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HYDROCARBON AND INSECTICIDE INDUCTION OF *BEAUVERIA BASSIANA* CATALYSIS OF ORGANOSULFUR COMPOUNDS

by

Felipe Nicolau-Manterola

A thesis submitted in partial fulfillment
of the requirements for the Doctor of Philosophy
degree in Chemical and Biochemical Engineering in the
Graduate College of
The University of Iowa

May 2016

Thesis Supervisor: Professor Tonya L. Peeples

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PH.D. THESIS

_____

This is to certify that the Ph.D thesis of

Felipe Nicolau-Manterola

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy in Chemical and Biochemical Engineering at the May 2016 graduation.

Thesis Committee: _____
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David Murhammer

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Jennifer Fiegel

_____
Tim Mattes

_____
David Cwiertny

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To my parents and my grandparents

"Niqitoa ni Nesaualkoyotl:
¿Kuix ok neli nemoua in tlaltikpak? An nochipa tlaltikpak:
san achika ya nikan.
Tel ka chalchiuitl no xamani,
no teokuitlatl in tlapani,
no ketsali posteki.
An nochipa tlaltikpak:
san achika ye nikan."

"Yo Nezahualcóyotl lo pregunto:
¿Acaso de veras se vive con raíz en la tierra?
Nada es para siempre en la tierra:
Sólo un poco aquí.
Aunque sea de jade se quiebra,
Aunque sea de oro se rompe,
Aunque sea plumaje de quetzal se desgarra.
No para siempre en la tierra:
Sólo un poco aquí."

Nezahualcoyotl (1402-1472)

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protein analysis. I thank the USDA for the donation of some fungal strains from their NRRL Culture Collection.

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ABSTRACT

Catalysts are utilized in 80% of all chemical synthesis operations. The industrial catalysts primarily used in oxidation reactions are highly polluting and expensive metal catalysts. Enzymes and whole cell biocatalysts are used to a lesser extent. Nowadays, several industrial sectors are developing bio-based technologies to reduce the high costs and environmental impact of traditional chemical processes. However, these applications are limited by the challenge of developing economically competitive biologically based systems. The key for adopting these sustainable advancements is the development of novel process designs, which assure robustness, simplicity, and sustainabile operations compatible with the current development of chemical reactions. In this regard, filamentous fungi may be considered good biocatalysts due to their natural biodiversity and their broad heterogeneous enzymatic pattern. The great selectivity of fungal catalysis is now well recognized for the production of commercially valuable steroids in the pharmaceutical industry. Although this inherent capacity is mainly used for functionalization of unactivated carbons, it can be further exploited for the oxidiation of heteroatoms, such as sulfur. Focusing on the oxidation of sulfur compounds, the widely used industrial processes are produced by an organometallic catalyst. This PhD project aims to overcome low substrate conversion and enzymatic expression by proving *that exposure of cells to insecticides and hydrocarbons increases cell's oxidative capacity expressed as higher substrate conversion and CYP450 content.* This study is focused in the application of pest management strategies, designed to enhance the biopesticide's efficacy, to induce and improve *Beauveria bassiana* oxidation. *B. bassiana* has a very flexible metabolism and is widely used as a biocontrol agent. It can metabolize hexadecane as a sole carbon source. In addition, it shows a synergistic effect over pest control efficacy when it is applied with low pesticides (carbaryl and/or imidacloprid) concentrations.

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A biocatalytic system was optimized to increase the conversion of organosulfur compounds under different fermentation conditions. Phenothiazine was used as our model substrate. Phenothiazine conversion was followed by GC-MS and HPLC. By NMR and MS fragmentation pattern product, phenothiazine metabolites were identified as (R)-hydroxyl metabolites (63% enatiomeric excess) and sulfoxide, the latter being the main metabolite. Phenothiazine conversions with growing cells resulted in 65±1.4% conversion with initial phenothiazine concentration of 500 ppm and final 325 ppm after 7 days. The highest conversion, 74±1 % was achieved with resting cells at the lowest cell concentration, 0.78 mg cell dry weight (cdw) /mL. Furthermore, the use of insecticides as inducers was an effective way to increase phenothiazine conversion from 47% to 64±3%. The major enzymes involved in catalysis of xenobiotic are heme-binding monooxygenases, in particular cytochrome P450. Heme positive proteins were identified by an SDS benzidine assay as well as the content of CYP450 by the CO difference spectrum. The P450 enzymes content was 12.3±1 pmol/µg protein for hexadecane adapted cells and 8.1± 1 pmol/µg protein for insecticides, respectively. The heme-positive proteins were characterized by MALDI-ToF and their peptide mass fingerprint compared to the available sequences on the SwissProt/Universal Protein Resource catalog of information on proteins (UniProtKB). Hemoproteins were found, including a cluster of catalase-peroxidase, alkane hydroxylase, and chloroperoxidase. The results from this project helped bridge the progress from agricultural biotechnology strain development into industrial biotechnology biocatalyst improvement. The success of this project helps us expand *B. bassiana's* catalysis and make it a better candidate for industrial biocatalysis.

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PUBLIC ABSTRACT

Filamentous fungi are used in biotechnology as cell factories for a wide range of products. The application of fungi in synthesis ranges from preparation of novel compounds in the milligram scale up to large-scale industrial production of bulk and fine chemicals. Since fungi live by absorbing nutrients, they possess the ability to rapidly adapt their metabolism to different suboptimal growth conditions, including nutrient deprivation, the presence of foreign and antifungal compounds. Fungi express a number of specific detoxifying enzymes, which degrade compounds in ways that eliminate the effect of toxic compounds or unfavorable conditions. However, these applications are limited by the challenge of developing economically competitive biologically based systems. The key for adopting these sustainable advancements is the development of novel process designs, which assure robustness, simplicity, and sustainability operations compatible with the current development of chemical reactions. A potential strategy for more sustainable chemicals production is the use of pest control fungi, such as *Beauveria bassiana*. *B.bassiana* research efforts had centered in virulence enhancement for better pest management, but the effect it has over oxidative catalysis has not been explored. Our work would provide evidence that oxidative performance is increased as consequence of virulence enhancement. The PhD project consisted of using insecticides and organic solvents to improve the oxidation of sulfur compounds, in particular phenothiazine. Results show that exposing cells to n-hexadecane and insecticides improved the conversion of phenothiazine. To further understand the proteins involved in sulfoxidation, the proteins were isolated from different cell fractions and their major expressed protein families were identified. In addition, to extend use of *B. bassiana*, it was necessary to optimize reaction conditions such as biocatalyst preparation, substrate concentration, inducers growth inhibition effects, and glucose concentration.

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TERMS AND ABBREVIATIONS

amu: Atomic Mass Unit, 1 Dalton

BSA: Bovine Serum Albumin

BCA: Bicinchoninic Acid

CDE: Cuticle degrading enzymes

CYP450: Cytochrome P450

Dunnett's test: Is a statistical procedure to compare multiple means of a number of treatments with tha mean of a single control, it is also refer as a many to one comparison.

EMS: Ethyl methanesulfonate

ER: Endoplasmic reticulum

ESI: Electron spray ionization

FCC: Flash Column Chromatography

GC: Gas Chromatography

HC: Linear alkanes, n-paraffin

HPLC: High Performance Liquid Chromatography

q-PCR: Quantitative Real Time Polymerase Chain Reaction

m/z: Mass to charge ratio

MALDI-TOF: Matrix assisted laser diffraction ionization time of flight

MASCOT: Software search engine that uses MS data to identify proteins from primary sequence databases

MIC: Minimum inhibitory concentration

NCBI: National Center for Biotechnology Information

NMR: Nuclear Magnetic Resonance

PDA: Potato dextrose agar

PMA: Phosphomolybdic acid

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R_f: Retention factor

RGI: Radial Growth inhibition

R_T: Retention time

SDA: Sabouraud dextrose agar

SDB: Sabouraud dextrose broth

SDS: Sodium dodecyl sulfate

Significant difference: Statistical difference is defined as the difference between the two means (D) divided by the standard error of that difference (computed from all the data) q=D/SED; the degree of among this difference will be how significant is the effect.

SIM: Selective Ion Monitoring Mode

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CHAPTER I

BEAUVERIA BASSIANA MORE THAN A CATALYST

Processing of industrial chemicals by microbial catalysts is a growing and important technology, but at present is more at the research and development stage than at the industrial-commercial stage. It is forecasted by Freedonia Group that the catalyst market will grow more into chiral catalyst technologies and into technologies that facilitate novel transformations; opportunities where fungal catalysts may have utility. *Beaveria bassiana* is a biocatalyst that may have a niche market for industrial processing. *B. bassiana* is a filamentous fungus widely used as biocatalyst; it can enhance the yield and conversion of oxidative biotransformations when it is grown in presence of chemical and/or environmental stresses. Adding interest to the potential characterization of *B. bassiana* is its wide application as a biopesticide. Using insect pathology and agricultural biotechnology information to guide research into the regulation of biocatalytic activity may lead to enhanced reactions. This may enable the development of better processing strategies important for the commercial application of fungal catalysis. Our research group has been working to develop processing strategies to increase the commercial relevance of fungal biocatalysts. In these research efforts it is necessary to: increase substrate conversion, increase the expression of key oxidases and enzymes, gain control over cell type, and improve protocols to handle mycelia.

The Market for Catalysts

Catalysts are utilized in 80% [1, 2] of all chemical synthesis operations and are responsible annually for approximately $4 trillion in goods and services worldwide[1]. Most demand comes from pharmaceutical and other fine chemical synthesis processes. In 2015 the total US merchant sales of these products was about $4.6 billion[3]. The greatest growth in market share occurred in chiral catalyst technologies and in technologies that facilitate novel transformations [1, 4]. The global chiral technology

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market was worth nearly $5.3 billion in 2011 and will approach $7.2 billion by the end of 2016. Chiral synthesis products accounted for the majority of the chiral technology market in 2011 with $3.9 billion in revenue and are expected to increase to $5.7 billion by 2016[1]. The presence of favorable government regulations promoting the use of chiral technology is one of the major drivers of the market. Although these incentives are beneficial for the early adoption and development of chiral catalysis, the lack of clear regulations between chemically synthesized products and biological synthesized ones have inhibited their growth. However, high technological innovation and high environmental awareness in North America and Europe is leading to high consumption of biocatalysts. Biocatalysis has replaced chemical synthesis processes and chemicals in a variety of industries such as starch processing, biofuel production, detergent industry, food and beverages industry, and pharmaceuticals industry[5, 6] (Figure 1-1). The global biocatalysts market demand is estimated to reach around 112 KT by 2019[6]. The biocatalysts market is anticipated to show a steady growth rate of 5.6% in the coming years with a higher growth in biopharmaceuticals and biofuel production than in other applications[6]. Among the major drivers for this increase are consumers in all end industries seeking and adopting greener and sustainable practices; thus a steady increase in the demand for different biocatalyst applications and types. These are commercial opportunities where microbial catalysts can thrive. However, lack of clear regulations, competition with chemically synthesized products, and lack of process cost optimization are inhibiting the growth of this sector[6]. Businesses are strengthening their position in the biocatalyst market by collaborating with bio-product manufacturers from different regions as well as focusing more in R&D for innovative high quality solutions to meet customer demands[1, 7].

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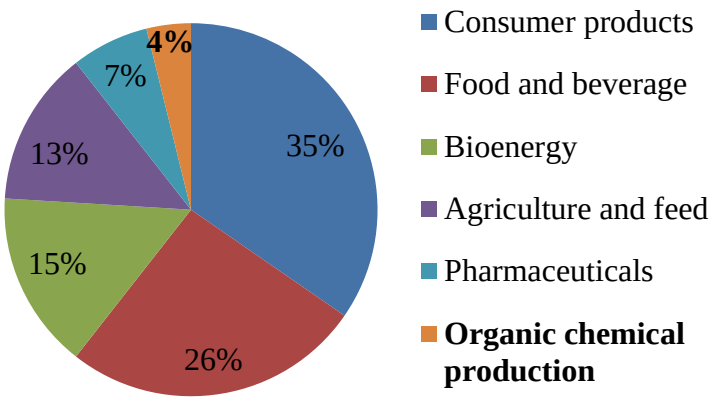

Figure 1-1 Percentage of US enzyme's market shares distributed into distinct industrial sectors, data is from total US enzyme market sales for 2014, worth $4.4 billion. The organic chemical production sector, bolded, is gradually becoming a more important sector in the enzyme market as the demand of greener technologies grows[6].

The processing of industrial chemicals by microbial catalysts has increased with the global trend for greener and more sustainable manufacturing processes[8, 9]. Moreover, 66% of the CEOs of chemical companies say they will increase their focus on reducing their environmental footprint [10]. Biocatalysts offer many advantages over chemical synthesis processes including highly chemoselective, regioselective, and stereoselective, high yield and purity at low costs, and yield enantiomeric excess of over 99%. They can act under mild conditions and can be adapted to organic solvents. In addition, whole cell catalysts and enzymes provide safer processing conditions and are environment friendly. As global manufacturing trends move toward increasing quality, safety, health, and environmental requirements of industrial chemical transformations, interest in advancing biocatalyst use in industrial processes is increasing. However, these applications are limited by the challenge of developing economically competitive biologically based systems [8]. The key for adopting these

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sustainable advancements is the development of novel process designs, which assure robustness, simplicity, and sustainability operations compatible with the current development of chemical reactions[4]. Since fungi subsist by absorbing nutrients, they possess the ability to rapidly adapt their metabolism to different suboptimal growth conditions, including nutrient deprivation, the presence of xenobiotics and antifungal compounds [11]. Fungi express a number of specific detoxifying enzymes, which degrade compounds in ways that eliminate the effect of toxic compounds or unfavorable conditions. A potential strategy for more sustainable chemicals production of industrially relevant compounds is the use of entomopathogenic fungi.

Beauveria bassiana

Beauveria bassiana is a filamentous and entomopathogenic fungus that belongs to the division *Ascomycota* and the *Clavicipitaceae* family [12]. *B. bassiana* is among the most frequently used biocatalysts; it is surpassed in application by *Aspergillus niger*, *Pseudomonas putida*, and *Saccharomyces cerevisiae*[2]. There are thirty four registered strains on the American Type Culture Collection (ATCC) which differ on their applications and isolation. We focus our study to ATCC 7159. ATCC 7159 is the most commonly used strain for biotransformation[13-15.] However, other strains isolated from their insect host have also been used in biocatalysis[16, 17].

B. bassiana a ubiquitous biocatalyst

B.bassiana is broadly used in catalysis to perform regio- and steroselective reactions due to its simplicity to handle and catalytic versatility. It can catalyze a variety of reactions[18] (Figure 1-2) including hydroxylations[19-29], epoxidations[30], glycosylation[28, 31], reductions[30], sulfoxidations[14, 19, 30], and lactonization[19, 30]. We are interested in the selective hydroxylation of unfunctionalized carbons in hydrocarbons and oxidation of sulfur compounds due to their functional and biochemical importance in the pharmaceutical industry for drug development and drug metabolism.

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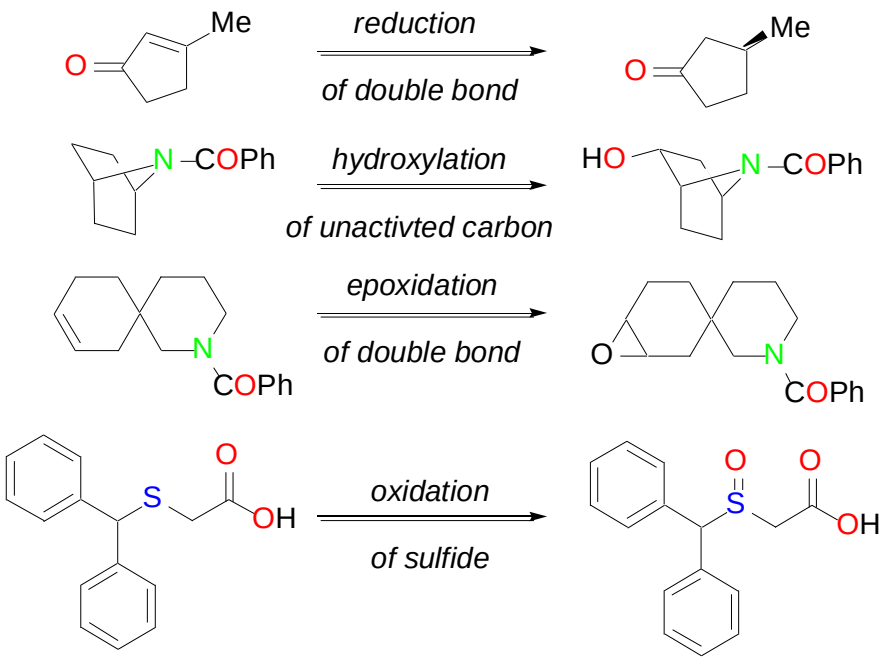

Figure 1-2 *B. bassiana* broad range of catalyzed reactions from hydroxylation to oxidation of heteroatoms.

In our group we have performed hydroxylation of azabicycloalkanes[15] and N-piperidinylacetophenone[13] and sulfoxidation of modafinil precursors[14] with *B. bassiana* in high yields and selectivity. A series of azabicycloalkanes (Figure 1-3 ,I-III) were hydroxylated at analogous endo sites in yields of 45%– 70% [15]. The products resulted from enzymatic attack at methylene groups within the substrate. The hydroxylation of N-piperidinylacetophenone (Figure 1-3, IV) yielded 34% after 3 days of fermentation, but also obtained a broad product distribution, eight biotransformation metabolites[13] . These oxidizd metabolites included Baeyer–Villiger oxidation, hydrolysis, and β-4-O-methylglucosidation of aromatic alcohol[13]. In comparison, Holland reported the hydroxylation of N-piperidinylacetophenone to yield N-(4-hydroxypiperidinyl)-acetophenone as a sole product in 20–40% yield after 3 days of fermentation [19]. This is one of many examples that shows the variability between researcher's yields and products which makes it harder to accurately predict the biotransformation products for

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optimization purposes and practicality. Current fungal hydroxylation may not be suitable for industry due to low reliability and yields lower than 50%. However, these hydroxylation and oxidation reactions offer the possibility to study eukaryotic xenobiotic metabolism and produce drug metabolites at a minor scale. Further, characterization of bio-oxidation capacity may enable the improvement of yield and reliability for industrial applications.

The majority of enzymes involved in drug metabolism (75%) are cytochrome P450s [32, 33] (CYP450s). The enzymes responsible for the hydroxylation and oxidation of xenobiotics are cytochrome P450 monooxygenases[33-35]. Cytochrome P450s are heme-thiolate proteins found in eukaryotic and prokaryotic organisms. They are expressed in low levels and in several isoforms, which makes the control of product distribution hard[35]. They catalyze regio- and stereospecific introduction of atomic oxygen into lipophilic compounds to increase their hydrophilicity[33]. Fungal cytochrome P450 are membrane bound on the microsomal fraction of cells[34, 36, 37] and are involved in primary and secondary metabolism as well as xenobiotic detoxification and/or degradation. The specificity and isoforms involved in CYP xenobiotic metabolism has been evaluated extensively for mammalian cells[32, 33]. In particular, CYP3A isoforms are the ones majorly induced by xenobiotics[38-40]. Evidence of CYP450 occurrence in filamentous fungi is limited because of difficulties in handling mycelia, low levels of these enzymes[2, 36] ,and poor understanding of their regulation[34]. In entomopathogenic fungi, these enzymes are involved in hydrocarbon catabolism[41]and are highly regulated by the chain length of the hydrocarbon where they grew[12, 42]. *B. bassiana* is still underutilized in biocatalysis because of its limitations, and its biocatalytic possibilities have not been explored completely. However, understanding the insect-fungal interactions and pathogenesis would facilitate better characterization of *B. bassiana's* potential as a selective catalyst and could lead to strategies to overcome its limitations. The studies of *B. bassiana* as a biocontrol agent allow us to understand fungal

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metabolic regulation and molecular biology essential for fungal growth and catabolism, which could lead to a better understanding of *B. bassiana* catalysis.

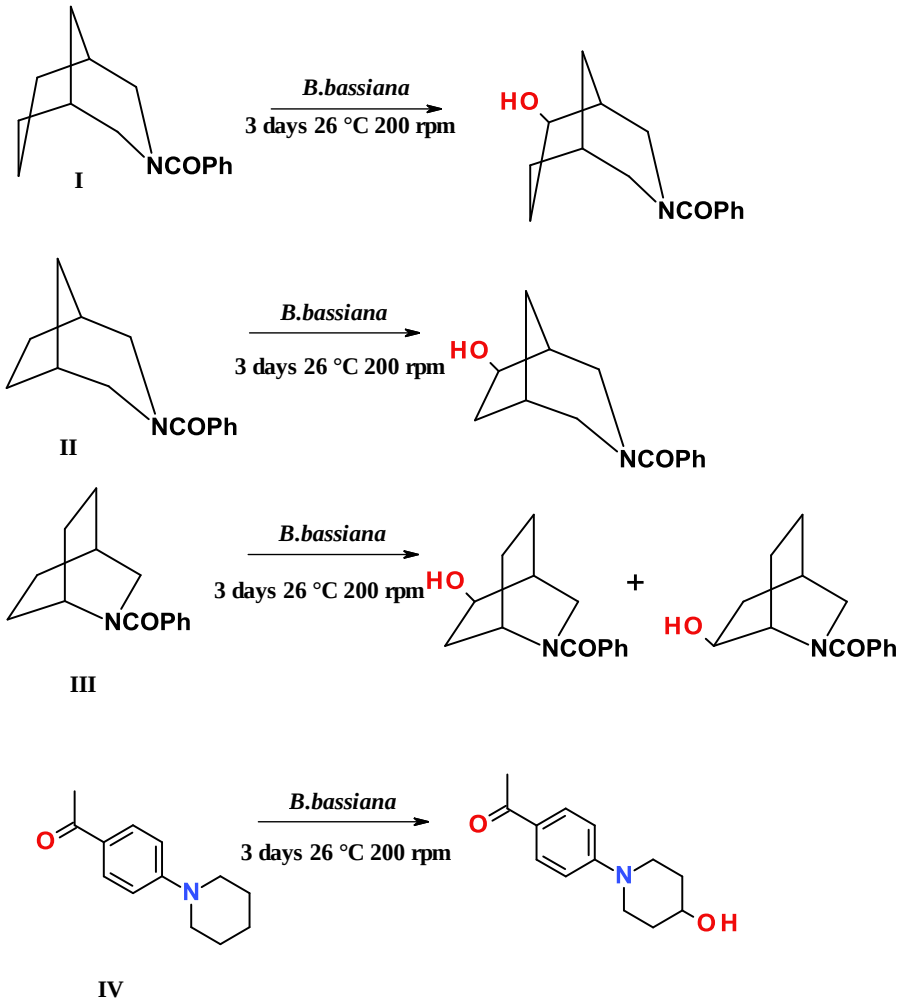

Figure 1-3 Biohydroxylation of azobicyloalkanes (I-III) and N-piperidnylacetophenone (IV) catalyzed by B. bassiana. Biotransformation assayed in a 250 mL Erlenmeyer flasks with 50 mL Corn Steep medium pH 4.5 at 26 C and 200 rpm

Biopesticide

B. bassiana has proved effective in controlling troublesome crop pests such as aphids, thrips and whitefly – even chemical pesticide-resistant strains. This fungus is

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one of the major mycoinsectides applied in the United States[43]. There are thirty four registered strains of *B. bassiana* in the American Type and Culture Collection (ATCC) that differ in their application and isolation. *B. bassiana* strains applied as biocontrol agents are *B.bassiana* GHA (Laverlam International Corporation), and ATCC 74040 (Troy Bioscience). *B. bassiana* mycoinsecticides can be used to control a broad range of persistent pests, species from the Lepitodptera and Coleoptera order[43]. These fungal catalysts initiate biocontrol upon contact with the insect cuticle[35] (Figure 1-4). The insect cuticle is a hydrocarbon-rich environment consisting of structural lipids and free lipids. Structural lipids are comprised of insoluble lipoproprotein, while free lipids are extractable from cuticles by organic solvents. The cuticle structure is layered, with a chitin-rich procuticle and a non-chitinous epicuticle. Within the rich hydrophobic epicuticle free lipid varies between insect species, but may contain hydrocarbons, fatty acids, fatty alcohols, waxy esters, glycerides, sterols, aldehydes, and ketones[44]. Hereby, cuticular hydrocarbons occur as a high percentage of free lipids and are complex mixtures of n-alkanes, olefins, and methylalkanes. *B. bassiana,* attacks the surface layers of the insect host, produces hydrocarbon-assimilating enzymes, and consumes hydrocarbons during growth in the epicuticle of the insect[42].

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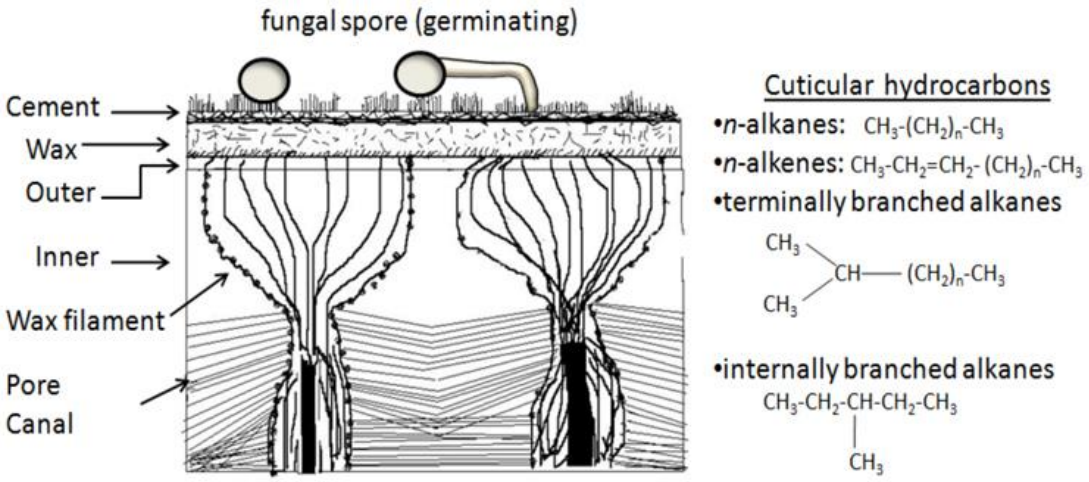

Figure 1-4 Fungal-insect interaction: the insect cuticle and its hydrocarbon contents. Image adapted from Frontiers Microbiology, Pedrini[35]. The inner, outer, wax, cement, and bloom layers are often considered as the epicuticle, with the pore canals, that transverse the exo-, plus the meso-, and endocuticle portions (not shown in figure) comprising the procuticle.

Efforts to optimize application and *B.bassiana* pesticide formulations involve the addition of molecules to help with spore adhesion to the cuticle and improve outbreak control. Additives include *Bacillus thurigienesis* toxin[45], plant extracts and/or oils, as well as stabilizers to enhance spore endurance[46] .Virulence enhancement aims are to induce and maintain optimal expression of key enzymes[47]. However, to prevent pest outbreaks it is necessary to overwhelm the insect population by inundation methods[46] and wait to elicit a response. Cuticle degrading enzymes (CDE) are hydrolytic enzymes, mainly chitinases, lipases and proteases. Their expression is highly dependent on the presence of the substrate they hydrolyze[48], otherwise, they remain at basal levels of expression[49] .In addition to CDE expression, biocontrol ability has been increased by use of sublethal concentrations of neonicotinoide and carbamate insecticides which have shown synergistic effects[50-52].

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Research motivation

The study of *Beauveria bassiana* oxidative performance would impact catalysis because it would lead to better control over product distribution and enzyme expression which eventually result in the ability to fine tune enzymes to product specifications. Lehman and Stewart in 2001 addressed that for a practical use of *B. bassiana* it is required to optimize hydroxylation conditions and to accurately predict its biotransformation products[2]. After 12 years there has not been enough progress on chemical selectivity of *B. bassiana.* The primary reason is that most work has been on virulence enhancement for pest management. However, these efforts give insight into the catalytic machinery involved in oxidation reaction; information necessary for prediction of regio- and stereoselectivity. The major challenges to *B. bassiana* catalysis are overcoming low yields[30 19] and low enzymatic expression [36,34], gaining control over cell type, and handling mycelia[53]. During the submerged cultivation of fungi, growth morphology can vary from discrete compact pellets of hyphae to homogeneous suspension of dispersed mycelia[2, 54-56]. These morphological differences are associated with significant differences in growth kinetics and physiology. In general, the filamentous morphology causes complex broth rheology and difficulty in mixing and aeration in STR and negatively affects mass transfer[57]. In addition, hyphae may exist in different physiological states and show different features such as vacuolated regions, growing apical cells, and lysated cells which are metabolically inactive[57-59]. All these drawbacks are detrimental for the industrial application of fungal catalysis. From the forementioned challenges, the current work is focused in overcoming low yields and enzymatic expression to enable better control over *B. bassiana* catalysis.

Specific aims

Our long-term goal is to exploit the inducibility of this fungal system for selective oxidation taking into consideration the major challenges of fungal catalysis by

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working under stress conditions, with hydrocarbons and insecticides, in order to overcome low oxidase content and low conversion. The specific objective of this work is to enhance the conversion of thioethers into sulfoxides by using insecticides and hydrocarbons as oxidase inducers. *The central hypothesis is that exposure of cells to insecticides and hydrocarbons will increase B. bassiana oxidative capacity expressed as higher substrate conversion and CYP activity*. We formulated this hypothesis, in part, based upon integrated pest management techniques studies, which show a synergistic effect between carbaryl/imidacloprid and *B. bassiana* as well as virulence enhacement with cells exposed to hydrocarbons. The impact of enhanced biocontrol efficacy on the oxidative biocatalysis mechanisms of *B. bassiana* has been effective in hydroxylation of dehyrdoepiandrosterone[27] by increasing yields from 41% to 60%. However, the effect these techniques have over the oxidation of heteroatoms has not been explored. The rationale for the proposed research is that once it is known how to consistently induce oxidases in *B. bassiana*, we will have a tunable enzymatic system of industrial interest. We pursue these studies in three Specific Aims:

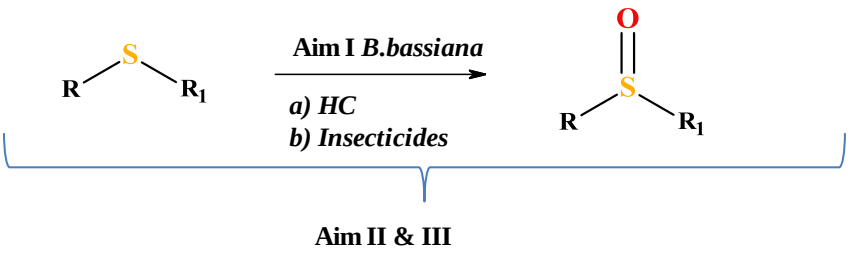

Figure 1-5 Biotransformation scheme, conversion of thioethers into sulfoxides.

Biotransformation: **Aim I** Enhance the catalytic performance in *B.bassiana* for the conversion of a model organosulfur compound using insecticides and hexadecane as oxidative inducers. The industrial oxidation of organosulfur compounds is catalyzed by

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a metal catalyst, so the development of a greener alternative as efficient as the current industrial process will allow to reduce its ecological footprint and manufacturing process costs. Details of this substrate will be presented in Chapter II. This aim tests the prediction that sub-lethal concentrations of carbaryl and/or imidacloprid, and hexadecane adapted cells increase sulfoxidation of our screening substrate, phenothiazine. Our underlying hypothesis is that *use of inducers, sub lethal amounts of insecticides or hexadecane, will increase the metabolism of organosulfur compounds.* The application of fungal isolates with minimum inhibitory concentrations (MIC) insecticides has been effective in the control of broad spectrum of pests, even towards insecticide resistant strains. The increase in efficacy is most likely due to an enhancement in metabolism through synergistic effects demonstrated in several bio-control experiments. *B. bassiana* can grow in hexadecane as it sole carbon source. Its great versatility allows it to adapt quickly to extreme conditions by the regulation of hydrolases and oxidases which are involved in xenobiotic metabolism and hydrocarbon catabolism. Consequently, the use of MIC of insecticides and hexadecane in conjunction with our substrates is expected to enhance *B.bassiana's* catalysis.

Proteins: **Aim II.** Determine the effect of insecticides and hexadecane on *B. bassiana's* oxidative activity. Oxidases are involved in in hydrocarbon catabolism and xenobiotic metabolism. They are responsible for the first oxidation step of hydrocarbon assimilation and xenobiotic detoxification. Therefore, the capacity to determine the cells' oxidative activity would enable the discernment of isolates with a higher catalytic potential. The underlying hypothesis is that *treated cells will have a higher content of CYP* expressed as a higher CO differential spectrum, which is the standard technique for CYP induction measurements.

Adaptation: **Aim III.** Evaluate *B. bassiana*'s physiological quality under nutrient deprivation and insecticide toxicity. We will validate that HC and MIC insecticide concentration are not detrimental for growth. In these studies we will

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examine the quality of the cells by defining that the HC and insecticides working concentration that does not affect cellular activity. The increased in catabolism/metabolism would permit cells to have higher content of oxidases and/or hydrolases; ideally, enzymes whose broad substrate specificity catalyze the conversion of thioethers into sulfoxides. Therefore it is necessary to prove that cells are metabolically active under stress conditions in order to use them as catalysts.

The work is innovative because it capitalizes on the advancements of green biotechnology, in particular insect pathogenesis and integrated pest management. Chapter 3 establishes that adapted cells remain catabolically active under stress; thus being stronger catalysts. The combined work presented in Chapters 2 and 4 validates the use of oxidase inducers. Chapter 5 takes us into our first look into the enzyme and proteins responsible for the variety of induced catalytic systems.In the last chapter, future experiments are presented to help us in our understanding of *B. bassiana* catalysis. By using microbial catalysts as substitutes in the place of chemical-based products, the manufacturing process can be redeveloped into a more environmentally friendly process.

Summary

Processing of industrial chemicals by microbial catalysts is a growing and important technology, but at present is more at the research and development stage than at the industrial-commercial stage. Biocatalysts offer a greener alternative to chemical catalysis; however, low substrate conversions, low yields and expensive processing are far from the ideal cost effective industrial routes. In extending the use of biocatalysts to commercial application, optimization of reaction conditions and process optimization are necessary. We meet these necessities by bridging agricultural technology advancements and biocatalyst development into the improvement of fungal catalysis for major industrial application. The overall purpose of this project is to improve fungal

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catalysis by studying the inherent effects of hydrocarbons and insecticides on *B.bassiana* oxidation of phenothiazine. *B. bassiana* can enhance the yield and conversion of oxidative biotransformations when it is grown in presence of chemical and/or environmental stresses. The underlying hypothesis is that exposure of cells to insecticides and hydrocarbons will increase the cells' oxidative capacity. The following chapters summarize our finding on *B. bassiana* biotransformation of phenothiazine with oxidative inducers and their effect on *B.bassiana's* catalytic ability and cell viability.

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CHAPTER II

CATALYTIC CAPACITY: EXPANDING *B. BASSIANA'S* OXIDATIONS BEYOND HYDROXYLATIONS

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Abstract

The great selectivity of fungal catalysis is now well recognized for the production of commercially valuable steroids in the pharmaceutical industry. Although this inherent capacity is mainly used for functionalization of unactivated carbons, it can be further exploited for the oxidization of heteroatoms, such as sulfur. The bioconversion of sulfur compounds has not been industrially scaled mainly due to the more efficient metal catalyzed alternatives. In this chapter *B. bassiana*'s catalysis is expanded beyond hydroxylations, its most common catalytic use. Fungal catalyzed phenothiazine oxidation was assayed with a wide selection of parasitic and degrading fungi. Phenothiazine sulfoxide was transformed in low yields and high selectivity, e.g 6.9% and 95 % with *Phellinus ignarius*, or high yields and low selectivity, e.g 33.3% and 54% with *Cunnighamella elegans*. The best performers were *Hirsutella citriformis* and *B. bassiana.* Furthermore, *B. bassiana* oxidized phenothiazine to its hydroxyl metabolites and sulfoxide, the latter being the main metabolite. Phenothiazine concentration varied according to the substrate concentration, resulting in the highest conversion (65±1.4%) after 7 days with an initial phenothiazine concentration of 500 ppm. In addition, *B. bassiana* can oxidize thioanisole and benzothiophene to sulfone, and thiophene to its sulfoxide. Fungal catalysts have been shown to be a promising alternative for greener organosulfur oxidation and desulfurization. However reaction conditions and process cost optimization need to be addressed in order to establish fungal bioprocessing as an industrially competitive alternative.

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Introduction

Fungal hydroxylation is the most used application in industrial bio-oxidation[8, 60, 61]. Bio-hydroxylation is involved on the functionalization of unactivated carbons of many intermediates, which are necessary for the synthesis of many drugs, fine chemicals, and bulk chemicals. Applications for the hydroxylation of unactivated and completely saturated hydrocarbons were the subject of the first systematic investigation on fungal oxidative catalysis. The research efforts in this field started around 1950, with the announcement of the pharmacological effects of steroids and with the identification of 11α-hydroxylase activity of *Rhizopus* species; a decisive step into the development of a cost effective and efficient synthesis of steroids with useful biological activity[62]. Fungal transformations of various steroids have been reviewed over the years[16, 17, 27, 63, 64]. While every site in a steroid molecule is accessible for microbial hydroxylation, the 11α-, 11β-, 15α- and 16α- hydroxylations are now accomplished mainly by microbial transformations[64]. *B. bassiana* has been successfully used for the hydroxylation[30] of substituted aromatic compounds[65], hydrocarbon substrates[13, 19, 21, 65], steroids[16, 17, 27, 66], natural products[60, 67], and synthetic cyclic amides[15]. Several microbial oxidations of unactived carbons have been reported; however, these selective reactions have not been fully explored in heteroatoms.

Sulfoxide importance

The past twenty years have seen an explosion in interest in the synthesis and utility of enantiopure heteronuclear molecules. An important subclass of these molecules are sulfoxides, in particular the compounds with different substituents, since the sulfur atom of the sulfoxide is a stereogenic center. The inherent chirality of sulfoxides is due to d-π bonding where the oxygen donates electron density from a lone pair into a d-orbital of sulfur[68], which allows the sulfur to assume a tetrahedral sp^3 hybridization with a lone pair of electrons from sulfur in the fourth quadrant.

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Sulfoxides are conformationally stable at room temperature. They will only racemize under extreme conditions—temperatures greater than 200°C, C-S bond scission, and radical transfer agents[69]. The barrier to inversion for most sulfoxide compounds is in the range of 38-41 kcal/mol[26, 68] which facilitates the separation of pure enantiomers.

Sulfoxides are found in a variety of natural products, drugs, catalysts, aromas, and flavors[25] (Figure 2-1). Enantiopure sulfoxides are present in high selling drugs such as sparsomycin and single enantiomer omeprazole. They are present in natural products such as sulforaphane and (+)-S-allyl-cysteine sulfoxides as well as chiral ligands, sulfoxide complexes and (S)-oxisurane. These molecules are utilized because of their enhanced therapeutic efficacy and biological activity based on the unique chemistry that sulfur imparts. Optical sulfoxides have emerged as potentially ideal ligand candidates for catalytic asymmetric transformations due to their ready availability, stability to moisture and air, and well-defined coordination features[70, 71]. Furthermore, these ligands have become competitors to more usual phosphorous or nitrogen containing ligands[72]. They possess the key advantage of being easier to synthesize from available commercial compounds, and they are more stable. The synthesis of highly efficient and selective ligands is an important part of developing enantioselective transition metal-catalyzed reactions which provide access to both enantiomers[71,73,74,75]. Although biocatalysis still remains a challenge in the chemical community, selective biocatalytic reactions can find a place to thrive as a suitable alternative for the design of different ligands from a single easily accessible chiral source.

