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DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

The isolation and characterization of novel pyrethroid hydrolases from biobeds using functional metagenomic approaches

A thesis submitted by

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Abstract

Insecticides are mainly used to control crop pest but also insects that are vectors of diseases threatening public health. However, their widespread use poses a major risk for the environment, due to accumulation of their residues in natural resources, and human health, due to presence of their residues in agricultural products. Our study aimed to isolate and characterize novel pyrethroid hydrolases from on-farm biobed systems with prior exposure to pyrethroid compounds using functional metagenomics. In earlier research, a 20,000-clone fosmid library was tested through fast-track phenotypic tests, revealing twelve positive clones for esterase activity. Further testing of these 12 clones identified one clone with the ability to degrade α-cypermethrin. Subsequent transposon mutagenesis and analysis on this particular clone identified BioH, an esterase, as a possible pyrethroid degrading enzyme. Expanding on these findings, the current thesis focused on heterologous overexpression of BioH in *E. coli,* followed by in vitro functional assays of the purified enzyme, which confirmed its ability to hydrolyze α-cypermethrin. Substrate specificity and kinetic parameters of BioH esterase were determined using p-nitrophenyl esters with various lengths of acyl chains. The results showed that BioH displayed higher activity towards substrate with an 8-carbon chain, with a Km and Vmax values of 539.8 μM and 21.3 μM/min, respectively. Optimal activity conditions were observed at 50 °C and pH 8. This study emphasizes the significance of functional metagenomics in identifying novel enzymes without the need for microbial cultivation. The newly identified enzymes have the potential to be utilized in the development of enzyme-based products for efficiently detoxifying pesticides from the environment.

Chapter 1 General Introduction

1.1 | Pyrethroid pesticides and the outcome of their excessive use

For nearly two centuries, there has been a continuous interest in protecting humans and animals against insects and diseases transmitted by insects, as well as crops against pests (Hołyńska-Iwan & Szewczyk-Golec, 2020). Without a doubt, as populations of humans, farm animals, and agricultural areas expand, the use of pesticides, including pyrethroid insecticides, is on the rise. Pyrethroids have been used for more than 30 years (since the 1980s) because of their high level of effectiveness and low toxicity in mammals compared to other insecticides, such as organophosphorus and carbamic ester compounds (Yoo et al., 2016; Zhai et al., 2012). Pyrethroids sales reached 2.8 billion dollars in 2019, representing 19% of the global insecticide market (Birolli et al., 2022). Nowadays, they find extensive application in crop protection, forestry, wood, and textile industries as well as in medicine and veterinary medicine to treat infestations of parasitic crustaceans. Moreover, they are utilized for personal insect protection through various means such as soaked mosquito nets, sprays, or gels (Hołyńska-Iwan & Szewczyk-Golec, 2020).

Hystorically, pyrethroids are a class of synthetic organic compounds derived from pyrethrins, toxins with insecticidal activity derived from the flowers of the plant *Tanacetum cinerariaefolium* (formerly *Chrysanthemum cinerariaefolium*) of the family *Asteraceae*. Natural pyrethroids are unstable substances that degrade fast when exposed to light; hence, derivatives that are more resistant to radiation and more poisonous to insects have been produced (Hodoșan et al., 2023). Chemically, pyrethroids are esters of chrysanthemic acid and they exist in two chiral systems: cis and trans, with cis stereoisomers being more active.

Pyrethroids are categorized into two types, Type I and Type II, based on their compound structure, mode of action, and associated adverse effects (Chrustek et al., 2018). Type I pyrethroids, such as allethrin, resmethrin,

and tetramethrin, lack the α-cyano group in their chemical structure, resulting in relatively lower toxicity levels (Figure 1). On the other hand, Type II pyrethroids, such as fenvalerate, cypermethrin, and tralomethrin, contain the α-cyano group, making them significantly more toxic. Regarding their mode of action, Type I pyrethroids change the conformation of sodium channels during their opening and closing in neuronal membranes. In contrast, Type II pyrethroids not only affect sodium channels but also influence chloride channels, including those dependent on GABA. Pyrethroids (both Type I and Type II) exhibit greater toxicity towards insects compared to mammals and birds, due to the more sensitive sodium channels in the insect nervous system (Hołyńska-Iwan & Szewczyk-Golec, 2020). In terms of the undesirable symptoms they induce, Type I pyrethroids cause the T (tremor) syndrome, characterized by marked behavioral arousal, aggressive sparring and fine body tremor while Type II pyrethroids cause the CS (choreoathetosis and salivation) syndrome. However, the connection between structure and syndrome isn't always straightforward, as some Type II compounds can cause either the T syndrome or a mix of T and CS syndrome (Costa, 2015).

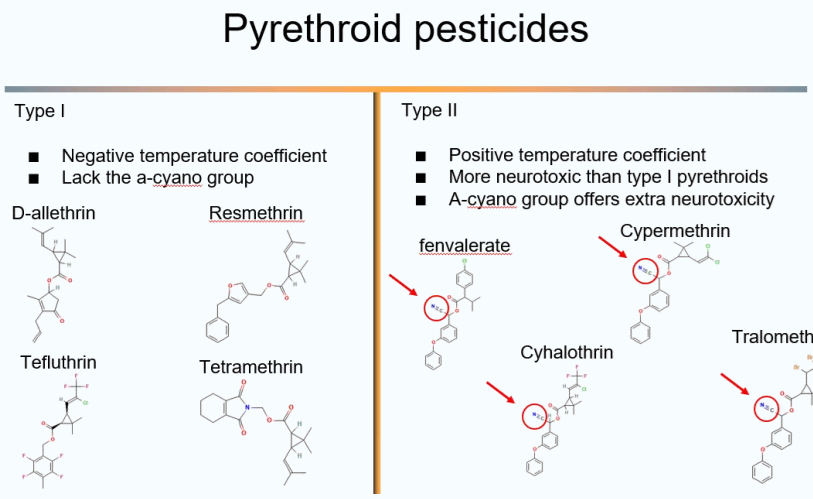

Figure 1. Pyrethroid pesticides are classified, based on their chemical structure, in Type I and Type II. Type II pyrethroids have an α-cyano group that offers extra toxicity.

The widespread and continuous use of pyrethroids has led to their detection in soil, sediment and surface water worldwide (Tang et al, 2018). Once in the soil, pyrethroids undergo diverse processes, including degradation, sorption-desorption, uptake by plants, runoff into surface waters, and transport into groundwater. Biodegradation and abiotic

degradation (e.g., oxidation, hydrolysis and photolysis) play significant roles in determining the environmental fate of pyrethroids (Cycoń & Piotrowska-Seget, 2016). In general, pyrethroids have half-lives in soil ranging from 5 to 1410 days, and their degradation is influenced by several factors, including the catabolic activity of soil microorganisms and various soil properties such as texture, organic matter content, moisture, pH, and temperature (Cycoń & Piotrowska-Seget, 2016). When they enter the aquatic environment, given their hydrophobic nature, pyrethroids quickly dissipate from the dissolved water phase and strongly bind to organic carbon in sediment particles (Ranatunga et al., 2023). According to Tang et al. (2018), who reviewed the reported levels of 31 pyrethroids and their metabolites in various environmental media (soils, water, and sediments), cypermethrin was the most frequently detected pyrethroid, followed by permethrin, deltamethrin, and fenvalerate. Regarding their presence in the atmosphere, their relatively low vapor pressure, suggests a low tendency to volatilize during application, and to revolatilize from soils or water bodies (Mejanelle, 2020).

1.2 | Microbial elimination of pyrethroids: microorganisms and involved enzymes

Contaminated environments such as wastewater, soils, and oceans represent significant reservoirs of pyrethroid-degrading strains, as they could utilize pyrethroids as energy sources for their growth (Cycón and Piotrowska-Seget 2016; Dash et al. 2013). Through the utilization of enrichment culture techniques, a broad spectrum of pyrethroid-degrading microorganisms has been isolated. These include a variety of bacterial and fungal species belonging to various genera, such as *Bacillus, Pseudomonas, Raoultella, Achromobacter, Acidomonas, Brevibacterium, Streptomyces, Serratia, Sphingobium, Clostridium, Klebsiella, Lysinibacillus, Acinetobacter, Aspergillus, Candida, Trichoderma*, and *Candida* (Cycoń and Piotrowska-Seget, 2016; Birolli et al., 2019; Hu et al., 2019; Zhao et al., 2019). It is noteworthy that almost 84% of the reported isolates consist of bacteria, with *Bacillus* spp. and *Pseudomonas* spp. playing prominent roles, while fungi, predominantly represented by

Aspergillus spp., constituted a smaller proportion of the degrading microbes (Zhao et al., 2021).

In general, the primary pathways for pyrethroid metabolism involve oxidation and hydrolysis processes catalyzed by P450 monooxygenases, glutathione S-transferases, phosphodiesterases, carboxylesterases, and peptidases. Among them, carboxylesterases are the enzymes responsible for the degradation of pyrethroid isomers in the environment. Bioelimination of pyrethroids by carboxylesterases usually leads to the breakage of ester bonds and generation of carboxylic acids and alcohols (Figure 2), which are further degraded into 3-phenoxybenzoic acid (3-PBA), 3-phenoxybenzaldehyde (PBAld), 3-phenoxybenzyl alcohol (PBAlc), and 2-(4-chlorophenyl)-3-methylbutyric acid (CLAc), and these are the most commonly observed metabolites in the microbial elimination of pyrethroids (Fang et al., 2022).

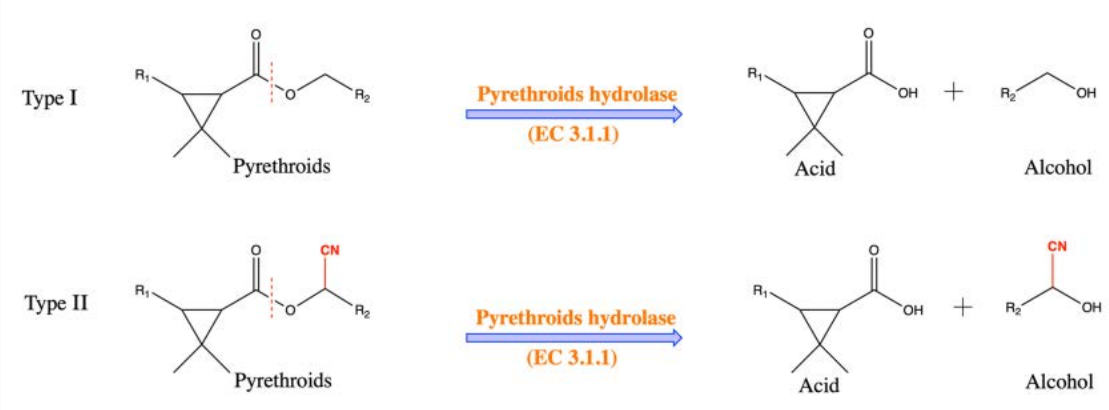

Figure 2. The catabolic reaction of Type I and Type II pyrethroid pesticides. R1 and R2 represent aliphatic and aromatic hydrocarbons aliphatic and aromatic hydrocarbons in different pyrethroid pesticides (Fang et al., 2022).

Pyrethroid carboxylesterases belong to the α/β superfamily of proteins. This group of enzymes shares conserved active site amino acids, which actively participate in catalytic reactions during pyrethroid degradation. Their catalytic sites include a triad of serine, histidine, and glutamine. Pyrethroid hydrolase primarily functions through acylation reactions in the presence of a nucleophile. The catalysis of pyrethroid carboxylesterase relies on an acylation reaction of the serine residue within the enzyme's active sites. This reaction leads to the release of alcohol from carboxyl ester and the formation of an acylated intermediate. Water acts as a nucleophile, hydrolysing acylated

intermediates by a nucleophilic attack mechanism (Bhatt et al., 2020).

Several microbial pyrethroid carboxylesterases have been reported for the degradation of pyrethroid such as CarCB2 from *Bacillus velezensis*, PytH from *Sphingobium faniae* JZ-2, Est3385 from *Rhodopseudomonas palustris* PSB-S, EstSt7 from *Sulfolobus tokodaii*, PytZ from *Ochrobactrum anthropi,* Est684 from the Mao-tofu metagenome and Sys410 from metagenomic library (Ding et al., 2022, Xu et al., 2020, Fan et al., 2012, Zhai et al., 2012, Wei et al., 2013, Fan et al., 2017, Luo et al., 2018). These enzymes exhibited their highest pyrethroid-degrading capability under slightly neutral or alkaline pH conditions (pH 7–9), with optimum temperatures ranging between 18 °C and 80 °C.

1.3 | Cypermethrin

Cypermethrin is a synthetic pyrethroid insecticide widely utilized in agriculture for controlling insects in vegetable crops, fruits, paddy, cereals, cotton, ornamental plants, and for treating cattle diseases. It is also used in household settings for managing ants and cockroaches. Cypermethrin is a broad-spectrum insecticide that kills both beneficial and target insects. Its intensive use could lead to insect resistance and causes environmental damage and adverse effects on human health (Indratin et al., 2019). Also, it has been shown to be hazardous to many fishes and aquatic invertebrates even at low quantities (Prusty et al., 2015). It acts on the central nervous system to produce a hyperexcitable state by interacting with sodium channels, by affecting voltage-dependent sodium channels and ATPase system in neuronal membranes that binds to nuclear DNA, destabilizing and unwinding the DNA (Indratin et al., 2019).

