for ZK102, respectively. These protein results said that CYP degradation was slower than 3PBA degradation.

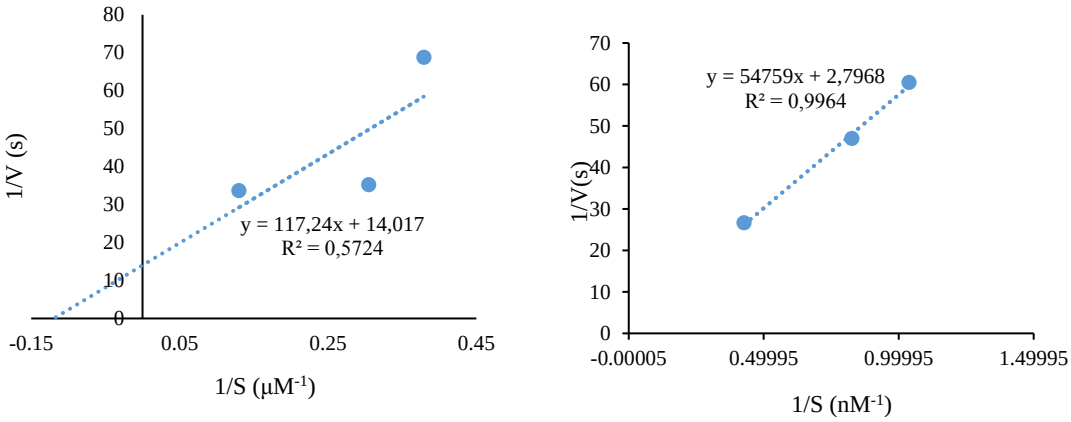
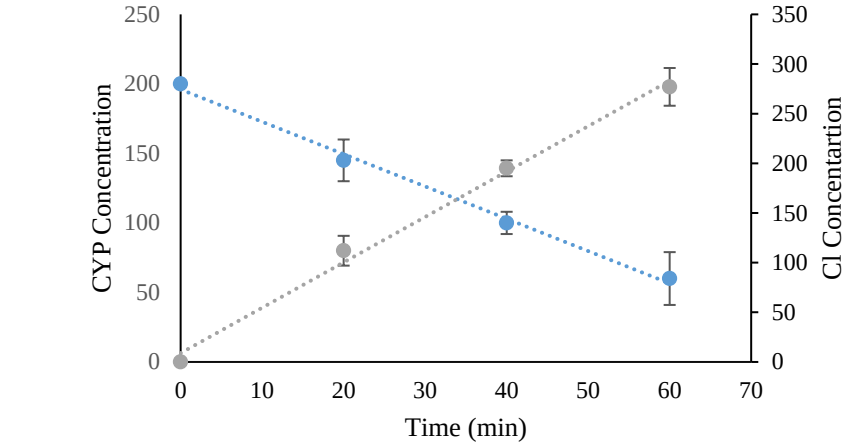


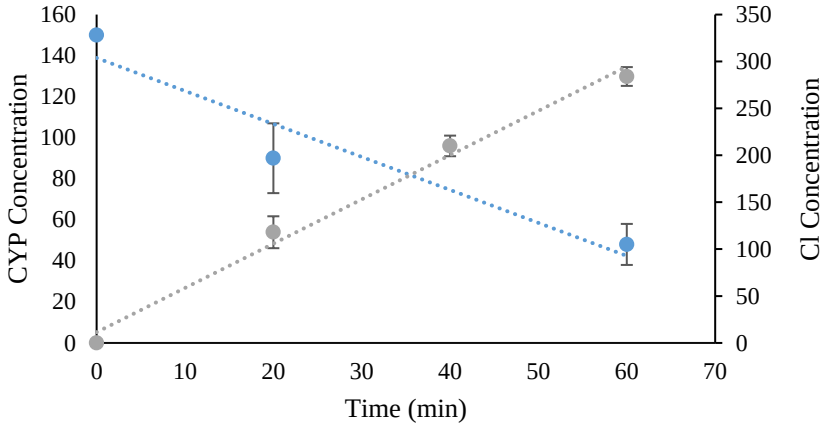
Figure 4.16. Lineweaver plots for ZK101 and ZK102

After finding the kinetic constants of enzymes, SDS-PAGE analysis was performed to find the size of the protein. To date, studies found the enzymes responsible for the degradation of CYP. Esterase from *Bacillus* sp (66.4 kDa), aminopeptidase from *Pseudomonas aeruginosa* GF31(53 kDa), monooxygenase from *Streptomyces* sp (41 kDa), carboxylesterase from *Sphingobium* sp. strain JZ-1 (31 kDa), 3PBA dioxygenase from *Sphingobium wenxiniae* JZ-1 (27.7 kDa), and phenol hydrolase from *Bacillus thermoglucosidasius* A7 (57 kDa) (Chen et al., 2013; Tang et al., 2007; Cheng et al., 2015; Kirchner et al., 2003).

Further dichlorination of CYP was studied with enzyme extracts. Cl study results were shown in Figure 4.17, and it showed that while 1 mol CYP degraded 2 mol Cl was produced. This would be consistent with the dichlorination and mineralization of the CYP degradation byproduct DCVA



a.



b.

Figure 4.17 Cl production with CYP degradation

The SDS-PAGE analysis (Figure 4.18) showed enzymes produced by ZK101 and ZK102. For ZK101 eight bands between 15-50 kDa (17, 22, 23, 27, 35, 37, 43, 66 kDa) and for ZK102 four bands between 15-50 kDa (17,23, 34, 37 kDa) were observed.