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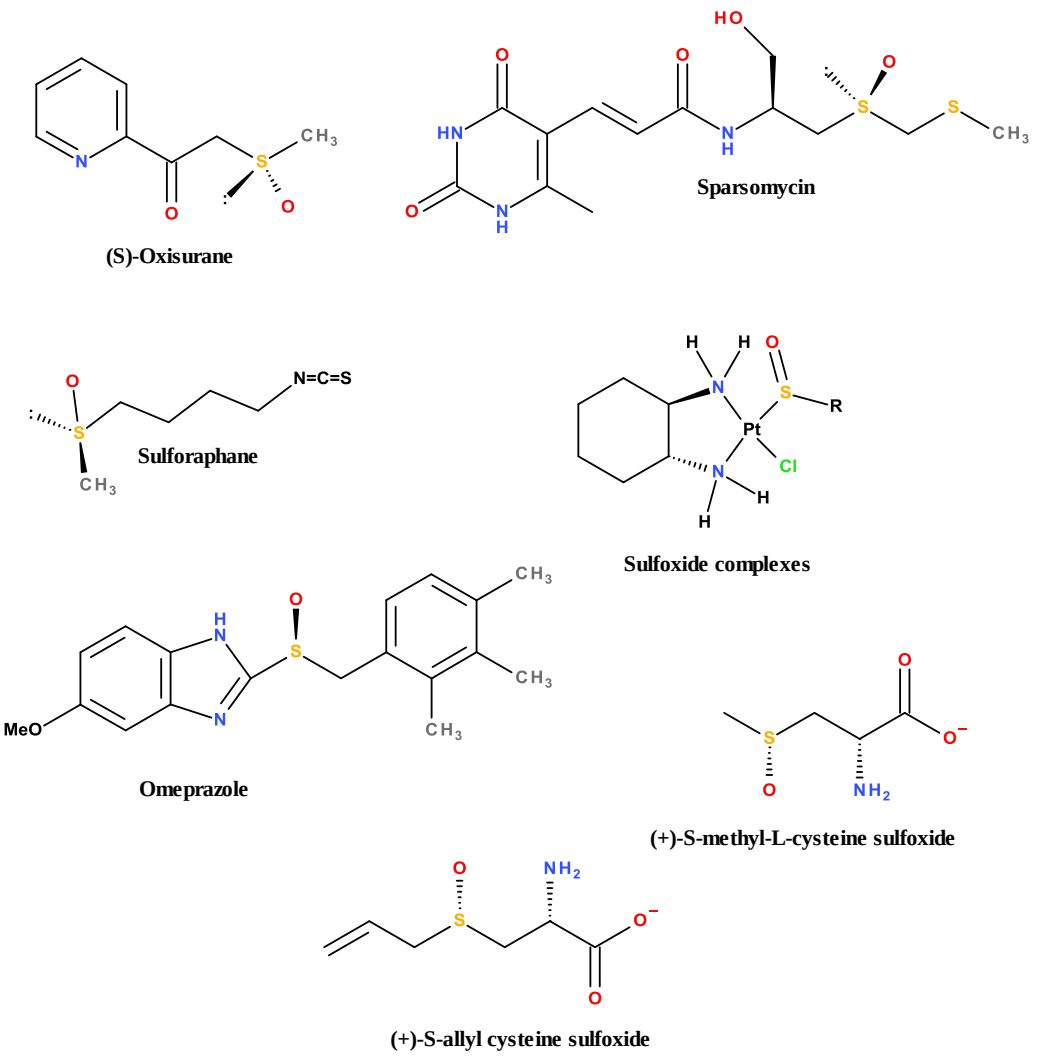

Figure 2-1 Significant sulfoxide compounds with industrial and biochemical value.

Sulfoxide synthesis at a micro- and macroscale

Much attention has been devoted in the pharmaceutical field for the preparation of biologically active chiral compounds with complete optical purity and high efficiency[71, 72]. The FDA policy statement regarding the development of new stereoisomeric drugs has led researchers to increasingly value synthesis of both enantiomers of pharmaceutically interesting chiral compounds. This FDA imperative

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has also impressed upon the scientific community the need to determine the biological activity of synthesized enantiomers. The enantioselective oxidation of a prochiral sulfide is the most direct and economical method for the synthesis of enantiomerically pure sulfoxides[68]. Other alternatives are the resolution of a racemic mixture, the transformation of diastereochemically pure sulfinates, and the structural modification of chiral sulfoxides without loss of stereochemistry[76](

Figure 2-2). Many asymmetric oxidations of achiral sulfides are catalyzed with a transition metal and a chiral ligand[69]or mild oxidants[26, 68]. This catalytic approach consists of the creation of the sulfur-oxygen bond by asymmetric oxidation of the parent thioether or formation of the carbon-sulfur bond by treatment of enantiopure sulfinyl derivatives with an organometallic reagent[71]. Despite high synthetic values, the production of optical sulfoxides has been achieved in low yields through chemical resolution[20,30]. Nevertheless, most of the sulfoxides are non-functionalized. The development of these products is limited due to the lack of general synthesis methods with high enantiomeric excess for the oxidation of non-functionalzed substrates. This opens a great opportunity for biocatalytic methods as suitable and greener alternatives at a micro and macroscale. Unfortunately, for the latter the current industrial sulfoxidation processes based on organometallic catalysts are more efficient (89-98% yields) and faster[26, 71-73] than biocatalytic processes; the wide industrial application demonstrates that sustainability needs are still surpassed by profitability targets.

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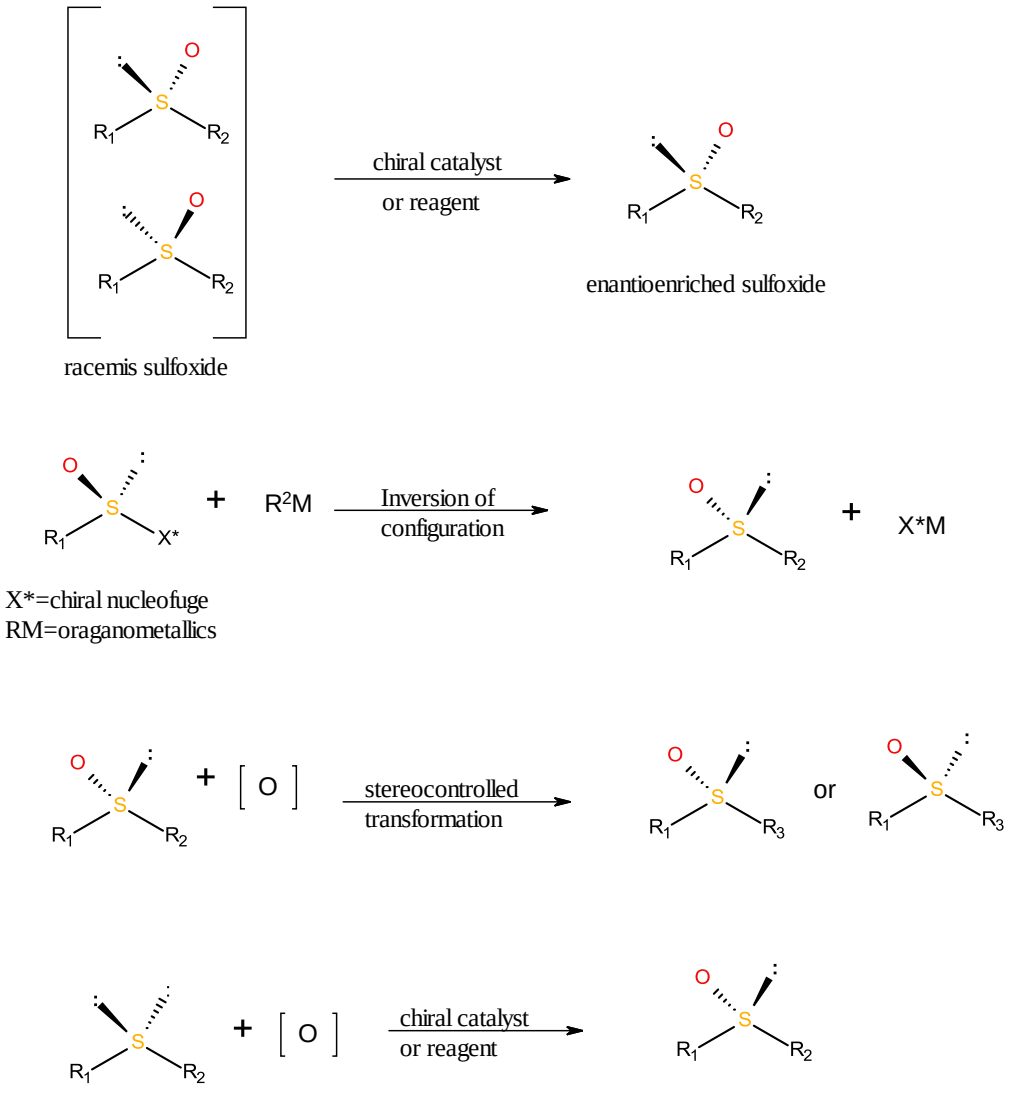

Figure 2-2 Main routes for the synthesis of chiral sulfoxides[76].

Industrial sulfoxidation is produced by the Modena oxidation with the modification of the Sharpless catalyst (Figure 2-3)[68, 69]. Other industrial methods include reactions catalyzed by isolated enzymes and whole-cells, although they are used on a smaller scale. Enzymes used in sulfoxide production include peroxidases, flavin-dependent monooxygenases, cytochrome P450 and semisynthetic

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metalloenzymes[26, 77]. Whole cell sulfoxidation has been catalyzed at a lab scale by *Cunnighamella elegans*, *Aspergillus niger*, *Penicillium verticillium*, *Mortierella isabellina*, *Helminthosporium sp.*, and *Pseudomonas putida*[9, 13, 78-81]. Currently, the microbial transformation of steroids is the only biocatalyst application that has been up scaled successfully with market values of 1000 ton/y for the controlled degradation of phytosterols with mutants of *Mycobacterium sp*. and <100 ton/y for regio- and steroselective hydroxylations with *Curvularia sp* (Figure A-1& A-2)[63].New government regulations and biotechnology advancements are facilitating the adoption of fungal reactions into major manufacturing processes. However, these applications are limited by the challenge of developing economically competitive biologically based systems[8]. The key for adopting these sustainable advancements is the development of novel process designs, which assure robustness, simplicity, and sustainability operations compatible with the current development of chemical reactions[4].

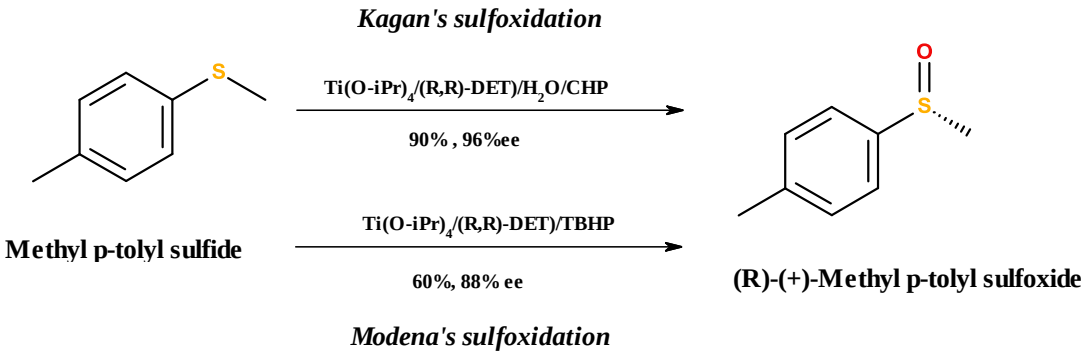

Figure 2-3 Industrial production of sulfoxides; DET=L(+)-Diethyl L-tartrate TBHP=tert-Butyl hydroperoxide CHP=cumene hydroperoxide, Ti(O iPr)4(RR)-DET =Sharpless catalyst, titanium tetra(isopropoxide) with diethyltartrate.

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Screening substrates

For these studies phenothiazine was used as a screening substrate, and the alternative substrates were thioanisole, thiophene, and dibenzothiophene. Phenothiazine ($C_{12}H_9NS$) is a thiazine class of heterocyclic compound (Figure 2-4, I). It is the parent molecule of antipsychotics, antiemetics, antihistamines (H1-receptor antagonists) drugs, and azo dyes such as methylene blue[82]. In addition phenothiazine is used as a stabilizer or inhibitor in the manufacture of monomers. The phenothiazine molecule induces flavin-containing monooxygenases and several cytochrome P450 in mammals[39], its metabolism has been thoroughly studied in mammals[82]. The oxidation of phenothiazine and substituted phenothiazine drugs has been studied in the lower fungi *C. elegans*[78] and endophytic fungi[79, 83, 84].

Thiophene (C_4H_4S), and dibenzothiophene ($C_{12}H_8S$) are heterocyclic thiothers present in large amounts in most fossil fuels (Figure 2-4, III-IV); up to 25% of the total 200-500 ppm natural fuel's thioether content is comprised of thiophene, dibenzothiophene and their derivatives[85]. The concentrations and nature of the organosulfur compounds change over the boiling range with the heaviest fraction containing the most recalcitrant sulfur compounds[85]. The most prevalent and recalcitrant are benzothiophene and dibenzothiophene. Dibenzothiophene is an aromatic thioether consisting of two benzene rings fused to a central thiophene ring. Thiophenes are building blocks in many agrochemicals, pharmaceuticals (lornoxicam), and electrically conductive polymers[86]. Studies on the biological conversion of these sulfur compounds have focused on the desulfurization to reduce the sulfur content of coal and oil within the refining process and to a lesser extent into their oxidation[86-88]. Thioanisole (C_7H_8S) is an aromatic thioether (Figure 2-4, II) used as a prochiral probe in sulfoxidation assays[89, 90]. It is a useful compound applied as a solvent and flavor additive, as well as a substrate for the synthesis of herbicides and insecticides.

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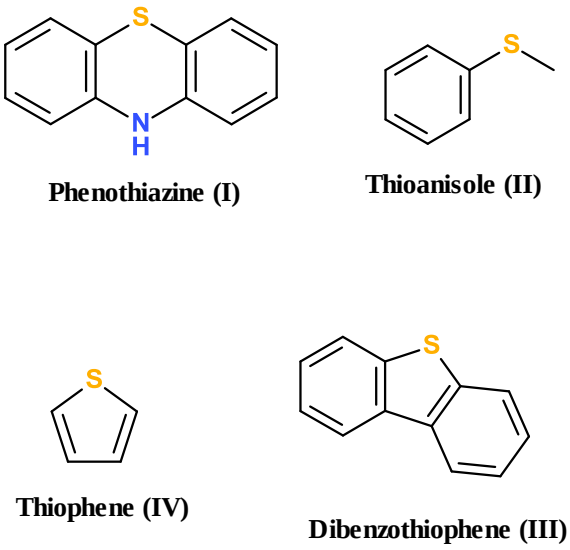

Figure 2-4 Screening substrates used for evaluating organosulfur conversion in *B.bassiana.*

Metabolite identification by Mass Spectroscopy

Several spectroscopic techniques are used in the identification and characterization of fungal metabolites such as mass spectrometry (MS), nuclear magnetic resonance (NMR), ultraviolet spectroscopy (UV), infrared spectroscopy (IR), and X-ray crystallography[91]. Recent advancements in MS have enabled MS to become an invaluable tool in the study of natural products[92, 93]. MS has long been used for identifying and quantifying compounds. MS is based on the production of gaseous, positively or negatively charged ions that are subsequently separated according to their mass-to-charge (m/z) ratio and detected [92, 94]. A typical mass spectrometer consists of an ion source, which generates ions, a mass analyzer, which separates ions based on their m/z, and a detector, which measures the separated ions[91, 94, 95].

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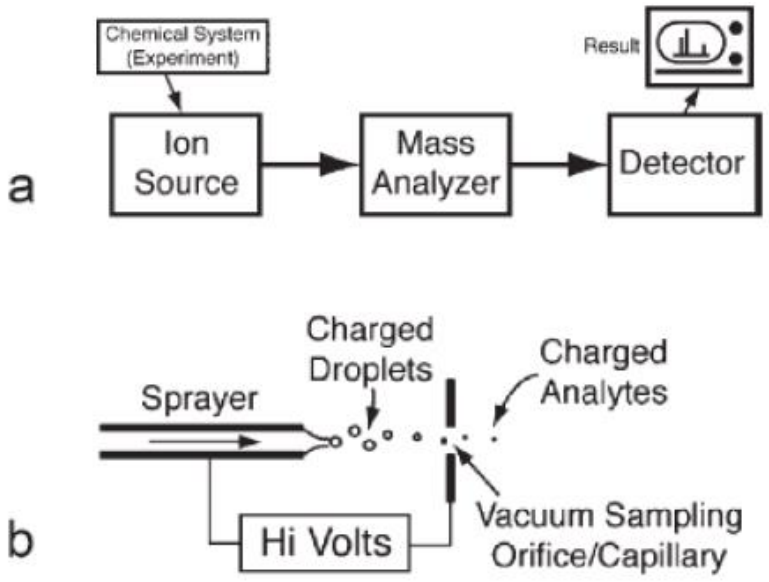

Figure 2-5 Schematic diagrams of mass spectrometry. (a) A generic MS system consisting of an ion source, a mass analyzer and a detector. (b) A schematic diagram of electrospray ionization (ESI).

The fragmentation of thioethers[96] mostly occurs as a α-cleavage at the longest chain; giving a $R_R^{\bullet}=CH_2$[97]. In case the R group contains a β-hydrogen, transfer to the $=CH_2$ would occur next with the loss of a neutral alkene, and therefore m/z 47 would be expected. Moreover, due to the extra stability of the RS• ion, cleavage occurs at the S-R link with RS• ions of fairly high relative abundances appearing in the series m/z 47, 61, 75, 89 [98, 99]. Sulfoxide fragmentation has a significant peak at m/z 16[15], which is diagnostic for the presence of either an N-oxide or an S-oxide function[100, 101] .The observed changes in the fragment ions, base peak, and MH+ will allow the identification of major catalyzed functionalities by *B. bassiana*. These variations will be due to the introduction of oxygen into the molecule. Though some glycosylated or acetylated conjugates can be formed from the major metabolites; the oxidized free forms are still more abundant. Therefore when MS is coupled with HPLC it will enable

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the determination of the time when the sulfoxide metabolites reach peak concentrations with respect to the small amount of conjugates or further oxidized products[96, 102].

For GC purposes it is necessary to derivatize compounds that have free hydrogen (i.e primary amines or primary or secondary hydroxyl groups) into their corresponding esters or silyates in order to facilitate volatilization, to avoid undesirable reaction and interaction with the GC column[103]. This adds to the complexity of the analysis, requiring additional sample preparation and the use of internal standards. Other analytical methods are used to circumvent this constrains. HPLC separations can be performed using a variety of chromatographic conditions, with reverse phase (RP) and normal phase (NP) being the most widely used. The use of HPLC allows for a simpler chromatographic method because derivatization is not required. The main procedure used does not require either chemical pretreatment or prior enrichment. The analysis parameters will be determined from the liquid-phase autoxidation of hydrocarbons carried out by Jensen[104] in the temperature range of 30 – 100°C. The HPLC detector most often used in is the fluorescence detector which is sensitive to polyaromatic hydrocarbons. Also UV detector may be used to measure compounds which do not fluoresce. For most hydrocarbon analyses, RP HPLC is used with an octadecyl or C_{18} bonded phase[105]. The mobile phase is commonly an aqueous mixture of either acetonitrile or methanol[106].

The goal of this chapter is to evaluate the biotransformation of phenothiazine with different fungal strains and to identify and characterize the oxidized metabolites by GC-MS, HPLC, and NMR.

Materials and Methods

Screening strains

B. bassiana, ATCC 7159, was purchased from the American Type Culture Collection (Manassas, VA). It was grown on potato dextrose agar (PDA) slants which

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were incubated at 26°C for 10-15 days and aerial conidia were harvested. NRRL 566 *Aspergillus niger,* NRRL 1369 *Cunninghamella blakesleeana,* NRRL 3655 *Cunninghamella echinulata,* NRRL 2310 *Cunninghamella elegans,* ARSEF 4954 *Harposporium anguillulae,* ARSEF 1035 *Hirsutella citriformis,* NRRL 5294 *Helminthosporium maydis,* ARSEF 2679 *Isaria fumosorosea,* ARSEF *23 Metarhizium anisopliae,* and NRRL 1757 *Mortierella isabellina* were ordered from the USDA-ARS Culture collection. *Coprinus comatus, Lactarius alnicola,* and *Phellinus igniarius* were respectively isolated from hardwood gardening mulch, *Quercus robur* leaf litter, and *Acer cappadocicum*. Isolated pure cultures were obtained by the direct transfer technique. The technique consisted in cutting the fruiting bodies with a sterile scalpel (No. 10 stainless steel), without touching the newly exposed flesh and a tissue fragment was transferred to a sterile PDA[107, 108]. The tissue was centered in PDA plate and gradually rose into a colony after a 10 days incubation in a humid chamber. The humid chamber consisted of a sterile Ziploc container (14 fl oz) with a sterile DI H_2O damp cotton plug. Fungal strains were grown on PDA slants incubated at 26°C for 10-15 days and aerial conidia were harvested.

Preparation of media

Fungal cultures were grown on Sabouraud Dextrose Emmons Broth (SDB); the medium was composed of glucose (20 g/L), yeast extract (5 g/L), and peptone (10 g/L). The pH was adjusted to 7 with HCl or NaOH. The biotransformation was done in a medium comprised of phosphate buffer amended with glucose as a carbon source. The medium included the following components: KH_2PO_4 (5.2 g/L), K_2HPO_4 (10.7 g/L), and glucose (5 g/L) for 0.5% (w/v) glucose potassium phosphate buffer solution (GPPB). Prior to inoculation, the medium was sterilized by autoclaving (121 °C, 15 psi) for 20 minutes. Phenothiazine oxidations with *B. bassiana* were conducted with either

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growing cells or resting cells (Figure 2-6). To validate that the desired oxidation was occurring, phenothiazine sulfoxide was chemically synthesized.

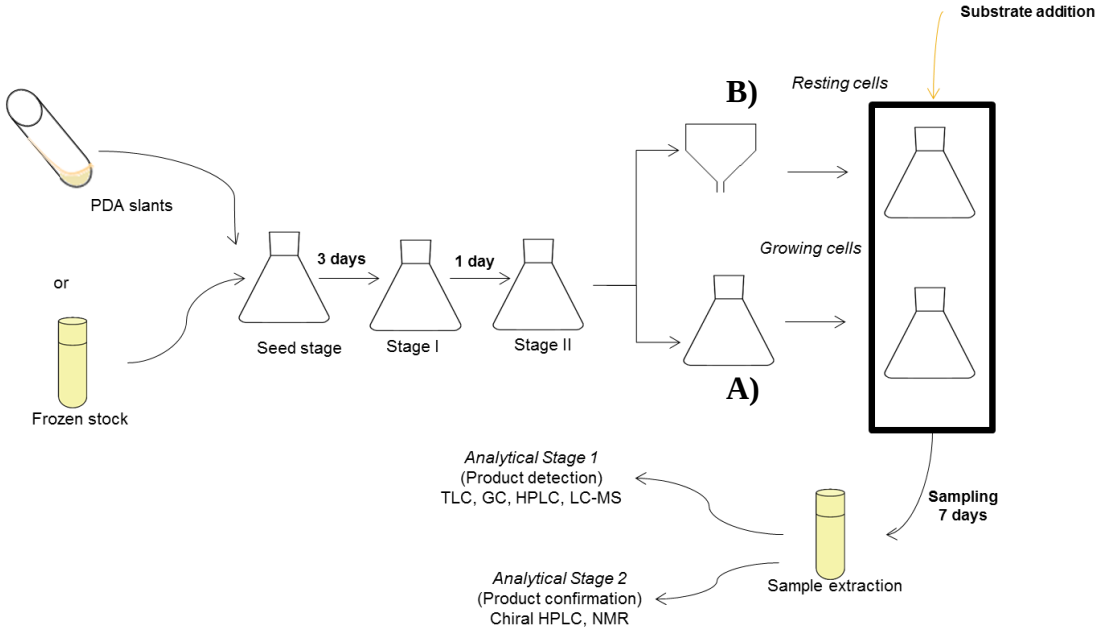

Figure 2-6 *B.bassiana* oxidation screening procedures. Spores are harvested from potato dextrose slants and grown in Sabouraud dextrose broth for 3 days (Stage I). Stage II is the biotransformation stage where the reaction can take place with growing cells (A) or resting cells (B) for 7 days. Throughout the reaction samples are collected for product detection and confirmation.

Synthesis of phenothiazine sulfoxide

Into a 50 mL round bottom flask hydrogen peroxide (820µL,8 mmol, 30%) was slowly added to phenothiazine (400mg ,2 mmol) diluted in glacial acetic acid (2 mL). The reaction mixture was stirred at room temperature until TLC indicated the reaction was complete. The resulting solution was neutralized with aqueous NaOH (4 M) and the product was extracted with CH_2Cl_2. The organic layer was dried over anhydrous

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Na_2SO_4 and then concentrated under reduced pressure to yield analytically pure product. The resulting product was a white powder.

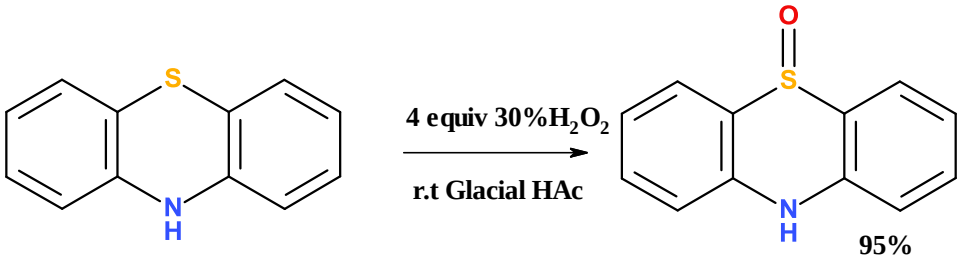

Figure 2-7 Phenothiazine sulfoxide synthesis.

Phenothiazine oxidation with fungal cells

Fungal conidia were harvested by flooding PDA slants with sterile detergent solution, 0.02% Tween 80, and were transferred into a 250 mL baffled flask with 50 mL Sabouraud Emmons Broth (SDB). The seeding stock was incubated for 72 h at 26°C and 180 rpm. To a 1 L baffled flask, 150 mL of SDB was added along with a 10% inoculum (15 mL of seeding stock). The cultures were grown for 72 h at 26° C and 180 rpm. After 3 days, 1.5 mL of a phenothiazine solution in acetone (50 mg/mL) was added into the flask(Figure 2-6 A). The reaction was monitored by thin layer chromatography in a developing chamber (glass tank, 6.5 cm O.D × 10.5 cm H) with 5 mL mobile phase, (1:1) petroleum ether: ethyl acetate (Figure 2-8). TLC plates (5 x 1.5 cm Silica gel 60 Merck, without UV indicator) were seeded with three 1µL spots; one spot was an ethyl acetate biotransformation extract and then two control spots, one with phenothiazine sulfoxide and the other one with phenothiazine. Compounds were visualized by spraying TLC plates with a solution of phosphomolybdic acid (1:10, v/v) and heating at 100°C until color developed.

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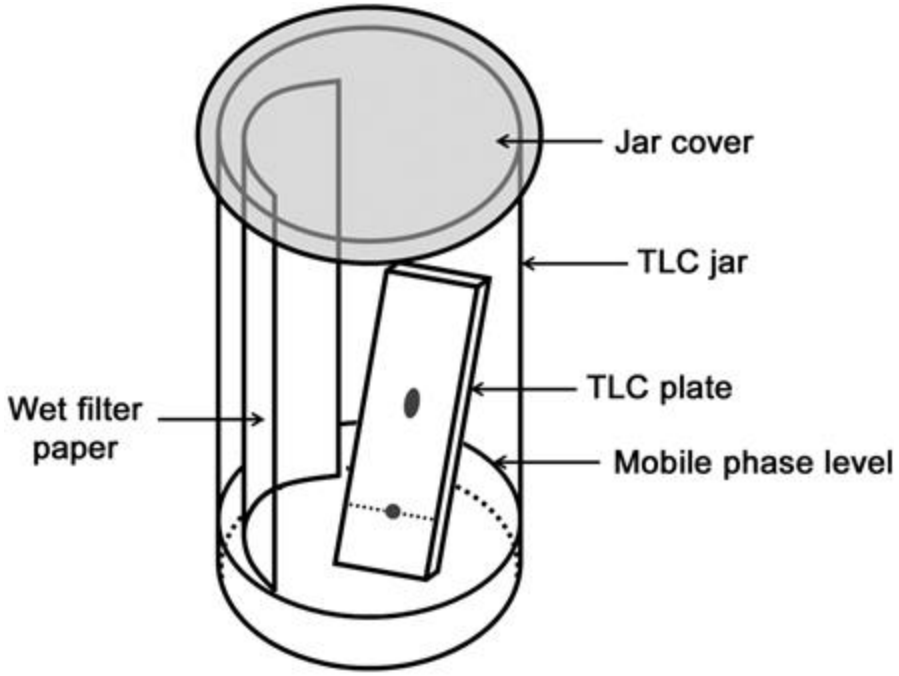

Figure 2-8 Thin layer chromatography experimental set up. The mobile phase level needs to be below the seeded spot.

Phenothiazine oxidation with *B. bassiana* Resting Cells.

For resting cell conversions, 72-h-grown *B. bassiana* cells were harvested and suspended in different volumes of GPPB (Figure 2-6 B) to have different cell concentrations (0.78-6.2 mg cdw/mL). A 10 mL cell suspension was washed and was dried at 65°C for 24 h for biomass dry weight determination. After the concentration was adjusted, 100 µL of a phenothiazine solution (5 mg) in acetone (50 mg/mL) was added into each 10 mL cell suspension. For the determination of the optimal substrate concentration, 1.5 mg cdw/mL cells were suspended in 10 mL GPPB and 50-2000 ppm phenothiazine was added to each reaction mixture, respectively. For the oxidation of the alternative substrates, a mixture of sulfur compounds containing 250 ppm each of dibenziothiophene, thiophene, and thioanisole were dissolved in 2 mL

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dimethylsulfoxide (DMSO) and were added into 150 mL of GPPB with 6.2 cdw/mL cells. For sulfoxide stability, 70 ppm of synthesized phenothiazine sulfoxide was added to 1.5 mg cdw/mL of cells suspended in 150 mL of GPPB. The reactions were run at 26° C and 180 rpm for 7 days.

Extraction and characterization

The cell broth was centrifuged at 1,956 G for 20 min. The supernatant was extracted with three volumes of 50 mL ethyl acetate each. The cell pellet was washed with two volumes of 15 mL ethyl acetate and incorporated into the total organic phase. The organic phase was dried over anhydrous Na_2SO_4 and vacuum distilled resulting in a brown solid. The metabolites were purified by silica gel flash column chromatography (2 cm x 12 cm) eluted with a petroleum ether-ethyl acetate gradient (80%-100%) followed by 10% methanol in ethyl acetate.

Phenothiazine conversion was followed by GC-MS. Samples (1µL) were injected into a SPB-5ms (60m x 0.25mm ID, 0.25 µm film thickness). Analyses were performed in a Thermo Voyager single quadrupole mass spectrometer interfaced with a Trace2000 GC, equipped with an AS3000 autosampler. Full scans were performed from 50-600 amu. Conversion was monitored via single ion monitoring (SIM) scans at [M+.] 199 with m/z reference peaks[$M^{+\cdot}$–32] 167 and [$M^{+\cdot}$ – 45] 154. The calibration curve for phenothiazine can be found in Appendix B (Figure B-2). Carbazole was used as an internal standard. Data were processed using Xcalibur 1.4 software. Ionization was performed using electron ionization (EI) with an electron energy of 70 eV. The temperature ramp was 100-250°C at 20°C/min and 250°C for 10 min[78, 109]. The injector and interface were at 250°C. For the conversion of phenothiazine sulfoxide, 10 µL samples were injected into an Agilent ZORBAX Eclipse Plus C18 (4.6x250 mm, 5 µ m). Analysis were performed in a Shimazdu 1200 HPLC equipped with a photodiode detector. Compounds were monitored at 254 nm in a gradient mode with MeOH: H_2O

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(55:45) to (95:05); 20 min gradient and 10 min hold at a 0.5 mL/min flow rate. . The calibration curve was determine based on known quantities of the phenothiazine sulfoxide is presented in Appendix B (Figure B-3).

The isolated metabolites were analyzed by LC-MS-TOF using both electrospray ionization (ESI) and in-source collision induced dissociation (CID) modes. Samples (15 µL) were injected into an Acquity BEH C18 (100mm x 2.1mm ID, 5 µm). Analysis was performed in an Acquity equipped with a Waters Q-TOF Premier detector. Full scans were performed from 120-1,000 m/z in gradient mode, 20 min gradient at a 0.2 mL/min flow rate[78], with ACN: 3 mM NH_4Acetate (20:80) to (80:20). Ionization was performed using positive electron spray ionization (ESI) at 100 V for accurate mass determination and low energy (20 eV) MS/MS collision energy for fragmentation pattern validation by in source CID. The enantiomeric excess for chiral-products, 3-hydroxy phenothiazine sulfoxide and thioansole sulfoxide, were analyzed by chiral HPLC (Figure D-5 & D-6). Samples (5 µL) were injected into a CHIRALPAK ®-MA+ (50mm x 4.6 mm ID, 3µm). Analysis was performed in a Shimazdu 1200 equipped with a photodiode detector. The compounds were monitored at 254 nm in an isocratic mode with 2 mM $CuSO_4$ solution for 15 min at a flow rate of 0.5 mL/min. All the collected analytical scans and NMR spectra for the products and reaction mixture are presented in Appendix D and E respectively. ^{1}H-NMR spectra were recorded on a 300 HZ Bücher NMR Spectrometer. Tetramethylsilane was used as internal standard. Samples were dissolved in deuterated chloroform.

High Resolution Mass Spectometry

The Facility provides information pertaining to the accurate mass, elemental composition, and molecular structure of small molecules. Equipments include a Waters Q-Tof Premier with electrospray ionization (ESI), atomospheric pressure chemical ionization (APCI), and tandem mass spectrometry experiments (MS/MS)

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capabilities. It is has LC-MS capabilities when interfaced with their Waters Acquity ultra-high pressure liquid chromatography system (UPLC). The Acquity UPLC includes a tunable wavelength detector (TUV). Waters GCT Premier is a gas chromatograph combined with a time-of-flight mass spectrometer. This instrument can be used to perform GC-MS with either electron ionization (EI) or chemical ionization (CI). If samples are not amendable to GC-MS, we have capabilities to use either a solids probe or desorption chemical ionization probe (DCI) to introduce a sample into the mass spectrometer. We can use this instrument to perform high resolution or accurate mass measurement experiments (HR-EI and HR GC-MS) Waters Acquity TQD which is a triple quadrupole mass spectrometer with a Acquity H Class UPLC. This instrument has electro spray ionization ESI capabilities, which is a softer ionization method very useful for electrophilic substrates. The TQD can be used to directly infuse samples for nominal mass determination. The TQD also has MS/MS capabilities that allow us to run precursor (parent) ion scans, product (daughter) ion scans, and constant neutral loss scans, all of which can be used for structure elucidation. Multiple reactions monitoring (MRM) can also be performed on the TQD for quantitative analysis. Thermo Voyager single quadrupole mass spectrometer in which samples are introduced into the instrument either by either GC or solids probe. Thermo LCQ Deca quadrupole ion trap mass spectrometer. The LCQ has both ESI and APCI available. Samples can be analyzed using direct infusion via a syringe pump and by using our Dionex Ultimate 3000 LC with TUV and autosampler.

NMR Research Center Facility

The available equipments for analysis are a Bruker Avance 600 Hz, Bruker Avance 300 Hz, Bruker DRX-400, Bruker DPX-300, Wide-Bore 300, and Bruker WM-360. The resolution of NMR increases with applied magnetic field; the 300 Hz's are fine for metabolite characterization purposes.

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Statistical analyses

Statistical evaluations of substrate conversion were conducted using GraphPad Prism 6 (GraphPad Software, La Jolla CA). The analyses were run in triplicates and were repeated twice for reproducibility purposes. The threshold for statistical significance was set at $P \leq 0.05$. The data were tested by one way analysis of variance (ANOVA); whenever a significant effect was indicated, a Dunnett multiple comparisons test was carried out. The upper and lower bars for each data point correspond to the 95% CI of the mean difference. If the bars intersect zero, the observed effect is not significant. The variations in significance are plotted below zero if the concentration had a negative effect and above zero if the concentration had an postive effect over phenothiazine conversion. The distance from zero of the difference between means relate to how significant that particular treatment had over conversion. Refer to Chapter III for more data analysis and interpretation. Time course experiments involved sampling three replicates at 4 distinct time points, at intervals of 48 h, after the addition of phenothiazine sulfoxide. Substrate conversion was plotted against time using GraphPad Prism and the rate was determined after curve-fitting to a one-phase association function. All data were expressed as mean ± standard deviation.

Results

Phenothiazine oxidation

Phenothiazine sulfoxidation was assayed with a broad selection of fungi (Table 2-1). Overall yields, 7 to 24%, were lower than the yields (89-98%) obtained by efficient metal catalyst oxidation yields. *H. citriformis* and *B. bassiana* were the best performers with higher selectivity and yields than any of the assayed strains. *Cunnighamella sp.* had the highest conversions, 28.3 and 33.3%, but very low selecitivies, 54-56%. *P. igniarius*

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had a very low conversion ,7.3%, but very high selectivity, 95%. The majority of strains had low yields with high selectivity or high yield with low selectivity.

Table 2-1 Phenothiazine conversion and phenothiazine sulfoxide yields after 7 days reaction in 1 L baffle flask with 150 mL SDB at 26°C and 180 rpm with different fungal strains.