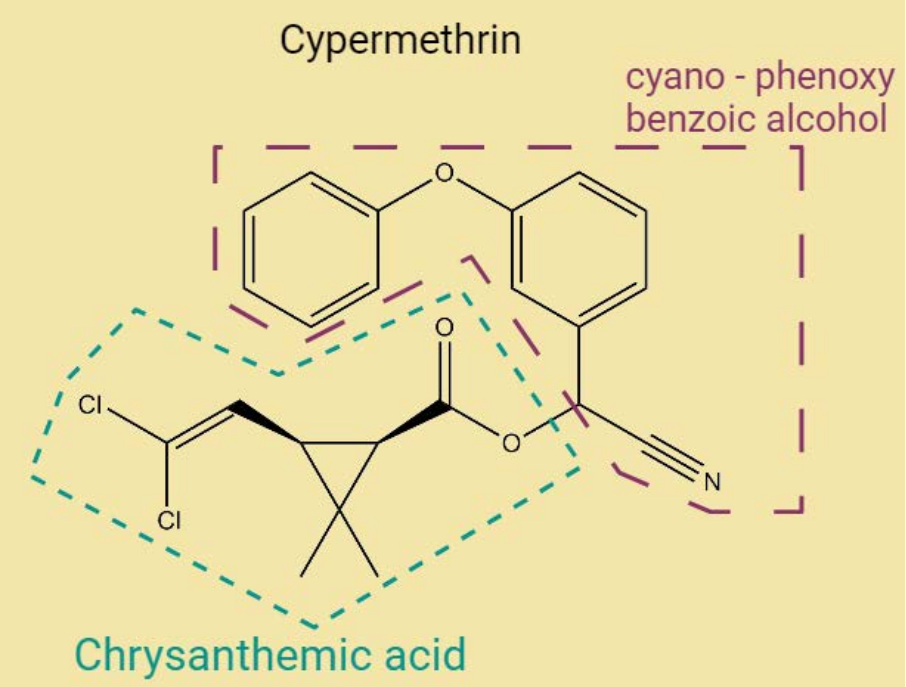

Figure 3. The chemical structure of α- cypermethrin derives from the chrysanthemic acid united with a cyano – phenoxybenzoic alcohol. Picture created by Biorender.

Cypermethrin is a non-polar compound adsorbed by the soil particles, in contrast with its metabolites that are mobile in the soil environment (Huang et al., 2018). It has a chemical structure of an ester (Figure 3), product of chrysanthemic acid with a cyano – 3 phenoxyboenzoic alcohol (Casida, 2010), and a molecular mass of 416.3 with a melting point at 78-81 ^{o}C. The physical form of the insecticide is a brown-yellowish thick liquid. Cypermethrin has very low solubility in water, 0.009 mg/L (Cypermethrin (PIM 163), n.d.), but it is easily soluble in organic solvents (Cypermethrin, 2017). Cypermethrin is a mixture of cis and trans isomers. The cis isomers are more active and stable than the trans isomers. Specifically, α-cypermethrin contains a mixture of the two most active isomers (Pronk, Speijers, Wouters & Ritter). Specialists from FAO/WHO in food additives confirms the level of non-observed adverse effect (NOAEL or No-observed-adverse-effect level) as the concentration 1.5 mg/kg per day, regarding α-cypermethrin ("alphα-cypermethrin", 2022).

A-cypermethrin exhibits medium persistence in the environment, with a degradation half-life (DT_{50}) of 86.9 days in soil under aerobic conditions and 96.3 days in aerobic water/sediment systems (“Alpha-

cypermethrin Classification and Endpoints," 2022). The degradation rate of cypermethrin in soil is increased in the presence of microorganisms (Chapman et al., 1981; Xie and Zhou, 2008). Various bacterial strains capable of degrading cypermethrin, including those from *Pseudomonas*, *Serratia, Streptomyces, Rhodobacter, Stenotrophomonas, Sphingomonas*, and *Bacillus* genera, have been isolated (Aguila-Torres et al., 2020; Cycoń & Piotrowska-Seget, 2016; Indratin et al., 2019). Like other pyrethroids, the principal transformation pathway of α-cypermethrin involves ester hydrolysis mediated by carboxylesterase activity. Genes encoding carboxylesterases responsible for degrading α-cypermethrin have been cloned and characterized (Diegelmann, Weber, Heinzel-Wieland & Kemme, 2015).

1.4 | Functional Metagenomic Analysis

All species, from humans to plants, have an influence on the microorganisms around them. For example, they serve as a catalyst for the continued evolution and well-being of the connection between man and the food he consumes. This notion raises several issues regarding the microbial communities that are concealed in the soil and that they may indirectly affect human health, since we are unable to grow them. Before Jo Handelsman introduced the term "metagenomics" in 1998, which has the potential to unlock the mysteries of the microbial world, there was no method to approach this idea. She defined the metagenome of the soil as the cloning and functional study of collective genomes of soil microorganisms (Navgire et al., 2022). Currently, metagenomics is known as the direct genetic examination of genomes included in an environmental sample.

Utilizing a variety of genomic technologies and bioinformatics tools, metagenomics provides direct access to the genetic information of whole organism communities. Over the past ten years, the area of metagenomics has significantly advanced microbial ecology, evolution, and diversity, and several research labs are currently actively working on it.

Metagenomes are examined according to their function (activity) or sequencing. Function-based screening is a simple technique aiming to

identify and discover new genes encoding for desirable enzymatic activities. However, it is frequently troublesome, owing to the skewed and inadequate expression of genomic segments in *Escherichia coli*. Sequence-based screening, on the other hand, is simpler but the genes retrieved this way are confined to those that have homologies to the probe sequence and may not allow us to discover novel genes and enzymes. The challenges due to heterologous gene expression have resulted in slow progress in functional metagenomics research over the past two decades, with a greater focus placed on sequence-based data compared to functional approaches (Chevrette & Handelsman, 2020).

Metagenomics allow access to the functional gene composition of microbial communities, providing a considerably wider description than phylogenetic surveys, which are frequently based solely on the diversity of a single gene, such as the 16S rRNA gene. Metagenomics, on its own, provides genetic information on possibly novel biocatalysts or enzymes, genomic links between function and phylogeny in uncultured species, and evolutionary profiles of community function and structure. So, metagenomics is an effective method for developing new theories about microbial activity (Thomas et al., 2012).

Specifically, the process of extracting DNA from microbial communities in order to study the activities of encoded proteins is known as functional metagenomics. Cloning DNA fragments, expressing genes in a surrogate host, and screening for enzyme activity are all part of the process. So, functional approach relies on the construction and screening of metagenomic libraries—physical libraries that contain DNA cloned from environmental metagenomes (Lam et al., 2015). The length of the inserts heavily influences the vector used in library development. Plasmids are appropriate for cloning DNA pieces less than 10 kb, whereas cosmids (25-35 kb), fosmids (25-40 kb), and Bacterial Artificial Chromosomes (BACs) (100-200 kb) can be used to clone longer fragments (Uchiyama & Miyazaki, 2009). Using this function-based method, it is possible to find new enzymes whose functions would not be expected based just on DNA sequence. Function-based analysis information may then be utilized to annotate genomes and metagenomes produced only from sequence-based analyses. Thus, functional metagenomics supplement sequence-based metagenomics in the same way that molecular genetics of model

organisms has supplied gene function information that is widely relevant in genomics.

1.5 | Purpose of the Thesis

The purpose of this thesis was to isolate and characterize a novel pyrethroid hydrolase from an on-farm biobed system previously exposed to pyrethroid compounds, including α-cypermethrin. In earlier research, functional metagenomic analysis of the biobed material identified twelve clones with esterase activity, one of which exhibited degradation capability towards α-cypermethrin. Subsequent transposon mutagenesis analysis pinpointed BioH, an esterase, as a potential pyrethroid-degrading enzyme. Building upon these findings, the current study aimed to isolate BioH through heterologous overexpression, to confirm its capability in degrading α-cypermethrin *in vitro* and subsequently proceeded with its biochemical characterization.

Chapter 2 Materials and Methods

2.1 | Previous research

A biobed system in the farm of the INRAE Institute in Dijon, France (French National Institute for Agricultural and Environmental Research) were exposed to a mixture of pesticides, containing mainly pyrethroids, triazoles, strobilurines and organophosphates (Figure 4). The initial hypothesis suggested that creating a stressful environment, like an environment with high and long exposure to pesticides, would create the proper conditions for the evolution of novel catabolic mechanisms.

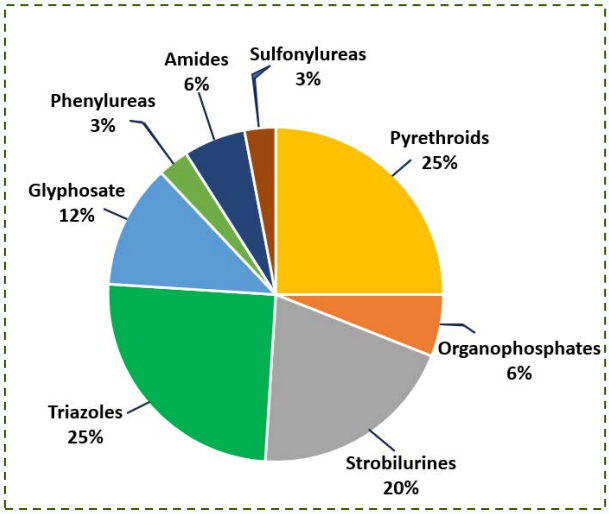

Figure 4. Exposure history of biobed samples to diverse pesticides (Perruchon et al., 2022).

Upon receiving biobed samples at the Laboratory of Plant and Environmental Biotechnology (Department of Biochemistry and Biotechnology, University of Thessaly), metagenomic DNA was extracted. The metagenomic DNA was used for the construction of a metagenomic library of 20,000 bacterial clones. These clones were tested for the presence of esterases through phenotypic screening tests. Twelve clones were identified as positive for esterases, nine from the tributyrin hydrolysis assay and three from the magenta-caprylate hydrolysis assay (Figure 5A and 5B).

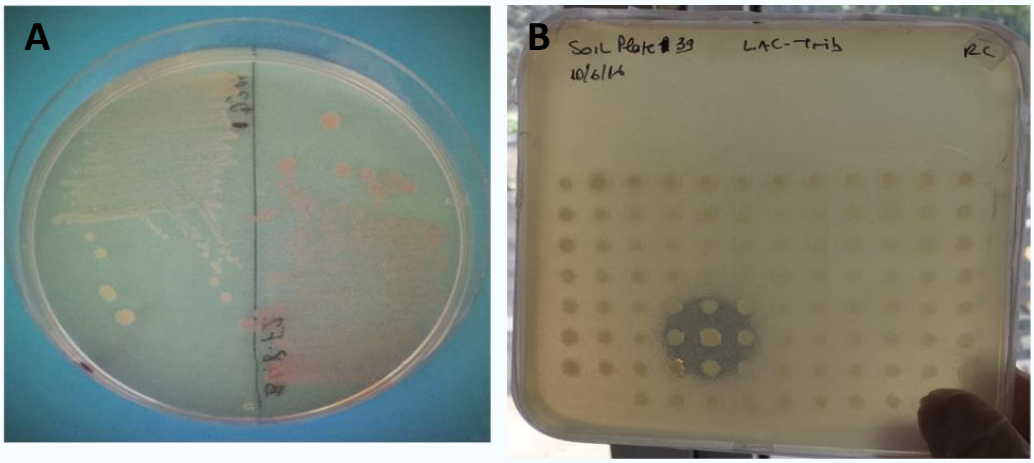

Figure 5. Phenotypic tests for esterases. **A.** Magenta caprylate hydrolysis assay and **B.** tributyrin hydrolysis assay.

Subsequently, the esterase-positive clones were assessed for their capability to degrade α-cypermethrin, indicating one clone with this ability (Figure 6). This clone was used in a transposon mutagenesis reaction, leading to the identification of the gene *bioH* that was potentially involved in α-cypermethrin degradation.

