FUNCTIONAL CHARACTERIZATION OF LIGNIN MODIFYING ENZYMES PRODUCED BY WHITE ROT FUNGI FOR THEIR MYCOREMEDIATION POTENTIAL OF PETROLEUM (XENOBIOTIC) POLLUTED SOIL

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Functional characterization of lignin modifying enzymes produced by white rot fungi for their mycoremediation potential of petroleum (xenobiotic) polluted soil

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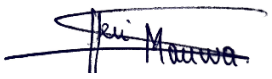

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ABSTRACT

Industrialization has resulted in the introduction of xenobiotics into the environment. These chemicals have polluted soil, water, and air, causing significant health concerns. White rot fungi possess the capability to produce unique enzymes known as lignin-modifying enzymes which can degrade not only lignocellulose, but also many xenobiotics. The aim of the study was to characterize LMEs using various bioassays and to apply the best performing fungi for an attempted bioremediation of a mixture of petroleum products. Using molecular characterization, fungal isolates were identified. These were qualitatively assayed for lignin peroxidase and laccase production. Enzyme production was quantitatively assayed by UV/Vis spectrophotometry using corn husk as growth medium; and common assay reagents were compared. Veratryl alcohol gave the best results for lignin peroxidase while manganese peroxidase activities were similar when using either 2,6 dimethoxyphenol or malonate/Mn^{3+}. ABTS was found to be more sensitive than guaiacol for laccase assay. *Trametes hirsuta* and *Schizophyllum commune* were selected as best performing fungi and were used for the attempted remediation PAHs-contaminated soil. Both isolates degraded petroleum fractions, particularly naphthalene by up to 100% and 67.47% respectively. Overall, the study proved the ability of LMEs to degrade xenobiotics.

Keywords: xenobiotic, white rot fungi, lignin-modifying enzymes, bioremediation, lignin peroxidase, manganese peroxidase, laccase, enzyme, lignocellulose, polycyclic aromatic hydrocarbons

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AOPs	Advanced oxidative processes
ATP	Adenosine 5′-triphosphate
BGC	Biosynthetic gene cluster
BLAST	Basic Local Alignment Search Tool
DDT	Dichlorodiphenyltrichloroethane
DMP	2,6-dimethoxyphenol
DNA	Deoxyribonucleic acid
EDTMP	Ethylenediamine tetra(methylene phosphonic acid)
ELISA	Enzyme-linked immunosorbent assay
EPA	United State Environmental Protection Agency
FPKM	Fragments per kilobase of exon model per million mapped reads
FPLC	Fast Protein Liquid Chromatography
GC-MS	Gas chromatography- Mass spectrometry
HBT	Hydroxybenzotriazole
HCH	Hexachlorocyclohexane
HPLC	High-performance liquid chromatography
IARC	Agency for Research on Cancer
ITS	Internal Transcribed Spacer
β-KAP	β-ketoadipate pathway
LMEs	Lignin-modifying enzymes
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass
MS	Spectrometry
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NHL	Non-Hodgkin lymphoma
NMR	Nuclear Magnetic Resonance
nrLSU-26S	Large ribosomal subunit
nrSSU-18S	Small ribosomal subunit
NZVI	Nanoscale zero-valent iron
OSMAC	One strain-many compounds

PAA	Polyacrylic acid
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCI	Probability of correct identification
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Protein Data Bank
PhACs	Pharmaceutically active compounds
PTMs	Post-translational modifications
PVX	Potato Virus X
QC	Quality control
RNA	Ribonucleic acid
RPKM	Reads per kilobase of exon model per million reads
rRNA	Ribosomal Ribonucleic acid
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SVE	Soil vapor extraction
TCA	Tricarboxylic acid
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TMV	Tobacco mosaic virus
UV-VIS	Ultraviolet–visible spectroscopy
VOCs	Volatile organic compounds
VP	Versatile peroxidase
WRFs	White rot fungi
YAC	Yeast Artificial Chromosome
YCps	Yeast Centromere Plasmids
YEp	Yeast episomal shuttle vectors
YRps	Yeast Replicative Plasmid

CHAPTER 1: INTRODUCTION

1.1. Background

The term xenobiotic refers to a compound, natural or synthetic, found within a biological system or an organism where it is not naturally produced. The term can also be used to identify compounds found in any system in concentrations higher than expected (Atashgahi et al., 2018; Embrandiri et al., 2016; Patel & Jyoti Sen, 2013). Besides them being foreign to environments where they are found, attention has been focused on xenobiotic compounds because of their toxic nature, making them hazardous to biological lives, especially to animals (Godheja et al., 2016). Moreover, they are often recalcitrant to partial or complete degradation (Narwal & Gupta, 2017).

While some naturally occurring compounds such as lignocellulose and phytotoxins are considered to be xenobiotic, the majority of xenobiotics are synthetically made, often resulting from anthropogenic activities (Panter & Stegelmeier, 2011). These include for example, pharmaceuticals, fossil-derived fuels, fertilizers, pesticides, cosmetics, dyes, food additives and many more. These manmade xenobiotics have accumulated in the environment, causing great concern (Patterson et al., 2010; Sinha et al., 2009). The accumulation of these toxic compounds presents a danger due to their recalcitrant and persistent nature which allows them to resist degradation and to remain within an environment for prolonged periods of time, while polluting it (Godheja et al., 2016). As they make their way inside organisms, xenobiotics cause not only serious physiological concerns in lower and higher order eukaryotes, but also disrupt the entire balance within the ecosystem as they pass through the food chain (Magan et al., 2010). In humans, they have been associated with many life-threatening diseases such as cardiovascular diseases and cancers (Gupta et al., 2018; Mansouri et al., 2017; Zhang et al., 2019).

Over the years, various strategies have been developed, and to some extent, successfully applied for the remediation of xenobiotic-contaminated environments. These can be grouped into two main categories: physicochemical or nonbiological approaches on one side, and biological remediation strategies on the other side

(Godheja et al., 2016). Unlike their physicochemical counterparts, biological remediation approaches have gained much attention in recent years because of the current global shift toward greener, safer, and sustainable processes. In this regard, plants and microorganisms –together with their enzymes, have been used to degrade xenobiotics resulting in simpler, less toxic compounds. In mycoremediation, a type of biological remediation using fungal cultures, a particular class of fungi known as white-rot fungi (WRFs) has gained much interest due to their unique capability to completely mineralize lignin into carbon dioxide and water (Shah et al., 1992; Su et al., 2018a; Tišma et al., 2010). These organisms have been shown to not only degrade wood, but rather a broad spectrum of compounds. This degrading ability is likely the result of the structural similarities observed between environmental pollutants and lignocellulose, thus making white-rot fungi a potential solution to pollution (Deshmukh et al., 2016; Rhodes, 2014).

White-rot fungi achieve this remediation process using different metabolic pathways through the action of a group extracellular, non-substrate specific enzymes referred to as lignin-modifying enzymes (LMEs) (Hatakka, 1994; Janusz et al., 2017; Tišma et al., 2010). As secondary metabolites, LMEs synthesis does not occur during the growth stage and requires particular circumstances such as carbon and nitrogen starvation to happen. As a result, only limited amounts of these enzymes are produced. A good deal of research has therefore been done in this regard with the objective of increasing LMEs production. While great knowledge has been gained to date, LMEs production is yet to be fully understood and a closer look into its molecular basis could provide valuable insight.

1.2. Problem statement

The industrial revolution has contributed greatly to life as we know it today. From agriculture to pharmaceuticals and specialty chemicals, the industrialization of processes has been one of the greatest advancements of the 20th century. Unfortunately, this industrialization has also resulted in the production of many toxic chemicals that have found their way into the environment. These xenobiotic compounds have been polluting soil, water, and air for decades, finding their way into living organisms. Xenobiotic contamination has been associated with some grave

consequences such as pollution of drinking water, extinction of certain animal species, irreversible damages to soils, and different types of cancers (Anderson, 2005; Bjerregaard, 2012; Gupta et al., 2018). While WRFs have shown great potential for the mycoremediation of different environments, their effectiveness is dependent on the action of lignin modifying enzymes (LMEs), which in turn, is directly proportional to the amount of enzyme produced. As biological systems, certain WRFs have been reported to produce insufficient amounts of LMEs which makes their industrial utilization non feasible. Therefore, the need to take a deeper look into the action of various WRFs during their degradation of different xenobiotic compounds. The particular interaction between WRFs and different components of xenobiotics such as polycyclic aromatic hydrocarbons will help shed more light on this complex mechanism which remains poorly understood to this day.

1.3. Rationale

White-rot fungi possess the capability to efficiently mineralize lignocellulose. Due to their unique set of non-specific, extracellular enzymes, these fungi can break down not just lignocellulose, but also other structurally similar compounds which include many xenobiotics. As such, WRFs present a vast array of benefits ranging from biofuel production to mycoremediation. As a remediation tool, WRFs could help us address the huge environmental challenge that is pollution in a simpler, greener, and sustainable way. Better understanding of fungal enzyme production and degradation would contribute greatly to xenobiotic bioremediation and environment restoration. By studying the production of LMEs in several fungal isolates and by assessing their effectiveness for bioremediation, this research aimed to gain valuable knowledge regarding fungal enzyme interaction with different xenobiotic components; a knowledge that would serve as starting point for the development of strategies for enzyme production and mycoremediation using monocultures.

CHAPTER 2: LITERATURE REVIEW

2.1. Xenobiotic compounds

2.1.1. What are they?

Xenobiotic compounds are defined as chemicals, natural or synthetic, found within a biological system or an organism where they are not normally produced and are, therefore, not expected to be present. At the same time, the term xenobiotic can also include compounds found in concentrations higher than expected within a given system (Atashgahi et al., 2018; Embrandiri et al., 2016; Patel & Jyoti Sen, 2013). These compounds therefore require elimination from the system they find themselves in. Although the term xenobiotic can be applied to any environment, it is usually employed for compounds that are foreign to animal, more specifically human life including plant components, drugs, additives, environmental pollutants such as dyes, pesticides, polycyclic aromatic hydrocarbons and many more (Patterson et al., 2010). Besides them being foreign to animal life, most xenobiotics also present a health hazard due to their toxic nature (Godheja et al., 2016). These exogenous compounds have various origins. While some are naturally found in an ecosystem, others have been artificially created and introduced to the environment through anthropogenic activities (Patel & Jyoti Sen, 2013; Sinha et al., 2009).

2.1.2. Natural xenobiotics

Due to either their abundance or their toxicity to animal life, certain naturally occurring chemicals are also considered to be xenobiotics (Panter & Stegelmeier, 2011). These include plant constituents such as lignocellulose and phytotoxins, as well as bacterial and fungal toxins (Bucheli, 2014; Panter & Stegelmeier, 2011; Sen et al., 2020). From the 2 000 billion tons of carbon estimated to be part of our terrestrial ecosystems, 550 billion tons are found in vegetations, usually in the form of lignocellulose or lignocellulosic biomass, with an annual world production estimated to be around 10^{11} tons (Smith, 2019). Lignocellulose is a complex, heterogenous material made of three main polymeric substances –cellulose, hemicellulose, and lignin, together with some other components such as acetyl groups, some minerals, and phenolic substituents

(Isikgor & Becer, 2015; Pawar et al., 2013). Lignocellulose is known to be very resistant to degradation. It has evolved to develop properties that confer resistance to most types of enzymatic and chemical degradations. Its recalcitrance to degradation, is as a result of not only the crystallinity of cellulose, but also its polymerization degree, the presence of acetyl groups bound to hemicellulose, the hydrophobicity of lignin as well as the encapsulation of cellulose by a hemicellulose and lignin matrix (Isroi et al., 2011). Due to its abundance and the inability of animals, except those with symbiotic microorganisms (King et al., 2010), to digest it, lignocellulose qualifies as a natural xenobiotic compound.

Contrary to lignocellulose, other plant components are not produced in high quantities but are, however, still considered xenobiotics when found in animal systems. This is the case of most phytotoxins –plant secondary metabolites that serve various roles from development to defense mechanism (Bucheli, 2014). Depending on their concentration within a biological system, phytotoxins can present a toxicological risk and require elimination. Research has shown the effect of plant toxins on humans and other animals and the associated dangers. Lopes et al. (2019) have reviewed the various toxins found in plants and mechanisms associated with their elimination through milk. On the other hand, Panter & Stegelmeier (2011) presented the dangerous effects of phytotoxins. While some toxins can induce abortion, others are known to affect fertility either temporary or permanently. Similar to those found in plants, fungal and bacterial toxins also present possible health hazards for animal life. Remarkably, even in humans, it is important to note that certain natural chemicals endogenous to humans can also be considered xenobiotics. This is the case of human sex hormones that are excreted in urine, pass through water treatment, end up in water bodies and inside fish resulting in physiological changes within fish (Bjerregaard, 2012; Patel & Jyoti Sen, 2013).

2.1.3. Manmade xenobiotics and their consequences to the environment.

Unlike the previously discussed chemicals, many xenobiotic compounds are not naturally found in the environment and were synthetically created by men. These are referred to as manmade or environmental xenobiotics. The 20th century's industrial revolution resulted in the development of many industries that never existed before and together with these, many toxic chemicals have found their way into the

environment (Embrandiri et al., 2016; Ojo, 2007). During the manufacturing of high value products, many chemical reactions take place, changing the properties of initial chemicals, transforming them into complex ones, and resulting in the production of high amount of toxic waste (Dubey et al., 2014). From pharmaceuticals to polycyclic aromatic hydrocarbons, polychlorinated biphenyls, fertilizers, pesticides, herbicides, cosmetics, fuels, dyes, food additives and others, manmade xenobiotics have accumulated in the environment, especially in water, air and soil, causing great concern (Patterson et al., 2010; Sinha et al., 2009).

Over the years, thousands of xenobiotic compounds have been reported to enter the environment every single year through anthropogenic activities. This has become an environmental issue as they result in environmental pollution since they cannot be used by most organisms (Mishra et al., 2019; Patterson et al., 2010). As the world population continues to increase, xenobiotic-producing industries have become essential to human survival causing their constant augmentation within the environment (Dubey et al., 2014). The accumulation of these foreign compounds presents a serious problem due to their persistent nature which allows them to remain in the environment for many years (Godheja et al., 2016). Xenobiotic compounds present in soil, air, and water eventually end up within organisms causing not only serious physiological concerns in lower and higher eukaryotes, but also disrupting the entire balance within the ecosystem as they pass through the food chain (Magan et al., 2010).

Polyaromatic hydrocarbons (PAHs) are a good example of harmful xenobiotics found as pollutants in air, water, and soil. Although they can be produced naturally through biological processes, PAH pollution is mostly the result of anthropogenic activities including fuel burning (petrogenic), oil spills, and other combustion processes (pyrogenic) (Honda & Suzuki, 2020). As environmental pollutants, PAHs have been shown to be toxic to life both on land and in aquatic environments causing neurotoxic, immunotoxic, carcinogenic and mutagenic effects (Abdel-Shafy & Mansour, 2016; Honda & Suzuki, 2020).

2.1.4. Characteristics of xenobiotics

Xenobiotic compounds are usually identified by certain physicochemical and behavioral characteristics. They are known to be large, complex structures (Mishra et

al., 2019). Most xenobiotics are aromatic or contain some aromatic moieties which makes them very stable, insoluble or only partially soluble in water (Dinka, 2018). Many of them are, however, lipophilic which favors their uptake in many organisms' fat tissues (Wang et al., 2015). The chemical structure of xenobiotics is closely related to their effects as the presence of certain functional groups dictates their toxic effects (Iovdijová & Bencko, 2010). The stable chemical nature of most xenobiotic compounds makes them very resistant to environmental breakdown (Singh, 2017). Xenobiotic compounds are known to be very persistent which worsens their environmental impact as they can survive within an environment for years, even decades without being degraded (Poursat et al., 2019; Rieger et al., 2002).

2.1.5. Scope of xenobiotic compounds in the modern era and their environmental effects

In recent years, xenobiotic compounds have received increasing attention mainly due to the need to strike a balance between the importance of the various processes that result in their production, and their dangerous effects on health and the environment. Out of the thousand xenobiotic compounds currently present in our ecosystem, some have received significantly more attention than others mostly due to their highly toxic nature. This is the case of pesticides, polychlorinated biphenyls, pharmaceuticals, and polycyclic aromatic hydrocarbons (**Figure 2.1**) (Dinka, 2018). The toxicologic effects of these compounds in different environments are also summarized in **Table 2.1**.

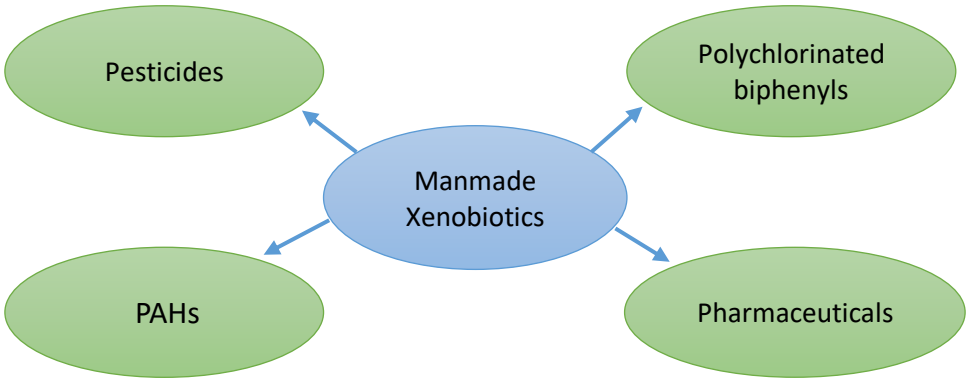

Figure 2. 1: Schematic representation of some of the most significant manmade xenobiotics

Pesticides

The ever-growing world population has resulted in a great deal of advancements in the field of agriculture with researchers developing more ways to optimize crops production. This has resulted in the introduction of pesticides, biologically active substances that prevent and destroy pests and weeds during crop growth and storage. Pesticide is a generic term that includes herbicides, insecticides, fungicides, and many other compounds (Iovdijová & Bencko, 2010). These compounds are currently found all over the world and can enter the food chain to bioaccumulate within organisms and cause serious concerns (Lushchak et al., 2018). From the different existing groups, organochlorines, consisting of chemicals such as DDT (Dichlorodiphenyltrichloroethane) have the most dangerous effects due to their lipophilic nature which allows them to accumulate inside most organisms (Dinka, 2018).

Pertinently, 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane, commonly known as DDT, was the first organochlorine insecticide formulated. It was later banned in the 70s due to its bioaccumulating and persistent nature with a half-life of over 30 years. DDT was the cause of many ecological problems including the extinction of many wildlife species (Anderson, 2005). In humans, cancer, neurological and immunodeficiency effects have been associated with this chemical (Mansouri et al., 2017).

Glyphosate or N-phosphomethyl[glycine], is an organophosphorus herbicide introduced by the agrochemical giant Monsanto in the 70s. Previously considered harmless, recent work has shown the hazardous nature of this chemical. In 2017, the International Agency for Research on Cancer (IARC) classified it as “possibly carcinogenic”. The carcinogenic effect of this herbicide was recently pointed out in a study by Zhang et al. (2019) where exposure to this herbicide and related ones resulted in a 41% increase in meta-relative risk of non-Hodgkin lymphoma (NHL). In an even more recent work, Peillex & Pelletier (2020) reviewed the toxicity of glyphosate and other related herbicides on human health. Evidence strongly suggests some cytotoxic and genotoxic effects. At the same time, this herbicide was found to increase oxidative stress, to impair cerebral functions, and correlates with certain types of cancer.

Another dangerous pesticide is Atrazine which has been reported to affect the reproduction of aquatic flora and fauna (Graymore et al., 2001). In humans and other

animals, Atrazine has been identified as an endocrine disruptor, the cause of different birth defects, and has been linked to various cancers from leukemia to lymphoma (Pathak & Dikshit, 2012).

Polychlorinated biphenyls

Once widely used in industries for their physical and chemical properties, polychlorinated biphenyls (PCBs) are a group of about 209 organic pollutants with various chlorine atoms attached to aromatic rings (Zhao et al., 2018). They are currently considered as some of the most persistent xenobiotics still found in the environment to this day, about 20 years after their restriction at the Stockholm convention on persistent organic pollutants in 2001 (Jing et al., 2018). With the ability to bioaccumulate within the food chain, these chemicals were able to contaminate humans causing chronic effects such as immune system damage, decreased pulmonary function, etc. (van den Berg et al., 2006). In a more recent study, Gupta et al. (2018) reported the effect of PCBs on human health even decades after their ban as PCBs are still linked to many cardiovascular diseases.

Pharmaceuticals

The medical field has also experienced many advancements in the past decades. With this, the pharmaceutical industry has seen a significant rise in the demand and consumption of its products (Kmmerer, 2010). Pharmaceuticals are usually considered to be “good” xenobiotics as they are intentionally introduced into the body to be taken up and used during the organism’s biochemical processes to combat a given disorder or pathology (Zimmermann et al., 2019). However, increased exposure to xenobiotic compounds is rapidly becoming a concern as these toxic chemicals are metabolized and then eliminated by the body into the environment where they inadvertently are still very active, earning them the collective terminology ‘pharmaceutically active compounds (PhACs)’. While humans are equipped, to a certain extent, with enzymes and microbiomes to prevent the harmful effects of these compounds when ingested, other organisms within the environment have not evolved these enzymes or abilities (Abdelsalam et al., 2020). Drugs such as hormones, anti-cancer medication and anti-depressants are among the mostly found pharmaceuticals in the environment (Devesh & Dayaram, 2015).

Estrogen –biological hormone derived from cholesterol and found in humans and other animals (Hamilton et al., 2017)– consumption has drastically increased since the introduction of contraceptive pills decades ago. This, together with its use to regulate growth in livestock, has resulted in an increased estrogen concentration in the environment including soil, plants, and water systems (Adeel et al., 2017). Estrogens have been reported to affect physiology of fish, domestical animals and humans. While male fish are made more feminine with low sperm count (Arnold et al., 2014), most domestic animals experience developmental abnormalities and humans run the risk of cardiovascular diseases and cancer when exposed to high concentrations (Adeel et al., 2017; Wocławek-Potocka et al., 2013).

Similarly to estrogens, many anticancer drugs' residues are eliminated by humans and find their way into aquatic environments where water treatment strategies have shown to have a limited to no effect on them (Heath et al., 2016; Patel & Jyoti Sen, 2013). Although they have the potential to be persistent, not a lot of work has focused on the environmental fate of anticancer drugs (Toolaram et al., 2014). To this day, research has demonstrated that cytostatic cancer drugs inhibit growth and damage DNA in aquatic species including algae, bacteria, crustaceans, and zebrafish (Elersek et al., 2016; Heath et al., 2016; Kovács et al., 2016). Moreover, inadvertent ingestion through potable water by humans exposes them to varying degrees of antagonistic and synergistic effects possibly from other medications they might be taking (A. Kumar et al., 2010).

Polycyclic aromatic hydrocarbons

Another important group of xenobiotics whose effects have been intensively studied during the past years is polycyclic aromatic hydrocarbons. PAHs are ubiquitous environmental pollutants produced mainly during the incomplete combustion of fuels although certain natural events such as wildfires can also produce them (Abdel-Shafy & Mansour, 2016). Composed of two or more benzene rings, PAHs are very stable chemicals which makes them highly persistent as pollutants (Guo et al., 2011). Depending on the number on benzene rings, they can exist either as gas or as solid particles (Haneef et al., 2020). Exposure to PAHs usually happens through vehicle and tobacco smokes as well as oil spills which affect water and soil (Guo et al., 2011). PAHs have been reported to affect reproduction, immunity and development in aquatic species and birds while having no toxic effect on terrestrial invertebrate unless found

in very high concentration (Abdel-Shafy & Mansour, 2016). Of the 100 plus identified, 16 PAHs have been identified as highly toxic, particularly to humans and are known as EPA (from the United States Environmental Protection Agency) PAHs (Anderson, 2005; Tongo et al., 2017) with carcinogenic effects reported in many studies after long-term exposure (Samburova et al., 2017; Tongo et al., 2017). This type of exposure can also result in decreased immune function, cataracts, kidney and liver damage, breathing problems, asthma-like symptoms, and lung function abnormalities (Abdel-Shafy & Mansour, 2016). In short-term exposure, Anthracene, benzo(a)pyrene and naphthalene have been reported to cause skin irritation or allergic reaction while other PAHs may cause eye irritation, nausea, vomiting, diarrhea, and confusion (Rengarajan et al., 2015).

Table 2. 1: Toxicologic effects of significant xenobiotics in different environments

Xenobiotic group	Chemicals	Effects	references
Pesticides	-Glyphosate (N-phosphomethyl[glycine])	-Possibly carcinogenic; cytotoxic and genotoxic effects; increased oxidative stress; impair cerebral functions.	(Peillex & Pelletier, 2020; Zhang et al., 2019)
	-Atrazine	-Affects the reproduction of aquatic flora and fauna; endocrine disruptor; birth defects; cancers like leukemia and lymphoma	(Graymore et al., 2001; Pathak & Dikshit, 2012)
Polychlorinated biphenyls	209 congeners	Immune system damage; decreased pulmonary function; cardiovascular diseases	(Gupta et al., 2018; M. van den Berg et al., 2006)
Pharmaceuticals	-Estrogens	-Make male fish more feminine with low sperm count; developmental abnormalities in domestic animals; cardiovascular	(Adeel et al., 2017; Arnold et al., 2014; Wocławek-

	-Anticancer drugs	diseases and cancer in humans -Growth inhibition and damage to DNA in aquatic species including algae, bacteria, crustaceans, and zebrafish	Potocka et al., 2013) (Elersek et al., 2016; Heath et al., 2016; Kovács et al., 2016)
Polyaromatic hydrocarbons	-Most -EPA PAHs	-Affect reproduction, immunity and development in aquatic species and birds (a) Short-term exposure: skin irritation, allergic reaction, eye irritation, nausea, vomiting, diarrhea, confusion (b) Long-term exposure: carcinogenic effects, decreased immune function, cataracts, kidney and liver damage, breathing problems, asthma-like symptoms, lung function abnormalities	(Abdel-Shafy & Mansour, 2016) (Abdel-Shafy & Mansour, 2016; Rengarajan et al., 2015; Samburova et al., 2017; Tongo et al., 2017)

2.2. Recalcitrance and persistence of xenobiotic compounds

In some cases, persistence of compounds is readily removed through natural processes of physical and weathering, as well as biodegradation. However, many xenobiotic compounds are known to be recalcitrant to breakdown which allows them to persist and accumulate within an environment for prolonged periods of time resulting in high levels of pollution (Singh, 2017). Xenobiotic compounds are known to possess certain physicochemical features and just like with many chemical

compounds, structure determines behavior (Katritzky & Fara, 2005). Many xenobiotic compounds are known to have high molecular sizes. This is the case of natural polymers such as lignocellulose, and synthetic ones such as plastics. Polymerization increases compound stability and durability which in turns decreases degradability (Webb et al., 2013). On one hand, polymers are made of tightly packed monomers linked together by strong bonds which gives them a crystalline structure that would require high amount of energy to be broken down (Malherbe & Cloete, 2002). On the other hand, the structures are too large to be ingested by most microorganisms that could break them down and therefore any degradation process will first require depolymerization of the huge polymer into its constituent monomers (Kawai, 2010). This was also presented by Huang et al. (2018) who established a correlation between the amount of aromatic rings in a PAH and its degradation rate. When only a couple of rings are present, a wide range of microorganisms, bacteria and fungi can use PAHs as carbon source. However, the more the aromatic rings, the larger the molecule gets, and lower the degradation. Depolymerization changes the physicochemical properties that give polymers their stability and resistance, therefore rendering them more susceptible to degradation. This explains the crucial role of pretreatment before lignocellulose utilization (Baruah et al., 2018).

Together with size, shape, and charge, the presence of functional groups within the structure also contributes greatly to the recalcitrance of xenobiotic compounds by giving them “unphysiological” features, mainly affecting bioavailability and solubility (Singh, 2017). As shown by Webb et al. (2013) and Kawai (2010), xenobiotics with a carbon backbone are made even more stable and resistant by the cyclic shape they have since the amount of energy required to break aromatic structures is much more than that of straight chains. The presence of aromatic groups also affects the solubility of the compounds making them highly hydrophobic and therefore insoluble or only partially soluble in aqueous environments (Makwana & Mahalakshmi, 2015). In the case of lignocellulose, due to its hydrophobicity and its specific binding with cellulose and hemicellulose, the aromatic structure of lignin contributes to the rigidity of the compound and its recalcitrance. Lignin creates a physical barrier preventing access to the polysaccharide content of lignocellulose (Zeng et al., 2014; Zoghlami & Paës, 2019).

Just like with aromatic rings, the presence and number of functional groups also brings great stability to xenobiotics and hence contributes to their recalcitrance and

persistence in the environment. Polychlorinated biphenyls (PCBs) are a prime example of this as the substitution of hydrogen atoms by chlorine ones in the benzene rings makes PCBs highly stable and persistent for decades in the environment (Dinka, 2018). As previously stated by Fewson (1988) and later confirmed by other researchers including Mathews & Sithebe (2018), the presence of chlorine substitution, especially at the ortho and meta positions makes compounds chemically and biologically inert which results in recalcitrance. This explains the need for reductive dichlorination —the replacement of chlorine substituents by hydrogens, in the degradation of halogenated xenobiotics (Wiegel & Wu, 2000) and the need for microorganisms capable of producing the necessary dehalogenases (Janssen et al., 2005).

The bioavailability of xenobiotic compounds is closely related to their chemistry and contributes greatly to their recalcitrance. In many cases, these compounds are highly hydrophobic but quite lipophilic (Dinka, 2018). This affinity for lipids usually results in the accumulation of these toxic compounds into fat tissues making them unavailable to catabolic enzymes that carry biochemical processes in aqueous environments (Abdel-Shafy & Mansour, 2016). In other cases, xenobiotic compounds are found to have a special affinity for soil and sediment particles to which they bind. They are therefore able to escape degrading microorganisms by hiding inside micro pores within soil aggregates and can bioaccumulate for years (Singh, 2017). However, in as much as bioavailability is necessary for degradation, having too much available toxic compounds does not solve the problem either. In fact, research has shown the toxic effect of many xenobiotic compounds on microbial species when present in high concentration.

In as much as recalcitrance depends on the physicochemical features of the compounds themselves, external factors also play an important role. This is the case of oxygen availability which is crucial to the degradation of various xenobiotics by serving as electron acceptor, for instance during the dehalogenation of PCBs (Bosma et al., 2005; Fewson, 1988). A good amount of xenobiotic biodegradation is carried under aerobic conditions making the presence of molecular oxygen a limiting factor not only as electron acceptor but also as prerequisite for microorganism survival (Janssen & Stucki, 2020). Other external parameters such as pH, temperature and water salinity also contribute to the biodegradability of xenobiotics (**Table 2.2**) and therefore their recalcitrance and resulting persistence (Singh, 2017).

Table 2. 2: Environmental factors that affect xenobiotic biodegradability

Environmental factor	Effect	Reference
Oxygen availability	-Serves as electron acceptor during biodegradation of PCBs	-(Bosma et al., 2005; Fewson, 1988)
	-Determines microbial communities found and the type of biodegradation (aerobic vs anaerobic)	-(Sinha et al., 2009)
Temperature	-Controls the rate of enzymatic reactions. Decrease in temp causes decrease in metabolic activity.	-(Eskander & Saleh, 2017)
	-Determines the types of microbe present (thermophilic vs mesophilic)	-(Moxley et al., 2019)
	-Affects solubility of pollutants i.e., PAHs	-(Sihag et al., 2014)
pH	-Affects enzyme catalytic activity as different enzymes have different optimal pH	-(Zhao & Yi, 2010)
	-Affects microbial population and richness	-(Liu et al., 2019)
Salinity	-Affects pH of the environment, accessibility of organic matter; might cause inhibition of microbes	-(Qin et al., 2012)
	-Affects types of microorganism present (halophiles)	-(Martins & Peixoto, 2012)
Moisture	-Determines the amount of soluble material present	-(Eskander & Saleh, 2017)

	-Affects soil respiration, enzymes and substrate motility; a decrease in water potential causes a decrease in metabolic activity	-(Manzoni et al., 2012; Moxley et al., 2019)

2.2.1. Current approaches to remediating xenobiotics

As more and more xenobiotics are continuously being introduced in the environment, scientists are constantly working on new strategies to either eliminate them or convert them into nontoxic compounds especially given the many dangers associated with their toxicity and persistence (Baldissarelli et al., 2019). Current advances in science and technology have introduced various remediation strategies than can be grouped into two main categories: physicochemical or nonbiological approaches on one side, and biological remediation strategies on the other side (Godheja et al., 2016).

2.2.1.1. Nonbiological remediation approaches

A myriad of strategies have been developed and to some extent successfully applied for the remediation of air, soil, or water using physical and/or physicochemical approaches (Baldissarelli et al., 2019; Cuerda-correa et al., 2020; Li et al., 2019). From the various developed approaches, some of the most effective include the following.

Soil vapor extraction

Also known as soil venting or vacuum extraction, soil vapor extraction (SVE) is one of the most common and cost-effective in-situ technologies used for the remediation of unsaturated soil contaminated with volatile or semi volatile organic compounds (Soares et al., 2010). This technique consists of applying a vacuum for air to circulate through a soil matrix, causing volatilization of pollutants, and transporting the produced vapors to extraction wells that are then treated before release into the atmosphere (Khan et al., 2004; Soares et al., 2010). Soil vapor extraction presents the advantages of being very efficient in soil decontamination, easy to carry out, and relatively fast with results being seen within months (Godheja et al., 2016). In their work, Soares et al. (2010) applied SVE for the remediation of benzene from sandy as well as humic soils. The technique was able to remove up to 92% benzene from sandy soil, and over 75% from humic soil. More recently, a full-scale case study was conducted by Labianca et

al. (2020) on the remediation of a petroleum hydrocarbon-contaminated site using SVE. Over a four-year period, the 13000 m^2 site had its pollutant concentration significantly deceased with an overall effectiveness of 73%.

Soil washing

Soil washing is a technique that uses solvents such water to scrub and separate soil components while decontaminating from pollutants, usually heavy metals. As the soil separated into fine and coarse particles, heavy metal contaminants are collected from the fines particles they usually bind to (Arwidsson et al., 2010). Although soil washing is effective in reducing the volume of contaminants which makes it easier for subsequent remediation technologies to be applied, the technique does not change the toxicity of the removed contaminants and still creates a disposal problem to be solved (Godheja et al., 2016). Zhai et al. (2018) used iron (iii) chloride as washing solvent for the remediation of metal-contaminated soil. When comparing remediation using soil washing alone with the combination of washing and immobilization, the later was found to be much more effective as the removed metal were later immobilized using lime. The effectiveness of soil washing depends on factors such as proper choice of solvent and chelating agent, pH, soil type, etc. (Arwidsson et al., 2010). As an example, when comparing ethylenediamine tetra (methylene phosphonic acid) (EDTMP) and polyacrylic acid (PAA), Feng et al. (2020) found PAA to be more effective in weak acidic and neutral conditions while EDTMP was found to act more as a chelator and required strong acidic or alkaline conditions.

Encapsulation

Encapsulation is a remediation technique used for water and soil remediation. It consists in entrapping contaminant molecules into a capsule made of a material they will be immobilized onto, usually silica, and therefore prevent their migration away from the site (Camenzuli & Gore, 2013). Encapsulation technology has successfully been used for both hydrocarbons and heavy metal contaminants in water and in soil environments (Akpoveta, 2020; Shebl et al., 2019). In as much as encapsulation has shown to be effective, its efficiency tends to decrease over time (Godheja et al., 2016), reason why many researchers are looking into novel materials for better results. Bezbaruah et al. (2011) introduced an alginate biopolymer with nanoscale zero-valent iron (NZVI) particles for the remediation of trichloroethylene. With this novel material, a 89-91% degradation was obtained after 2 hours and the material was found to last

for 4 months with minimal decrease in efficiency. Shebl et al. (2019) also introduced a zeolite-encapsulated Cu(II) complex material that was found to be less toxic than previously used materials.