Strain	Phenothiazine Sulfoxide			
	Concentration (ppm)	Conversion (%)*	Yield (%)+	Selectivity (%)#
A. niger	84	28.2	15.6	55
B. bassiana	100	22.0	18.6	85
C. blakesleeana	104	27.7	19.4	70
C. comatus	98	28.3	18.2	64
C. echinulata	100	33.3	18.6	56
C. elegans	97	33.3	18.1	54
H.anguillulae	86	21.2	16.0	75
H. citriformis	127	24.2	23.6	98
H. maydis	44	9.3	8.2	88
I. fumosorosea	45	10.1	8.4	83
L. alnicola	36	12.0	6.7	56
M. anisopliae	41	8.0	7.0	88
M. isabellina	43	11.2	8.0	71
P. igniarius	37	7.3	6.9	95

*Conversion= $(S_0-S_f)/S_0$
+$Yield_{Sulfoxide}=P_f/P_{teo}$
#Selectivity = $Yield_{Sulfoxide}$ / Conversion

Throughout the course of experimentation, only two metabolites were isolated from the *B. bassiana* mediated conversion of phenothiazine. The isolated metabolites were phenothiazine sulfoxide (II) and 3-hydroxy phenothiazine (III) (Figure 2-9). Phenothiazine sulfoxide positive ESI-MS with CID had significant ions[110] at m/z 216^+, 199 (MH^+ - 17), 167 (MH^+- 49) and 166 (MH^+ -50), which were consistent to losses of oxygen and sulfur. 3-Hydroxyphenothiazine sulfoxide positive ESI-MS with CID had significant ions[110] at m/z 232^+, 215 (MH^+- 17), 186 (MH^+- 46), 183 (MH^+- 49), 182

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(MH^+- 50) and 154, which were consistent to losses of oxygen, sulfur, and CO fragments. The extrusion of oxygen from the molecular ion gave the base peak on both cases. The enantiomeric excess for 3-hydroxy phenothiazine sulfoxide was 63±4% (R)-3-hydroxy phenothiazine sulfoxide. The major quantifiable metabolite was phenothiazine sulfoxide even after an additional 7 days of reaction.

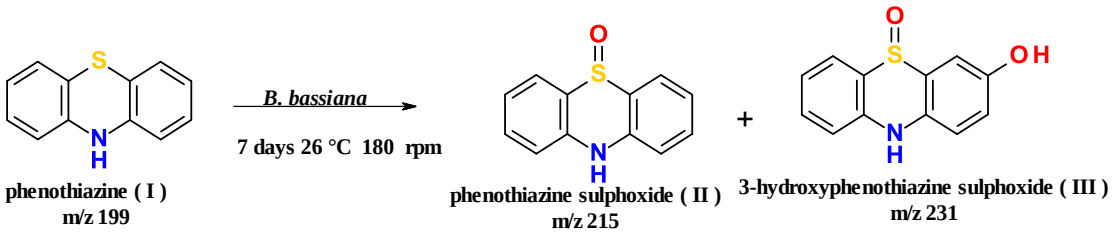

Figure 2-9 Isolated and characterized metabolites from the degradation of phenothiazine by *B. bassiana*.

Phenothiazine sulfoxide stability was verified by measuring its conversion over 7 days. The consistent data from these recurrent experiments indicates that oxidation of phenothiazine sulfoxide is slower, $5.77x10^{-03}$±$3x10^{-03}h^{-1}$, than phenothiazine conversion[111], 0.11±0.09 h^{-1} which gives a wider window for product extraction without major losses (Figure 2-10).

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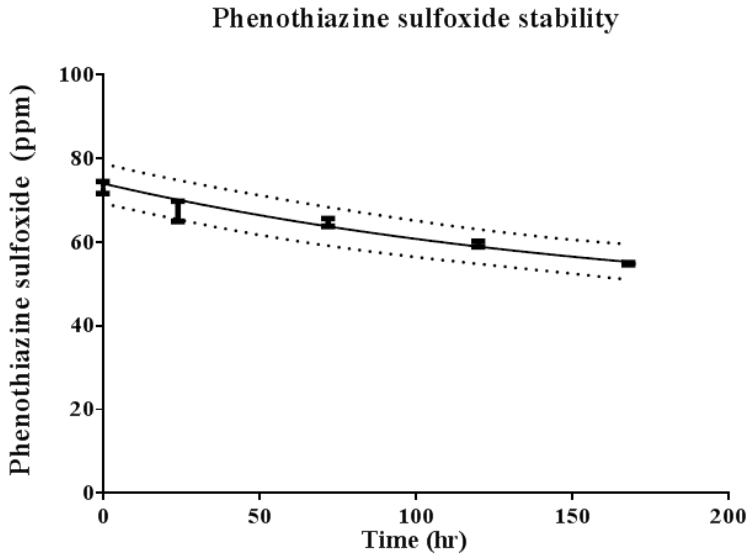

Figure 2-10 Phenothiazine sulfoxide time course reaction in GPPB at 26°C and 180 rpm, fitted to a one phase exponential decay ($y=30.2e^{-5.7E-03*t} + 44, R^2$ 0.94), dotted lines represent 95% confidence interval,error bars are ±SD of the 3 replicates average.

The effect of different phenothiazine concentrations, 50 – 2,000 ppm, was compared to the control group, 500 ppm. The optimal initial substrate concentration was selected for higher conversion. As starting substrate concentrations increased from 100 to 2000 ppm, a reduction in conversion was observed. Initial concentrations of 100 -500 ppm did not result in differences that were statistically significant while higher starting values of 750 - 2,000 ppm produced a significant reduction in conversion. The maximum conversion was reached after 72 hours (Figure 2-11).

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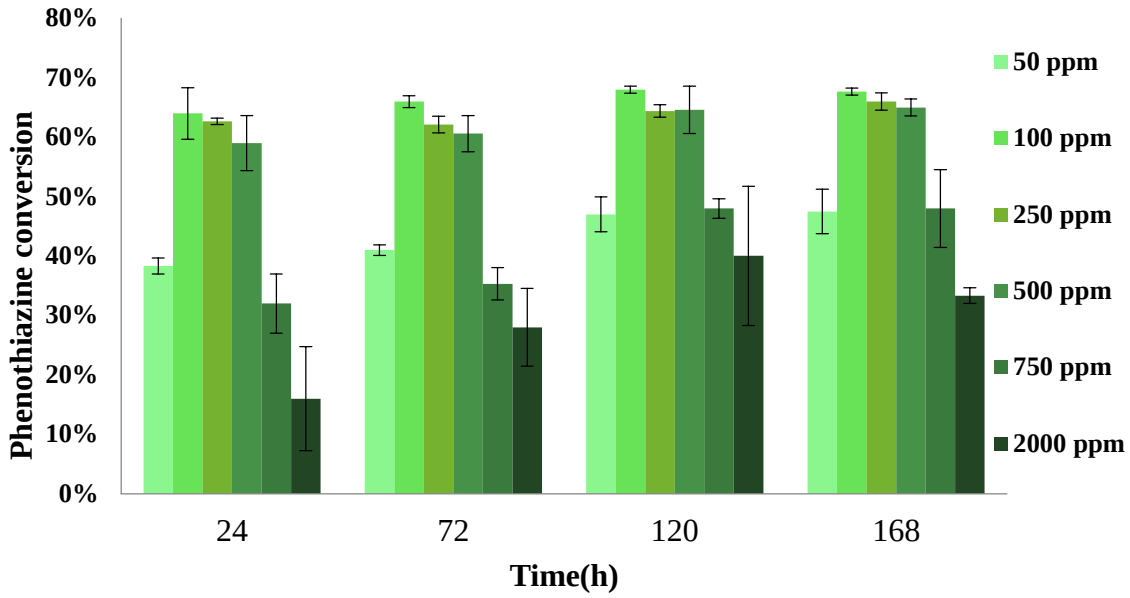

Figure 2-11 Time course conversion of the biotransformations with different phenothiazine concentrations catalyzed by 1.5 mg cdw/mL suspended in 10 mL GPPB in 70mL cell culture tubes assayed at 26°C and 180 rpm, bars represent standard deviation of 3 replicates average.

Phenothiazine concentration varied according to the substrate concentration, resulting in the highest conversion (65±1.4%) at phenothiazine concentrations of 100-500 ppm after 7 days (Figure 2-12). The 500 ppm phenothiazine concentration was selected to carry out further analysis since it facilitated downstream processing and metabolite isolation in comparison to the lower concentrations, 100 and 250 ppm.

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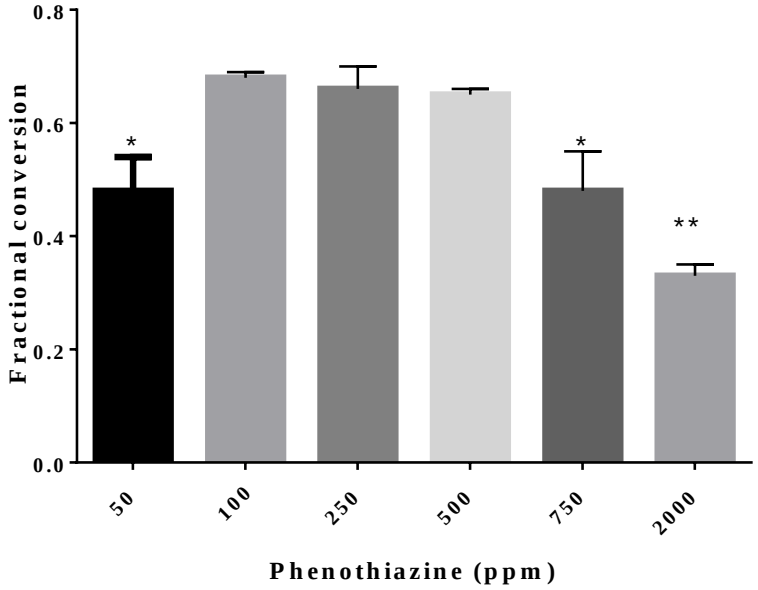

Figure 2-12 Phenothiazine fractional conversion in 1.5 mg cdw/mL cells in GPPB after 7 days with different substrate concentrations, grown in 70 mL cell culture tubes at 26°C and 180rpm, *statistical significant difference to the control reaction with 500 ppm assay by Dunnett multiple comparisons test; bars represent standard deviations of the 3 replicate average.

Metabolite characterization

Incubation of phenothiazine with *B.bassiana* gave two metabolites that were separated by flash liquid chromatography. The metabolites were identified by comparison of their proton shifts and published NMR data[78, 112]. Phenothiazine sulfoxide (II) ^{1}H NMR spectrum had a set of three peaks between δH 7.21-7.89 ppm consistent with a symmetrical heterocyclic compound. The ^{1}H NMR spectrum of 3-hydroxyphenothiazine sulfoxide (III) had a several peaks between δH 7.13-7.84 ppm that integrated for 1H consistent with an aromatic substitution where each H has a different chemical shift (Table 2-2). Details of the NMR analysis are as follows:

Phenothiazine sulfoxide Rf 0.28 (1:9 petroleum ether–ethyl acetate); 1H NMR (300 MHz, $CDCl_3$) 10 (NH broad s) 7.89(2H, dd, J=7.7,1.5 Hz), 7.56(2H,ddd, J= 8.2, 7.7,1.5 Hz) 7.38(2H, dd, J=8.2, 1.1 Hz) 7.21(2H,ddd, J=7.7, 7.7, 1.1 Hz); HRMS (MALDI-TOF) calc. from$C_{12}H_9NSO$+H $[M+H]^+$ calc m/z 215.0556, found 215.0478.

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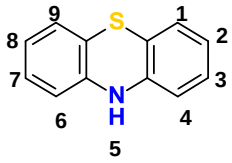
3-Hydroxy phenothiazine sulfoxide (III**)** Rf 0.15 (1:9 petroleum ether–ethyl acetate); 1H NMR (300 MHz, $CDCl_3$) 9.69 (1NH s) , 7.84(1H J=8,1.5), 7.52 (1H, J=8.4,1.5), 7.33(1H, J=2.8),7.31 (1H J=8.4, 1.1), 7.28 (1H J= 8.8), 7.14 (1H, J=8, 1.1), 7.13(1H, J=8.8,2.8) HRMS (MALDI-TOF) calc. from $C_{12}H_9NSO_2$+H $[M+H]^+$ calc m/z 231.055, found 231.0391.

Table 2-2 Hydrogen NMR chemical shifts (ppm) and coupling constant for phenothiazine and its metabolites.



Assignment	Phenothiazine	Phenothiazine sulfoxide (II)	3-hydroxyphenothiazine sulfoxide (III)
H_1	-	7.38	7.28
H_2	6.97	7.56	7.13
H_3	6.77	7.21	-
H_4	6.94	7.89	7.33
H_6	6.94	7.89	7.84
H_7	6.77	7.21	7.14
H_8	6.97	7.56	7.52
H_9	-	7.38	7.31
H_{10} (Nitrogen)	11.8	10 (broad s)	9.69 (broad s)
Coupling constants			
Phenothiazine $J_{2,3}$=7.7; $J_{3,4}$=7.7			
Phenothiazine sulfoxides $J_{1,2}$=8.2; $J_{1,3}$=1.1; $J_{2,3}$=7.7; $J_{2,4}$=1.5; $J_{3,4}$=7.7			
3-hydoxyphenothiazine sulfoxide $J_{1,2}$=8.8; $J_{2,4}$=2.8; $J_{6,7}$=8; $J_{6,8}$=1.5; $J_{7,9}$=1.1;$J_{8,9}$=8.4			

Sulfoxidation of alternative substrates

B. bassiana sulfoxidation of different heterocyclic sulfocompound was assayed after 7 days of reaction in GPPB (Figure 2-13). The metabolite's spectra were confirmed

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with NIST mass spectra and published data.With 250 ppm of thioanisole, sulfone is observed as the only product with no sulfoxide although by chiral HPLC chromatography a very small amount of S-thioanisole sulfoxide, 53% ee, was observed. GC−MS spectra of the organic products display molecular ion peak at m/z 156 and fragmentation pattern at m/z 140(M^+-16) and 124 (M+-16) corresponding to the excisions of oxygen. The unreacted thioanisole molecular ion peak occurs at m/z 124 exhibits an intense peak at m/z 109 (M^+-CH_3) corresponding to an alpha cleavage of the C-S bond. It further expels C=S to yield cyclopentadienyl m/z 65[100, 113, 114].

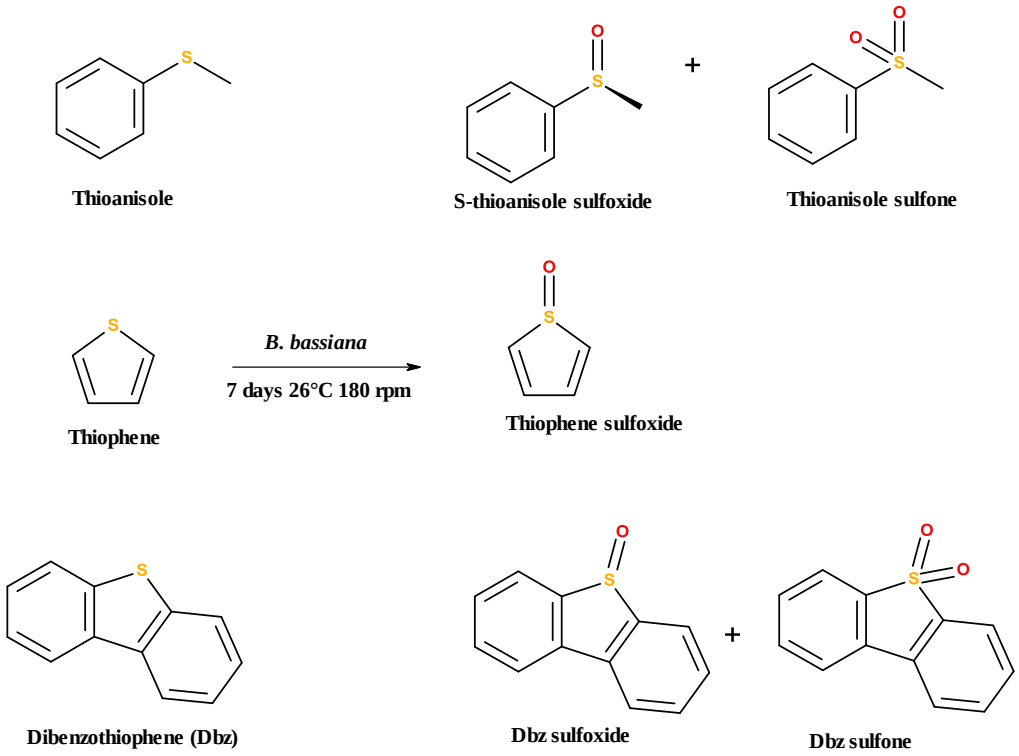

Figure 2-13 Sulfoxidations of recalcitrant sulfur compounds by *B.bassiana.*

The oxidation products of dibenzothiophene were dibenzothiophene sulfoxide and for thiophene was thiophene sulfoxide confirmed by the presence of molecular ion peaks at m/z 216 and 100, respectively.

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Discussion

Phenothiazine sulfoxidation results (Table 2-1) showed that *H. citriformis* and *B. bassiana* were the best performers with higher selectivity and yields than any of the assayed strains. It was surprising that *Cunnighamella sp.* had the highest conversions, buy yet had very low selecitivies. The low selectivity of this starin is most likely due to a larger number of side reactions taking place; hence their major use as microbial models[40, 115]. *P. igniarius* had a very low conversion and high selectivity values characteristic of wood parasitic fungi since their metabolism and growth rates are slower than other parasitic fungi[108]. However, the majority of strains had low yields with high selectivity or high yield with low selectivity which in an industrial setting are parameters that need to be further optimized to develop processes of commercial relevance.

B. bassiana oxidized phenothiazine, thioanisole, dibenzothiophene and thiophene into their corresponding sulfoxides (Figure 2-9 and Figure 2-13). Other byproducts were detected in a minor concentration with respect to phenothazine sulfoxide. The chemical stability of phenothaize sulfoxide was analyzed over a period of 7 days, the oxidized metabolites were minimal. Because sulfoxides are metabolic intermediates which can enter different reactions, variation in conversions are common; phenothiazine can be further oxidized to more hydrophilic metabolites or reduced to the parent molecule[82]. The sulfoxide can be further oxidized into sulfones but in the case of phenothiazine sulfoxide it happens at a lower rate, $5.77x10^{-03}\pm3x10^{-03}h^{-1}$, than the conversion of phenothiazine, $0.11\pm0.09\ h^{-1}$. Over the assay time frame phenothiazine sulfoxide was the major isolated metabolite (Figure 2-10). These results are similar to bioconversions of phenothiazine with the yeast *C. elegans*[78], *B. bassiana* [111], and the functional group conversions identified in the oxidation of phenothiazine drugs[79, 83, 84, 116].

The 500 ppm phenothiazine concentration facilitated downstream processing and metabolite isolation in comparison to the lower concentrations, 100 and 250 ppm. These

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results further demonstrate the value of exploring the concentration variations experimentally rather than assuming lower concentrations will result in the optimal conversion.

The oxidation of sulfides is the most straightforward synthetic route to produce sulfoxides, and numerous reagents and oxidative procedures are available for this transformation. The MS fragmentation of molecular ions into fragment ion provides clues into the molecular structure of our products. The fragmentation of thioethers mostly occurs as an α-cleavage at the longest chain; giving a $R_R^{\bullet}=CH_2$. The extra stability of the $RS^{\bullet}$ ion, cleavage occurs at the S-R link with $RS^{\bullet}$ ions of fairly high relative abundances appearing in the series m/z 47, 61, 75, 89[98]. Sulfoxide fragmentation has a significant peak at m/z 16, which is diagnostic for the presence of either an *N*-oxide or an *S*-oxide function[100, 101]. The observed changes in the fragment ions, base peak, and MH^+ allowed the identification of major catalyzed functionalities by *B. bassiana.*

Immobilized *Mortierella isabellina* in calcium alginate beads oxidized thioanisole to the (*R*)-sulfoxide with high enatioselectivity 92–94% ee[117], only when methanol is used as permeabilizing agent. Also baker's yeast can oxidize thioanisole and benzyl-alkyl sulfides to their corresponding (R)-sulfoxides when the transformations are carried out under semi-anaerobic conditions[20]. The majority of the metabolites give evidence of over-oxidation to the corresponding sulfones, as observed by the fragmentation patterns losses of M^+-16 and M^+-32 of their respective molecular ion peak (M+). Mixed cultures of oil degrading bacteria degraded alkyl thiophenes and supported their growth after 15 day incubation; 50% of the sulfur was completely mineralized into sulfate[118, 119]. The main oxidation product from the conversion of thiophene with *Rhodococcus* and mixed bacterial culture were thiophene sulphoxide dimers[120] under nutrient limited conditions with hydrocarbons as the carbon source. *C. versicolor* utilized thiophenes, such as 2-hydroxymethyl-, 2-formyl-, and 2-carboxyl-thiophenes, as a sulfur source for growth;

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thus, it is possible to mineralize these compounds and to partially degrade them[86]. Overall, fungi metabolize thiophene as a sulfur nutrient and can be used to adapt cells to better degrade it and other aromatic sulfides. Dibenzothiophene is a more recalcitrant compound so it harder to oxidize in comparison to thiophene which is oxidized by *Agrocybe aegerita* into mainly S-oxidation products and in lower amounts, ring-hydroxylation compounds. *Coprinellus radians* converted 60% of dibenzothiophene into sulfoxide and sulfone as the sole metabolites[87]. In most of the aforementioned cases product distribution, conversion and selectivity had a lot variations. However, control of the reaction conditions plays an important role in avoiding the formation of side products, but this is often hard to achieve and therefore there is still considerable interest in the development of selective oxidants for this transformation.

B. bassiana has the catalytic capacity to oxidize sulfur compounds into stable sulfoxide compounds. The ability to transform sulfur compounds by several fungal strains (Table 2-1) opens an industrial opportunity for biocatalyst to thrive. However, there is still a lot of room of improvement for the oxidation to be as efficient as the current industrial catalyzed processes. Although, the difference in selectivity and yield variations between lower and upper fungi, presents an abundant catalyst repertoire from where to choose.

Conclusion

These results demonstrate that *B. bassiana* converts phenothiazine into phenothiazine sulfoxide and hydroxyl phenothiazine sulfoxide. Furthermore, these results demonstrate *B. bassiana's potential* catalytic use for the desulfurization of fossil fuels by the oxidation of the most recalcitrant compounds present, dibenzothiophene and thiophene. The ability of *B. bassiana* to oxidize phenothiazine provides further foundation for a well-characterized biocatalyst of possible industrial interest. The following chapter explores the effects adaptation and biotransformation have over cell

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growth. The goals is to ensure that *B. bassiana* remains catalytically active in order to use it as a biocatalyst.

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CHAPTER III

ADAPTIVE CAPACITY: ENDURING THE EXTREMES, HYDROCARBONS AND INSECTICIDE TOLERANCE

Abstract

B. bassiana has a very flexible metabolism and is widely used as a biocontrol agent. It can metabolize hexadecane as a sole carbon source. In addition, it shows a synergistic effect over pest control efficacy when it is applied with lower concentrations of pesticides. In the current chapter the effect pesticides, imidacloprid and carbaryl, have on *B. bassiana* growth was evaluated by measuring radial growth in ¼ Sabouraud dextrose agar plates. In addition, hexadecane consumption was monitored by GC-MS. Hexadecane adapted cells had a steadier consumption rate of 0.18± 0.04 ppm/day in comparison to non-adapted cells, 0.27± 0.04 ppm/day. Different pesticide concentrations had diverse degrees of induction and/or reduction of *B. bassiana's* radial growth. Imidacloprid had a moderate radial growth inhibition in comparison to carbaryl. Furthermore, only lower concentrations of the pesticides caused a slight reduction in colony size. Therefore, it is possible to use the tested pesticides as inducers for phenothiazine oxidation.

B.bassiana metabolism

Many microorganisms require an external carbon source for their growth, usually glucose is the preferred source. However, it is well known that certain bacteria, yeast, and fungi are able to grow on n-alkane enriched media as their sole carbon source[121-125]. Microbes grown under nutrient limited conditions demonstrate higher tolerance to other stress conditions, a phenomenon referred to as cross tolerance[126]. Fungi have the capacity to express any of several phenotypes in response to these environmental and physical variations. It is likely that cells under nutritive stress have

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many, if not most, of their stress-related genes induced, thereby providing better protection against other stress conditions[12,126].

Other than bioremediation related studies on heavy oil sediments containing very long chain alkanes, limited biochemical evidence is available on microorganism utilization of very long chain alkanes, such as those usually present in the insect epicuticle. Uptake and utilization of n-octacosane and n-nonacosane by *Arthrobacter sp.*[118, 127], were reported to produce same chain length fatty acids[41]. The ability of filamentous fungi and yeast to utilize straight chain alkanes, from n-decane to n-octadecane, for growth is well-documented[122,123, 128]. *B. bassiana* can metabolize n-alkanes into corresponding alcohols[65] by inducible oxidases[12, 41] (Figure 3-1). These alcohols are further metabolized by terminal and sub-terminal oxidation, with subsequent entrance to the β oxidation pathway [123,41, 42,65].

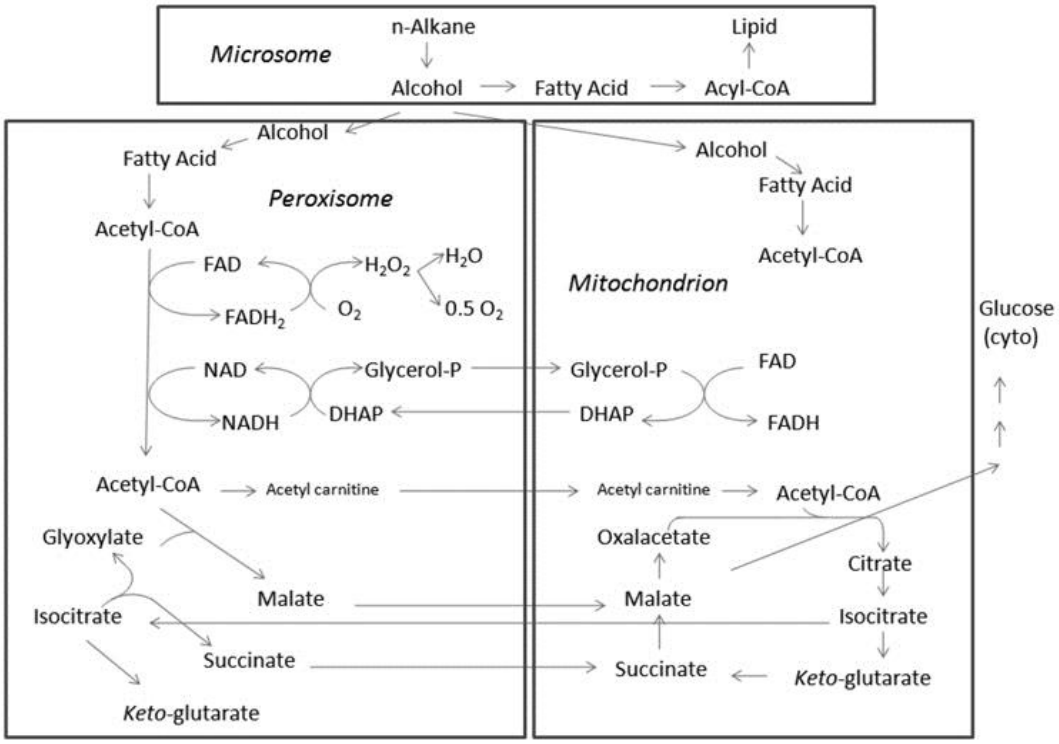

Figure 3-1 Alkane catabolism pathway in *B. bassiana.* The assimilation of hydrocarbons of medium chain alkanes (10 – 16 carbon atoms) goes through β-oxidation pathway. As the number of carbons increases the β-oxidation becomes defective and the metabolism favors ω-oxidation which is carried in the ER instead of the mitochondria [52].

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Fungi respond differently against foreign structures, xenobiotics. They are either assimilated as growth factors or degraded to reduce their metabolic toxicity. Most of the toxic compounds are lipophilic in nature, so fungi first distinguish whether the substrate contains an aromatic moiety. In the case of sulfur compounds, if it is non-aromatic, then driving force for the metabolism is an acquisition of a sulfur nutrient so the pathways involved in transformation are more active when fungi are cultured in non-sulfur-containing medium. But when the substrate is aromatic, the fungus distinguishes whether the aromatic moiety contains free hydroxyl groups in order to conjugate them with sugars. Interestingly, *P. chrysosporium*[129, 130] and *B. bassiana.*[13, 30] have a tendency to catalyze the glucosylation and *P. ostraeatus* and *C. versicolor* to catalyze the xylosilation[86]. However when no hydroxyl groups are found, the fungus converts the substrate to more hydrophilic products via sulfur oxidation or hydroxylation reactions[50](Figure 3-2).

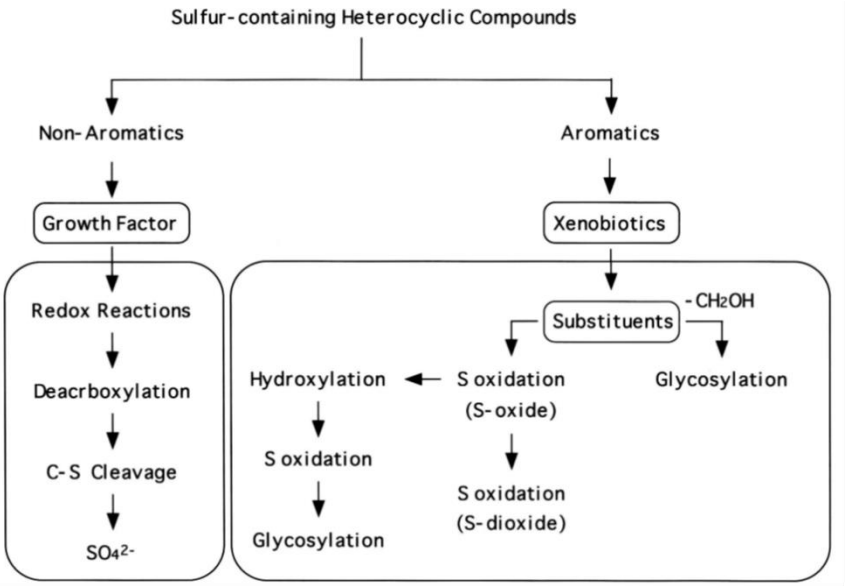

Figure 3-2 Fungal degradation of sulfur-containing heterocyclic compounds, metabolic response and substrate recognition. Taken from Ichinose et al[86].

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B. bassiana strains are widely used as biological control agents against a broad range of insect and arachnid pests. However, the control efficacy of entomopathogenic fungi is variable because of unfavorable and fluctuating environmental and formulation conditions[131, 132]. One strategy to enhance efficacy is a combined use of entomopathogenic fungi and low dosages of pesticides. These sub-lethal dosages of chemicals can increase the control efficiency of entomopathogenic fungi but only if they do not affect the fungi [133, 134]. Adverse effects could include the inhibition of germination and/or vegetative growth as well as conidiogenesis[51, 134-136], which affect the overall efficacy of the bio-pesticide as well as its use as a bio-catalyst. The goal of this chapter is to evaluate if the assaying concentration of insecticides and phenothiazine have negative effects over cell growth. Since, it is necessary to validate that cells remaine metabolically active under nutrient deprivation and insecticide toxicity in order to use them as catalysts.

Material and Methods

Seeding strain

B. bassiana ATCC 7159 was purchased from the American Type Culture Collection (Manassas, VA). It was incubated in potato dextrose agar slants in humid chamber at 26°C for 10 days. The agar slants were kept at 3°C in the refrigerator. Spores were harvested and suspended in 2 mL of sterile DI H_2O with 0.02% Tween solution. The spore suspension was transferred into a 250 mL baffled flask with 50 mL of SDB and grown for 5 days at 26°C and 180 rpm in an incubator (New Brunswick Scientific C25 Incubated Floor Model Shakers). After 5 days of growth, the cell suspension was used as an inoculum in order to analyze cell growth and pesticide tolerance.

Preparation of media

B. bassiana's substrate tolerance and pesticide were evaluated on Sabourad Dextrose-Emmons Broth (SDB) and ¼ Sabourad Dextrose Agar (SDA), respectively.

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SDB consisted of glucose (20 g/L), yeast extract (5 g/L), and peptone (10 g/L). SDA consisted of glucose (5 g/L), yeast (2.5g/L) and peptone (2.5 g/L), and agar (15 g/L) for SDA. The adaptation was done in a minimum salt medium (MM) comprised with 10%(v/v) hexadecane as a carbon source. The medium included KH_2PO_4 (0.4 g/L) , Na_2HPO_4 (1.4 g/L) , $MgSO_4.7H_2O$ (0.6 g/L), KCl (1 g/L), NH_4NO_3 (0.7 g/L), and $FeSO_4$ x $7H_2O$ (0.06 g/L). The pH was adjusted to 7 with either HCl or NaOH. Prior to inoculation, the media were sterilized by autoclaving (121 °C, 15 psi) for 20 minutes.

Growth induction cycle

B. bassiana was grown on SDB for 5 days (FS_0). A 10% inoculum of these*B. bassiana* cells were added to minimum medium supplemented with 10 % (v/v) hexadecane (FS_n). These cells were grown in presence of n-C_{16} for 15 days at 180 rpm and 26°C. After the first 15 days, the adaptation process started with a 10% v/v inoculum from this culture seeded into a new flask with MM (FS_{n+1}). The resulting cell suspension was grown under similar conditions for another 15 day period. This repetitive process has been carried out for 80 cycles (Figure 3-3).

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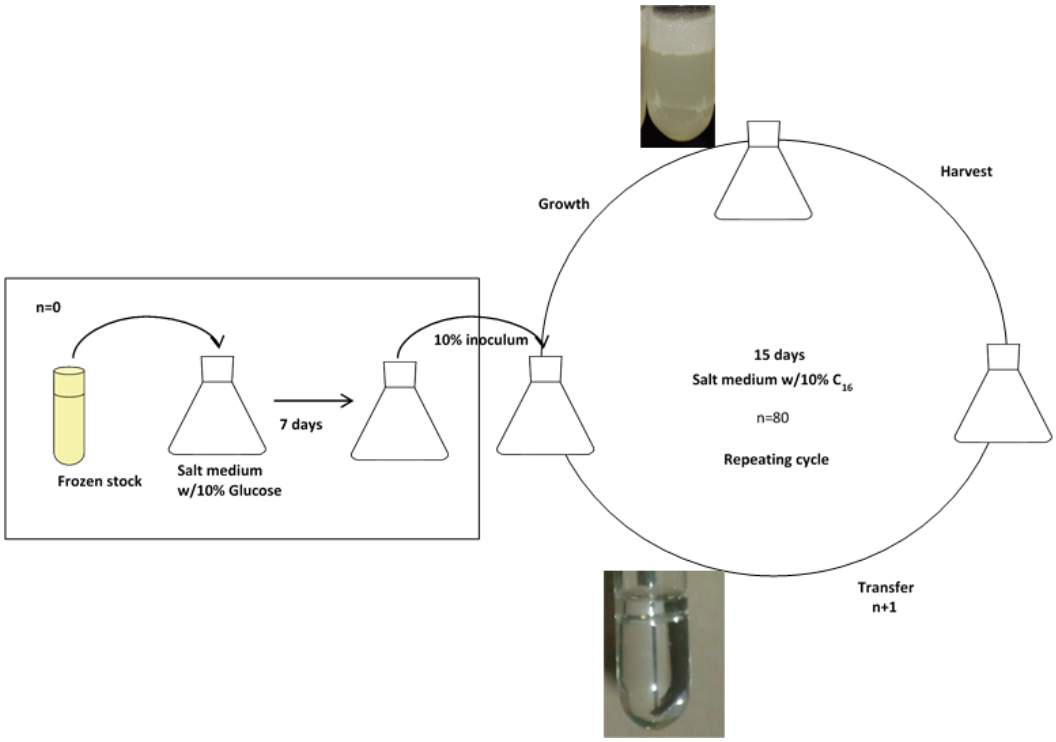

Figure 3-3 *B. bassiana* growth adaptation process to grow in hexadecane as sole carbon source. Cells are grown in minimum medium (MM)suplmented with 10% hexadecane for 15 days, then cells are harvested and transferred into MM to repeat the adaptation cycle.

Hydrocarbon quantification by Mass Spectroscopy

The most widely used analytical method for hydrocarbon analysis is GC[137 104, 138, 139]; GC is the standard[106 105, 140] method of analysis for n-alkane compounds. In particular GC-FID[128, 141] is used for quantitative analysis of all the non-polar hydrocarbons and semi-quantitative assessment of hydrocarbon decomposition. Flame ionization detection (FID) is not selective and of modest sensitivity, which are serious drawbacks in view of the broad patterns of unresolved peaks. However, the response of an FID is proportional to the mass of hydrocarbon present and is insensitive to the type of hydrocarbon (e.g., aromatic, n-alkane and olefin). A mass spectrometer, however, may have very different responses for two different hydrocarbon compounds. GC parameters allow the

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measurement of a hydrocarbon range of n-hexane (C_6) to n-octacosane (C_{28}), a boiling point range of approximately 65 °C to 450 °C[140].[141] The main drawback of GC-MS is that isomeric compounds and other compounds have similar mass spectra [141].

Mass Spectroscopy (MS) gives additional information about compound characteristics such as molecular weight (MH+) and fragmentation patterns. The fragmentation of molecular ions into fragment ion provides clues into the molecular structure of our products. Hydrocarbons (HC) mass spectra are often complex with many possible fragmentation patterns; saturated hydrocarbons their $M^{+\bullet}$peaks are quite weak, thus making it very difficult to identify long-chain hydrocarbons. For characterization purpose the main distinction are an odd-numbered series m/z (14n+1),from CH_3+ and CH_3CH_2+ to $CH_3(CH_2)9$+, where n is the number of carbon atoms in the fragment. Therefore, the typical ions in saturated hydrocarbon spectra are m/z 15,29,43,57,71,85,99,…(M-CH_2CH_3), differing by 14 amu[95].The second characteristic feature is the distribution of the relative intensities of ions. The most intense ion is m/z 57 (C_4 fragment), while C_3 and C_5 fragments, 43 and 71 respectively, are also intense; on either side of this range, the ion intensities fall of rapidly, so the whole spectrum falls within an envelope shown by a smooth curve[104].

B. bassiana's hexadecane consumption

Assays were performed on MM saturated with hexadecane. MM was transferred into a 250 mL separation funnel and 50 mL of hexadecane was added. It was mixed and separated into two phases. The organic and the saturated MM phases were separated. The process was repeated twice. The resulting MM was used to evaluate hexadecane consumption with adapted and non-adapted cells. The adapted cells were taken from an FS_n stock while the non-adapted cells were an inoculum grown in SDB for 5 days. Cells were harvested, and the cell concentration was adjusted with MM to 1.5 mg cell dry weight (cdw)/mL. A 1 mL cell suspension in saturated MM was added to 10 mL

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incubation tubes. Triplicate cultures were assayed as well as an abiotic control, medium, and hexadecane. The cultures were grown at 26° C and 180 rpm for 15 days.

Residual n-hexadecane was analyzed after 3, 6, 9, 12, and 15 days of incubation. The samples were centrifuged and the supernatant was extracted with 2 volumes of 250 µL of petroleum ether. The cell pellet was washed and was filtered for biomass wet weight determination. The organic phase was dried in a RapidVap vacuum dry evaporation system. The dried extract was suspended in dichloromethane. Hexadacane consumption was followed by GC-MS. The calibration curve for hexadecane can be found in Appendix B (Figure B-1). Samples (1µL) were injected into a SPB-5ms (60m x 0.25mmID, 0.25 um film thickness). Analyses were performed in a Thermo Voyager single quadrupole mass spectrometer interfaced with a Trace2000 GC equipped with an AS3000 autosampler. Full scans were performed from 50-600 amu. Conversion was monitored via single ion monitoring (SIM) scans at [M+.] 226 with m/z reference peaks 71 and 57, corresponding to C_4 fragment and C_5 fragments, respectively. Data were processed using Xcalibur 1.4 software. Ionization was performed using electron ionization (EI) with an electron energy of 70 eV. The temperature was ramped up from 100 to 250°C at 20°C/min ramp held at 250°C for 10 min[137]. The injector and interface were at 250°C. Helium was used as the carrier gas with a constant flow rate of 1 mL/min.