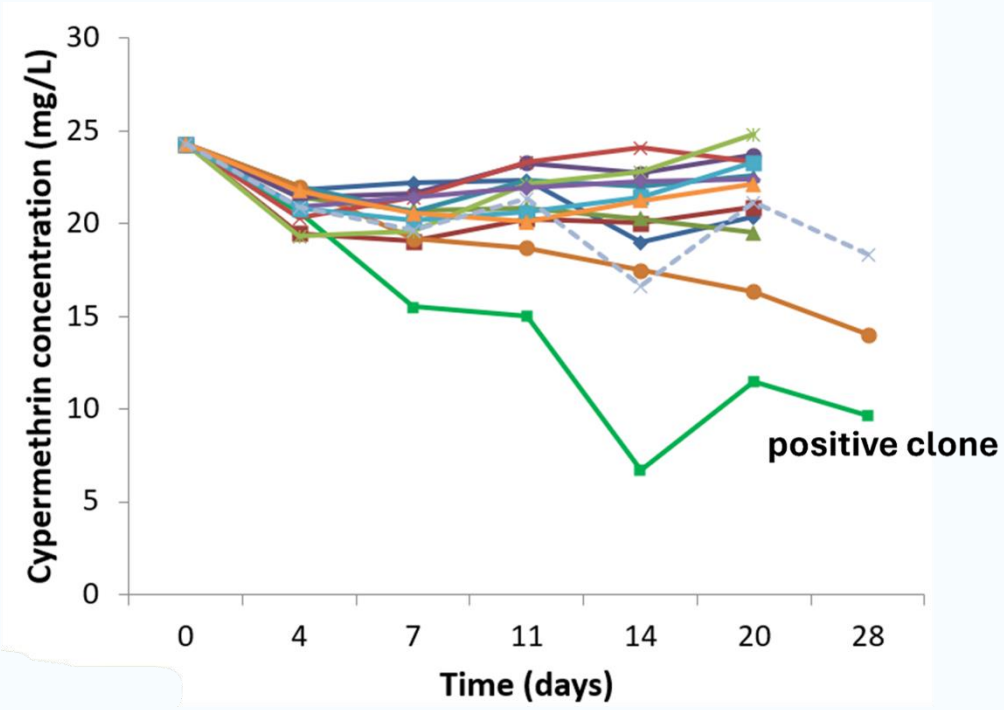

Figure 6. The 12 clones positive for esterases were tested for their ability to degrade α-cypermethrin. Only one clone had this ability.

2.2 |Phylogenetic analysis

NCBI protein-protein BLAST (blastp) was used to identify the 100 most similar protein sequences to BioH from the protein database (search 21.04.2023). Prior to the phylogenetic analysis, the redundancy within the dataset was reduced using CD-HIT with a 70% identity threshold on clusters. Multiple alignments of the representatives of each cluster were conducted using MUSCLE algorithm implemented in Seaview (Wu, 2018). The phylogenetic tree was then constructed using the maximum likelihood estimation method and the reliability of the tree was determined by bootstrap analysis with 100 replicates.

2.3 |Gene Isolation and amplification

The full length *bioH* gene encoding for a putative pyrethroid degrading enzyme was amplified from fosmid DNA using appropriate primers for easy cloning into pET21a vector. The NCBI Primer-BLAST tool was utilized for primer design, and the primers were obtained from Eurofins Scientific. Details on the primers used for the amplification of the target gene are given in Table 1. Amplification was carried out in 50 µl reactions with Q5 High Fidelity DNA Polymerase (New England Biolabs) and 0.1 ng fosmid DNA as template (Table 2). The thermocycling conditions used for the amplification of the target gene are shown in Table 3.

PCR products were subsequently analyzed by 1% agarose gel electrophoresis, and the DNA bands were visualized through UV transilluminator. The desired DNA band was purified through gel extraction (NucleoSpin Gel and PCR cleanup by Macherey-Nagel) and quantified with UV-Vis spectrophotometer (Quawell) to assess the recovery of DNA.

Table 1. Sequences of primers used for PCR amplification and cloning of *bioH* gene

bioH.NdeI.F	**5' – ACTGACATATGAGCCTGCGCGTGGACATC – 3'**
Extra bases for efficient cleavage	**ACTGA**
NdeI recognition site	**CATATG**

Start of *bioH* gene sequence without ATG	**Black Letters**
bioH.NotI.R	**5' – ACTATGCGGCCGCTGGATGCACTGCCCCCTC – 3'**
Extra bases for efficient cleavage	**ACTAT**
NotI recognition site	**GCGGCCGC**
End sequence of *bioH* gene without stop codon	**Black Letters**

Table 2. Reaction setup for *bioH* gene amplification

Component	50 µl Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	25 µl	1X
10 µM Forward Primer	2.5 µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
Template DNA	0.1 ng	
Nuclease-Free Water	to 50 µl	

Table 3. Thermocycling conditions for *bioH* amplification.

	Temperature	Time
Initial denaturation	98°C	30 sec
25-35 cycles	98°C	10 sec
	72°C	30 sec
	72°C	30 sec
Final extension	72°C	2 min
Hold	4-10°C	∞

2.4 |Cloning and Transformation

The gene *bioH* was cloned into pET21a vector. It was cloned downstream of a T7-lac promoter and with a C-terminal His-Tag sequence. Initially, the cloning products were confirmed through *in silico* cloning using the SnapGene program. Following the *in silico* confirmation, the experimental procedure was employed, where both the *bioH* gene and the pET21a vector were digested with the enzymes NdeI and NotI for 30 minutes at 37 ^{o}C (Table 4). The products were checked by agarose gel and purified by gel extraction (Nucleospin Gel and PCR cleanup by Macherey-Nagel).

Afterwards, the compatible ends of the digested insert and vector were ligated together in a ligation reaction (Table 5). The reaction was gently mixed and then incubated overnight at 16 ^{o}C. The following day, the reaction was heat-inactivated at 65 °C.

Subsequently, the recombinant pET21a-BioH vector was transformed into *Escherichia coli* DH5α according to the following protocol:

1. 3.5 µl of ligation reaction was mixed into 100 µL of *E. coli* DH5a competent cells in a microcentrifuge.
2. The competent cell - DNA mixture was incubated on ice for 20-30 mins.
3. The transformation tube was heat shocked by placing the tube into a 42 °C heat block for 60 secs.
4. The tube was put back on ice for 2-5 min.
5. 400 µl LB (without antibiotic) was added to the bacteria, and they were incubated in a 37°C shaking incubator for 1h.
6. The cells were plated onto an LB agar plate containing carbenicillin (50 µg/ml)
7. Plates were incubated at 37 °C overnight.

The following day, 4 colonies were picked, and they were incubated separately in falcon tubes with liquid LB containing 50 µg/ml carbenicillin at 37°C overnight. Then, the plasmids were extracted from the bacterial cells, and they were quantified using a UV-Vis Spectrophotometer (Quawell). The isolated plasmids were checked for the proper insertion of the *bioH* gene through diagnostic digestion reactions (Table 6). After incubating at 37 °C for 1 hour, the reaction mixture was analyzed using agarose gel electrophoresis.

Table 4. Digestion reaction for the pET21a plasmid and *bioH* gene.

Component	**30 µl Reaction**
10x Buffer rCutsmart	3 µl (1X)
NotI-HF enzyme (20,000 units/ml)	0.75 µl (15 units)
NdeI enzyme (20,000 units/ml)	0.75 µl (15 units)
Plasmid pET21a (50.3 ng/µl) Or *bioH* gene (171.2 ng/µl)	22 µl (1,1 µg) 8 µl (1,4 µg)
Nuclease-Free Water	to 30 µl

Table 5. Ligation reaction for the insertion of *bioH* gene into the pET21a plasmid.

Component	**20 µl Reaction**
10x T4 DNA Ligase Buffer	2 ul (1X)
T4 DNA ligase (400,000 units/ml)	1 ul (400 units)
Insert gene *bioH* (37.5ng/µl)	1 ul (37.5 ng)
Plasmid pET21a (50 ng/µl)	4 ul (200 ng)
Nuclease-Free Water	to 20 µl

Table 6. Diagnostic Digestion Reaction for confirmation of *bioH* gene insertion into pET21a plasmid.

Restriction enzymes: EcoRV and NotI		
	BioH- pET21a (91.2ng/μl)	BioH- pET21a (73,9ng/μl)
DNA	4.38 μl (400ng)	5.4 μl (400ng)
10X Buffer rCutsmart	1 μl	1 μl
NotI	0.5 μl	0.5 μl
EcoRV	0.5 μl	0.5 μl
ddH_2O	3.62 μl	2.6 μl

2.5 | Protein overexpression and purification

For the BioH overexpression, the recombinant pET21a-BioH vector was transformed into *E. coli* BL21-Gold (DE3) cells. BL21-Gold (DE3) is an all-purpose strain for high-level protein expression and easy induction using IPTG (Isopropyl-1-thio-β-D-galactopyranoside). We began with small-scale expression studies to determine the optimal expression conditions for BioH. Specifically, we examined two parameters: the amount of inducer and the growth temperature. Once the optimal conditions for BioH expression were determined, we proceeded with the large-scale expression experiment.

Small-scale bacterial expression

The experimental procedure for the small-scale experiment proceeded as follow:

1. Starting from a single colony, 5 ml LB starter cultures were prepared with carbenicillin (50 μg/ml) and tetracycline (12,5 μg/ml). These cultures were grown overnight at 37 °C with shaking at 210 rpm.
2. The following day, fresh 5 ml cultures were inoculated with the starter cultures at a 1/50 dilution, and they were incubated at 37 °C for about 2 hrs with shaking at 210 rpm.
3. Once the OD_{600} reached 0.6-0.8, IPTG was added to achieve final concentrations of 0 mM, 0.05 mM, 0.1 mM, 0.3 mM, 0.5 mM, 1 mM.

Each concentration was then incubated in three different temperatures: 16 °C, 25 °C, 37 °C for 16 hrs.

4. At the end of the incubation period, 1 ml cell samples were harvested by centrifugation at 6,000 rpm for 15 min. The cell pellets were resuspended in lysis buffer (pH 7.0, 50 mM NaH_2PO_4, 150 mM NaCl). The suspension was sonicated on ice (6 x 15 bursts with 15 s cooling break)

5. After lysis, an aliquot (80 µl) of the bacterial lysate (crude extract) was set aside for SDS-PAGE. The soluble and insoluble fractions were isolated by centrifugation (9,500 rpm, 4 °C for 30 min). Aliquots (80 µl) of the soluble fraction (supernatant) and insoluble fraction (pellet) were collected for SDS-PAGE analysis.

6. The samples were mixed with 5X Sample Loading Buffer (225 mM Tris-HCl, pH 6.8, 50% glycerol, 5% SDS, 0.05% Bromophenol blue, 250 mM DTT), and they were heated at 95 °C for 5-10 min before being loaded into the wells of the SDS-PAGE gel. The gel was stained with 0.1% Coomassie Blue R250 solution containing 10% acetic acid, 50% methanol, and 40% H2O. Destaining of the gel was done by soaking it in a solution of 10% acetic acid, 50% methanol, and 40% H_2O.

Large-scale bacterial expression

Following the identification of a suitable expression clone with high BioH expression levels and good solubility, we proceeded with the growth of cultures for large-scale expression. Specifically, a starter LB culture (25 ml) supplemented with carbenicillin (50 µg/ml), and tetracycline (12.5 µg/ml) was inoculated by a glycerol stock (BL21-GoldDE3 +pET21a-BioH) and incubated overnight at 37 °C. The starter culture of 25 mL was used to inoculate 1L of LB in 2L flask. The 1L LB was incubated at 37 °C until OD_{600} reached 0.6-0.8. The culture was further incubated for 14-16 hrs at the optimal IPTG concentration (0.5 mM) and temperature (37 °C) as they had been determined from the small-scale expression tests.

After this phase, we continued with the preparation of cleared *E. coli* lysates under native conditions following the instruction outlined in the user manual ''Purification of His-tag proteins, Macherey-Nagel''. Specifically, *E. coli* expression culture cells were harvested by

centrifugation at 6,000 x g for 15 minutes at 4 °C and the pellet was resuspended in the lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7). Cells were lysed by adding lysozyme to a final concentration of 1 mg/mL, followed by stirring the solution on ice for 60 minutes. Subsequently, the suspension was sonicated (6 x 15 bursts with 15 s cooling breaks). The crude lysate was then clarified by centrifugation at 9,500 x g for 30 minutes at 4 °C to remove cellular debris. The supernatant was carefully transferred to a clean tube without disturbing the pellet and stored on ice.

The polyhistidine-tagged BioH protein was purified using the Protino Ni-NTA product manufactured by Macherey-Nagel, which is based on the immobilized metal ion affinity chromatography (IMAC). This involves the selective binding of histidine-tagged proteins to immobilized nickel ions, allowing for their efficient purification from complex protein mixtures.

We followed the protocol described in the "Gravity-flow purification of polyhistidine-tagged proteins under native conditions" section of the user manual, with minor modification. Briefly, the Ni-NTA resin was equilibrated with NPI-20 buffer. Subsequently, the supernatant was added to the resin and incubated for 1 hour. Following incubation, the resin was washed twice with NPI-20. The bound proteins were then eluted using elution buffer NPI-250. The buffers are shown on table 7.

Table 7. The NPI- 20 washing buffer and NPI-250 elution buffer were used through the purification of the BioH protein.

NPI - 20	NPI - 250
NaH_2PO_4 (50mM)	NaH_2PO_4 (50mM)
NaCl (300 mM)	NaCl (300 mM)
Imidazole (20 mM)	Imidazole (250 mM)
pH = 7	pH = 7

After the protein purification, a dialysis step was performed to remove the imidazole from the protein solution. The purified protein was loaded into a dialysis membrane (Spectra/Por® 3 - MWCO 3500) and immersed in the dialysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, pH 7) at a ratio 1:100 (protein to dialysis buffer vol:vol). The dialysis was allowed

to run overnight at 4 °C. The following day the dialysate was replaced with fresh buffer, and dialysis was continued for another 4 hours.