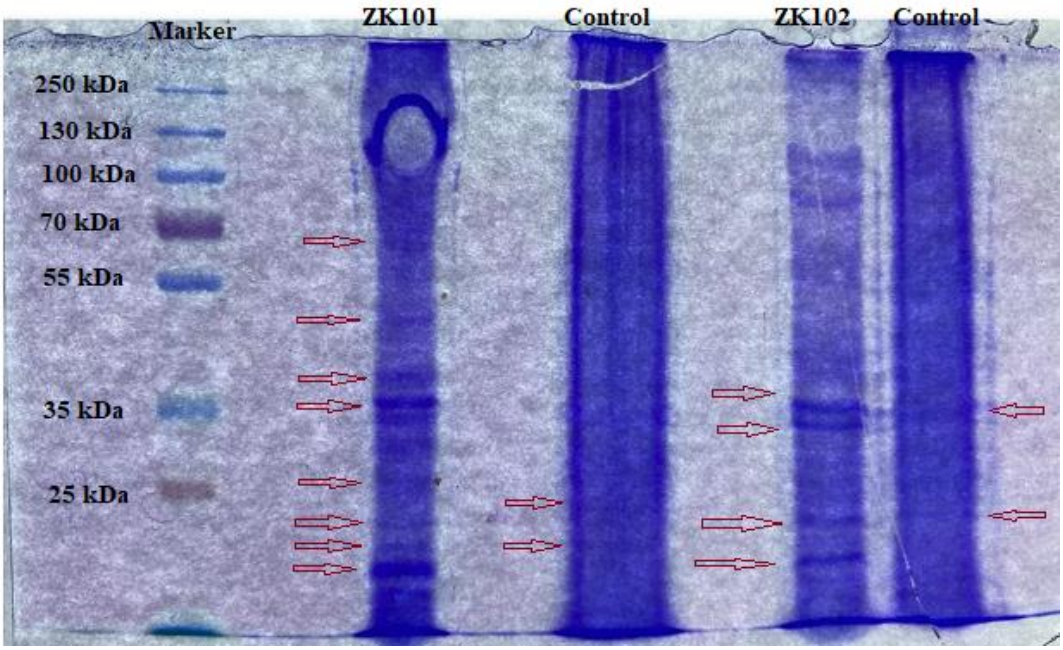


Figure 4.18 SDS-PAGE profile of ZK101 and ZK102 cell-free extracts

Figure 4.15 explain the CYP degradation pathways started by three enzymes, this study found the size of the enzymes that break down the ester bond was closer to aminopeptidase and esterase for ZK101 and ZK102, respectively. In addition, other enzymes in the biodegradation pathways can be phenoxy benzaldehyde dehydrogenase, 3PBA dioxygenase, 3-Phenoxybenzoate dioxygenase, and 3PBA hydroxylase, protocatechuate-3, 4-dioxygenase.

4.6 Stoichiometric Calculation and Oxygen Uptake Results

The oxygen uptake experiments were done to see the oxygen consumption rates of the responsible enzymes. The whole cells of ZK101 and ZK102 oxygen uptake rates showed that cells used oxygen while CYP, 3PBA, and Phenol degradation. The cell extracts results showed that oxygen does not require CYP degradation but 3PBA and Phenol degradation oxygen is required. ZK101 used 2.7±0.2 and 3.2±1.2 μmoles of O_2. μg min^{-1} for 3PBA and Phenol, respectively. ZK102 used

7.6±0.9 and 5.3±0.8 μmoles of O_2. μg min^{-1} for 3PBA and Phenol, respectively. The amount of oxygen consumed per minute was tabulated in Table 4.7

Table 4.7 Oxygen uptake of CYP degrading ZK101 and ZK102 (μmoles of O_2/min/μg), measurements performed in duplicates

Assay substrate/culture [a]	LB grown whole cells	CYP grown whole cells	LB grown cell extract	CYP grown cell extract
CYP/ ZK101	ND[b]	11.8 ± 5.1	ND	ND
CYP/ ZK102	ND	6.4±1.1	ND	ND
3PBA/ ZK101	ND	1.6± 0.8	ND	2.7±0.2
3PBA/ ZK102	ND	4.2±1.0	ND	7.6±0.9
Phenol/ ZK101	0.3±0.1	2.4±0.3	0.9±0.2	3.2±1.2
Phenol/ ZK102	1.2±0.1	3.6±0.7	1.7±0.2	5.3±0.8

[a] The experiment was performed with 120nM of total substrate concentration

[b]ND: not detected

To see the needed amount of nitrogen and oxygen for degradation, stoichiometric balance in the microbial oxidation reaction was performed (Eq. (1)). Bacterial cultures use half of the CYP for biomass ($C_5H_7NO_2$) and the other half is used for oxygen consumption, and the theoretically found stoichiometric balance of the reaction was shown in Eq. (2,3,4).

$$a\ (Substrate) + b(NH_3) + c(O_2) \rightarrow d(C_5H_7NO_2) + e(CO_2) + f(H_2O) \quad \text{Eq. (1)}$$

$$3\ (C_{22}H_{19}Cl_2NO_3) + 8(NH_3) + 18(O_2) \rightarrow 11(C_5H_7NO_2) + 11(CO_2) + (H_2O) \quad \text{Eq. (2)}$$

$$3\ (C_{13}H_{10}O_3) + 6.5(NH_3) + 9.5(O_2) \rightarrow 6.5(C_5H_7NO_2) + 6.5(CO_2) + 2(H_2O) \quad \text{Eq. (3)}$$

$$3\,(C_6H_6O) + 3(NH_3) + 6(O_2) \rightarrow 3(C_5H_7NO_2) + 3(CO_2) + 3(H_2O) \quad \text{Eq. (4)}$$

The bacterial cultures consume 6 mol of oxygen per CYP, 3.33 moles of oxygen per 3PBA, and 3 moles of oxygen per Phenol needed. The exact amount of oxygen needed by whole cells was calculated and shown in Table 4.8. The experimental results were similar to the theoretically found stoichiometric ratio.

Table 4.8 Stoichiometry of resveratrol degrading ZK101 and ZK102, measurements performed in duplicates

Chemical/ culture	LB grown whole cells	CYP grown whole cells
CYP/ ZK101	ND[b]	5.93±0.22
CYP/ ZK102	ND[b]	5.15±0.11
3PBA/ ZK101	ND[b]	4.73±0.19
3PBA/ ZK102	ND[b]	4.33±0.28
Phenol/ ZK101	NM[c]	2.42±0.13
Phenol/ ZK102	NM	2.29±0.05
LB/ ZK101	4.93±0.62	NM
LB/ ZK102	5.26±0.17	NM

[b]ND: not detected

[c]NM: not measured

4.7 Discussion

CYP has been in use for decades for many purposes like agriculture or domestic insect control, however, CYP and its some metabolites have toxicity to non-target organisms, so elimination of CYP from the environment is important. In this study,

CYP degrading two new bacterial strains were isolated from a fig tree farm which had a long history of several pyrethroids and other pesticides application. The reason behind not using previously found strains was that isolating strains from agricultural farms were heavily CYP used, and to see what the consequences of will be using CYP in the farms.

The main purpose of the study was to find the biodegradation pathway of commercial-grade CYP, so all experiments were designed to step by step to find the degradation pathway.

Firstly, pure bacterial strains were isolated from the soil by Enrichment method for further studies. 16s rDNA results showed that *Enterobacter* (99.9%) and *Stenotrophomonas* (100%) species are responsible for the degradation of commercial-grade CYP. ZK101 is a gram-negative, rod-shaped, facultatively anaerobic, non-spore-forming, flagella-containing, urease-positive, and lactose fermenting species. ZK102 is a gram-negative bacillus, aerobic and non-fermenting species. However, both species cause many nosocomial infections. It is the first report about ZK101 CYP degradation, and only Chen et al. (2011) showed that ZK102 can degrade CYP.

ZK101 and ZK102 showed the capability of degradation of both commercial-grade CYP and 3PBA. Strain ZK101 degraded 57.2%, 65.4% and 91.5% of 1, 2 and 4 mg. L^{-1} commercial-grade CYP within 6 days of incubation. Strain ZK102 degraded 63.7%, 67.9% and 79.7% of 1, 2 and 4 mg. L^{-1} commercial-grade CYP in 6 days. The differences in the biodegradation rates of ZK101 and ZK102 are caused by the secretion of different enzymes.