B. bassiana growth on phenothiazine

To a 1 L baffled flask, 150 mL of SDB was added along with a 10% inoculum (15 mL seeding stock). The culture was grown for 72 h at 26° C and 180 rpm. After 3 days, 1.5 mL of a phenothiazine solution in acetone (50 mg/mL) was added into phenothiazine flask while 1.5 mL acetone was used for the control flask. To evaluate the effect of phenothiazine on growth, $OD_{@600nm}$ was monitored with a Beckman DU 640 spectrophotometer. The reaction was run at 26°C and 180 rpm for 7 days.

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B. bassiana's radial growth on pesticides

The effect imidacloprid and carbaryl have on B. bassiana growth is defined by spore yield and viable germination which is ideal for biocontrol purposes1-4. However, for biocatalysis, it is important to maintain viable cell growth to retain catalytic activity. While it may be desirable to determine the ability of xenobiotics to induce catalytic activity, it is also necessary to define the potential of these compounds to inhibit cell growth. The insecticides evaluated were 75 WP ® , imidacloprid, (Baeyer, Monheim am Rhein, Germany) and Sevin SL, carbaryl, (Baeyer, Monheim am Rhein, Germany). The selected pesticide concentrations are 2 ppm to 4 ppm for imidacloprid[51] and 100 to 400 ppm for carbaryl[134]. The aformentioned concentrations correspond the the concentration reanges used in literature in bipesticide synergistic studies[51, 134]. The effect of insecticide on fungal growth can be determined by evaluating the radial growth of cultures on plates in comparison to the growth of cultures in the absence of inhibitory compounds. Fungal growth is expressed as a rate in mm/day or total extension after 12 day in mm. The radial growth was determined according to the methods of Shapiro and Bidochka, with the following modifications[59, 134, 142]. *B. bassiana* spores were harvested from PDA plates directly into sterile distilled water and were washed twice with the same solution. *B. bassiana* was purifiied by single spore isolation technique. This technique was carried out by streaking the mycelia on water agar in a zigzag manner. Its purpose is to assure the attainment of a pure culture, one that is not capable of genetic segregation, no variability, and that is unisexual. Once spore germinated, a single conidium was transferred onto ¼ SDA plates and was incubated at 26°C for 10 days . After 10 days, a disk of 6 mm diameter was cut out from the fully sporulating colony with a sterile cork borer. All replicates were taken from the same plate to reduce experimental variability and spore germination deviations. The 6 mm size plugs were placed in the center of sterile ¼ SDA plates with and without insecticides. Four perpendicular straight lines were drawn on the

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bottom of each Petri dish. The crossing point coincided with the center of the 6 mm initial fungus disc plug. Radial growth measurements were recorded each 2 days from one extreme of fungus mycelia development to the opposite extreme, following the four segments formed by the perpendicular lines (Figure 3-3). The colony diameters were measured with a caliper. The diameters were averaged and compared to the average diameter of the control to evaluate the extent of the effect each concentration had over growth (Figure 3-3). The control plate was ¼ SDA without pesticides. The plates were prepared in triplicate, and incubated in the dark at 26°C for 12 days.

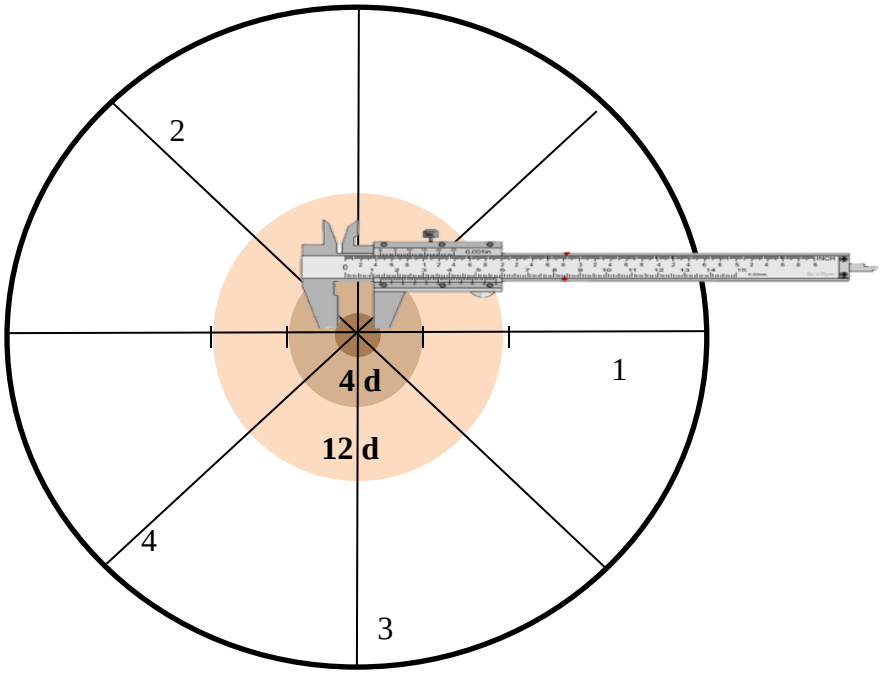

Figure 3-4 Radial growth expansion schematics. Growth is evaluated by measuring the diameter's (mm) dispersion from 4 perpendicular locations (1-4) with a caliper at after 12 days grown in the dark in ¼ Sabourad dextrose agar.

Statistical analysis

Statistical evaluations were conducted using GraphPad Prism 6 (GraphPad Software, La Jolla CA). The analyses were run in triplicates and were repeated twice for

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reproducibility purposes. The threshold for statistical significance was set at $P < 0.05$. P constitutes the probability that the true value will be between the upper and the lower limits of a 95% confidence interval. The data were tested by one way analysis of variance (ANOVA); whenever a significant effect was indicated, a Dunnett multiple comparisons test was carried out. Dunnett's test is pairwise comparisons of group means by t-student statistics. It determines which pairs are significantly different from the designated control group (1/4 SDA w/o insecticides) after the ANOVA null hypothesis of equal means has been rejected. This procedure reduces the multiple-comparisons price you pay of comparing all group means, while taking into account that all these comparisons are correlated since they all use the same "control" data[143, 144]. Since each comparison has the same control in common, the procedure incorporates the dependencies between these comparisons. In particular, the t-statistics are all derived from the same estimate of the error variance. For example when you compare the mean of 2.5 ppm to the mean of control, the test compares difference between means to the amount of scatter, quantified using information from all the groups, not just groups 2.5 and control (Table 3-1). The results are a set of decisions (Figure 3-5) : "statistically significant" or "not statistically significant". These decisions take into account multiple comparisons. Both tests can compute a confidence interval for the difference between the two means that accounts for multiple comparisons. In addition, Graphad Prism™ program gives a q ratio which is the ratio of the difference for each treatment divided by the standard error of the difference. It is used to compare the results with other studies and text. The optical density at 600nm were used to construct fungal growth curves by non-linear regression with the GraphPad Prism 4 software fitted to the Gompertz equation $Y=YM*\exp(\ln(Y0/YM)*\exp(-\mu_{max}*t))$. In this sigmoidal function YM is the upper asymptote of the growth curve (i.e. the maximum biomass produced). Y_0 is the lower asymptote, originally defined by the initial

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biomass concentration at t_0, here reflecting the spectophometiric detection level, t is the time course [145]. All data were expressed as mean ± standard deviation.

Table 3-1 Dunnet multiple comparison of different imidcloprid concentration (ppm) to a control w/o insecticide at $P \leq 0.05$. The following is the difference tables obtained from Graphad Prism 6 after the multiple comparison analysis was run. The degree of the significance is dependent on how big is the 95% CI of the mean difference between one imidacloprid concentration and the control. The asterisks correspond to the level of significant difference, SE standard error.

Number of families			1		
Number of comparisons per family			5		
Alpha			0.05		
Difference degrees of freedom			12		
Comparison	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
2 vs. Control	-1.967	-2.570 to -1.363	Yes	****	< 0.0001
2.5 vs. Control	-1.200	-1.803 to -0.5967	Yes	***	0.0004
3 vs. Control	-0.2667	-0.8700 to 0.3366	No	ns	0.5930
3.5 vs. Control	-1.100	-1.703 to -0.4967	Yes	***	0.0008
4 vs. Control	-1.850	-2.453 to -1.247	Yes	****	< 0.0001
Test details					
Comparison	Mean 1	Control Mean	Mean Diff.	SE of diff.	q=Diff/SE
2 vs. Control	10.68	12.65	-1.967	0.2079	9.458
2.5 vs. Control	11.45	12.65	-1.200	0.2079	5.771
3 vs. Control	12.38	12.65	-0.2667	0.2079	1.282
3.5 vs. Control	11.55	12.65	-1.100	0.2079	5.290
4 vs. Control	10.80	12.65	-1.850	0.2079	8.897

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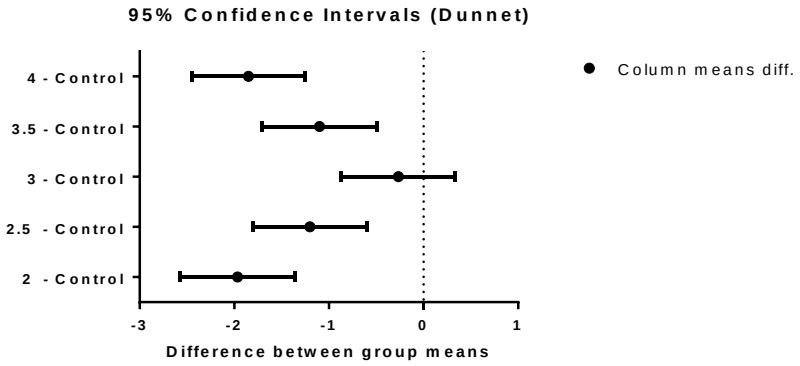

Figure 3-5 Graphical representation of the Dunnet multiple comparison between the different imidacloprid concentrations and control w/o imidacloprid. The upper and lower bars for each data point correspond to the 95% CI of the mean difference. If the bars intersect zero, the observed effect is not significant. The variations in significance are plotted below zero if the concentration had an inhibitory effect and above zero if the concentration had an enhancing effect. The distance from zero of the difference between means relate to how significant that particular concentration had over radial growth.

Results

B. bassiana's hexadecane consumption

The aim of adaptation efforts was to enhance the oxidative capacity of *B. bassiana* by gradual exposure to hydrocarbons. In the first period of adaptation after 15 days of growth the hexadecane organic phase layer was scanned with a 640 Beckman UV/Vis spectrophotometer and a new peak at 230 nm appeared which was different from the hexadecane wave scan (Figure 3-6).

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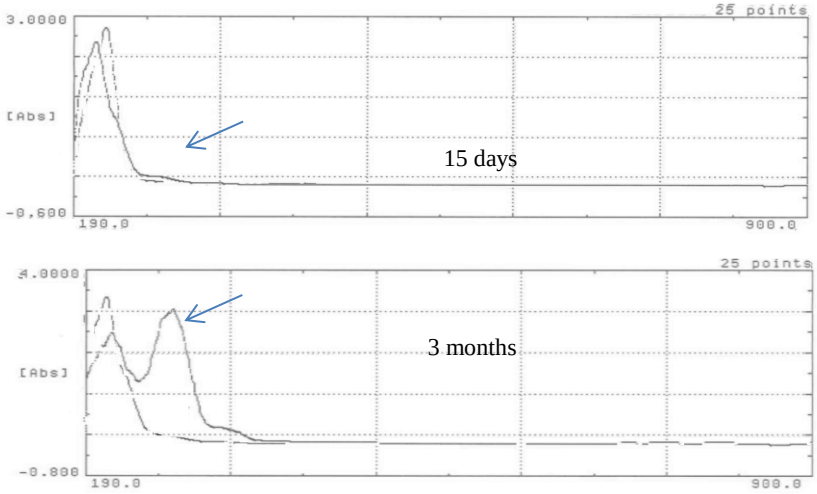

Figure 3-6 Wavelength scan of the organic phase from the HC adapting cells of adapting cells.The arrow indicates the appearance of another peak after 15 days of growth, which increased after 3 months.

Adapted cells had a shorter phase followed by a steady first order kinetics decline in hexadecane concentration, 0.18± 0.04 ppm/day. In comparison, non-adapted cells had a longer phase followed by a faster decay of 0.27± 0.04 ppm/day (Figure 3-7). After the 6^{th} day adapted cells, 2.5±0.01 mg, and non adapted cells, 2±0.01mg, reached the maximum biomass weight which plateued to 0.9±0.001 mg (Figure C-1). The cell broth from non-adapted cells formed clusters of cells suspended in MM while the adapted cells were well dispersed throughout. Degradation rates were faster based on the longer lag phase and abrupt hexadecane reduction after the 5^{th} day (Figure 3-7).

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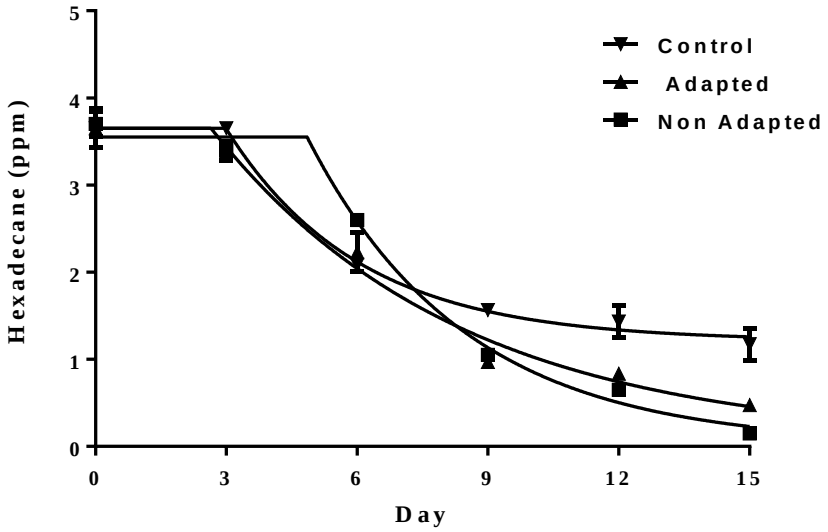

Figure 3-7 *B. bassiana* hexadecane consumption measured by GC-MS fitted to a one phase exponential decay graph, bars represents ±SD of a 3 replicates average, control is medium without cells, adapted is medium inoculated with cells grown in hexadecane as sole carbon source while non-adapted where cells grown on glucose.

Phenothiazine tolerance

B. bassiana was able to grow in the presence of phenothiazine with no observable morphological differences. In the presence of phenothiazine, fungal growth was slower an apparent growth rate of μ_{max}=2.57 day^{-1}, but reached higher optical densitiy OD_{600nm} 1.5, than in cultures without phenothiazine μ_{max}=2.76 day^{-1}, OD_{600nm} 1.25 (Figure 3-8). Similar variation of higher cell growth in presence of different substrates had been reported in the biotransformations of cyclohexanone and terpenes[27, 146]. Though it is unclear the metabolic effects played by the different substrates on fungal growth[7]. In addition, biomass and activity correlations are not always defined since several factors may play single or combinatorial effects (i.e primary or secondary metabolism, carbon catabolism, toxic substrate or product, stress response, transport and diffusion into mycelium bundle) in substrate conversion[146].

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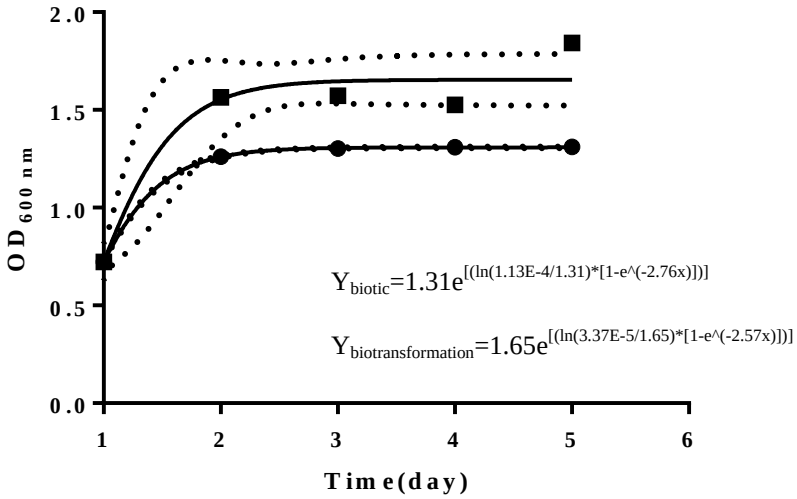

Figure 3-8 Phenothiazine effect on fungal growth; best fitted to the Gompertz growth function; squares are for cells grown with phenothiazine R^2 = 0.99 & circles represent cells grown w/o phenothiazine R^2=0.97; dotted lines represent the 95% CI, n=3, optical densiy measured from 1 mL aliquots takedn at different time intervals from cells grown in 250 mL baffled flasks with 50mL of sabouraud dextrose dextrose with and w/o phenothiazine at 26°C and 180 rpm.

In vitro effects of pesticides on *B. bassiana growth*

From the collected data, it was determined that imidacloprid and carbaryl could stimulate *B. bassiana* growth to different degrees (Figure 3-9), and their stimulation was related to their concentration. Carbaryl had a positive effect over the 12 days vegetative growth as seen by higher radial dispersion than the control 13±0.15 mm for most of the assay concentration 100-400 ppm with the exception of 200 ppm which was below the control at 11±0.21 mm (Figure 3-10 and Figure 3-11). Imidacloprid, a second generation insecticide, lowered cell radial expansion to aprox 11mm, inhibited growth 10-18% (Figure 3-10). This inhibition was not substantial to cause major effect over the catalytic capacity, was between the 95 confidence interval of the control cell growth curve.

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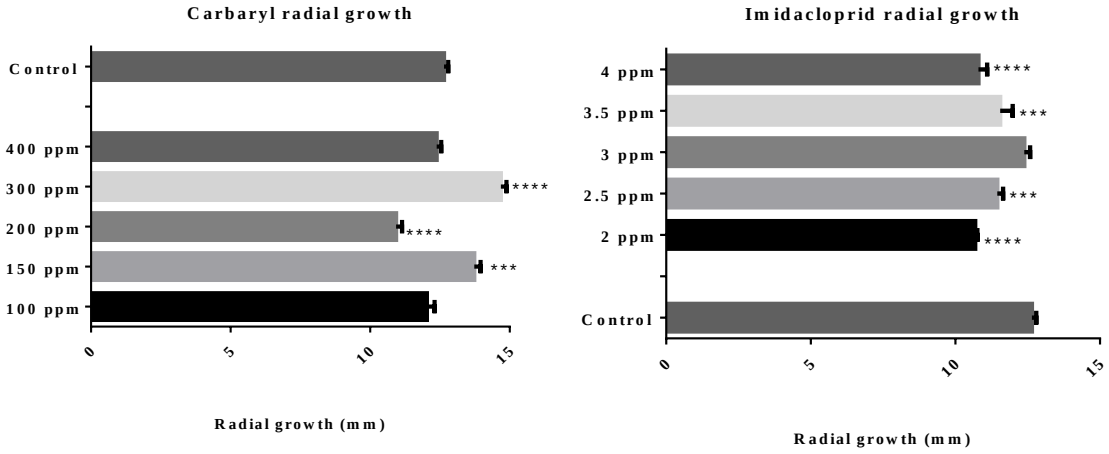

Figure 3-9 Comparison of *B.bassiana* radial extension after 12 days grown in the dark in ¼ SDA with different concentrations of insecticide (ppm) ; bars represent SD, n=3, control is ¼ SDA w/o insecticides, and the numbers of stars represent the level of significant difference by the Dunnett multiple comparisons test between the control and the concentration.

The lower pesticide concentrations, 2 ppm and 200 ppm, had a negative effect on cell growth while higher concentration appeared to have an inductive growth effect. In some cases, the metabolic effect of lower concentrations of pesticides and/or toxins is more latent than the immediate acute effects of higher concentrations, so the effects are gradual and cumulative and in some cases even more severe than higher exposures[147].

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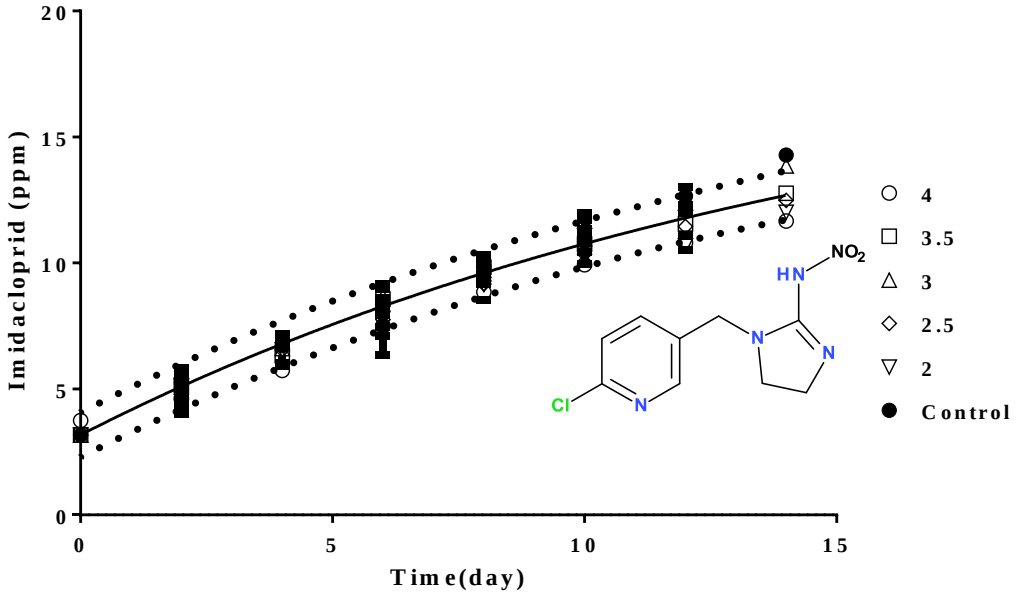

Figure 3-10 Imidacloprid effect over *B. bassiana* radial growh expansion, cells grown in the dark on ¼ SDA with different concetration of pesticides (ppm); dotted line represents 95% CI from the control growth curve, error bars are ±SD of n=3

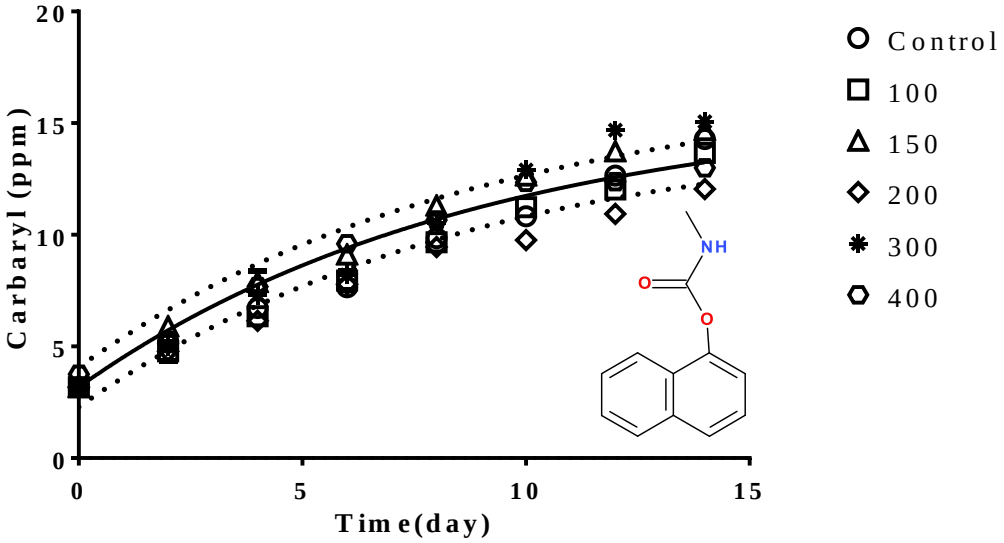

Figure 3-11 Carbaryl effect over *B. bassiana* radial growh expansion, cells grown in the dark on ¼ SDA with different concetration of pesticides (ppm); dotted line represents 95% CI from the control growth curve, error bars are ±SD of n=3.

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Discussion

A period of growth induction on a hydrocarbon enriched medium markedly enhances the fungal ability for hexadecane consumption. The aim of adaptation efforts was to enhance the oxidative capacity of *B. bassiana* by gradual exposure to hydrocarbons. Upon initial adaptations, the growth from non adapted cells is due in part to residual glucose and carbon sources presented inside the cell and autolysis, which are common responses in filamentous fungi during nutrition deprivation[59]. Differences in the appearance of cells in adapted and non-adapted cultures (clusters of non-adapted versus dispersed adapted cells) are common among cells that had a prolonged exposure to an alternative carbon source, such as hexadecane, since their metabolism has been optimized for its steady consumption[59, 148] while cells first exposed to hexadecane take longer to consume or degrade it. However, cells under alternative carbon sources, entered a nutrient stress state where mycelia shrank and formed cell shear sensitive cell bundles named gosammers, so a lot more data variability is observed[146]. Usually, degradation rates are faster than consumption rates in order to avoid cellular damage once the toxicity threshold is surpassed, which was observed in our results by the longer phase and abrupt hexadecane reduction. In *B. bassiana,* most of the hydrocarbons are incorpored into the cell membrane and used as energy source as established by Crespo[41, 149] and Juarez.[41]

B. bassiana's exposed to hexadecane and broad range of imidacloprid and carbaryl concentrations were not detrimental for growth. We report the presence of a longer ‘‘lag time’’ in non adapted cells which corresponds to the switch from growth on exogenous carbon to re-growth on endogenous carbon[59, 148] and alternative carbon source (Figure 3-7). While starvation induced changes may be detrimental in some processes, for example decreasing second metabolite production[150] a series of recent studies has shown that periodic carbon strarvation can lower process' costs. During fed batch fermentation, carbon deprivation can reduce the size of fungal mycelia which leads to lower power

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input and higher productivity due to the reduction in viscosity and the higher rate of mass transfer[148, 151, 152].

Biomass growth is an important parameter that is not reported by authors in biocatlysis and organic chemistry journals, reason for which it is not easy to make comparison among studies and a lot of product and conversion variability are reported. Radial growth rate of filamentous fungi on solid media has been shown to relate directly to dry weight[153, 154]. This method of growth assessment was preferred to dry weight determination because the latter does not account for the substantial quantities of stored carbohydrates that may have been present in some treatments during this study [153 154]. The dry weight of such storage material does not represent structural growth. It was surprising that even though the concentrations of carbaryl were higher than imidacloprid the overall effect on radial growth dispersion were higher than the control groups. This variation may be in part due to the metabolic and genetic effects new generation pesticides such as imidacloprid have over persistent pests and other hosts in comparison to reversible competitive inhibition from more widely used pesticide such as carbaryl[155]. In addition the wider application of carbaryl[134], the third most used pesticide brings a higher exposure to it for soil fauna and entomopathogenic fungi, which develops a higher tolerance to it and/or metabolize it faster. The cells remained metabolically active under stress as observed by the lower inhibition in radial dispersion (Figure 3-9) and the apparent increase in cell growth with phenothaizine (Figure 3-8). *B. bassiana* was able to grow in presence of phenothiazine. Fungal growth was often higher than in culture without substrate. Similar variations of higher growth rate and biomass development had been reported with cyclohexanone and botanicals [7]. Further analyses are necessary to understand the metabolic effect played by phenothiazine and different metabolites in fungal growth. The increase in co-metabolism during phenothiazine and insecticide tolerance will permit to have cells with a higher content of oxidases and/or hydrolases;

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ideally, enzymes whose broad substrate specificity catalyze the conversion of thioethers into sulfoxides. Entomopathogenic fungi play an important natural role in controlling insect pests, but few have been successfully commercialized due to low virulence and sensitivity to environmental stresses that produce inconsistent results upon applications[46, 156] . The use of fungi in integrated pest management (IPM) when used in combination with selective chemical provide satisfactory control against many agricultural insect pests without affecting fungal growth[157]. This outcome of insecticides on the growth of fungi can be different due to the chemical nature of products and the fungal species that are interacting with it. Therefore, improved pest management techniques where small amount of insecticide are applied in junction with the *B. bassiana* spores had been studied by several groups [52, 156, 158, 159]. The relatively less toxic effect of imidacloprid and carbryl to mycelial growth and spore production (P = 0.05) had been reported in *Metharhizium anisopliae*, *B.bassiana*, and other entomopathogenic fungi [134, 158]. Salem et al[2] observed that imidacloprid had minimal effects over maximum radial growth of *M. anisopliae* with values of r = 25.81±0.21 and r = 24.81±0.61 respectively when compared with control r = 29.50±0.59 . In addition the high in vitro toxicity of insecticides will not always be the same in the more diluted industrial application setting. The synergism between *B. bassiana* and insecticides has also been observed with other synthetic pyr otenoid[6] and herbal oils[7,133]which opens additional substrates that can be assayed as suitable oxidative inducers.

Researchers have shown that imidacloprid[50],[136, 160, 161] and carbaryl[134]enhance the effectiveness of fungi in killing a variety of insect species. It is postulated that these additives not only impact the insect population but also have an impact on the entomopathogenic fungi[51, 161]. Neonicotinoids, in particular imidacloprid, are combined with certain fungicides or other agrochemicals that block detoxifying enzymes (i.e.,

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cytochrome P450, monooxygenases, FMN); their toxicity increases by factor from 1.52 to 1141 depending on the combination [160].

Conclusions

These results demonstrate that *B. bassiana* can grow in the presence of carbaryl, imidacloprid and phenothiazine. Phenothiazine had an apparent inductive effect over cellular growth as well as the higher concentration of insecticides. Imidacloprid had a higher inhibitory effect than carbaryl. Under the current assay condition, the effect of insecticides and phenothiazine are not significantly limiting for catalysis. The next chapter covers *B.bassiana* phenothiazine oxidation under different fermentation conditions and the use of inducers to improve conversion and oxidative capacity.

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CHAPTER IV

CATALYTIC PERFORMANCE: ENHANCING *B.BASSIANA'S* SULFOXIDATION CONVERSION

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Abstract

The ability of filamentous fungi to grow on rather simple and inexpensive substrates as well as their capacity to produce a wide range of valuable metabolites has been exploited for decades in biotechnology. Since fungi subsist by absorbing nutrients, they possess the ability to rapidly adapt their metabolism to different suboptimal growth conditions, including nutrient deprivation, the presence of xenobiotics and antifungal compounds. Fungi express a number of specific detoxifying enzymes, which degrade compounds in ways that eliminate the effect of toxic compounds or unfavorable conditions. However, to get chemical diversity expressed in filamentous fungi, sources rich in amino acids, vitamins, trace metals, cofactors, and inducers have to be added. Although, there is no consensus on which media are optimal for selective oxidation of commercially relevant compounds, a series of botanicals, pesticides, and hydrocarbons have been shown to enhance the oxidative capacity of filamentous fungi. A biocatalytic system was optimized to increase the conversion of phenothiazine under different fermentation conditions. The highest conversion, 74±1 % at a rate of $0.16\pm1.7x10^{-2}$ h^{-1}, was achieved at the lowest cell concentration, 0.78 mg cdw /mL. The consistent data from these current experiments indicates that reduction of phenothiazine sulfoxide into phenothiazine has a lesser impact on the conversion in the more diluted cells, 74±1 %, in comparison to more concentrated cells, 62%±2. The effect different glucose concentration had over conversion, 34-39%, wasn't statistically significant. In addition, the use of insecticides as inducers was the most effective way to increase phenothiazine

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conversion from 47% to 64±3%. The success of this project helps us expand the oxidation capacity of *B. bassiana* as a fungal biocatalyst while improving its utility for industrial purposes.

Introduction

Economic, reliable, and controllable bioprocesses for filamentous fungi are of utmost importance for the large scale production of a wide range of products including food additives, bulk chemicals, organic acids, enzymes, and antibiotics. Therefore, bioprocess engineers seek to double the productivity and to reduce bioprocess development time to 50%, summarized as the two overall goals "two-times 50%" [162]. In order to meet these requirements, it is necessary to understand and control the biological system used; hence, elevating the importance for strain improvement and development.

The use of improved microbes is not new; it has been practiced for centuries in the preparation of fermented foods and beverages where specialized strains are used. The expansion and improvement of the commercial application of microbial strains has been exploited for decades by culturing specific microbes with unique hereditary qualities. The advent of industrial mycology and mycology in the 19th century made possible the application of pure cultures and strain development[162]. Afterwards with the advancements in biochemistry, engineering, and molecular biology techniques, the development of microbial and cell cultures expanded and led to more targeted approaches for the production of valuable compounds, proteins, and biopharmaceuticals[163]. Further improvements in bioreactor design, liquid fermentation, product recovery and process control played an important role to shape today's large-scale production of pharmaceutical and fermentation products[162]. The manipulation and improvement of microbial strains in order to enhance their metabolic capabilities for biotechnological applications is referred to as strain improvement. Classical strain development has typically relied on naturally or chemically induced mutations and random screening of

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improved strains. This empirical approach is best exemplified by the improvements achieved in penicillin production titers of 50g/L at least a 4,000–fold over the original parent and also fungal cultures capable of over-producing metabolites in quantities as high as 80 g/L[164]. One part of this continued interest in strain improvement has been the union of classical techniques, molecular genetics, and biochemical engineering to create a synergistic effect on strain and catalytic improvements. Fermentation processes have benefited from this combinatorial approach. However, to get chemical diversity expressed in filamentous fungi, sources rich in amino acids, vitamins, trace metals, cofactors, and inducers have to be added[165, 166].

Strain improvement

B. bassiana has been widely used in insect pest management programs. In considering the suitability of a fungal isolate for practical and commercial purposes the most relevant aspects to be improved are virulence, i.e. speed of kill[167]. *B. bassiana*, *M. anisopliae*, and *I. fumosoroseus* produced on media with low water activity or with a high glycerol concentration were more virulent than conidia produced on a rich medium without stress[153, 168]. Osmotic pressure can exert a profound influence on cell growth and its metabolism; as seen in the osmophilic yeast strain *Yarrowia lipolytica* grown in glycerol and its increase in erythritol production[169].In addition, other studies demonstrate that carbon starvation, UV radiation, and osmotic stress during growth improved virulence of the resulting *M. anisopliae* and *B.bassiana* conidia[126, 170]. Different approaches have been employed to develop enhanced *B. bassiana* strains, including the recovery of mutants after UV-light irradiation, site directed mutagenesis, and engineered genetic constructs[110, 163, 164].

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Catalytic improvement

Protective groups, e.g. esters, silyl esters, and/or oxoazoline, have been used to increase product yields and product selectivity[25, 171] in microbial bio-oxidations. In case of sulfoxidations of chondrine with individual growing cutlures of *B. bassiana* or *Beauveria caledonica*, the yields were improved with the use of N-protecting groups and achieved yields higher than 90%[25]. Weber and coworkers investigated the use of chemical auxiliaries, termed docking/protecting (d/p) groups, mostly containing an aromatic moiety for improving the hydroxylation range by various monooxygenases and biocatalyst[24] (Figure A-3). They demonstrated that cyclic- and hetero-cyclic hydrocarbons were not hydroxylated or did not undergo side reactions in the absence of the d/p groups, while the presence of d/p groups resulted in more selective hydroxylations[172]. Subsequently, it was learned that regio- and enantioselectivity can be altered by changing the functional groups of the auxiliary[173, 174]. The reaction of cyclopentanone, (R)-3-methylcyclohexanone, with (R)-2-amino-1-propanol and subsequent in situ benzoylation afforded the corresponding N-benzoylated oxazolidine derivative, which was diastereoselectively hydroxylated by *B. bassiana* with 60% yield, 99% diastereomeric excess[171]. The d/p application route distribution has been used successfully in other cyclic ketones, acyclic ketones, fatty acids, and terpenes. *B. bassiana* hydroxylated fluorinated alcohol derivatives using N-phenylcarbamoyl as the d/p group; racemic 2-fluorocyclohexanols gave 15% yield of (S, S)-hydroxylation products, whereas racemic 2-fluorocycloheptanols gave 41.5% yield (S, S) [171, 175].

The use of decoy molecules, such as branched carboxylic acids both with and without a chiral center, provides a simple and quick way for increasing the substrate pool of biocatalyst without the need for mutagenesis[173]. Sulfoxidation of thioanisole was catalyzed in the presence of various n- and branched- carboxylic acids and generally R-sulfoxide with no over-oxidation to the sulfone was seen[26, 173]. Interestingly, methyl aryl

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substitution position had an effect over stereo selectivity of thioanisole sulfoxidation to favor the S enantiomer (11 % ee) when p-substituted methylphenylacetic acid (MPAA) was used while m- and o-substitutions maintained R-stereoselectivity.

Gradual exposure of fungal cells to complex carbohydrates, metal cofactors, alcohols, or hydrocarbons have positive effects over the catalysis of steroids and heterocyclic compounds[27, 176]. Polyphenol precursors, gallic acid and tannic acid, have shown an inductive effect over the oxidative activity in ectomycorrhizal and wood decaying fungi[177]. A large diversity of substrates is recognized, particularly by xenobiotic-metabolizing enzymes, many of these substrates are suboptimal and/or have multiple binding modes. These substrate promiscuity are possibly due to an increase in dissociation rates, which are detrimental for substrate conversion. However, oxidase selectivity and substrate conversion can be improved using chemical auxiliaries, such as fatty acids, Schiff's base, and/or alkoxides[171, 173, 178-180] in two ways. The first approach uses decoy molecules to control the site of oxidation by blocking a part of the active site, while the second strategy applies substrate engineering to control oxidation by the enzymes[173].

Although there is no consensus on which media are optimal for selective oxidation of commercially relevant compounds, a series of botanicals, pesticides, and hydrocarbons have been shown to enhance the oxidative capacity of filamentous fungi. *B. bassiana* exhibited enhanced virulence towards persistent pests when grown in the presence of hexadecane as sole carbon source[12]. In addition, *B. bassiana* exposed to a sub-lethal concentration of imidacloprid and carbaryl has shown a synergistic effect in virulence enhancement although its impact in catalysis has not been explored[50, 134]. We have examined pest management strategies, designed to enhance the biopesticide efficacy, to induce and improve oxidations. In previous works, botanicals and hydrocarbons were found to be effective inducers of hydroxylase activity; tomatidine and

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Primula veris root extract in *Rhizopus nigricans* [176] and hexadadecane in *B. bassiana* [27]. The following chapter presents different processing strategies to increase substrate conversion in *B. bassiana,* using inducers and different fermentation conditions.

Materials and Methods

Growth and spore harvest

B. bassiana, ATCC 7159 was grown on potato dextrose agar (PDA) slants were incubated at 26°C for 10-15 days, and aerial conidia were harvested. *B.bassiana* conidia were harvested by flooding PDA slants with sterile detergent solution, 0.02% Tween 80. The suspended conidia were transferred to a 250 mL baffled flask with 50 mL of Sabouraud Emmons Broth (SDB), which served as the seeding stock.