The quantity of the purified protein was measured with an Invitrogen Qubit fluorometer using the Invitrogen Qubit Protein Assay kit. The protein stored at -20 °C temperature with 50% glycerol.

2.6 | In vitro assessment of the activity of BioH against α-cypermethrin

The hydrolytic activity of the purified BioH enzyme towards α-cypermethrin was assessed with an enzymatic assay following the method described by Wang et al (2009). Specifically, the assays were performed in 1 ml reactions consisting of 50 mM monosodium phosphate buffer (pH 7.5), 150 mM NaCl and 10 mg/L α-cypermethrin. The reaction mixtures were incubated at 37 °C.

Degradation of α-cypermethrin was determined with 200 μg BioH protein at 0 hrs, 9 hrs and 72 hrs by analysing samples in an HPLC-UV system equipped with a reversed-phase C18 column. The mobile phase was a mixture of acetonitrile and water (80:20, v/v). The column temperature was 25 °C; the flow rate was 1 mL/min; the injection volume was 20 μL; UV detection was at 235 nm. Under these conditions the retention time of α-cypermethrin was 9.3 minutes. For the extraction of a-cypermethrin from the assay reactions, aliquots of 0.2 ml were mixed with 0.2 ml of methanol. Pesticide concentration was quantified by the external standard method using calibration curve obtained by injection of standard solutions.

2.7 |Enzymatic Characterization

After confirmation of BioH activity against α-cypermethrin, we proceeded with the characterization of the enzyme. The first step was to evaluate the esterase activity against a common substrate for esterases, such as p-

nitrophenyl decanoate. Optimal enzyme concentration and assay timing were established through a time-course experiment, in which various enzyme amounts (10 µg, 20 µg, 50 µg, 100 µg) were tested. The hydrolysis of the substrate was performed with 100 µg BioH protein at 50 °C for 120 min in 50 mM monosodium phosphate buffer (pH 7.0) containing 150 mM NaCl. The degradation of p-nitrophenyl decanoate results in the production of decanoate and p-nitrophenol (Figure 7). P-nitrophenol exhibits a distinct bright yellow color, enabling its detection via spectrophotometry at a wavelength of 400 nm.

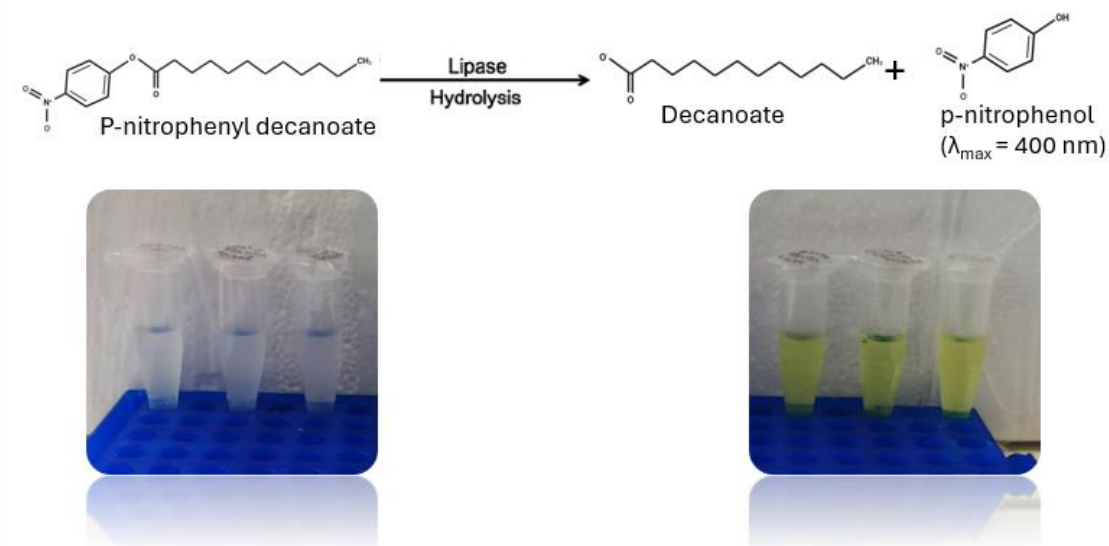

Figure 7. Enzymatic hydrolysis reaction of p-nitrophenyl decanoate.

Substrate specificity against various p-nitrophenyl esters was assessed using esters containing aliphatic chains of different lengths, including p-nitrophenyl acetate (C2), p-nitrophenyl caprylate (C8), p-nitrophenyl decanoate (C10), and p-nitrophenyl laurate (C12). The esterase activity was then measured separately for each substrate at a final concentration of 1.2 mM at 50 °C for 5 min in 50 mM phosphate buffer (pH 7) containing 150 mM NaCl.

The effects of temperature and pH on the esterase activity were examined using pNP-C8 (p-NP caprylate) as the substrate. The optimum temperature was studied in a temperature range of 10 °C – 80 °C, utilising a 50 mM Monosodium phosphate buffer containing 150 mM NaCl with pH 7. The optimum pH was studied at 50 °C in the pH range of 4.0–10.0. The

following buffers (50 mM) were used: disodium phosphate (pH 4.0–7.0), Tris-HCl (pH 7.0–10.0).

Enzyme kinetics of BioH were determined using p-nitrophenyl caprylate as a substrate at different concentrations ranging from 0.05 to 1.2 mM in 50 mM monosodium phosphate buffer (pH 7.0) containing 150 mM NaCl at 50 °C. The affinity constant (Km) and maximal velocity (Vmax) were calculated through Lineweaver-Burk plots.

Chapter 3 Results

3.1 | Phylogenetic analysis

The amino acid sequence analysis and BLAST search identified WP_246455038.1 pimeloyl-ACP methyl ester esterase [BioH] (*Thermomonas brevis*), as the protein with the highest amino acid identity (percentage identity 80.4%) to BioH isolated from our metagenomic library. As it is shown in Figure 8 the phylogenetic tree was divided into 3 main groups, with robust bootstrap values (>70%), which were further subdivided into 6 subgroups.

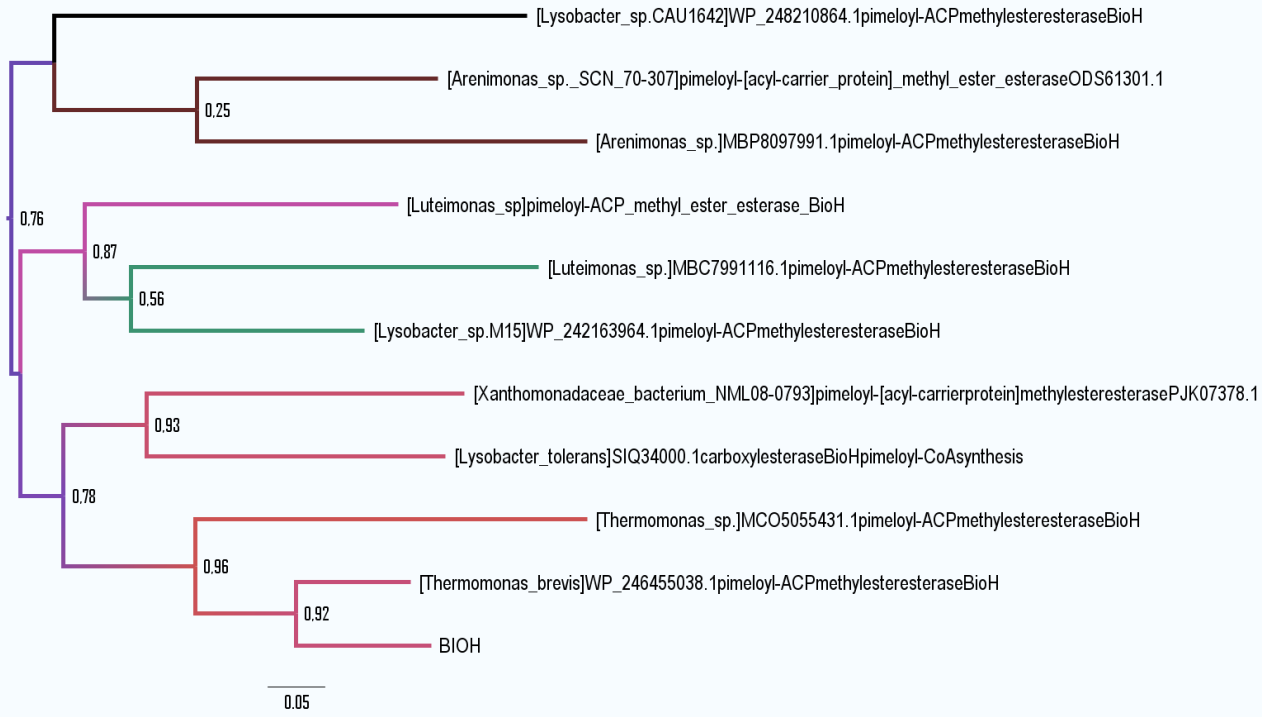

Figure 8. Phylogenetic tree of BioH and related proteins.

3.2 ||Gene amplification and bioinformatic analysis

Following the identification of the *bioH* gene, which encodes for an esterase potentially involved in pyrethroid degradation, we designed appropriate primers for amplification and gene overexpression. The *bioH* gene was amplified through PCR, and the resulting products were visualized in 1% agarose gel. The desired PCR product had a DNA length of **839 bp** as shown in Figure 9.

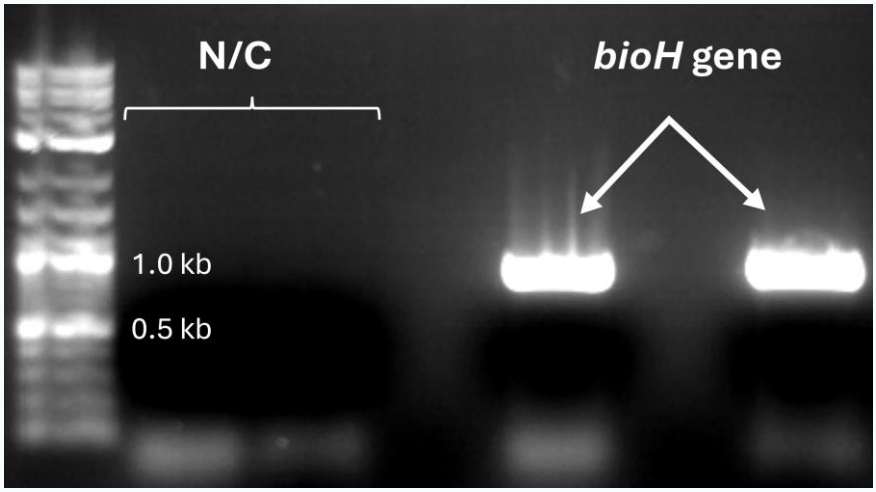

Figure 9. Agarose gel (1%) electrophoresis showing the results of PCR amplification targeting the *bioH* gene. Lane 1: DNA ladder, Lanes 2 and 3: negative controls (N/C), Lanes 4 and 5: amplified *bioH* gene.

The amplified product from the 3 different initial gene quantities were cleaned and united into one tube. Quantification of the amplified product indicated that 91.2 ng/ul of the gene was isolated, through the PCR amplification process.

3.3 |Cloning and Transformation

The subsequent step involved cloning of the amplified *bioH* product into pET21a plasmid, resulting in the creation of a pET21a-BioH plasmid. The target gene was inserted downstream of a T7-lac promoter and fused with a C-terminal His-Tag sequence, facilitating protein purification process (Figure 10A). Then the resulting recombinant plasmid was transformed into *E. coli* DH5a competent cells. Subsequently, the plasmid was digested with NotI and EcoRV to check the insert presence.