Moreover, in the optimization studies, 4 mg. L^{-1} commercial-grade CYP was mixed with ZK101 and ZK102 in two different flasks and flasks were incubated at 40 °C and pH 8 for 5 days. After, 75.8% and 76.6% of CYP were degraded by ZK101 and ZK102, respectively. Unlike the previously found CYP degrading bacteria, ZK101 and ZK102 degraded lower CYP concentrations for a longer time. For example, *Ochrobactrumanthropi* JCm1 degraded 90.5% of 100 mg. L^{-1} CYP in 10 days (Akbar et al., 2015b), *Bacillus thuringiensis* strain ZS-19 degraded 80.8% of 100 mg. L^{-1}

CYP in 72 h (Chen et al., 2014), *Streptomyces aureus* HP-S-01 degraded 87.8% of 50 mg. L^{-1} CYP in 10 days (Chen et al., 2012b).

ZK101 and ZK102 also degraded 3PBA. Strain ZK101 and ZK102 degraded 49.4% and 48.9% of 4 mg. L^{-1} 3PBA in 5 days, lower than CYP degradation. *Streptomyces aureus* HP-S-01 degraded 79.3% of 50 mg. L^{-1} 3PBA in 10 days (Chen et al., 2012b). *Acinetobacter calcoaceticus* MCm5 degraded 84.5% of 100 mg. L^{-1} 3PBA, *Brevibacillus parabrevis* FCm9 degraded 100% of 100 mg. L^{-1} 3PBA, and *Sphingomonas* sp. RCm6 degraded 86.8% of 100 mg. L^{-1} 3PBA in 10 days (Akbar et al., 2015b). The kinetic results of two strains and comparing results with previously found strains showed that ZK101 and ZK102 need more time for the complete removal of CYP and 3PBA. The specific activities of CYP and 3PBA degradation enzymes proved that 3PBA in the MSM supplemented with commercial-grade CYP did not accumulate, however, in the enrichment culture (MSM with soil) 3PBA accumulated. That is important because in the agricultural field CYP is applied more than once a year and causes a risk of 3PBA accumulation. That is the study knows that CYP is converted to 3PBA by bacterial enzymes.

Chlorination studies showed dichlorination of CYP i.e., two moles of chlorine were produced per mol of CYP.

Oxygen uptake studies showed that whole cells of cultures needed oxygen for CYP, 3PBA, and Phenol degradation that is because there are cofactors in whole cells. However, cell extracts showed that CYP did not require oxygen for degradation, unlike 3PBA and Phenol so the initial enzymes responsible for the breakage of the ester bond in CYP do not need oxygen to initiate degradation. Moreover, theoretically found and calculated stoichiometric rate of oxygen gave the same results. Theoretically found rates were 6, 3.33, and 3 for CYP, 3PBA, and Phenol, respectively. Calculated rates were approximately 6, 4, and 2 for CYP, 3PBA, and Phenol, respectively.

Esterase is widely distributed in animals, plants, and microorganisms. Enzyme activity assay and SDS-PAGE showed that CYP degradation enzymes were inducible because it shows activity when exposed to CYP or its metabolites.

Aminopeptidase, Esterase, monooxygenase, and carboxylesterase (a subgroup of esterase) play an important role in the initial conversion of CYP through cleavage of the ester bond. Moreover, carboxylesterase, dioxygenase, and phenol hydrolase are aerobic enzymes. Up to now, Pyrethroid hydrolase *PytZ* (25KDa) from *Ochrobactrum anthropi* YZ-1 (Zhai et al., 2012), *PytH* (31KDa) from *Sphingobium* JZ-1 and *Sphingobium* JZ-2 (Wang et al., 2009; Gu et al., 2009), pyrethroid hydrolyzing esterase *pye3* (31kDa) from mutagenic DNA (Li et al., 2008), esterase *EstP* (73 kDa) from *Klebsiella* sp. ZD112 (Wu et al., 2006) and esterase (26 kDa) from *B. subtilis* 1D (Gangola et al., 2018) have been purified. Moreover, Phenol hydroxylase (41KDa) and Catechol-1,2-dioxygenase (34KDa) have been purified. (Kirchner et al., 2003). The enzymes operate optimally between 25-40 °C and pH between 6 to 8 (Wang et al., 2009). In the SDS-PAGE analysis for ZK101 and ZK102, eight bands between 35-50 kDa and four bands between 15-35 kDa were observed, respectively.

Considering all these findings a biodegradation pathway was proposed and showed that there could be two different pathways for CYP degradation in two different strains isolated from the same areas (Figure 4.19). Carboxylesterase converted CYP to cyano-3-phenoxybenzyl alcohol and DCVA with 1:1 ratio. Then 3PBH and 3PBA were formed. Aminopeptidase and esterase also converted CYP to cyano-3-phenoxybenzyl alcohol and DCVA. The study detection of 3PBH, 3PBA, phenol, and catechol by ZK101 and ZK102 is consistent with pathways of CYP found by Bhatt et al. (2020) and Tallur et al. (2008).

In the predicted pathway, CYP is firstly converted to DCVA and cyano-3-phenoxy benzaldehyde by the activity of aminopeptidase and esterase produced by ZK101 and ZK102, respectively. DCVA is reported as a non-harmful chemical for humans and it completely mineralized when it was formed, and DCVA was converted to CO_2, H_2O, and some other chemicals (Sundaram et al.,2013). Cyano-3-phenoxy benzaldehyde is an unstable chemical and produces 3PBH, and then 3PBA was produced from 3PBH by oxidization reaction. After 3PBA formation, oxidization, and cleavage of diaryl ether formed phenol.