Preparation of media

B. bassiana was grown on SDB. The medium was composed of glucose (20 g/L), yeast extract (5 g/L), and peptone (10 g/L). The pH was adjusted to 7 with HCl or NaOH. The biotransformation was done in a medium comprised of phosphate buffer amended with glucose as a carbon source. The medium included the following components: KH_2PO_4 (5.2 g/L), K_2HPO_4 (10.7 g/L), and glucose (5 g/L) for 0.5% (w/v) glucose potassium phospate buffer solution (GPPB). The adaptation was done in a minimum salt medium comprised of 10% (v/v) hexadecane as a carbon source. The medium included the following components: KH_2PO_4 (0.4 g/L), Na_2HPO_4 (1.4 g/L), $MgSO_4.7H_2O$ (0.6 g/L), KCl (1 g/L), and NH_4NO_3 (0.7 g/L), and $FeSO_4$ x $7H_2O$ (0.06 g/L) . The pH was adjusted to 7 with either HCl or NaOH. Prior to inoculation the media were sterilized by autoclaving (121 °C, 15 psi) for 20 minutes.

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Adaptation process

A 10% inoculum of *B. bassiana* cells grown in a 250 mL Erlenmeryer flask with 50 mL SDB were trasnfered to 250 mL baffled flasks with 50 mL MM supplemented with 10 % (v/v) hexadecane. These cells were grown in presence of n-C_{16} for 15 days at 180 rpm and 26°C. After 15 days, a 10% v/v inoculum from this culture was used to inoculate a new flask with MM. This process was repeated for 80 cycles. In the case of insecticides, *B. bassiana* cells were grown in 1 L baffled flask with 150 mL SDB insecticide mixture with 5 ppm each of imidacloprid (Merit 75 WP) and carbaryl (Sevin SL). The insecticide adapted cells were grown for 5 days at 180 rpm and 26°C. The insecticides for the study were manufactured by Baeyer AgroScience (Monheim am Rhein, Germany). All other reagents were obtained from Sigma Aldrich. Refer to Chapter III for more details.

Phenothiazine biotransformation with growing cells

For growing cell conversion, the seeding stock of *B. bassiana* was incubated for 72 h at 26°C and 180 rpm. To a 1 L baffled flask, 150 mL of SDB was added along with a 10% inoculum (15 mL of seeding stock). The culture was grown for 72 h at 26° C and 180 rpm. After 3 days, 1.5 mL of a phenothiazine solution in acetone (50 mg/mL) was added into the flask. The reaction was monitored by thin layer chromatography. To evaluate the effect of glucose on the conversion, 1 mL of 72 h cells was harvested and suspended into 10 mL SDB with different glucose concentrations 5-40 g/L. After addition of cells, 100 µL of a phenothiazine solution (5 mg) in acetone (50 mg/mL) was added into each reaction mixture. The reaction was run at 26° C and 180 rpm for 7 days.

Phenothiazine biotransformation with resting cells

For resting cell conversions, 72-h-grown cells were harvested and suspended in different volumes of GPPB to obtain cell concentrations (0.78-6.2 mg cdw/mL). The

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dilutions were prepared as serial dilutions (1-, 2-, 4- and 8-fold) of the original cell stock. A 10 mL cell suspension was washed and was dried at 65°C for 24 h for cell dry weight determination. To evaluate the effect of inducers, a 150 mL suspension of adapted cells, and non-adapted cells were washed and adjusted accordingly to 1.5 mg cdw/mL with GPPB. After the concentration was adjusted, 100 µL of a phenothiazine solution (5 mg) in acetone (50 mg/mL) was added to each 10 mL cell suspension. The reactions were run at 26° C and 180 rpm for 7 days.

Extraction and characterization

The cell broth was centrifuged at 1,952 G for 20 min. The supernatant was extracted with three volumes of 2 mL ethyl acetate. The cell pellet was washed with two volumes of 1 mL ethyl acetate and incorporated into the total organic phase. The organic phase was dried over anhydrous Na_2SO_4 and vacuum distilled into a brown solid. The reaction extracts were dissolved in dichloromethane for GC-MS phenothiazine conversion analysis. Samples (1µL) were injected into a SPB-5ms (60m x 0.25mmID, 0.25 µm film thickness). Analyses were performed in a Thermo Voyager single quadrupole mass spectrometer interfaced with a Trace2000 GC, equipped with an AS3000 auto sampler. Full scans were performed from 50-600 amu. Conversion was monitored via single ion monitoring (SIM) scans at [M+.] 199 with m/z reference peaks [M+ . – 32] 167 and [M+. – 45] 154 (Figure B-2). Carbazole was used as internal standard. Data were processed using Xcalibur 1.4 software. Ionization was performed using electron ionization (EI) with electron energy at 70 eV. The temperature ramp was 100-250°C at 20°C/min ramp and 250°C for 10 min[78, 109]. The injector and interface were at 250°C.

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Statistical analysis

The analyses were run in triplicates and were repeated twice for reproducibility. Evaluations were done using GraphPad Prism 6 (GraphPad Software, La Jolla CA). The threshold for statistical significance was set at $P \leq 0.05$. The data were tested by one way analysis of variance (ANOVA); whenever a significant effect was indicated, a Dunnett multiple comparisons test was carried out. Substrate conversion was plotted against time and the rate was determined after curve-fitting. Substrate concentration was calculated from an external standard calibration curve. Time course experiments involved sampling three replicates at 4 distinct time points, at intervals of 48 h, after the addition of phenothiazine. All data were expressed as mean ± standard deviation. Refer to Chapter III for more details.

Results

Resting and growing whole-cells were employed as catalysts for the biotransformation of phenothiazine. The following are the results of efforts to increase the catalytic performance of *B. bassiana's* sulfoxidation.

Effects of cell density on phenothiazine conversion

Whole-cell sulfoxidation reactions were performed with different cell concentrations in triplicate experiments for each concentration. Values of the cell concentrations tested ranged from 0.78 to 6.2 mg cdw /mL. The highest conversion, 74±1 % at a rate of $0.16 \pm 1.7 \times 10^{-2}$ h^{-1}, was achieved at the lowest cell concentration, 0.78 mg cdw /mL (Table 4-1). This value was demonstrated to be statistically distinct from the conversion experiments at other concentrations by the Dunnett multiple comparisons test.

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Table 4-1 Kinetic parameters derived from biotransformation of phenothiazine with different cell concentrations in glucose buffer solution after 7 days, assayed in 10 mL glucose buffer solution at 26°C and 180 rpm.

Variable	0.78 mg/mL	1.55 mg/mL #	3.1 mg/mL	6.2 mg/mL
kobs(h^{-1})	0.16±0.02	0.11±0.09	0.12±0.055	0.08±0.03
Plateau (ppm)	1.3±0.02	2±0.05	2±0.12	2±0.16
Max Conversion	74%±1*	64%±1	62%±2	60%±7
R^2	0.99	0.99	0.98	0.97
*p<0.05 significant difference, ±SD, # control group				

Higher concentrations, 1.5-6.2 mg cdw /mL, suffered slight variations that were not statistically significant throughout the 7 days (Figure 4-1).

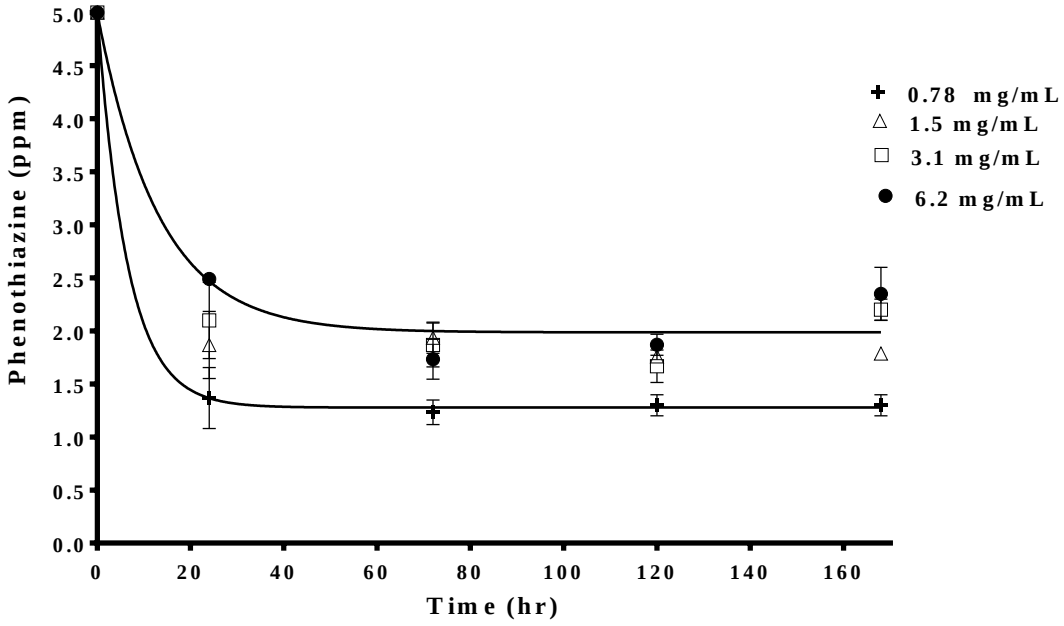

Figure 4-1 Phenothiazine time course reaction with different cell concentrations in GPPB at 26°C and 180 rpm, fitted to a one phase –exponential decay; bars represent standard deviations, n=3

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Glucose effects on phenothiazine conversion

The effect of initial glucose concentration, 5 – 40 g/L, was compared to the control group, 20 g/L. Glucose concentration effects on phenothiazine conversion were not seen over the time course of the experiments despite the impact that glucose levels have on cellular growth, affecting bioenergetics (Table 4-2). Based on the experimental results from this present study, the oxidase expression involved in phenothiazine conversions, 34-39% , arenot as sensitive to glucose fluctuations throughout the 7 days of reaction (Figure 4-2).

Table 4-2 Kinetic parameters of the biotransformation of phenothiazine after 7 days reaction in 70mL cell culture tubes with 10 mL SDB with different glucose concentration run at 26°C and 180 rpm.

Variable	5 g/L	10g/L	20 g/L#	30 g/L	40 g/L
kobs(h^{-1})	0.083±0.02	0.052±0.001	0.07±0.02	0.06E-02±0.01	0.06±.0.008
Plateau (ppm)	1.6±0.09	1.6±0.01	1.6±0.13	1.5±0.14	1.6±0.08
Max Conversion	36%	34%	38%	39%	37%
R^2	0.99	0.99	0.98	0.98	0.99
No statistical significance, P<0.05 ±SD, # control group					

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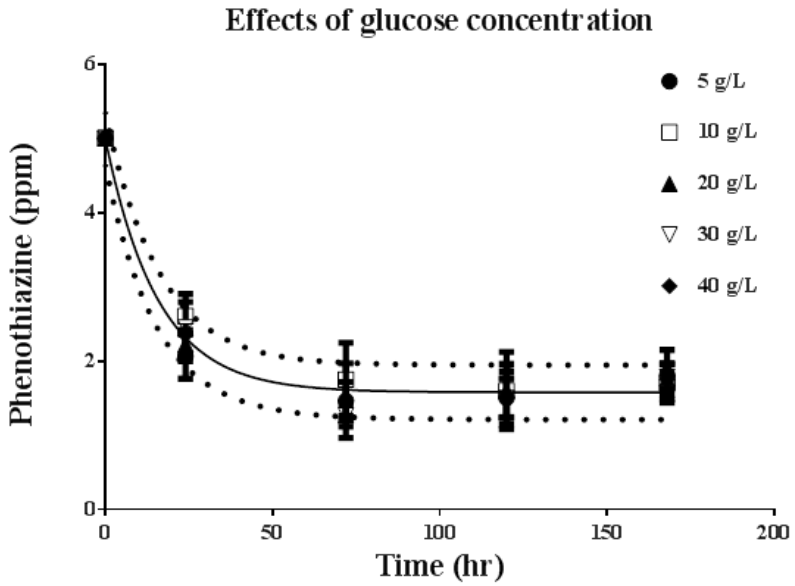

Figure 4-2 Effects of glucose concentration on the conversion of phenothiazine time course reaction plot fitted to a one phase exponential decay , dotted lines $p<0.05$ confidence intervals. Reaction ran for 7 days at 26°C and 180 rpm in 70 mL cell culture tubes with 10 mL SDB with different glucose concentrations, error bars represent ±SD of n=3

Effects of inducers in phenothiazine biotransformation

Whole-cell sulfoxidation reactions were performed with 1.5 mg cdw/mL in triplicate experiments. The highest conversion, 64±3%, was achieved with insecticide adapted cells (Figure 4-3). This value was statistically significant for the conversion experiments without inducers but not for the hexadecane adapted cells. Based on the experimental results from this present study, the oxidase expression involved in phenothiazine conversions is sensitive to inducers.

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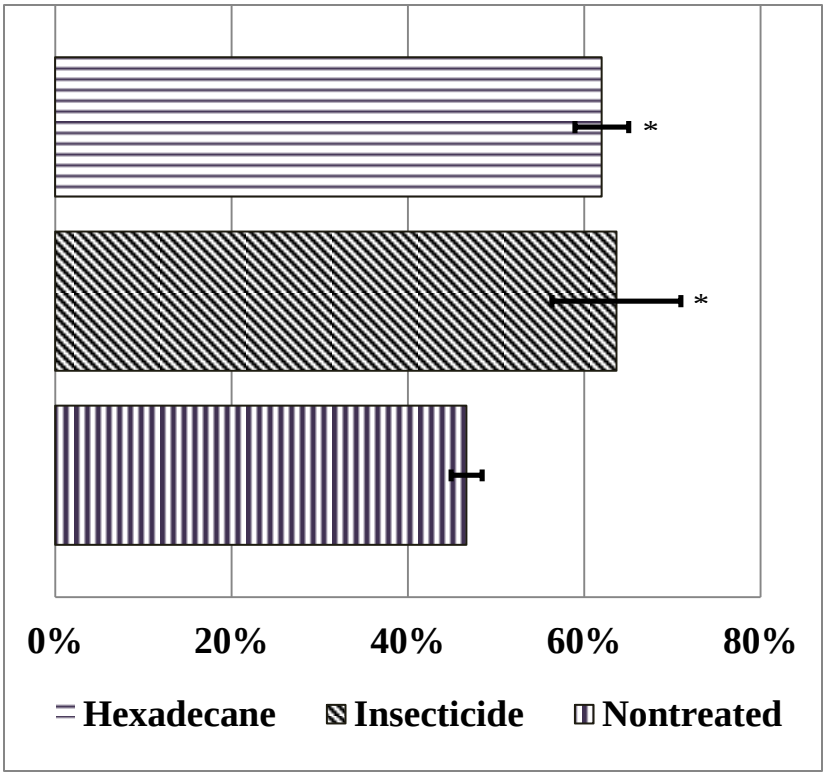

Figure 4-3 Effect of inducers over the conversion of phenothiazine with *B. bassiana* ,1.5 mgcdw/mL , after 7 days, the reactions were run in 70 ml cell culture tubes with 10 mL GPPB at 26°C and 180 rpm *statistical significant different from nontreated assay by Dunnett multiple comparison test ; bars represent standard deviations of 3 replicates.

Discussion

Growing cells are considered more favorable than resting-cells when expressing a protein with low stability, since they permit sustained protein expression during biotransformation. However, resting-cells have the advantage that the desired reaction can be investigated independently of growth phenomena and at higher cell densities. Furthermore, biotransformation conditions can be chosen independently from growth conditions minimizing side reactions and allowing identification of potential limitations[181]. The limitations in *B. bassiana* catalysis were physical variations that affected broth's rheology and overall mass transfer rather than biochemical limitations.

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The oxidation of phenothiazine had higher conversions (Figure 4-1) with resting cells in comparison to growing cells (Figure 4-2). The variations in conversions on resting cells highlighted the importance fungal morphology and biomass played in the overall bioprocess optimization rather than bioenergetics, glucose concentrations. Therefore, the next step in process improvement will target bioprocess control parameter. One alternative that has been successful for the control of pellet morphology in filamentous fungi are fluidized bed bioreactors with pulsing flow[182]. The pulsing flow helps control pellet morphology which improves rheology and enhances catalysis. For instance, in the case of citric acid strains of *A. niger*, the pellet diameter corresponding to a 0.35 s^{-1} pulsed system was kept between 3.3 ± 0.1 mm while in the nonpulsed bioreactor pellets were produced with a diameter of 6.7 ± 0.3 mm[182].

Since sulfoxides are metabolic intermediates which can enter different reactions, variation in phenothiazine conversion is common; the phenothiazine can be either further oxidized to alternative metabolites or reduced to the parent molecule[82]. One possible explanation in the low cell density phenomena, is the differential regulation of enzyme activities in response to increased cell population[86, 183].This might be verified through evaluation of proteomics or gene expression, but was beyond the scope of the current study. The consistent data from these current experiments indicates that reduction of phenothiazine sulfoxide into phenothiazine has a lesser impact on the conversion in the more diluted cells, 74±1 %, in comparison to more concentrated cells, 62%±2. In addition to potential metabolic phenomena at higher cell densities, physical variations, diffusion, and viscosity play a larger role in conversion sometimes being factors which impose greater limitations than low catalytic power or low enzymatic expression. As cell density gets higher, oxygen mass transfer limitation become a greater problem for catalysis. To harness the reducing power of NADH and $FADH_2$ into energy, the cells convert the reducing equivalents to energy (ATP) in the presence of O_2 by cellular

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respiration. Since tighter mycelium bundles make substrate and O_2 permeability harder to get to all the available biocatalyst, lower conversion and overall efficiencies are reported at higher cell densities[58, 59, 147]. In addition oxidation of substrates can have a large impact on central metabolism if it requires significant reducing equivalents (NADH and NADPH), and free energy equivalents (ATP). Previous studies on penicillin G production in a high-producing industrial strain of *P. chrysogenum* have shown that constraints in central metabolism may reside in the supply and regeneration of the cofactor NADPH rather than in the supply of the carbon precursors[184].These results further demonstrate the value of exploring the concentration space experimentally rather than assuming higher cell density will result in higher conversion.

Fungi have been reported to turn off a large number of genes in the presence of glucose as an energy saving response. This phenomenon primarily affects catabolic enzymes without affecting other cellular functions, such as xenobiotic metabolism or virulence of *B. bassiana* to aphids[185, 186]. Based on the experimental results from this present study, the oxidase expression involved in phenothiazine conversions is not as sensitive to glucose fluctuations. Quantitative effects of glucose in fungal biotransformation had been demonstrated during the conversion of meloxicam[187] and cyproheptadine hydrochloride[188] and with alternative carbon sources in the conversion of ferulic acid[189] and albendazole[190]. An increase in reversible reactions lowers phenothiazine conversion at higher glucose concentrations. More detailed experiments are required to assay the effects isolated enzymes have over conversion.

The carbon source and nitrogen source are two important factors affecting cell growth and product formation in microorganisms. In the biotransformation of modafinic acid, different nitrogen sources, i.e., yeast extract, peptone, soy meal, and ammoniun nitrate, were assayed and no significant differences in conversion and yields were observed among the screened nitrogen sources. Carbon and nitrogen sources may have

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either repressing or inducing effects on enzyme productions. It was reported that the production of extracellular digestive enzymes by *Aspergillus japonicus* could vary by 100 times on different carbon sources, i.e., corn starch, maltose, fructose, glucose, galactose, sorbitol, and sucrose, the latter of which was found to be the best for β-fructofuranosidase production[191]. Metabolic enzymes such as glucoamylase, amylase, and glucosidase are all induced by starch and repressed by glucose[186]. Also, most extracellular proteases are repressed under the conditions of high glucose and ammonium levels in the medium[37]. For example, the extracellular proteases of *Aspergillus nidulan*s are subjected to carbon, nitrogen and sulfur metabolite repression[192]. The depletion of low molecular weight sources of nitrogen, carbon, phosphorus, and sulfur elevates protease activity[183, 193].

Chemical inducers affect fungal biotransformation to different degrees. These inducers serve as co-substrates that increase oxidative capacity or mutagens that improve strain's catalytic efficiency. Previous studies have shown nearly a 10-fold increase in steroid hydroxylase activity in *R. nigricans* with selective induction by rich *Primula veris* root extract[176], and a 5-fold increase in *B. bassiana* with selective induction by hexadecane[27]. Strain improvements by chemical mutagenesis with colchicine and ethyl methanesulfonate have shown a 2- and 8-fold increase in conversion of α-pinene to verbenol in *Aspergillus* sp.[146]. The inductive effects of pesticides in fungal biotransformation had been demonstrated in the conversion of progesterone[176] and dehydroepiandrosterone[27]. Fungal sulfoxadtion of phenothiazine was improved using insecticides and hexadecane as oxidative inducers. The used of of insecticides or hexadecane as inducers increased the conversion of phenothiazine from 47% to 64% (Figure 4-3). The practice of using pro-substrates, such as lignin, ethanol, polyaromatics or bezopyrene have been effective to increase the expression of oxidases and enzymes necessary for the oxidation of xenobiotics and/or alternative carbon sources[86, 194-197]. In addition, it enables the optimization and improvement of wild type fungal catalysis and

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the development of greener oxidation methods for the conversion of pro-drugs and organosulfur compounds. Furthermore, nutrition limitation was explored to improve the conversion of phenothiazine. It was expected that fluctuation in glucose came with variations in phenothiazine conversion, as specific growth is a function of glucose concentrations and correlates to the overall energy levels[186](Table 4-2) . However, these fluctuations did not affect catalysis, although the effect over fungal growth, in particular specific growth rate, hyphal vacuolation and diameter needs to be explored further[57, 148, 151]. In an ideally mixed bioreactor, all dispersed hyphae and fungal cells experience identical environmental conditions and temporal profiles can be monitored and controlled by process parameters. Many evolutionary acquired traits contributing to the natural fungal life style such as hyphal growth, secretion of hydrolases, cometabolism up- & down-regulation, cell death and conidiation are dispensable during industrial processes and might affect production yields [198-200].

Conclusion

This chapter highlights the strategies to enhance *B. bassiana* catalytic performance. The fluctuations in glucose concentrations did not have a significant effect on conversion while lower cell densities resulted in a significant increase in conversion from 62% to 74±1 %. Also hexadecane and insecticides had a positive inductive effect on phenothiazine conversion in comparison to non-induced cells. The ability to use inducers to increase phenothiazine conversion in *B. bassiana* provides further support for the use of biocatalysts to develop sustainable industrial practices. In the next chapter we take a deeper look into *B. bassiana's* oxidative induction and the effect inducers have on CYP450 content is taken.

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CHAPTER V

OXIDASE CAPACITY: ELUCIDATING WHICH PROTEINS ARE INVOLVED ON *B.BASSIANA'S* INDUCTION

Abstract

Fungal species are a very important source of many different enzymes. Currently, fungal oxidations may not be suitable for industry due to their low yields and product selectivity, although they are used for drug metabolite transformations and studies of eukaryotic xenobiotic metabolism. The major enzymes involved in catalysis of xenobiotics are heme-binding monooxygenases, in particular cytochrome P450 (CYP450) enzymes. In this chapter, heme positive proteins were identified by an SDS benzidine assay and the content of CYP450 by the carbon monoxide difference spectrum. The CYP450 content was 12.3±1 pmol/µg protein for hexadecane-adapted cells and 8.1± 1 pmol/µg protein for insecticide-adapted cell. The heme-positive proteins were further characterized by MALDI-ToF, and their peptide mass fingerprint compared to the available protein sequences in UniProtKB. *B. bassiana* was under oxidative stress under the assayed conditions as shown by the expression of thioredoxin and catalase. Hemoproteins were also found, including a cluster of catalase-peroxidase, alkane hydroxylase, and chloroperoxidase. These results show our progress to better understand and expand *B. bassiana's* catalytic performance.

Introduction

Fungi are used as cell factories to produce antibiotics, enzymes, secondary metabolites and to oxidize prodrugs and fine chemicals for industrial and/or synthetic uses. In the environment, fungi are the major decomposers of biomass and play an essential role in biogeochemical cycling. They possess a robust metabolism which is highly adaptable and inducible. In nature, several fungi such as entomopathogenic

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Sordariomycetes contain hydroxylases and hydrolases that help them degrade insect's epicuticle and are responsible for the natural control of persistent pests[201]. With the advent of genomics and proteomics studies, fungal xenobiotic metabolism has slowly been demystified from a black box into a series of induced and regulated oxidation and conjugation enzymatic reactions. Moreover, now that half of the 1000 genomes in the Fungal Genomes Project (www.1000.fungalgenome.org) are completed, it is possible to identify the specific enzymes involve in catabolism and xenobiotic metabolism pathways[202].

Oxidoreductases

Oxidoreductases comprise a class of enzymes that has received much attention due to the ability of these enzymes to catalyze the transfer of electrons among different substrates in redox reactions. They need a cofactor, usually NADPH or $NADP^+$, which activates the enzyme. Oxidoreductases play a major role in anabolism and catabolism. They can be found in glycolysis, the TCA cycle, oxidative phosphorylation, and in fatty acid, amino acids, and secondary metabolites metabolism[203]. The industrial application of isolated redox enzymes is very low mainly because some of the most promising ones have only been discovered, isolated, and/or expressed[5, 203, 204]. Therefore, most of the reactions and studies are done with whole cells which allow cofactor regeneration and better protein stability. There are 6 major subclasses of oxidoreductases (Figure 5-1) which vary by their reaction products, coenzyme requirements, and the nature of the reducing/oxidizing pairs[205], they are oxidases, dehydrogenases, peroxidases, hydroxylases, oxygenase and reductases.

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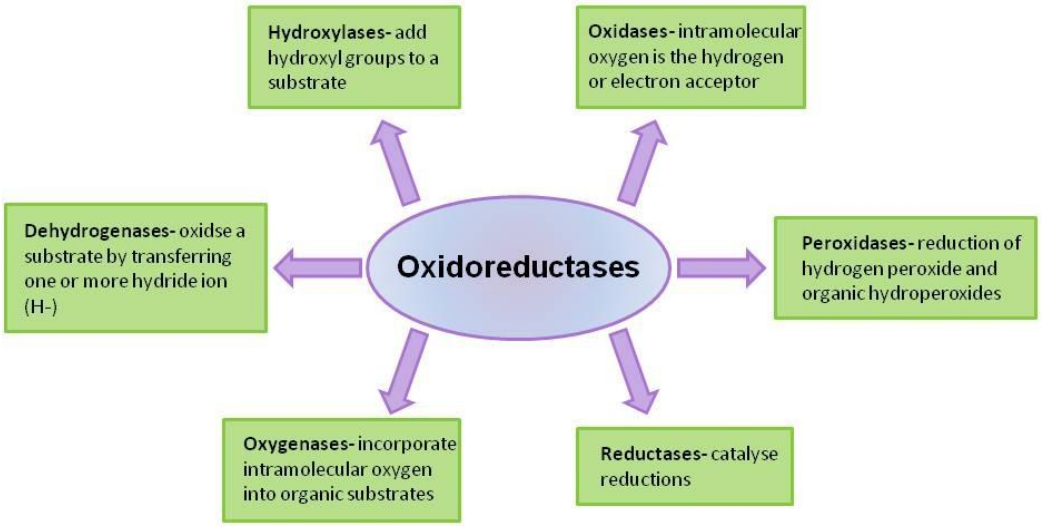

Figure 5-1 Oxidoreductases sub-classes

Fungal oxidases

Xenobiotics are generally hydrophobic compounds that are toxic or therapeutic to organisms. From the microbial model, *Cunninghamella elegans*, is now known that the oxidation and subsequent elimination of xenobiotics occurs through reactions mediated by cytochrome P450, epoxide hydrolase, glutathione S-transferase, and UDP-glucuronosyltransferase[115, 206]. In contrast, few published data are available pertaining to the characterization, isolation or function of these enzymes in higher order fungi[62, 176, 207, 208]. Cytochrome P450s have been detected in several fungi. Fungal CYPs are major induced in the biotransformation of aromatic and aliphatic homo- and heterocompounds[12, 53, 62, 209]. They catalyze regio- and stereospecific introduction of atomic oxygen into lipophilic compounds to increase their hydrophilicity[2]. CYP450s utilize O_2 and two electrons supplied by NAD(P)H, mediated by redox partner

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flavoproteins and iron-sulfur proteins, to catalyze the oxygenation of numerous substrates[33, 34]. The overall equation for the reaction adheres to the following formula ,where A is the substrate:

$$A + O_2 + NAD(P)H + H^+ + 2e^- \xrightarrow{CYP450\ monooxygenase} AO + H_2O + NAD(P)^+$$

Cytochrome P450s (CYP450s) are heme-thiolate monooxygenases found in eukaryotic and prokaryotic organisms. Fungal CYP450s are associated with the endoplasmic reticulum on the microsomal fraction of cells[34, 36, 210], and a single reductase drives all the CYPs, cytochrome b_5, as in humans. Fungal CYPs are involved in primary and secondary metabolism as well as xenobiotic detoxification and/or degradation[2,35]. In recent years, 276 putative CYP families[36] have been identified from 6,000 fungal genes of 113 species by fungal genome sequencing projects[37], and often these fungal P450s are first members of new families[12] with unknown catalytic function. These leave great opportunities to explore novel enzymes. Evidence of P450's occurrence in filamentous fungi is limited because of difficulties handling mycelia, low levels of these enzymes[36], and poor understanding of their regulation[12]. The fungal CYP450 database possesses at least 40 CYP450 sequences. Pedrini et al. found that n-alkane solvents induced the gene expression of 8 oxidative cytochrome P450 enzymes (CYP) in *B. bassiana*[12]. They identified the genes that codified for the eight cytochromes and successfully cloned them into *E.coli*. These induced CYP450 are involved in hydrocarbon catabolism[41] and are highly regulated by the chain length of the hydrocarbon where they grew[12 123]. Crespo, Juarez and Pedrini et al[42, 123] identified them as alkane monoxygenases a type of cytochrome P450 which are essential for the assimilation of hydrocarbons in *B. bassiana.* Alkane monooxygenases are involved in the terminal and subterminal hydroxylation of n-alkane, which is necessary for the complete alkane assimilation via the β-oxidation pathway[41, 211].

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The only cluster of fungal enzymes that had been thoroughly studied and exploited for decades due to their selectivity as well as their industrial and medical value are the ones involving steroid transformation. Steroid transforming enzymes have been extracted, purified and expressed in *Rhizopus sp*[62, 204, 212, 213], *P.chrysosporium*[38, 209,209, 214], and *Aspergillus sp.*[207, 208, 215, 216] Some of the purified enzymes are involved in the ergosterol biosynthetic pathway, which includes 14α-demethylase, C-24 methyltransferase, C-22 desaturase, and C-24 reductase[64, 217]. In addition, steroid-transforming enzymes with a higher commercial value had been isolated, including different hydroxylases (7 α-, 11 α-,11β-, 14 α-hydroxylase), oxidoreductases (5α-reductase, 3β- hydroxysteroid dehydrogenase/isomerase, 17β-hydroxysteroid dehydrogenase, C-1/C-2 dehydrogenase) and C-17–C-20 lyase[63, 64]. Reports of chiral sulfoxidation by microbial P450 are surprisingly rare. The major findings are from bacterial enzymes since the enzyme have not been isolated and/or characterized in fungi. Recently Lin and coworkers have shown that the sulfoxidation activity of the strain *Rhodoccus* is attributed to a fused CYP related to P450-Rhf, which possed a high ee up to 99% for (S)-sulfoxides[26]. The widely studied P450cam from *P. putida* catalyzed the oxidation of thioanisole to yield an (R) : (S) ratio of 72:28 demonstrated experimentally and modelling by Ortiz de Montellano[26, 90]. Also other oxidases have shown sulfoxidation activity including monooxygenease and diooxygenases from the bacteria *Rhodocoeccus eryhthripolis*[26, 194] and desaturases from *Chlorella vulgaris*[77]. In the case of desaturases, oxidation takes place as long as the compound's sulfur atom is at the site where the dehydrogenation reaction of fatty acyl analogue is initiated[77].

Protein identification by Mass Spectroscopy

Today Mass Spectroscopy (MS) is a central technique for protein analysis in research and clinical diagnostics. MS is the basic technique for global proteomic analysis due to its accuracy, resolution, and sensitivity (in the femtomole to attomole

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concentration range), and due to the fact that is has the capacity for a high throughput screening[218]. Not only does MS allow one to profile a proteome, but more importantly, it allows one to identify the protein species and characterize post-translational modifications and interactions. This started with the invention of ionization techniques compatible with proteins and peptides[219]. Two different techniques are available to create an ion source that can transform proteins and peptides into gas phase ions: matrix assisted laser desorption ionization (MALDI) and electron spray ionization (ESI)[220, 221]. By combining an ion source with mass analyzers of different kinds (time of flight (ToF), ion trap, quadrupole, and ion cyclotron or hybrid combinations) the molecular species present are separated in gas phase and detected with accurate mass values (Figure 5-2) [222, 223]. Some of these mass analyzers (tandem mass spectrometers, or MS/MS) are also capable of selecting specific ions and initiating structure specific fragmentation followed by mass measurements of the resulting ions[219]. Proteins are identified from mass spectra of intact proteins (top-down proteomics), or peptide fragments obtained after enzymatic or chemical treatment (bottom-up proteomics). Currently, bottom-up approaches are most common for detailed analysis. Proteins are digested with sequence-specific proteases, commonly trypsin, to generate amino acid sequences that are then analyzed to build up information about the original proteins present[42]. Three main modes of MS based on this approach can be defined:

1. Protein Identification of gel bands/spots

Gel bands or spots of interest are excised from the gel, in-gel digested and made compatible with the selected ionization technique. Normally, identification is performed using the process of peptide mass fingerprinting (PMF) on MALDI-ToF instruments[220, 224]. In PMF, a list of peptide masses are compared to theoretical lists generated from sequence databases. Based on the degree of similarity, protein identities are assigned with different degrees of probability. This approach is only valid for pure proteins, and the

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more stringent process of using sequence-specific fragmentation data is increasingly replacing PMF for identification, for example using MALDI-ToF/ToF[223].

2. Global LC-MS/MS

Protein mixtures are digested and fed to tandem mass spectrometers after separation of the peptide mixtures using nano-LC (generally reverse phase chromatography (RPC) or a combination of ion exchange chromatography and RPC). Protein identification is enabled by acquiring sequence-specific fragmentation data from the eluted peptides[225]. Mass spectrometers with fast scan rates are used to enable interrogation of as many peptides as possible at each point in the chromatography. Generally, samples are labeled, either at the protein or peptide level, with isotope-code tags, and depending on the technique used, two or more samples are merged before analysis[39]. By comparing peak areas (MS or MS/MS level) for peptides that are identical except for their different tags, relative quantitation data can be extracted[42].

3. Targeted LC-MS/MS

In contrast to the global approach, targeted LC-MS/MS analysis is tailored for a defined set of proteins. For each targeted protein, one or a few tryptic peptides are selected as quantitation probes. Optimally, probes should be present in only one protein in the assayed sample (proteotypic) and have favorable analytical properties[39]. To achieve absolute quantitation, an internal standardization approach is used; stable isotope-labeled analogs of the proteotypic peptides are spiked into the samples in defined amounts before LC-MS/MS analysis[222, 224]. Spiked samples are separated in similar ways as in global approaches, but mass spectrometers can be programmed to focus on the defined proteotypic peptides only. Instrument types (triple quadrupole) capable of selected reaction monitoring are especially suitable in this approach. Signals from specific

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fragmentation pairs are monitored, whereby quantitation accuracy, sensitivity, and dynamic range can be improved compared with global approaches[219].

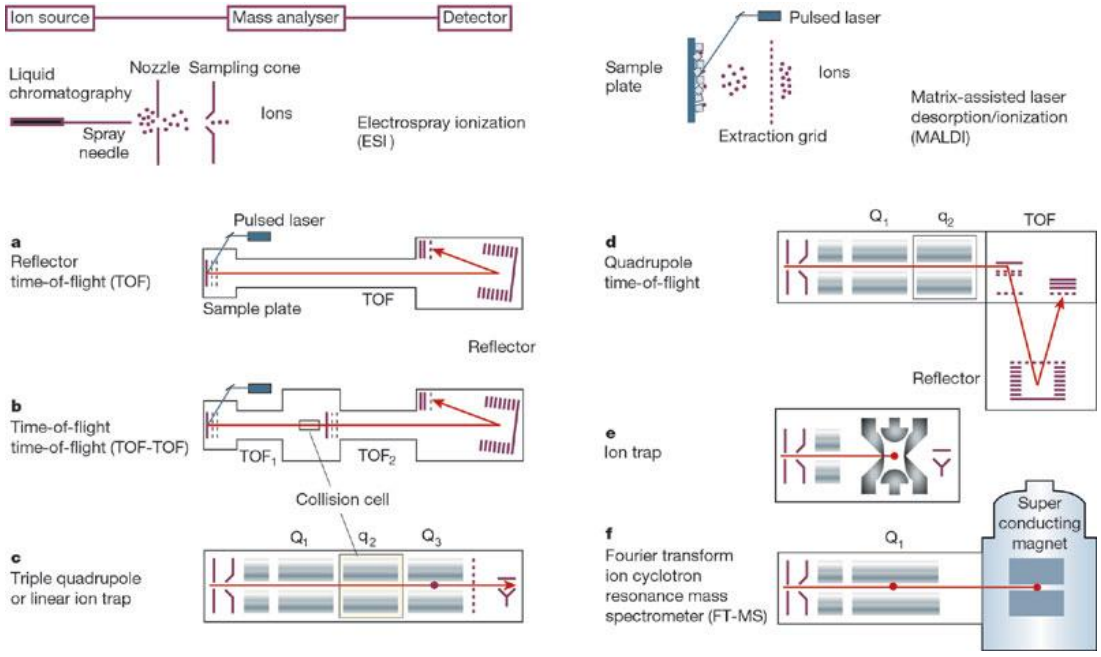

Figure 5-2 Mass Spectrometers used for protein analysis. The most common MS spectrometers used for protein research are TOF and ion trap[223].

As high-performance mass spectrometers continue to evolve, top down approaches to protein analysis in complex samples are starting to emerge[223]. Full length proteins are introduced and their structures deduced by complex fragmentation reactions in the gas phase. However, sensitivity is substantially lower compared to bottom-up approaches, and large proteins or truly complex mixtures cannot currently be handled[223]. Fungal proteomics research has experienced great advances over the last years, because of the availability of powerful proteomics technologies and the increasing number of fungal genome sequencing projects[218, 220, 226]. Currently, more than 50 pathogenic fungal genomes have been sequenced. The goal of this chapter is to determine the effect of insecticides and hexadecane on *B. bassiana's* protein and CYP450 content. The

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underlying hypothesis is that hexadecane and insecticide will induced the expression of CYP450s.

Materials and Methods

Screening strain

B. bassiana, ATCC 7159, was purchased from the American Type Culture Collection (Manassas, VA). It was grown on potato dextrose agar (PDA) slants which were incubated at 26°C for 10-15 days and stored at 4 ◦C. Seven days before fermentation, 6ml of sterile distilled water mixed with 0.01% (v/v) Tween 80 was added on a slope. Aerial conidia were harvested from the slopes by cautiously scraping of the slope's surface with a wire loop. Subsequently, 1 mL of this spore suspension was transferred into 250 mL baffles flask with 50 mL SDB. These seeding stock flasks were incubated for 7 days at 26 ◦C and 180 rpm. After the end of the incubation period, 10 mL of cell suspensions were used to inoculate the fermentation assay flask, Stage II.