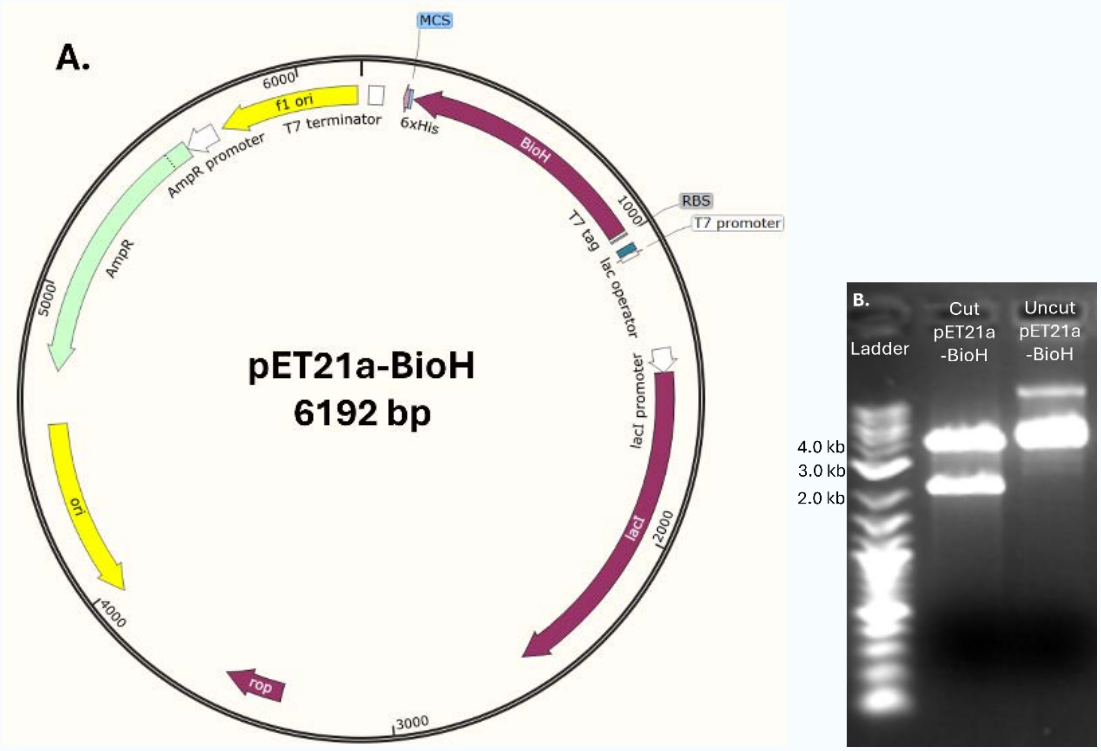

Figure 10. A. Schematic map of recombinant plasmid pet21a-BioH **B.** A diagnostic digestion verified the proper insertion of the target gene. The restriction enzymes EcoRV and NotI were used creating two fragments of 2097 and 4095 base pairs in the second well. In the third well, the different folds of the plasmid are depicted.

3.4 |Protein overexpression

Recombinant pET21a-BioH was subsequently used to transform *E. coli* BL21-Gold (DE3) chemical competent cells, chosen for their suitability for protein overexpression. The optimization of the overexpression procedure

involved varying IPTG concentrations, incubation temperatures, and durations. To confirm enzyme overexpression, SDS-PAGE analysis was performed on crude, supernatant, and pellet samples.

The molecular weight of the BioH protein was calculated to be approximately 30 kDa using the ExPASy's "compute pI/mw" tool (Figure 12). Analysis revealed that BioH was overexpressed to a greater extent in the supernatant fraction when induced with 0.5 mM IPTG at 37°C for 16 hours, as observed in Figure 13.

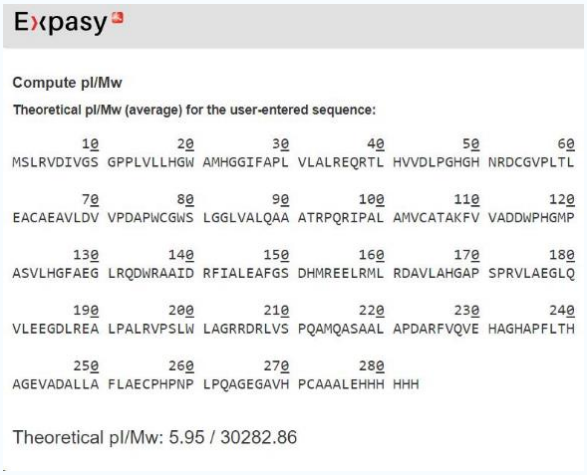

Figure 12. BioH protein has a molecular weight of 30.282 kDa as measured in the Expasy program.

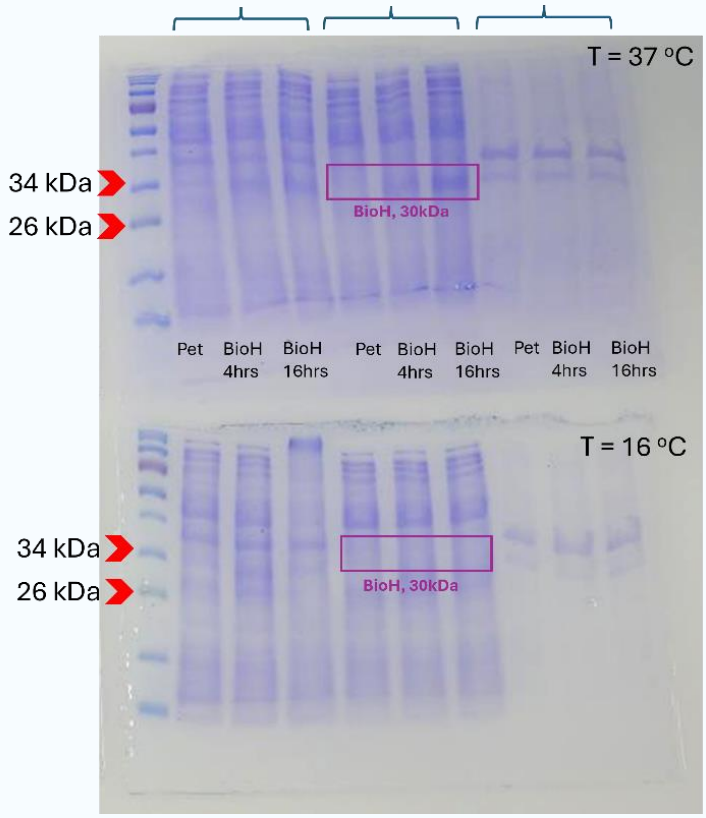

Figure 13. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (12%) was used to visualise the separated bacterial proteins according to their molecular weights. The BioH protein was overexpressed after 0.5 mM IPTG induction in 37 °C and 16 °C incubation, respectively.

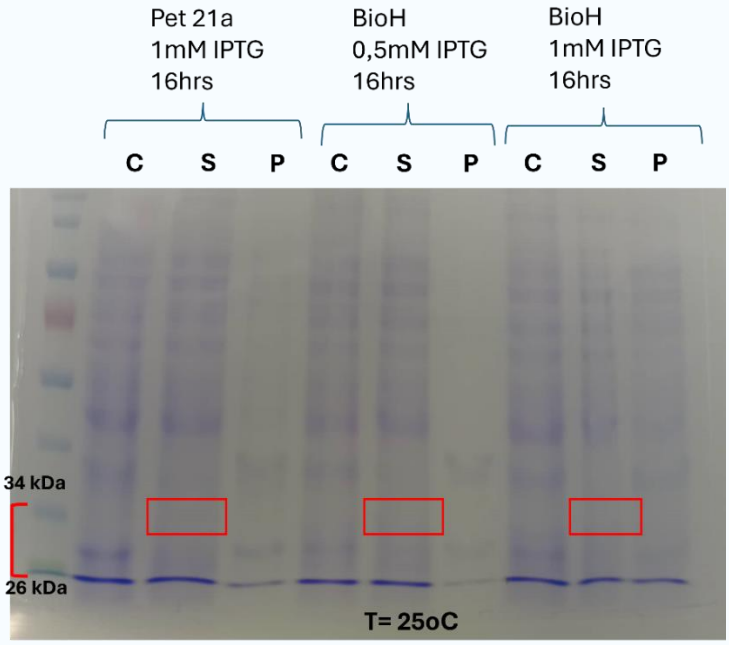

Figure 14. SDS-page gel (12%) indicating the bacterial proteins after adding different IPTG concentrations in 25 °C incubation. C = Crude, S = Supernatant, P = Pellet, L = Ladder.

3.4 | Protein purification

Before verifying the BioH enzyme's ability to degrade α-cypermethrin, the enzyme was purified. To achieve maximum purity, the purification steps were optimized. Buffers with varying imidazole concentrations were tested, with the most effective purification result achieved through a two-step wash using the NPI buffer containing 30 mM imidazole (Figure 15).

After the protein purification process, a dialysis step was performed to remove imidazole from the protein's buffer. The quantity of the purified protein was measured at 1.16 mg of protein per millilitre of protein buffer derived from 2-liter culture using the Invitrogen Qubit fluorometer with the Invitrogen Qubit Protein and Protein Broad range Assay kit. The purified protein was stored at -20 °C temperature with 50% glycerol for preservation.

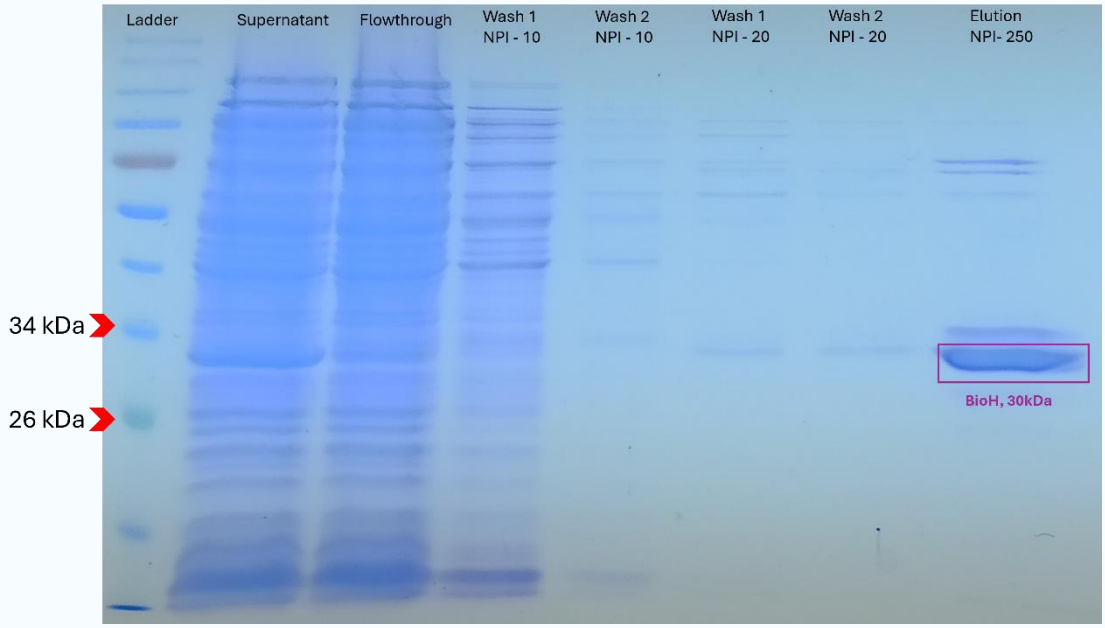

Figure 15. SDS-page gel (12%) indicating the washing steps before the protein isolation with the elution buffer (NPI-250)

3.5 | In Vitro Degradation of α-cypermethrin

The hydrolytic activity of the purified BioH enzyme towards α-cypermethrin was evaluated using an enzymatic assay. Degradation of α-cypermethrin was monitored at 0 hours, 9 hours, and 72 hours by analysing samples in an HPLC-UV system. As depicted in Figure 16, 200 μg of purified BioH catalysed the hydrolysis of cypermethrin under assay conditions of pH 6.5 and 37 °C within 9 hours.

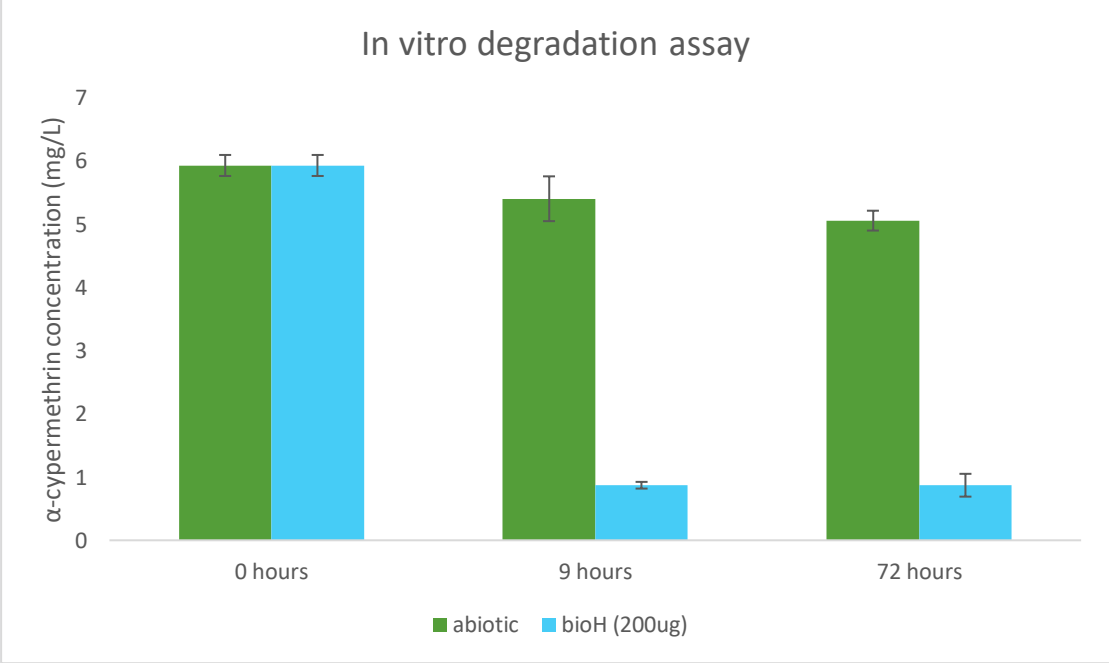

Figure 16. In vitro degradation of α-cypermethrin by the BioH enzyme. The diagram shows the pesticide's concentration over the passing of time after treatment with the BioH enzyme, indicating the pesticide's degradation over 72 hours.