Air stripping

Air stripping is another quite effective method of the remediation of volatile organic compounds (VOCs) from wastewaters. In this method, an air stripper is used to remove contaminants from a wastewater body by increasing the surface area of the wastewater and transferring the contaminants to the stripper which can be of various kinds (Abdullahi et al., 2014). Air stripping has been found to be quite effective with up to 95% removal for contaminants that are not water soluble (Godheja et al., 2016). The technique is similar to steam stripping which instead of air, uses steam for VOCs removal. Both techniques have been found effective for the removal of halogenated compounds from wastewater, but steam stripping was found to present the added advantage of being more sustainable (Toth & Mizsey, 2015).

Advanced oxidative processes (AOPs)

This is a collection of technologies that use strong oxidizing agents such as hydroxyl and sulfate radicals to remediate soil, water, or air by mineralizing pollutants into water, carbon dioxide, and other inorganic components (Baldissarelli et al., 2019). Unlike some techniques that only remove contaminants, AOPs convert them into less toxic or even non-toxic products without the need of any subsequent treatment (Deng & Zhao, 2015). Various AOPs, including physical, physicochemical, catalytic, ozone or UV-based, have been used, especially for wastewater treatment (Miklos et al., 2018; Oturan & Aaron, 2014). Fenton processes are some of the most common AOPs. Their principle resides on the oxidation of iron ions in the presence of hydrogen peroxide, permanganate, persulfate, or ozone to produce hydroxyl radicals that oxidize environmental contaminants forming products that are less toxic (Baldissarelli et al., 2019; Cuerda-correa et al., 2020).

Aside from the few discussed here, many more physicochemical remediation technologies have been employed including flocculation, microfiltration, electrodialytic remediation, dehalogenation, etc. (Godheja et al., 2016).

2.2.1.2. Biological remediation approaches

These include all remediation approaches that employ biological systems for the removal and/or transformation of xenobiotic contaminants from the environment. Research is continuously evolving and the use of novel solutions to current issues in on-going. With that came the focus on biological means of remediating our environment from xenobiotic compounds. Compared to physicochemical ones, biological strategies present the advantage of being eco-friendly, economical, sustainable, and do not result in the formation of other potentially toxic by-products (Peter, 2011; Rambabu et al., 2020).

Plant remediation or phytoremediation

Phytoremediation is the use of plants, usually in associating with microorganisms, to remove, store and/or degrade xenobiotic environmental contaminants (Peter, 2011; Peuke & Rennenberg, 2005). This technology can be used to decontaminate soil and water environments polluted with heavy metals, organic and inorganic compounds, petroleum hydrocarbons, pesticides, explosives, etc. (Favas et al., 2014). Phytoremediation has all the advantages associated with biological processes plus the advantage of being applicable to a large-scale field, prevent soil erosion and metal leaching, and improve soil fertility (Yan et al., 2020). Seven phytoremediation approaches can be distinguished based on the fate of contaminants once taken by the plant and its destination. These are phytoextraction, phytotransformation, phytovolatilization, rhizofiltration, phytostimulation, phytostabilization, and phytoscreening (Anawar et al., 2017; Godheja et al., 2016; Peuke & Rennenberg, 2005). Various plant species have successfully been used for phytoremediation including water hyacinth (Saha et al., 2017), Indian mustard (Rathore et al., 2019), sunflower (Adesodun et al., 2010), and many more.

Microbial remediation

Microbial remediation is the use of microorganisms, bacteria or fungi, to remove and degrade environmental pollutants. These organisms use pollutants as carbon or nitrogen source and using metabolic pathways to produce enzymes necessary for the degradation (Bilal & Iqbal, 2020a; Jaiswal & Shukla, 2020). With their ability to naturally degrade a broad range of xenobiotic compounds, microorganisms are to this day the most promising remediation strategy. And therefore, a great deal of research

has been done on their metabolic pathways and molecular features that give them the capability to degrade these contaminants. Jin et al. (2018) reviewed the mechanisms of microbial remediation on heavy metal-contaminated soils and found biosorption to be the most used mechanism in microbes. The efficacy of these mechanisms was also found to depend on parameters such as temperature, pH, and the nature of the substrate. Novel microorganisms are also being discovered and introduced to contaminated environments to optimize remediation. A fluoride-resistant bacterium was isolated by Mukherjee et al. (2017) from contaminated groundwater and found to remove 82% of fluoride when the starting concentration was 20 mg/L.

Fungi have been successfully used for the mycoremediation of various xenobiotics, especially PAHs and other similar compounds (Liu et al., 2017; Ma et al., 2021; Xu et al., 2017). Compared to bacteria, fungi present the advantage of producing extracellular degradative enzymes which can hydrolyze xenobiotic polymers into simpler forms that can then be used by these organisms as carbon source (Ellouze & Sayadi, 2016; Karigar & Rao, 2011).

Whether with fungi or bacteria, microbial remediation is usually carried in two possible ways. In biostimulation, organic and inorganic nutrients are added to the environment to enhance indigenous microorganisms as they degrade contaminants (Peter, 2011). This is done through processes such as biosparging which consists of injecting air and nutrients into soil saturated zones in order to enhance the biological activity of microorganisms found within that soil (Azubuike et al., 2016). Fertilization and composting are other biostimulation strategies used principally for soil remediation (Godheja et al., 2016; Peter, 2011). In bioaugmentation however, microorganisms possessing biodegradation abilities are added to an environment to assist indigenous ones with the remediation process (Cycoń et al., 2017; Jiang et al., 2016). This approach has made possible the bioremediation of various environments through the isolation and addition of adapted and/or mutated microorganisms from extreme environments (Mukherjee et al., 2017). Although important advances have been made, research is still on-going to develop better remediation strategies to tackle xenobiotics.

2.3. The role of biocatalysis in bioremediation

Biocatalysis can be defined as the use of a biological catalyst to transform or convert a substrate into a product. This catalyst can be a purified enzyme, a solution of

enzymes in a lysate, or wholes cells (Hughes & Lewis, 2018). Present for centuries, the use of biocatalysis has seen a significant increase since the second half of the 20th century with the introduction of industrial bioprocessing to shift from traditional concepts of only focusing on production yields, but to also consider sustainability with waste management, utilization of available raw material and avoidance of hazardous reagents (Sheldon & Woodley, 2018). From pharmaceuticals to commodity chemicals and many other industries, the shift to biocatalysis presents various advantages including milder reaction parameters resulting in less energy consumption, the use of aqueous environments in most cases which minimizes the amount of toxic waste produced, stability and high level of specificity, etc. (Abdelraheem et al., 2019). Biocatalysis has also become of crucial importance in remediation due to the various advantages it presents over traditional remediation strategies as previously discussed. Therefore, the need to take a closer look at this process.

2.3.1. Function of enzymes in biodegradation

In biodegradation, living organisms, usually bacteria and fungi, use their cellular machinery to mineralize or transform a chemical compound into a simpler form. When dealing with xenobiotic compounds, the transformation process usually results in a product that is less toxic or non-toxic (Joutey et al., 2013; Urbanek et al., 2018). Microorganisms degrade xenobiotic compounds by introducing them into their metabolic processes where a series of enzymatic reactions use the xenobiotic compound as substrate. This is usually done in two different ways: growth-linked biodegradation and cometabolism. In growth-linked biodegradation, microorganisms use the pollutant as a sole carbon source to provide growth and energy (Joutey et al., 2013). In some cases, however, certain pollutants can be degraded by microorganisms and not be used as carbon source, usually due to the presence of another substrate that serves as preferred carbon source. This process is known as cometabolism (Nzila, 2013).

As biological catalysts, microbial enzymes are the prime actors in biodegradation since it is through their catalytic actions that compounds are transformed (Gurung et al., 2013; Rao et al., 2010). Enzyme action is of crucial value to microbial life because enzymes catalyze all processes that are essential to life including growth, energy production, signaling, defense mechanism, and many more (Gurung et al., 2013).

During growth-linked biodegradation, microorganisms introduce the pollutant into biochemical pathways that direct growth and energy production.

Based on the type of pollutant being used, different types of enzymes are necessary. These are usually divided into two main categories: extracellular and intracellular enzymes (Karigar & Rao, 2011). Extracellular enzymes are those that are produced by microorganisms but then excreted into the outside environment to carry out their catalytic action (Li, Sun, et al., 2019; Traving et al., 2015). Conversely, intracellular enzymes are produced and function within the microorganism (Amblee & Jeffery, 2015). The type of enzyme produced depends not only on the microorganism's genetic abilities, but also on the pollutant to be degraded. In the case of large structures such as PAHs, lignocellulose, synthetic plastics, and other high molecular weight xenobiotics, extracellular enzymes are needed to breakdown these polymers into their constituent monomers which can then pass the cytoplasmic membrane and be introduced into catabolic pathways that produce energy (Agrawal et al., 2018; Andlar et al., 2018; Li, Sun, et al., 2019). Mohanan et al. (2020) recently reviewed the biodegradation of plastics and established that the process takes place in two stages namely depolymerization and mineralization. Degradation begins with extracellular action to hydrolyze the plastic into shorter intermediates which are then assimilated intracellularly to release CO_2 after complete mineralization. In their work, Muthukumar & Veerappapillai (2015) present different microorganisms, both fungi and bacteria, reported to degrade different types of plastics.

Different groups of microbial enzymes play important roles during biodegradation of xenobiotic compounds with hydrolases and oxidoreductases being the main classes (Rao et al., 2010).

a) Hydrolases

Hydrolytic enzymes play a vital role in the biodegradation of xenobiotic compounds, especially larger ones with molecular weight above 600 Da which makes them unable to pass through the cell membrane (Karigar & Rao, 2011). These enzymes catalyze the cleavage of chemical bonds which causes size reduction and detoxification of pollutants. The biodegradation of petroleum hydrocarbons, plastics, insecticides, and many other xenobiotics depends primarily on the action of hydrolytic enzymes such as lipases, cellulases, and proteases, produced by fungi and bacteria, especially those produced extracellularly (Karigar & Rao, 2011; Mohanan et al., 2020). For instance,

Mislan & Gates (2019) investigated the biodegradation of bitumen with hydrolytic enzymes that efficiently convert the pollutant into glucose and fatty acids. Hydrolytic enzymes are usually the first group of enzymes used in biodegradation creating intermediates that can further be broken down by the action of other enzymes. Zafra & Cortés-Espinosa (2015) reviewed the biodegradation of PAHs and pointed out the importance of hydrolytic enzymes, together with oxidoreductases, in the first steps of the degradation process.

b) Oxidoreductases

Microbial detoxification of many xenobiotic compounds is carried through oxidative coupling reactions that are catalyzed by oxidoreductases. These enzymes catalyze oxidation/reduction reactions with electron transfer from a donor to an acceptor resulting in the production of energy and of an oxidized harmless compound (Karigar & Rao, 2011; Khatoon et al., 2017). Mahmood et al. (2016) have reviewed the role of bacterial oxidoreductases in the detoxification of azo dyes. Bacteria biodegrade azo dyes in two phases. After the first step which results in decolorization, an energy producing oxidative process detoxifies the intermediates by means of oxidoreductases such as peroxidases and tyrosinases. Fungal oxidoreductases, especially peroxidases and laccases, have also been intensively studied for their role in the biodegradation of lignocellulose and structurally similar compounds including PAHs (Kadri et al., 2017; Steffen, 2003). For instance, Zhang et al. (2016) characterized a manganese peroxidase from the fungus *Trametes* sp. 48424 with great degradative abilities on broad range of dyes and PAHs. Oxidoreductases also contribute to the biodegradation of various halogenated pollutants including pesticides and PCBs (Rao et al., 2010).

2.3.2. Biocatalysis: Biodegradation vs Bioremediation

As primary decomposers and recyclers in the ecosystem, fungi and bacteria perform biodegradation as part of their normal functioning (Berg & Laskowski, 2005). To ensure their survival, heterotrophic microorganisms must feed on available organic matter within their surroundings. These are usually plants and animals, dead or alive that serve as carbon source for microbial growth and energy production (Khatoon et al., 2017). Microorganisms are equipped with metabolic pathways, usually catabolic

ones that enable the successful utilization of organic matter. These catabolic pathways depend on the action of various enzymes like those previously discussed. Microorganisms carry out biodegradation under aerobic or anaerobic conditions but much focus has been given to aerobic biodegradation since the vast majority of microorganisms function in the presence of oxygen and are able to output greater amounts of energy (ATPs) under this condition (Ghattas et al., 2017; Singh, 2017). Whether under aerobic or anaerobic conditions, biodegradation usually consists of 3 main steps. In the first step of the process, microorganisms take in the organic matter to be degraded. Depending on the complexity of the substrate, hydrolytic and/or oxidative enzymes might be necessary to convert the complex matter into a simpler form. Next, the formed intermediate enters the main degradative pathway which can be glycolysis or acidogenesis. And lastly, energy, CO_2, water, or methane are produced either via TCA cycle and oxidative phosphorylation in the case of aerobes, or via fermentation and methanogenesis in the case of anaerobes (Alshehrei, 2017; Eskander & Saleh, 2017; Ortiz-Hernandez et al., 2013).

The accumulation of xenobiotic compounds in the environment, especially those produced by anthropogenic activities, has resulted in their utilization by various microorganisms as biodegradation substrates (Bilal & Iqbal, 2020b; Janssen et al., 2005). Either through their traditional mechanisms or newly evolved ones, microorganisms can recognize major pollutants and convert them into harmless products (Sinha et al., 2009). This natural ability has been exploited by humans in the process known as bioremediation to remove environmental pollutants including heavy metals (Tarekegn et al., 2020), dyes (Mahmood et al., 2016), plastics (Urbanek et al., 2018), pesticides (Baldissarelli et al., 2019), PAHs (Labianca et al., 2020), and many more. In the bioremediation of PAHs for instance, their structural resemblance to the naturally occurring lignin has allowed their utilization by lignin-degrading microorganisms (Kadri et al., 2017; Zafra & Cortés-Espinosa, 2015). In most cases, PAHs undergo catalytic attack by oxygenases that break their aromatic rings, forming intermediates that are converted to cis-dihydrodiols, catechols, and then aliphatic acids or TCA intermediates used for energy production (Gupte et al., 2016; Sharma et al., 2016). This is a similar scheme to that of lignin biodegradation as demonstrated by (Khatoon et al., 2017).

2.3.3. Limitations to biocatalysis

Although bioremediation has been successfully used for the removal of environmental pollutants, its application is not without flaws. In certain cases, bioremediation cannot be performed or completed due to the presence of a pollutant or an intermediate that cannot be assimilated by a given microorganism (Nzila, 2013). In most of these cases, the absence of enzymes capable of converting the pollutant or intermediate into a harmless product is usually the underlying issue, therefore the use of microbial consortia to increase the diversity of available enzymes (Nzila, 2013; Sharma et al., 2016). Enzyme specificity —the characteristic of enzymes to discriminate among substrates, even those that are closely related (Hedstrom, 2010)— is an important aspect of biocatalysis. Enzymes catalyze reactions in three main steps. First, the enzyme recognizes one of many specific substrate(s), brings them into its active site following a particular orientation and forms an enzyme-substrate complex. Next, reactive amino acid within the enzyme active site catalyze a particular reaction which converts reagent(s) into product(s). And finally, the enzyme releases the product(s) and returns to its initial state (Blow, 2000; Kürten, 2018). Formation of the enzyme-substrate complex is a very selective mechanism that has been described using two models. Introduced by Emil Fischer in 1894, the “lock and key” model was the only accepted enzyme-substrate binding model for a long time. In this model, the enzyme and substrate are viewed as geometrically complementary and fit into each other as a key inside a lock (Kürten, 2018; Tripathi & Bankaitis, 2017). Years later, the “induced-fit” model was proposed by Koshland in 1958 where he suggested that upon arrival within the enzymatic active site, the substrate undergoes conformational changes that result in a perfect fit between the two entities (Schmitt et al., 2009). Both of these models are currently accepted as plausible explanation of enzyme activity, further, these models put an emphasis on the high level of specificity required for catalysis to occur as demonstrated by Hedstrom (2010) with serine proteases which require chemo-, regio-, and stereospecificity.

While it can be viewed as an advantage, especially in significant industrial processing where only a particular reaction is targeted, high specificity presents a drawback in bioremediation as it limits the extent to which pollutants can be utilized by a given microorganism (Abdelraheem et al., 2019; Tanokura et al., 2015). Based on the particularity of their active sites, many microbial enzymes can only effectively bind

certain pollutants, often requiring the introduction of bioaugmentation in order to introduce new biocatalysts capable of biodegrading the remaining recalcitrant compounds (Cycoń et al., 2017; Sharma et al., 2016). This was explained by Lyon et al. (2013) in their review on bioaugmentation for groundwater bioremediation. Anaerobic bioremediation of vinyl chloride, a highly toxic pollutant, has shown to be possible only when microorganisms known as *Dehalococcoides* spp. are added to the environment. These microbes are able to degrade chlorinated organic compounds through the action of specific dehalogenases (Taş et al., 2011).

However, not all microbial biocatalysts demonstrate absolute specificity. Some enzymes, particularly those produced extracellularly, demonstrate low substrate specificity which has enabled their application for the bioremediation of a wide range of xenobiotic compounds (Mahmood et al., 2016; Rugabber & Talley, 2006). These enzymes have group specificity where they recognize functional groups or particular bonds which they act upon regardless of the rest of the molecule (Robinson, 2015). This is the case of lignin-modifying enzymes (LMEs) whose low substrate specificity has allowed their application in the bioremediation of a wide range of xenobiotic pollutants (Kumar & Chandra, 2020).

2.4. Fungi in xenobiotics degradation

2.4.1. White-rots fungi (WRFs)

White-rot fungi are a diverse group of fungi that belong primarily to the phylum *Basidiomycota*. They constitute more than 90% of all wood-rotting basidiomycetes and are the only identified organisms with the ability to efficiently mineralize lignin (Rodríguez-Couto, 2017). White-rot fungi are made of branching hyphae, usually 1 to 2 µm in diameter, growing from each tip (**Figure 2.2**). They begin as spores that invade the cells of wood to be located within lumen walls. From there, they develop hyphae that quickly invade the entire wood structure and secrete a great number of enzymes and metabolites necessary to the degradation process (Kirk & Cullen, 1998). Unlike other wood-decaying fungi which are usually associated with the decay of coniferous wood (gymnosperms), white-rot fungi can efficiently decay hardwood (angiosperms). This has been demonstrated by many studies such as that of Rudakiya & Gupte (2017)

where two white-rots, *Pseudolagarobasidium acaciicola* and *Tricholoma giganteum* were used to degrade two Indian hardwoods.

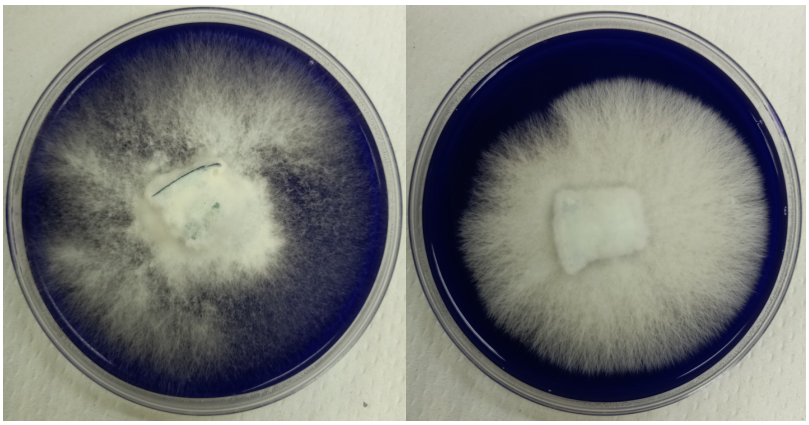

Figure 2.2: Picture of growing white-rot fungi on petri dishes (own images)

What makes white-rot fungi unique is their capability to completely mineralize lignin into carbon dioxide and water (Shah et al., 1992; Su et al., 2018a; Tišma et al., 2010). This is done through the action of a group extracellular, non-substrate specific enzymes referred to as lignin-modifying enzymes (LMEs) (Hatakka, 1994; Janusz et al., 2017; Tišma et al., 2010). Due to this, white-rot fungi have had multiple applications throughout the years. One of the many roles they play is as recyclers. First considered by Lawson and Still in 1957, the use of white-rot fungi for biological delignification has been shown to play a major role in the recycling of organic matter within the ecosystem. With their exceptional ability to decompose lignocellulose, white-rot fungi release important nutrients back into the environment (Zahmatkesh et al., 2018). They can also degrade a variety of chemicals similar in structure to lignin and can access many non-polar and insoluble compounds which makes them essential contributors to terrestrial life as we know it (Levin et al., 2003; Riley et al., 2014).

This lignin degrading capability has made white-rot fungi a potential solution to the world's energy and pollution problems. Species such as *Phanerochaete chrysosporium* and *Pleurotus ostreatus* have been intensively studied for biological pre-treatment (Madadi & Abbas, 2017; Su et al., 2018b; Yao & Nokes, 2014). Just like bacteria, fungi present an effective, cheaper, less energetic and more eco-friendly alternative to traditional pre-treatment methods (Madadi & Abbas, 2017). However, white-rot fungi have demonstrated a higher efficiency of degradation as compared to

bacteria (Janusz et al., 2017). For this reason, they can also be applied to various industries. In the pulp and paper industry, white-rot fungi present a low cost, environmentally friendly alternative to traditional pulping. Pre-treatment of wood chips with white-rot fungi (biopulping) requires less equipment, reduces the amount of energy being consumed, therefore being environmentally friendly (Husaini et al., 2011). Furthermore, white-rot fungi have shown to be useful for the biodegradation of recalcitrant organic pollutants structurally similar to lignin which has earned them an important place in bioremediation (Xu & Zhou, 2017; Yadav & Yadav, 2015).

2.4.1.1. Lignin-modifying enzymes (LMEs)

Also known as ligninolytic enzymes or ligninases, LMEs are a group of catalysts, with the most commonly identified being lignin peroxidase (LiP) (1.11.1.14), manganese peroxidase (MnP) (1.11.1.13) and laccase (1.10.3.2), produced by white-rot fungi and other lignocellulose degrading microorganisms (Andlar et al., 2018; Ellouze & Sayadi, 2016; Gai et al., 2014; Plácido & Capareda, 2015). Due to their low substrate specificity, these enzymes have also shown the ability to degrade many xenobiotics structurally similar to lignin (Isroi et al., 2011; Rodríguez-Couto, 2017). Fungal LMEs are preferred to their bacterial counterpart for various reasons with the most important ones being their extracellular nature, their applicability to a broad range of substrates, and their adaptability to various extreme environments and pollutant concentrations (Ellouze & Sayadi, 2016; Kumar & Chandra, 2020; Rhodes, 2014). In bioenergy production for instance, ligninolytic enzymes have been reported in various studies for their application in biological pre-treatment using different feedstocks. In their work, Su et al. (2018b) as well as Wang et al. (2013) have studied the ability of ligninolytic enzymes to degrade corn stover. Using different white-rot strains, both studies were able to efficiently delignify the substrate by over 50% more than the control. Other feedstocks including wheat straw (Gai et al., 2014), corn straw (Li et al., 2020), rubberwood (Nazarpour et al., 2013), pulp and paper sludge (Yunqin et al., 2010), and many more have also been used.

The efficiency of LMEs on environmental pollutants have also been demonstrated throughout the years. In their recent work, Henn et al. (2020) have reported the biodegradation of atrazine, a widespread chlorinated herbicide, using white-rot fungi and their lignin-degrading system. Over a period of 20 days, close to 40% of the

pollutant was degraded, showing high potential for atrazine remediation using white rots. Yadav & Yadav (2015) reviewed the application of these enzymes on various environmental pollutants ranging from wastewater to dyes, coal, and polymers. This extensive research once again put forth the enormous potential of ligninases for various industries and environmental remediation.

2.4.1.2. Catalytic mechanisms of LMEs

Although the complete mechanism that governs the activity of LMEs is yet to be elucidated, model-based experiments have been able to bring some insights into their processes (Kumar & Chandra, 2020).

Lignin peroxidase

Lignin peroxidase (LiP) is a monomeric homoprotein containing a Fe^{3+} that is penta-coordinated to its 4 heme tetrapyrrole nitrogens and to a histidine residue (Plácido & Capareda, 2015). In its catalytic reaction, LiP is oxidized by hydrogen peroxide resulting in the formation of a 2-electron-oxidized intermediate (Lip-I). This intermediate then oxidizes aromatic substrates by one electron giving the second intermediate (Lip-II). A third oxidation occurs which brings the enzyme back to its resting state (Cullen & Kersten, 2004; Isroi et al., 2011). This mechanism can be seen in the following reactions:

$$LiP[Fe(III)] + H_2O_2 \longrightarrow LiP\text{-}I\ [Fe(IV)=O^+] + H_2O$$

$$LiP\text{-}I + AH \longrightarrow LiP\text{-}II\ [Fe(IV)] + A^+$$

$$LiP\text{-}II + AH \longrightarrow LiP + A^+$$

Among the many reactions catalyzed by lignin peroxidase, the oxidation of benzyl alcohols to their corresponding aldehydes or ketones has been the most exploited in the formulation of assays, with the veratryl alcohol assay being a prime example (Wong, 2009). This reaction has been exploited to measure lignin peroxidase activity in various studies. For instance, Yasmeen et al. (2013) exploited this reaction for the optimization of lignin peroxidase production using response surface methods. Other

researches including (Cerniglia, 1993; Ćilerdžić et al., 2011) had previously confirmed the efficacy of this assay for lignin peroxidase activity.

Manganese peroxidase

Similar to lignin peroxidase, manganese peroxidase (MnP) is also a heme-containing protein requiring hydrogen peroxide for its oxidation. The enzyme requires manganese which acts as a mediator during its activity (Chen et al., 2011). As Mn^{2+} is oxidized to Mn^{3+}, the latter is then free to mediate the oxidation of various organic substrates. The reaction mechanism includes two one-electron oxidations coupled with the formation of an intermediate cation radical (Xu et al., 2017b).

$$MnP + H_2O_2 \longrightarrow MnP\text{-}I + H_2O$$

$$MnP\text{-}I + Mn^{2+} \longrightarrow MnP\text{-}II + Mn^{3+}$$

$$MnP\text{-}II + Mn^{2+} \longrightarrow MnP + Mn^{3+} + H_2O$$

$$\text{Then, } Mn^{3+} + RH \longrightarrow Mn^{2+} + R^{\cdot} + H^{+}$$

This mechanism governs all manganese peroxidase assays. Although various organic substrates can be used, hydrogen peroxide and manganese ions are essential to all assays (Dhouib et al., 2005; Yasmeen et al., 2013).

Laccase

Laccase is a copper-containing enzyme that belongs to the oxidoreductase enzymatic group. This enzyme oxidizes a variety of substrates as a consequence of the presence of a mediator which facilitates reactions to occur without the need of the substrate to enter the enzyme's active site (Chen et al., 2011; Datta et al., 2017). The laccase-catalyzed reaction is a one-electron oxidation coupled with the reduction of O_2 to H_2O. Laccase substrates can be phenolics, aromatic amines or any other electron-rich substrates (Cullen & Kersten, 2004).

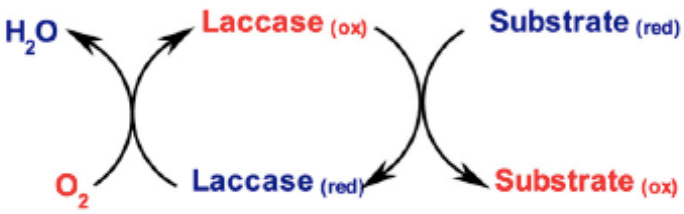

Figure 2.3: Schematic representation of laccase-catalyzed redox cycles for substrate oxidation. Adapted from Bassanini et al. (2021).

Due to its low redox potential, laccase requires a mediator which can be natural or artificial. Some of its common mediators include ABTS, TEMPO and HBT (Kumar & Chandra, 2020). Laccase activity has been studied over the years using these mediators. Dhouib et al. (2005) used ABTS for laccase activity, while Cerniglia, 1993) utilized the hydrogen peroxide-independent oxidation of syringaldazine to monitor laccase activity.

2.4.1.3. Multi-specificity characteristics of white-rot fungi (WRFs) and its implication to bioremediation

WRFs have been shown to be capable of not only degrading wood, but rather a broad spectrum of compounds. In bioremediation, structural similarities observed between environmental pollutants and lignocellulose have made white-rot fungi a potential solution to pollution through a process called mycoremediation (Deshmukh et al., 2016; Rhodes, 2014). Compounds such as PAHs, explosives, alkanes, and fuels can be degraded by white-rot fungi thanks to the low specificity of their degradative enzymes. Ellouze & Sayadi (2016) have reported on the ability of white-rot fungi to utilize xenobiotic compounds as nutrients and reduce them to simpler, less, or non-toxic forms. In a recent study, Dao et al. (2019) investigated the ability of various white-rot fungi to degrade the pollutant 2,3,7,8- tetrachlorodibenzo-p-dioxin and a *Rigidoporus* species was found to be most efficient in bioremediation with a 73% degradation rate after 28 days. A good amount of work has also been done on the bioremediation of PAHs using white-rot fungi (Gupte et al., 2016; Rao et al., 2010; Zafra & Cortés-Espinosa, 2015; Zhang et al., 2016). The multi-specificity of WRFs is a direct consequence of their LMEs and the mechanisms that govern their catalytic actions.

The observation with WRFs' catalytic mechanisms is that their LMEs' activity is not limited to a given substrate, but they rather recognize certain functional groups like aromatic rings which they cleave through oxidative reactions. The low specificity of LMEs is as a result of limited or sometimes no direct interaction between the enzyme's active site and the substrate (Kumar & Chandra, 2020). In the case of laccase for example, catalysis can be done on phenolic compounds in a direct way, or on non-phenolic substrates in an indirect way, facilitated by the presence of a mediator (Plácido & Capareda, 2015). During direct reaction, the phenolic substrate enters the active site and only encounters one of the three copper atoms (T1) within the site which oxidizes it to a radical form. The oxidized substrate is immediately released from the site and further non-enzymatic reactions result in the phenolic ring cleavage. This limited interaction between enzyme and substrate explains the low specificity as any phenolic can follow the same reaction scheme (Chaurasia et al., 2013; Matera et al., 2008). In the presence of a non-phenolic substrate on the other hand, no contact occurs between enzyme and substrate. In this case, laccase is converted to its oxidized version through the action of molecular oxygen. The oxidized enzyme then reacts with a mediator by oxidizing it, and it is this mediator that will then act non-enzymatically on the substrate which will never come in direct contact with the enzyme (Chaurasia et al., 2013; Plácido & Capareda, 2015).

Similar reactions are also observed with peroxidase enzymes. Both enzymes require oxidation from hydrogen peroxide which converts them to highly reactive intermediates that can in turn oxidize aromatic and non-aromatic substrates through single electron transfer making radicals that will further react non-enzymatically (Järvinen et al., 2012; Romero et al., 2019). Similar to laccase, manganese peroxidase also catalyzes reactions by using the redox couple Mn^{2+}/Mn^{3+} which facilitates substrate attack with no contact with the enzyme. The highly reactive Mn^{3+} can oxidize a substrate without it entering the active site which increases the substrate range of the enzyme (Kumar & Chandra, 2020; Xu et al., 2017b).

Since many recalcitrant xenobiotic compounds are aromatics or at least contain aromatic moieties, the reaction schemes followed by LMEs explain the capacity of these enzymes to degrade a broad range of pollutants. From PAHs to dyes, pesticides, PCBs and many more, LMEs can catalyze the bioremediation of xenobiotic pollutants by starting with radical formation and aromatic ring cleavage which premises their

subsequent utilization as carbon sources leading to mineralization (Janssen et al., 2005; Sharma et al., 2016).

2.4.2. Fungi metabolism

Fungi have developed many metabolic pathways in order to successfully perform their role as decomposers and recyclers. Their catabolic pathways break down a wide range of substrates such as cellulose and lignin to be used as food source (Ferdeş et al., 2020). They are also brilliant engineering factories for the synthesis of diverse compounds including peptides, pigments, amino acids as well as some toxic products like antibiotics and mycotoxins (Scharf & Brakhage, 2013; Wisecaver et al., 2014). Studies of specialized fungal metabolic pathways have revealed an important feature also existing in animal genes which is the presence of gene clusters (Rokas et al., 2018). These are genes physically linked on chromosomes (Wisecaver & Rokas, 2015). However, fungal gene clusters differ from animal ones in that they are made of evolutionary unrelated genes whereas those in animals are tandemly duplicated. These gene clusters are found to take part in many important processes such as the synthesis of secondary metabolites, catabolism of xenobiotics and the metabolism of various compounds including vitamins, amino acids, vitamins, and carbohydrates (Rokas et al., 2018; Wisecaver et al., 2014).

Fungal metabolic pathways have been categorized into two main groups depending on their products. On one hand we have primary metabolic pathways which produce primary metabolites necessary for nutrition, growth, and other vital functions (Goyal et al., 2016), and the other hand we have secondary metabolic pathways which give rise to secondary metabolites mainly used for defense, interactions with environment, and other stress-induced mechanisms (Keller, 2019). In fungi, primary metabolites are principally synthesized during the initial growth phase of the organism as they are needed for replication and growth to happen. Maximum amounts of these compounds are measured towards the end of the growth phase (Chroumpi et al., 2020). However, secondary metabolites are usually synthesized during spore formation (Calvo et al., 2002). They form a chemically diverse group of compounds which plays a rather crucial role in our ecosystem either by their undesirable interactions with plants and animals as toxins or their pharmaceutical attributes (Goyal et al., 2016).

Genes responsible for the synthesis of secondary metabolites are commonly found in clusters which has facilitated their identification from entire genomes (Leitão & Enguita, 2014). Under normal conditions, these genes could be silent and require either a genetic modification or variation in growth conditions for them to be induced (Boruta, 2018). In their work, Keller (2019) looked at the impact of environmental signals on biosynthetic gene cluster (BGC) regulation in fungi and presented environmental stimuli as being responsible for transcriptional activation of certain BGCs and subsequent secondary metabolite production. This has been extensively proven with fungal strains especially in drug discovery where changes in nutritional inputs result in different secondary metabolomes due to a process referred to as one strain-many compounds (OSMAC) (Gubiani et al., 2016; Hewage et al., 2014). Manipulation of growth media and/or physical parameters could therefore act as an inducer for certain BGCs while acting as a repressor for others (Boruta, 2018; Calvo et al., 2002).

In the case of WRFs, secondary metabolism resulting in LMEs synthesis has been observed to occur during stress or carbon/nitrogen starvation (Ayeronfe et al., 2019; Silva et al., 2010). In their research, Irshad & Asgher, (2011) investigated the effect of C:N ratio in the optimization of LMEs production by *Schizophyllum commune* and observed that an increase in C:N ratio resulted in repression of LMEs activities due to growth inhibition. Physiological studies of fungal growth have also shown that LMEs production commences only after depletion of nitrogen which also slows linear growth Wu & Zhang (2010). Various authors have associated LMEs synthesis with a catabolic repression. Tonon et al. (1990) had already suggested that nitrogen repression could serve as the regulatory trigger for LMEs production. The regulation of LMEs as well as other extracellular proteins through nitrogen and/or carbon catabolic repression have further been presented (Bonnarme et al., 1991; Kobakhidze et al., 2016).