Preparation of media

Fungi were grown on Sabouraud Dextrose Emmons Broth (SDB). The medium was composed of glucose (20 g/L), yeast extract (5 g/L), and peptone (10 g/L). The pH was adjusted to 7 with HCl or NaOH. The biotransformation was done in a medium comprised of phosphate buffer amended with glucose as a carbon source. The medium included the following components: KH_2PO_4 (5.2 g/L), K_2HPO_4 (10.7 g/L), and glucose (5 g/L) for 0.5% (w/v) glucose potassium phospate buffer solution (GPPB).The adaptation was done in a minimum salt medium comprised of 10%(v/v) hexadecane as a carbon source. The minimum salt medium (MM) included the following components: KH_2PO_4 (0.4 g/L), Na_2HPO_4 (1.4 g/L), $MgSO_4.7H_2O$ (0.6 g/L), KCl (1 g/L), NH_4NO_3. $7H_2O$ (0.7 g/L), and $FeSO_4.H_2O$ (0.06 g/L). The pH was adjusted to 7 with either HCl or

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NaOH. Prior to inoculation the media were sterilized by autoclaving (121 °C, 15 psi) for 20 minutes.

Cytochrome P450 (CYP450) content was analyzed in 100 mM potassium phosphate buffer pH 7.4 (PPB) with 1mM EDTA, 20% glycerol (v/v), 0.5 % sodium cholate (w/v), and 0.4% Triton N-100.

Adaptation conditions

A 10% inoculum of *B. bassiana* cells grown in 250 mL Erlenmeryer falsk with 50 mL SDB were inoculated into 250 mL baffled falsks with 50 mL MM amended with 10 % (v/v) hexadecane. These cells were grown in presence of n-C_{16} for 15 days at 180 rpm and 26°C. After 15 days, a 10% v/v inoculum from this culture was used to inoculate a new flask with MM. This process was repeated for 80 cycles. In the case of insecticides, *B. bassiana* cells were grown in 1 L baffled flask with 150 mL SDB insecticide mixture with 5 ppm each of imidacloprid (Merit 75 WP) and carbaryl (Sevin SL). The insecticide adapted cells were grown for 5 days at 180 rpm and 26°C. The insecticides for the study were manufactured by Baeyer AgroScience (Monheim am Rhein, Germany). All other reagents were obtained from Sigma Aldrich.

Protein extraction and identification

Filamentous fungi have an exceptionally robust cell wall, consisting largely of chitin, which makes up the majority of the cell mass[227]. Because of its rigidity, cell lysis is an important element in fungal proteomics. This type of fungi can be considered, similarly to plants, recalcitrant biological material, so the preparation of protein samples is a critical step. To overcome this challenge, cell lysis was performed with freeze dry cells using sonication, because these methods are more efficient than those based on chemical or enzyme extraction[227]. No universal set of protocols exists due to the large chemical/physical fungal heterogeneity of both proteins and sample sources. For protein

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extraction the cell broth was centrifuged at 10,000 rpm for 25 min at 3°C. The supernatant was discharged and the cell pellet was washed twice with 25 mL of PPB. The washed pellet was resuspended in 15 mL of PPB and was lyophilized. The freeze dried pellet was pulverized and the powder, 100 mg, was transferred into a 15 mL centrifuge tube. Fractionation was performed following the instruction provided in the ReadyPrepTM Protein Extraction Kit, Membrane 1 (BioRad, Hercules, CA, USA). The kit is based on temperature-dependent phase partitioning using Triton X-114[228]. In brief, samples are mixed in the membrane protein extraction buffers with 10µL of 1,000 mg/mL protease inhibitors cocktail. The samplers were sonicated 30s with 1 min rest intervals on ice 3 times. The homogenates are incubated at 37°C for 30 min, centrifuged at 13,000 rpm for 5 min, producing an upper aqueous phase and a low detergent-rich phase. Proteins are partitioned into an aqueous phase enriched in cytoplasmic proteins, a detergent rich phase enriched in membrane and transmembrane proteins, and an insoluble pellet enriched in more complex membrane proteins. The detergent fractions were furtherly analyzed for CYP450 content.

Protein concentration was measured with the BioRad RC/DC assay; using bovine serum albumin as standard. Proteins in the mixtures were first separated by electrophoresis using 1-D PAGE. 1-DE is a simple, reliable technique for finger-printing crude extracts, and it is especially useful in the case of hydrophobic and low-molecular-weight proteins[221]. Non reduced protein isolates, 25 µg, were run in 4-10% MOPS PAGE at 150 V for 60 min. The gels were rinsed with deionized water for 5 min and stained with GelCode™ Blue Stain Reagent (Thermo Scientific, Waltham, MA, USA) or 100 mL 6mM tetramethylbenzidine in 250 mM sodium acetate solution, respectively. The heme stain samples were incubated in the dark for 2 hrs followed by the addition of 500 µL of 30% hydrogen peroxide. The stain was visible as a blue band within 5 min. Background was removed with (3:7) isoproponal: 0.25M sodium acetate[229, 230]. After the

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staining of proteins, the selected bands were cut out and digested with trypsin. Tryptic peptides were analyzed in the University of Iowa Proteomics facility. PMF and combined (+MS/MS) searches were performed in the SwissProtUniProtKB database of proteins using the MASCOT algorithm. Protein species were identified by comparison of the experimental spectra with the theoretical ones obtained *in silico* from protein, genomic, or MS spectra public databases[222]. All sequences were processed in an open license software Scaffold Version 4. Tandem mass spectra were extracted by UI Proteomic Facility Personal. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search the SwissProt_2015_03 database (547964 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 0.50 Da. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification. Scaffold (version Scaffold_4.5.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm[231] with Scaffold delta-mass correction and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were annotated with GO terms[232] from NCBI (downloaded Mar 5, 2016). The sequence homology were tested against the NCBI protein database and classified according to gene ontology (GO) annotations for metabolic function and cellular components.

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Quantification of cytochrome P450

Cytochrome P450 content and activity were measured on the detergent and pellet fractions. CYP450 was detected by the carbon monoxide difference spectra in a BioMate 5 double bean UV/Vis spectrophotomer (Thermo Scientific, Waltham, MA, USA) as described by Omura and Sato[233-235] with minimal modifications. These assays were carried out in the absorbance range of 0.05–1.0 in order to have sufficient absorbance to measure accurately and remained on the operating range of the instruments. Difference spectra are useful for determining total CYP450 content, examining substrate interaction with P450, and investigating mechanism of inhibition. The method measures all of the P450s in a particular biological sample.. The protein samples were diluted with PPB to a concentration of 1,500 µg/mL. Freshly prepared 0.5M KCN, pH 7.7, was added to the samples, 10µl/ ml per isolate, to mask the spectral interference of cytochrome oxidase (negative absorption at 445 nm) with the CYP peak at 450 nm in the CO difference spectrum. The mixture was transferred into polystyrene cuvettes and a reference spectrum was recorded from 400 to 500 nm. The mixture was slowly bubbled with CO, 30-40 bubbles, into the cuvettes for about 1 minute and 1 mg of sodium dithionite was added and gently mixed to reduce CYP. The difference spectra were recorded between 400 to 500 nm until the 450 peak stopped increasing. A useful aspect is that the loss of activity of P450 enzymes is associated with a loss of the 450-nm spectrum and conversion to a form with a wavelength maximum at 420 nm[233, 234, 236]. Thus, the assays serve as a check on the integrity of the enzyme, which is useful in routine research activities. The P450 content is determined as follows:

$$[(A_{450-490})_{observed}\,] \,/\, \Delta\varepsilon_{450-490} = \mu M\ CYP450$$

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In addition, CYP 420, was estimated which consists of denatured forms of P450, using the following formulas:

$$(\mu M\ CYP450)x(\Delta\varepsilon_{420-450}) = (A_{420-490})_{theoretical}$$

$$[(A_{420-490})_{observed} - (A_{420-490})_{theoretical}]/_{0.110} = \mu M\ CYP420$$

The CYP concentration was calculated using molar extinction coefficients of ε_{450nm}=91 mM^{-1} cm^{-1} and $\varepsilon_{420\text{-}450nm}$ = - 41$mM^{-1}cm^{-1}$, respectively, and the measured absorbances (A) at the corresponding wavelengths of 490,450, and 420 nm.

Sample calculation for P450 spectral assay

Sample data is from the insecticide pellet fraction's CO difference spectrum. The absorbance is measured at 420 nm to estimate the content of denatured forms of P450. This particular sample exhibited a high absorbance at 420 nm which indicates the relevance of proper sample handling and preparation to avoid further degradation. Foremost, the high level of absorbance at 420 nm is an indication of how labile these enzymes are. One of the problems with this assay is that it does not readily distinguish between (cytochrome) P420, a conglomerate forms of P450 that have lost the characteristic thiol ligand (protein cysteine), and contaminating hemeproteins that often show spectral peaks at 420 nm. Notorious problems are seen with some heterologous expression vector systems showing low CYP450 expression levels[233, 234, 236].

Fraction	450 nm	420 nm	448 nm	490 nm
Insecticide pellet	0.345	0.28	0.283	0.234

$$[(A_{450-490})_{observed}]/\Delta\varepsilon_{450-490} = nmol\ P450\ /\ mL$$

$$0.345/0.091 = 3.79\mu M\ P450\ x\ 10\ (dilution\ factor) = 37.9\mu M\ P450$$

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$$(nmol\ P450/mL)x(\Delta\varepsilon_{420-450}) = Theoretica\ A_{420-490}$$

$$Theoretical\ A_{420-490} = 3.79x(-0.041) = -0.155$$

$$Observed\ A_{420-490} = -0.046$$

$$[Observed\ A_{420-490} - Theoretical\ A_{420-490}]\Big/_{0.110} = \mu M\ P420$$

$$(-0.046) - (-0.155) = 0.109$$

$$0.109\ /\ 0.110 = 0.99\mu M\ P420\ x\ 10 = 10\ \mu M\ of\ P420$$

$$\therefore\ total\ CYP\ content = \mu MP450 + \mu MP420 = 48\ \mu M\ CYP$$

$$37.8\ nmol\ mL\ /\ 1{,}500\ mL\ \mu g = 25.26\ pmol\ /\ \mu g\ protein$$

Also, the carbon monoxide difference spectrum was carried out with non- induced cells isolates and no cytochrome P450 was detected. Even the characteristic red color from the positive presence of CYP450 was not observed.

Thermal method for carbon monoxide production

Carbon monoxide was produced by dehydrating formic acid with concentrated sulfuric acid as shown below:

$$H_2SO_4(l) + HCOOH(l) \xrightarrow{\Delta} CO(g) + H_2SO_4{\cdot}H_2O(l)$$

Carbon monoxide is combustible and burns in air with a bluish flame, changing to carbon dioxide. Set CO system in the fume hood (Figure 5-3). Into a test tube, 10 mL of H_2SO_4 conc was added, followed by 20 mL of formic acid. The test tube was plugged with a perforated rubber stopper containing two bents. The plunger's rubber diaphragm was lubricated with silicone spray or oil. The bents were connected into two 50 mL syringes, one CO reservoir and the other one as waste. The test tube was heated with a small flame, alcohol burner. The entry to CO reservoir was clipped. 10 mL of gas was collected in the waste container and then clipped. Finally the desire amount of CO was collected or bubbled into the sample.

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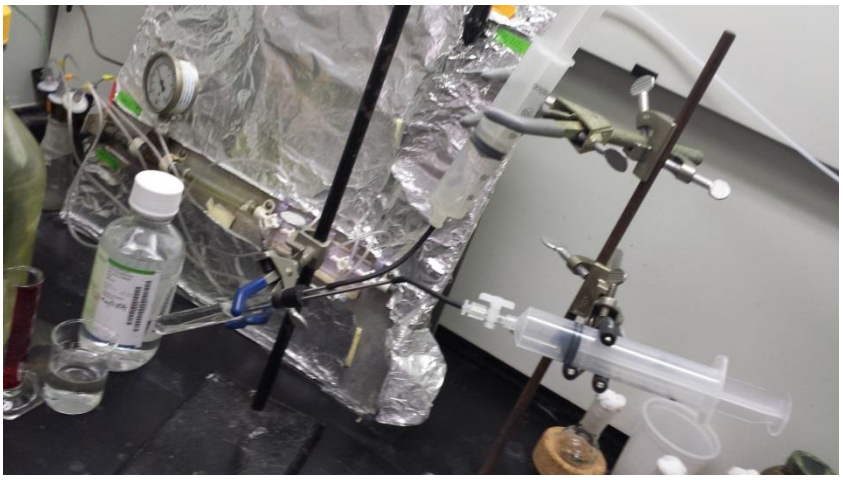

Figure 5-3 Carbon monoxide production by thermal dehydrogenation of formic acid with heated concentrate sulfuric acid for CO production

Proteomics Facility

The Facility, provides services and state-of-the-art instrumentation for discovery proteomics or more in-depth quantification studies. Services currently available in the facility include: molecular weight determination of intact proteins and macromolecules, small molecule structural and quantitative analyses, protein identification following proteolysis in PAGE gels or solution applying MASCOT, SEQUEST or SpectrumMill search engines as well as characterization of certain PTM. Equipment in the Facility includes a Bruker UltrafleXtreme MALDI TOF/TOF, a Thermo LTQ XL linear ion trap with electron transfer dissociation (ETD) capabilities and an atmospheric MALDI source, an Agilent 6520 quadropole time-of-flight (Q/TOF) with routine 10 ppm mass accuracy, an Agilent 6400 triple quadropole (3Q) for tandem MS/MS scanning, especially multiple reaction monitoring. The Agilent instruments are interfaced to ChipCube platforms driving by Agilent 1200 nano- and capillary HPLC pumps. Matched Dionex 3000 RSLC two dimensional capillary systems are interfaced to an Proteineer II robotic fraction collector (for off-line separation prior to MALDI MS/MS) and the LTQ. The matched LC

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for ESI and MALDI are used to build accurate mass and retention time libraries for tissue archives. Associated equipment includes a Dionex ICS 3000 for glycoproteins or chromatofocusing, an automated digester, electrophoresis units, an OffGel Fractionator and an Eksigent 2D nano LC to partition complex peptide mixtures using IEF or SCX.

Results

The results of the induction biotransformation from Chapter IV indicate that the oxidases involved in sulfoxidation are part of a highly inducible enzyme system, greatly dependent on the presence of xenobiotics. The fungal system was further characterized with respect to its peptide mass fingerprinting of selected bands as well as its CYP450 inducibility.

Hemoprotein identification

Two oxidase bands from the detergent phase protein fraction were detected in the 45-60 kDa range after incubation with benzidine and H_2O_2 (Figure 5-4). No heme-positive bands were observed in the aforementioned mass range in the aqueous phase fraction, so under the assay conditions no heme-binding monoxygenases were detected. The additional bands lower than 25kDa are dissociated heme groups. The variability observed in lane 2-4 was due to the origin of the sample. Lane 2 was the solubilized protein pellet which is composed of more complex membrane bound proteins while lane 3 and 4 are the proteins from the hydrophobic fraction.

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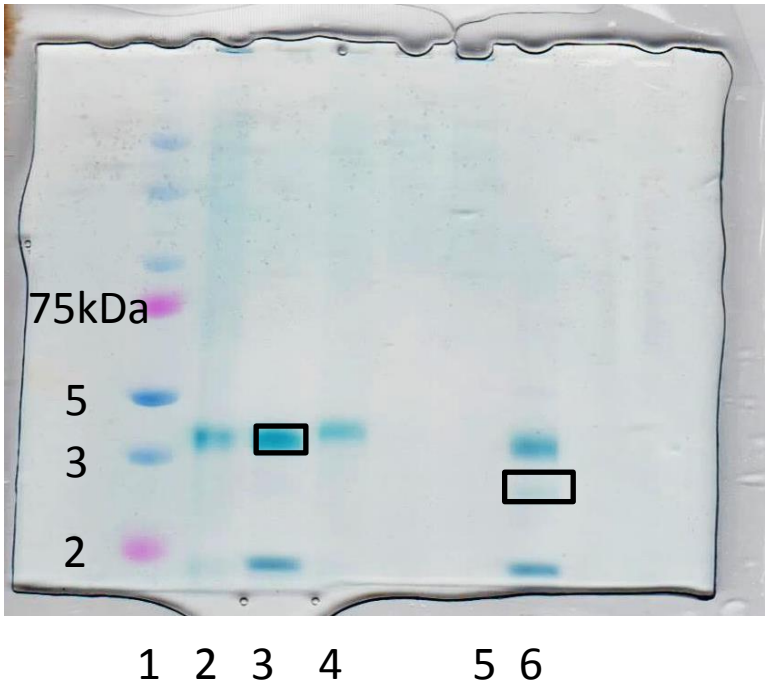

Figure 5-4 Benzoidine-SDS PAGE of hydrophobic protein fractions from hexadecane and insecticide adapted *B.bassiana* (seeded 25 µg of each unreduced samples) 1) Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards 2) C_{16} induced fraction pellet 3) C_{16} induced hydrophobic fraction, 4) C_{16} induced hydrophobic fraction-acetone precipited, 5)Non induced fraction, 6)Insecticide induced fraction; box are the excised band

The two selected bands were analyzed and identified by peptide mass fingerprinting MALDI-TOF MS/MS analysis. Proteins were classified according to NCBI GO annotation for metabolic function and cellular components (Figure 5-5). Through this approach, 31 proteins were identified from the two hemoprotein bands, B1 and B2, but after the removal of common contaminats, keratin and trypsin, 26 proteins remained. Proteins involved in metabolic functions such as catalytic activity, binding activity, and antioxidant activity were most abundant. Other classes of identified proteins include transporter activity and molecular transducer activity. When determining the sub-cellular localization of our identified proteins we observed 27% membrane or transmembrane proteins as well as 39% of cytoplasmic proteins which were carried over from the aqueous layer chaotropic fractionation process. Catalytic activity forms the largest class of proteins with known functions, 41% of the totally identified proteins.

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Among them, significant portions had antioxidant activity 6% and 4% heme binding capacity. Multiple enzymes involved in fatty acid metabolism were identified, including pantothenate synthase and acyltransferases. *B. bassiana* was under oxidative stress under the assayed conditions shown by the expression of thioredoxin and catalase. Hemoproteins were also found, including a cluster of catalase-peroxidase, alkane hydroxylase, and chloroperoxidase. In addition a sulfate binding protein was identified.

CYP450 content

The content of cytochrome P450 was quantified by the CO-binding difference spectroscopy to validate the inductive effect hexadecane and insecticides had in *B. bassiana's* CYP450. The CYP450 enzyme content was 12.3±1 pmol/µg protein for hexadecane adapted cells and 8.1± 1 pmol/µg protein for insecticides, respectively.

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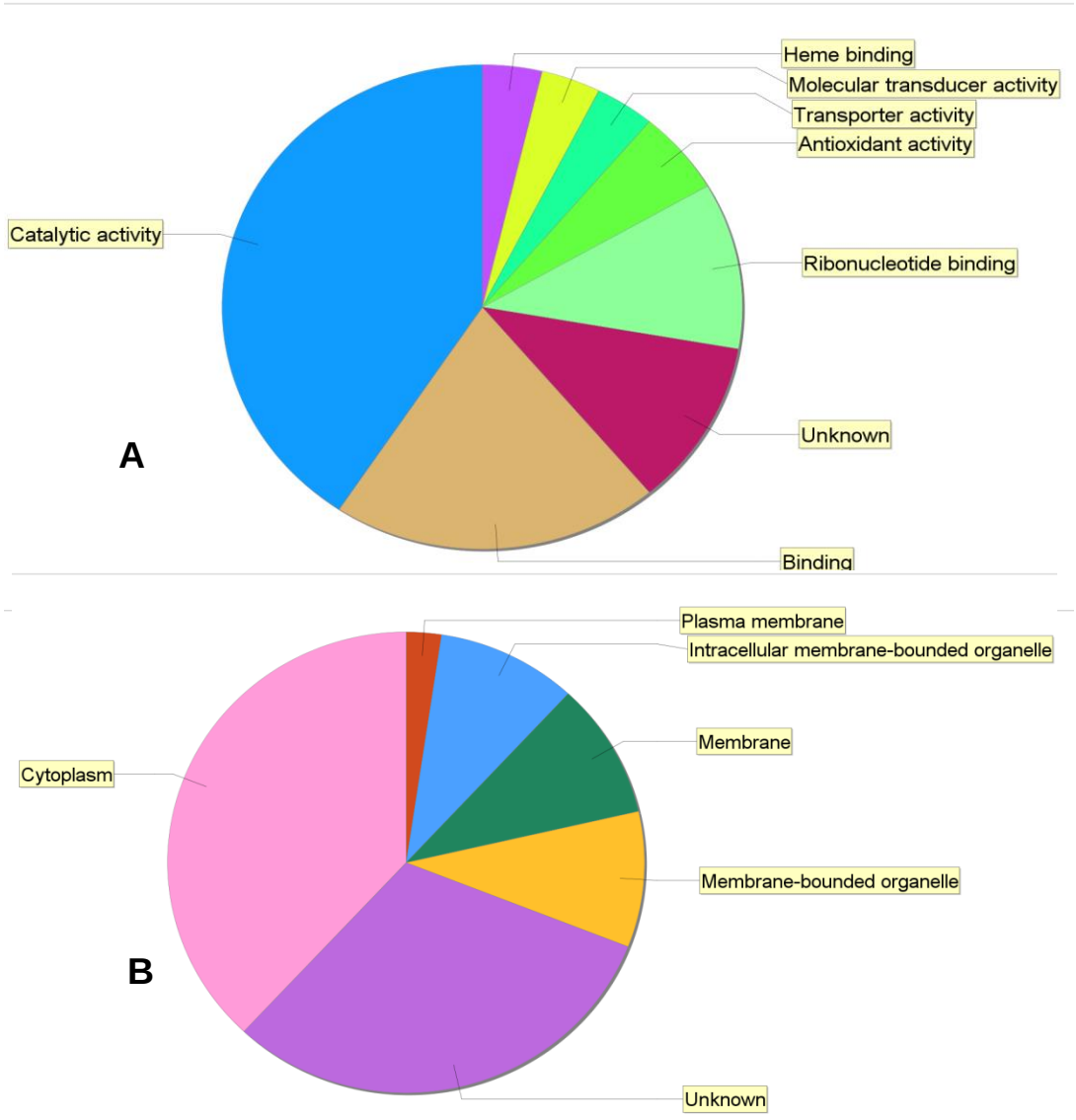

Figure 5-5 Functional categorization of proteins identified in the heme positive bands from adapted *B.bassiana's* hydrophobic protein fraction were classified on the basis of their metabolic functions (A) and cellular components (B).

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Table 5-1 Proteins identified in heme-positive bands by searching spectra against the the UniProtKB database. Peptides with a score less than 20 were filtered and proteins were identified having a significance threshold of $p < 0.05$ by the Peptide Prophet algorithm with Scaffold delta-mass correction. Italicized proteins are unique to only one heme-band (B1) when searched with Blastp and the UniProtKB database. Bold, italicized proteins are identified to bind heme when searched with Blastp against the UniProtKB database. The number identified for each cluster are in parenthesis. Underlined protein are shared by B1 and B2 heme positive bands, protein mass ranged from 31-64 kDa . The search criteria was kingdom Fungi, order Hypocreales.

Protein	Molecular Weight	Biological process	Accesion Number	Taxonomy	Total spectrum count	Percentage of total spectra	Percentage sequence coverage
HPr kinase/phosphorylase	35 kDa	regulation of carbohydrate metabolic process	A0KAL7	*Burkholderia cenocepacia HI2424*	5	0.05%	11.50%
Transaldolase	35 kDa	pentose-phosphate shunt	Q39E45	*Burkholderia lata*	7	0.06%	5.68%
Cluster of Malate dehydrogenase (2)	35 kDa	tricarboxylic acid cycle	B2T9P8	*Burkholderia mallei SAVP1*	85	0.79%	30.90%
			A2S105		133	1.23%	48.60%
Acetyl-CoA acetyltransferase	49 kDa	tricarboxylic acid cycle	A0AYH4	*Burkholderia cenocepacia HI2424*	16	0.16%	16.50%
Acetylglutamate kinase	32 kDa	arginine biosynthetic process	B1YPR7	*Burkholderia ambifaria MC40-6*	18	0.17%	21.10%
Argininosuccinate synthase	48 kDa	arginine biosynthetic process	A1JID7	*Yersinia enterocolitica subsp. enterocolitica 8081*	8	0.08%	14.60%

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Table 5-1 continued

2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	29 kDa	lysine biosynthetic process	A0K8F4	*Burkholderia cenocepacia HI2424*	45	0.42%	25.50%
4-hydroxy-tetrahydrodipicolinate synthase	32 kDa	lysine biosynthetic process	Q21WN2	*Rhodoferax ferrireducens T118*	17	0.16%	7.74%
2-dehydro-3-deoxyphosphooctonate aldolase	31 kDa	ketodeoxyoctanoate biosynthetic process	A0K8N2	*Burkholderia cenocepacia HI2424*	7	0.06%	21.10%
Lipid-A-disaccharide synthase	42 kDa	lipid A biosynthetic process	P14611	*Burkholderia cenocepacia HI2424*	7	0.07%	10.50%
Cluster of Pantothenate synthetase	31 kDa	pantothenate biosynthetic process	B1YUT9	*Burkholderia ambifaria MC40-6*	6	0.06%	12.20%
Cluster of 3-oxoacyl-[acyl-carrier-protein] synthase 3 (2)	35 kDa	fatty acid biosynthetic process	A0K5U6	*Burkholderia cenocepacia HI2424*	43	0.40%	23.70%
D-alanine--D-alanine ligase	33 kDa	peptidoglycan biosynthetic process	B4E6J0	*Burkholderia cenocepacia HI2424*	52	0.48%	28.80%
Cluster of catalase-peroxidase (2)	80 kDa	hydrogen peroxide catabolic process	A9AGE5	*Burkholderia multivorans ATCC 17616*	21	0.21%	6.04%
	79 kDa		Q39JF1		16	0.16%	4.67%
Chloroperoxidase	30 kDa	antibiotic biosynthetic process	P25026	*Burkholderia pyrrocinia*	95	0.88%	33.10%

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Table 5-1 continued

Elongation factor Ts	31 kDa	binding	A0K8E2	*Burkholderia cenocepacia HI2424*	340	3.14%	53.90%
Elongation factor Tu	43kDa	binding	A0K3L0	*Burkholderia cenocepacia HI2424*	18	0.17%	14.60%
Cluster of Protein TolB (3)	37 kDa	protein import	P02906	*Salmonella enterica subsp. enterica serovar Typhimurium str. LT2*	136	1.37%	28.10%
	46 kDa		A0K4X6	*Burkholderia cenocepacia HI2424*	181	1.82%	35.00%
sn-glycerol-3-phosphate-binding periplasmic protein UgpB	48 kDa	ABC transporter ATP binding	A1JID7	*Yersinia enterocolitica subsp. enterocolitica 8081*	7	0.07%	4.10%
Sulfate-binding protein	37 kDa	sulfate transport	P02906	*Salmonella typhimurium*	17	0.16%	5.47%

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Discussion

The enzymatic composition of protein isolates varies greatly depending on the exposure of cells to different xenobiotics. The benzidine method of identification of proteins containing heme is possible because hemeproteins retain their peroxidase activity after SDS-denaturation[230]. Heme is a porphyrin heterocyclic found in metalloenzymes. It is a prosthetic group that allows proteins, hemoproteins[230], to carry out functions of biological importance. The heme prosthetic group is as a source of electrons during electron transfer and redox reaction and diatomic gases binding site. Hemeproteins have diverse biological functions which include the transportation of gases, chemical catalysis, gas detection, and electron transfer. In the case of oxidases the heme group is involved in the catalytic ability[237]. The major inducible oxidases are hemeproteins, which enables this indirect in gel identification, to highlight active bands for further protein analysis. A mass range between 45-55 kDa was selected because the major fungal oxidases[37, 108] lie within this range.

B. bassiana was under oxidative stress under the assayed conditions shown by the expression of thioredoxin and catalase. A significant increment in the catalase activity of the peroxisomal fraction (12.6-fold) has been reported when *B. bassiana* is grown in insect-like hydrocarbon[41, 123, 149]. In addition, a peroxisomal and cytosolic catalase, 54.7 and 84.0 kDa per subunit, was identified and purified which lies within our selected mass range[24]. Hemoproteins were also found, including a cluster of catalase-peroxidase, alkane hydroxylase, and chloroperoxidase. In addition a sulfate binding protein was identified, which is involved in sulfur compound metabolism[183]. The identified catalase activity is indicative of *B. bassiana* ability to degrade hydrocarbons. In addition, the identification of catalases in our protein mixture is the link between the efforts of fungal strains development for insect control and the fungal biocatalyst improvement. The early up regulation of catalase plays an important role in the fungal-insect infection process[238], so

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it may mediate responses to oxidative and other stresses, as well as participate in the remediation of host toxic metabolites. Furthermore, it is a sign of enhanced fatty acid metabolism which in entomopathogenic fungi is assisted by CYP450[41, 211].

These results with *B. bassiana* are consistent with results for other fungi. The first step in β-oxidation of hydrocarbons involves CYP450 which comes with an increase in the inventory of organelles responsible for fatty acids catabolism and peroxidase-catalase activity[123]. Metabolism of some insecticides and toxic compounds is known to involve cytochrome P450-dependent monooxygenase enzymes. Fungal CYP450s are membrane bound hemoproteins that are induced in different degrees by xenobiotics. CYPs are involved in many essential cellular processes and play diverse roles in primary metabolism, degradation of xenobiotics, and biosynthesis of secondary metabolites. Multiple CYP450 isoforms are involved in catalysis. *B. bassiana* CYP450 levels are similar with observation on CYP activity increases in other fungi, *M. isabellina, Cunninghamella blakesleeana,* and *Rhizopus nigricans*[12, 53, 239]. Daliang achieved a 55 % increase in CYP450s, 34 to 62 pmol/μg of protein, in *Phanerochaete chrysosporium* by optimizing different benzoic acid concentrations and medium conditions in the induction of CYP450[209]. For instance, the addition of xenobiotics, cyclopentyl-1,3-benzo-xazole, or n-hexane into *Mortierella alpina* cultures resulted in induction of CYP450 although the hydroxylation of 2-cyclopentyl-1,3-benzo-xazole correlated with the content of substrate-induced P450 but not with the level of n-hexane-induced CYP450[53]. *R. nigricans* exposed to progesterone had a 2 - 4 fold increase in CYP450 content[240]. The higher concentration of CYP450 in hexadecane adapted cells relates to the major expression of oxidases required to start the catabolism of alkanes as shown by Pedrini[12] and Crespo[41, 123]. Fungi have been reported to turn on and off a large number of genes in the presence of alternative carbon sources and xenobiotics. This regulatory phenomenon primarily affects catabolic enzymes or detoxifying enzymes[185, 186]without affecting other cellular functions. *B. bassiana* genome encodes 83 CYP genes, which are involved in

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detoxification, degradation of xenobiotics, secondary metabolism, and biosynthesis[241]. Over-expression of the CYP450 enzymes has been associated with hexadecane exposure in *B.bassiana*[12] however, not much else is known about the enzymes themselves or the action of these detoxification enzymes in entomopathogenic fungal metabolism. *B. bassiana* grown in hexadecane as sole carbon source showed an enhancement on the conversion of dehydroepiandrosterone[27] from 54.6% to 72% as well as 250–350-fold induction levels in P450alk genes[12]. The selective induction of CYP450 has a transformative potential for the oxidation of valuable compounds. However, peptide mass fingerprinting by MALDI-TOF MS alone was insufficient for protein identification because of the lack of a genome database for *B. bassiana*. A wider range proteome analysis will undoubtedly be helpful to further understand *B. bassiana's* hydroxylation and sulfoxidation.

The amino acid sequences were compared to the reviewed proteome of the soil bacteris *Burkholderia sp.* since it was the one with the highest degree of similarity and convergence ($p<0.05$) with our digested protein mixture. Among the various bacterial species present in fungal affected soil habitats members of the genus *Burkholderia,* have been found to be very successful in colonizing the fungal surfaces[230]. It is not surprising to find some positive proteins from *Burkholderia* in our protein mixture. We couldn't find any similarities within the 580 reviewed proteins sequences from the Hypocreales order species. It also highlighted that at the moment the strains' of particular interest are plant pathogens such as *Trichoderma sp.* and *Fusarium sp.* rather than insect pathogens from the Clavicipitacea and Cordycipiticeae families. There are still 843,429 non reviewed proteins that haven't been fully annoted and registered into the UniProtKB database (Figure 5-6). So, there is still room for improvement to expand the database search in order to get a more torough analysis. In recent years, soil-colonizing fungi such as *B. bassiana* and associated bacteria have gained renewed attention as consortia that may increase the biodegradation potential of soil for recalcitrant pollutants[242, 243]. These

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opens opportunity to study mix culture bioreactors and their overall effect over degradation.

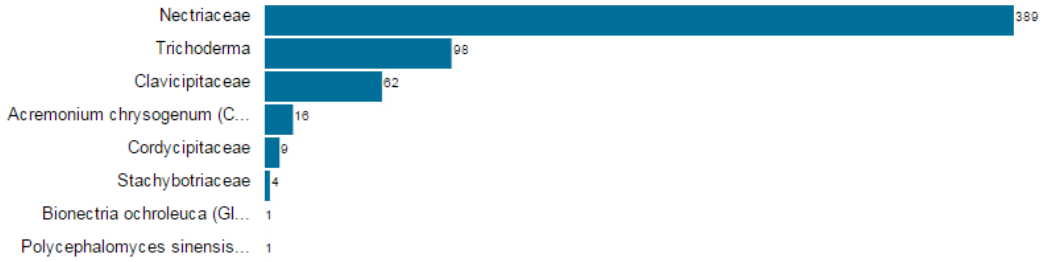

Figure 5-6 Reviewed protein sequences (580) registered in UniProtKB sorted by families from the Hypocreales order. The number are the reviewed proteins from each family. The major findings are from plant pathogens. There are few reviewed proteins from insect pathogens, Clavicipitacea and Cordycipiticeae.

Conclusion

B. bassiana is sensitive to the presence of inducers as was shown by the CYP450 content and presence of oxidative stress proteins and heme-binding proteins. These results demonstrate that CYP450s are induced during phenothiazine oxidation at different levels depending on the inducers. In addition, catalytic enzymes involved in metabolism and catalysis were identifined, including a cluster of catalase-peroxidase, alkane hydroxylase, and chloroperoxidase. The preliminary identification of proteins involve in sulfoxidation will help in the development of better industrial bio-catalyst and enable future expression of this enzyme in *B.bassiana* or host microbes.

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CHAPTER VI

FUTURE WORK EXPLORING MORE INTO *B. BASSIANA'S* INDUCTIVE CAPACITY

B. bassiana is a very robust catalyst that showed to be an effective oxidizer of sulfur compounds. The oxidation of phenothiazine with *B. bassiana* was studied under different fermentation conditions. The product distributions, together with data obtained from selective induction experiments suggested that *B. bassiana* contains a range of oxidases with different substrate specificities. The summarized results from this work helped us to validate our hypothesis that exposure of cells to insecticides and hydrocarbons increased cell oxidative capacity expressed as higher substrate conversion from 47% to 64±3% and CYP content up to 12.3 ±1 pmol CYP450/µg protein with hexadecane. The use of pesticide and hexadecane proved effective as CYP inducers. More importantly, these results suggest that this fungus has a promising future as biocatalyst in the production of drug metabolites. We successfully developed a more efficient process to oxidize organo sulfur compounds, using phenothiazine as our screen substrate. Moreover, the use of biocatalyst for the conversion of recalcitrant sulfur compounds makes these a more attractive approach for biodesulfurization and transitional displacement of metal catalyzed chiral sulfoxidation. The process modifications presented will help in addressing problems associated with production of valuable products using biotransformation.

The data from Chapter II and IV showed that *B.bassiana's* catalytic performance was enhanced using insecticides and hexadecane as oxidative inducers; validating their positive effects over organosulfur compound's metabolism. In addition, *B. bassiana* catalytic performance was impacted by cell density which opens an opportunity for further optimization as it involves a suitable way for maximum conversion and high product selectivity without the associated cost of the adaptation process and/or inducers.

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The CO difference spectrum collected from Chapter V validated that adapted cells had a higher content of CYP which made them better oxidizers than non-adapted growing cells. Furthermore, the protein identification and benzidine assay highlighted that hemeproteins in fact are involved to a certain degree in the oxidation of phenothiazine. Finally, *B. bassiana*'s remained catalytically active under the assayed conditions. In addition, the different pesticide concentration didn't have a significant effect over cell growth, so cells remain catabolically active under stress as presented in Chapter III. Surprisingly, imidacloprid had a more pronounced effect over cell growth than carbaryl did. Overall, phenothiazine and pesticide effects were more inductive than repressive on cellular growth. These apparent inductive growth effect needs to be further study to rule out experimental fluctuations and strain variabilities.

Some other work that could help us better understand the inductive effect of xenobiotics and hexadecane is to assay the induction fold of selected CYP genes, which will correlate to the protein expression of desirable oxidases that can catalyze sulfoxidations. We are in the brink of a major transition from the traditional retrosynthetic analytical approach into the design of total enzymatic assisted fine chemicals synthesis. However in order to achieve this, a lot more work needs to be done on strain improvement in particular expanding *B. bassiana*'s substrate specificities as well as product yields. While oxidases in particular CYP450 & FMO may be attractive biocatalysts because of their high substrate promiscuity, predicting the structures of their products remains challenging. This promiscuity paradox is an important obstacle hindering their use as biocatalysts. This can be done either by selected mutagenesis or less invasive techniques such as substrate engineering. In substrate engineering the attention is focused on improving the catalysis by better understanding the enzymatic process involved in the oxidation of your desirable compounds and locking the desirable configuration and chemistry in order to favor selective oxidation. This approach has been successful in the hydroxylation of recalcitrant substrates. It is clear that whole-cell

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oxidation, which is most often used, can be a disadvantage, since cells have a vast number of enzymes and the formation of by-products is very common. Thus, elucidation of primary structure, cloning and overexpression of the desired enzymes in the appropriate expressed vector would be a better approach, as this will provide higher yields and fewer by-products.

The following is a more detail approach about some future work that can help in our understanding of *B. bassiana* xenobiotic metabolism and make a more attractive industrial catalyst. Further details are expressed below.

Screen additional fungal screening strains

Other filamentous fungi from the *Polyporales*, *Hypocreales*, *Mucorales*, and *Mortierellales* orders have been studied as suitable catalysts. These covers species from the phyla *Ascomycota*, *Basidomycot*a and *Zygomycota* whose evaluation allows the induction of variable oxidases far from the conserved CYP clans, CYP 51 and CYP 61[244]. These studies may open the opportunity for the expression of unique CYP families or enzymes particular to an order or phyla. In chapter III we assayed a wider range of fungi, however a lot more optimization is needed to use them as suitable biocatalyst

Express the induction fold of identified CYP450's isoforms

Quantitative real time PCR (qPCR) is one of the most accurate methods to measure small changes in mRNA levels for individual genes; in our case changes in the expression of 8 CYP450s. However, the quality of the results is directly related to normalization with reference genes whose expression is stable with experimental procedures[245], so failure to choose a stable reference gene will lead to erroneous results[246]. The most common reference genes are non-coding ribosomal components, actin, or tubulin (Table C-1).