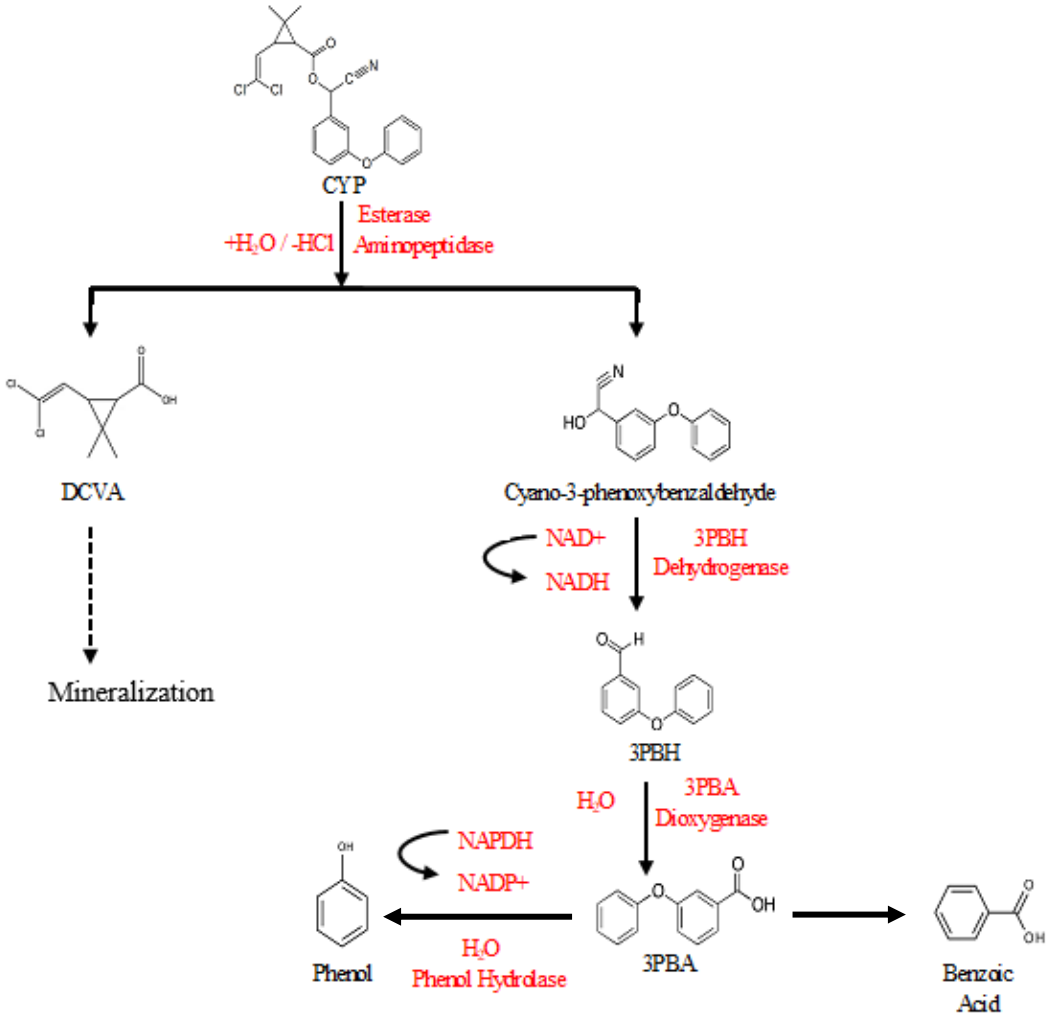


Figure 4.19 Proposed degradation pathway of commercial-grade CYP by esterase and carboxylesterase

CHAPTER 5

CONCLUSION

In the study, CYP biodegradation mechanisms were investigated. To be able to test CYP biodegradation HPLC was used, and two different methods with two different columns were applied. The first method was applied with Eclipse plus C18 column (100, 4.60 mm, 3.5 μm), and CYP and 3PBA were detected around 6.095 and 2.427 min, respectively. The second method was applied with Ascentis C18 column 5μm particle size and 150 x 4.6 mm, and CYP and 3PBA were observed around 3.2 and 2.8min, respectively. The reason behind using two columns was to see one peak for CYP means all isomers of commercial-grade CYP with one peak to discriminate other metabolites from isomers of CYP.

With the enrichment method, the two novel strains ZK101 from *Enterobacter* genus and ZK102 from the *Stenotrophomonas* genus were isolated from heavily CYP used agricultural area located in Adana. The phylogenetic tree was created with MEGAX software to compare two novel strains with previously isolated CYP degraders. Kinetic studies showed that ZK101 and ZK102 can degrade CYP efficiently and convert it to 3PBA. The RSM experiment found optimum conditions (initial CYP concentration, incubation temperature, and pH) for biodegradation of CYP, and variables were analyzed with Minitab software. According to the quadratic polynomial equations found by RSM, the strain ZK101 degraded 5.69 mg. L^{-1} CYP was efficiently at 33.9°C, and pH 6.66, and strain ZK102 degraded 6.62 mg. L^{-1} CYP at 38.1 °C and pH 6.66 efficiently. Under these optimum conditions, ZK101 and ZK102 degraded 67.7% (5.69 mg. L^{-1}) and 81.9% (6.62 mg. L^{-1}) of CYP, respectively.

The previously purified enzymes from CYP degrading bacteria showed that initial enzymes can be aminopeptidase, carboxylesterase, esterase, or monooxygenase.

This study found that 3PBA formation and dichlorination so the initial enzyme could not be monooxygenase. The oxygen uptake rate of cell extracts showed that oxygen did not require CYP degradation, unlike 3PBA and Phenol. The stoichiometric calculations of oxygen for degradation of one mole of CYP, 3PBA, and phenol are 6, 3.33, and 3 moles. The calculations also found that approximately 6, 4, and 2 moles of oxygen were consumed per mole of CYP, 3PBA, and phenol, respectively. The chlorination and oxygen uptake rate studies showed that enzymes could be aminopeptidase or esterase. Moreover, SDS-PAGE results showed that aminopeptidase and esterase are the responsible enzymes to initiate degradation.

In the proposed biodegradation pathway CYP was converted to 3PBH through cleavage of the ester bond. Then, 3PBA was produced by phenoxy benzaldehyde dehydrogenase through an oxidization reaction. 3PBA was converted to phenol, and phenol was converted into catechol.

LIMITATIONS AND RECOMMENDATIONS

During enrichment culture, the CYP should be added when the concentration is higher than 20 mg. L^{-1} to prevent bacterial shut down due to limited carbon source, and measurement should be done after one day of feeding.

During the kinetic analysis of CYP and enzyme studies, adjusting the concentration of CYP was challenging due to low water solubility, so stock solutions with a higher concentration of substrate should be prepared.

To date, many biodegradation pathways of CYP by bacterial strains were proposed and a few studies were found exact degradation pathways. However, the studies related to specific activities of enzymes are very rare. Knowing enzyme properties is important to see whether metabolites from the mother compound accumulate or not.

ZK101 and ZK102 are efficient in CYP degradation in the agricultural field. However, CYP metabolite 3PBA accumulated in the soil can cause neurotoxicity and endocrine disruption. For that reason, these two strains can be used in the field to remove the toxic and harmful effects of CYP. However, Enterobacter and Stenotrophomonas genus were reported as pathogenic so whole-genome sequences should be applied to see if the pathogenic genes are present in ZK101 and ZK102. In addition, disc experiments also need to be applied to see whether ZK101 and ZK102 have antimicrobial resistance or not. If they have antimicrobial resistance, two strains can not be used in the field.

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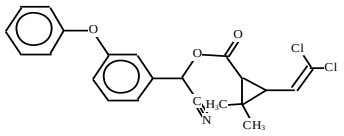
APPENDICES

A. Appendix I- EPI Suite Results

EPI Suite Results For CAS 052315-07-8



SMILES : CLC(CL)=CC1C(C)(C)C1C(=O)OC(C(#N))c2cccc(Oc3ccccc3)c2

CHEM : **Cypermethrin**

MOL FOR: C22 H19 CL2 N1 O3

MOL WT : 416.31

---------- EPI SUMMARY (v4.11) ----------

Physical Property Inputs:

Log Kow (octanol-water): ------

Boiling Point (deg C) : ------

Melting Point (deg C) : ------

Vapor Pressure (mm Hg) : ------

Water Solubility (mg/L): ------

Henry LC (atm-m3/mole) : ------

Log Octanol-Water Partition Coef (SRC):

Log Kow (KOWWIN v1.68 estimate) = 6.38

Log Kow (Exper. database match) = 6.60

Exper. Ref: TOMLIN,C (1997)