2.4.3. Biochemical pathways in fungi metabolism

Fungal metabolism is made of numerous biochemical pathways necessary for growth, energy production, signaling, defense, etc. Although, catabolic pathways are some of the most important ones as they contribute to microbial growth and nutrition for energy production (Khosravi et al., 2015). These are also pathways involved in biodegradation and bioremediation of xenobiotic compounds (Khatoon et al., 2017). The catabolic pathway used by fungi depends principally on the substrate they are feeding on. When

feeding on simple sugars such as pentoses and hexoses, fungi will use glycolysis as main catabolic pathway followed by the TCA cycle and oxidative phosphorylation for energy production (Deveau et al., 2008; Khosravi et al., 2015).

However, when dealing with more complex substrates, fungal metabolism involves different catabolic pathways. These routes generally involve the metabolism of organic substances through depolymerization, intermediates formation, and their utilization in central pathways (Liu et al., 2019). Catabolism of aromatics usually requires the use of “upper pathways” which serve as funnels that convert various substrates into intermediates like catechol and protocatechuate (Khatoon et al., 2017; Liu et al., 2019). The β-ketoadipate pathway (β-KAP) is the most common pathway that then performs ring cleavage to convert these intermediates into TCA metabolites, mainly acetyl-CoA (**Figure 2.4**) (Bilal & Iqbal, 2020; Wells & Ragauskas, 2012). These nine enzymes pathway is of major importance in mycoremediation because it is thanks to it that most xenobiotic compounds can be utilized by fungi and be converted into harmless products.

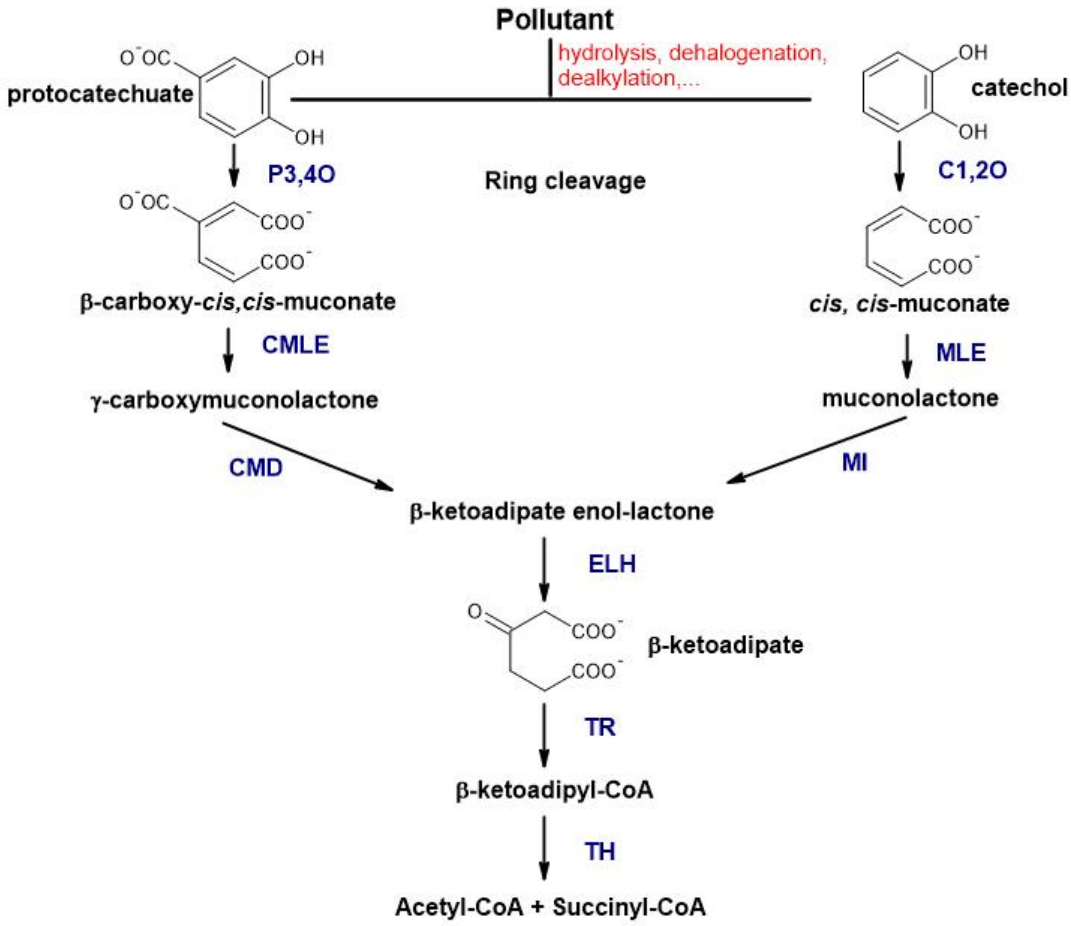

Figure 2.4: biodegradation of aromatic pollutants using the β-ketoadipate pathway. Adapted from Wells & Ragauskas (2012).

2.4.4. Bioremediation pathways used in fungi.

As previously discussed, fungi play an important role in environmental cleanup of xenobiotic contamination. Depending on the pollutant to be degraded and the fungal strain, various pathways have been described (Deshmukh et al., 2016). Morelli et al. (2013) studied the role of fungi in the bioremediation of PAHs contaminated soils and concluded that the biochemical pathway used depends primarily on two factors: the size of the pollutant and the enzyme system used by the fungi. Many non-ligninolytic fungi have been shown to use the cytochrome P450 monooxygenase pathway for the bioremediation of low molecular weight PAHs as demonstrated by various authors (Abdel-Shafy & Mansour, 2016; Ostrem Loss et al., 2019). On the other hand, high molecular weight PAHs are degraded by ligninolytic fungi and first require the action of the extracellular enzymatic system (**Figure 2.5**) (Deshmukh et al., 2016; Morelli et al., 2013).

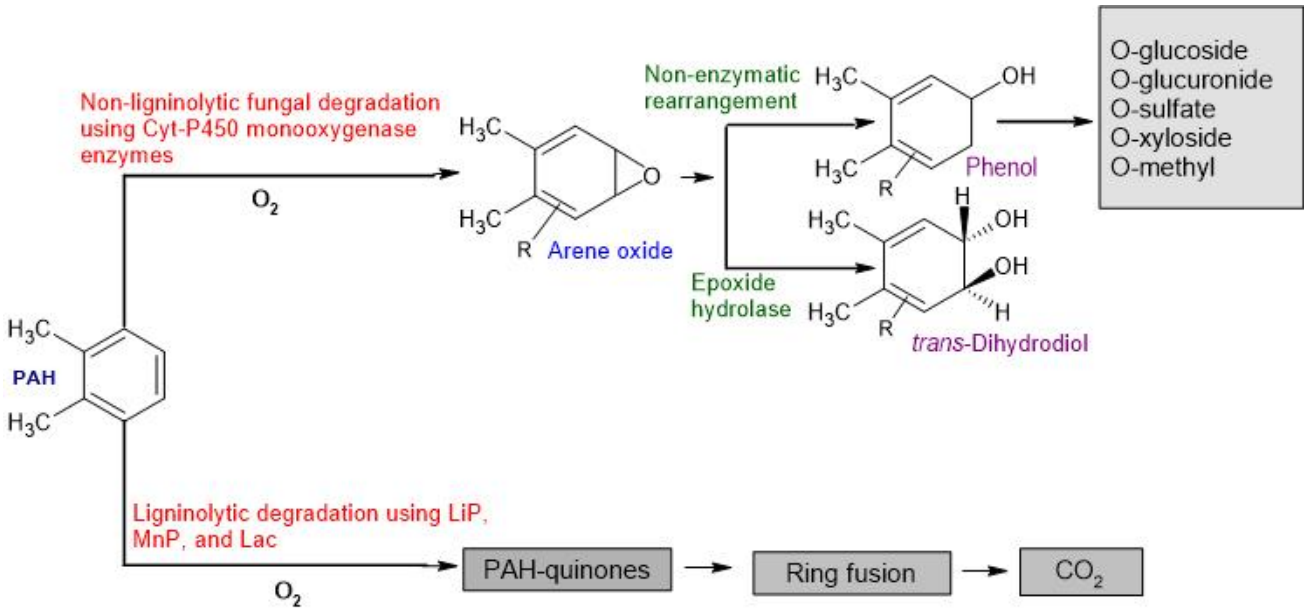

Figure 2.5: Pathways used for the degradation of PAHs by fungi. Adapted from Shahsavari et al. (2015).

When dealing with aliphatic hydrocarbons, fungi and other microorganisms use a common pathway which begins with terminal methyl group oxidation to form a primary alcohol which is further oxidized to form a fatty acid that enters the β-oxidation pathway after being conjugated to coenzyme A (**Figure 2.6**) (Koshlaf & Ball, 2017). The oxidation could also be sub-terminal forming successively secondary alcohols,

ketones, esters, and finally fatty acids to enter the β-oxidation pathway. Many reports have shown the application of fungi, especially *Aspergillus sp*. in the bioremediation of these straight chain hydrocarbons (Asemoloye et al., 2020; Steliga, 2012).

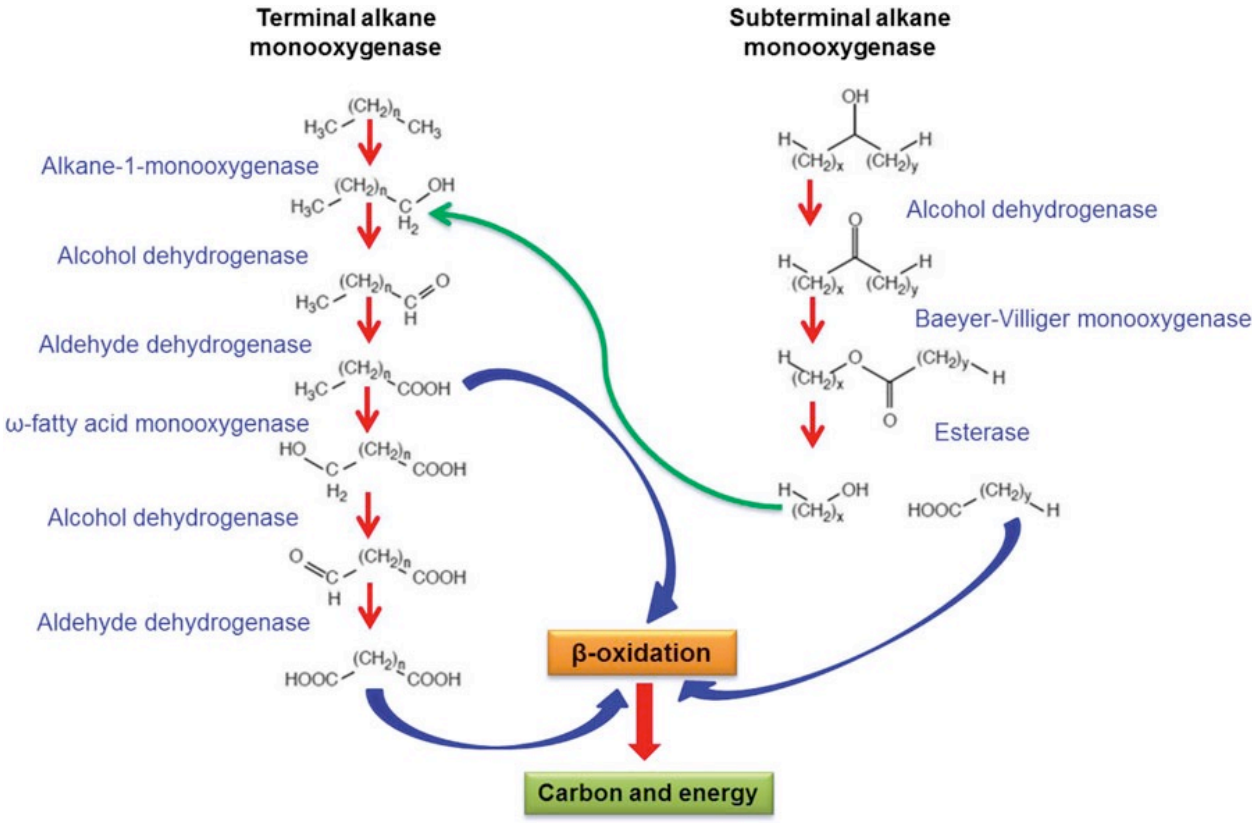

Figure 2.6: Bioremediation pathway of aliphatic hydrocarbons from terminal or subterminal methyl oxidation (Shahsavari et al., 2015)**.**

In the presence of other organic pollutants such as pesticides, dyes or PCBs, various pathways could be used for bioremediation but all of them will usually follow the same scheme (**Figure 2.7**) which starts with hydrolysis, dehalogenation, or dealkylation to remove active groups. The produced aromatic intermediate then passes through the β-ketoadipate pathway before being further degraded to CO_2 (Mohapatra et al., 2018). This has been shown in various studies including that of Diez (2010) looking at various organopollutants, and that of Maqbool et al. (2016) whose focus was pesticides. Some common fungal strains used in the bioremediation of various xenobiotic compounds and their metabolic pathways are presented in **Table 2.3**.

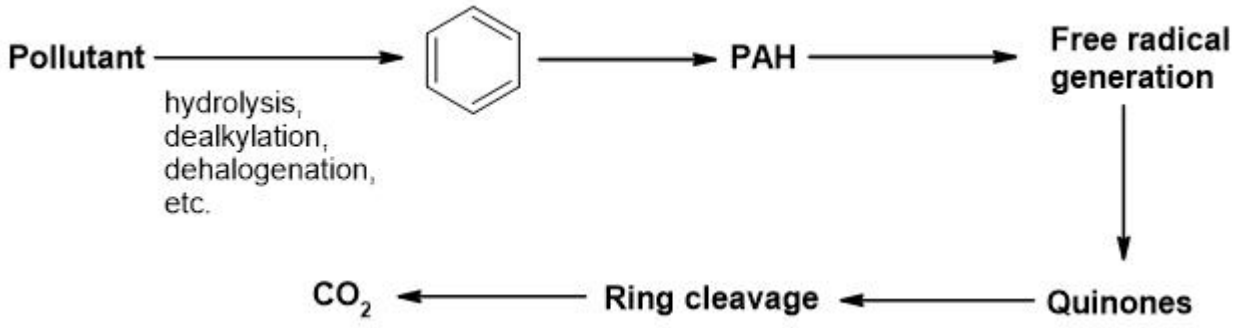

Figure 2.7: general scheme for the biodegradation of organic pollutants. Adapted from Mohapatra et al. (2018).

Table 2.3: Common fungal strains used for the bioremediation of xenobiotic compounds.

Fungi	Xenobiotic	Reference
***Aspergillus* sp.**	PCBs	(Marco-Urrea et al., 2015)
	Dyes	(Jebapriya & Gnanadoss, 2015)
	Crude oil	(Damisa et al., 2013)
	Leather tanning effluents	(Bennet et al., 2013)
	Heavy metals	(Akhtar et al., 2013)
***Trichoderma* sp.**	Organic solvents	(Oros et al., 2011)
	PAHs	(Argumedo-Delira et al., 2012)
	Pesticide-polyresistance	(Hatvani et al., 2006)
	Agrochemicals	(Katayama & Matsumura, 1993)
	Dyes	(Jebapriya & Gnanadoss, 2015)
Pleurotus ostreatus	DDT	(Purnomo et al., 2010)
	PCBs	(Chun et al., 2019)
	Heavy metals	(Kapahi et al., 2017)
	PAHs	(Hestbjerg et al., 2003)
	Lindane	(Rigas et al., 2009)
Trametes versicolor	Atrazine	(Bastos et al., 2009)
	Pentachlorophenol	(Walter et al., 2005)
	Diphenyl ether	(Rosales et al., 2013)
Penicillium sp.	PAHs	(Zehra et al., 2018)
	Chlorophenols	(Aranciaga et al., 2012)
	Heavy metals	(Leitão et al., 2009; Xu et al., 2015)
	Pesticides	

		(Sondhia et al., 2013; Zehra et al., 2018)
***Bjerkandera* sp.**	Hexachlorocyclohexane (HCH)	(Quintero et al., 2007)
	Textile dye wastewater	(Robinson et al., 2008)
	Dyes	(Gao et al., 2020)
	PAHs	(Kotterman et al., 1998; Gupte et al., 2016)

2.4.5. Regulation of fungi biochemical pathways

Fungal metabolic pathways, especially those involved in xenobiotic metabolism are regulated internally by genes, and externally by substrate availability and type, as well as environmental factors. Many transcription factors have been identified to control both primary and secondary metabolism in fungi. These are divided into broad-domain regulatory proteins and pathway-specific proteins (García-Estrada et al., 2018). In fungal metabolism, carbon catabolite repression is one of the most common regulatory mechanisms which enables fungi to adapt to their environment by suppressing certain enzymes in order to use the most readily available carbon source in their surroundings and reduce ATP exertions necessary for synthesis (Adnan et al., 2018). This was studied by Mogensen et al. (2006) using *Aspergillus nidulans* grown on glucose or ethanol. Carbon catabolite repression was found to depend on CreA (Cre1), a zinc finger transcription factor that regulates the expression of over 100 genes.

Unlike genes responsible for primary metabolism that are found dispersed throughout the genome, those responsible for secondary metabolism are found in clusters which allows them to be co-regulated (Keller, 2019). These gene clusters are controlled through a complex network of proteins in response to environmental factors. One of their regulatory approaches is the previously discussed one strain-many compounds (OSMAC) regulation process which determines which secondary metabolites are produced based on the selected growth medium and conditions (Hewage et al., 2014). Environmental stimuli including carbon and nitrogen types and concentration, light, pH, temperature, all influence secondary metabolism (Brakhage, 2013; Keller, 2019). In their work, Tudzynski (2014) reviewed nitrogen regulation of fungal secondary

metabolism and demonstrated that nitrogen influences physiological and morphological characteristics in fungi. The transcription factor *AreA* and its co-repressor *Nmr* have been identified as crucial to nitrogen regulation as they control not only growth, but also secondary metabolites production (García-Estrada et al., 2018).

Environment-induced regulation of fungal metabolism is also important as it allows microorganisms to respond to changes in their environment. In their work, (Martins et al., 2019) studied the metabolic and developmental regulation of the pH signaling transcription factor PAC-3 in pathogenic fungi. PAC-3 was found to directly regulate genes involved in the synthesis of enzymes needed for fungal virulence. Studies have also focused on light regulation of fungal metabolism. Exposure to light was found to trigger alterations in fungi that affect the metabolism of carotenoid, polysaccharide, fatty acid, etc. (Tisch & Schmoll, 2010).

Although many fungal regulatory mechanisms have been elucidated, many uncertainties still surround the mechanisms of expression and regulation of LMEs and this is mostly due to the presence of isozymes. Over the years, research has shown that a number of LMEs isoforms (isozymes) are synthesized by different white-rot fungi and one organism may possess multiple genes coding for different isozymes (Chandra et al., 2017; Kumar & Chandra, 2020). This is a quite common phenomenon in eukaryotes. The presence of multiple isozymes has been linked to the need for large quantity of a given enzyme or the catalytic sub-functionalization of each isozyme (Janusz et al., 2017; Vasina et al., 2017). Goudopoulou et al. (2010) looked at the differential gene expression of LMEs in the model white-rot fungus *Pleurotus ostreatus*. Their results demonstrated that while in certain cases all genes coding for a given ligninolytic enzyme undergo the same regulation process, it is also possible to find different regulation pattern for different isozymes. For instance, while the manganese peroxide gene *mnp2* was up regulated from day 12 to day 18 in their study, *mnp3* reached its maximum on day 10 and started to decrease thereafter.

2.4.6. Limitation to LMEs production

As secondary metabolites, LMEs synthesis does not occur during the growth stage and requires particular circumstances i.e., carbon and nitrogen starvation. Also, their synthesis is regulated by carbon catabolite repression which prevents utilization of

complex carbon sources when simpler forms are available (de Assis et al., 2021). As a result, only limited amounts of these enzymes are produced. A good deal of research has therefore been done in this regard with the objective of increasing LMEs production. Usha et al. (2014) investigated the addition of inducers, surfactants, and copper sulphate in the production on LMEs by *Stereum Ostrea*. The addition of 0.02% veratryl alcohol, 300 uM copper sulphate, Tween 20, Tween 80, and Triton X 100 was found to significantly increase LMEs production during solid state fermentation. In another study, Lettera et al. (2011) investigated increasing laccase production through breeding of monokaryotic compatible strains. This approach was found to be effective in enhancing laccase production while also avoiding mutagenic transformations. In yet another study, Yasmeen et al. (2013) looked at factors such as pH, temperature, incubations, etc. for the optimization of LMEs production using response surface methodology.

In as much as all these approaches have been successful to some extent, LMEs production is yet to be fully understood and a closer look into its molecular basis could provide valuable insight. New technological advances have provided scientists with tools capable of investigating LMEs production from genes to proteins. Korripally et al. (2015) studied the regulation of LMEs gene expression. Whole transcriptome shotgun sequencing revealed 356 genes whose amounts increased by four times from the moment ligninolytic enzyme production began. Of the 356 up-regulated genes, 165 were of unknown function. Similar results have been observed in other studies (Liu et al., 2019; Minami et al., 2009) which indicate the need for more transcriptomic studies in order to bring more knowledge on the topic and possibly serve as starting point for the elucidation of LMEs gene expression and regulation. However, before looking at genes and their expression, a good understanding of fungal isolation, growth and characterization is necessary.

2.5. Techniques used in fungal studies

Over the years, various approaches have been used by scientists to study fungi especially focusing on biological diversity, behaviors, metabolites, and enzyme production. Several scientific innovations have also had great impact on techniques of organisms and metabolites characterizations. More recent advances have brought about great progress particularly in specific aspects of omics such as proteomics and

metabolomics easily witnessed in the varied bioproducts in various industries. These improved techniques are pertinent to the bioprospecting and exploration of bioproducts from these economically important organisms.

2.5.1. Isolation techniques

As a diverse group, fungi grow in various environments and under various conditions. For instance, wood-decaying fungi are some of the most abundant fungi found in forests and wild environments. In their review, Lonsdale et al. (2008) presented the importance of wood-decaying fungi in maintaining forest ecosystems and the need to preserve species-richness. In a recent study, Park et al. (2020) investigated the diversity of wood-decaying fungi in Central Siberia, Russia and found over fifty different species of wood-colonizing fungi.

In all natural and extreme environments, fungi and other microorganisms are found to co-exist, displaying different types of interactions from mutualism to antagonism (Ijoma & Tekere, 2017; Perotto et al., 2013). For this reason, any fungal study usually begins with isolation of the fungus of interest from its environment, away from other microorganisms to allow for optimal characterization without interference. Fungal isolation techniques therefore involve growing the microorganism in a medium that will favor its growth while preventing that of other microorganisms that will be considered as contaminants (Papke & Ward, 2004). According to Nevalainen et al. (2014), fungal isolation is most commonly done through plating on petri dishes containing a rich medium. This medium is usually supplemented with antibiotics to prevent the growth of other opportunistic and contaminating microorganisms, especially bacteria (Shi et al., 2019). Nagano et al. (2008) and later Black (2020) have compared fungal growth on various media with and without antibiotic supplementation and both researches concluded that antibiotics, especially chloramphenicol, contribute greatly to bacterial growth inhibition.

Several growth media have been developed to grow and isolate different fungal strains. Basu et al. (2015) reviewed fungal media evolution. While all fungal media have the particularity of being rich in carbon: nitrogen ratio, with pH ranging from 5 to 6, various compositions are available. These media are broadly divided into two: natural media made of natural substrates such as stems, seeds, corn meal, etc. with no exact composition, and synthetic media made of all necessary components with known

composition. However, depending on the strain being isolated, a specific media could be needed. In their research, Kostadinova et al. (2009) used Cooke Rose Bengal agar, a highly selective media with a range of bacterial inhibitors, for the isolation of fungal strains in Antarctica. In a different study, (Smithee et al., 2014) proposed a fungal growth media containing hydrochloride salt of creatinine for even better selection of fungi from clinical and environmental sources. Although many specialized media have been proposed and successfully used, potato dextrose agar (PDA) and malt extract agar (MEA) are currently the most popular for fungal cultures due to their high C:N ratio which is advantageous for growth, sporulation, and pigmentation of many fungal taxa (Black, 2020; Griffith et al., 2007). However, not all taxa have been successfully cultured, despite advances made in culture media and techniques, as such there are ongoing research developing novel technologies towards improving isolation of fungi. Some of these, including laser printing, are reviewed by Cheptsov et al. (2019).

While growth and isolation are typically done on solid, agar-containing media, fungal growth in liquid media is also possible, especially for the purpose of enrichment to increase the population of a target microorganism (Nevalainen et al., 2014). Although, submerged culture approaches tend to be challenging due to oxygen constraints and its effects on these aerobic eukaryotic cells. Prenafeta-Boldú et al. (2001) reported the isolation and characterization of fungi feeding on PAHs using liquid media containing mineral salts. This approach is quite common when the goal is the production of enzymes or antibiotics, especially when using a particular carbon source (Clemente et al., 2001; Dhouib et al., 2005).

Combined with media selection, fungal isolation tediously requires subculturing and purification which involves the transfer of a strain onto new media with the objective of ultimately obtaining pure microbial cultures (de Vero et al., 2019). Isolate purification has traditionally been done through single spore isolation for spore producing fungi. This involves preparing a spore suspension from which a single spore will be collected, plated on agar after germination, and repeatedly transferred to a new plate until a pure isolate is obtained (Choi et al., 1999). However, this approach is time consuming, fastidious, and involves the manipulation of tiny fungal spores. This has caused scientists to come up with a simpler technique referred to as hyphal tip isolation which involves cutting a tip of a growing fungal hyphae to be transferred onto a new plate as discussed by Leyronas et al. (2012). Since their introduction, these techniques have seen considerable advancements, all with the objective of making them more effective

and less time-consuming. In their work, Noman et al. (2018) describe an improved approach to single spore isolation. This method does not require the preparation of a spore suspension, but rather direct plating on solid media, followed by transfer of spores onto new plates the same day which speeds up the procedure. Once pure cultures have been obtained, their identification is done based on their observable traits.

2.5.2. Characterization techniques

The establishment of axenic mycelial growth on agar plates allows for the important steps of characterisation and taxonomic identification of the microorganism(s). It also ensures storage and reproducibility. Fungal stains are characterized based on their morphological, or molecular and functional features.

2.5.2.1. Morphological characterization

As they grow, fungi form colonies of hypha fibers with distinctive phenotypes such as shapes, colors, and sizes that can be differentiated at macroscopic or microscopic level. Fungal identification based on phenotypic characteristics is a common, traditional method for fungi classification and has contributed greatly to taxonomic studies. First reported in the 1700s, phenotype-based characterization has seen much improvement with the introduction of microscopes (Senanayake et al., 2020). Currently, various microscopes including light, electron, fluorescence, phase contrast, etc. are available for morphological characterization. In their work, Alsohaili & Bani-Hasan (2018) reported the morphological identification of fungi from the desert of Jordan. Using a compound light microscope with a digital camera, lactophenol cotton blue-stained fungal slides were observed and characterized. A similar approach was used by Toledo et al. (2013) for the characterization of a fungus isolated from planthoppers in Argentina. Although, lactophenol cotton blue is the most popular stain used for fungal morphological characterization, various other stains are available (**Table 2.4**) and can be used depending on the desired reaction.

Morphological characterization depends greatly on comparative analysis with previously reported features. For instance, Rachmania et al. (2018) used morphological characterization to identify fungi from deteriorated old Chinese

manuscripts up to genus level. This was achieved through comparison of observed structures with those reported in various monographs like the one developed by Bentivenga & Morton (1995). Although, advances in microscopy and computational analysis have improved the accuracy of morphological characterization, it remains an imprecise method, especially when differentiating members of the same genus, and requires individual expertise further increasing biases in identification (Lutzoni et al., 2004). Therefore, there is a need to investigate beyond the phenotype because genotype studies provide a non-discriminatory characterization.

Table 2.4: Example of stains used for fungal morphological characterization.

Stain	Observation	References
Lactophenol cotton blue	Intense blue color	(Leck, 1999)
10% KOH	Purple color on stromatic tissues	(Afshar et al., 2018)
Melzer's reagent	Blue to black color on Amyloid structures Brown color on pseudo-amyloid structures Faint yellow color on non-amyloid structures	(Senanayake et al., 2020)
Congo red	Red color	(Shamly et al., 2014)
India ink	Black background	(Kwizera et al., 2017)
Schultze's reagent	Blue to black color	(Senanayake et al., 2020)

2.5.2.2. Molecular characterization

Progress in the field of molecular biology has been important for the evolution of fungal characterization. Introduced over two decades ago, the use of molecular techniques for fungal identification has rapidly gained popularity as a gold standard approach and has overtaken or are use as validation alongside morphological characterization. Several authors including Ab Majid et al. (2015) have employed molecular characterization in the taxonomic identification of pathogen fungi with resolutions to species level after unsuccessful identified with the use of only morphological characterization. Using sequencing techniques and specific DNA markers, fungal isolates can easily be identified to species level and their phylogeny established.

Developed in 1977, Sanger sequencing is the current standard molecular tool for identification (Slatko et al., 2018). Sanger sequencing is made possible through polymerase chain reaction (PCR), a molecular technique that enables synthesis of a complementary DNA strand from a template using the enzyme *taq* polymerase (Valones et al., 2009). In 1990, a ground-breaking advancement in fungal molecular characterization was introduced with the identification of fungal nuclear ribosomal RNA (rRNA) operon primers (White et al., 1990). The DNA sequences associated with the large ribosomal subunit (nrLSU-26S or 28S), the small ribosomal subunit (nrSSU-18S), and the whole internal transcribed spacer region (ITS1, 5.8S, ITS2; 650-900 bp) have since become the target region of fungal identification by Sanger sequencing (Raja et al., 2017). Different evolution rates have been observed in the region, resulting in different levels of variation from one organism to the next. As the fastest evolving and most variable segment, the ITS region has become the golden standard for fungal identification using ITS1 and ITS4 primers (Cadez et al., 2002; Raja et al., 2017; Schoch et al., 2012). Molecular characterization also owes its success to the development of bioinformatics tools and databases that enable identification.

This process involves the initial amplification of a conserved DNA region using a genomic DNA template and specific primers that targets specific regions. Amplification is then terminated using di-deoxynucleotides. The derived sequences are matched to other previously submitted sequences in a comprehensive database. These comparisons characterize and identify through matching to the closest relatives on the database, by allocating a value range of between 0 – 100; the latter being an exact match (Slatko et al., 2018; Totomoch-Serra et al., 2017).

Databases such as the NCBI-BLAST are crucial to molecular characterization as it is through them that identification is done by means of comparison with previously recorded organisms as reviewed by Federhen (2012). Other fungi specific databases have also been developed. Nilsson et al. (2019) as well as Kõljalg et al. (2019) describe UNITE, a database designed for fungi molecular identification curating all public fungal sequences as well as those that have not successfully been assigned taxonomic lineage beyond phylum. The latter are assigned a unique digital object identifier (DOI).

Although genomic data is to a certain extent sufficient for molecular characterization, it has been found to give limited insight into an organism's physiology for the simple reason that even though the same genes are present, they do not get expressed at

the same time, nor in the same way (Guo, 2014). This therefore brings the need to look deeper into the succession of events from genes to metabolites.

2.5.3. Transcriptomics

2.5.3.1. Principle and techniques

The central dogma of molecular biology resides on the transcription of genes into messenger RNA (rRNA) and the translation of the latter into peptides that are later rearranged into proteins (Guo, 2014; Koonin, 2015). For many years, research had been focused on the genomic aspect which was important in connecting genes with morphological and physiological characteristics. However, the understanding of gene expression and its particularity has shifted focus from the genome to the transcriptome (Manzoni et al., 2018). Defined as the study of mRNA transcripts, the field of transcriptomics bridges the gap between genes and proteins according to Lowe et al. (2017). The introduction of transcriptomics has been of great benefit as it makes possible the monitoring and measurement of gene expression in different tissues, as well as the responses to different conditions, or at different time points. It is also an important step in the functional characterization of an organism. Considering that there is an array of genes, some expressions are a consequence of the activity of a single gene, a cluster of genes, even some genes having no assigned function. Transcriptomics has been applied in research such as that of Noriega et al. (2019) to identify genes that are crucial to different developmental stages in the coffee berry borer *Hypothenemus hampei*. Many previously unannotated genes have also had their function elucidated using transcriptomics. While some were reviewed by Evans (2015), Kim et al. (2020) used a similar approach to identify genes associated with a particular phenotype. In their work, transcriptomics studies were conducted to elucidate the yellow leaf color of a mutant *Cymbidium* orchid. RNA sequencing was able to identify over 2000 genes differently expressed in the mutant compared to the wild type. Among these genes, some were associated with chlorophyll metabolism as well as ion transport and were suggested to be responsible for leaf color.

With regards to WRFs, transcriptomics brings forth the possibility of getting a better understanding of the process of LMEs production. Techniques such as reverse transcription-quantitative PCR (RT-qPCR) have been found very useful in identifying

and quantifying mRNA at any given time in biological systems (Pombo et al., 2017). Compared to other PCR techniques, RT-qPCR gives accurate quantification of mRNA as measurement is being done in real time. This technique allows the investigation of changes in gene expression as a result of different treatments. RT-qPCR also presents advantages such as no need of further handling after amplification, easier automation, and the ability to process larger samples (Adams, 2020; Bleve et al., 2003). As a quantitative method, RT-qPCR can look at a target mRNA, convert it to cDNA which is more stable than RNA, and amplify it while emitting fluorescence which enables real-time quantification (Arya et al., 2005). Fernández-Fueyo et al. (2014) used RT-qPCR to investigate the effect of environmental parameters temperature and pH on gene expression and regulation in *Pleurotus ostreatus*. By monitoring gene expression under various conditions, the authors were able to present certain predictions on the correlation between temperature and pH in gene regulation. With the help of RT-qPCR, various ligninolytic genes have also been identified like in the case of Stuardo et al. (2004) who identified the peroxidase genes *lip* and *mnp* from soil fungi, or more recently, Vasina et al. (2017) where the absolute expression of 18 peroxidase genes encoding class II peroxidases in *T. hirsuta* was quantified. The characterization of this multigene family will allow for the design of specific primers to be used for further studies.

Although fast and effective, RT-qPCR remains a mid-throughput technique only able to look at few genes at a time. Especially for the study of fungi and other eukaryotes who have multiple genes involved in the production of a given compound, RT-qPCR is unable to give the full picture (Smith & Osborn, 2009). According to Castanera et al. (2015), RT-qPCR for fungal work also presents the challenge of choosing a correct reference gene as the expression of many genes commonly used as references can also be affected by growth conditions and other external parameters. This has motivated the introduction of novel, high-throughput technologies in order to overcome these limitations (Lowe et al., 2017).

Hybridization techniques such as microarrays were able to partially address these issues by increasing the amounts of genes to be analyzed per run. However, they still required prior knowledge of these genes to develop complementary probes (Lowe et al., 2017). RNA sequencing (RNA-seq) has been able to solve these challenges by allowing the analysis of thousands of known or unknown genes at once without the need for a reference (Wang et al., 2009). This technique uses next-generation

sequencing principle to obtain whole transcriptome profile (type and quantity) of a cell, tissue, organ, or entire organism (Jazayeri et al., 2015). Since its introduction, various sequencing platforms have been developed and although they all use the same principle, they differ in terms of read lengths, throughput, error, and price. Different authors have reviewed and compared these platforms for various applications (Jazayeri et al., 2015; Loman et al., 2012; Quail et al., 2012; Wang et al., 2019). Ravichandran et al. (2020) used RNA-seq on an Illumina platform to get insight on the degradative ability of a WRF based of the expression of genes related to degradative enzymes. Ma et al. (2016) used the same tool to study fungal metabolic regulation by identifying all genes under the regulation of a given transcription factor such as the Xyr factor in charge of carbohydrate metabolism. In yet another study, Henske et al. (2018) have used RNA-seq to investigate differential expression of LMEs in the presence of different substrates.