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We will extract and purify the mRNA with PureLink® RNA Mini Kit (Invitrogen), we will follow manufacturer instructions. First stranded cDNA will be synthesized using the RevertAidTM Firstr Strand cDNA synthesis Kit (MBI Feremntas), as directed by the manufacturer manual. Initially, the mRNA content will be assayed throughout the reaction period in order to identify the change in induction through time. The fold induction levels are a ratio between the mRNA expressions of induced cells and control calculated by the comparative threshold (C_T) quantitation method.

$$\Delta\Delta C_T = \Delta C_{T(interestgene;CYP\ gene)} - \Delta C_{T(reference\ gene,\ 5.8\ S\ RNA)}$$

$$Induction\ fold = 2^{-\Delta C_T\ (induced\ cells)} / 2^{-\Delta C_T\ (control)}$$

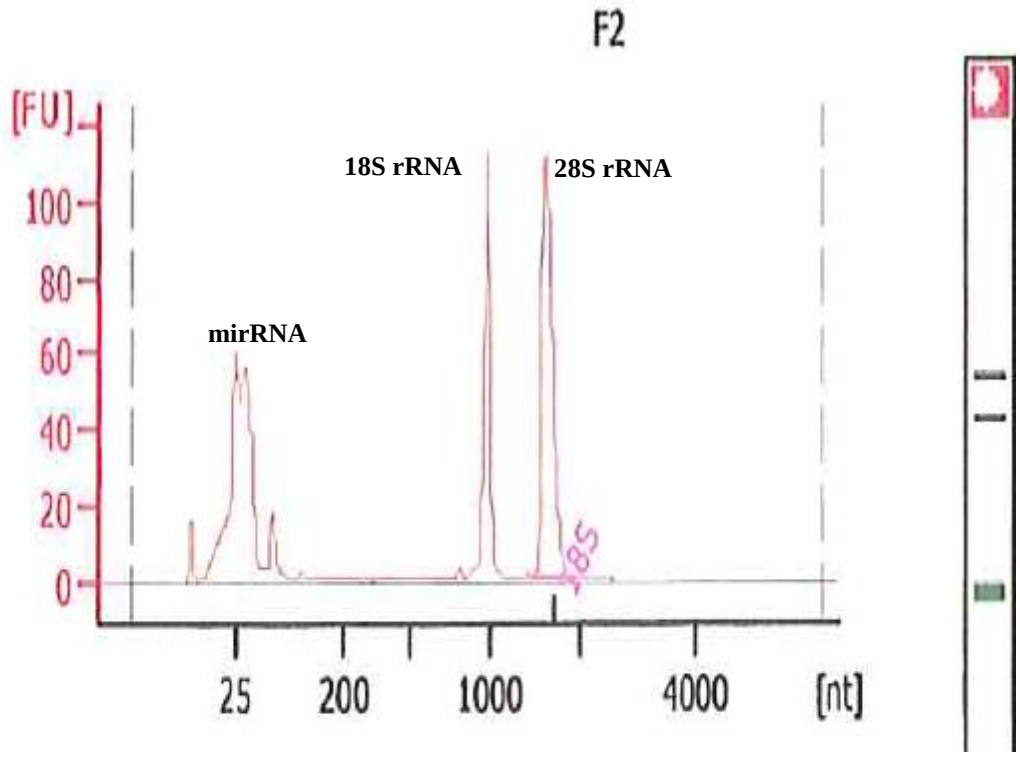

Figure 6-1 Resolution of fungal RNA on Bioanalyzer (CE) resolved on an Agilent Lab-on-a-Chip. Total RNA was isolated with TRIAZOL Reagent. It showed high RNA purity, 337 ng/µL, as well as integrity, RIN ≥9.2.

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A gene is considered to be induced if the positive control shows greater than 1.5 to 5 fold induction. We will check RNA purity (Figure 6-) in a TE buffer (10 mM Tris-Cl, pH 7.5 1 mM EDTA for pH=8)before amplification by A_{260}/A_{280} (UV/Vis Spectrophotometer Shimadzu Corp) ratio 2:1, and we will repurify if ratio is less than 1.98 and will discharge if RIN (RNA integration number) is less than 7. Further purification of the RNA is required in order to get a better quality RNA for We will use the primers identify by Pedrini et al. for the eight CYP450 involve in hydrocarbon catabolism[12]. We will use as housekeeping gene the 5.8 S RNA, a non-coding ribosomal RNA component associated to ribosome translocation, and as blank controls cDNA generated without RT as well as primer plates without templates. We will run duplicates for each of the replicates. In our case it is useful to validate pesticide CYP induction. It is consider an inducer according to FDA recommendations if the induction fold change is equal or greater than 40% of the positive control (cells with substrate w/o inducer).

A whole cell proteome analysis by LC-MS/MS

Based upon band identification and benzidine analysis from Chapter V, we were able to identified hemeproteins in the hydrophobic fraction. A more in depth proteomic analysis is required order to get a more detailed understanding of *B. bassiana* catalysis and metabolic pathways. This includes further fractionation by strong cation exchange (SCX) chromatography and profiling the whole proteome under different environmental and physical stress conditions. Shin et al.[247] and Park et al.[37]protein analysis procedures are followed to compare the difference in expressed oxidases, hemoproteins, between treated and non-treated cells. Peptide identification is performed using a nano- LC–MS system (Agilent 6410B triple quadrupole / Agilent 6530 quadrupole time-of flight) equipped with a electros spray ionization (ESI) source. Peptides will be eluted directly off a C_{18} column with an organic gradient[37, 247] of 2 to 80% mobile phase II over 60 minutes

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at flow rate. The mobile phase is composed of 2% acetonitrile with 98% of 0.1% formic acid (v/v) in water (mobile phase I) and 98% acetonitrile with 2% of 0.1% formic acid (v/v) in water (mobile phase II). The MS scan sequence is programmed for a full scan, a zoom scan to determine the charge of the peptide, and a MS/MS scan of the most abundant peak in the spectrum will be done in order to select the three most abundant MS ions for comparison purposes[224, 248]. Dynamic exclusion[37, 226, 247] is used to exclude intense ions, 50 mDa, from continually being selected for MS/MS analysis of the same peptide; also to allow data acquisition on lower intensity ion. The sequence MS/MS data is processed into a merged file in order to use MASCOT (Matrix Science, London, UK) peptide search against the National Center for Biotechnology Information (NCBI), Fungal/GOLD database ($>3 \times 10^5$ sequences), and Fungal cytochrome P450 databases[37]. These databases searches will be limited to the taxon Fungi in particular to the order *Hypocreales* with special attention to the genera identified as insect pathogens. They are employed because a completely sequenced and annotated genome of *B. bassiana* has yet to be publicly released; sequencing of the *B. bassiana* genome is 96.1 % complete[241]. Although the protein databases are growing, the lists are far from complete. Hence, correct functional characterization of putative oxidative proteins is necessary. The general search parameters take into account methionine oxidation, the modifications of N-terminal Gln to pyroGlu, acetylation of protein N terminus, carbamidomethylation of cysteine, and acrylamide modified cysteine. In addition, two missed cleavages for the enzyme are considered [37, 226, 247]. These parameters are adjusted accordingly throughout the search in order to filter peptides with a score less than 20 and to tolerate peptide/fragment mass tolerance of ± 0.5 Da for the MS/MS ion search[37, 247]. Proteins are identified having a significance threshold of $p < 0.05$ by their probability based MASCOT scores.

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Expand the oxidase screening scope

The contribution of Flavin monooxygenase (FMO) to xenobiotic metabolism is often overlooked or underestimated since these enzymes thermolabile in the absence of NADPH. These oxygen-dependent enzymes convert nucleophilic nitrogen, sulfur, phosphorous and selenium compounds to oxygenated products[249]. Strategies to identify FMO catalysis require the quenching of CYP components. In Lassic studies a cocktails of inhibitory compounds were used to cover as many CYP isoforms as possible[250 30]. Biotransformation metabolites were analyzed with respect to a control run where no inhibitor was used. From this work enzyme specificity was suggested. The current work will further identify key enzymes through the proteomic evaluation of enzyme inventory. FMO will be assayed by the detection and quantification of the resultant (R) and (S) methyl p-tolyl sulfoxide using an HPLC with a chiral column and UV detector set at 254 nm[249].

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APPENDIX A

STEROID BIOTRANSFORMATIONS

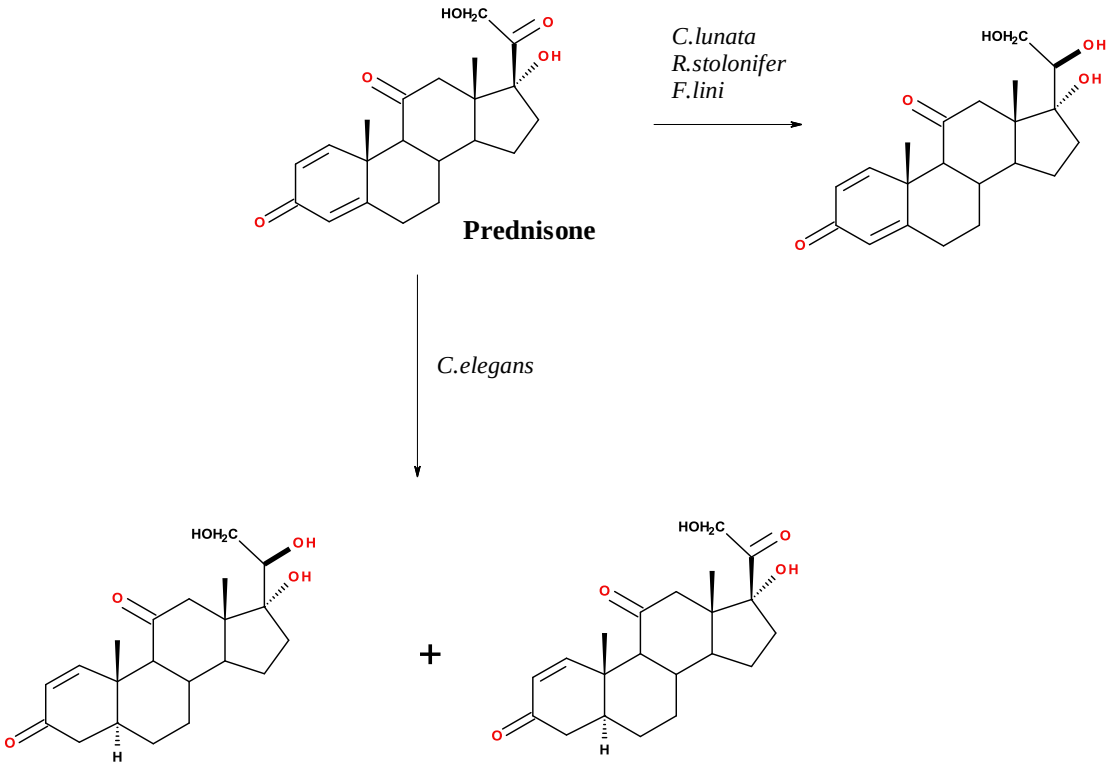

Figure A-1 Biotransformation of prednisone.

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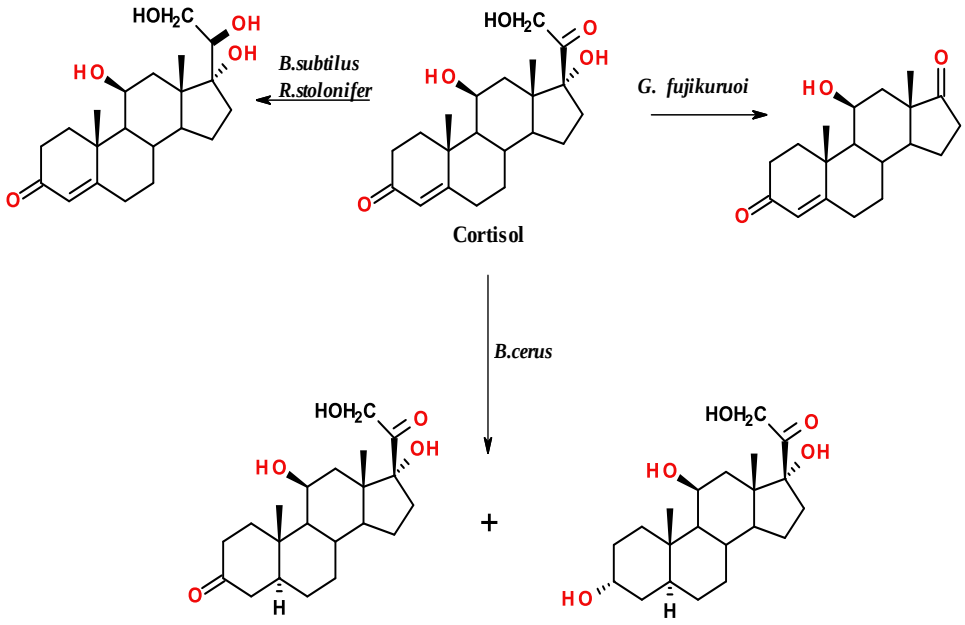

Figure A-2 Biotransformation of cortisol.

Table A-1 Distribution of sulfur compounds over the distillation of oil with total sulfur compound of 1.2% [85].

Distillation range (°C)	Sulfur content (%)	Sulfur compounds distribution (%)			
		Thiols	Sulfides	Thiophenes	Other [a]
70-180 (naphtha)	0.02	50	50	Trace	-
160-240 (kerosene)	0.2	25	25	35	15
230-350 (distillate)	0.9	15	15	35	35
350-550 (vacuum gel oil)	1.8	5	5	30	60
>550 (vacuum residue)	2.9	Trace	Trace	10	90

a Benzothiophenes, dibenzothiophenes, and heavy sulfides

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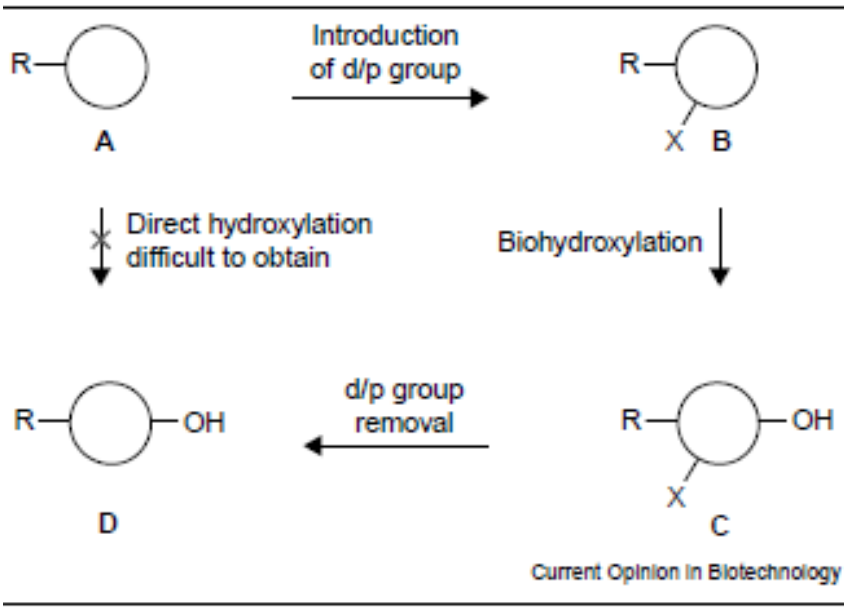

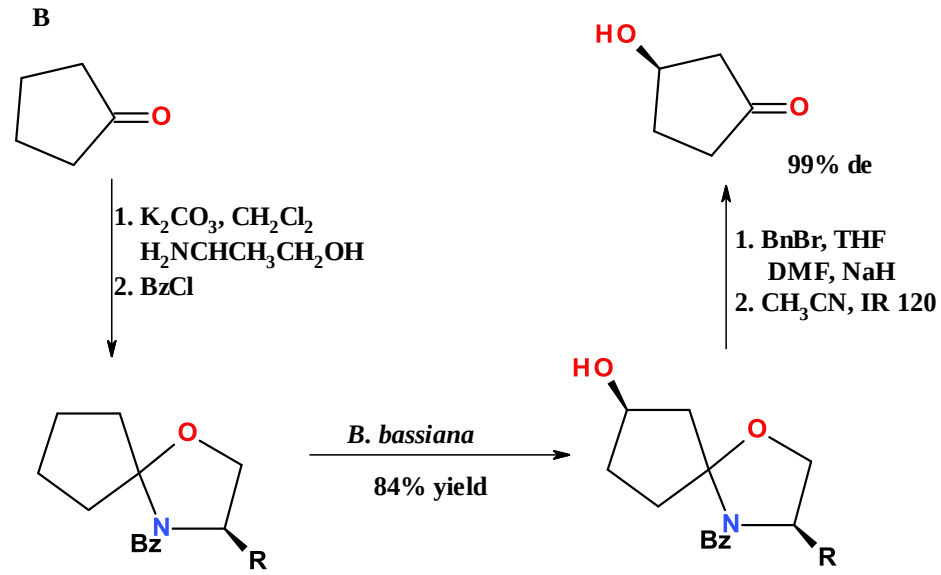

Figure A-3 Substrate engineering by the docking/protecting goup scheme. The d/p gives better control over the product distribution and facilitates hydroxylation in comparison to the traditional route w/o using these groups. A) The d/p group (X) is conjugated with the substrate A to give derivate B. Then compound B is more easily catalyzed in the biohydroxylation reaction to poduct C and the d/p group also protect against side reactions. The d/p group is finally removed to produce hydroxylated product D. [171] B) Application of the d/p group scheme for the hydroxylation of cyclopentanone.

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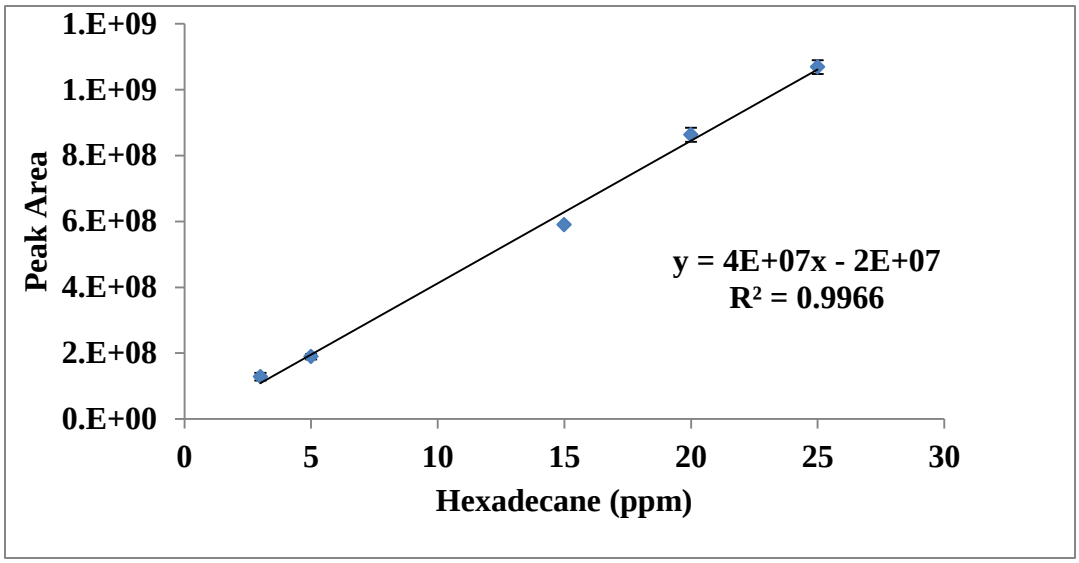
APPENDIX B

SUBSTRATE AND HEXADECANE CALIBRATION CURVES



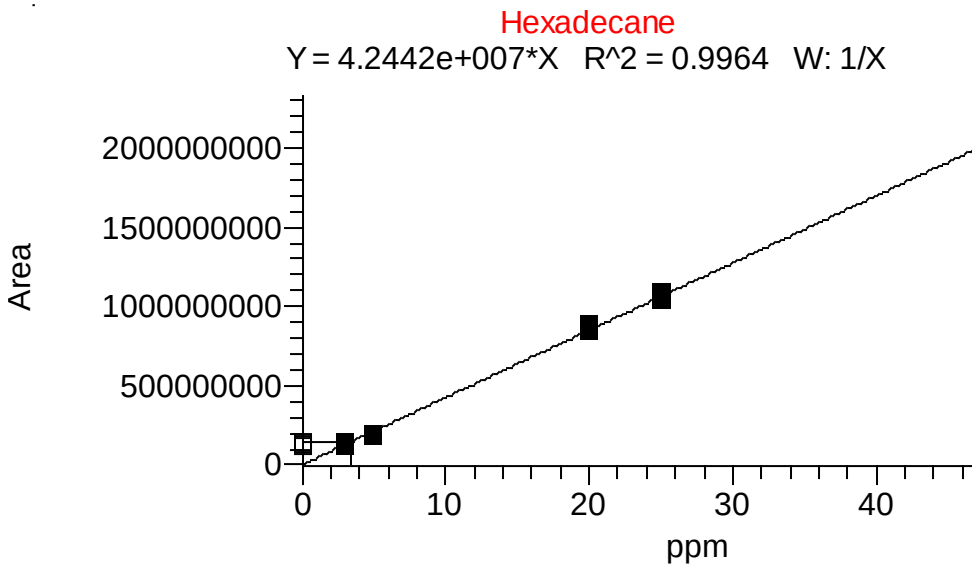


Figure B-1 GC calibration curve for hexadecane (ppm) by single ion monitoring (m/z 226) in a SPB-5ms at a temperature ramp was 100-250°C at 20°C/min and 250°C for 10 min; bars represent ±SD, n=3.

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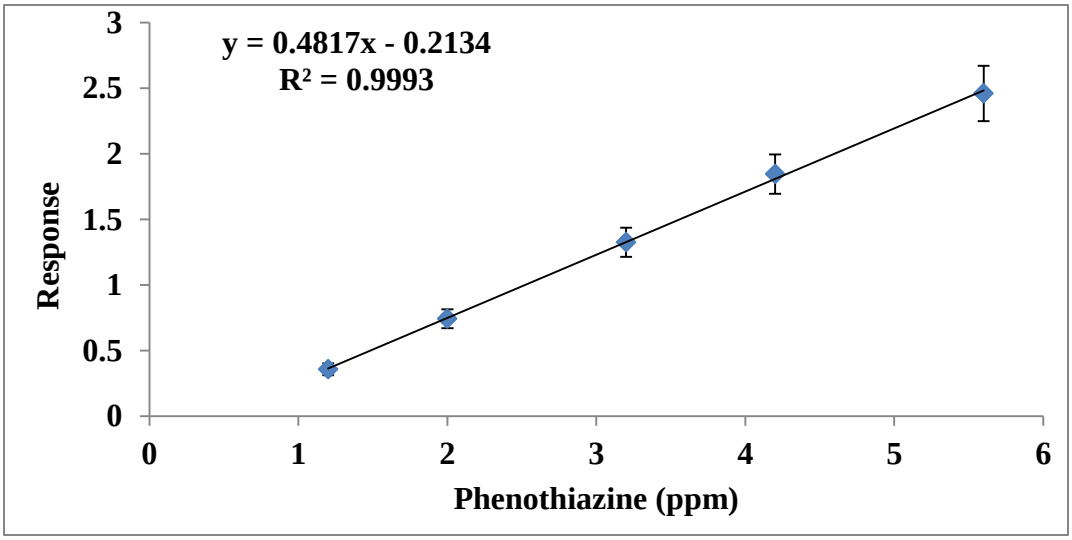

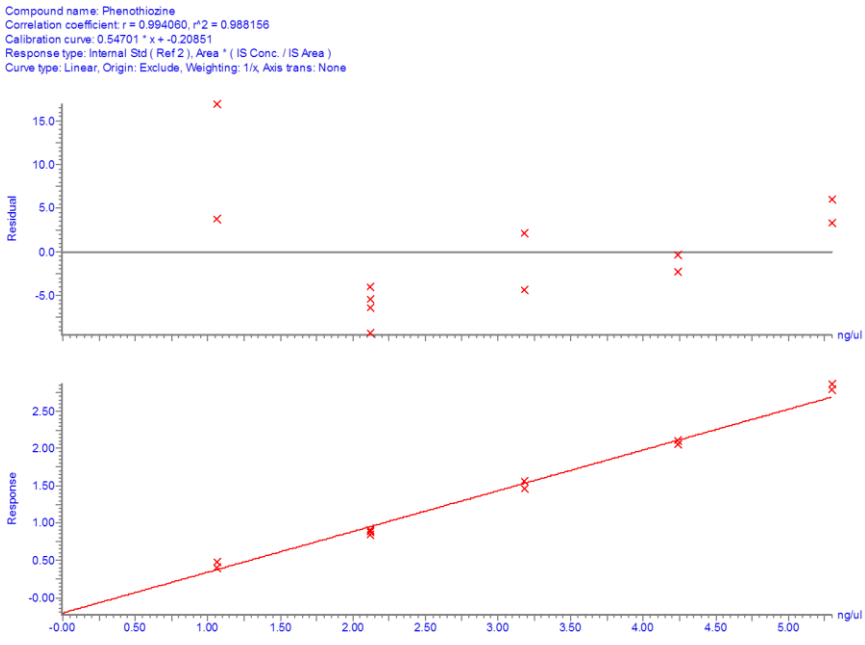

Figure B-2 GC calibration curve for phenothiazine (ppm) by single ion monitoring (m/z 199) in a SPB-5ms at a temperature ramp was 100-250°C at 20°C/min and 250°C for 10 min; ISTD carbazole bars represent ±SD, n=3.

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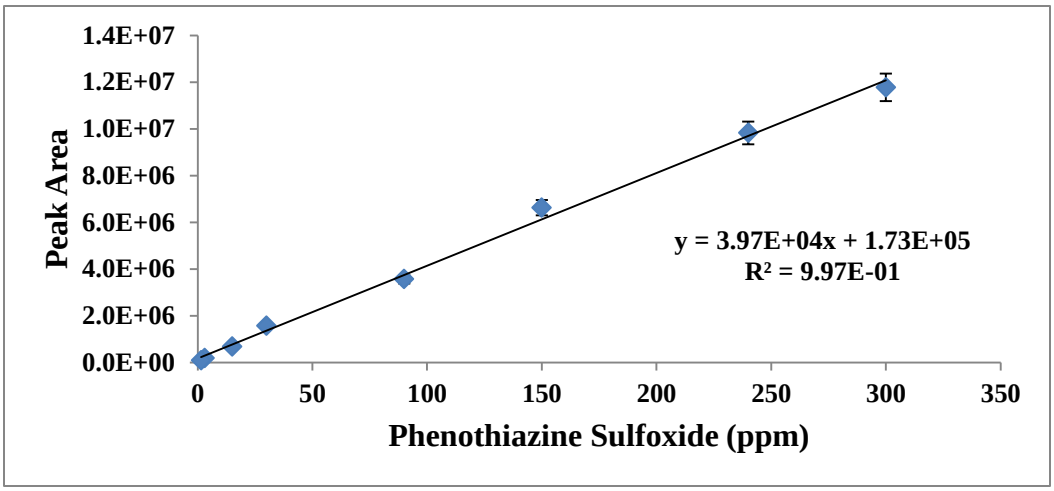

Figure B-3 HPLC calibration curve of phenothiazine sulfoxide (ppm) analyzed in a Agilent ZORBAX Eclipse Plus C_{18} (4.6x250 mm, 5 µ m). Analysis was performed in a Shimazdu 1200 HPLC equipped with a photodiode detector. Metabolites were monitored at 254 nm in a gradient mode with MeOH: H_2O (55:45) to (95:05); 20 min gradient and 10 min hold at a 0.5 mL/min flow rate; bars represent ±SD, n=3.

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APPENDIX C

B. BASSIANA ADAPTATION AND CATALYSIS STRATEGIES

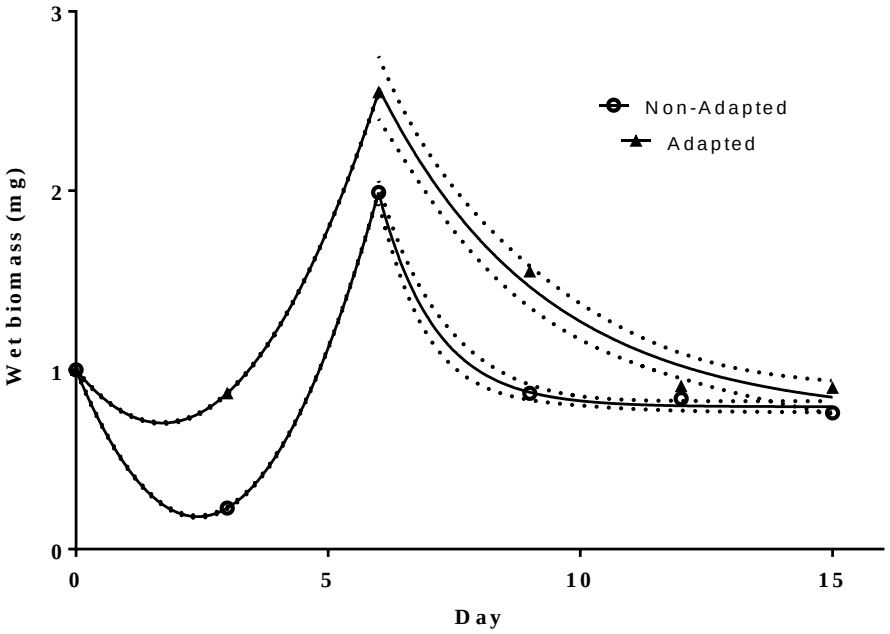

Figure C-1 Variations in wet weight between hexadecane adapted and non-adapted cells ; fitted to a one phase decay exponential decay after the 6th day dotted line represents the 95% CI, n=3

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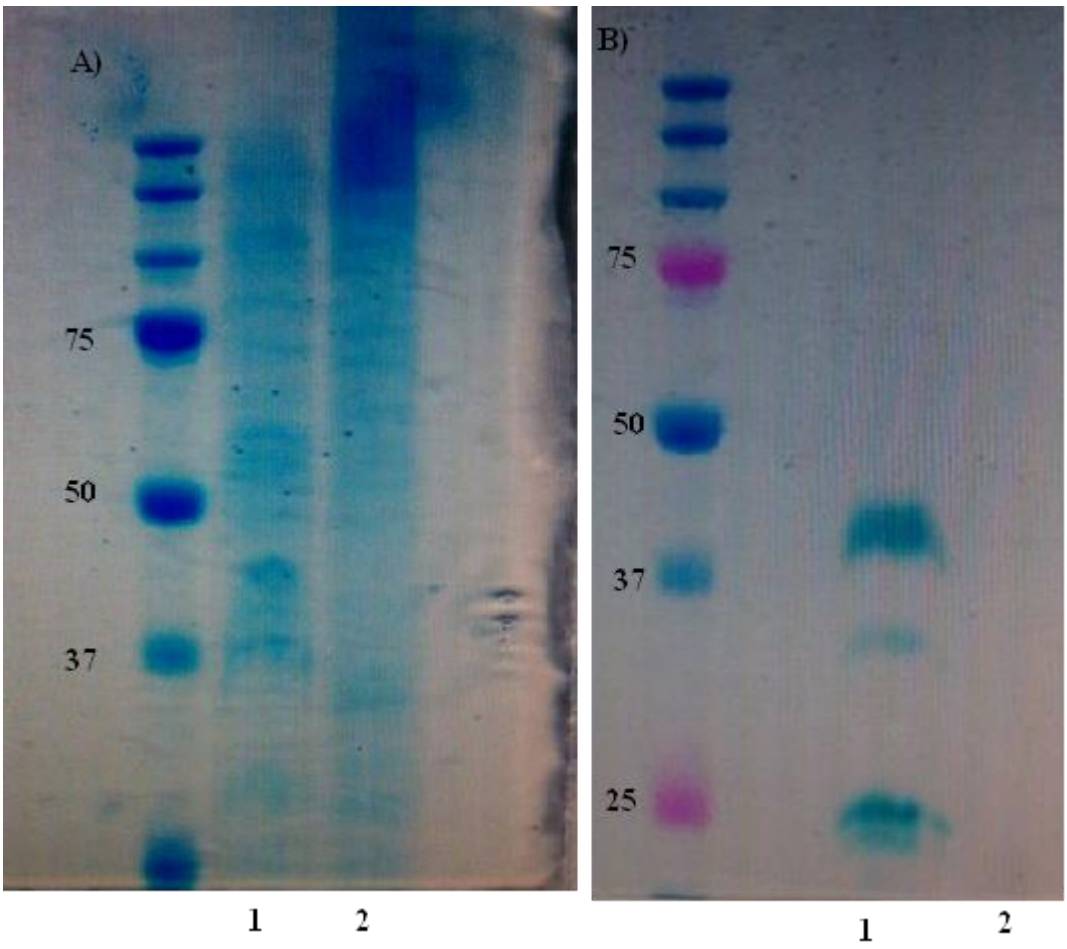

Figure C-2 Coomassie stained (A) and heme-stained (B) SDS-PAGE of C16 and non-adapted cells hydrophobic protein fraction extracted via the Triton X-114 chaotropic fractionation method. Loaded 40 µg of proteins , unreduced samples and no heat treatment. 1) Protein ladder 2) C16 fraction 3)Biotic control. Coomasie stained with GelCode™ Blue Stain Reagent and heme-stained with benzoidine

The reference genes mentioned below are a selection of stable genes assayed in *B. bassiana.* The reference stability was evaluated by Zhuo and collegues[92] against thermal, nutritional and osmotic stress.

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Table C-1 Reference housekeeping genes that can be used for *B. bassiana* qPCR.

Gene	Accession No.	Protein/RNA	Primer sequence(5′ → 3′)
TBP	HQ23239	TATA box binding protein	TBP-F: CCGAGTATAACCCAAAGCGT
			TBP-R: GTAGATCAGACCAGGGAACA
TEF	EF193181	Translation elongation factor	TEF-F: AGAAGTTCGAGAAGGAAGCC
			TRF-R: GGTGGTGTCCATCTTGTTGA
HGPT	HQ232401	Hypoxanthine guanine phosphoribosyl transferase	HGPT-F: CGTCACCTATAACGATGTGC
			HGPT-R: GATGCTGAATTCCGTCTTGG
CrzA	HQ248182	CrzA, a C2H2 type zinc finger	CrzA-F: GAGGTCCTTGGTATTGGTTC
			CrzA-R: ATTTGGGACGCCCTCTATCT
28S	EU334679	28S rRNA	28-F: TCAACGTCAAGAGGGTCAAG
			28-R: CGATTCTGTTCAGGGTCTTC
18S	EU334679	18S rRNA	18-F: AACATGGTCGCAACGGGTAA
			18-R: AGTGAAGGGCATGAGGTTCC
5.8S	EU673371.1	5.8S rRNA	5.8 F - AAGAACGCAGCGAAACGCGATAAG
			5.8R- TCGTTAAGTTCAGCGGGTAGTCCT

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Table C-1 continued

His3	HQ232402	Histone 3	His3-F: CAGACAGCAAGAAAGTCCAC
			His3-R: TTGGTGTCCTCGAAGAGAGA
His2A	HQ232403	Histone 2A	His2A-F: AGAAGAAGCAGCAGAGCCAC
			His2A-R: CTCGACTTTGAGTAGCAGTG
GAPD	AY679162	Glyceraldehyde-3-phosphate dehydrogenase	GAPD-F: GTGTCTTCACCACTACTGAG
			GAPD-R: TGTAGCCGAGAATGCCCTTG
PP1	HQ248176	Ser/Thr protein phosphatase 1	PP1-F: TCGAGGCTCCCATCAAGATT
			PP1-R: GCAGTCGGGAATATCAGTAG
CypB	HQ123503	Cyclophilin B	CypB-F: ATGTTCAACCTCCGTCGCTT
			CypB-R: ACTTGTTGCCGTAGATGGAC
CypA	HQ610831	Cyclophilin A	CypA-F: ATGGCTAACCCCAAGGTCTT
			CypA-R: AACTGGGAGCCGTTGGTGTT
β-Tub	AJ312228	β-Tubulin	β-Tub-F: ATGCGTGAGATCGTTCACCT
			β-Tub-R: ACCGAGAGAATGGGTGATCT
γ-Act	HQ232398	γ-Actin	γ-Act-F: ATGGAGGAAGAAGTTGCTGC
			γ-Act-R: ACACGGAGCTCGTTGTAGAA

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APPENDIX D

PRODUCT CHARACTERIZATION ANALYTICAL METHODS

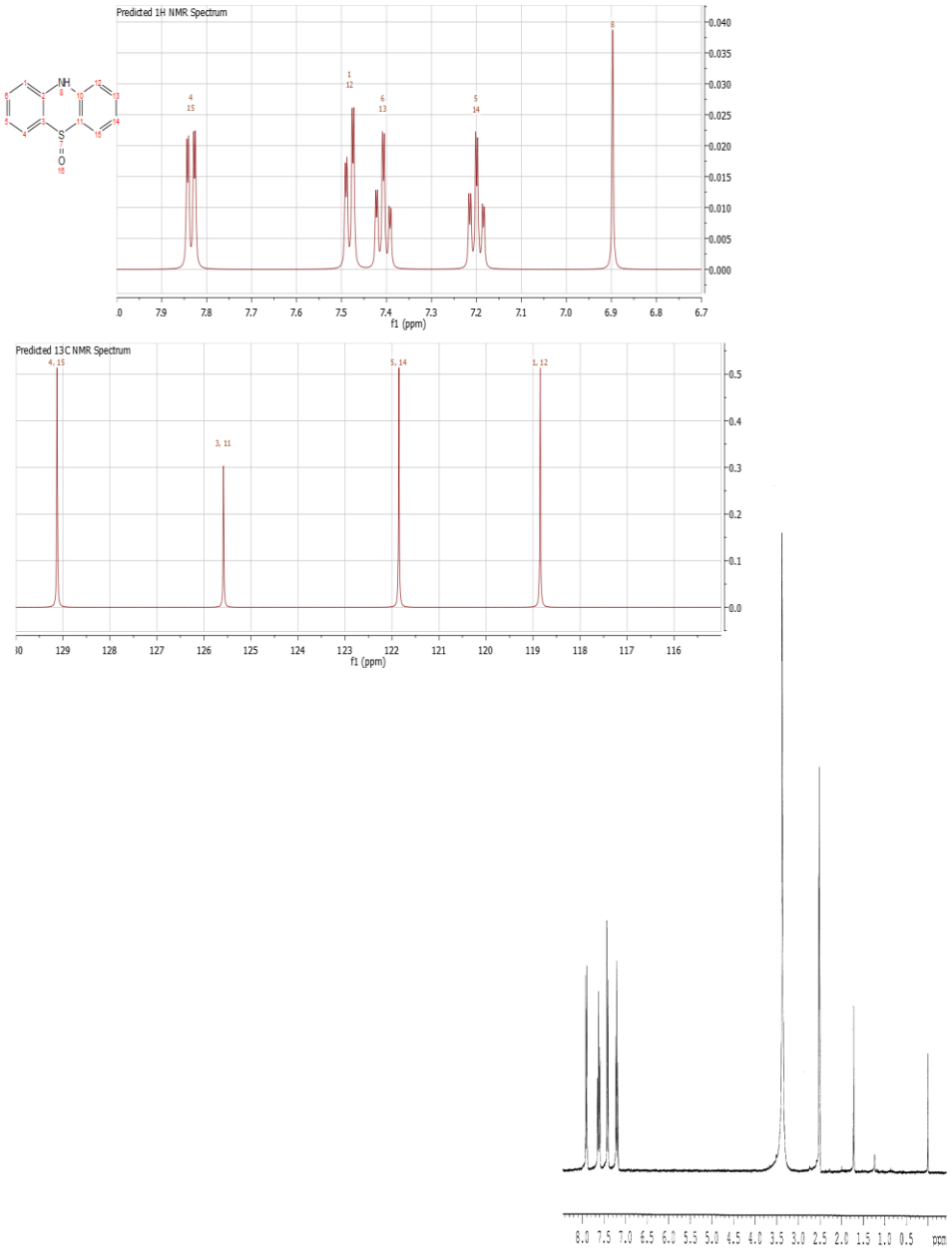

Figure D-1 ^{13}C and ^{1}H NMR spectrum of phenothiazine sulfoxide in $CDCl_3$ and d_6-DMSO.