Log Kow (Exper. database match) = 6.05

Exper. Ref: SANGSTER (1993)

Log Kow (Exper. database match) = 6.06

Exper. Ref: SANGSTER (1993)

Log Kow (Exper. database match) = 6.94

Exper. Ref: TOMLIN,C (1997)

Boiling Pt, Melting Pt, Vapor Pressure Estimations (MPBPVP v1.43):

Boiling Pt (deg C): 450.48 (Adapted Stein & Brown method)

Melting Pt (deg C): 82.07 (Mean or Weighted MP)

VP(mm Hg,25 deg C): 1.3E-007 (Modified Grain method)

VP (Pa, 25 deg C) : 1.74E-005 (Modified Grain method)

MP (exp database): 84 deg C

BP (exp database): 200 @ 0.07 mm Hg deg C

VP (exp database): 1.30E-03 mm Hg (1.73E-001 Pa) at 20 deg C

Subcooled liquid VP: 0.00498 mm Hg (20 deg C, exp database VP)

: 0.664 Pa (20 deg C, exp database VP)

Water Solubility Estimate from Log Kow (WSKOW v1.42):

Water Solubility at 25 deg C (mg/L): 0.001559

log Kow used: 6.94 (expkow database)

no-melting pt equation used

Water Sol (Exper. database match) = 0.004 mg/L (20 deg C)

Exper. Ref: WAUCHOPE,RD ET AL. (1991A)

Water Sol (Exper. database match) = 0.01 mg/L (25 deg C)

Exper. Ref: TOMLIN,C (1997)

Water Sol (Exper. database match) = 0.115 mg/L (25 deg C)
Exper. Ref: TOMLIN,C (2003); pH7

Water Sol Estimate from Fragments:
Wat Sol (v1.01 est) = 0.016524 mg/L

ECOSAR Class Program (ECOSAR v1.11):
Class(es) found:
Esters
Vinyl/Allyl Halides
Benzyl Nitriles
Pyrethroids

Henrys Law Constant (25 deg C) [HENRYWIN v3.20]:
Bond Method : 7.89E-007 atm-m3/mole (8.00E-002 Pa-m3/mole)
Group Method: Incomplete
Exper Database: 4.20E-07 atm-m3/mole (4.26E-002 Pa-m3/mole)
For Henry LC Comparison Purposes:
User-Entered Henry LC: not entered
Henrys LC [via VP/WSol estimate using User-Entered or Estimated values]:
HLC: 4.568E-005 atm-m3/mole (4.628E+000 Pa-m3/mole)
VP: 1.3E-007 mm Hg (source: MPBPVP)
WS: 0.00156 mg/L (source: WSKOWWIN)

Log Octanol-Air Partition Coefficient (25 deg C) [KOAWIN v1.10]:
Log Kow used: 6.94 (exp database)
Log Kaw used: -4.765 (exp database)
Log Koa (KOAWIN v1.10 estimate): 11.705
Log Koa (experimental database): None

Probability of Rapid Biodegradation (BIOWIN v4.10):

Biowin1 (Linear Model) : 0.8838

Biowin2 (Non-Linear Model) : 0.9883

Expert Survey Biodegradation Results:

Biowin3 (Ultimate Survey Model): 1.7424 (recalcitrant)

Biowin4 (Primary Survey Model) : 3.1382 (weeks)

MITI Biodegradation Probability:

Biowin5 (MITI Linear Model) : 0.2501

Biowin6 (MITI Non-Linear Model): 0.0054

Anaerobic Biodegradation Probability:

Biowin7 (Anaerobic Linear Model): 0.0610

Ready Biodegradability Prediction: NO

Hydrocarbon Biodegradation (BioHCwin v1.01):

Structure incompatible with current estimation method!

Sorption to aerosols (25 Dec C)[AEROWIN v1.00]:

Vapor pressure (liquid/subcooled): 0.664 Pa (0.00498 mm Hg)

Log Koa (Koawin est): 11.705

Kp (particle/gas partition coef. (m3/ug)):

Mackay model : 4.52E-006

Octanol/air (Koa) model: 0.124

Fraction sorbed to airborne particulates (phi):

Junge-Pankow model : 0.000163

Mackay model : 0.000361

Octanol/air (Koa) model: 0.909

Atmospheric Oxidation (25 deg C) [AopWin v1.92]:

Hydroxyl Radicals Reaction:

OVERALL OH Rate Constant = 21.4274 E-12 cm3/molecule-sec

Half-Life = 0.499 Days (12-hr day; 1.5E6 OH/cm3)

Half-Life = 5.990 Hrs

Ozone Reaction:

OVERALL Ozone Rate Constant = 0.023261 E-17 cm3/molecule-sec

Half-Life = 49.268 Days (at 7E11 mol/cm3)

Fraction sorbed to airborne particulates (phi):

0.000262 (Junge-Pankow, Mackay avg)

0.909 (Koa method)

Note: the sorbed fraction may be resistant to atmospheric oxidation

Soil Adsorption Coefficient (KOCWIN v2.00):

Koc : 7.975E+004 L/kg (MCI method)

Log Koc: 4.902 (MCI method)

Koc : 1.401E+005 L/kg (Kow method)

Log Koc: 5.146 (Kow method)

Experimental Log Koc: 5 (database)

Aqueous Base/Acid-Catalyzed Hydrolysis (25 deg C) [HYDROWIN v2.00]:

Total Kb for pH > 8 at 25 deg C : 6.124E-003 L/mol-sec

Kb Half-Life at pH 8: 3.587 years

Kb Half-Life at pH 7: 35.867 years

(Total Kb applies only to esters, carbmates, alkyl halides)

Bioaccumulation Estimates (BCFBAF v3.01):

Log BCF from regression-based method = 2.987 (BCF = 970.3 L/kg wet-wt)

Log Biotransformation Half-life (HL) = 0.3658 days (HL = 2.322 days)

Log BCF Arnot-Gobas method (upper trophic) = 2.850 (BCF = 707.8)

Log BAF Arnot-Gobas method (upper trophic) = 3.816 (BAF = 6542)
log Kow used: 6.94 (expkow database)

Volatilization from Water:
Henry LC: 4.2E-007 atm-m3/mole (Henry experimental database)
Half-Life from Model River: 2846 hours (118.6 days)
Half-Life from Model Lake : 3.122E+004 hours (1301 days)

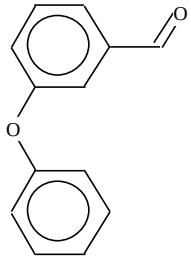
Removal In Wastewater Treatment:
Total removal: 93.82 percent
Total biodegradation: 0.78 percent
Total sludge adsorption: 93.04 percent
Total to Air: 0.00 percent
(using 10000 hr Bio P,A,S)

Level III Fugacity Model:

Mass Amount (percent)	Half-Life (hr)	Emissions (kg/hr)
Air 0.0537	11.9	1000
Water 3.53	4.32e+003	1000
Soil 73.3	8.64e+003	1000
Sediment 23.2	3.89e+004	0