Although transcriptomics tools have deepened our understanding of the expressional patterns followed by fungi, especially with regards to LMEs, several aspects of functionality are yet to be elucidated. Korripally et al. (2015) used RNA-seq to study the regulation of LMEs gene expression. Using whole transcriptome shotgun sequencing, an increase of up to four-fold from an initial 356 genes, was observed from the moment ligninolytic enzyme production began. Of the 356 up-regulated genes, 165 were of unknown function. Similar results have been observed in other studies (Liu et al., 2019; Minami et al., 2009).

Where studies do not extend to transcriptomics, and to reduce expenses associated with comprehensive transcriptomics studies, it is possible to utilize whole genome sequences and sequence scaffolds as well as the vast array of databases to predict possible functions for a specific gene or gene clusters (Hansen et al., 2018; Zhao et al., 2020). However, this does not replace transcriptomics as some of these genes may be present but due to environmental conditions, they may not be active. Therefore, only real-time biochemical activities can identify their correlation with metabolite production and/or transformation (Ijoma et al., 2021).

2.5.3.2. Bioinformatics tools used for transcriptomics studies

While previous transcriptomics techniques did not require much data analysis, current ones, especially RNA-seq, use large amounts of data which requires computational tools for their analysis to produce accurate and reproducible results. RNA-seq data analysis involves three main steps namely quality control of the raw reads (1), mapping and alignment (2), quantification of reads and differential expression analysis (3); each of them involving a number of bioinformatics tools (Hernández-Domínguez et al., 2019; Lowe et al., 2017). As a technology that uses huge amounts of complex data, RNA-seq is susceptible to many variations including from technical and random sources (Merino et al., 2016). Quality control, especially that of raw data, is therefore important in increasing the accuracy of analysis by tackling biases such as those of nucleotide composition and GC content (Wang et al., 2012). To date, various bioinformatics tools have been developed for RNA-seq quality control (QC). In their work, Hernández-Domínguez et al. (2019) present FastQC, as one of the most popular QC tools for Illumina platform. FastQC reports quality information based on reads or sequence, also giving the proportion of each nucleotide base in the reads. This was also reported by Qi et al. (2017). Other QC tools, including FASTX-Toolkit, QC-Chain, and NGS QC Toolkit, are discussed by Zhou et al. (2018) who also introduced RNA-QC-chain, a novel comprehensive tool for QC which comes with the advantages of trimming, automatic rRNA detection, and contaminating species identification. Quality control not only takes place at the beginning of data analysis, but also precedes each analysis step.

Initial quality control is followed by mapping and alignment where all reads are located either with respect to a reference genome or using de *novo* assembly. In their work, Schaarschmidt et al. (2020) evaluate seven different mapping tools (bwa, CLC Genomics Workbench, HISAT2, kallisto, RSEM, salmon and STAR) using experimental data from *Arabidopsis thaliana* and similar results are obtained with all of them showing high reproducibility. According to Hernández-Domínguez et al. (2019), three strategies could be followed during the mapping process. When the goal is to identify new transcripts, reads are aligned with gaps to a reference genome. Tools such as STAR have been found to be best adapted for this type of mapping as they can map spliced sequences of any length (Dobin & Gingeras, 2016). When new transcripts are not the goal, reads are aligned to the reference genome without gaps

using tools such as RSEM. In other cases, a reference genome is not available, and reads are used for de *novo* assembly. Haas et al. (2014) describe Trinity, one of the most popular tools used for de *novo* assembly. Trinity is an “assembly-first” tool for transcriptome reconstruction consisting of three modules namely Inchworm, Chrysalis, and Butterfly. Trinity is able to assemble transcriptomes by separating the data into many de Bruijn graphs that are processed separately before using parallel computing to reconstruct the transcriptome (Grabherr et al., 2011).

Transcript quantification is an important step in data analysis as it gives RNA-seq its quantitative aspect which allows comparison between different expression points. Jin et al. (2017) evaluated different quantification methods using tools such as TopHat, RSEM, HTSEq and featureCounts. The authors also differentiated between alignment-based and alignment-free methods depending on the presence or absence of a reference genome. Another important step in transcript quantification is normalization of data to remove the influence of all possible biases. Normalized measures such as RPKM (reads per kilobase of exon model per million reads), FPKM (fragments per kilobase of exon model per million mapped reads), and TPM (transcripts per million) are then used to report expression values (Conesa et al., 2016). Based on the obtained data, differential expression analysis can then be carried out, by comparing values from different samples. Various tools are also available for differential expression including DESeq, Cufflinks, PoissonSeq, UpperQuartile, etc. (Conesa et al., 2016; Jazayeri et al., 2015). Although most currently used tools have been found to be very effective for differential expression, Assefa et al. (2018) reported low performance when assessing differential expression of long non-coding RNAs (lncRNAs) using 25 different pipelines. The authors correlated this with sample levels and variability as lncRNAs are expressed at low levels and are quite variable. For fungal work, Wang et al. (2016) describe a workflow for differential expression in fungal species using the Bioconductor package DESeq2. In a recent study, Pawlik et al. (2019) use RNA-seq to study differential expression in the fungus *Cerrena unicolor* FCL139 when grown under different lighting conditions. Mapping was done using a reference genome and the DESeq 2 package was also used to identify differentially expressed genes.

2.5.4. Proteomics

Translation of mRNA into functional proteins is the second step of the central dogma, and the one that allows all biological activities to proceed. Depending on the complexity and size of the organisms' protein; the final product can be simple or complex involving the folding of peptide molecules, which requires further steps before the formation of the final, functional compound (Wang et al., 2014). Proteomics looks at the identification and quantification of all proteins found in a given cell, tissue, or organism (Aslam et al., 2017; Graves & Haystead, 2002). The study of protein expression, modification, structure and function by means of proteomics has brought progress in various fields of science and technology, because proteins are usually the end products of gene expression and the sought after bioproducts. Amiri-dashatan et al. (2018) reviewed the application of proteomics to food technology, biomarker, and drug target identification. A deeper look at the applications of proteomics in pharmaceuticals was taken by Yokota (2019) including the use of proteomics to study expression profiling, protein-protein interactions, and post-translational modifications. Others such as Champer et al. (2016) have focused their proteomics studies on fungi specifically in order to identify potential vaccines and drug targets. In the work, the authors used quantitative Mass Spectrometry- Elevated Collision Energy to identify fungal proteins with no significant homology with human ones to use as vaccine candidates. Ball et al. (2019) review advancements in MS-based proteomics as it relates to fungal pathogenesis and interactions between these fungi and the host.

2.5.4.1. Protein production and analysis

As eukaryotes, fungi protein production is more complex than that of simpler organisms such as bacteria for example. Post-translational modifications (PTMs), including phosphorylation, glycosylation, acetylation, etc. have been reported to be important for fungal proteins function, affecting even virulence in certain medically important strains Leach & Brown (2012). These are covalent modifications that change properties of proteins and therefore impact their functions. Whether reversible or irreversible, they are generally seen in proteins of particular importance such as those in the cell membrane responsible for cell-cell interactions, or secretory ones to be used extracellularly (Ramazi & Zahiri, 2021).

Wang et al. (2020) present filamentous fungi as having a mature PTMs machinery, especially glycosylation. Unlike yeast that mainly produce glycoproteins with high mannose content, filamentous fungi use many other monosaccharides for their glycosylation, making them more appropriate for the production of mammalian-like proteins for pharmaceutical uses. In their work, Wang et al. (2017) look at the evolution of glycosylation in eukaryotes. The authors differentiate between N- and O-glycosylation. While N-glycoproteins have glycans attached to the amide group of their asparagine residues, O-glycoproteins have this modification attached to the carboxyl group of their serine, lysine, threonine, and proline residues. Additionally, glycosylation is here presented as one of the most complex PTMs, playing a crucial role in excretory proteins' folding. This is confirmed by Ramazi and Zahiri (2021) who also link glycosylation, or the lack thereof, to conditions such as cancer and diabetes in human. In fungi, Goto (2007) reviewed structures and functions of O-glycosylation. Most fungal secretory proteins are glycosylated through the action of O-mannosyltransferase and several other glycosyltransferases as they move from the endoplasmic reticulum to the golgi apparatus before reaching the cell exterior. These modifications have been reported to add stability and solubility to extracellular proteins (Deshpande et al., 2008; Goto, 2007; Ramazi & Zahiri, 2021). PTMs therefore play an important role in enzyme production strategies, especially when artificial means are used. In this regard, Tokmakov et al. (2012) looked at the correlation between PTMs and the success of heterologous protein synthesis. Results suggested that prior identification of potential PTMs using protein sequences could predict and optimize heterologous synthesis. Proteomics technologies such as mass spectrometry (MS) are able to study these modifications with the goal of identifying their location and the resulting data is stored on different databases including PhosphoGRID, PHOSIDA, PhosphoELM, iPTMNet, etc. (Aslam et al., 2017; Cruz et al., 2019).

Filamentous fungi have the particularity of producing extracellular enzymes which allows them to play their role in the environment. This has largely contributed to their industrial application as these extracellular enzymes require minimal processing for their collection (Asemoloye et al., 2020; Ellouze & Sayadi, 2016; Rugabber & Talley, 2006). Recently, Arnau et al. (2020) presented strategies and challenges for the production of industrial enzymes using the extracellular machinery of fungi. The authors describe the use of classical mutagenesis and screening in order to identify and/or develop mutant organisms with the ability to produce higher enzyme titers.

Together with that, many other strategies including the use of stronger promoters, codon optimization of gene sequences, deletion of protease-coding genes, addition of artificial transcription factor, etc. have been employed for industrial enzyme production.

Enzyme production through recombinant DNA technology

Recombinant DNA technology involves the modification of an organism's genetic material in order to obtain a desired result. This usually implies the insertion, into a host organism, of a gene coding for a desired product through a vector (Khan et al., 2016). Since its introduction in the 70s, the technology has contributed greatly to the large-scale production of many important proteins in fields ranging from agriculture to pharmaceuticals and cosmetics (Gifre et al., 2017; Gupta et al., 2017). Over the years, different expression hosts have been developed for recombinant protein production. In their work, Tripathi & Shrivastava (2019) review these expression hosts including bacteria, mammalian cells, yeasts, insects, and transgenic plants. The authors also point out the importance of using eukaryotic hosts to produce therapeutical proteins as these require post-translational modifications, especially glycosylation, for their efficacy. As previously discussed, PTMs play a crucial role in eukaryotic protein stability, solubility and functionality. As such PTMs must therefore be considered during the selection of the host for recombinant protein production. von Schaewen et al. (2018) further explain this as they discuss the limitations of bacterial hosts such as *E.coli* for the production of recombinant eukaryotic proteins.

Besides host selection, recombinant protein production also relies greatly on the effectiveness of cloning. As explained by Gupta et al. (2016), expression of recombinant eukaryotic proteins involves cloning of the cDNA of interest into an appropriate expression vector, followed by its insertion into the host cell. Therefore, a number of cloning vectors are available depending on the intended use. Various cloning methods have also been developed. Jia & Jeon (2016) describe different cloning methods and their possible application for high-throughput recombinant protein production. These include restriction enzyme-based cloning which utilizes restriction enzymes to determine and cut the beginning and the end of the insert gene, recombination-based cloning where a site-specific recombinase is used to make the recombinant vector without using restriction enzymes, and ligation-independent cloning which enables direct directional cloning of any insert with no restriction enzyme, nor recombinase needed.

Different vectors can be designed to introduce a recombinant gene into the selected host. Depending on the chosen host, these vectors contain elements or motifs necessary for the optimal expression of the recombinant protein. This includes a promoter region, affinity tags, fluorescence tag, and many more as described by Hartley (2006) and Rocco et al. (2008). Various authors have reported different vectors compatible with eukaryotic hosts. Some of the most common are summarized in **Table 2.5**.

Table 2.5: Eukaryotic expression vectors

Host	Vector	Reference
Yeast	-Yeast episomal shuttle vectors (YEp type)	(Hohnholz et al., 2017)
	-Yeast Integrative Plasmid (YIps)	(Siddiqui et al., 2014)
	-Yeast Replicative Plasmid (YRps)	(Falcon et al., 2005)
	-Yeast Centromere Plasmids (YCps)	(Gnügge & Rudolf, 2017)
	-Yeast Artificial Chromosome (YAC)	(Larionov et al., 1996)
Plant	- Tobacco mosaic virus (TMV) vectors	(Hefferon, 2017)
	-Potato Virus X (PVX) vectors	(Lacomme & Chapman, 2008)
	- Ti plamids	(Christie & Gordon, 2014)
Mammalian	- Adenoviral vectors	(Edholm et al., 2001)
	- pSV plasmid vectors	(Cerqueira et al., 2017)
	- baculovirus vector	(Naik et al., 2018)

Industrial strategies for enzyme production in fungi

Enzyme production in fungi can be done using two strategies: submerged fermentation and solid-state fermentation although most industrial processes use submerged fermentation (McKelvey & Murphy, 2018). Enzyme production with

submerged fermentation was reported by Hu et al. (2011) using *Aspergillus niger* inoculum submerged in liquid culture containing carbon, nitrogen and other important nutrients for fungal growth. In solid-state fermentation however, the inoculum is placed onto a humid matrix made usually of a carbon source. This matrix is on top of a media and the secreted enzymes diffuse through the matrix and into the media (Castanera et al., 2012; Yasmeen et al., 2013). Comparative studies between these two strategies have shown that their usual differences in growth patterns, pellets vs mycelial, are a result of different gene expression (te Biesebeke et al., 2002) likely due to changes in water activity and the necessary adaptation responses. Also, although submerged fermentation is most commonly used in industry, solid-state fermentation usually produces high enzyme yields due to its resemblance to fungi natural habit and the reduced water activity important to fungal optimal growth (Gowthaman et al., 2001; Li et al., 2013).

Expression analysis

Depending on the objective, protein expression analysis can be done using high-throughput methods such as mass spectrometry (MS) or protein arrays. According to Alharbi (2020), protein arrays are less preferred in comparison to MS due to the huge amounts of proteins they produce, making it difficult to work with. On the other hand, MS brings accuracy and sensitivity, two features that have contributed to its popularity. Data obtained through analysis by LC-MS/MS, MALDI-TOF/MS, etc. enables the discovery and identification of protein biomarkers, most of which can be found on databases such as Mascot, MS-Tag, and PepProb. In certain cases, selected proteins can be analyzed using low-throughput techniques such as ELISA and western blotting which depend on the reaction between a protein and a complementary tag (Alharbi, 2020; Cruz et al., 2019). In their work, Braitbard et al. (2006) used ELISA to assay human proteins using specific peptides and antibodies. These techniques not only confirm the presence or absence of a given protein, but they also, to some extent, give a quantitative idea. Although not common in fungal studies, ELISA can be effective for the serological detection of fungi. In this regard, some of its first applications go back to the 90s with works such as that of (Kim et al., 1991) where ELISA was used to identify white and brown rot fungi with their ligninolytic metabolites serving as antigens. In a more recent work, Martin-Souto et al. (2020) used ELISA to detect fungi from cystic fibrosis patients. Using whole protein extract from *S. boydii* as antigenic extract,

Scedosporium and *Lomentospora* fungal species were serologically detected in patients' sera with 100% sensitivity. Similar work has also been done using western blotting to identify fungi, mostly those causing diseases (Santana et al., 2018).

2.5.4.2. Protein purification and separation

As many different fungal proteins and mostly enzymes are produced extracellularly, purification is necessary to separate the enzyme(s) of interest from the rest of the secretome. Purification is a prerequisite for most protein work. On one side, pure proteins are crucial for the effectiveness of subsequent studies such structural and functional determination (Kim et al., 2008). On the other hand, pure proteins are important for applications in various industries as the presence of contaminants could result in different unwanted reactions. In the pharmaceutical industry for instance, protein therapeutics require high levels of purity to prevent unwanted interferences and reactions.

Purification aims to exploit differentiating physico-chemical characteristics of enzymes in order to separate them from a mixture (Thiemann et al., 2004). Various techniques have been applied for protein purification as summarized in **Table 2.6**, most of them using liquid chromatography. More et al. (2011) reported the chromatographic purification of a laccase enzyme using anionic exchange followed by gel filtration by means of Fast Protein Liquid Chromatography (FPLC). Using this approach, purification was first done based on the net charge of the proteins, followed by their molecular weights. Mukhopadhyay & Banerjee (2015) also used anionic exchange and gel filtration chromatography for their purification. Additionally, they had it followed by an ultrafiltration step with a Amicon system for further separation. This extra step enabled concentration of the pure protein. In their studies, Irfan et al. (2018) and Carrasco et al. (2017) used ammonium sulfate precipitation based on the biochemical process of salting in/out which allows proteins to precipitate out of solution as a result of a change in ammonium sulfate ionic strength.

Table 2.6: Protein purification techniques

Technique	Principle	Reference
Salting in/out	Exploiting protein solubility by increasing salt concentration in the solution	(Duong-Ly & Gabelli, 2014)
Dialysis	Using size exclusion to separate proteins from small molecules and ions that pass through a semi-permeable membrane	(Bhat et al., 2018)
Gel filtration chromatography	Separation of proteins based on size	(Ó'Fágáin et al., 2017)
Ion-exchange chromatography	Separation of proteins based on their net charge	(Acikara, 2013)
Affinity chromatography	Exploiting the affinity of proteins for given chemical groups to separate them.	(Rodriguez et al., 2020)
High-Pressure Liquid Chromatography (HPLC)	Can use different principles of column chromatography to separate proteins using high pressure to give better resolution	(Mant et al., 2007)

Regardless of the method used, purification is most often coupled with a separation technique for monitoring. Cruz et al. (2019) reports gel-based techniques as being the main separation techniques for proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2-DE), and two-dimensional differential gel electrophoresis (2D-DIGE) allow separation and visualization of proteins within a mixture. Furthermore, 2-DE gives the protein profile of a sample which is of valuable use in comparative studies using databases like the World-2DPAGE (Dias et al., 2010; Encarnación et al., 2005).

2.5.4.3. Structural studies and sequencing

After purification, especially when working with a novel protein, structural studies are usually done in order to predict function (Najmanovich et al., 2005). X-ray crystallography and NMR are the principal techniques used to determine the 3-D structure of proteins (Cruz et al., 2019; Yee et al., 2005). Although they can be used alone, Yee et al. (2005) describe these techniques as complementary. This is also shown in the work of Bryn Fenwick et al. (2014) where using these techniques in synergy gives more accuracy in the structure. This structure can then be deposited in the different structure databases such as Protein Data Bank (PDB), PDBsum, or ModBase (Chen et al., 2017). From these structures, functions can be predicted using the various approaches reviewed by Mills et al. (2015). Together with structure, protein sequence can also be determined. Miyashita et al. (2001) describe protein sequencing using Edman degradation, a classical technique used to determine the amino acid sequence of proteins. Although effective in many cases, Edman degradation is limited to proteins without post-translational modifications on the N-terminal. This has shifted focus to mass spectrometry to become the main protein sequencing technology even though more novels approaches are continuously being developed (Restrepo-Pérez et al., 2018; Steen & Mann, 2004). Using the sequence, bioinformatic tools such as UnitProt and RefSeq can be used (Chen et al., 2017). As a consortium of databases, UniProt is the go-to platform for protein sequences. Its constituent databases, UniProt Knowledgebase, UniProt Archive, UniProt Reference Clusters, and UniProt Proteomes, cater to all protein inquiries from functional information, annotations, publications, homology, and full proteome information (Pundir et al., 2015). Similarly, RefSeq gives non-redundant sequence information of proteins including conserved regions and variations. As part of NCBI, RefSeq can be accessed from all NCBI tools such as BLAST (Pruitt et al., 2007).

2.6. Research Objectives

The purpose of this study was to characterize LMEs found in different white rot fungi using various bioassays. Further, based on enzyme activity, the best performing fungi

were selected for bioremediation testing using a mixture of petroleum products. This research had the following objectives:

1. To acquire, culture, and characterize fungal isolates using microbiological and mycological techniques of plate cultivation as well as molecular sequencing for identification.
2. To qualitatively assess the production of LMEs in the characterized isolates by means of plate cultivation in the presence of enzyme substrates
3. To assess the growth of isolates on lignocellulosic material and quantitatively assay LMEs production using spectrophotometric analysis
4. To compare commonly used substrates in LMEs activity assay
5. To determine which of the isolates are good LMEs producers
6. To apply these selected isolates in the bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated soil and evaluate their efficiency using bench-type approach to the testing of polluted soils

CHAPTER 3: METHODOLOGY

3.1. Fungal isolates used for the study

A total of twelve (12) fungal isolates were obtained from Dr. Grace Ijoma (University of South Africa, Institute for the Development of Energy for Africa Sustainability). These isolates were previously used in studies and stored as stock cultures (Ijoma & Tekere, 2017).

The isolates were cultivated on potato dextrose agar (PDA) (Sigma Aldrich) for the duration of the research. Every three (3) months, fresh plates were prepared, and the isolates were transferred from old plates to new ones. This was achieved by adding 39 g of PDA (Sigma) to sufficient distilled water to make 1 L of media. After ensuring that all PDA has dissolved, the media was autoclaved at 121°C for 15 min. When media had cooled to approximately 60°C, it was aseptically poured into 90 mm sterile petri dishes and allowed to solidify before a 1x1 cm fungal plug was transferred from the old plate to the freshly prepared one. Inoculated plates were incubated at 25°C for at least 7 days to allow mycelial proliferation and then stored in the fridge at 4°C for later use.

3.2. Molecular characterization of organisms

Molecular characterization to ensure axenic character of all acquired isolates was done by Sanger sequencing using ITS1/ITS4 primers (White et al., 1990) as follows:

3.2.1. Genomic DNA extraction

From each fungal isolate, genomic DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research). About 100 mg of fungal cells were obtained by scrapping the surface of a 7-day old plate. These cells were added to a ZR BashingBead ™ Lysis tube together with 750 µl of BashingBead ™ Buffer. The tube was placed on a Disruptor Genie ™ and processed for 20 minutes. After processing, the lysis tube was centrifuged for 1 min at 10 000xg in a microcentrifuge. Up to 400 µl of supernatant was to a Zymo-Spin ™ III-F Filter in a collection tube and centrifuged for 1 min at 8 000xg. To the filtrate in the collection tube, 1 200 µl of genomic lysis buffer supplemented with beta-mercaptoethanol (0.5% v/v) were added.

From there, 800 µl of the obtained mixture was transferred to a Zymo-Spin ™ IICR Column[3] in a collection tube and centrifuged for 1 min at 10 000xg. The flowthrough from the collection tube was discarded and centrifugation was repeated using the remaining amount of the mixture. Following that, 200 µl of DNA Pre-Wash buffer was added to the Zymo-Spin ™ IICR Column in a new collection tube and centrifuged for 1 min at 10 000xg. Next, 500 µl of g-DNA Wash Buffer was added to the Zymo-Spin ™ IICR Column and centrifuged for 1 min at 10 000xg. The collection tube was then discarded, the Zymo-Spin ™ IICR Column was transferred to a clean 1.5 ml microcentrifuge tube, and 100 µl of DNA Elution Buffer was added directly to the column matrix. The tube containing the column was centrifuged for 30 sec at 10 000xg and pure genomic DNA was collected in the microcentrifuge tube. This DNA was stored at -20°C until before use.

3.2.2. Polymerase Chain Reaction (PCR) and sequencing

The ITS target region was amplified by PCR using OneTaq® Quick-Load® 2x Master Mix (NEB) with the ITS1/ITS4 primer pair (**Table 3.1**). PCR products were run on a gel and extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye ™ Terminator Cycle Sequencing Kit V3.1, BRD 3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™). The purified fragments were then analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. CLC Bio Main Workbench v7.6 was used to analyze the .ab1 files generated by the ABI 3500xl Genetic Analyzer. As quality filtering, each generated DNA sequence (chromatogram) was trimmed at the beginning as well as the end. On average, the first 40 bp of the sequence were removed due to overlapping peaks, and the end portion of the sequence showing low peak intensity was also trimmed away. This left around 500-600 bp of “good” data which was then aligned (BLAST) against the NCBI nucleotide database.

Table 3. 1: ITS Primers sequences

Name of primer	Target	Sequence (5’ to 3’)	Reference

ITS1	Small sub-unit	TCCGTAGGTGAACCTGCGG	(White et al., 1990)
ITS4	Large sub-unit	TCCTCCGCTTATTGATATCG	(White et al., 1990)

3.3. Qualitative screening of the production of LMEs using fungal isolates

Twelve (12) characterized isolates were screened for their capability to produce two of the most industrially relevant LMEs, namely Lignin peroxidase and Laccase.

3.3.1. Lignin peroxidase screening

To screen for the capability of fungal isolates to produce Lignin peroxidase, a method was adapted from (Falade et al., 2017). For 1 L of media, 39 g of PDA (Sigma Aldrich) were dissolved in dH_2O and supplemented with 0,01% (w/v) of Azure B (Sigma). The media was sterilized at 121°C for 15 min and aseptically poured into sterile 90 mm petri dishes after cooling to below 50°C. Following media solidification, a mycelial plug of about 1x1 cm, obtained from a 7-day old plate, was transferred to each petri dish. The plates were incubated at 25°C for 7 days. Lignin peroxidase production was identified by the decolorization of Azure B on plates.

3.3.2. Laccase screening

Isolates were screened for the production of laccase using the method by (Rao et al., 2019). Media was prepared by dissolving 39 g of PDA in enough dH_2O to make 1 L of solution. This was sterilized by autoclave at 121°C for 15 min. Once the media had cooled to about 50°C, it was supplemented with 0.02% (v/v) Guaiacol (Sigma) and properly mixed. Media was then aseptically poured into 90 mm petri dishes and allowed to solidify. A 1x1 cm mycelial plug obtained from a 7-day old plate was transferred to the freshly prepared one which was then incubated at 25°C for 7 days. Laccase production was identified by the appearance of an orange-brown ring on plates.

3.4. Production of LMEs on lignocellulosic biomass and quantitative assays

3.4.1. Lignocellulosic biomass preparation

Corn husk was chosen as lignocellulosic biomass to be used in this study due to its availability. Sufficient amount of fresh corn husk was obtained from local maize sellers at the Roodepoort market in Johannesburg, Republic of South Africa. The corn husk was cleaned with distilled water to remove dirt and any other small particles before being placed in an oven at 70°C to dry overnight. The dried husks were then pulverized using a lab blender and sieved to obtain particle sizes of below 2 mm. This pulverized husk was stored at 4°C before use to prevent any microbial growth.

3.4.2. Microbial growth on corn husk

From the screened isolates, nine (9) fungi were selected from the total twelve (12) for LME production. These were grown on solid media containing lignin in the form of corn husk as sole carbon source. The growth media composition was adapted from Rao et al. (2019) and contained for every liter of media, 20 g agar (Sigma), 10 g corn husk, 1 g KH_2PO_4 (Sigma), 1 g yeast extract (Sigma), 0.5 g $MgSO_4.7H_2O$ (Merck), 0.5 g $(NH_4)_3PO_4$ (Sigma), 0.1 g $CaCl_2.2H_2O$ (Sigma), 4 mg $ZnSO_4.7H_2O$ (Sigma), 1 mg $CuSO_4.5H_2O$ (Sigma), and 1 mg $Fe_2(SO_4).7H_2O$ (Sigma). The media was autoclaved at 121°C for 15 min and 1 mL of 2 mg/mL filter sterilized Thiamine HCl solution was added once the media temperature had dropped to 50°C. Media was then poured into 90 mm sterile petri dishes in triplicate and a 2x2 cm mycelial plug was cut from 7 days old plates and added to each petri dish accordingly. The plates were incubated at 25 ±1°C for a total of 21 days with collection every third day. All experiments were run in triplicate and ensure both biological and technical replication and to determine variance.

3.4.3. Crude enzyme extraction and activity assays

During sample collection, crude enzyme was extracted from plates by washing the mycelia growth with 10 mL of the appropriate buffer for each targeted enzyme. These were 125 mM sodium tartrate buffer (pH 3.0), 200 mM sodium malonate buffer (pH 4.5), and 50 mM sodium malonate buffer (pH 4.5) for lignin peroxidase, manganese peroxidase, and laccase, respectively. Activity assays were carried using a

spectrophotometric instrument, Thermo Scientific GENESYS^{TM} 10S UV-VIS. Two sets of activity assays methods were applied for each enzyme and enzyme activity was expressed as U/ml, defined as the amount of enzyme needed to convert 1 mmol of substrate into product in 1 minute. This was calculated using the formula **$EA = \Delta A \times V/t \times \varepsilon \times v$**

Where EA: Enzyme activity (U/ml)

ΔA: change in absorbance

V: total volume of reaction mixture (ml)

t: time (min)

ε: Molar extinction coefficient of substrate ($M^{-1}cm^{-1}$)

v: volume of crude enzyme extract (ml)

Lignin peroxidase

Method 1: the veratryl alcohol assay as described by Arora and Gill (2001) is briefly described. In a reaction cell, 1 ml of 125 mM sodium tartrate buffer (pH 3.0) (Sigma), 500 µl of 10 mM veratryl alcohol (Sigma Aldrich), and 500 µl of enzyme extract were added. Absorbance of the mixture was measure at 310 nm, and the reaction was initiated by the addition of 500 µl of 2 mM hydrogen peroxide (Merck). Absorbance was again measured after 1 min of reaction.

Method 2: the dye Azure B method, also previously used by Arora and Gill (2001) was employed. In a reaction cell, 1 ml of 125 mM sodium tartrate buffer (pH 3.0) (Sigma), 500 µl of 0.160 mM Azure B (Sigma), and 500 µl extract were added. Absorbance of the mixture was measure at 651 nm, and the reaction was initiated by the addition of 500 µl of 2 mM hydrogen peroxide (Merck). Absorbance was again measured after 1 min of reaction.

Manganese peroxidase

Method 1: enzyme assay as described according to Couto et al. (1998) was used. In a reaction cell, 500 µl of 200 mM sodium malonate buffer (pH 4.5) (Sigma), 500 µl of 4 mM 2,6 dimethoxyphenol (Sigma), 500 µl of 4 mM $MnSO_4$ (Sigma), 200 µl of extract, and 33 µl of dH_2O were added. Absorbance of the mixture was measured at 468 nm, and the reaction was initiated by the addition of 267 µl of 3 mM hydrogen peroxide (Merck). Absorbance was again measured after 1 min of reaction.

Method 2: enzyme assay as described according to the method by Yasmeen et al. (2013) was applied. In a reaction cell, 1 ml of 1 mM $MnSO_4$ (Sigma), 1 ml of 50 mM sodium malonate buffer (pH 4.5) (Sigma), and 100 µl crude extract were added. Absorbance of the mixture was measure at 270 nm, and the reaction was initiated by the addition of 500 µl of 1 mM hydrogen peroxide (Merck). Absorbance was again measured after 1 min of reaction.

Laccase

Method 1: enzyme assay as it was adapted from Yasmeen et al. (2013) is described briefly. A reaction mixture was made by adding 1 mL of 1 mM ABTS (Sigma), 1 ml of 50 mM malonate buffer (pH 4.5) (Sigma), and 100 µl of crude extract. Absorbance was measured at 436 nm and the mixture was incubated at 25°C for 10 min before measuring absorbance again.

Method 2: enzyme assay as it was adapted from Abd El Monssef et al. (2016) was employed. The reaction mixture was made of 400 µl of 2 mM Guaiacol (Sigma), 1200 µl of 10 mM sodium acetate buffer (pH 5.0) (Sigma), and 400 µl of crude enzyme. The mixture was incubated at 35°C for 15 min. A blank was prepared using dH_2O instead of crude enzyme and was used to zero the instrument at 450 nm. After incubation, the reaction mixture was measured at the same wavelength.

3.4.4. Pure enzyme assay (Experiment Controls)

Pure forms of the 3 enzymes were used as positive control to also compare the different activity substrates. Enzyme solutions were prepared by mixing the dry enzyme with distilled water. These solutions were then used to run activity assays according to the previously described methods.

Lignin peroxidase

To begin, 1 g of Lignin peroxidase (0.12 U/mg) was obtained. A 1 U/ml solution was prepared by dissolving 83.3 mg of enzyme in 10 ml of solution (calculation in Appendix B). This enzyme solution was used to run both the veratryl alcohol assay and the Azure B.

Manganese peroxidase

A 0.134 U/ml manganese peroxidase solution was prepared using 10 mg of pure enzyme (13.4 U/g) (calculation in Appendix B).
This enzyme solution was used to run both manganese peroxidase assays.

Laccase

A 1 U/mg laccase solution was prepared by dissolving 12.82 mg of enzyme (0.78 U/mg) in 10 ml of solution (calculation in Appendix B).
This enzyme solution was used to run laccase assay using both ABTS and Guaiacol.

3.5. Application of fungal isolates in the bioremediation of polycyclic aromatic hydrocarbons (PAHs) contaminated soil.

The ability of LMEs to bioremediate PAHs-contaminated soil was studied using *Schizophyllum commune* and *Trametes hirsuta*, the two fungi that demonstrated the best production of LMEs from the previous experiments. Two remediation conditions were assessed for maximum remediation based on literature (Magan et al., 2010; Sukarta & Sastrawidana, 2014): remediation with corn husk supplementation, as well as remediation without corn husk supplementation.

3.5.1. Sample preparation

Soil was collected locally and sterilized at 121°C for 20 min prior to use. Polyaromatic hydrocarbons were obtained in the form of diesel and unleaded petrol which were mixed together. Samples were then prepared according to the following conditions:

Condition 1: 30 g of soil and 2% hydrocarbons in a sterile container.
Condition 2: 30 g of soil, 3 g of pulverized corn husk and 2% hydrocarbon.
Samples were left uncovered overnight in a sterile environment (fumehood) to allow the evaporation of volatile components. Each sample container was then inoculated with a 2x2 cm fungal plug of *Schizophyllum commune* (SC) or *Trametes hirsuta* (TR) and the samples were incubated at room temperature for a total of 7 weeks.

3.5.2. Assessment of the biodegradation of PAHs

The biodegradation of PAHs in soil was assessed by GC-MS

3.5.2.1. Extraction of PAHs from soil and sample preparation

PAHs were extracted from the weekly collected soil samples by ultrasonication using a method adapted from Oluseyi et al. (2011). For this, 5 g of PAH contaminated soil was weighed and added to 50 ml brown, glass bottle. The extraction solvent used was 99% GC grade methanol (Sigma) of which 15 ml was added to the glass bottle. The bottle was sealed and placed in an ultrasonic bath (Elmasonic) at 50°C, at maximum frequency for 30 min. The bottle was vigorously shaken every 10 min to allow resuspension of the soil and optimal extraction. After sonication, the bottle was allowed to rest for 10 min for larger soil particles to settle at the bottom. The supernatant was decanted into a new glass tube which was centrifuged for 1 min at 4 000xg. The supernatant was filtered into 2 ml GC vials using a 0.45 µm filter and stored at 4°C before use.