3.6 | Enzymatic Characterization

After confirming the activity of BioH towards α-cypermethrin *in vitro*, we proceeded with its characterization. Initially, we evaluated its esterase activity using p-nitrophenyl decanoate (1.2 mM) as a substrate. Optimal enzyme concentration and assay duration were determined through a time-course experiment involving varying enzyme amounts (10 μg, 20 μg, 50 μg, 100 μg).

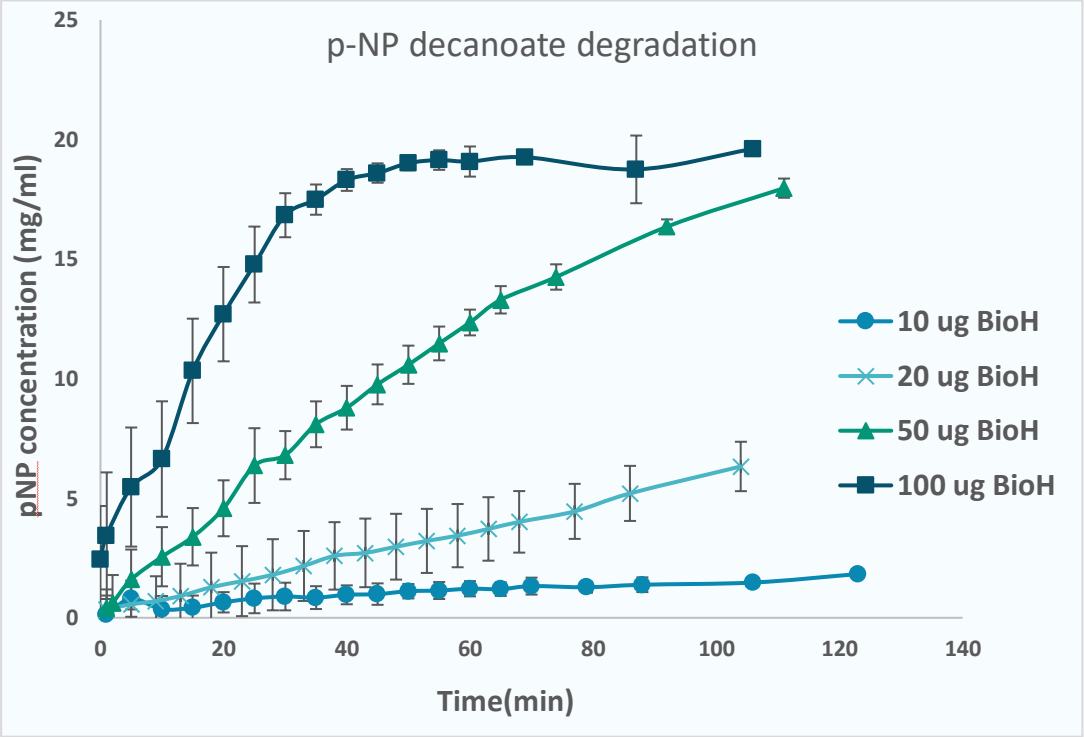

Figure 17. Degradation of p-nitrophenyl decanoate by different concentration of protein BioH. The diagram shows the p-nitrophenol that is produced from p-NP decanoate over minutes of time.

In Figure 17 we see the reaction progress curves obtained by mixing the enzyme and the substrate together and measuring the production of p-nitrophenol. We chose to proceed to subsequent assays using 100 μg of enzyme as this reaction demonstrated a higher initial reaction rate, maintained linearity for a sufficient duration to be assayed effectively, and produced a measurable amount of product.

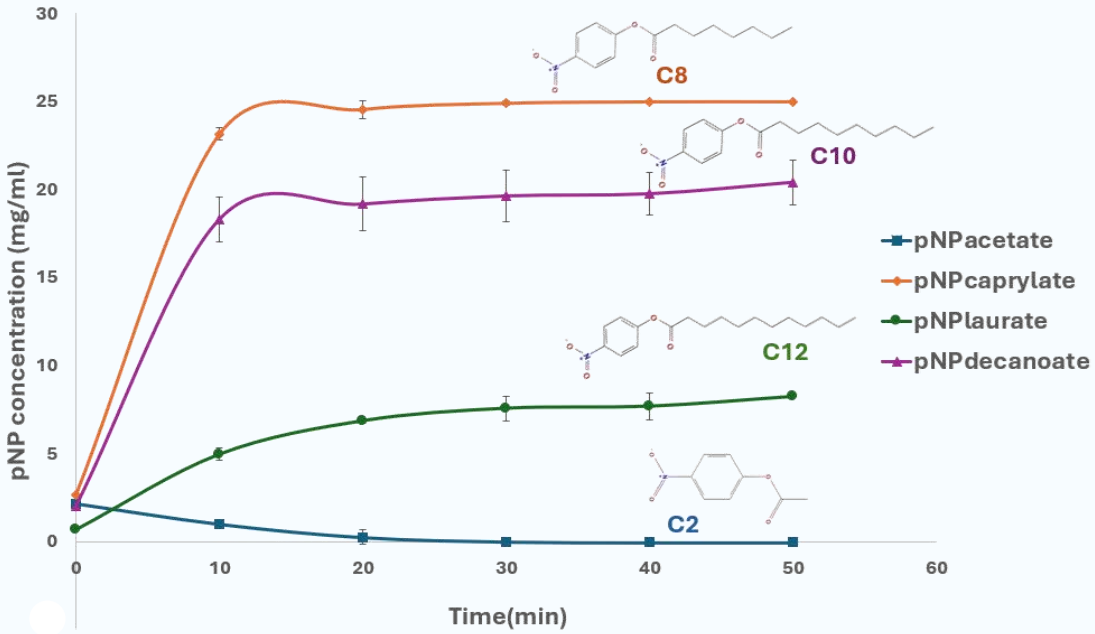

Figure 18. In vitro assay for the degradation of different p-nitrophenyl ester substrates by BioH. Four different substrates with aliphatic chains with 2 (p-NP acetate), 8 (p-NP caprylate), 10 (p-NP decanoate) and 12 (p-NP laurate) carbohydrates were used. The diagram shows p nitrophenol concentration over minutes of time.

In the next step, the substrate specificity of BioH was determined using pNP-esters with aliphatic chains of different lengths. Based on the findings of the previous assay, 100 μg of BioH were added to each reaction. As depicted in Figure 18, the enzyme showed the highest activity with p-nitrophenyl caprylate (C8), with activity decreasing as the aliphatic chain length increased. No activity was observed with esters containing aliphatic chain of 2 carbon atoms.

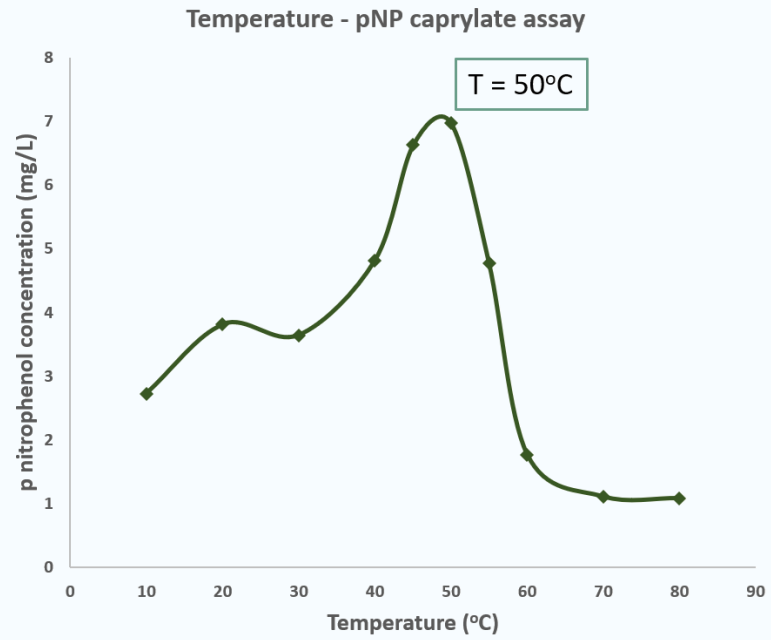

Figure 19. In vitro assay for the effect of temperature in p-NP caprylate degradation. The diagram shows p-nitrophenol production in different temperature values.

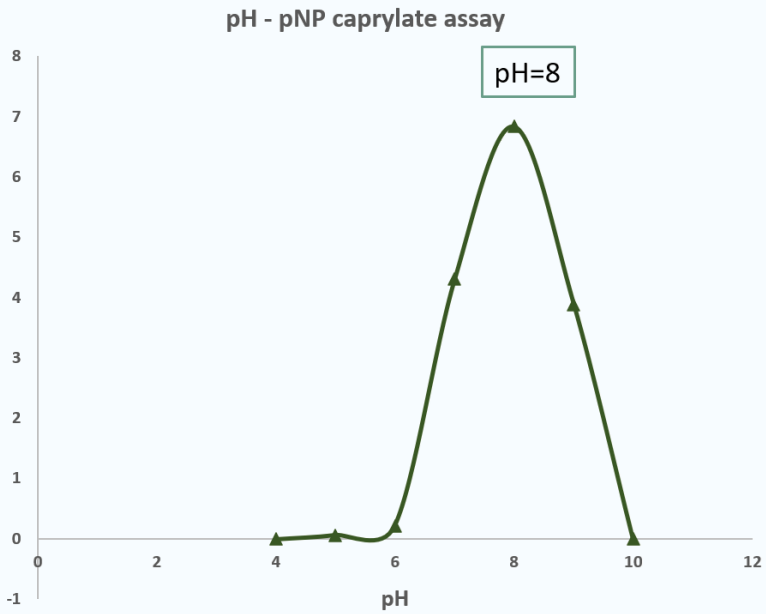

Figure 20. In vitro assay for the effect of pH in p-NP caprylate degradation. The diagram shows p-nitrophenol production in different pH values.

The following step focused on determining the optimal conditions of pH and temperature for BioH activity. Various temperature and pH values were tested and as depicted in the Figures 19 and 20 the BioH enzyme

exhibited its highest hydrolytic activity at a temperature of 50 ^{o}C and pH of 8.

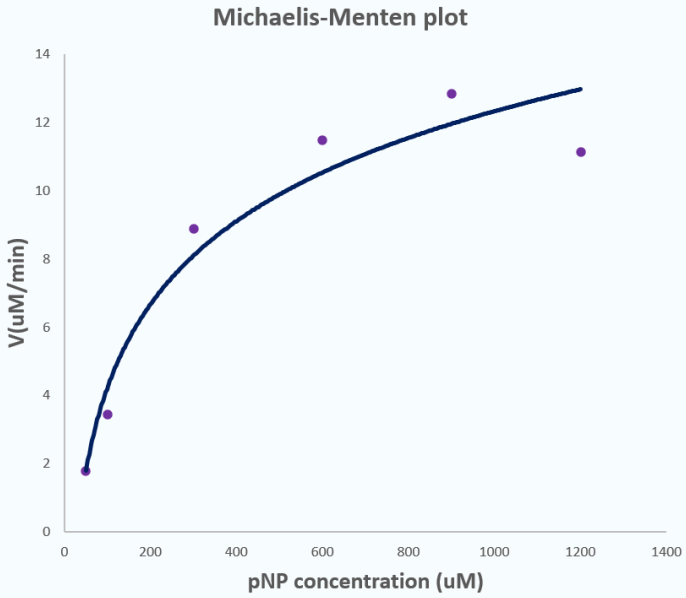

Figure 21. Michaelis-Menten plot indicating the velocity of p-NP caprylate degradation that changes with the different concentration of substrate.

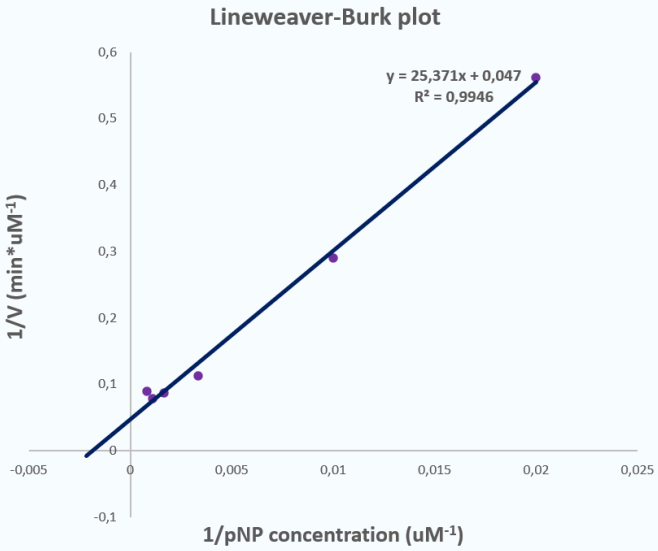

Figure 22. Lineweaver-Burk plot indicating the reverse velocity of the p-NP caprylate degradation that changes with the reversed difference in substrate concentration.