Persistence Time: 6.89e+003 hr

EPI Suite Results For CAS 039515-51-0



SMILES : O=Cc(cc(Oc(cccc1)c1)cc2)c2

CHEM : **Benzaldehyde, 3-phenoxy-**

MOL FOR: C13 H10 O2

MOL WT : 198.22

---------- EPI SUMMARY (v4.11) ----------

Physical Property Inputs:

Log Kow (octanol-water): ------

Boiling Point (deg C) : ------

Melting Point (deg C) : ------

Vapor Pressure (mm Hg) : ------

Water Solubility (mg/L): ------

Henry LC (atm-m3/mole) : ------

Log Octanol-Water Partition Coef (SRC):

Log Kow (KOWWIN v1.68 estimate) = 3.77

Log Kow (Exper. database match) = 3.38

Exper. Ref: HANSCH,C ET AL. (1995)

Boiling Pt, Melting Pt, Vapor Pressure Estimations (MPBPVP v1.43):
Boiling Pt (deg C): 322.15 (Adapted Stein & Brown method)
Melting Pt (deg C): 83.57 (Mean or Weighted MP)
VP(mm Hg,25 deg C): 0.000164 (Modified Grain method)
VP (Pa, 25 deg C) : 0.0219 (Modified Grain method)
BP (exp database): 140 @ 0.1 mm Hg deg C
Subcooled liquid VP: 0.000597 mm Hg (25 deg C, Mod-Grain method)
: 0.0796 Pa (25 deg C, Mod-Grain method)

Water Solubility Estimate from Log Kow (WSKOW v1.42):
Water Solubility at 25 deg C (mg/L): 58.02
log Kow used: 3.38 (expkow database)
no-melting pt equation used

Water Sol Estimate from Fragments:
Wat Sol (v1.01 est) = 75.167 mg/L

ECOSAR Class Program (ECOSAR v1.11):
Class(es) found:
Aldehydes (Mono)

Henrys Law Constant (25 deg C) [HENRYWIN v3.20]:
Bond Method : 2.94E-007 atm-m3/mole (2.98E-002 Pa-m3/mole)
Group Method: 1.47E-006 atm-m3/mole (1.49E-001 Pa-m3/mole)
For Henry LC Comparison Purposes:
User-Entered Henry LC: not entered
Henrys LC [via VP/WSol estimate using User-Entered or Estimated values]:
HLC: 7.372E-007 atm-m3/mole (7.470E-002 Pa-m3/mole)
VP: 0.000164 mm Hg (source: MPBPVP)

WS: 58 mg/L (source: WSKOWWIN)

Log Octanol-Air Partition Coefficient (25 deg C) [KOAWIN v1.10]:
Log Kow used: 3.38 (exp database)
Log Kaw used: -4.920 (HenryWin est)
Log Koa (KOAWIN v1.10 estimate): 8.300
Log Koa (experimental database): None

Probability of Rapid Biodegradation (BIOWIN v4.10):
Biowin1 (Linear Model) : 1.1978
Biowin2 (Non-Linear Model) : 1.0000
Expert Survey Biodegradation Results:
Biowin3 (Ultimate Survey Model): 2.7473 (weeks-months)
Biowin4 (Primary Survey Model) : 3.8404 (days)
MITI Biodegradation Probability:
Biowin5 (MITI Linear Model) : 0.8030
Biowin6 (MITI Non-Linear Model): 0.8755
Anaerobic Biodegradation Probability:
Biowin7 (Anaerobic Linear Model): 0.4960
Ready Biodegradability Prediction: NO

Hydrocarbon Biodegradation (BioHCwin v1.01):
Structure incompatible with current estimation method!

Sorption to aerosols (25 Dec C)[AEROWIN v1.00]:
Vapor pressure (liquid/subcooled): 0.0796 Pa (0.000597 mm Hg)
Log Koa (Koawin est): 8.300
Kp (particle/gas partition coef. (m3/ug)):
Mackay model : 3.77E-005

Octanol/air (Koa) model: 4.9E-005

Fraction sorbed to airborne particulates (phi):

Junge-Pankow model   : 0.00136

Mackay model   : 0.00301

Octanol/air (Koa) model: 0.0039

Atmospheric Oxidation (25 deg C) [AopWin v1.92]:

Hydroxyl Radicals Reaction:

OVERALL OH Rate Constant = 24.2941 E-12 cm3/molecule-sec

Half-Life =   0.440 Days (12-hr day; 1.5E6 OH/cm3)

Half-Life =   5.283 Hrs

Ozone Reaction:

No Ozone Reaction Estimation

Fraction sorbed to airborne particulates (phi):

0.00218 (Junge-Pankow, Mackay avg)

0.0039 (Koa method)

Note: the sorbed fraction may be resistant to atmospheric oxidation

Soil Adsorption Coefficient (KOCWIN v2.00):

Koc   : 145.9 L/kg (MCI method)

Log Koc: 2.164   (MCI method)

Koc   : 418.1 L/kg (Kow method)

Log Koc: 2.621   (Kow method)

Aqueous Base/Acid-Catalyzed Hydrolysis (25 deg C) [HYDROWIN v2.00]:

Rate constants can NOT be estimated for this structure!

Bioaccumulation Estimates (BCFBAF v3.01):

Log BCF from regression-based method = 1.897 (BCF = 78.91 L/kg wet-wt)

Log Biotransformation Half-life (HL) = 0.1093 days (HL = 1.286 days)
Log BCF Arnot-Gobas method (upper trophic) = 2.237 (BCF = 172.6)
Log BAF Arnot-Gobas method (upper trophic) = 2.237 (BAF = 172.7)
log Kow used: 3.38 (expkow database)

Volatilization from Water:
Henry LC: 1.47E-006 atm-m3/mole (estimated by Group SAR Method)
Half-Life from Model River: 562.2 hours (23.42 days)
Half-Life from Model Lake : 6251 hours (260.5 days)

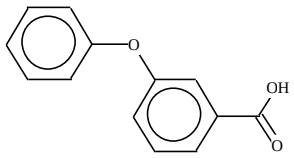
Removal In Wastewater Treatment:
Total removal: 10.65 percent
Total biodegradation: 0.16 percent
Total sludge adsorption: 10.41 percent
Total to Air: 0.08 percent
(using 10000 hr Bio P,A,S)

Level III Fugacity Model:

Mass Amount (percent)	Half-Life (hr)	Emissions (kg/hr)
Air 0.466	10.6	1000
Water 22.6	900	1000
Soil 76.8	1.8e+003	1000
Sediment 0.203	8.1e+003	0

Persistence Time: 955 hr

EPI Suite Results For CAS 003739-38-6



SMILES : OC(=O)c2cccc(Oc1ccccc1)c2

CHEM : **m-Phenoxybenzoic acid**

MOL FOR: C13 H10 O3

MOL WT : 214.22

---------- EPI SUMMARY (v4.11) ----------

Physical Property Inputs:

Log Kow (octanol-water): ------

Boiling Point (deg C) : ------

Melting Point (deg C) : ------

Vapor Pressure (mm Hg) : ------

Water Solubility (mg/L): ------

Henry LC (atm-m3/mole) : ------

Log Octanol-Water Partition Coef (SRC):