3.5.2.2. GC-MS analysis

Chromatographic analysis was done using a 7890B Agilent Technologies GC system coupled with a 5977B Agilent technologies mass spectrometer. For the analysis, 2 µl of sample was injected into the instrument equipped with a HP-5MS capillary column (30 m length, 0.250 mm diameter, 0.25 µm film thickness) with helium as carrier gas at the constant flowrate of 1 ml/min. The injection was done using split-less mode with an inlet temperature set at 180°C. The oven program was started at 70°C and held there for 1 min, then slowly increased to 300°C at a rate of 3°C/min. Detection was done using the scan mode, ranging from 30 m/z to 600 m/z with MS source at 230°C and quadrupole at 150°C. Data analysis was done using MassHunter 10.0.368 software.

CHAPTER 4: RESULTS

4.1. Introduction to results

The degradation of xenobiotic compounds using fungal monocultures depends greatly on the ability of these organisms to produce lignin modifying enzymes (LMEs). A total of twelve fungal monocultures were grown on potato dextrose agar. Using their extracted genomic DNA, the cultures were molecularly characterized by means of Sanger sequencing. A preliminary qualitative screening was carried to assess the ability of the various isolates to produce LMEs on solid PDA media. This informed the selection of isolates for subsequent steps. Selected isolates were used to study the production of LMEs on solid media supplemented with ligninolytic biomass as sole carbon source. Using UV/Vis spectrophotometry, enzyme production was measured by means of activity assays. A comparative study of different substrates used for LMEs activity assays was also done. Two of the best enzyme producers were used for an attempted bioremediation of PAHs-contaminated soil. Using GS-MS, the degradation profile of some of these hydrocarbons was obtained.

4.2. Growth and molecular characterization of fungal isolates

PDA media was used to grow and conserve fungal isolates throughout the research. Characterization of the isolates was done through Sanger sequencing. Extracted genomic DNA was amplified by PCR using ITS1/ITS4 primers. **Figure 4.1** shows an agarose gel which was used to determine the efficacy of the amplification process with lanes 1 to 12 representing isolates 1 to 12. The size of the DNA fragments was estimated through comparison with the DNA ladder. These DNA fragments were extracted and sequenced in the forward and reverse direction. The generated sequencing files were analyzed with CLC Bio Main Workbench v7.6 and results obtained by BLAST search on NCBI. **Table 4.1** shows results of the BLAST search and predicted organisms based on the similarity between the queried sequences and those found with the NCBI database.

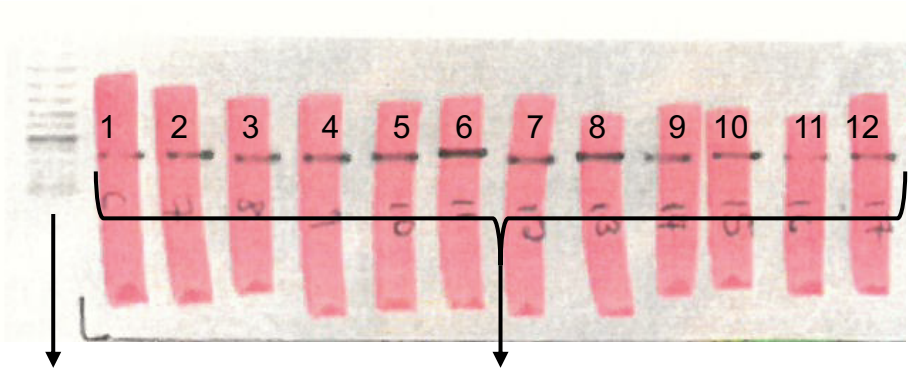

Figure 4. 1: Photographic image of a 1% (w/v) agarose gel indicating the fragments generated by the ITS1/ITS4 PCR. Numbers 1 to 12 represent the 12 isolates.

Table 4. 1: BLAST results and predicted organisms

Sample no	No of base pairs	Query description	Pred organisms	% Similarity	Query coverage	E-value	GenBank Accession
1	558	*Myrmaecium rubricosum* strain VRJ 18S ribosomal RNA gene, partial sequence	*Myrmaecium rubricosum*	100	100%	0.0	KP687882.1
2	602	*Trichoderma harzianum* isolate NEFU43 18S ribosomal RNA gene, partial sequence	*Trichoderma harzianum*	99.8	100%	0.0	KJ028794.1
3	675	*Rhizopus microsporus* isolate 8273 small subunit ribosomal RNA gene, partial sequence	*Rhizopus microsporus*	100	100%	0.0	MF176403.1
4	615	*Trametes hirsuta* genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: NBRC 7038	*Trametes hirsuta*	100	100%	0.0	AB733170.1
5	647	*Fomitopsis meliae* isolate FM1C20 small	*Fomitopsis meliae*	100	100%	0.0	MW221272.1

		subunit ribosomal RNA gene, partial sequence					
6	541	*Fusarium fujikuroi* isolate GX28 18S ribosomal RNA gene, partial sequence	*Fusarium fujikuroi*	100	100%	0.0	KJ000434.1
7	597	*Curvularia coatesiae* isolate 582947 small subunit ribosomal RNA gene, partial sequence	*Curvularia coatesiae*	100	100%	0.0	MT341911.1
8	504	*Schizophyllum commune* isolate MF-O1 internal transcribed spacer 1, partial sequence	*Schizophyllum commune*	92.4	100%	0.0	MN178556.1
9	565	*Penicillium oxalicum* strain DUCC5744 small subunit ribosomal RNA gene, partial sequence	*Penicillium oxalicum*	77.9	81%	0.0	MT582784.1
10	513	*Talaromyces sp*. Xz2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	*Talaromyces sp.*	95.8	92%	0.0	KJ935026.1
11	558	*Bjerkandera sp.* JCM 28456 genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA, partial and complete sequence	*Bjerkandera sp.*	97.5	100%	0.0	LC133859.1
12	578	*Aspergillus sp.* MR55 18S ribosomal RNA gene, partial sequence;	*Aspergillus sp.*	98.3	100%	0.0	KT374059.1

		internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence					

4.3. Qualitative screening of the production of LMEs by characterized isolates

A qualitative screening of the twelve isolates was carried out to assess their ability to produce lignin peroxidase and laccase. Screening was done on solid PDA plates and results assessed visually.

4.3.1. Lignin peroxidase screening

The qualitative screening for lignin peroxidase was done using 0.01% Azure B. In the presence of lignin peroxidase, the blue dye is decolorized, and this can be visually observed on solid media. **Figure 4.2a** shows the results of the screening. It can be seen that nine (9) out of the twelve (12) isolates presented lignin peroxidase production though in varied quantities as seen from the different decolorization levels observed on different plates.

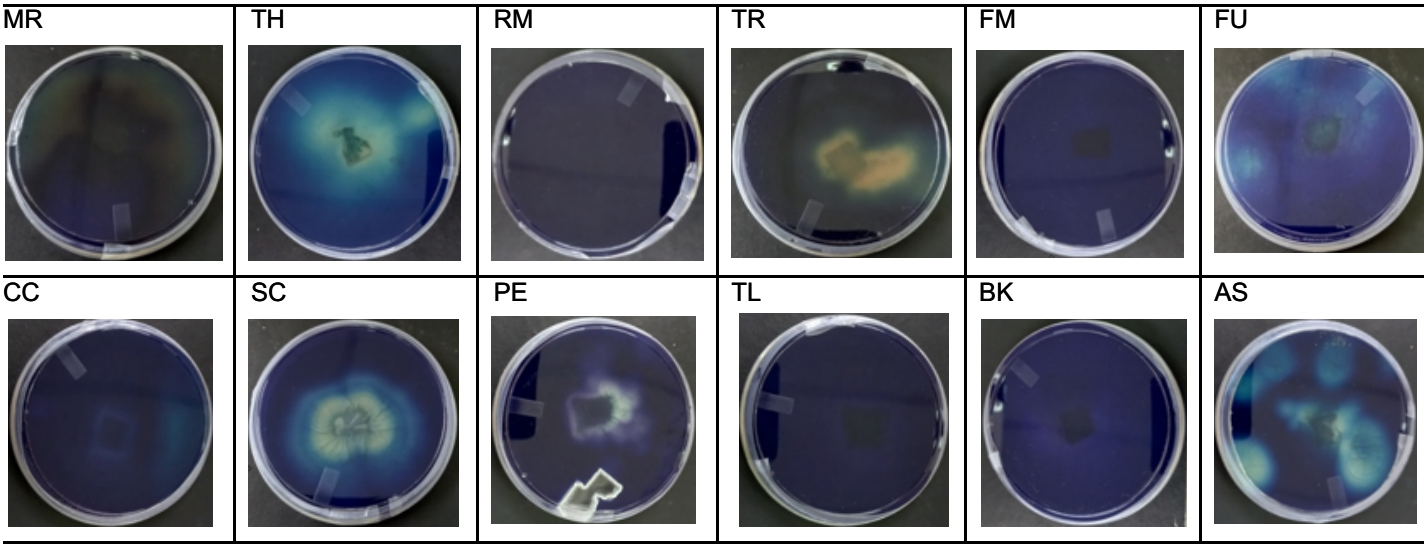

Figure 4. 2 a: Qualitative screening for Lignin peroxidase using 0.01% Azure B. MR: *Myrmaecium rubricosum*, TH: *Trichoderma harzianum*, RM: *Rhizopus microspores*, TR: *Trametes hirsuta*, FM: *Fomitopsis meliae*, FU: *Fusarium fujikuroi*, CC: *Curvularia coatesiae*, SC: *Schizophyllum commune*, PE: *Penicillium oxalicum*, TL: *Talaromyces sp.*, BK: *Bjerkandera sp.*, AS: *Aspergillus sp.*

4.3.2. Laccase screening

The qualitative screening for laccase, on the other hand, was done using 0.02% Guaiacol. In the presence of laccase, guaiacol is oxidized and produces a brown color on solid media. In this experiment, less than half of the tested isolates gave positive results as seen in **Figure 4.2b**, and very minimal amounts of laccase could be observed on some of them. **Table 4.2** shows cumulative results of both screening experiments and the isolates selected for further studies are mentioned as (1) to (9).

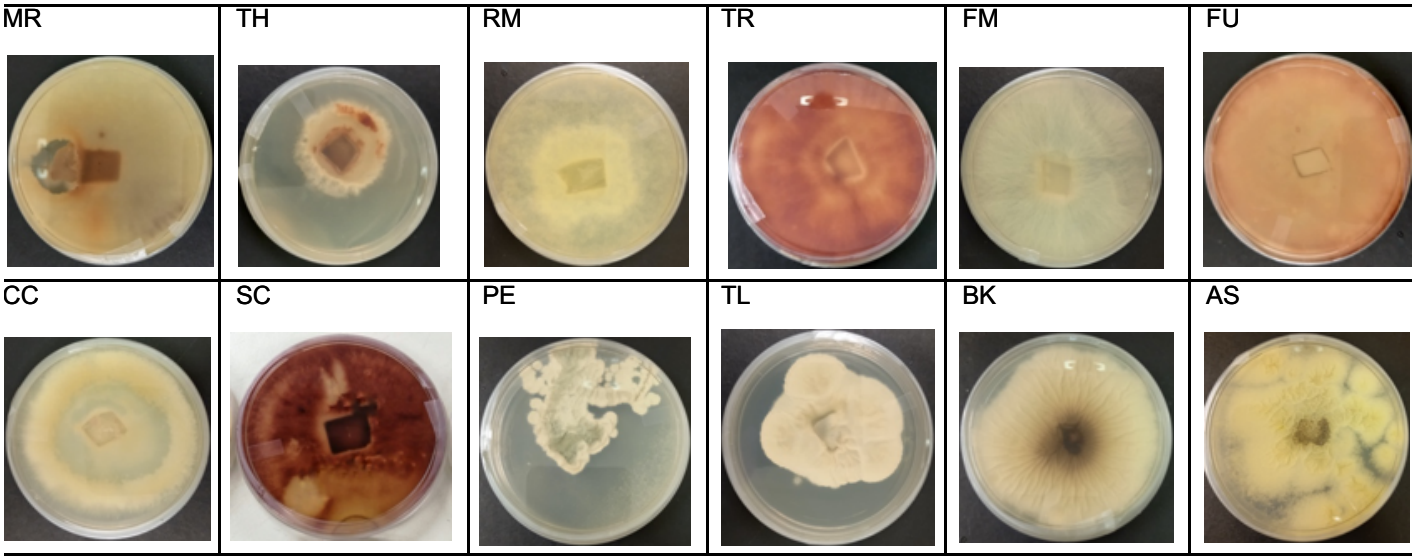

Figure 4. 2 b: Qualitative screening for Laccase using 0.02% Guaiacol. Isolates names as in Figure 4.2 a.

Table 4. 2: Screening results for Lignin peroxidase and Laccase

Isolate name	Azure B test (LiP)	Guaiacol test (Lacc)
Myrmaecium rubricosum (1) MR	+	+
Trichoderma harzianum (2) TH	+	+ (small)
Rhizopus microspores RM	-	-
Trametes hirsuta (3) TR	+	+
Fomitopsis meliae (4) FM	- (-ve control)	- (-ve control)
Fusarium fujikuroi (5) FU	+	+

Curvularia coatesiae (6) CC	+	-
Schizophyllum commune (7) SC	+ (+ve control)	+ (+ve control)
Penicillium oxalicum (8) PE	+	-
Talaromyces sp. TL	-	-
Bjerkandera sp. BK	+ (small)	-
Aspergillus sp. (9) AS	+	-

4.4. Production of LMEs on lignocellulosic biomass and quantitative assays

From the twelve (12) initial isolates, nine (9) were selected for quantitative assays based on the screening results (**Figures 4.2a and 4.2b)**. These isolates were grown on solid media containing lignocellulose in the form of corn husk as the only carbon source. For each of the 3 enzymes of interest, two different assays were executed employing some of the most commonly used substrates from previous studies. Enzyme production was monitored and quantified over a period of 21 days and results recorded. All experiments were run in triplicates and a negative control was represented by substituting the enzyme extract with double distilled water (see Appendix A for detailed results).

4.4.1. Lignin peroxidase

Lignin peroxidase was quantitatively assayed using first the veratryl alcohol assay, and later the Azure B assay. In the first assay, change in absorbance was recorded at 310 nm and the activity was calculated using an extinction coefficient of 9 300 $M^{-1}cm^{-1}$. **Table 4.3a** presents average lignin peroxidase activities for each of the 9 isolates and the negative control from day 3 to day 21. **Figure 4.3a** gives a cumulative graphical representation of lignin peroxidase production by the isolates over 21 days. Careful examination of this figure reveals that enzyme production happened rapidly, as values of up to 18 U/ml can be observed from day 3. It was also observed that while some isolates reached maximum enzyme production levels on day 12 (TR, TH, FU),

few others reached theirs on day 15 (PE, CC, SC) followed by a steady drop. Highest activity reached is about 60 U/ml observed on day 12 for TR.

The second lignin peroxidase assay was carried using azure B as substrate at 651 nm with an extinction coefficient of 48800 $M^{-1}cm^{-1}$. Average activities are presented in **Table 4.3b** and used for the cumulative graph in **Figure 4.3b**. Similar to the veratryl alcohol assay, **Figure 4.3b** shows enzyme production already at day 3. However, all isolates show decreasing values from that point onward with many isolates approaching zero values.

Table 4. 3 a: Average values for lignin peroxidase activities over 21 days using the veratryl alcohol assay.

	MR	TH	TR	FM	FU	CC	SC	PE	AS	NC
Day 3	0.54 ± 0	0 ± 0	0 ± 0	3.76 ± 1.07	14.87 ± 2.17	16.49 ± 4.51	15.59 ± 4.30	11.83 ± 2.46	6.45 ± 1.61	0
Day 6	7.71 ± 0.31	9.86 ± 5.80	11.83 ± 3.36	9.68 ± 5.69	33.69 ± 7.22	24.37 ± 3.66	18.46 ± 2.76	21.33 ± 5.95	22.22 ± 2.76	0
Day 9	32.79 ± 1.08	34.41 ± 3.52	42.83 ± 14.37	17.74 ± 2.69	23.65 ± 1.94	33.69 ± 1.89	18.1 ± 4.51	21.15 ± 3.06	12.54 ± 6.46	0
Day 12	43.37 ± 1.35	56.81 ± 8.09	59.86 ± 7.53	18.46 ± 10.57	19.17 ± 2.96	15.95 ± 1.73	17.74 ± 2.99	30.11 ± 6.86	16.66 ± 3.72	0
Day 15	17.56 ± 1.12	42.83 ± 2.71	39.78 ± 11.67	18.1 ± 3.28	41.22 ± 3.28	44.62 ± 3.27	33.69 ± 5.75	53.05 ± 7.34	41.39 ± 3.36	0
Day 18	11.83 ± 2.69	25.81 ± 1.07	32.26 ± 9.96	13.44 ± 2.46	20.61 ± 8.09	25.63 ± 2.24	23.48 ± 5.99	20.07 ± 1.35	11.11 ± 5.65	0
Day 21	9.678 ± 3.52	25.27 ± 1.61	23.29 ± 3.96	11.83 ± 3.27	19.35 ± 3.36	22.04 ± 1.42	13.62 ± 3.58	11.83 ± 3.88	15.41 ± 4.17	0

Table 4. 3 b: Average values for lignin peroxidase activities over 21 days using Azure B.

	MR	TH	TR	FM	FU	CC	SC	PE	AS	NC
Day 3	4.85 ± 3.18	3.48 ± 2.81	2.18 ± 0.86	4.41 ± 0.67	5.81 ± 4.07	7.99 ± 5.07	2.93 ± 0.97	5.19 ± 3.04	6.21 ± 1.03	0
Day 6	0 ± 0	0 ± 0	0.24 ± 0.26	1.195 ± 0.68	2.05 ± 0.45	3.69 ± 0.80	1.98 ± 1.07	1.77 ± 0.31	1.09 ± 0.26	0
Day 9	1.33 ± 0.41	1.23 ± 0.92	1.09 ± 0.16	1.95 ± 0.47	1.60 ± 0.56	1.19 ± 0.67	1.16 ± 0.62	1.84 ± 0.37	1.77 ± 0.39	0
Day 12	1.33 ± 0.27	1.91 ± 0.51	1.02 ± 0.21	1.64 ± 0.37	1.57 ± 0.48	1.093 ± 0.48	1.13 ± 0.18	0.96 ± 0.26	1.13 ± 0.27	0
Day 15	1.29 ± 0.15	1.57 ± 2.05	1.36 ± 0.21	1.50 ± 0.16	1.36 ± 0.16	1.23 ± 0.18	0.99 ± 0.56	0.37 ± 0.16	1.06 ± 0.51	0
Day 18	0.99 ± 0.06	0.38 ± 0.36	0.72 ± 0.10	1.02 ± 0.35	1.50 ± 0.26	1.39 ± 0.16	1.093 ± 0.16	1.29 ± 0.21	0.92 ± 0.54	0
Day 21	0.96 ± 0.41	0.85 ± 0.46	0.89 ± 0.77	0.85 ± 0.78	0.99 ± 0.51	1.95 ± 1.01	2.73 ± 1.22	1.13 ± 0.37	1.57 ± 0.48	0

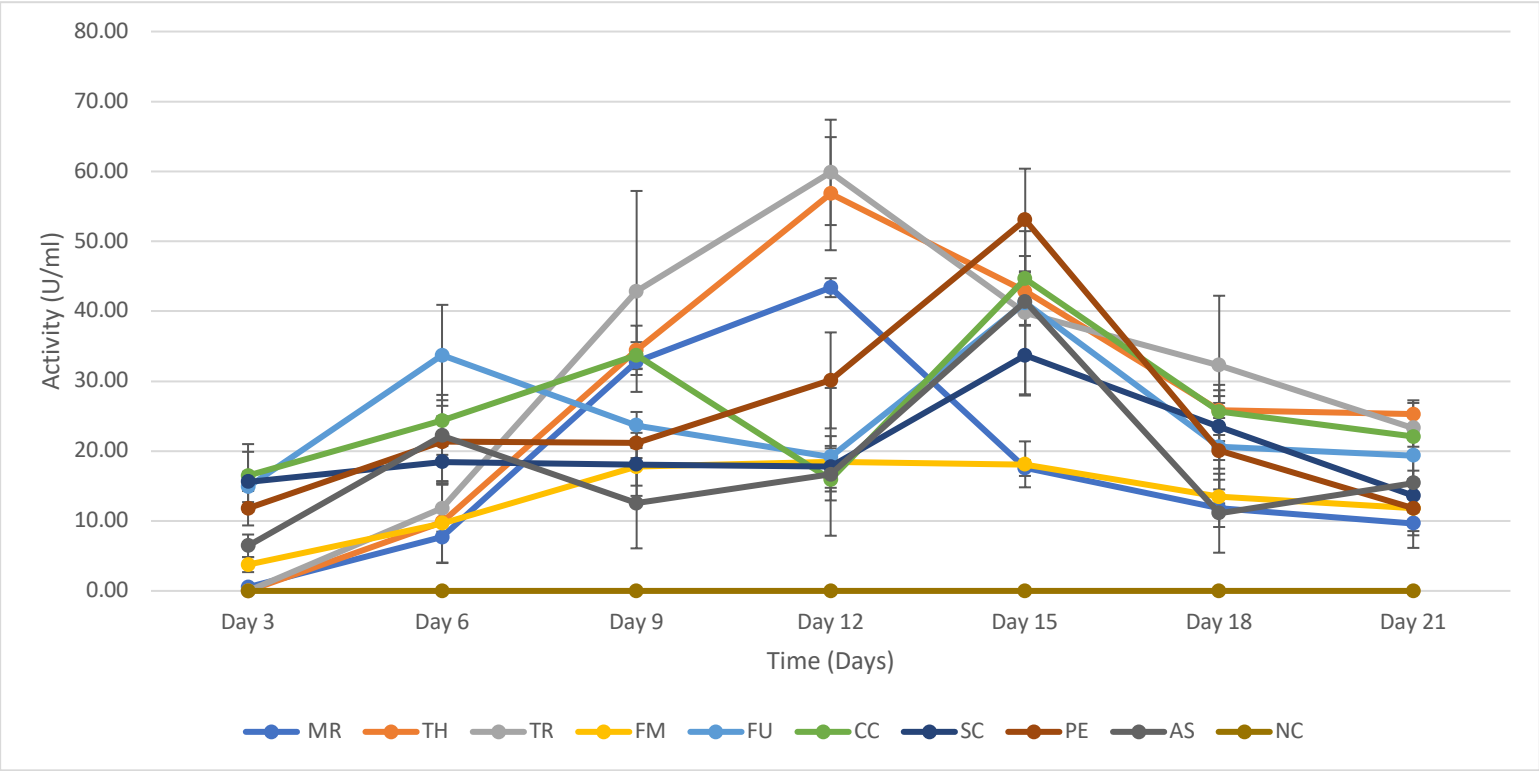

Figure 4. 3 a: Cumulative graph of lignin peroxidase activity in the different 9 isolates over 21 days using the veratryl alcohol assay. MR: *Myrmaecium rubricosum*, TH: *Trichoderma harzianum*, TR: *Trametes hirsuta*, FM: *Fomitopsis meliae*, FU: *Fusarium fujikuroi*, CC: *Curvularia coatesiae*, SC: *Schizophyllum commune*, PE: *Penicillium oxalicum*, AS: *Aspergillus sp.*

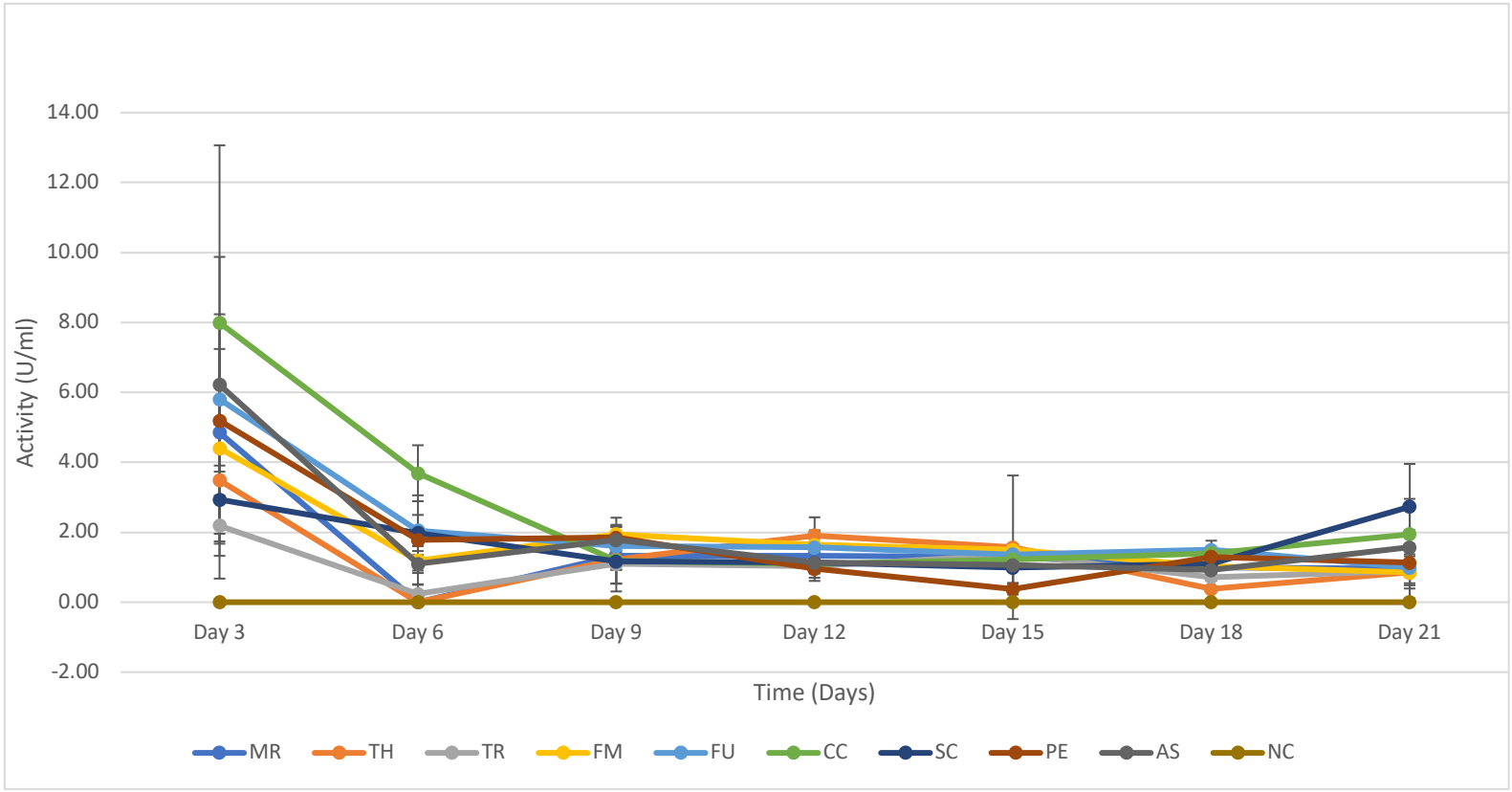

Figure 4. 3 b: Cumulative graph of the lignin peroxidase activity in the different 9 isolates over 21 days using the Azure B assay. MR: *Myrmaecium rubricosum*, TH: *Trichoderma harzianum*, TR: *Trametes hirsuta*, FM: *Fomitopsis meliae*, FU: *Fusarium fujikuroi*, CC: *Curvularia coatesiae*, SC: *Schizophyllum commune*, PE: *Penicillium oxalicum*, AS: *Aspergillus sp.*

4.4.2. Manganese peroxidase

Spectrophotometric assay of Manganese peroxidase was firstly done using 2,6 dimethoxyphenol at 468 nm with an extinction coefficient of 49 600 $M^{-1}cm^{-1}$. **Table 4.4a** presents average activities for each isolate over the 21 days period. These values are then used to plot **Figure 4.4a**. Most isolates show minimal activity up to day 9 at which point, they slowly start increasing reaching maximum values less than 100 U/ml. On the contrary, TR and SC stand out with higher values with TR reaching a maximum of 218 U/ml on day 18.

In a similar manner, manganese peroxidase was also assayed using the combination of manganese ions and sodium malonate at 270 nm with an extinction coefficient of 8 000 $M^{-1}cm^{-1}$. The observed activities are seen in **Table 4.4b** and used to plot **Figure 4.4b**. This figure shows similar trend compared to **Figure 4.4b** with TR and SC producing highest activities of manganese peroxidase while the remaining isolates show much lower values.

Table 4. 4 a: Average manganese peroxidase activities for 21 days using 2,6 dimethoxyphenol at 468 nm.

	MR	TH	TR	FM	FU	CC	SC	PE	AS	NC
Day 3	37.74 ± 5.56	38.14 ± 9.72	90.02 ± 30.91	13.95 ± 2.78	25.58 ± 12.0	33.77 ± 7.25	31.72 ± 0.56	27.63 ± 6.52	26.57 ± 5.72	0
Day 6	15.33 ± 0.83	15.66 ± 3.28	120.82 ± 37.33	7.73 ± 0.20	19.83 ± 1.57	11.04 ± 1.99	46.29 ± 7.99	19.17 ± 1.09	11.10 ± 3.52	0
Day 9	16.19 ± 2.48	14.28 ± 2.88	168.67 ± 13.23	2.84 ± 0.41	13.62 ± 2.35	12.23 ± 2.89	97.55 ± 7.85	20.69 ± 4.52	14.74 ± 4.52	0
Day 12	33.71 ± 7.74	34.50 ± 5.26	174.09 ± 23.60	8.26 ± 1.84	30.53 ± 7.34	24.59 ± 7.29	60.08 ± 14.02	39.23 ± 3.56	21.48 ± 4.24	0
Day 15	57.17 ± 7.24	48.84 ± 7.16	171.12 ± 50.30	9.85 ± 1.71	59.29 ± 15.65	36.48 ± 3.90	107.87 ± 8.13	59.82 ± 7.55	61.07 ± 3.82	0
Day 18	59.75 ± 1.69	66.89 ± 12.59	218.71 ± 69.60	17.25 ± 6.11	72.57 ± 10.84	43.62 ± 10.16	78.72 ± 13.18	44.48 ± 3.09	45.67 ± 7.37	0
Day 21	16.65 ± 4.48	20.09 ± 1.20	147.59 ± 62.99	2.77 ± 0.59	19.17 ± 1.49	15.53 ± 3.67	26.77 ± 6.73	18.70 ± 2.53	16.92 ± 1.59	0

Table 4. 4 b: Average manganese peroxidase activities over 21 days using manganese ions and sodium malonate at 270 nm.