Under these optimum conditions, the Km and Vmax values were calculated for p-nitrophenyl caprylate substrate. Utilizing Michaelis-Menten and Lineweaver-Burk plots (Figures 19 and 20), the K_m was found to be 539,8 μM while the V_{max} was calculated as 21,3 μM/min.

Chapter 4 Discussion

In this thesis we further pursue the isolation of novel pyrethroid hydrolase from a metagenomic library containing DNA derived from biobed samples. The clones of this library were submitted into phenotypic tests revealing 12 clones positive for the presence of esterases/hydrolases. The 12 clones were tested for their ability to degrade α-cypermethrin; one of them carried the desired phenotype, the degradation of α-cypermethrin. Transposon mutagenesis in the positive fosmid pointed to *bioH* gene as responsible for the degradation of α-cypermethrin. The BioH protein was overexpressed and an *in vitro* assay for the α-cypermethrin degradation indicated that this enzyme was able to degrade α-cypermethrin. The isolation of BioH indicates that the microbial community of biobed systems, expected to be heavily and regularly exposed to pesticides, could evolve generic enzymatic capacities for pesticide degradation (Papazlatani, Karas, Tucat & Karpouzas, 2019).

Many studies indicate that bacteria (like *Bacillus spp., Raoultella ornithinolytica, Pseudomonas fluorescens*) and fungi (like *Aspergillus sp., Candida sp., Trichoderma sp*.) have a pyrethroid degrading ability in liquid cultures or in soil using them as carbon source (Birolli et al., 2016b; Cycoń and Piotrowska-Seget, 2016; Chen and Zhan, 2019; Birolli et al., 2019; Hu et al., 2019; Zhao et al., 2019). Ester hydrolysis is the first step in pyrethroid degradation pathway (Zhao et al., 2015; Tang et al., 2017; Bhatt et al., 2020a). Purified enzymes, like carboxylesterases, monooxygenases and aminopeptidases have been characterized as potent in the hydrolysis of the ester bond of pyrethroids. Carboxylesterases involved in the degradation of pyrethroids have been previously isolated by soil microbes including EstP (*Klebsiella sp. ZD112*), Pye3 (derived from soil microbiome), PytH (*Sphingobium sp. JZ-1*), PytZ and PytY (derived from the genomic ligrary of *Ochrobactrum anthropic sp. YZ-1*) (Wu et al., 2006; Li et al., 2008; Wang et al., 2011; Zhai et al., 2012; Ruan et al., 2013). It should be noted that these enzymes are quite distant phylogenetically suggesting that they have evolved in parallel from different ancestors under the environmental pressure of high pyrethroid exposure. Nevertheless, all of them have the same ability in degrading pyrethroid pesticides, cypermethrin included (Cycoń & Piotrowska-Seget, 2016).

As aforementioned, the *bioH* gene codes for an α/β hydrolase necessary for the biosynthetic pathway of biotin in many bacteria, as shown in Figure 23. Biotin, known as vitamin H, is an essential cofactor for the primary metabolism of microbes, including fatty acid synthesis and amino acid degradation (Cao, Zhu, Hu & Cronan, 2017). Biotin biosynthesis involves six genes that belong to the bioABFCD operon (Hu & Cronan, 2020). The BioH enzyme catalyzes the removal of methyl groups allowing the synthesis of the protein carrier of pimeloyl-alkyl groups through the biosynthetic pathway of fatty acids, through ester hydrolysis.

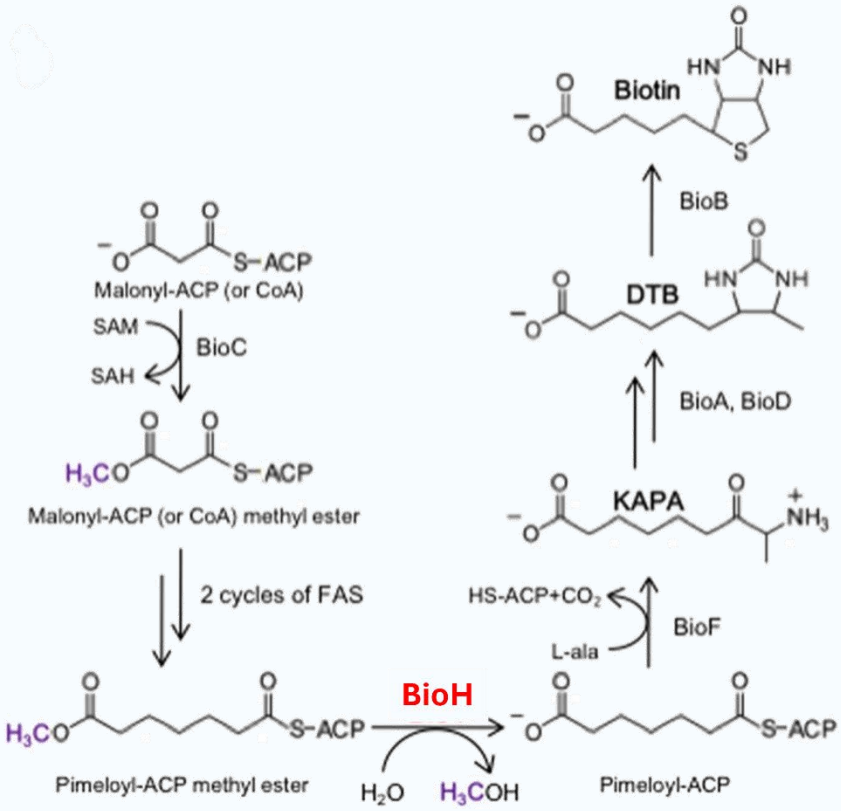

Figure 23. Biotin biosynthetic pathway consists of six genes, including BioH, which catalyses the degradation of pimeloyl-Acp methyl ester into Pimeloyl-ACP.

BioH, also, has a weak thioesterase activity as it prefers ester bonds from short-chain fatty acids and p-nitrophenyl esters (Tomczyk et al., 2002). Zhou et al (2022) reported the potential involvement of BioH in the degradation of fenvalerate, another pyrethroid, by *Citrobacter freundii CD-9*. In parallel, this same strain had the capacity to degrade other pyrethroids like β-cypermethrin (Zhou et al., 2022). The amino acid sequence of this enzyme was compared (NCBI Blast) with the BioH enzyme of this research thesis revealing a 40% identity. This suggests that BioH, like many carboxylesterases involved in the degradation of

pyrethroids, may be phylogenetical distant but they can share a common hydrolytic activity against pyrethroids. Based on the inherent role of BioH in the biosynthesis of biotin and its proven capacity to degrade pyrethroids, we could speculate that under continuous exposure of microorganisms to pyrethroids bacteria could mobilize hydrolases being involved in the primary metabolism to degrade toxicants like pyrethroids.

After identification of the BioH as a pyrethroid degrading enzyme, the enzyme was characterized, and the highest enzymatic activity was observed at temperature 50 ^{o}C and pH 8. Among environmental factors, temperature and pH have significant effects on the degradation efficiency of enzymes, which is consistent with the previous studies on optimization of degradation conditions (Liu et al., 2001; Chen et al., 2022; Tang et al., 2017). Under these conditions, the K_m and V_{max} for p-NP caprylate were calculated as 539,8 μM and 21,3 μM/min, respectively. Considering other studies, the K_m value suggested a high affinity for p-nitrophenyl caprylate substrate, which was used as an optimum substrate for testing (Luo et al., 2018), while these values will be next derived for alpha-cypermethrin. Substrate specificities revealed that BioH had optimum activity towards medium-chain p-nitrophenyl esters. So, BioH may be an enzyme with lipase activity, according to the different preferences of substrates between carboxylesterases and lipases.

Taken together, a new pyrethroid degrading enzyme, BioH, has been identified and cloned in this study. The enzyme had the ability to degrade α-cypermethrin, a synthetic pyrethroid pesticide, over 9 hours. The enzyme has the highest degrading capacity over substrates with medium aliphatic chains, like p-nitrophenyl caprylate, at 50 ^{o}C temperature and pH 7. The kinetics indicate a medium affinity for p-NP caprylate, as it is not a substrate that the enzyme utilizes naturally. Finally, further studies are necessary to identify the full breadth of activity of BioH against other pyrethroids.

Chapter 5 Future perspectives

Future perspectives

The on-going research is related to the characterization of the activity range of BioH against different pyrethroids. First, second and new generation pyrethroids will be used in order to identify the range of BioH activity towards pyrethroids and potential chemical structures that increase or decrease the activity of BioH toward pyrethroid compounds. The compounds tested will be the following:

- ***Acrinathrin***
- ***Bifenthrin***
- ***Cyfluthrin***
- ***Etofenprox***
- ***Tau-fluvalinate***

References

- A, A. K. (2018). Epidemiology of Pesticides in Developing Countries. Advances in Clinical Toxicology, 3(1). https://doi.org/10.23880/act-16000128
- Aguila-Torres, P., Maldonado, J., Gaete, A., Figueroa, J., González, A., Miranda, R., González-Stegmaier, R., Martin, C., & González, M. (2020, May 13). Biochemical and Genomic Characterization of the Cypermethrin-Degrading and Biosurfactant-Producing Bacterial Strains Isolated from Marine Sediments of the Chilean Northern Patagonia. Marine Drugs, 18(5), 252. https://doi.org/10.3390/md18050252
- alphα-cypermethrin. (2022). Retrieved 10 August 2022, from https://pubchem.ncbi.nlm.nih.gov/compound/alphα-cypermethrin
- Andreu, V. and Picó, Y. (2012) 'Determination of currently used pesticides in biota', Analytical and Bioanalytical Chemistry [Preprint]. https://doi.org/10.1007/s00216-012-6331-x
- Bhatt, P., Bhatt, K., Huang, Y., Lin, Z., & Chen, S. (2020). Esterase is a powerful tool for the biodegradation of pyrethroid insecticides. Chemosphere, 244, 125507. https://doi.org/10.1016/j.chemosphere.2019.125507
- Birolli, W. G., Vacondio, B., Alvarenga, N., Seleghim, M. H. R., and Porto, A. L. M. (2018). Enantioselective biodegradation of the pyrethroid (±)-lambda-cyhalothrin by marine-derived fungi. Chemosphere 197, 651–660. https://doi.org/10.1016/j.chemosphere.2018.01.054
- Casida, J. (2010). Michael Elliott's billion dollar crystals and other discoveries in insecticide chemistry. Pest Management Science, 66(11), 1163-1170. https://doi.org/10.1002/ps.1982
- Castillo, M. D. P., & Torstensson, L. (2008). Biobeds - Biotechnology for Environmental Protection from Pesticide Pollution. In Methods and Techniques for Cleaning-up Contaminated Sites (pp. 145–151). Springer Netherlands. https://doi.org/10.1007/978-1-4020-6875-1_13
- Chapman, R. A., & Harris, C. R. (1981, January). Persistence of four pyrethroid insecticides in a mineral and an organic soil. Journal of

Environmental Science and Health, Part B, 16(5), 605–615. https://doi.org/10.1080/03601238109372282

- Chen, C. et al. (2022) 'Expression, characterization, fermentation, immobilization, and application of a novel esterase Est804 from metagenomic library in pesticide degradation', Frontiers in Microbiology, 13. https://doi.org/10.3389/fmicb.2022.922506
- Chevrette, M. G., & Handelsman, J. (2020). From Metagenomes to Molecules: Innovations in Functional Metagenomics Unlock Hidden Chemistry in the Human Microbiome. Biochemistry, 59(6), 729–730. https://doi.org/10.1021/acs.biochem.0c00033
- Chrustek, A., Hołyńska-Iwan, I., Dziembowska, I., Bogusiewicz, J., Wróblewski, M., Cwynar, A., & Olszewska-Słonina, D. (2018). Current Research on the Safety of Pyrethroids Used as Insecticides. Medicina, 54(4), 61. https://doi.org/10.3390/medicina54040061
- Ding, J., Liu, Y., Gao, Y., Zhang, C., Wang, Y., Xu, B., Yang, Y., Wu, Q., & Huang, Z. (2022, February). Biodegradation of λ-cyhalothrin through cell surface display of bacterial carboxylesterase. Chemosphere, 289, 133130. https://doi.org/10.1016/j.chemosphere.2021.133130
- Lucio G. Costa, Chapter 9 - The neurotoxicity of organochlorine and pyrethroid pesticides, Editor(s): Marcello Lotti, Margit L. Bleecker, Handbook of Clinical Neurology, Elsevier, Volume 131, 2015, Pages 135-148, ISSN 0072-9752, ISBN 9780444626271, https://doi.org/10.1016/B978-0-444-62627-1.00009-3
- Cycoń, M., and Piotrowska-Seget, Z. (2016). Pyrethroid-degrading microorganisms and their potential for the bioremediation of contaminated soils: a review. Front. Microbiol. 7:1463. https://doi.org/10.3389/fmicb.2016.01463
- CYPERMETHRIN CAS no. 52315-07-8 Evaluation report according to Regulation 528/2012 Arysta LifeScience Benelux sprl, Belgium. (2017). Retrieved 13 September 2022, from https://echa.europa.eu/documents/10162/716f554b-b973-1a61-5bf2-eff87b36a237
- Diegelmann, C., Weber, J., Heinzel-Wieland, R., & Kemme, M. (2015). Characterization of a cypermethrin-degrading Methylobacterium sp. strain A-1 and molecular cloning of its

carboxylesterase gene. Journal Of Basic Microbiology, 55(11), 1245-1254. https://doi.org/10.1002/jobm.201500186