Log Kow (KOWWIN v1.68 estimate) = 3.93

Log Kow (Exper. database match) = 3.91

Exper. Ref: HANSCH,C ET AL. (1995)

Boiling Pt, Melting Pt, Vapor Pressure Estimations (MPBPVP v1.43):
Boiling Pt (deg C): 365.14 (Adapted Stein & Brown method)
Melting Pt (deg C): 127.48 (Mean or Weighted MP)
VP(mm Hg,25 deg C): 3.16E-006 (Modified Grain method)
VP (Pa, 25 deg C) : 0.000421 (Modified Grain method)
MP (exp database): 149-150 deg C
Subcooled liquid VP: 5.76E-005 mm Hg (25 deg C, Mod-Grain method)
: 0.00767 Pa (25 deg C, Mod-Grain method)

Water Solubility Estimate from Log Kow (WSKOW v1.42):
Water Solubility at 25 deg C (mg/L): 16.91
log Kow used: 3.91 (expkow database)
no-melting pt equation used

Water Sol Estimate from Fragments:
Wat Sol (v1.01 est) = 20.526 mg/L

ECOSAR Class Program (ECOSAR v1.11):
Class(es) found:
Neutral Organics-acid

Henrys Law Constant (25 deg C) [HENRYWIN v3.20]:
Bond Method : 2.37E-009 atm-m3/mole (2.40E-004 Pa-m3/mole)
Group Method: 2.39E-009 atm-m3/mole (2.42E-004 Pa-m3/mole)
For Henry LC Comparison Purposes:
User-Entered Henry LC: not entered
Henrys LC [via VP/WSol estimate using User-Entered or Estimated values]:
HLC: 5.267E-008 atm-m3/mole (5.337E-003 Pa-m3/mole)
VP: 3.16E-006 mm Hg (source: MPBPVP)

WS: 16.9 mg/L (source: WSKOWWIN)

Log Octanol-Air Partition Coefficient (25 deg C) [KOAWIN v1.10]:
Log Kow used: 3.91 (exp database)
Log Kaw used: -7.014 (HenryWin est)
Log Koa (KOAWIN v1.10 estimate): 10.924
Log Koa (experimental database): None

Probability of Rapid Biodegradation (BIOWIN v4.10):
Biowin1 (Linear Model) : 1.0824
Biowin2 (Non-Linear Model) : 0.9984
Expert Survey Biodegradation Results:
Biowin3 (Ultimate Survey Model): 2.7775 (weeks)
Biowin4 (Primary Survey Model) : 3.6284 (days-weeks)
MITI Biodegradation Probability:
Biowin5 (MITI Linear Model) : 0.7210
Biowin6 (MITI Non-Linear Model): 0.7668
Anaerobic Biodegradation Probability:
Biowin7 (Anaerobic Linear Model): 0.6390
Ready Biodegradability Prediction: YES

Hydrocarbon Biodegradation (BioHCwin v1.01):
Structure incompatible with current estimation method!

Sorption to aerosols (25 Dec C)[AEROWIN v1.00]:
Vapor pressure (liquid/subcooled): 0.00768 Pa (5.76E-005 mm Hg)
Log Koa (Koawin est): 10.924
Kp (particle/gas partition coef. (m3/ug)):
Mackay model : 0.000391

Octanol/air (Koa) model: 0.0206

Fraction sorbed to airborne particulates (phi):

Junge-Pankow model   : 0.0139

Mackay model   : 0.0303

Octanol/air (Koa) model: 0.622

Atmospheric Oxidation (25 deg C) [AopWin v1.92]:

Hydroxyl Radicals Reaction:

OVERALL OH Rate Constant =   6.7827 E-12 cm3/molecule-sec

Half-Life =   1.577 Days (12-hr day; 1.5E6 OH/cm3)

Half-Life =   18.923 Hrs

Ozone Reaction:

No Ozone Reaction Estimation

Fraction sorbed to airborne particulates (phi):

0.0221 (Junge-Pankow, Mackay avg)

0.622 (Koa method)

Note: the sorbed fraction may be resistant to atmospheric oxidation

Soil Adsorption Coefficient (KOCWIN v2.00):

Koc   : 217.8 L/kg (MCI method)

Log Koc: 2.338   (MCI method)

Koc   : 236.8 L/kg (Kow method)

Log Koc: 2.374   (Kow method)

Aqueous Base/Acid-Catalyzed Hydrolysis (25 deg C) [HYDROWIN v2.00]:

Rate constants can NOT be estimated for this structure!

Bioaccumulation Estimates (BCFBAF v3.01):

Log BCF from regression-based method = 0.500 (BCF = 3.162 L/kg wet-wt)

Log Biotransformation Half-life (HL) = -0.0153 days (HL = 0.9653 days)
Log BCF Arnot-Gobas method (upper trophic) = 2.441 (BCF = 275.8)
Log BAF Arnot-Gobas method (upper trophic) = 2.441 (BAF = 275.9)
log Kow used: 3.91 (expkow database)

Volatilization from Water:
Henry LC: 2.39E-009 atm-m3/mole (estimated by Group SAR Method)
Half-Life from Model River: 3.585E+005 hours (1.494E+004 days)
Half-Life from Model Lake : 3.912E+006 hours (1.63E+005 days)

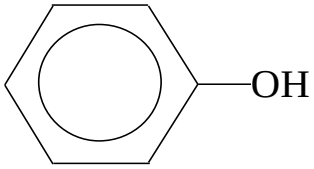
Removal In Wastewater Treatment:
Total removal: 26.13 percent
Total biodegradation: 0.29 percent
Total sludge adsorption: 25.84 percent
Total to Air: 0.00 percent
(using 10000 hr Bio P,A,S)

Level III Fugacity Model:

Mass Amount (percent)	Half-Life (hr)	Emissions (kg/hr)
Air 0.0429	37.8	1000
Water 17.8	360	1000
Soil 82	720	1000
Sediment 0.181	3.24e+003	0

Persistence Time: 756 hr

EPI Suite Results For CAS 000108-95-2



SMILES : Oc(cccc1)c1

CHEM : **Phenol**

MOL FOR: C6 H6 O1

MOL WT : 94.11

---------- EPI SUMMARY (v4.11) ----------

Physical Property Inputs:

Log Kow (octanol-water): ------

Boiling Point (deg C) : ------

Melting Point (deg C) : ------

Vapor Pressure (mm Hg) : ------

Water Solubility (mg/L): ------

Henry LC (atm-m3/mole) : ------

Log Octanol-Water Partition Coef (SRC):

Log Kow (KOWWIN v1.68 estimate) = 1.51

Log Kow (Exper. database match) = 1.46

Exper. Ref: HANSCH,C ET AL. (1995)

Boiling Pt, Melting Pt, Vapor Pressure Estimations (MPBPVP v1.43):