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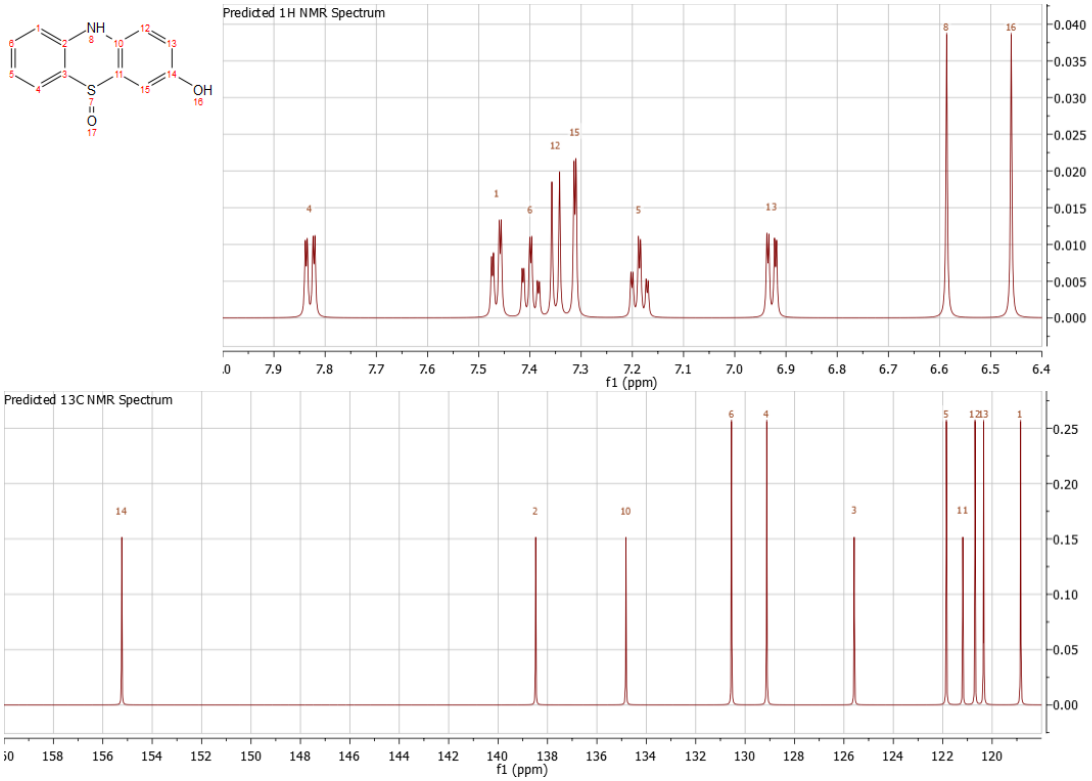

Figure D-2 ^{13}C and ^{1}H spectrum of 3-hydroxy-phenthozine sulfoxide in $CDCl_3$.

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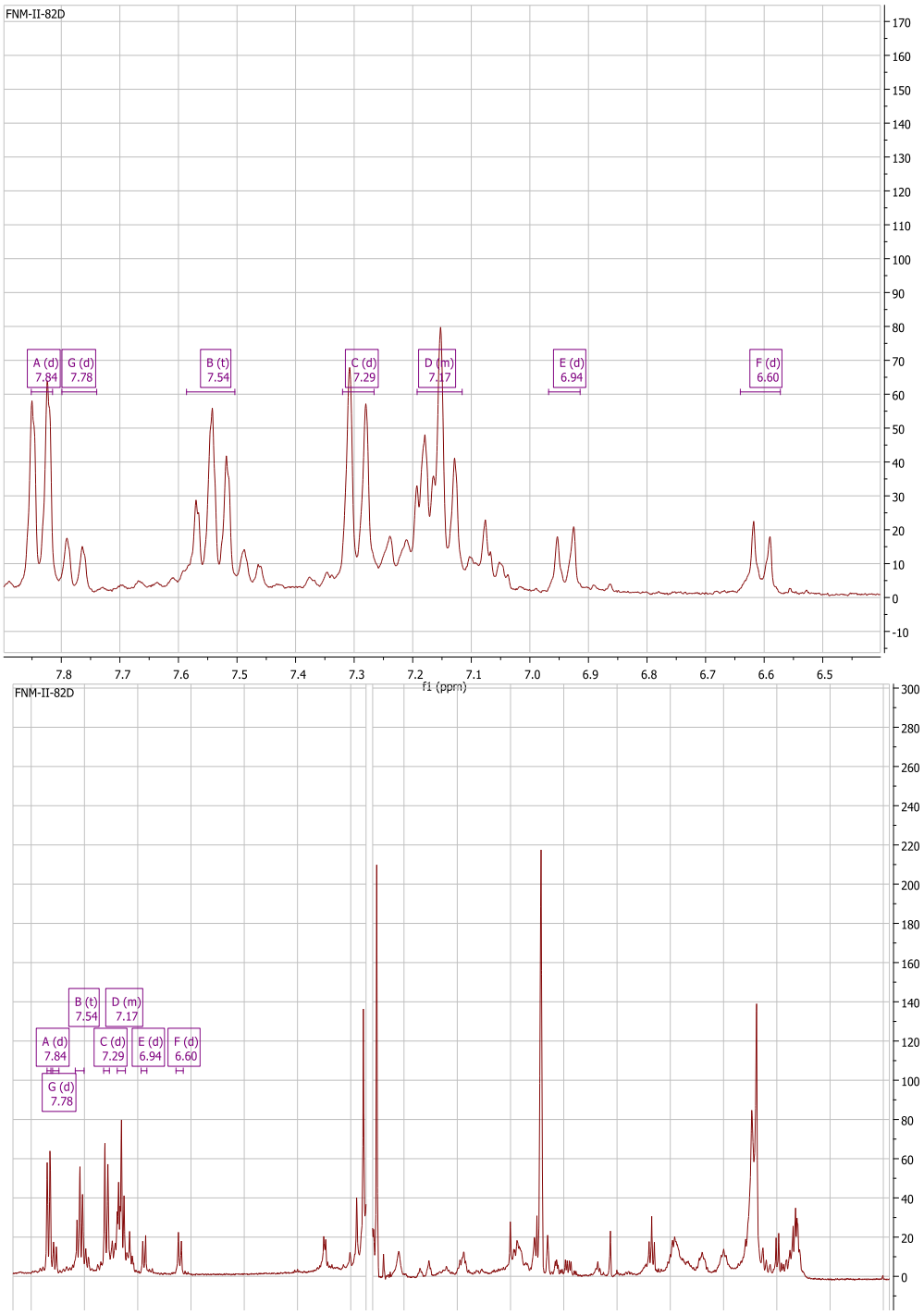

Figure D-3 ^{1}H spectrum of 3-hydroxy-phenthozine sulfoxide in d6-DMSO; the single H2O peak at 3.3 ppm, some residual Ethyl Acetate (1.99 (s), 4.03 (q),1.17 (t)) H_2O 3.33 (s),2.54.

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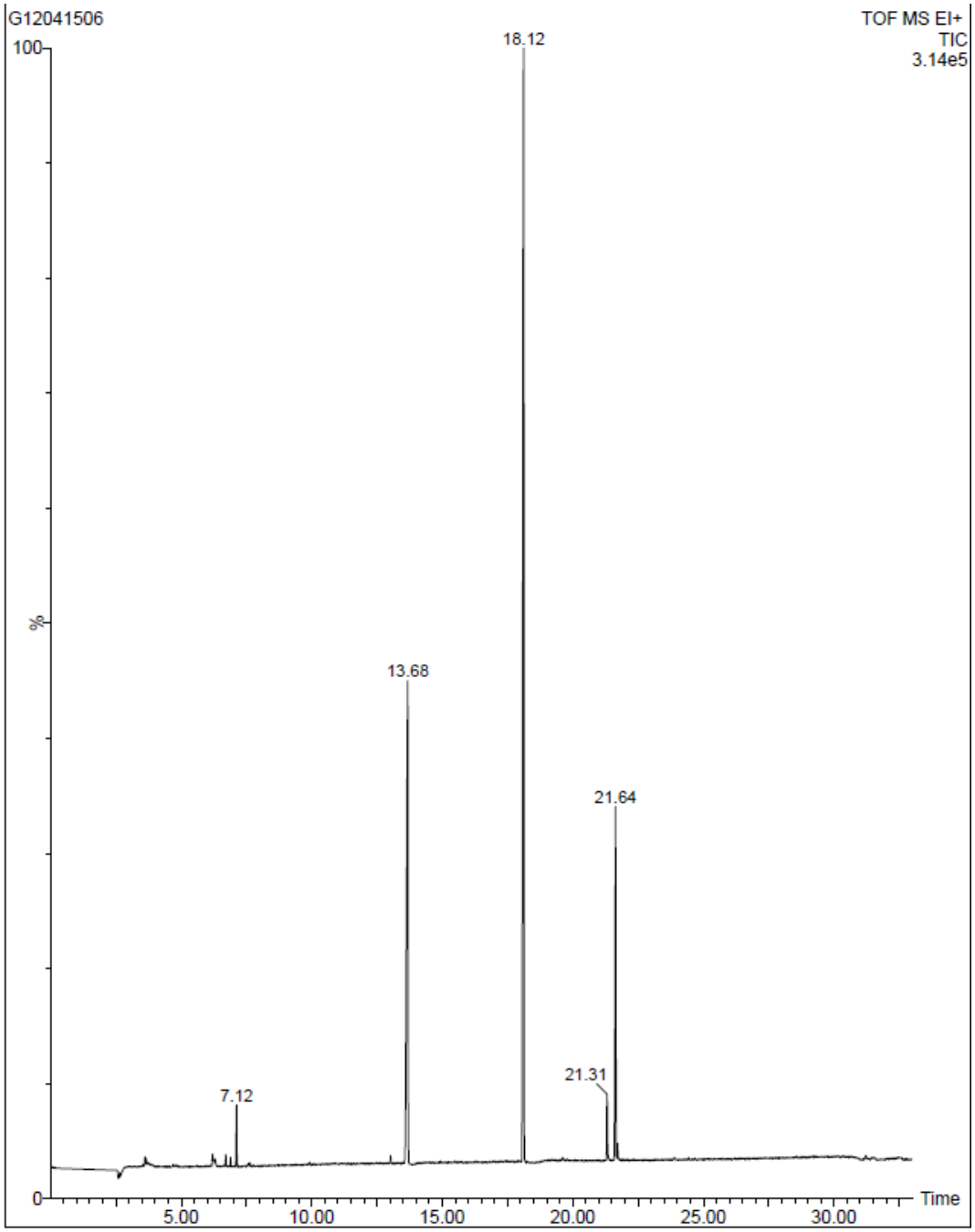

Figure D-4 GC-MS Total ion chromatogram (TIC) of the sulfoxidation of thioanisole, thiophene, and dibenzothiophene after 7 days of reaction at 26°C and 180 rpm.

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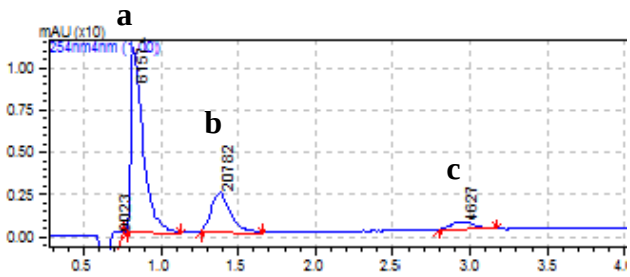

Figure D-5 Chiral chromatogram of 3-hydroxy phenothiazine sulfoxide run in a CHIRALPAK ®-MA+ at a flow rate of 0.5 mL/min of 2mM $CuSO_4$ soln. a) phenothiazine sulfoxide, b) (R)-3-hydroxy phenothiazine sulfoxide and c) (S)-3-hydroxy phenothiazine sulfoxide.

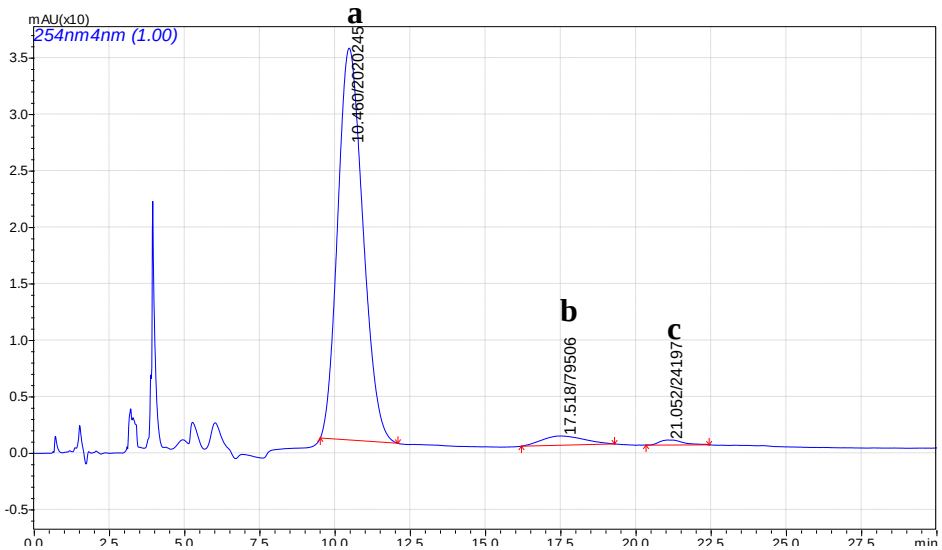

Figure D-6 Chiral chromatogram of thioanisole sulfoxide run in a DIACEL CHIRALPAK-OB at a flow rate of 1mL/min of hexane/i-PrOH (95:05) S-Thioansiole sulfoxide (53%S ee) c) R-Thioanisole sulfoxide.

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APPENDIX E

MASS SPECTRA OF PHENOTHIAZINE OXIDIZED METABOLITES

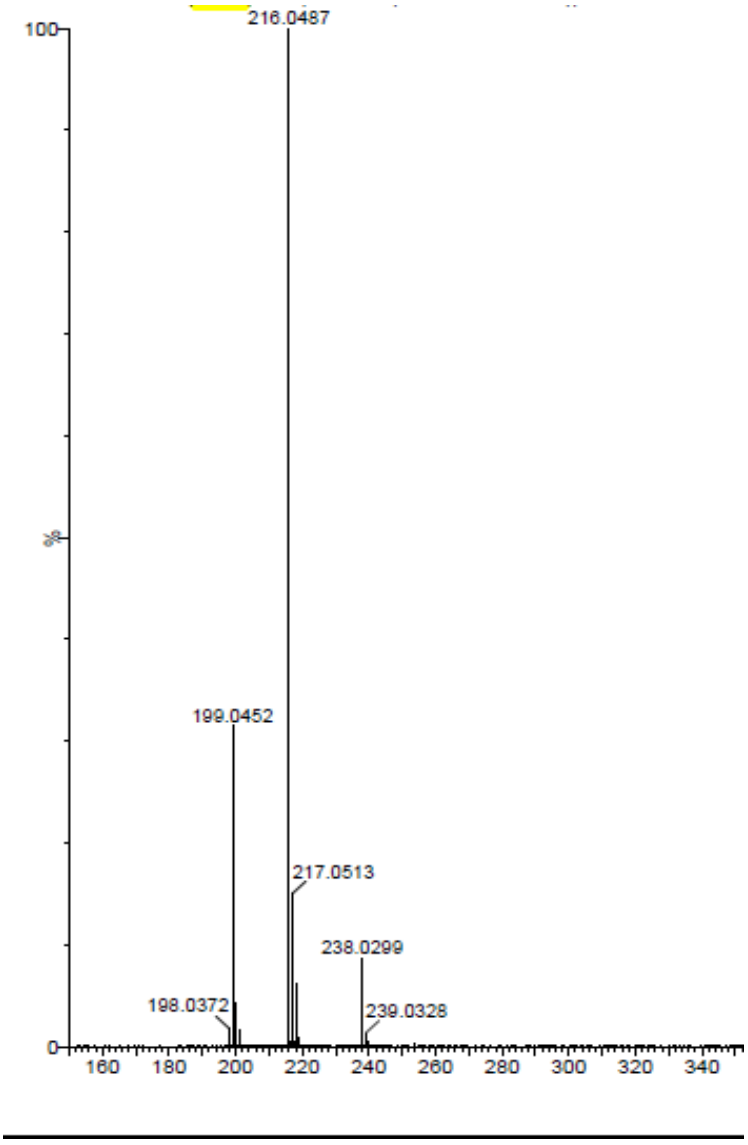

Figure E-1 Phenothiazine sulfoxide positive ESI-MS at 100V.

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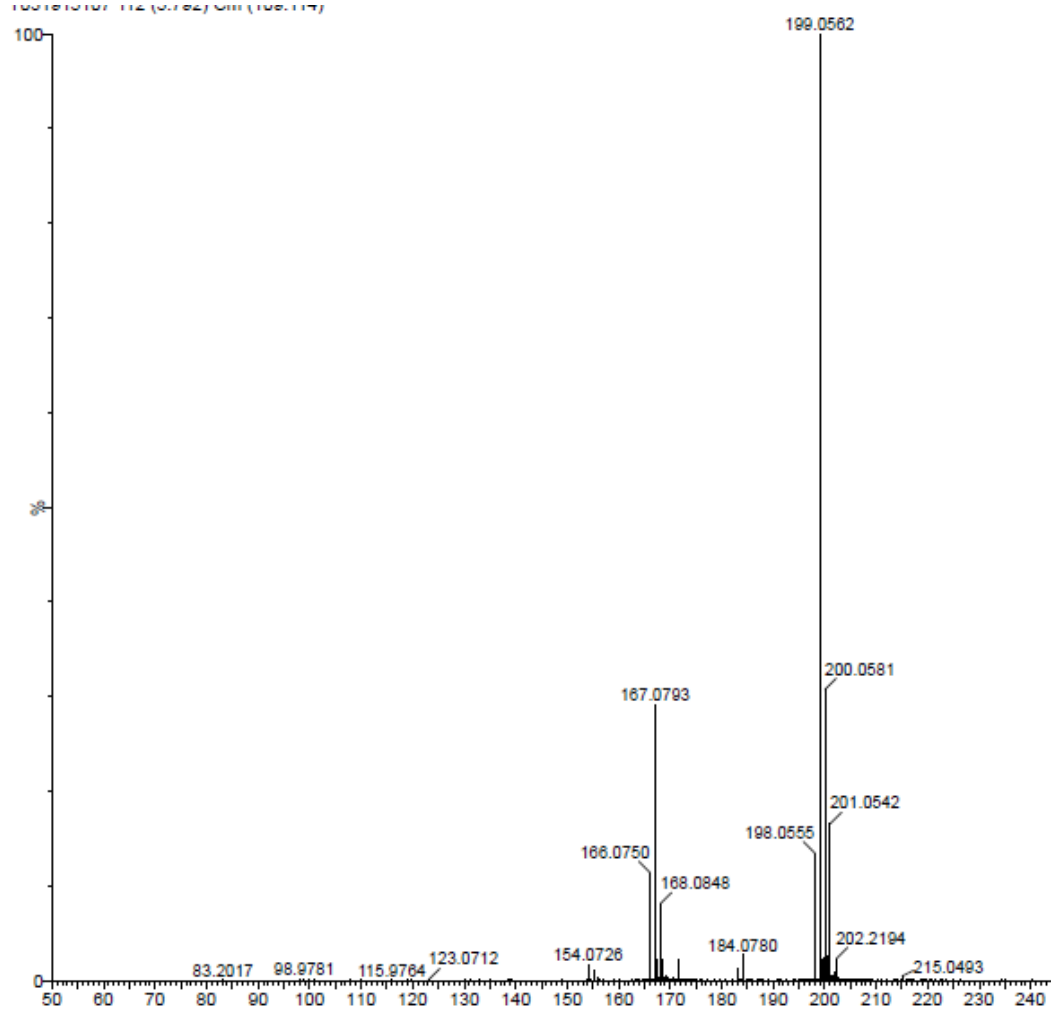

Figure E-2 Phenothiazine sulfoxide positive ESI-MS with low energy in-source CID at 20 eV.

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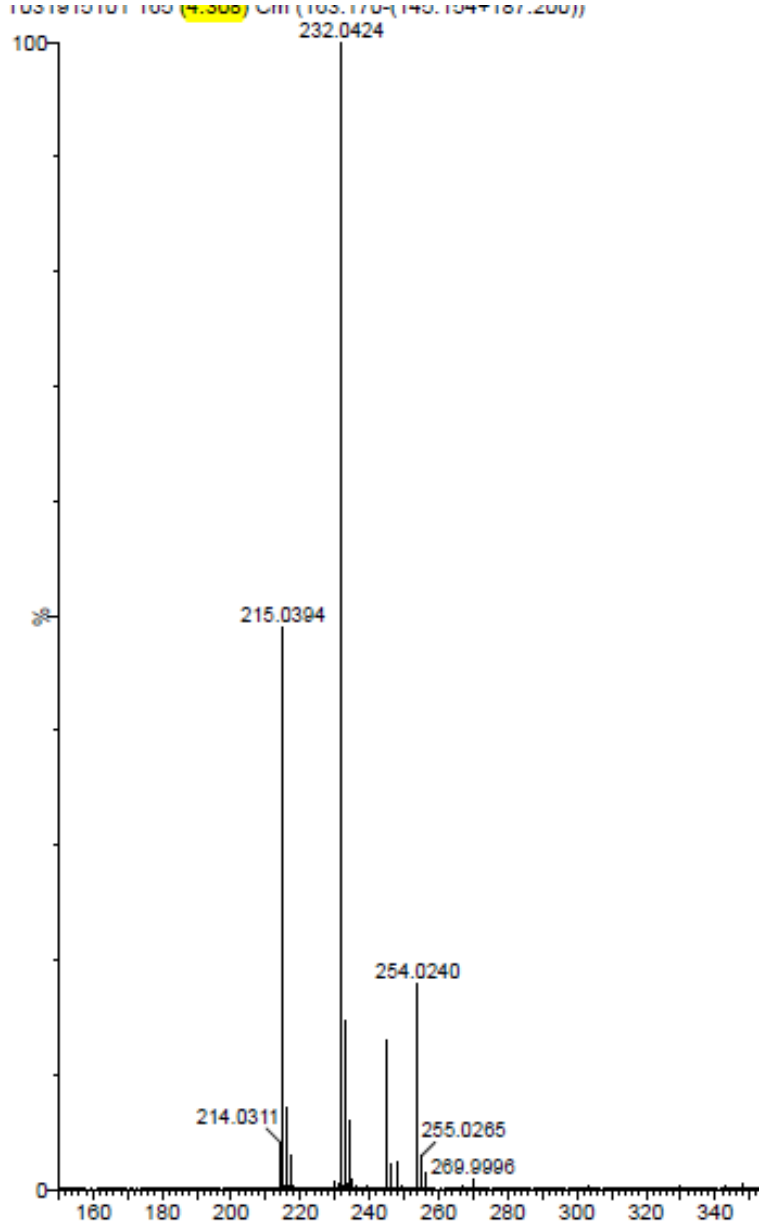

Figure E-3 3-hydroxy- phenothiazine sulfoxide positive ESI –MS at 100 V.

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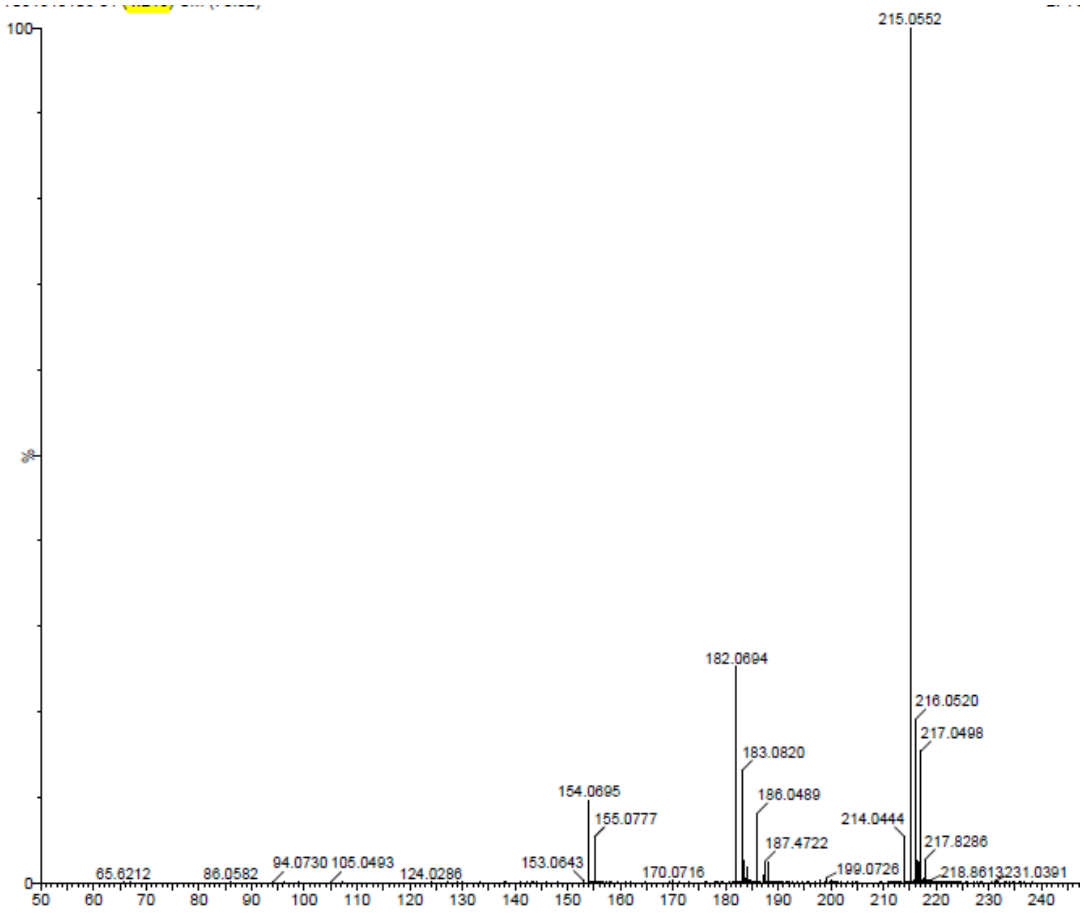

Figure E-4 3-hydroxy- phenothiazine sulfoxide positive ESI –MS with low energy in-source CID at 20 eV.

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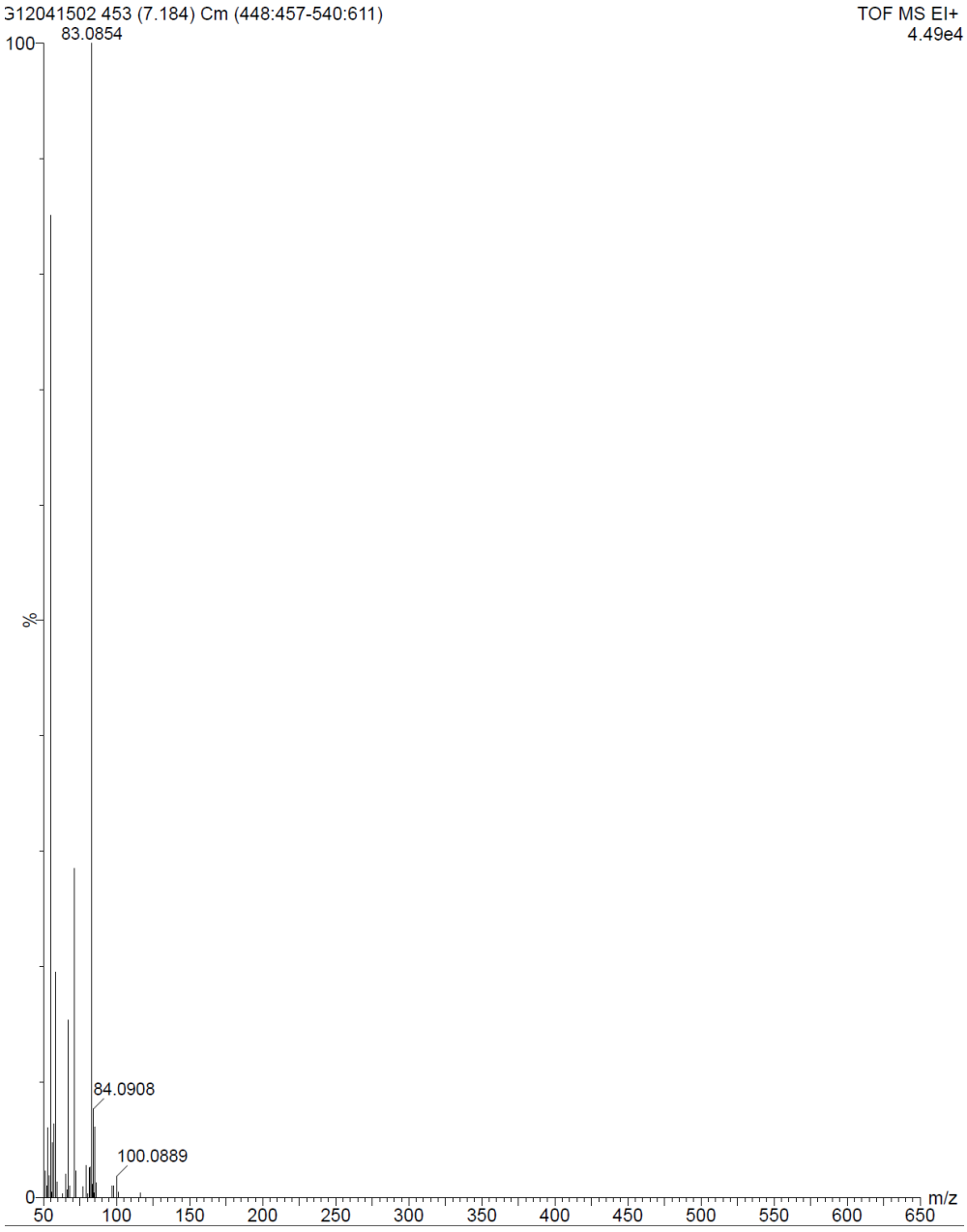

Figure E-5 GC-MS time of flight: Thiophene sulfoxide (m/z 100) fragmentation pattern.

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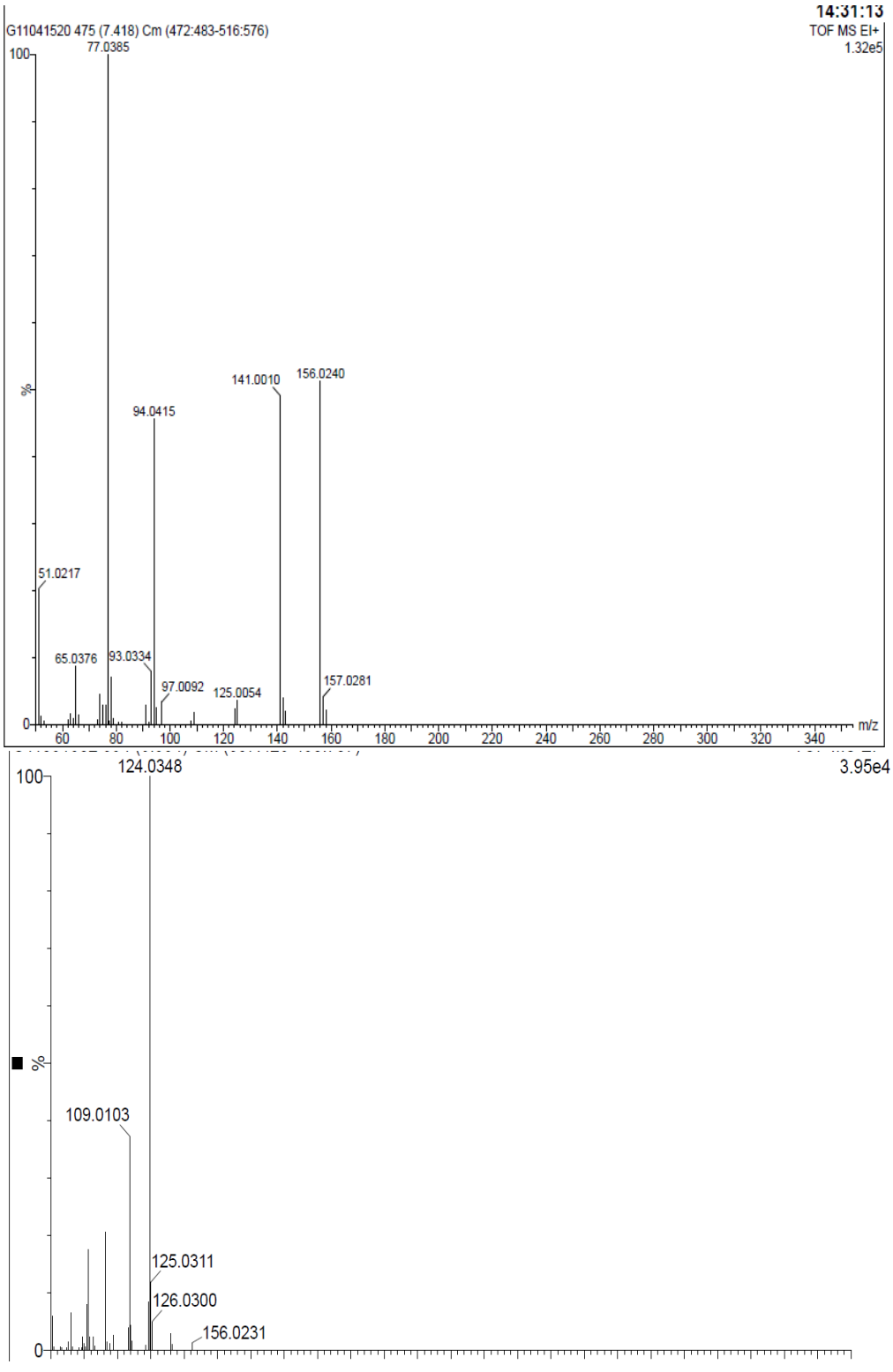

Figure E-6 GC-MS time of flight: Thioanisole sulfone (m/z 156.02) and thioanisole (m/z 124.03) fragmentation patterns

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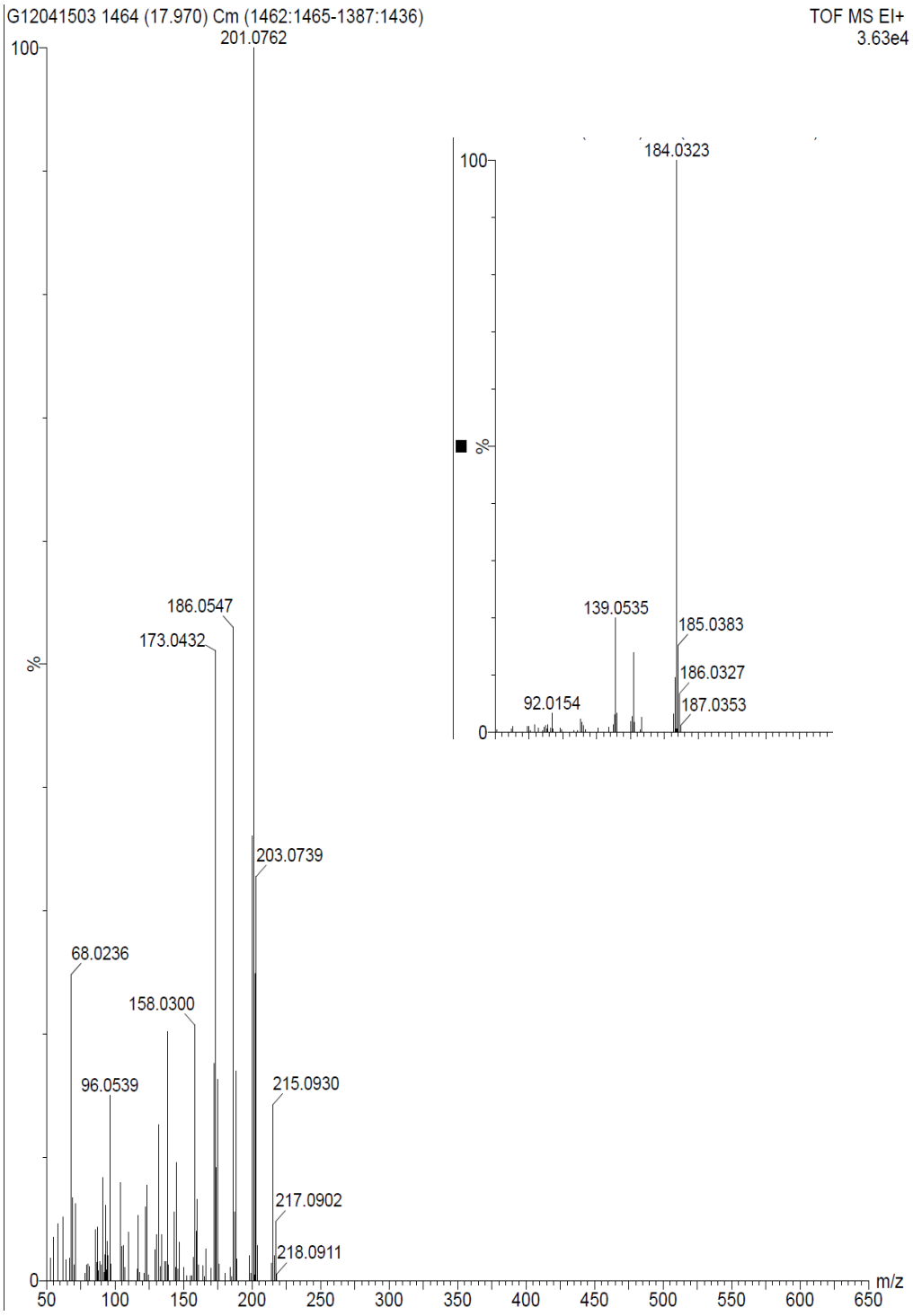

Figure E-7 GC-MS time of flight: dibenzothiophene sulfone (m/z 216) and dibenzothiophene (m/z 184) fragmentation pattern

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Dreams

Hold fast to dreams
For if dreams die
Life is a broken-winged bird
That cannot fly.
Hold fast to dreams
For when dreams go
Life is a barren field
Frozen with snow.
Langston Hughes (1902-1967)

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