	MR	TH	TR	FM	FU	CC	SC	PE	AS	NC
Day 3	37.25 ± 1.95	36.95 ± 8.26	102.94 ± 16.61	11.28 ± 1.01	30.62 ± 2.85	30.62 ± 2.82	32.63 ± 3.32	22.64 ± 3.09	21.79 ± 2.76	0
Day 6	15.44 ± 2.36	13.82 ± 3.37	134.02 ± 13.52	7.261 ± 2.09	20.10 ± 2.65	11.16 ± 1.35	60.86 ± 15.58	17.86 ± 1.74	10.74 ± 1.57	0
Day 9	17.04 ± 0.39	14.08 ± 2.47	169.67 ± 11.83	2.766 ± 0.77	26.74 ± 2.84	11.53 ± 2.03	98.27 ± 8.54	22.31 ± 2.56	13.42 ± 1.56	0
Day 12	35.08 ± 7.66	33.72 ± 6.24	172.79 ± 23.57	7.80 ± 1.49	42.31 ± 8.45	19.18 ± 2.27	99.21 ± 8.54	39.79 ± 5.05	19.76 ± 2.69	0
Day 15	56.01 ± 9.40	48.02 ± 6.59	174.49 ± 52.63	8.77 ± 1.88	63.59 ± 8.17	37.26 ± 3.34	110.14 ± 10.19	58.74 ± 3.32	40.30 ± 8.15	0
Day 18	58.87 ± 0.94	68.04 ± 10.40	184.40 ± 49.34	15.92 ± 2.05	71.27 ± 8.60	45.09 ± 9.16	77.91 ± 9.53	44.08 ± 3.73	35.51 ± 2.27	0
Day 21	14.69 ± 3.15	21.03 ± 2.41	138.49 ± 67.71	3.153 ± 0.99	18.47 ± 1.66	11.55 ± 2.36	28.54 ± 4.58	16.95 ± 1.19	16.32 ± 1.81	0

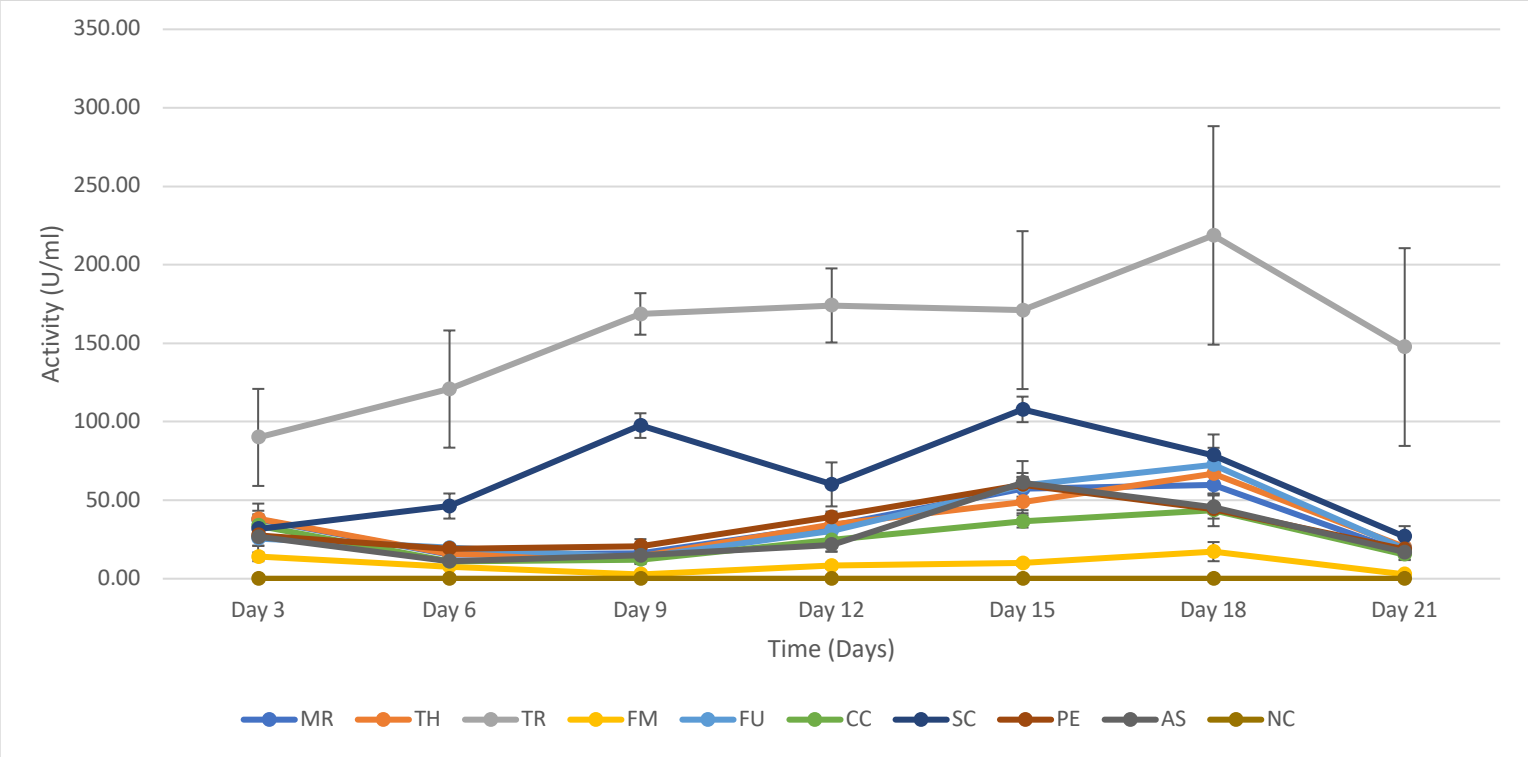

Figure 4. 4 a: Cumulative graph of the manganese peroxidase activity in the different 9 isolates over 21 days using 2,6 dimethoxyphenol at 468 nm. MR: *Myrmaecium rubricosum*, TH: *Trichoderma harzianum*, TR: *Trametes hirsuta*, FM: *Fomitopsis meliae*, FU: *Fusarium fujikuroi*, CC: *Curvularia coatesiae*, SC: *Schizophyllum commune*, PE: *Penicillium oxalicum*, AS: *Aspergillus sp.*

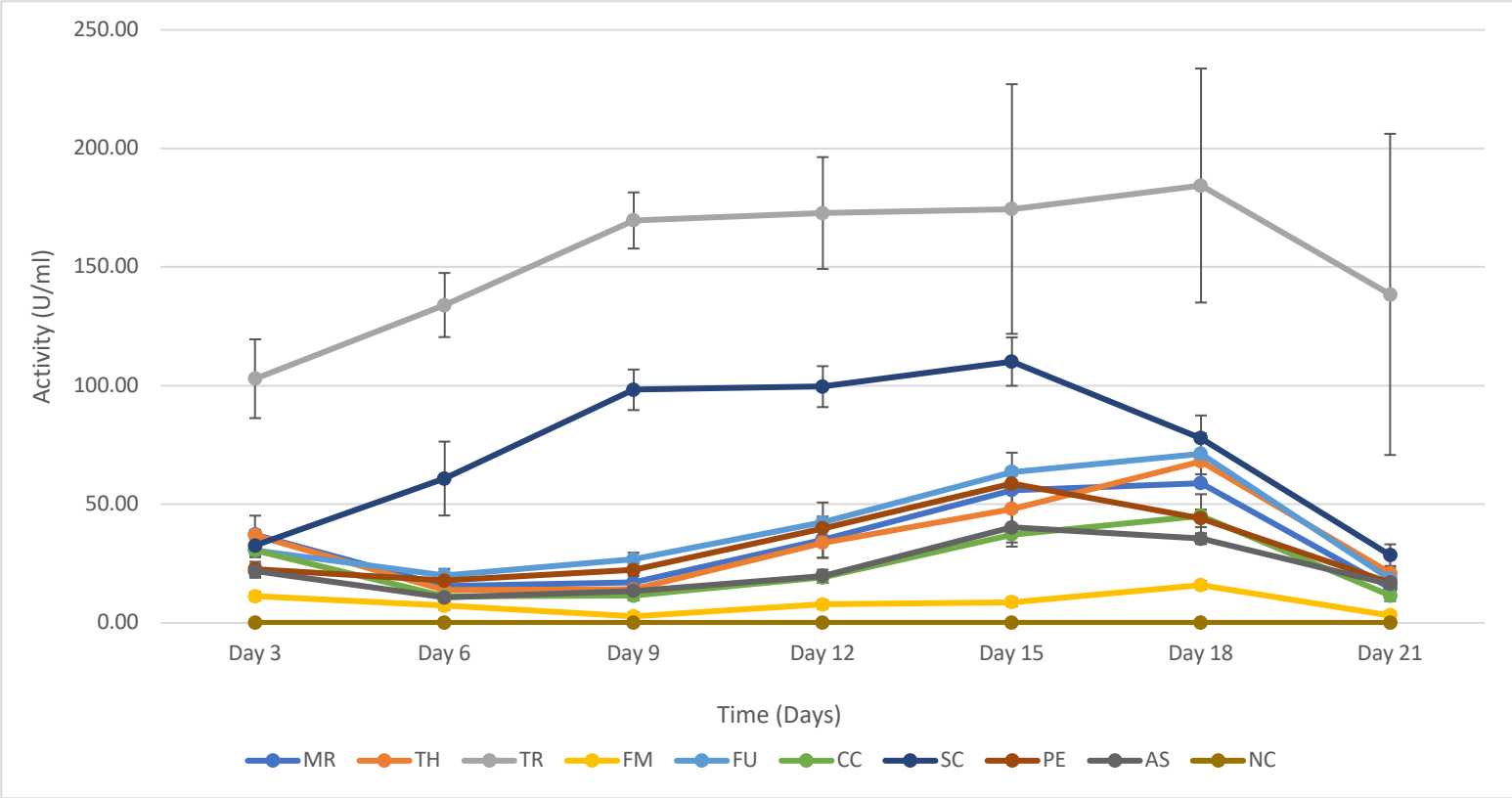

Figure 4. 4 b: Cumulative graph of the manganese peroxidase activity in the different isolates over 21 days using manganese ions and sodium malonate at 270 nm. MR: *Myrmaecium rubricosum*, TH: *Trichoderma harzianum*, TR: *Trametes hirsuta*, FM: *Fomitopsis meliae*, FU: *Fusarium fujikuroi*, CC: *Curvularia coatesiae*, SC: *Schizophyllum commune*, PE: *Penicillium oxalicum*, AS: *Aspergillus sp.*

4.4.3. Laccase

Laccase was the third LME to be quantitatively assayed. ABTS and guaiacol, two common reagents used for the spectrophotometric assessment of laccase were compared. Using ABTS, laccase production was quantified at 436 nm (ε = 29 300 $M^{-1}cm^{-1}$). Average values are seen in **Table 4.5a** and a cumulative activity graph is seen in **Figure 4.5a**. More than half of the isolates showed no laccase activity throughout the 21 days. TR showed a more or less constant activity of about 200 U/ml throughout while SC reached its highest values between days 9 and 12. Some limited activity is also observed from FU with a maximum of 13.78 U/ml on day 15 and from MR starting at day 15 with less than 4 U/ml.

Using guaiacol, laccase was quantified at 450 nm (ε = 6 740 $M^{-1}cm^{-1}$) after 15 min of reaction at 35°C. Average activity values for the organisms over the 21 days period are summarized in **Table 4.5b**. The values are used to plot **Figure 4.5b** where TR stands out once again as the highest laccase producer increasing from 60 U/ml on day 3 to just below 120 U/ml from day 12 onwards. All other isolates also show some activity, although marginal, contrasting with the observation done in the first assay.

Table 4. 5 a: Average activities for laccase activities over 21 days using ABTS at 436 nm.

	MR	TH	TR	FM	FU	CC	SC	PE	AS	NC
Day 3	0	0	161.79 ± 15.82	0	0	0	2.65 ± 0.71	0	0	0
Day 6	0	0	201.61 ± 6.46	0	4.18 ± 0.21	0	46.58 ± 5.27	0	0	0
Day 9	0	0	190.24 ± 10.92	0	8.38 ± 2.55	0	91.99 ± 14.65	0	0	0
Day 12	0	0	198.08 ± 4.22	0	10.03 ± 1.58	0	89.16 ± 17.33	0	0	0
Day 15	3.39 ± 0.32	0	193.66 ± 1.01	0	13.78 ± 3.96	0	41.24 ± 1.78	0	0	0
Day 18	2.84 ± 0.32	0	201.71 ± 5.31	0	10.89 ± 1.88	0	35.55 ± 14.29	0	0	0
Day 21	2.44 ± 0.36	0	201.66 ± 4.20	0	8.40 ± 2.37	0	21.54 ± 9.88	0	0	0

Table 4. 5 b: Average activities for laccase activities over 21 days using guaiacol at 450 nm.

	MR	TH	TR	FM	FU	CC	SC	PE	AS	NC
Day 3	5.51 ± 0.52	6.25 ± 2.00	60.12 ± 4.64	1.63 ± 0.39	5.52 ± 0.52	2.47 ± 0.73	6.13 ± 2.08	2.97 ± 0.35	2.36 ± 0.66	0
Day 6	6.69 ± 1.05	7.10 ± 2.47	86.37 ± 12.09	2.55 ± 0.46	6.35 ± 0.76	3.19 ± 0.72	6.59 ± 2.30	3.362 ± 0.36	2.93 ± 0.59	0
Day 9	5.93 ± 0.99	7.91 ± 4.28	109.36 ± 7.20	1.71 ± 0.22	10.50 ± 2.57	3.92 ± 0.74	8.90 ± 3.97	6.36 ± 1.41	5.46 ± 1.27	0
Day 12	4.75 ± 0.30	10.45 ± 2.03	118.33 ± 15.90	1.27 ± 0.08	7.93 ± 1.88	6.31 ± 1.51	6.09 ± 2.00	13.25 ± 2.59	4.04 ± 2.09	0
Day 15	4.12 ± 0.27	8.26 ± 1.32	114.74 ± 7.64	1.27 ± 0.59	5.88 ± 1.67	3.92 ± 0.64	4.63 ± 1.55	5.13 ± 1.19	3.16 ± 1.17	0
Day 18	4.33 ± 0.40	13.06 ± 2.30	119.01 ± 10.26	1.32 ± 0.06	6.02 ± 1.39	4.27 ± 0.80	6.76 ± 3.26	8.67 ±2.27	2.31 ± 1. 27	0
Day 21	4.14 ± 0.15	4.94 ± 0.23	114.85 ± 7.87	1.24 ± 0.42	4.38 ± 0.31	3.53 ± 1.79	4.61 ± 1.07	5.14 ± 1.82	2.88 ± 1.37	0

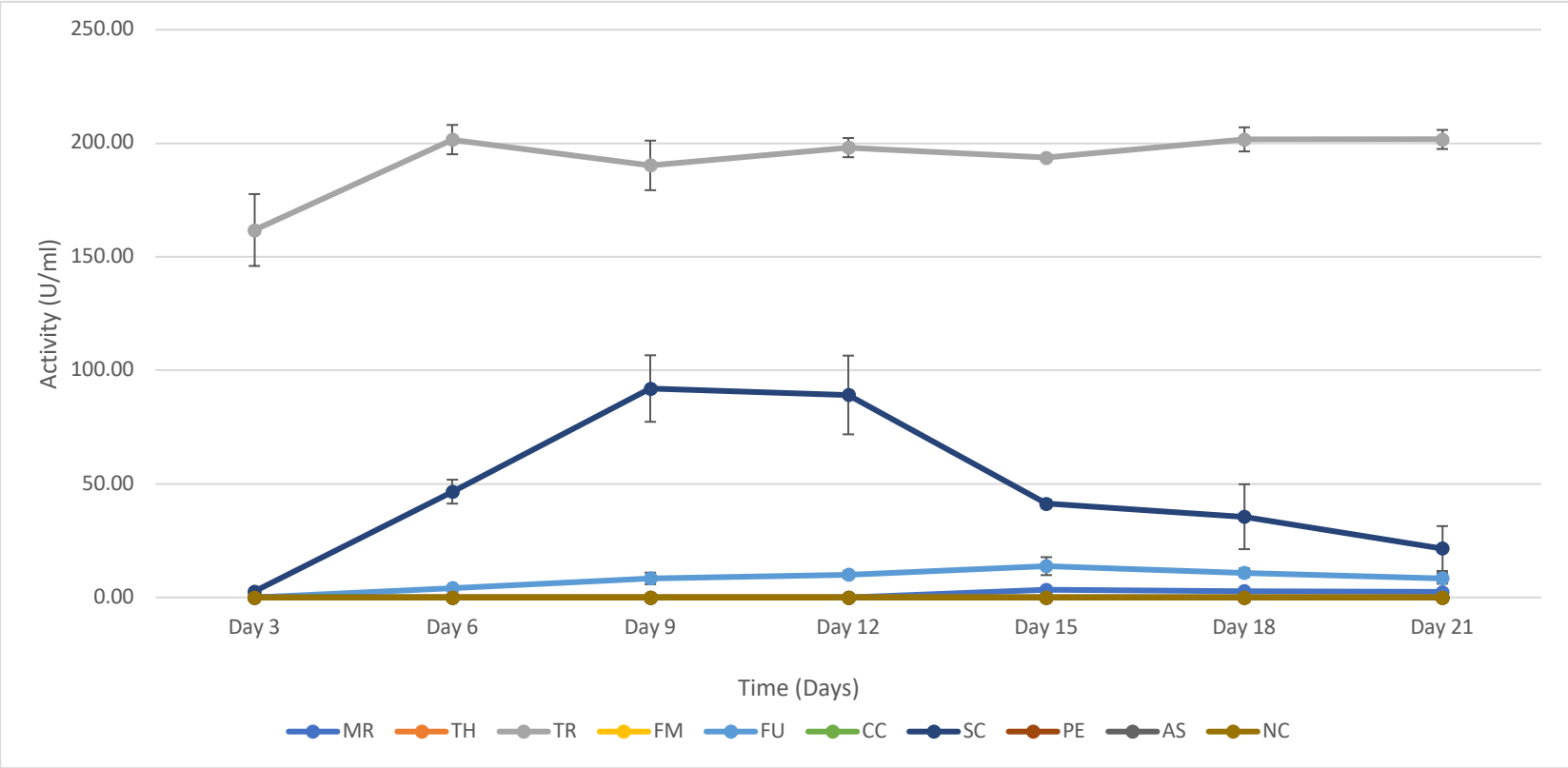

Figure 4. 5 a: Laccase activity in the different isolates over 21 days using ABTS at 436 nm. MR: *Myrmaecium rubricosum*, TH: *Trichoderma harzianum*, TR: *Trametes hirsuta*, FM: *Fomitopsis meliae*, FU: *Fusarium fujikuroi*, CC: *Curvularia coatesiae*, SC: *Schizophyllum commune*, PE: *Penicillium oxalicum*, AS: *Aspergillus sp.*

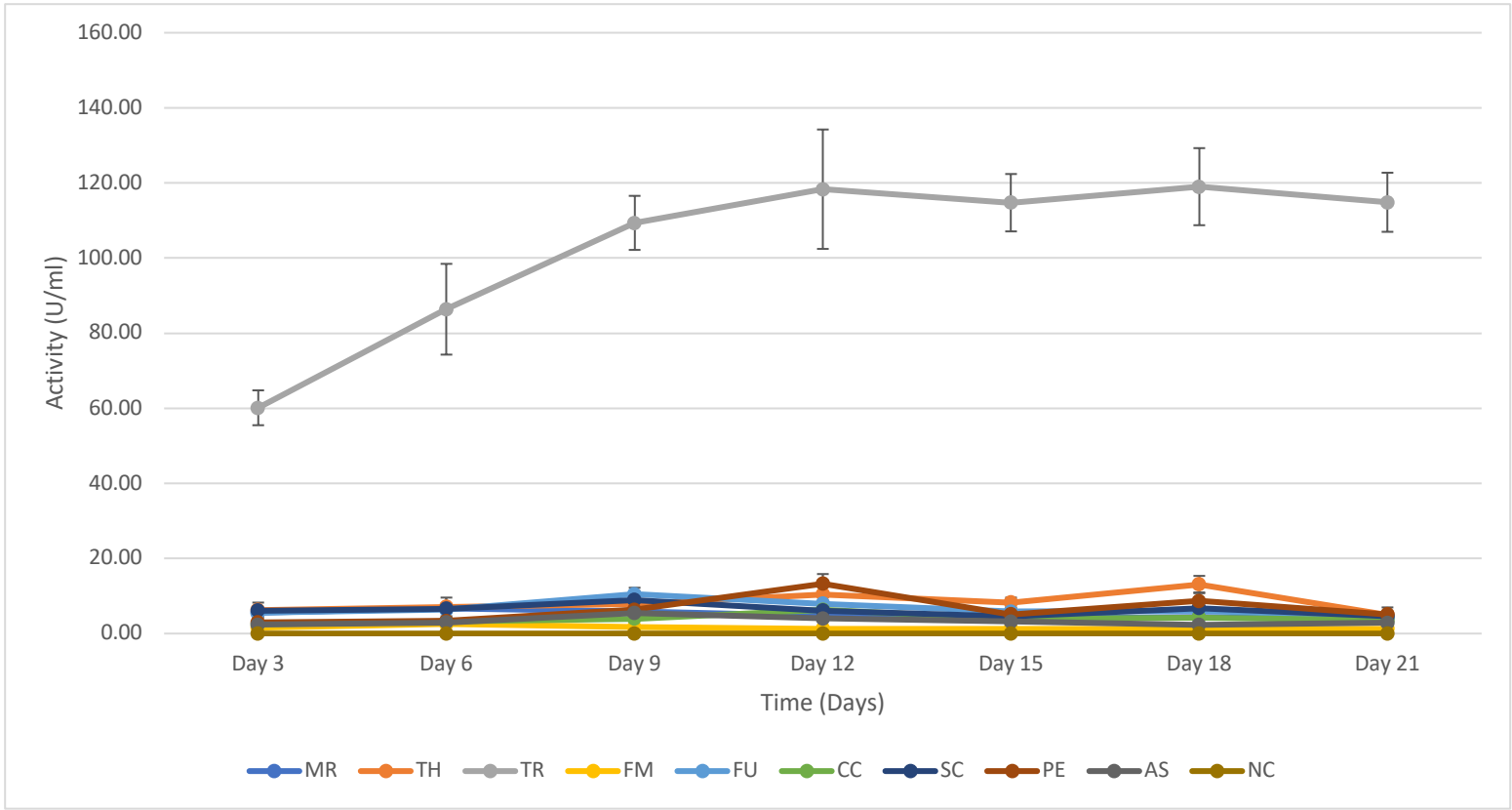

Figure 4. 5 b: Laccase activity in the different isolates over 21 days using guaiacol at 450 nm. MR: *Myrmaecium rubricosum*, TH: *Trichoderma harzianum*, TR: *Trametes hirsuta*, FM: *Fomitopsis meliae*, FU: *Fusarium fujikuroi*, CC: *Curvularia coatesiae*, SC: *Schizophyllum commune*, PE: *Penicillium oxalicum*, AS: *Aspergillus sp.*

4.4.4. Activity assay using pure enzymes

Pure, commercial enzymes were also used to compare the different activity assay substrates as well as to serve as positive controls.

Lignin peroxidase

A 1 U/ml solution was prepared and used to run both the veratryl alcohol assay and the Azure B. The following activities were obtained:

Table 4. 6 a: Calculated lignin peroxidase activity for pure enzyme

Veratryl alcohol	Azure B
57.53 ± 2.46	66.33 ± 0.77

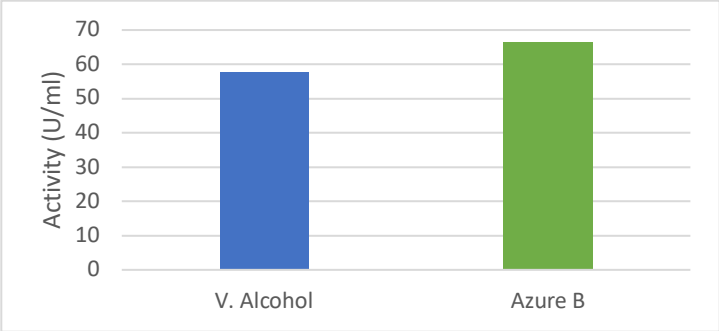

Figure 4. 6 a: Lignin peroxidase activity of pure enzyme using Veratryl alcohol vs Azure B

Manganese peroxidase

A 0.134 U/ml solution was prepared using commercially acquired manganese peroxidase. This enzyme solution was used to run both manganese peroxidase assays giving the following values:

Table 4. 6 b: Calculated manganese peroxidase activity for pure enzyme

2,6 dimethoxyphenol (DMP)	Mn3+ and sodium malonate
50.3 ± 3.97	52 ± 8.6

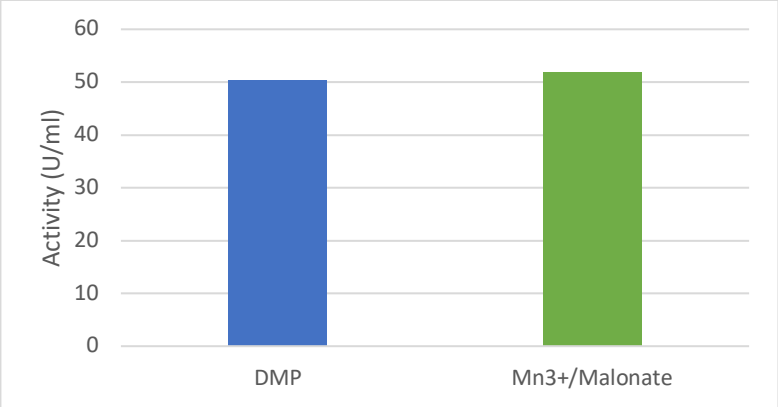

Figure 4. 6 b: Manganese peroxidase activity of pure enzyme using DMP vs Mn3+/Malonate

Laccase

A 1 U/mg laccase solution was prepared using commercial enzyme. This solution was used to run laccase assay using both ABTS and guaiacol and the results were as follows:

Table 4. 6 c: Calculated laccase activity using pure enzyme

ABTS	Guaiacol
237.02 ± 1.21	140.32 ± 0.87

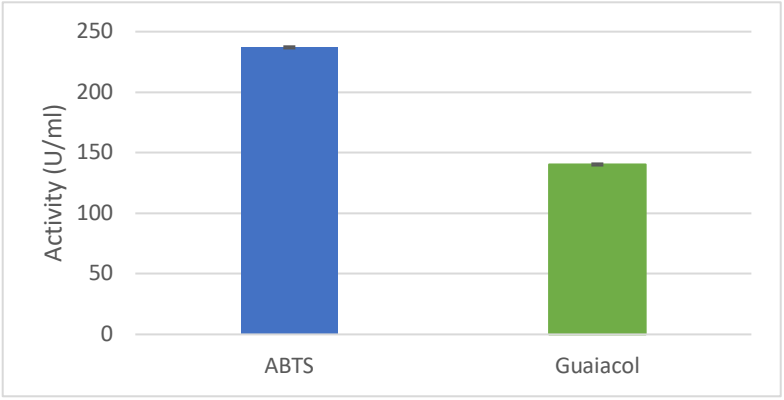

Figure 4. 6 c: Laccase activity of pure enzyme assayed with ABTS vs Guaiacol

4.5. Application of fungal isolates in the bioremediation of polycyclic aromatic hydrocarbons (PAHs) contaminated soil.

Two of the best LMEs producers from this study, *Schizophyllum commune* (SC) and *Trametes hirsuta* (TR), were used for the bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated soil. The isolates were grown on spiked soil samples contaminated with a mixture of unleaded petrol and diesel to allow for LMEs to breakdown these pollutants. PAHs degradation was assessed using GC-MS. Given the large variety of PAHs found in these fuels, a more generic approach was followed where degradation was expressed in terms of decrease of the area under the various PAHs peaks on the chromatogram. Using each fungus, degradation was studied both in the presence and absence of corn husk in the contaminated soil. **Figure 4.7a** shows chromatograms for PAHs-contaminated soil bioremediation using SC over a period of 7 weeks. A striking evolution of the chromatogram is observed from week 1 to week 7 with some of the main initial peaks either disappearing or losing height over the course of the experiment. The appearance of “new” peaks was also observed, mainly at the beginning and end of the chromatogram. Using the area under the peaks of the GC-MS chromatograms as an indication of the amounts of each compounds, evolution profiles were obtain**Table 4.7a** represents the degradation of some aromatic compounds through the action of SC over 7 weeks.

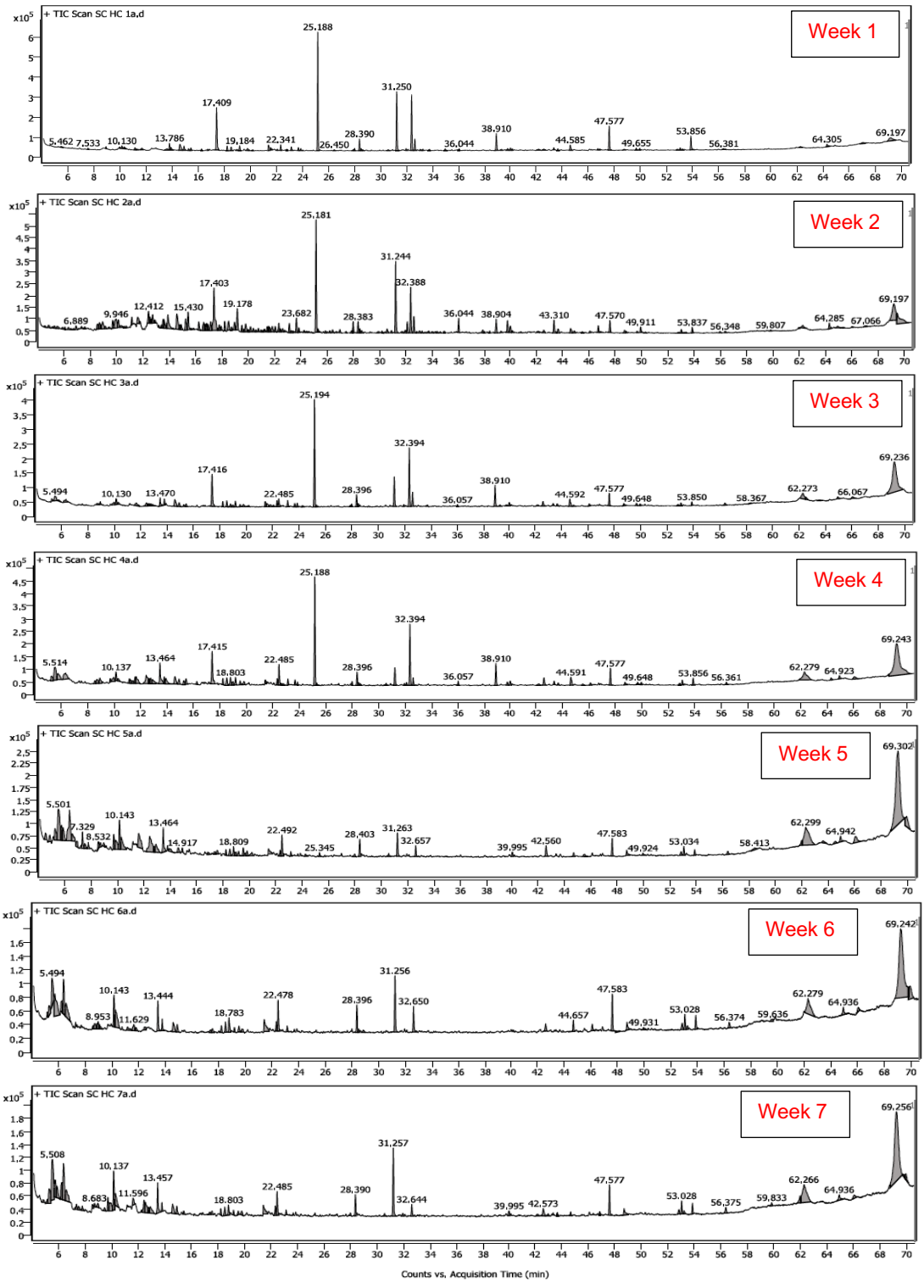

Figure 4. 7 a: GC-MS chromatograms of the degradation of PAHs using *Schizophyllum commune* (SC) over 7 weeks

Table 4. 7 a: Evolution profile of aromatic compounds in soil samples during remediation with *Schizophyllum commune* (SC)

RT	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Compound
5.5	38398	82648	161089	327832	755732	444256	210458	Benzene, 1-3-dimethyl
8.9	29133	282344	38757	188185	45339	62340	40647	Ethanone, 2-(formyloxy)-1-phenyl
9.9	35426	460749	79496	161449	223955	0	0	Benzene trimethyl
13.7	135021	23324	66500	113293	46066	68164	47662	p-Toluic acid, 4-nitrophenyl ester
15.4	61378	87202	46026	45089	44156	40316	29018	1-Pentanone, 1-(4-methylphenyl)
16.2	35195	191990	0	53245	0	0	0	Benzene, 4-ethenyl-1,2-dimethyl
17.4	1078210	1416275	593173	740677	90107	0	0	Cyclopentasiloxane, decamethyl
18.2	92998	151739	71756	68398	51784	50139	42784	Naphthalene
18.5	93538	93541	93091	89365	84761	68957	68116	Benzenepropanenitrile
22.3	118455	171834	92171	108626	63424	52796	48230	1,4-Benzenedicarboxaldehyde, 2-methyl
23.14	82451	84441	54510	44318	44051	38176	33322	Naphthalene, 2-methyl
32.6	249247	270595	207368	129484	100208	167977	87718	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters
42.5	54339	75539	103111	173373	132854	64258	86443	Benzenesulfonamide, N-butyl

While some important PAHs such as naphthalene and 2-methylnaphthalene are seen to clearly decrease over the course of the experiment, a fluctuation in amounts is observed for other aromatic compounds. Following the same approach, GC-MS was used to study bioremediation of PAHs-contaminated soil supplemented with ligninolytic biomass in the form of corn husk. Resulting chromatograms are presented in **Figure 4.7b** and compound evolution profiles in T**able 4.7b.**

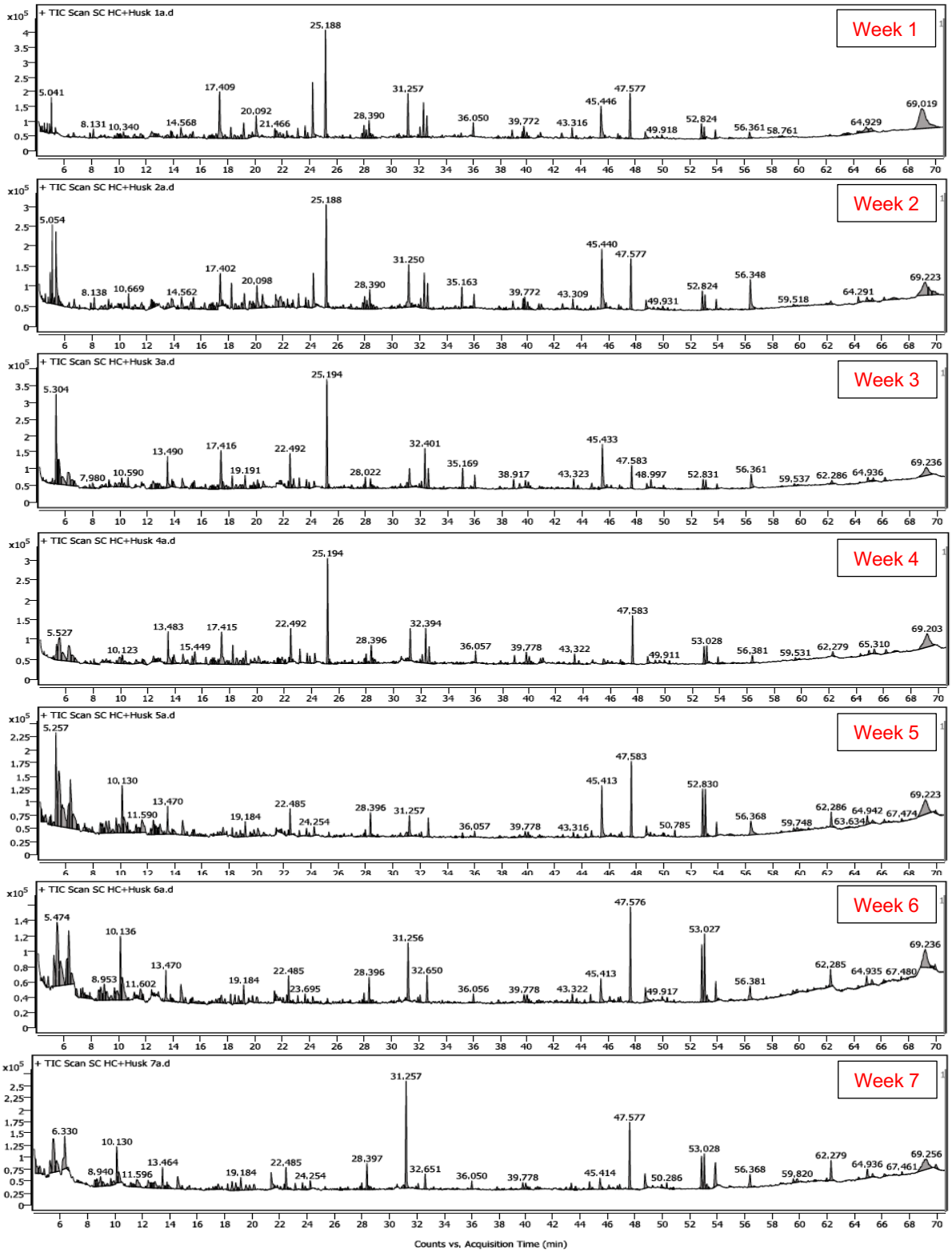

Figure 4. 7 b: GC-MS chromatograms of the degradation of PAHs using *Schizophyllum commune* (SC) supplemented with corn husk over 7 weeks

Table 4. 7 b: Evolution profile of aromatic compounds in soil samples during remediation with *Schizophyllum commune* (SC) supplemented with corn husk

RT	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Compound
5.5	0	0	397437	683479	1197474	861305	752301	Benzene, 1,3-dimethyl
8.6	50788	83633	55552	36898	0	0	0	Benzene, 1,2,3-trimethyl
8.9	44063	86637	48903	55404	182376	155509	182177	Ethanone, 2-(formyloxy)-1-phenyl
9.9	102268	70052	123824	171353	182579	145705	82868	1-Hexanone, 5-methyl-1-phenyl
13.8	109003	191938	74056	59597	41178	32780	58113	p-Toluic acid, 4-nitrophenyl ester
15.4	120548	187408	114874	192260	57950	46260	70865	Benzene, 1,2,3,5-tetramethyl-
17.4	1050383	451650	669258	633758	47906	0	0	Cyclopentasiloxane, decamethyl-
18.2	162270	239575	164242	207142	69422	50855	52783	Naphthalene
18.5	104915	103402	86601	76589	76068	63934	24878	Benzenepropanenitrile
20.1	339243	336175	122188	135070	112934	61515	64672	Benzaldehyde, 4-methyl
23.1	158855	152374	135205	139724	57055	43317	14991	Naphthalene, 2-methyl
28.5	71273	62418	52329	65393	20608	26058	39418	Naphthalene, 1,2-dimethyl
32.6	283942	256578	252418	191478	161449	153859	143661	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters
42.5	101768	81953	36192	35407	26181	15828	0	Benzenesulfonamide, N-butyl

Similar to the first condition, naphthalene and its derivatives show an overall steady decrease from week 1 to week 7 while some simpler aromatics such as benzene di-, tri-, and tetra-methyl present fluctuating values from week to week.

The remediation experiment was repeated using *Trametes hirsuta* (TR) both with and without corn husk and results are seen in **Figures 4.7c & 4.7d** as well as **Tables 4.7c & 4.7d**.