Boiling Pt (deg C): 170.04 (Adapted Stein & Brown method)
Melting Pt (deg C): -2.27 (Mean or Weighted MP)
VP(mm Hg,25 deg C): 0.323 (Modified Grain method)
VP (Pa, 25 deg C) : 43 (Modified Grain method)
MP (exp database): 40.9 deg C
BP (exp database): 181.8 deg C
VP (exp database): 3.50E-01 mm Hg (4.67E+001 Pa) at 25 deg C
Subcooled liquid VP: 0.503 mm Hg (25 deg C, exp database VP)
: 67 Pa (25 deg C, exp database VP)

Water Solubility Estimate from Log Kow (WSKOW v1.42):
Water Solubility at 25 deg C (mg/L): 2.616e+004
log Kow used: 1.46 (expkow database)
no-melting pt equation used
Water Sol (Exper. database match) = 8.28e+004 mg/L (25 deg C)
Exper. Ref: SOUTHWORTH,GR & KELLER,JL (1986)

Water Sol Estimate from Fragments:
Wat Sol (v1.01 est) = 45856 mg/L

ECOSAR Class Program (ECOSAR v1.11):
Class(es) found:
Phenols

Henrys Law Constant (25 deg C) [HENRYWIN v3.20]:
Bond Method : 5.61E-007 atm-m3/mole (5.68E-002 Pa-m3/mole)
Group Method: 6.58E-007 atm-m3/mole (6.67E-002 Pa-m3/mole)
Exper Database: 3.33E-07 atm-m3/mole (3.37E-002 Pa-m3/mole)
For Henry LC Comparison Purposes:

User-Entered Henry LC: not entered

Henrys LC [via VP/WSol estimate using User-Entered or Estimated values]:

HLC: 1.529E-006 atm-m3/mole (1.549E-001 Pa-m3/mole)

VP: 0.323 mm Hg (source: MPBPVP)

WS: 2.62E+004 mg/L (source: WSKOWWIN)

Log Octanol-Air Partition Coefficient (25 deg C) [KOAWIN v1.10]:

Log Kow used: 1.46 (exp database)

Log Kaw used: -4.866 (exp database)

Log Koa (KOAWIN v1.10 estimate): 6.326

Log Koa (experimental database): None

Probability of Rapid Biodegradation (BIOWIN v4.10):

Biowin1 (Linear Model)   :   0.9466

Biowin2 (Non-Linear Model)   :   0.9876

Expert Survey Biodegradation Results:

Biowin3 (Ultimate Survey Model):   3.0696 (weeks   )

Biowin4 (Primary Survey Model) :   3.7565 (days   )

MITI Biodegradation Probability:

Biowin5 (MITI Linear Model)   :   0.5375

Biowin6 (MITI Non-Linear Model):   0.7105

Anaerobic Biodegradation Probability:

Biowin7 (Anaerobic Linear Model): 0.6578

Ready Biodegradability Prediction: YES

Hydrocarbon Biodegradation (BioHCwin v1.01):

Structure incompatible with current estimation method!

Sorption to aerosols (25 Dec C)[AEROWIN v1.00]:

Vapor pressure (liquid/subcooled): 67.1 Pa (0.503 mm Hg)

Log Koa (Koawin est): 6.326

Kp (particle/gas partition coef. (m3/ug)):

Mackay model : 4.47E-008

Octanol/air (Koa) model: 5.2E-007

Fraction sorbed to airborne particulates (phi):

Junge-Pankow model : 1.62E-006

Mackay model : 3.58E-006

Octanol/air (Koa) model: 4.16E-005

Atmospheric Oxidation (25 deg C) [AopWin v1.92]:

Hydroxyl Radicals Reaction:

OVERALL OH Rate Constant = 33.4673 E-12 cm3/molecule-sec

Half-Life = 0.320 Days (12-hr day; 1.5E6 OH/cm3)

Half-Life = 3.835 Hrs

Ozone Reaction:

No Ozone Reaction Estimation

Reaction With Nitrate Radicals May Be Important!

Fraction sorbed to airborne particulates (phi):

2.6E-006 (Junge-Pankow, Mackay avg)

4.16E-005 (Koa method)

Note: the sorbed fraction may be resistant to atmospheric oxidation

Soil Adsorption Coefficient (KOCWIN v2.00):

Koc : 187.2 L/kg (MCI method)

Log Koc: 2.272 (MCI method)

Koc : 79.34 L/kg (Kow method)

Log Koc: 1.900 (Kow method)

Experimental Log Koc: 1.9 (database)

Aqueous Base/Acid-Catalyzed Hydrolysis (25 deg C) [HYDROWIN v2.00]:
Rate constants can NOT be estimated for this structure!

Bioaccumulation Estimates (BCFBAF v3.01):
Log BCF from regression-based method = 0.630 (BCF = 4.269 L/kg wet-wt)
Log Biotransformation Half-life (HL) = -1.5015 days (HL = 0.03152 days)
Log BCF Arnot-Gobas method (upper trophic) = 0.384 (BCF = 2.419)
Log BAF Arnot-Gobas method (upper trophic) = 0.384 (BAF = 2.419)
log Kow used: 1.46 (expkow database)

Volatilization from Water:
Henry LC: 3.33E-007 atm-m3/mole (Henry experimental database)
Half-Life from Model River: 1707 hours (71.11 days)
Half-Life from Model Lake : 1.87E+004 hours (779.1 days)

Removal In Wastewater Treatment:
Total removal: 1.98 percent
Total biodegradation: 0.09 percent
Total sludge adsorption: 1.87 percent
Total to Air: 0.02 percent
(using 10000 hr Bio P,A,S)

Level III Fugacity Model:

Mass Amount (percent)	Half-Life (hr)	Emissions (kg/hr)
Air 0.788	9.76	1000
Water 25.4	360	1000
Soil 73.6	720	1000

Sediment 0.231 3.24e+003 0

Persistence Time: 478 hr

B. Appendix II 16s rDNA of CYP degrading bacteria in the phylogenetic tree

>zk101

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>zk102

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ACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTA

> Brevibacillus sp. clone BCP09

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAA
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>Bacillus cereus strain BCC01

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> Bacillus subtilis strain BSF01

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CGGTGGAGCATGTGGTTTAATTTGAAGCAACGCGAAGAACCTTACCAGGTCTT
GACATCCTTTGACAACCCTAGAGATAGGGCTTTTCCTTTGGGAGCAGAGTGAC
AGGTGGTGCATGGTTGTTGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGATTTTAGTTGCCATCATTTAGTTGGGCACTTTAAGG
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> Pseudomonas aeruginosa strain GF31

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> Bacillus cereus strain SG2

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GGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCT
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> Azoarcus indigens strain HZ5

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> Bacillus thuringiensis strain SG4

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> Brevibacillus parabrevis strain FCm9

CGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGGGTTTTCGGACCCTAG
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> **Acinetobacter calcoaceticus strain MCm5**

AGTCGAGCGGAGAGAGGTAGCTTGCTATCGATCTTAGCGGCGGACGGGTGAGT
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> **Sphingomonas sp. RCm6**

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> Bacillus subtilis strain 1d

CGTGRRWCGTTTSTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTT
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> JC1

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> Flavobacterium haoranii strain LQY-7

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> Rhodopseudomonas palustris strain GJ-22

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> JCN13

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