- Fan, X., Liang, W., Li, Y., Li, H., & Liu, X. (2017, September 11). Identification and immobilization of a novel cold-adapted esterase, and its potential for bioremediation of pyrethroid-contaminated vegetables. Microbial Cell Factories, 16(1). https://doi.org/10.1186/s12934-017-0767-9
- Fan, X., Liu, X., Huang, R., & Liu, Y. (2012, March 13). Identification and characterization of a novel thermostable pyrethroid-hydrolyzing enzyme isolated through metagenomic approach. Microbial Cell Factories, 11(1). https://doi.org/10.1186/1475-2859-11-33
- Fang, Y. et al. (2022) 'Microbial elimination of pyrethroids: Specific strains and involved enzymes', Applied Microbiology and Biotechnology, 106(21), pp. 6915–6932. https://doi.org/10.1007/s00253-022-12200-w
- Gangola, S., Sharma, A., Bhatt, P., Khati, P., & Chaudhary, P. (2018, August 24). Presence of esterase and laccase in Bacillus subtilis facilitates biodegradation and detoxification of cypermethrin. Scientific Reports, 8(1). https://doi.org/10.1038/s41598-018-31082-5
- Hodoșan, C., Gîrd, C. E., Ghica, M. V., Dinu-Pîrvu, C. E., Nistor, L., Bărbuică, I. S., Marin, T. C., Mihalache, A., & Popa, L. (2023, November 29). Pyrethrins and Pyrethroids: A Comprehensive Review of Natural Occurring Compounds and Their Synthetic Derivatives. Plants, 12(23), 4022. https://doi.org/10.3390/plants12234022
- Hołyńska-Iwan, I., & Szewczyk-Golec, K. (2020). Pyrethroids: How They Affect Human and animal health? Medicina, 56(11), 582. doi:10.3390/medicina56110582
- Huang, Y., Xiao, L., Li, F., Xiao, M., Lin, D., Long, X., & Wu, Z. (2018). Microbial Degradation of Pesticide Residues and an Emphasis on the Degradation of Cypermethrin and 3-phenoxy Benzoic Acid: A Review. Molecules, 23(9), 2313. https://doi.org/10.3390/molecules23092313
- Indratin, I., Kurnia, A., & Wahyuni, S. (2019, December 20). Degradation of Cypermethrin by Indigenous Bacteria from

Contaminated Soil. Makara Journal of Science, 210–216. https://doi.org/10.7454/mss.v23i4.7998

- King, A.M. and Aaron, C.K. (2015) 'Organophosphate and carbamate poisoning', Emergency Medicine Clinics of North America, 33(1), pp. 133–151. https://doi.org/10.1016/j.emc.2014.09.010
- Koureas, M., Tsakalof, A., Tsatsakis, A., & Hadjichristodoulou, C. (2012). Systematic review of biomonitoring studies to determine the association between exposure to organophosphorus and pyrethroid insecticides and human health outcomes. Toxicology Letters, 210(2), 155–168. https://doi.org/10.1016/j.toxlet.2011.10.007
- Lam, K. N., Cheng, J., Engel, K., Neufeld, J. D., & Charles, T. C. (2015). Current and future resources for functional metagenomics. Frontiers in Microbiology, 6. https://doi.org/10.3389/fmicb.2015.01196
- Li, G., Wang, K., & Liu, Y. (2008). Molecular cloning and characterization of a novel pyrethroid-hydrolyzing esterase originating from the Metagenome. Microbial Cell Factories, 7(1). https://doi.org/10.1186/1475-2859-7-38
- Liu YChung Y, Xiong Y. 2001. Purification and Characterization of a Dimethoate-Degrading Enzyme of Aspergillus niger ZHY256, Isolated from Sewage. Appl Environ Microbiol 67:. https://doi.org/10.1128/AEM.67.8.3746-3749.2001
- Luo, X. et al. (2018) 'Cloning and characterization of a pyrethroid pesticide decomposing esterase gene, EST3385, from rhodopseudomonas palustris PSB-S', Scientific Reports, 8(1). https://doi.org/10.1038/s41598-018-25734-9
- Méjanelle, L., Jara, B., & Dachs, J. (2020). Fate of Pyrethroids in Freshwater and Marine Environments. The Handbook of Environmental Chemistry, 81–107. https://doi.org/10.1007/698_2019_433
- New England Biolabs. (2012, December 7). Protocol for Q5® High-Fidelity 2X Master Mix | NEB. https://www.neb.com/en/protocols/2012/12/07/protocol-for-q5-high-fidelity-2x-master-mix-m0492

- Navgire, G. S., Goel, N., Sawhney, G., Sharma, M., Kaushik, P., Mohanta, Y. K., Mohanta, T. K., & Al-Harrasi, A. (2022). Analysis and Interpretation of metagenomics data: an approach. Biological Procedures Online, 24(1). https://doi.org/10.1186/s12575-022-00179-7
- Perry, J. D., & Freydière, A. M. (2007). The application of chromogenic media in clinical microbiology. Journal of Applied Microbiology, 103(6), 2046–2055. https://doi.org/10.1111/j.1365-2672.2007.03442.x
- Primer designing tool. (n.d.). National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi
- Prusty, A. K., Meena, D. K., Mohapatra, S., Panikkar, P., Das, P., Gupta, S. K., & Behera, B. K. (2015). Synthetic pyrethroids (Type II) and freshwater fish culture: Perils and mitigations. International Aquatic Research, 7(3), 163–191. https://doi.org/10.1007/s40071-015-0106-x
- Ranatunga, M., Kellar, C., & Pettigrove, V. (2023, July). Toxicological impacts of synthetic pyrethroids on non-target aquatic organisms: A review. Environmental Advances, 12, 100388. https://doi.org/10.1016/j.envadv.2023.100388
- Singh, B., & Walker, A. (2006). Microbial degradation of organophosphorus compounds. FEMS Microbiology Reviews, 30(3), 428-471. https://doi.org/10.1111/j.1574-6976.2006.00018.x
- Sirithanakorn, C., & Cronan, J. E. (2021, January 11). Biotin, a universal and essential cofactor: synthesis, ligation and regulation. FEMS Microbiology Reviews. https://doi.org/10.1093/femsre/fuab003
- Tang, A., Wang, B., Liu, Y., Li, Q., Tong, Z., & Wei, Y. (2015, April 30). Biodegradation and extracellular enzymatic activities of Pseudomonas aeruginosa strain GF31 on β-cypermethrin. Environmental Science and Pollution Research, 22(17), 13049–13057. https://doi.org/10.1007/s11356-015-4545-0
- Tang, A.-X. *et al.* (2017) 'Purification and characterization of a novel β-cypermethrin-degrading aminopeptidase from *pseudomonas aeruginosa* GF31', *Journal of Agricultural and Food Chemistry*, 65(43), pp. 9412–9418. https://doi.org/10.1021/acs.jafc.7b03288

- Tang, W., Wang, D., Wang, J., Wu, Z., Li, L., Huang, M., Xu, S., & Yan, D. (2018). Pyrethroid pesticide residues in the global environment: An overview. Chemosphere, 191, 990–1007. https://doi.org/10.1016/j.chemosphere.2017.10.115
- Thomas, T., Gilbert, J., & Meyer, F. (2012). Metagenomics - a guide from sampling to data analysis. Microbial Informatics and Experimentation, 2(1). https://doi.org/10.1186/2042-5783-2-3
- Tomczyk, N. H., Nettleship, J. E., Baxter, R. L., Crichton, H. J., Webster, S. P., & Campopiano, D. J. (2002, February). Purification and characterisation of the BIOH protein from the biotin biosynthetic pathway. FEBS Letters, 513(2–3), 299–304. https://doi.org/10.1016/s0014-5793(02)02342-6
- Uchiyama, T., & Miyazaki, K. (2009). Functional metagenomics for enzyme discovery: challenges to efficient screening. Current Opinion in Biotechnology, 20(6), 616–622. https://doi.org/10.1016/j.copbio.2009.09.010
- Wang, B., Guo, P., Hang, B., Li, L., He, J., Wang, B., et al. (2009). Cloning of a novel pyrethroid-hydrolyzing carboxylesterase gene from Sphingobium sp. strain JZ-1. Appl. Environ. Microbiol. 75, 5496–5500. https://doi.org/10.1128/AEM.01298-09
- Wang, Y., Fan, W., Zhang, X., Guan, Q., Li, J., & Wang, M. et al. (2022). Enhanced Biodegradation Of Triadimefon by Newly Isolated Enterobacter Hormaechei Strain Ty18: Performance Optimization, Degradation Pathways and Transcriptome Profiles. SSRN Electronic Journal. https://dx.doi.org/10.2139/ssrn.4118349
- Wei, T., Feng, S., Shen, Y., He, P., Ma, G., Yu, X., Zhang, F., & Mao, D. (2013, December). Characterization of a novel thermophilic pyrethroid-hydrolyzing carboxylesterase from Sulfolobus tokodaii into a new family. Journal of Molecular Catalysis B: Enzymatic, 97, 225–232. https://doi.org/10.1016/j.molcatb.2013.07.022
- Wu, P. C., Liu, Y. H., Wang, Z. Y., Zhang, X. Y., Li, H., Liang, W. Q., et al. (2006). Molecular cloning, purification, and biochemical characterization of a novel pyrethroid-hydrolyzing esterase from Klebsiella sp. strain ZD112. J. Agric. Food Chem. 54, 836–842. https://doi.org/10.1021/jf052691u
- Wu, Y.-W. (2018) 'EzTree: An automated pipeline for identifying phylogenetic marker genes and inferring evolutionary relationships

among uncultivated prokaryotic draft genomes', BMC Genomics, 19(S1). https://doi.org/10.1186/s12864-017-4327-9

- Xu, Y., Yang, J., Li, W., Song, S., Shi, Y., Wu, L., Sun, J., Hou, M., Wang, J., Jia, X., Zhang, H., Huang, M., Lu, T., Gan, J., & Feng, Y. (2022, July 11). Three enigmatic BioH isoenzymes are programmed in the early stage of mycobacterial biotin synthesis, an attractive anti-TB drug target. PLOS Pathogens, 18(7), e1010615. https://doi.org/10.1371/journal.ppat.1010615
- Xie, W., & Zhou, J. (2008, December). Cypermethrin persistence and soil properties as affected by long-term fertilizer management. Acta Agriculturae Scandinavica, Section B - Plant Soil Science, 58(4), 314–321. https://doi.org/10.1080/09064710701743096
- Xu, D., Gao, Y., Sun, B., Ran, T., Zeng, L., He, J., He, J., & Wang, W. (2020, June 2). Pyrethroid Carboxylesterase PytH from Sphingobium faniae JZ-2: Structure and Catalytic Mechanism. Applied and Environmental Microbiology, 86(12). https://doi.org/10.1128/aem.02971-19
- Yoo, M., Lim, Y. H., Kim, T., Lee, D., & Hong, Y. C. (2016, January 13). Association between urinary 3-phenoxybenzoic acid and body mass index in Korean adults: 1st Korean National Environmental Health Survey. Annals of Occupational and Environmental Medicine, 28(1). https://doi.org/10.1186/s40557-015-0079-7
- Zhai, Y., Li, K., Song, J., Shi, Y., & Yan, Y. (2012). Molecular cloning, purification and biochemical characterization of a novel pyrethroid-hydrolyzing carboxylesterase gene from Ochrobactrum anthropi YZ-1. Journal of Hazardous Materials, 221-222, 206–212. https://doi.org/10.1016/j.jhazmat.2012.04.031
- Zhan, H., Huang, Y., Lin, Z., Bhatt, P., & Chen, S. (2020). New insights into the microbial degradation and catalytic mechanism of synthetic pyrethroids. Environmental Research, 182, 109138. https://doi.org/10.1016/j.envres.2020.109138
- Zhao, J., Jia, D., Chi, Y., & Yao, K. (2020). Co-metabolic enzymes and pathways of 3-phenoxybenzoic acid degradation by Aspergillus oryzae M-4. Ecotoxicology And Environmental Safety, 189, 109953. https://doi.org/10.1016/j.ecoenv.2019.109953
- Zhao, T., Hu, K., Li, J., Zhu, Y., Liu, A., Yao, K., & Liu, S. (2021, September). Current insights into the microbial degradation for

pyrethroids: strain safety, biochemical pathway, and genetic engineering. Chemosphere, 279, 130542. https://doi.org/10.1016/j.chemosphere.2021.130542