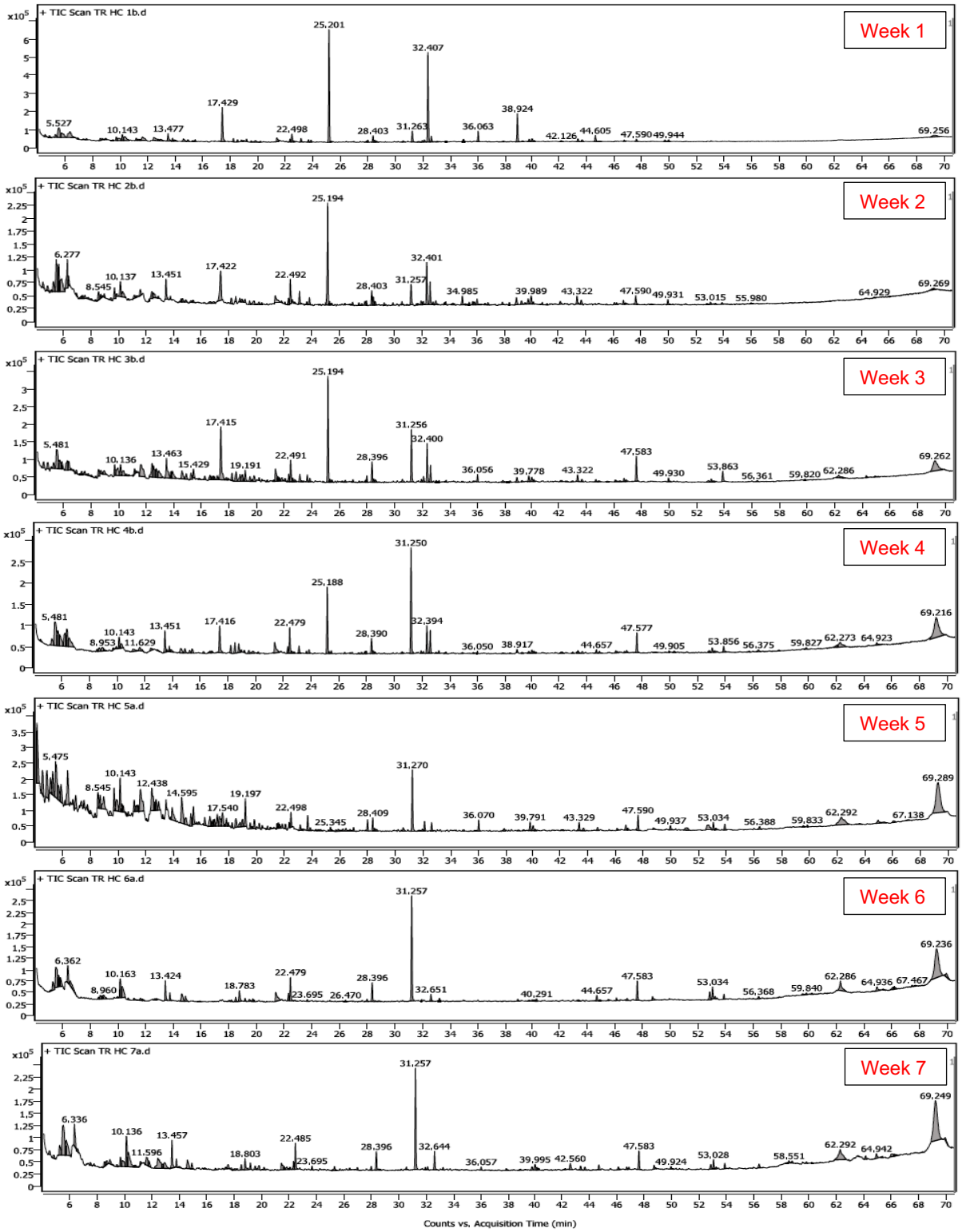

Figure 4. 7 c: GC-MS chromatograms of the degradation of PAHs using *Trametes hirsuta (TR)* over 7 weeks

Table 4. 7 c: Evolution profile of aromatic compounds in soil samples during remediation with *Trametes hirsuta (TR)*

RT	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Compound
5.5	532468	727577	585825	406603	987736	463370	730398	Benzene, dimethyl
13.8	50395	49132	49837	42554	22924	0	0	p-toluic acid, 4-cyanophenyl ester
15.4	44474	48851	40117	40053	0	0	0	1-Pentanone, 1-(4-methylphenyl)
18.2	62461	75282	80826	81349	66205	14788	0	Naphthalene
18.5	51941	106436	129965	132459	177687	47880	54639	Benzenepropanenitrile
19.7	0	17043	22973	39754	30947	26129	35934	Isophthalaldehyde
22.3	56932	49326	95794	95547	48341	70000	67373	1,4-Benzenedicarboxaldehyde, 2-methyl
23.1	170113	136104	110006	84549	83155	15198	0	Naphthalene, 2-methyl
32.6	109991	184238	208161	246693	116536	66437	16645	Pentanoic acid, 5-hydroxy-2,4-di-t-butylphenyl esters

Soil bioremediation with TR shows a similar pattern to that with SC. In both cases, naphthalene and its derivatives show consistent decrease from the beginning of the experiment to its end. Quite a number of benzene derivatives are also detected throughout the remediation process with their concentrations not following a particular pattern. While many aromatics were detected ,and to some extent, successfully degraded, it would appear that the organisms fed on other non-aromatic compounds, primarily.

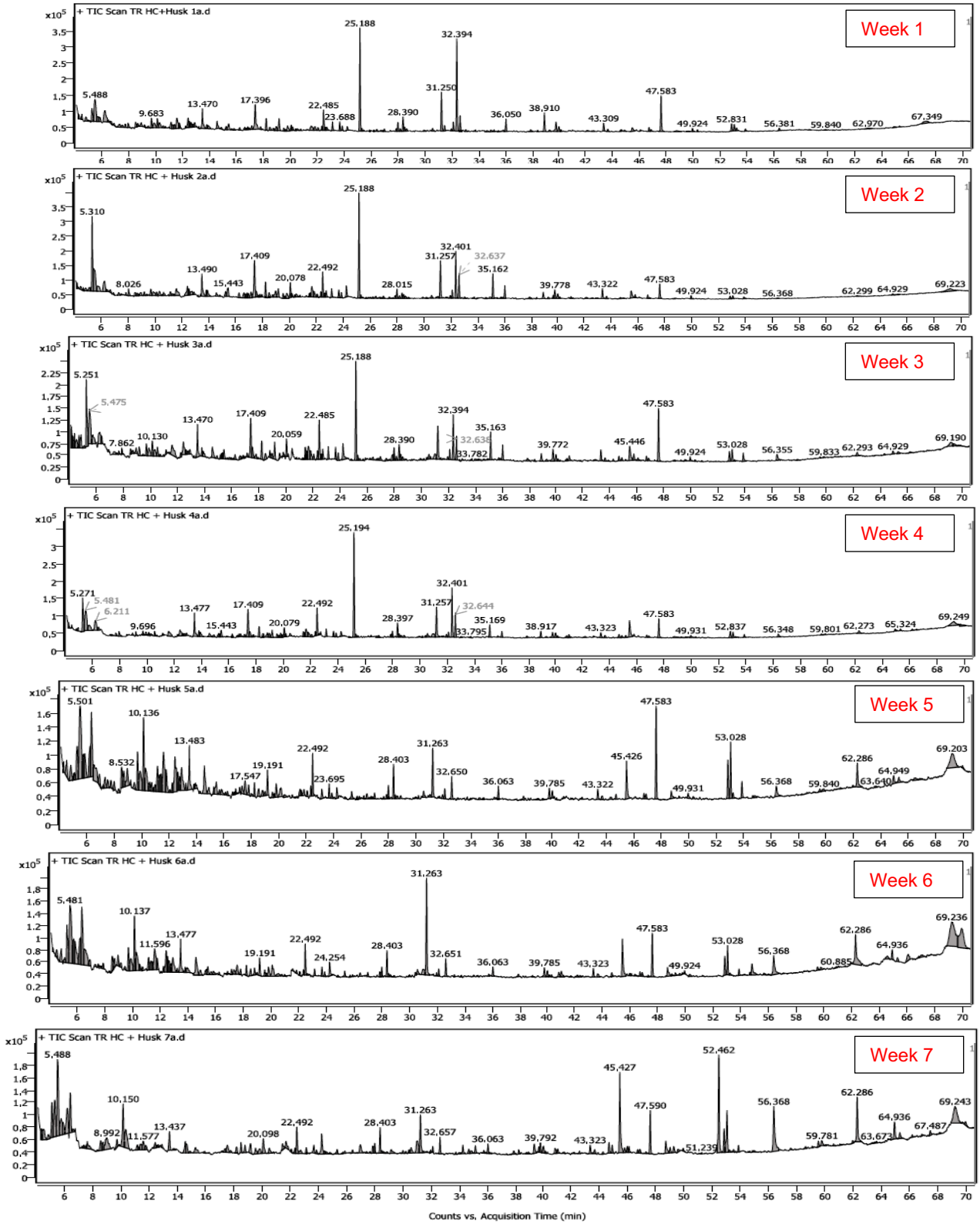

Figure 4. 7 d: GC-MS chromatograms of the degradation of PAHs using *Trametes hirsuta (TR)* supplemented with corn husk over 7 weeks

Table 4. 7 d: Evolution profile of aromatic compounds in soil samples during a 7 week remediation with *Trametes hirsuta (TR)* supplemented with corn husk

RT	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Compound
5.4	664255	869538	705956	609926	1161680	1071414	891606	Benzene, 1,3-dimethyl
8.9	80887	100408	70189	27917	20510	92521	30841	Ethanone, 2-(formyloxy)-1-phenyl
13.8	95558	102989	88550	67464	18482	28857	14920	p-toluic acid, 4-cyanophenyl ester
15.2	95558	95673	88550	62265	35752	0	0	1-Pentanone, 1-(4-methylphenyl)
15.4	140046	214371	106165	101358	143596	75087	0	Benzene, tetramethyl
16.2	44061	92958	51595	38292	25463	11429	0	Benzene, 4-ethenyl-1,2-dimethyl
18.2	148315	218571	173152	130815	76582	68656	55803	Naphthalene
18.5	62774	96540	90747	68036	70898	68305	69410	Benzenepropanenitrile
20.1	93378	227191	147229	127664	126288	164047	154209	Benzaldehyde, 4-methyl
23.1	111597	126114	101707	86155	76380	47023	42763	Naphthalene, 2-methyl
32.6	196981	348950	197699	190990	140670	125146	118536	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters
45.7	0	36490	67704	14840	33419	0	40810	6-methoxy-3-methylbenzofuran

4.5.1. Naphthalene degradation

As the only major EPA PAH detected, naphthalene degradation was compared across all 4 remediation conditions. Highest percentage of degradation was found to occur with *Trametes hirsuta* (TR) without corn supplementation as seen in **Table 4.8**.

Table 4. 8: Naphthalene degradation in all 4 conditions

Condition	Initial count	Final count	% degradation
SC	92998	42784	53.99%
SC + Husk	162270	52783	67.47%
TR	62461	0	100%
TR + Husk	148315	55803	62.38%

CHAPTER 5: DISCUSSION

5.1. Introduction to discussion

The elimination of xenobiotic compounds from the environment using fungal cultures has gained significant attention over the past years. White-rot fungi in particular have shown great potential, as a result of the set of lignin modifying enzymes that they produce (Dao et al., 2019; Ellouze & Sayadi, 2016). However, different fungal isolates could have different remediation efficiencies. Similarly, various types of interactions would occur between LMEs and different fractions of complex xenobiotics such as PAHs. Therefore, this presents the need to compare the action of different isolates with the goal of identifying the best bioremediators and study their interactions with with complex compound structures.

5.2. Molecular characterization

Molecular characterization was done in order to identify and ensure the axenic nature of each isolate as the purpose of the research was to study remediation using fungal monocultures. Sanger sequencing was used for this as this method is considered the golden standard for single taxon identification in different scientific fields (Banos et al., 2018; Crossley et al., 2020; Totomoch-Serra et al., 2017). In this method, a DNA conserved region or barcode is amplified and compared to those previously recorded in databases. In the case of fungi, the ITS region has been identified as the fastest evolving DNA portion, showing the highest barcoding gap as interspecific variations exceed infraspecific variations. This is why it has been chosen globally as the standard fungal barcode for molecular identification all the way to species level (Raja et al., 2017; Schoch et al., 2012). In this study, pure fungal isolated were grown and primers ITS1 and ITS4 (**Table 3.1**) were used for sequencing as they include the entire ITS region (~ 550-600 bp). This sequencing approach has been validated in multiple studies including the recent work of Gencheva and Beev (2021) where Sanger sequencing using the ITS region enabled the identification of various *Fusarium* spp. Ezeonuegbu et al. (2022), Oetari et al. (2018), as well as Yin et al. (2017) have also reported using the same method in their works. The advantage of using primers ITS1 and ITS4 was reported in the work of Badotti

et al. (2017) where amplification of the entire ITS region resulted in a higher probability of correct identification (PCI) compared to the amplification of ITS sub-regions. As results in **Table 4.1** show, ITS sequencing was effective in the identification of all fungal isolates with percentage of similarity being exactly or close to 100% in most cases. The use of pure isolates significantly contributed to this by giving good sequences used to query the database. In the case of *Penicillium oxalicum* which gave the lowest percentage similarity (77.9%), Yin et al. (2017) discussed similar results and attributed this to the fact that the *Penicillium* genus is large with many close-related species which makes their identification challenging when using the ITS region alone, and therefore requiring a combination of DNA markers. Low similarity could also mean an isolate that has not yet been studied and therefore not yet present in the database although different parameters are to be considered in this case (Jeewon & Hyde, 2016).

5.3. Qualitative assay

Qualitative assays serve the purpose of determining the presence or absence of a compound of interest within a given environment (Bisswanger, 2014). Enzyme qualitative assays are generally used as preliminary studies that enable an informed selection of organisms and/or conditions that are optimal for the intended study. By exploiting the reaction between enzymes and certain chromogenic substrates, rapid screening is possible as a visual indication of the enzyme's presence or absence is easily obtained (Kaur et al., 2018). In this work, all characterized isolates were qualitatively assayed for their capability to produce lignin peroxidase and laccase using azure B and guaiacol, respectively.

Azure B is a thiazine blue dye that undergoes decolorization only in the presence of high redox potential agents such as lignin peroxidase (Falade et al., 2017). In the presence of the enzyme, azure B has been found to be converted into a colorless compound and has therefore been used to qualitatively assay lignin peroxidase production (Kameshwar & Qin, 2017). In this study, fungal PDA plates were supplemented with 0.01% of azure B. After 7 days of incubation, LiP production was visualized on the plates by the decolorization of the deep blue azure B dye as seen in **Figure 4.2a**. Of the 12 studied isolates, 9 showed positive lignin peroxidase production. Various decolorization levels

were observed with TH and SC showing the most changes while MR, CC and BK had minimal decolorization.

Laccase on the other hand was qualitatively assayed on PDA plates using 0.02% guaiacol. Laccase is known to oxidize guaiacol, resulting in a brownish color with size and intensity being proportional to the concentration of laccase present (Pisacha et al., 2020; Yuliana et al., 2020). The results in **Figure 4.2b**, show that 5 of the 12 isolates exihibited some level of laccase production after 7 days of incubation. Of these, TR, SC and FU showed change in color on the entire plates which most likely means high amounts of the enzyme were produced.

The popularity of azure B and guaiacol as well as their easy reactivity with LiP and laccase, respectively, informed their selection for the qualitative assay. This has also been reported by (Agrawal et al., 2017; Kameshwar & Qin, 2017; Kaur et al., 2018). As a screening mechanism, qualitative analysis plays an important role in enzyme experiments' design by informing the selection of potential enzyme producers, particularly when starting with large numbers of samples. This was the case for Megersa et al. (2017) who started with 56 fungal cultures that were qualitatively assayed for laccase production, and of these, only 11 cultures showed significant enzyme production potential and were selected for quantitative studies.

5.4. Quantitative assay

Based on the results of the qualitative assay, nine of the initial twelve isolates were selected to quantitatively assay LMEs production. This selection included primarily isolates that had given positive qualitative results for at least one of the enzymes. The experiment was once again done on solid media. However, pulverized corn husk with its high lignocellulose content was used as sole carbon source. Corn husk has extensively been studied and found to contain about 31%-47% cellulose, 34%-44% of hemicellulose, 1%-14% lignin (Akinfemi et al., 2009; Mendes et al., 2015; Mohammed et al., 2022) with only minimal amounts of reducing carbohydrates including glucose and fructose (Bernhardt et al., 2019). This limited amount of readily available simple sugars forces microbes into secondary metabolism and the production of enzymes capable of utilizing lignocellulose. As secondary metabolites, LMEs synthesis is associated with stress as

well as carbon/nitrogen starvation (Ayeronfe et al., 2019; Silva et al., 2010). So, while the limited readily available carbon and nitrogen allowed for initial fungal growth to occur, these were fast depleted, forcing the organisms to activate secondary metabolism and LMEs production in order to utilize carbon found in the corn husk (lignocellulose). Quantitative assay results show that this was indeed the case, as LMEs production was recorded from as early as day 3 in most cases, thus confirming the importance of catabolic repression in LMEs synthesis as previously reported (Bonnarme et al., 1991; Kobakhidze et al., 2016; Tonon et al., 1990; Wu & Zhang, 2010).

LiP was assayed using the veratryl alcohol assay, as well as the azure B assay. **Tables 4.3a&b** as well as **Figures 4.3a&b** summarize these results. Using veratryl alcohol, activity was observed from day 3 with a highest value of 15.59 U/ml for CC. Activities continued to increase, reaching maximum values close to 60 U/ml for TR and TH on day 12, 53 U/ml for PE on day 15, and above 40 U/ml for CC and AS on day 15 as well before slowly dropping as nutrients were being depleted and cells dying. Different observations were however made when the azure B assay was carried. First, activities started quite low compared to the veratryl alcohol experiment with a highest value of 8 U/ml recorded for CC on day 3. From there, a sharp drop was recorded in all isolates with values remaining mostly below 2 U/ml throughout the experiment. While the veratryl alcohol assay has been extensively reported as main LiP assay (Asgher et al., 2016; Ergun & Urek, 2017; Rai et al., 2016; Tien & Kirk, 1984), some authors have used the azure B assay introduced by Archibald (1992). In their work, Arora & Gill (2001) compared both methods and found the azure B method best appropriate when using agricultural residues. The authors suspected the UV wavelength of veratryl alcohol (310 nm) to be a drawback for the method as possible phenolic compounds present in the enzyme extract could also absorb light at the same wavelength. However, various authors have successfully used the veratryl alcohol assay with agricultural wastes. For instance, Ergun & Urek (2017) used this method with potato peel waste, Asgher et al. (2016) used the same method with banana stalk.

Differences in growth conditions as well as interferences with different reagents are possibly the main reason for these discrepancies. While Ten et al. (1997) identified peptone and yeast extract as possible inhibitors of veratryl alcohol oxidation, Tariq &

Irvine (1995) were unable to replicate (Archibald, 1992) results with azure B even when using the same organism and suspected the use of extracellular culture filtrate instead of pure enzyme to be the reason for the observed differences. To further investigate this, the two methods were used to assay the activity of pure LiP (**Table 4.6a** and **Figure 4.6a**). Closer activity readings were measured (57.53 ± 2.46 U/ml vs 66.33 ± 0.77 U/ml) emphasizing the possible presence of compounds that reduced the sensitivity of azure B activity in the initial experiment where crude extracts were used.

Focusing on the results from the veratryl alcohol assay, *Trametes* (TR) and *Trichoderma* (TH) were found to be the best LiP producers. The genus *Trametes* has been reported to be effective LiP producers (Liu et al., 2019; Vrsanska et al., 2016). In the case of *Trametes hirsuta* particularly, Vasina et al. (2016) reported peroxidase activity using the veratryl alcohol assay. Although the authors pointed out the possibility that the observed activity could be a result of different peroxidase enzymes such as versatile peroxidase (VP), they later on were able to identify nine putative LiP genes present in this organism in their subsequent work (Vasina et al., 2017). Similarly, *Trichoderma* species have also been reported to be good producers of LiP. Lisboa et al. (2017) conducted extensive research on Brazilian *Trichoderma* strains and most of them were found to produce LiP as their primary LME.

MnP was quantitatively assayed using first 2,6 dimethoxyphenol at 468 nm, and then a combination of manganese ions and sodium malonate at 270 nm as seen in **Tables 4.4a&b** as well **Figures 4.4a&b**. Although not identical, results showed similar enzyme production trends with TR and SC giving the highest values with maxima between days 15 (SC) and 18 (TR). MnP production by *Trametes* species has been reported in the work of Vasina et al. (2016, 2017). Furthermore, *Trametes* strains have been identified as outstanding LiP, MnP and Laccase producers, with some showing diversity of up to 10 different MnP genes (Zhang et al., 2021). In the case of *Schizophyllum commune* (SC), high MnP production on corn husk is in agreement with literature as this organism has been found to have one of the most complete polysaccharide breakdown systems of all basidiomycetes facilitating its growth on a variety of substrates (Asgher et al., 2016) Although many studies have reported MnP production by SC (Irshad & Asgher, 2011;

Kumar & Arora, 2022), in their review, (Tovar-Herrera et al., 2018) argued the absence of a MnP gene in SC and presented these reported enzymes as possible members of a multi-copper oxidase and hydroxyl radical generation system with MnP-like activity.
In this study, however, when commercially acquired MnP was assayed using the same methods (**Table 4.6b** and **Figure 4.6b**), similar results were obtained (50.3 ± 3.97 U/ml vs 52.0 ± 8.6 U/ml) therefore showing correlation between the two methods irrespective of the probability that enzymes other than MnP could have also contributed to the readings observed in the initial experiments.

Following the same approach, laccase was quantitatively assayed using ABTS and guaiacol as seen in **Tables 4.5a&b** as well as **Figure 4.5a&b**. In the ABTS experiment, TR, SC, FU and MR were the only isolates to show laccase activity with a more or less stable production of 200 U/ml by TR throughout. When guaiacol was used however, all isolates showed some level of enzyme production, although minimal, with TR's maximum being close to half of that recorded with ABTS. As one of the main laccase producers studied to date, TR behavior was in alignment with previous research (Ancona-Escalante et al., 2018; Jia et al., 2022; Rodríguez Couto et al., 2006; Vasina et al., 2016). In the case of SC however, results obtained with guaiacol did not correlate with literature as this organism has also been reported to be a good laccase producer (Asgher et al., 2016; Irshad & Asgher, 2011; Kumar et al., 2022). Tovar-Herrera et al. (2018) reported the presence of 2 laccase genes in the organism and Hirai et al. (2008) even reported the expression of a SC laccase gene in a transgenic tobacco plant. It is however important to note that most reports of SC laccase activity have been done using ABTS and not guaiacol (Asgher et al., 2016; Han et al., 2021; Irshad & Asgher, 2011a; Kumar et al., 2022) and to the best of our knowledge, no comparative study has been conducted for this organism using both methods.
When comparing ABTS and guaiacol as laccase substrates, an interesting insight is found in literature. Robles et al., (2000) investigated laccase activity from strains of *Chalara paradoxa* found in olive mill wastewater. When comparing 15 different laccase substrates including ABTS, guaiacol, syringaldazine, and 2,6-dimethoxyphenol, the authors found great differences in oxidation rates among the substrates. They attributed this not only to

differences in redox potentials between the enzyme and the substrates, but also to steric discrimination due to substrate structure. A further possibility of enzyme inactivation by reaction products was presented for certain monophenols, including guaiacol. Similar to this work, Sheikhi et al., (2012) compared ABTS and guaiacol for laccase activity in *Bacillus subtilis*. Results showed higher values with ABTS (2.28 U/L) compared to guaiacol (1.6 U/L). Authors stated a higher laccase sensitivity and affinity towards ABTS, as well as enzyme inactivation by reaction products as reasons for these differences. This correlate with our own findings in **Table 4.6c** and **Figure 4.6c** where laccase activity was found to be higher with ABTS compared to guaiacol even when using a pure enzyme; therefore, confirming a higher catalytic efficiency for ABTS than guaiacol (Li et al., 2008). While these reasons could give meaning to the decrease of values observed for TR and SC in the presence of guaiacol, they were unable to justify values recorded using guaiacol for all other isolates that had given zero values with ABTS. Further investigation is thus needed to better understand how each isolate reacted with guaiacol and ABTS in these conditions.

Although we can agree that these results did show correlation with literature in many ways, it is important to emphasize the likely influence that environmental growth conditions play on enzyme production which may account for differences observed not only in this work, but across literature (Li et al., 2008; Robles et al., 2000; Sheikhi et al., 2012; Vasina et al., 2016). This is so much more important as some of our least performing isolates (FS, AS, or PE) have been reported to produce high amounts of LMEs in different conditions (Ayla et al., 2018; Dhakar et al., 2015; Huy et al., 2017). The vast size of the fungi kingdom is also to keep in mind as many different strains of organisms could behave differently (Nayan et al., 2018; Robles et al., 2000). The lack of consensus regarding activity assay substrates and conditions is also an important limitation to current research as it prevents the determination of a “true value” when comparative studies are carried. In this study, TR and SC were identified as best overall LMEs producers. In their transcriptomic study of a *Trametes hirsuta* (TR) strain, Vasina et al. (2017) identified and characterized 18 peroxidase genes encoding 9 LiP, 7 MnP, and 2 VP. Majority of these genes were found to be active under different conditions and at different developmental stages. Similarly, Liu et al. (2022) recently characterized laccase genes of a *Schizophyllum commune* (SC)

strain from deep sediment below seafloor. A total of 6 putative genes, grouped into 3 phylogenic classes were characterized. These genes' transcription was found to be differently induced by various elements including substrate type and stress. LMEs expressional patterns appear to be greatly dependent on external factors which highlights the need for further transcriptional work to gain better understanding of mechanisms that dictate enzyme production. As current research aims at the application of WRFs and their LMEs for xenobiotic degradation and bioremediation, this knowledge will be essential in the selection of organisms and conditions for optimal breakdown of different xenobiotic compounds, or fractions of complex xenobiotics such as PAHs.

5.5. Application of fungal isolates in the bioremediation of polycyclic aromatic hydrocarbons (PAHs) contaminated soil

Due to their ability to degrade a wide variety of xenobiotic compounds, WRFs have gained much attention in research as their industrial application could represent a great biotechnological advancement (Rodríguez-Couto, 2017). However, the lack of comprehensive understanding of molecular and biochemical processes that govern LMEs production has led scientists to continuously investigate new organisms for their capability to degrade different types of xenobiotics, as well as understand what conditions are optimal for this to happen. Following the same approach, our two best overall LMEs producers, *Trametes hirsuta* (TR) and *Schizophyllum commune* (SC) were used for the attempted remediation of soil contaminated with PAHs in a 7-week benchtop experiment. The goal was to investigate the capability of these organisms to individually degrade various PAHs found in a mixture of petrol and diesel, with and without corn husk supplementation. A number of studies have investigated the use of agricultural waste to increase LMEs production during fungal remediation and have suggested that this supplementation could promote LMEs production and activity (Magan et al., 2010; Sukarta & Sastrawidana, 2014). In this study, both fungi had already shown the capability to grow on agricultural waste through the production of LMEs. Corn husk supplementation was therefore included in one of the remediation conditions in order to assess its ability to boost remediation. Remediation was investigated using GC-MS to measure the level of degradation of PAHs after extraction from soil samples. Ultrasonication and methanol

were respectively selected as extraction method and solvent based on the comparative study of Oluseyi et al. (2011) where it was found that this particular approach gave best results for PAHs extraction from soil. Lau et al. (2010) also did present ultrasonication as an efficient method for PAHs extraction from soil, although the extent of efficiency tends to vary from one study to the next.

Petrol and diesel are complex mixtures made of aliphatic and aromatic hydrocarbons in different concentrations (Chin & Batterman, 2012; Suppajariyawat et al., 2019). WRFs have extensively been reported to degrade these hydrocarbons through the action of LMEs (Agrawal et al., 2018; Ameen et al., 2016; Liu et al., 2017; Young et al., 2015). During hydrocarbon degradation, fungi and other microorganisms have been reported to prioritize the utilization of simpler hydrocarbons compared to the more complex ones. Short-chain alkanes are readily mineralized before long-chain or branched ones. Similarly, PAHs with fewer rings are degraded before those with higher number of rings (McFarlin et al., 2014; Nzila, 2018; Pereira et al., 2019).
As large and complex structures, PAHs resist degradation, accumulate in the environment, and cause mutagenic and carcinogenic concerns as they find their way into our food supply and ultimately our bodies (Kadri et al., 2017; Pereira et al., 2019). Research has shown particular interest in the use of fungi for the bioremediation PAHs-contaminated soils mainly because of their ability to produce a wide array of extracellular degradative enzymes which, unlike bacteria, allows them to be more effective in the breakdown of large structures. Fungi also have the advantage of having hyphae that can penetrate soil and reach pollutants that have spread deep within the soil layers (Cajthaml et al., 2008; Husaini et al., 2008). Fungi degrade PAHs through a process of depolymerization followed by intermediates formation before subsequent utilization in central pathways (Liu et al., 2019). The β-ketoadipate pathway (β-KAP) has been found to play a crucial role in ring cleavage and the formation of TCA metabolites from pollutants like PAHs (**Figure 2.4**) (Bilal & Iqbal, 2020; Wells & Ragauskas, 2012).

In this work, the complexity and abundance of PAHs present in the soil samples made the quantitative measurement of the degradation of each fraction non-feasible. As a result,

the approach taken by Ameen et al. (2016) as well as Asemoloye et al. (2020) was adopted and PAHs degradation was measured as an expression of peak variation on the GC-MS chromatogram over time. **Figures 7.4a-d** show this as peak reduction (and augmentation) was observed over the course of the experiment. **Tables 7.4a-d** present evolution profiles of some of the aromatics measured. A clear distinct trend is seen in that some of the largest initial peaks (compounds) slowly disappear, while new, smaller peaks appear mainly at the beginning of the chromatogram. Ameen et al. (2016) associated this with the degradation of larger hydrocarbons as well as the production of breakdown products and presumed metabolites. Compounds including 1-pentanone, 1-(4-methylphenyl), naphthalene, benzenepropanenitrile, and naphthalene, 2-methyl showed a net decrease in their areas under the peak, relating to their utilization by the organisms in all conditions.

Ligninolytic fungi degrade PAHs through a multi-step mechanism proportional to the number of aromatic rings present. This process generally begins with PAH oxidation by LMEs and possibly cytochrome P450 monooxygenase, resulting in some quinone intermediates. Ring cleavage and further oxidation catalysed by LMEs convert PAH-quinones into phthalic acid intermediates which undergo more ring cleavage and oxidation by LMEs to form simpler compounds such as pyruvic acid that can enter the central metabolic pathway to produce carbon dioxide (Al-Hawash et al., 2018; Elyamine et al., 2021).

As one the most hazardous PAHs in the environment, particular interest was given to naphthalene, a 2 ring PAH found in fuel, agricultural pesticides as well as household products and various other industrial wastewaters. Due to its cytotoxic, mutagenic, and carcinogenic effects, naphthalene has been classified as a priority pollutant by the United State Environmental Protection Agency (EPA) and strategic guidelines have been established to ensure its disposal and removal from the environment (Asemoloye et al., 2020; Mohapatra & Phale, 2021). Authors have reported naphthalene degradation using different fungi. Mohammed et al., (2014) studied optimal conditions for naphthalene degradation using different fungi and found highest results with a *Fusarium* strain for 8 days at 30°C and pH 7.0. In this study, naphthalene was degraded by 100% with TR, 67.47% with SC + Husk, 62.38% with TR + Husk, and 53.99% with SC + Husk (**Table**

4.8). The low molecular weight of this pollutant is likely responsible for this, making its utilization relatively easy.

Although able to degrade PAHs, some studies have claimed that fungi do not typically utilize PAHs as their sole carbon source, but rather convert them into less toxic compounds through co-metabolism while using other available substrates as main carbon sources (Abd El-Aziz et al., 2021; Mohammed et al., 2014). This presents PAHs degradation as an indirect result of fungal metabolism, requiring a primary carbon source. This assertion has been challenged by other studies such as that of Mohammed et al. (2014) and Stoyanova et al. (2022) where fungi grown solely in PAHs have shown growth and degradative behavior. Therefore, to investigate the direct or indirect degradation of PAHs by the fungi and to assess the claim of increased LMEs production with agricultural waste supplementation by Magan et al. (2010) and Sukarta & Sastrawidana (2014), corn husk was added to PAHs-contaminated soils and remediation in the presence and absence of corn husk was measured. For the degradation of naphthalene, the addition of corn husk was found to be advantageous for SC (increase degradation from 53.99% to 67.47%) while having the opposite effect for TR (decrease degradation from 100% to 62.38%) (**Table 4.8**). The results show that PAHs degradation was obsersed in both the presence and absence of corn husk, which agrees with the claims of direct degradation and utilization of PAHs. The results also show that agricultural waste supplementation did not always have a boosting effect on enzyme production as claimed by Magan et al. (2010) and Sukarta & Sastrawidana (2014). Simultaneously, diauxic growth could have also contributed to the observed results. When presented with two different carbon sources, microorganisms have been known to follow a two-phase growth separated by a lengthy lag phase (Chu & Barnes, 2016). This could have been the case for TR when corn husk was added to the soil.

When comparing the ability of different ligninolytic fungi to degrade naphthalene, Clemente et al. (2001) pointed out different enzyme production patterns among the studied microbes. It was also found that MnP was the main enzyme produced in the condition that resulted in highest naphthalene degradation. Similar results were found by Rathankumar et al. (2020) during the remediation of PAHs using a *Trametes hirsuta* strain

where MnP was found to be the most performing enzyme, followed by laccase and LiP. It could therefore be inferred that the high amount of MnP produced by TR in our case might have contributed to naphthalene degradation. But as complex eukaryotes, fungal metabolism follows a rather multifaceted pattern. So, while certain causal relationships could be speculated, the bulk of these processes remains understood, especially when dealing with complex chemicals such PAHs

CHAPTER 6: CONCLUSIONS

6.1. Conclusion

White rot fungi possess the ability to produce unique, non-specific extracellular lignin-modifying enzymes (LMEs) that present great potential in xenobiotic degradation and environmental bioremediation. This study has been able to use molecular techniques, sanger sequencing particularly, to successfully characterize twelve white rot fungi. Using Azure B and guaiacol, qualitative screening has been able to establish LMEs production in several isolates, thus informing their selection for quantitative studies. The studied fungi successfully produced lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase and were able to breakdown ligninolytic biomass in the form of corn husk. The study has also established the importance of substrate selection in activity assay experiments with different substrates giving different results under the same conditions. In the studied conditions, veratryl alcohol and ABTS were found to be best appropriate for LiP and laccase, respectively. However, no substrate preference could be established for MnP. These results, together with those of previous studies, reveal the possible need to revisit standard activity assay substrates for LMEs due to their lack of consensus and important variation in the results they give. *Trametes hirsuta* and *Schizophyllum commune* produced the highest levels of enzymes and were used for the remediation of PAHs-contaminated soil. Both fungi were able to successfully degrade a number of petroleum fractions including the toxic xenobiotic naphthalene through direct utilization. Addition of corn husk to the polluted soil was found to increase the degradative ability of *Schizophyllum commune*, marginally, while decreasing that of *Trametes hirsuta*. While differences in gene activation based on substrate type and stress could have been responsible for this, a transcriptomic study would be required to confidently understand this behavior. Overall, this research has proven the capability of white rot fungi and their LMEs to degrade and utilize xenobiotic compounds including lignocellulose and PAHs.

6.2. Recommendations

As eukaryotic systems, fungi possess complex mechanisms that dictate their behaviors, particularly enzyme production. Further research focusing on fungal transcriptional and

expressional patterns relating to LMEs production will provide much needed knowledge to better understand these organisms. Grasping the impact of various environmental factors on enzyme production would bring insight on how to optimize production for industrial applications.

CHAPTER 7: REFERENCES

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APPENDICES

Appendix A: Media preparation

PDA

For 1 L of media,

39 g of PDA powder were weighed and added to sufficient ddH_2O to make 1 L of media. The solution was microwaved to dissolve all the PDA before being autoclaved at 121°C for 15 min.

PDA supplemented with 0.01% (w/v) azure B

1% means 1 g in 100 mL of solution

= 10 g in 1000 mL of solution

0.01% means 10 g x 0.01 = 0.1 g

Weigh 0.1 g azure B and 39 g PDA and add to sufficient ddH_2O to make 1 L of solution. Microwave to dissolve PDA and autoclave at 121°C for 15 min.

PDA supplemented with 0.02% (v/v) guaiacol

1% means 1 mL in 100 mL of solution

= 10 mL in 1000 mL of solution

0.02% means 10 mL x 0.02 = 0.2 mL

Prepare PDA media and autoclave, once media cools below 50°C, add 0.2 mL of filter-sterilized guaiacol solution.

Table A 1: Corn husk supplemented minimal media (1 L)

Reagent	Amount
Agar	20 g
Corn husk	10 g
KH_2PO_4	1 g
Yeast extract	1 g

$MgSO_4.7H_2O$	0.5 g
$(NH_4)_3PO_4$	0.5 g
$CaCl_2.2H_2O$	0.1 g
$ZnSO_4.7H_2O$	4 mg
$CuSO_4.5H_2O$	1 mg
$Fe_2(SO_4).7H_2O$	1 mg
Thiamine HCl	1 mL (2mg/mL)

All reagents except thiamine were added to sufficient ddH_2O to make 1 L of solution. After autoclaving, 1 mL of filter-sterilized thiamine HCl solution was added.

Appendix B: Reaction solutions

125 mM Tartrate buffer pH 3.0

500 mL tartaric acid

m= Mr x C x V

m= 150.09 g/mol x 0.125 mol/L x 0.5 L

m= 9.38 g

Add 9.38 g of tartaric acid to some ddH_2O to dissolve, then top up to 500 mL with more ddH_2O

500 mL of Sodium Potassium tartrate

m= Mr x C x V

m=282.22 g/mol x 0.125 mol/L x 0.5 L

m= 17.64 g

Dissolve 17.64 g of Na-K-tartrate in sufficient ddH_2O to make 500 mL of solution.

While monitoring pH with a pH-meter, slowly mix acid and base solution until a pH of 3.0 is reached.

50 mL of 10 mM veratryl alcohol

m= Mr x C x V

m= 168.19 g/mol x $10x10^{-3}$ mol/L x $50x10^{-3}$ L

m= 84.1 mg

density of veratryl alcohol is 1.157 g/mL

volume= mass/density

v= 0.0841 g/1.157 g.mL^{-1} = 0.073 mL or 73 μL

Add 73 μL of veratryl alcohol to sufficient ddH_2O to make 50 mL of solution and check vigorously.

50 mL of 10 mM hydrogen peroxide stock solution

m= Mr x C x V

m= 34.01 g/mol x 10x10^{-3} mol/L x 50x10^{-3} L

m= 0.017005 g

density of hydrogen peroxide is 1.45 g/mL at room temp

volume= mass/density

v= 0.017005 g/1.45 g.mL^{-1} = 12 μL

Add 12 μL of hydrogen peroxide to sufficient ddH_2O to make 50 mL of solution

For a 1 mM solution, add 1 part stock with 9 parts of ddH_2O.

For a 2 mM solution, add 2 parts stock with 8 parts of ddH_2O.

For a 3 mM solution, add 3 parts stock with 7 parts of ddH_2O.

50 mL of 16 mM azure B (100x stock)

m= Mr x C x V

m= 305.8 g/mol x 16x10^{-3} mol/L x 50x10^{-3} L

m= 0.245 g

Dissolve 0.245 g of azure B in sufficient ddH_2O to make 50 mL of solution.

For a 0.160 mM solution, add 1 part stock with 99 parts ddH_2O.

200 mM sodium malonate buffer pH 4.5

500 mL of 200 mM malonic acid

m= Mr x C x V

m= 104.06 g/mol x 0.2 mol/L x 0.5 L

m=10.4 g

Add 10.4 g of malonic acid to some ddH_2O and top up to 500 mL of solution with more ddH_2O.

500 mL of 200 mM sodium malonate

m= Mr x C x V

m= 148.03 g/mol x 0.2 mol/L x 0.5 L

m= 14.8 g

Dissolve 14.8 g of sodium malonate in sufficient ddH_2O to make 500 mL of solution.

While monitoring with a pH meter, slowing mix acid and base to reach a pH of 4.5.

50 mL of 40 mM 2,6 dimethoxyphenol (10x stock)

m= Mr x C x V

m= 154.16 g/mol x $40x10^{-3}$ mol/L x $50x10^{-3}$ L

m= 0.308 g

Dissolve 0.308 g of 2,6 dimethoxyphenol in sufficient ddH_2O to make 50 mL of solution

For a 4 mM solution, add 1 part stock to 9 parts ddH_2O.

50 mL of 40 mM $MnSO_4$ (stock)

m= Mr x C x V

m= 169.02 g/mol x $40x10^{-3}$ mol/L x $50x10^{-3}$ L

m= 0.338 g

Dissolve 0.338 g of $MnSO_4$ in sufficient ddH_2O to make 50 mL of solution

For a 4 mM solution, add 1 part stock to 9 parts ddH_2O.

For a 1 mM solution, add 1 part 4 mM solution to 3 parts ddH_2O.

50 mM sodium malonate buffer pH 4.5

500 mL of 50 mM malonic acid

m= Mr x C x V

m= 104.06 g/mol x 0.05 mol/L x 0.5 L

m=2.60 g

Add 2.60 g of malonic acid to some ddH_2O and top up to 500 mL of solution with more ddH_2O.

500 mL of 50 mM sodium malonate

m= Mr x C x V

m= 148.03 g/mol x 0.05 mol/L x 0.5 L

m= 3.7 g

Dissolve 3.7 g of sodium malonate in sufficient ddH_2O to make 500 mL of solution.

While monitoring with a pH meter, slowing mix acid and base to reach a pH of 4.5.

50 mL of 10 mM ABTS (10x stock)

m= Mr x C x V

m= 548.68 g/mol x $10x10^{-3}$ mol/L x $50x10^{-3}$ L

m= 0.274 g

Dissolve 0.274 g of ABTS in sufficient ddH_2O to make 50 mL of solution.

For a 1 mM solution, add 1 part stock to 9 parts ddH_2O.

50 mL of 2 mM guaiacol

m= Mr x C x V

m= 124.14 g/mol x $2x10^{-3}$ mol/L x $50x10^{-3}$ L

m= 0.012414 g

volume= mass/density

volume= 0.012414 g/1.11 $g.mL^{-1}$ = 0.011 mL or 11 μL

Add 11 μL of guaiacol to some ddH_2O and top up to 50 mL with more ddH_2O.

10 mM sodium acetate buffer pH 5.0

500 mL of 10 mM acetic acid

Using a 17.4 M stock solution of acetic acid

$C_1 \times V_1 = C_2 \times V_2$

$V_1 = 10x10^{-3}$ M x 500 mL/17.4 M

V_1= 0.287 mL

Add 0.287 mL of stock solution to some ddH_2O and top up to 500 mL with more ddH_2O.

500 mL of 10 mM sodium acetate

m= Mr x C x V

m= 82.03 g/mol x $10x10^{-3}$ mol/L x 0.5 L

m= 0.401 g

Dissolve 0.401 g of sodium acetate in sufficient ddH_2O to make 500 mL of solution.

While monitoring with a pH meter, slowing mix acid and base to reach a pH of 5.0.

LiP pure solution

We had 1 g of 0.12 U/mg and needed 10 mL of a 1 U/ml solution

0.12 U is found in 1 mg of powder

1 U is found in 1/0.12 = 8.33 mg of powder

8.33 mg added to 1 mL of ddH_2O gives a 1 U/ml solution

For 10 mL, 8.33 mg x 10 = 83.3 g

83.3 mg of pure enzyme was dissolved in enough ddH_2O to make 10 mL of solution.

MnP pure solution

We had 10 mg of a 13.4 U/g

In 1 g we have 13.4 U

In 10 mg we have 13.4/100 = 0.134 U

Sufficient ddH_2O was added to 10 mg of pure enzyme to make 1 mL of solution.

Laccase pure solution

We had 1 g of 0.78 U/mg and needed 10 mL of a 1 U/ml solution

0.78 U is found in 1 mg of powder

1 U is found in 1/0.78 = 1.282 mg of powder

1.282 mg added to 1 mL of ddH_2O gives a 1 U/ml solutiob

For 10 mL, 1.282 mg x 10 = 12.82 mg

12.82 mg was added to sufficient ddH_2O to make 10 mL of solution.

Appendix C: Activity assay using crude extracts

I. Lignin peroxidase using veratryl alcohol

Table C 1: Change in Abs after 1 min of reaction with veratryl alcohol

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	0.001	0.001	0.001	-0.025	-0.087	-0.066	-0.008	-0.008	-0.007	0.009	0.007	0.005	0.032	0.024	0.027
Day 6	0.014	0.015	0.014	0.026	0.006	0.023	0.02	0.017	0.029	0.022	0.006	0.026	0.057	0.078	0.053
Day 9	0.063	0.059	0.061	0.065	0.057	0.07	0.092	0.098	0.049	0.038	0.028	0.033	0.047	0.045	0.04
Day 12	0.083	0.078	0.081	0.123	0.096	0.098	0.112	0.125	0.097	0.057	0.024	0.022	0.03	0.036	0.041
Day 15	0.035	0.032	0.031	0.085	0.079	0.075	0.088	0.085	0.049	0.035	0.039	0.027	0.078	0.07	0.082
Day 18	0.027	0.022	0.017	0.046	0.048	0.05	0.074	0.067	0.039	0.03	0.021	0.024	0.048	0.046	0.021
Day 21	0.025	0.017	0.012	0.05	0.044	0.047	0.049	0.046	0.035	0.029	0.019	0.018	0.041	0.038	0.029

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	0.035	0.021	0.036	0.037	0.021	0.029	0.023	0.017	0.026	0.012	0.009	0.015	0
Day 6	0.043	0.04	0.053	0.03	0.033	0.04	0.05	0.028	0.041	0.037	0.047	0.04	0
Day 9	0.063	0.059	0.066	0.024	0.038	0.039	0.041	0.044	0.033	0.024	0.011	0.035	0
Day 12	0.026	0.031	0.032	0.034	0.027	0.038	0.067	0.059	0.042	0.035	0.023	0.035	0
Day 15	0.079	0.08	0.09	0.056	0.075	0.057	0.105	0.108	0.083	0.075	0.072	0.084	0
Day 18	0.051	0.049	0.043	0.048	0.052	0.031	0.04	0.035	0.037	0.021	0.031	0.01	0
Day 21	0.038	0.043	0.042	0.027	0.031	0.018	0.028	0.024	0.014	0.035	0.02	0.031	0

Table C 2: Calculated enzyme activities with veratryl alcohol

MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3

Day 3	0.538	0.538	0.538	0	0	0	0	0	0	4.838	3.763	2.688	17.204	12.903	14.516
Day 6	7.527	8.065	7.527	13.978	3.225	12.365	10.752	9.139	15.591	11.827	3.225	13.978	30.645	41.935	28.494
Day 9	33.871	31.72	32.796	34.946	30.645	37.634	49.462	52.688	26.344	20.43	15.053	17.741	25.268	24.193	21.505
Day 12	44.624	41.935	43.548	66.129	51.612	52.688	60.215	67.204	52.15	30.645	12.903	11.827	16.129	19.354	22.043
Day 15	18.817	17.204	16.667	45.698	42.473	40.322	47.311	45.698	26.344	18.817	20.967	14.516	41.935	37.634	44.086
Day 18	14.516	11.828	9.14	24.731	25.806	26.881	39.784	36.021	20.967	16.129	11.29	12.903	25.806	24.731	11.29
Day 21	13.441	9.14	6.451	26.881	23.655	25.268	26.344	24.731	18.817	15.591	10.215	9.677	22.043	20.43	15.591

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	18.817	11.29	19.354	19.892	11.29	15.591	12.365	9.139	13.978	6.451	4.838	8.064	0
Day 6	23.118	21.505	28.494	16.129	17.741	21.505	26.881	15.053	22.043	19.892	25.268	21.505	0
Day 9	33.87	31.72	35.483	12.903	20.43	20.967	22.043	23.655	17.741	12.903	5.913	18.817	0
Day 12	13.978	16.666	17.204	18.279	14.516	20.43	36.021	31.72	22.58	18.817	12.365	18.817	0
Day 15	42.473	43.01	48.387	30.107	40.322	30.645	56.451	58.064	44.623	40.322	38.709	45.161	0
Day 18	27.419	26.344	23.118	25.806	27.956	16.666	21.505	18.817	19.892	11.29	16.666	5.376	0
Day 21	20.43	23.118	22.58	14.516	16.666	9.677	15.053	12.903	7.526	18.817	10.752	16.666	0

II. Lignin peroxidase using azure B

Table C 3: Change in Abs after 1 min of reaction with azure B

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	0.078	0.048	0.016	0.013	0.065	0.024	0.031	0.017	0.016	0.042	0.037	0.05	0.1	0.022	0.048
Day 6	-0.054	-0.076	-0.063	-0.018	-0.107	-0.02	0.002	0.005	-0.011	0.015	0.016	0.004	0.023	0.022	0.015
Day 9	0.017	0.009	0.013	0.012	0.003	0.021	0.011	0.012	0.009	0.024	0.015	0.018	0.013	0.022	0.012
Day 12	0.016	0.012	0.011	0.024	0.018	0.014	0.008	0.01	0.012	0.02	0.013	0.015	0.017	0.019	0.01
Day 15	0.013	0.014	0.011	0.038	-0.067	0.008	0.015	0.014	0.015	0.016	0.015	0.013	0.015	0.013	0.012
Day 18	0.009	0.01	0.01	0.007	-0.002	0.004	0.006	0.007	0.008	0.012	0.012	0.006	0.015	0.017	0.012
Day 21	0.005	0.01	0.013	0.004	0.013	0.008	0.012	-0.032	0.014	-0.003	0.015	0.01	0.004	0.013	0.012

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	0.116	0.096	0.022	0.036	0.032	0.018	0.027	0.041	0.084	0.051	0.071	0.06	0
Day 6	0.031	0.032	0.045	0.019	0.03	0.009	0.018	0.02	0.014	0.013	0.008	0.011	0
Day 9	0.005	0.018	0.012	0.018	0.01	0.006	0.019	0.021	0.014	0.013	0.02	0.019	0
Day 12	0.007	0.016	0.009	0.01	0.013	0.01	0.012	0.009	0.007	0.014	0.009	0.01	0
Day 15	0.011	0.014	0.011	0.007	0.016	0.006	0.005	0.004	0.002	0.016	0.008	0.007	0
Day 18	0.015	0.012	0.014	0.012	0.009	0.011	0.011	0.012	0.015	0.015	0.005	0.007	0
Day 21	0.011	0.03	0.016	0.032	0.035	0.013	0.008	0.01	0.015	0.019	0.017	0.01	0

Table C 4: Calculated enzyme activities with azure B

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	7.991	4.918	1.639	1.331	6.659	2.459	3.176	1.741	1.639	4.303	3.79	5.122	10.245	2.254	4.918
Day 6	0	0	0	0	0	0	0.204	0.512	0	1.536	1.639	0.409	2.356	2.254	1.536
Day 9	1.741	0.922	1.331	1.229	0.307	2.151	1.127	1.229	0.922	2.459	1.536	1.844	1.331	2.254	1.229
Day 12	1.639	1.229	1.127	2.459	1.844	1.434	0.819	1.024	1.229	2.049	1.331	1.536	1.741	1.946	1.024
Day 15	1.331	1.434	1.127	3.893	0	0.819	1.127	1.434	1.536	1.639	1.536	1.331	1.536	1.331	1.229
Day 18	0.922	1.024	1.024	0.717	0	0.409	0.614	0.717	0.819	1.229	1.229	0.614	1.536	1.741	1.229
Day 21	0.512	1.024	1.331	0.409	1.331	0.819	1.229	0	1.434	0	1.536	1.024	0.409	1.331	1.229

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	11.885	9.836	2.254	3.688	3.278	1.833	2.766	4.2	8.606	5.225	7.274	6.147	0
Day 6	3.176	3.278	4.61	1.946	3.073	0.922	1.844	2.049	1.434	1.331	0.819	1.127	0
Day 9	0.512	1.844	1.229	1.844	1.024	0.614	1.946	2.151	1.434	1.331	2.049	1.946	0
Day 12	0.717	1.639	0.922	1.024	1.331	1.024	1.229	0.922	0.717	1.434	0.922	1.024	0
Day 15	1.127	1.434	1.127	0.717	1.639	0.614	0.512	0.409	0.204	1.639	0.819	0.717	0
Day 18	1.536	1.229	1.434	1.229	0.922	1.127	1.127	1.229	1.536	1.536	0.512	0.717	0
Day 21	1.127	3.073	1.639	3.278	3.586	1.331	0.819	1.024	1.536	1.946	1.741	1.024	0

III. Manganese peroxidase using 2,6 dimethoxyphenol

Table C 5: Change in Abs after 1 min of reaction with 2,6 dimethoxyphenol

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	0.219	0.163	0.189	0.242	0.191	0.144	0.622	0.426	0.314	0.084	0.071	0.056	0.177	0.149	0.061
Day 6	0.082	0.074	0.076	0.09	0.087	0.06	0.743	0.691	0.394	0.04	0.039	0.038	0.103	0.106	0.091
Day 9	0.096	0.073	0.076	0.086	0.073	0.057	0.901	0.876	0.775	0.012	0.016	0.015	0.075	0.076	0.055
Day 12	0.208	0.172	0.13	0.197	0.18	0.145	1.014	0.793	0.827	0.048	0.031	0.046	0.192	0.152	0.118
Day 15	0.324	0.29	0.251	0.284	0.243	0.212	1.148	0.779	0.662	0.059	0.048	0.042	0.373	0.313	0.214
Day 18	0.31	0.301	0.293	0.409	0.316	0.288	1.452	1.107	0.75	0.113	0.053	0.095	0.423	0.341	0.361
Day 21	0.09	0.103	0.059	0.102	0.107	0.095	1.104	0.627	0.502	0.017	0.014	0.011	0.104	0.097	0.089

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	0.21	0.163	0.138	0.162	0.128	0.158	0.145	0.169	0.104	0.165	0.129	0.108	0
Day 6	0.066	0.055	0.046	0.262	0.058	0.205	0.102	0.097	0.091	0.076	0.05	0.042	0
Day 9	0.077	0.06	0.048	0.52	0.092	0.464	0.117	0.118	0.078	0.099	0.07	0.054	0
Day 12	0.158	0.129	0.085	0.353	0.125	0.253	0.192	0.183	0.218	0.133	0.096	0.096	0
Day 15	0.181	0.205	0.166	0.573	0.283	0.515	0.343	0.268	0.294	0.3	0.33	0.294	0
Day 18	0.201	0.278	0.181	0.444	0.227	0.35	0.208	0.226	0.239	0.273	0.213	0.205	0
Day 21	0.097	0.06	0.078	0.159	0.095	0.111	0.109	0.088	0.086	0.093	0.077	0.086	0

Table C 6: Calculated enzyme activities with 2,6 dimethoxyphenol

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	43.424	32.32	37.476	47.985	37.872	28.553	123.334	84.469	62.261	16.656	14.078	11.104	35.096	29.544	12.095
Day 6	16.259	14.673	15.069	17.845	17.25	11.897	147.326	137.015	78.124	7.931	7.733	7.534	20.423	21.018	18.044
Day 9	19.035	14.474	15.069	17.052	14.474	11.302	178.655	173.698	153.671	2.379	3.172	2.974	14.871	15.069	10.905

Day 12	41.243	34.105	25.777	39.062	35.691	28.751	201.062	157.241	163.982	9.517	6.146	9.121	38.07	30.139	23.397
Day 15	64.244	57.503	49.769	56.313	48.183	42.036	227.632	154.465	131.265	11.698	9.517	8.328	73.365	62.063	42.433
Day 18	61.468	59.684	58.097	81.099	62.46	57.106	287.911	219.502	148.714	22.406	10.509	18.837	83.875	62.261	71.581
Day 21	17.845	20.423	11.698	20.225	21.216	18.837	218.908	124.325	99.539	3.37	2.776	2.181	20.621	19.233	17.647

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	41.64	32.32	27.363	32.122	25.38	31.329	28.751	33.51	20.621	32.717	25.578	21.414	0
Day 6	13.086	10.905	9.121	51.951	11.5	40.648	20.225	19.233	18.044	15.069	9.914	8.328	0
Day 9	15.268	11.897	9.517	103.108	18.242	92.004	23.199	23.397	15.466	19.63	13.88	10.707	0
Day 12	31.329	25.578	16.854	69.995	24.785	50.166	38.07	36.386	43.226	26.372	19.035	19.035	0
Day 15	35.889	40.648	32.915	113.618	56.115	102.117	68.012	53.14	58.296	59.485	65.434	58.296	0
Day 18	39.855	55.123	35.889	88.039	45.01	69.4	41.243	44.812	47.39	54.132	42.234	40.648	0
Day 21	19.233	11.897	15.466	31.527	18.837	22.009	21.613	17.449	17.052	18.44	15.268	17.052	0

IV. Manganese peroxidase using manganese ions and sodium malonate

Table C 7: Change in Abs after 1 min of reaction with manganese ions and sodium malonate

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	0.017	0.015	0.017	0.021	0.016	0.014	0.053	0.042	0.048	0.005	0.004	0.005	0.015	0.014	0.011
Day 6	0.008	0.006	0.006	0.008	0.007	0.005	0.066	0.059	0.057	0.004	0.003	0.002	0.009	0.01	0.008
Day 9	0.007	0.008	0.008	0.007	0.008	0.005	0.079	0.078	0.068	0.001	0.002	0.001	0.008	0.007	0.006
Day 12	0.019	0.016	0.012	0.018	0.014	0.011	0.089	0.068	0.072	0.004	0.003	0.004	0.014	0.012	0.012
Day 15	0.03	0.024	0.022	0.025	0.02	0.019	0.103	0.072	0.057	0.005	0.003	0.003	0.032	0.028	0.025
Day 18	0.027	0.026	0.032	0.035	0.029	0.026	0.125	0.1	0.066	0.008	0.006	0.007	0.035	0.028	0.032
Day 21	0.005	0.007	0.009	0.01	0.006	0.011	0.096	0.05	0.039	0.002	0.002	0.001	0.01	0.008	0.008

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	0.015	0.015	0.013	0.014	0.016	0.015	0.011	0.01	0.009	0.009	0.011	0.009	0

Day 6	0.006	0.005	0.005	0.032	0.03	0.019	0.009	0.008	0.008	0.006	0.005	0.004	0
Day 9	0.006	0.006	0.004	0.047	0.04	0.044	0.011	0.009	0.01	0.007	0.005	0.005	0
Day 12	0.009	0.008	0.007	0.035	0.03	0.023	0.017	0.015	0.02	0.01	0.008	0.009	0
Day 15	0.015	0.018	0.017	0.054	0.049	0.045	0.028	0.025	0.019	0.022	0.015	0.017	0
Day 18	0.019	0.025	0.017	0.04	0.035	0.03	0.018	0.012	0.021	0.017	0.015	0.01	0
Day 21	0.006	0.005	0.004	0.015	0.012	0.01	0.006	0.008	0.008	0.008	0.005	0.005	0

Table C 8: Calculated enzyme activities with manganese ions and sodium malonate

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	39.245	35.338	37.163	45.816	35.582	29.467	120.273	87.167	101.376	12.365	10.367	11.104	33.573	30.387	27.892
Day 6	17.964	13.278	15.069	16.992	14.183	10.289	148.278	132.376	121.397	9.35	7.258	5.174	19.173	23.1	18.044
Day 9	16.766	16.87	17.488	14.943	16.002	11.302	176.98	176.003	156.02	2.567	3.619	2.113	18.487	15.278	13.467
Day 12	42.398	35.711	27.119	40.811	31.336	29.027	200.012	158.824	159.554	8.124	6.176	9.11	31.836	29.365	25.718
Day 15	66.472	53.287	48.273	55.521	45.364	43.176	231.936	162.934	128.593	10.835	7.153	8.328	71.498	64.092	55.176
Day 18	59.73	57.872	59.019	79.402	65.72	59.001	280.271	220.11	146.825	17.935	13.835	15.976	79.395	62.261	72.17
Day 21	12.547	18.309	13.227	23.012	21.729	18.342	215.284	112.825	87.365	4.167	3.11	2.181	20.387	17.387	17.647

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	32.189	32.32	27.363	29.179	35.801	32.901	25.801	22.498	19.627	20.824	24.902	19.643	0
Day 6	12.712	10.478	10.287	72.498	66.914	43.165	19.498	16.034	18.044	12.091	11.098	9.023	0
Day 9	12.286	13.08	9.237	106.398	89.37	99.046	25.185	20.279	21.472	15.092	12.013	13.165	0
Day 12	21.379	19.318	16.854	79.209	67.153	52.472	39.016	35.186	45.187	22.653	17.345	19.276	0
Day 15	33.961	40.648	37.163	120.389	110.03	100.01	61.703	55.157	59.375	49.554	34.187	37.175	0
Day 18	42.981	55.123	37.173	87.9	76.916	68.92	40.42	43.925	47.885	37.012	32.902	36.62	0
Day 21	13.709	11.897	9.035	32.651	29.37	23.613	15.668	18.026	17.163	18.409	15.268	15.278	0

V. Laccase using ABTS

Table C 9: Change in Abs after 10 min of reaction with ABTS

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	-0.014	-0.015	-0.025	-0.019	-0.025	-0.025	2.37	2.003	2.399	-0.003	-0.002	-0.004	-0.06	-0.011	-0.015
Day 6	0	-0.007	-0.01	-0.002	-0.013	0	2.78	2.744	2.915	-0.001	-0.003	-0.002	0.055	0.06	0.06
Day 9	-0.034	-0.02	-0.021	-0.02	-0.032	-0.021	2.512	2.636	2.815	-0.002	-0.006	0	0.099	0.158	0.094
Day 12	-0.013	-0.004	-0.001	-0.023	-0.023	-0.028	2.696	2.803	2.792	-0.005	-0.007	-0.004	0.123	0.165	0.132
Day 15	0.043	0.052	0.047	-0.018	-0.036	-0.024	2.687	2.704	2.715	-0.03	-0.003	-0.018	0.158	0.256	0.163
Day 18	0.044	0.035	0.04	-0.029	-0.018	-0.026	2.811	2.742	2.89	-0.002	-0.006	-0.004	0.141	0.182	0.133
Day 21	0.039	0.034	0.029	-0.021	-0.016	-0.029	2.846	2.746	2.849	-0.005	-0.003	-0.015	0.086	0.114	0.149

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	-0.034	-0.071	-0.047	0.03	0.023	0.044	-0.019	-0.018	-0.028	-0.01	-0.006	-0.009	0
Day 6	-0.001	-0.003	-0.023	0.702	0.551	0.598	-0.014	0	-0.026	-0.002	-0.001	-0.025	0
Day 9	-0.007	-0.017	-0.017	1.428	1.255	1.139	-0.064	-0.047	-0.029	-0.015	-0.013	-0.019	0
Day 12	-0.018	-0.031	-0.008	1.415	1.137	1.073	-0.069	-0.017	-0.055	-0.005	-0.008	-0.007	0
Day 15	-0.009	-0.016	-0.024	0.516	0.521	0.593	0	-0.004	-0.003	-0.015	-0.013	-0.014	0
Day 18	-0.01	-0.011	-0.01	0.637	0.451	0.355	-0.011	-0.027	-0.018	-0.001	-0.004	-0.008	0
Day 21	-0.001	-0.006	-0.018	0.398	0.257	0.203	-0.033	-0.021	-0.047	-0.011	-0.013	-0.015	0

Table C 10: Calculated enzyme activities with ABTS

	MR 1	MR 2	MR 3	TH 1	TH2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	0	0	0	0	0	0	169.863	143.559	171.941	0	0	0	0	0	0
Day 6	0	0	0	0	0	0	199.249	196.668	208.924	0	0	0	3.941	4.3	4.3
Day 9	0	0	0	0	0	0	180.04	188.928	201.757	0	0	0	7.095	11.324	6.737
Day 12	0	0	0	0	0	0	193.228	200.897	200.109	0	0	0	8.815	11.825	9.46
Day 15	3.081	3.726	3.368	0	0	0	192.583	193.802	194.59	0	0	0	11.324	18.348	11.682

Day 18	3.153	2.508	2.866	0	0	0	201.47	196.525	207.133	0	0	0	10.105	13.044	9.532
Day 21	2.795	2.436	2.078	0	0	0	203.979	196.812	204.194	0	0	0	6.163	8.17	10.879

	CC1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	0	0	0	2.15	1.648	3.153	0	0	0	0	0	0	0
Day 6	0	0	0	50.313	39.491	42.86	0	0	0	0	0	0	0
Day 9	0	0	0	102.348	89.948	81.634	0	0	0	0	0	0	0
Day 12	0	0	0	101.416	81.491	76.904	0	0	0	0	0	0	0
Day 15	0	0	0	39.982	37.341	42.501	0	0	0	0	0	0	0
Day 18	0	0	0	45.65	32.324	25.443	0	0	0	0	0	0	0
Day 21	0	0	0	28.525	18.419	14.549	0	0	0	0	0	0	0

VI. Laccase using guaiacol

Table C 11: Abs readings after 15 min of reaction with guaiacol

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	0.101	0.111	0.122	0.112	0.095	0.172	1.165	1.158	1.324	0.03	0.042	0.027	0.123	0.11	0.102
Day 6	0.118	0.129	0.159	0.143	0.094	0.194	1.964	1.793	1.482	0.044	0.062	0.049	0.146	0.121	0.118
Day 9	0.139	0.099	0.122	0.112	0.26	0.108	2.207	2.359	2.068	0.039	0.03	0.035	0.157	0.26	0.22
Day 12	0.099	0.089	0.1	0.167	0.219	0.248	2.301	2.75	2.127	0.024	0.027	0.026	0.135	0.204	0.142
Day 15	0.086	0.077	0.087	0.139	0.17	0.192	2.306	2.481	2.173	0.033	0.032	0.012	0.138	0.139	0.08
Day 18	0.097	0.084	0.082	0.263	0.218	0.311	2.377	2.627	2.215	0.026	0.028	0.026	0.125	0.148	0.092
Day 21	0.081	0.083	0.087	0.095	0.104	0.101	2.317	2.484	2.166	0.033	0.026	0.016	0.085	0.096	0.085

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	0.043	0.067	0.04	0.154	0.076	0.142	0.065	0.063	0.052	0.063	0.039	0.041	0
Day 6	0.051	0.08	0.063	0.165	0.08	0.155	0.074	0.07	0.06	0.073	0.054	0.051	0
Day 9	0.075	0.096	0.067	0.217	0.088	0.235	0.101	0.127	0.158	0.106	0.087	0.138	0

Day 12	0.11	0.163	0.11	0.136	0.078	0.156	0.324	0.22	0.26	0.128	0.072	0.045	0
Day 15	0.074	0.07	0.094	0.099	0.06	0.122	0.124	0.11	0.077	0.091	0.054	0.047	0
Day 18	0.072	0.083	0.104	0.145	0.067	0.198	0.224	0.169	0.133	0.076	0.028	0.036	0
Day 21	0.048	0.053	0.113	0.095	0.071	0.114	0.146	0.077	0.089	0.09	0.047	0.038	0

Table C 12: Calculated enzyme activities with guaiacol

	MR 1	MR 2	MR 3	TH 1	TH 2	TH 3	TR 1	TR 2	TR 3	FM 1	FM 2	FM 3	FU 1	FU 2	FU 3
Day 3	4.995	5.489	6.033	5.539	4.698	8.506	57.616	57.27	65.479	1.483	2.077	1.335	6.083	5.44	5.044
Day 6	5.835	6.379	7.863	7.072	4.648	9.594	97.131	88.674	73.293	2.176	3.066	2.423	7.22	5.984	5.835
Day 9	6.874	4.896	6.033	5.539	12.858	5.341	109.149	116.666	102.274	1.928	1.483	1.73	7.764	12.858	10.88
Day 12	4.896	4.401	4.945	8.259	10.83	12.265	113.798	136.003	105.192	1.186	1.335	1.285	6.676	10.089	7.022
Day 15	4.253	3.808	4.302	6.874	8.407	9.495	114.045	122.7	107.467	1.632	1.582	0.593	6.824	6.874	3.956
Day 18	4.797	4.154	4.055	13.006	10.781	15.38	117.556	129.92	109.545	1.285	1.384	1.285	6.181	7.319	4.549
Day 21	4.005	4.104	4.302	4.698	5.143	4.995	114.589	122.848	107.121	1.632	1.285	0.791	4.203	4.747	4.203

	CC 1	CC 2	CC 3	SC 1	SC 2	SC 3	PE 1	PE 2	PE 3	AS 1	AS 2	AS 3	NC
Day 3	2.126	3.313	1.978	7.616	3.758	7.022	3.214	3.115	2.571	3.115	1.928	2.027	0
Day 6	2.522	3.956	3.115	8.16	3.956	7.665	3.659	3.461	2.967	3.61	2.67	2.522	0
Day 9	3.709	4.747	3.313	10.731	4.352	11.622	4.995	6.28	7.814	5.242	4.302	6.824	0
Day 12	5.44	8.061	5.44	6.726	3.857	7.715	16.023	10.88	12.858	6.33	3.56	2.225	0
Day 15	3.659	3.461	4.648	4.896	2.967	6.033	6.132	5.44	3.808	4.5	2.67	2.324	0
Day 18	3.56	4.104	5.143	7.171	3.313	9.792	11.078	8.358	6.577	3.758	1.384	1.78	0
Day 21	2.373	2.621	5.588	4.698	3.511	5.637	7.22	3.808	4.401	4.451	2.324	1.879	0

Appendix D: Activity assay using pure enzymes

I. Lignin peroxidase using veratryl alcohol

Table D 1: Change in Abs after 1 min of reaction with veratryl alcohol

Sample 1	Sample 2	Sample 3
0.104	0.105	0.112

Table D 2: Calculated enzyme activities with veratryl alcohol

Sample 1	Sample 2	Sample 3
55.91	56.45	60.24

II. Lignin peroxidase azure B

Table D 3: Change in Abs after 1 min of reaction with azure B

Sample 1	Sample 2	Sample 3
0.646	0.656	0.641

Table D 4: Calculated enzyme activities with azure B

Sample 1	Sample 2	Sample 3
66.17	67.18	65.65

III. Manganese peroxidase using DMP

Table D 5: Change in Abs after 1 min of reaction with DMP

Sample 1	Sample 2	Sample 3
0.274	0.254	0.234

Table D 6: Calculated enzyme activities with DMP

Sample 1	Sample 2	Sample 3
54.33	50.36	46.33

IV. Manganese peroxidase using manganese ions and sodium malonate

Table D 7: Change in Abs after 1 min of reaction with manganese ions and sodium malonate

Sample 1	Sample 2	Sample 3
0.015	0.019	0.014

Table D 8: Calculated enzyme activities with manganese ions and sodium malonate

Sample 1	Sample 2	Sample 3
48.75	61.75	45.5

V. Laccase using ABTS

Table D 9: Change in Abs after 10 min of reaction with ABTS

Sample 1	Sample 2	Sample 3
3.288	3.32	3.313

Table D 10: Calculated enzyme activities with ABTS

Sample 1	Sample 2	Sample 3
235.66	237.95	237.45

VI. Laccase using guaiacol

Table D 11: Abs readings after 15 min of reaction with guaiacol

Sample 1	Sample 2	Sample 3
2.819	2.837	2.855

Table D 12: Calculated enzyme activities with guaiacol

Sample 1	Sample 2	Sample 3
139.43	140.33	141.20

Appendix E: GC-MS chromatograms

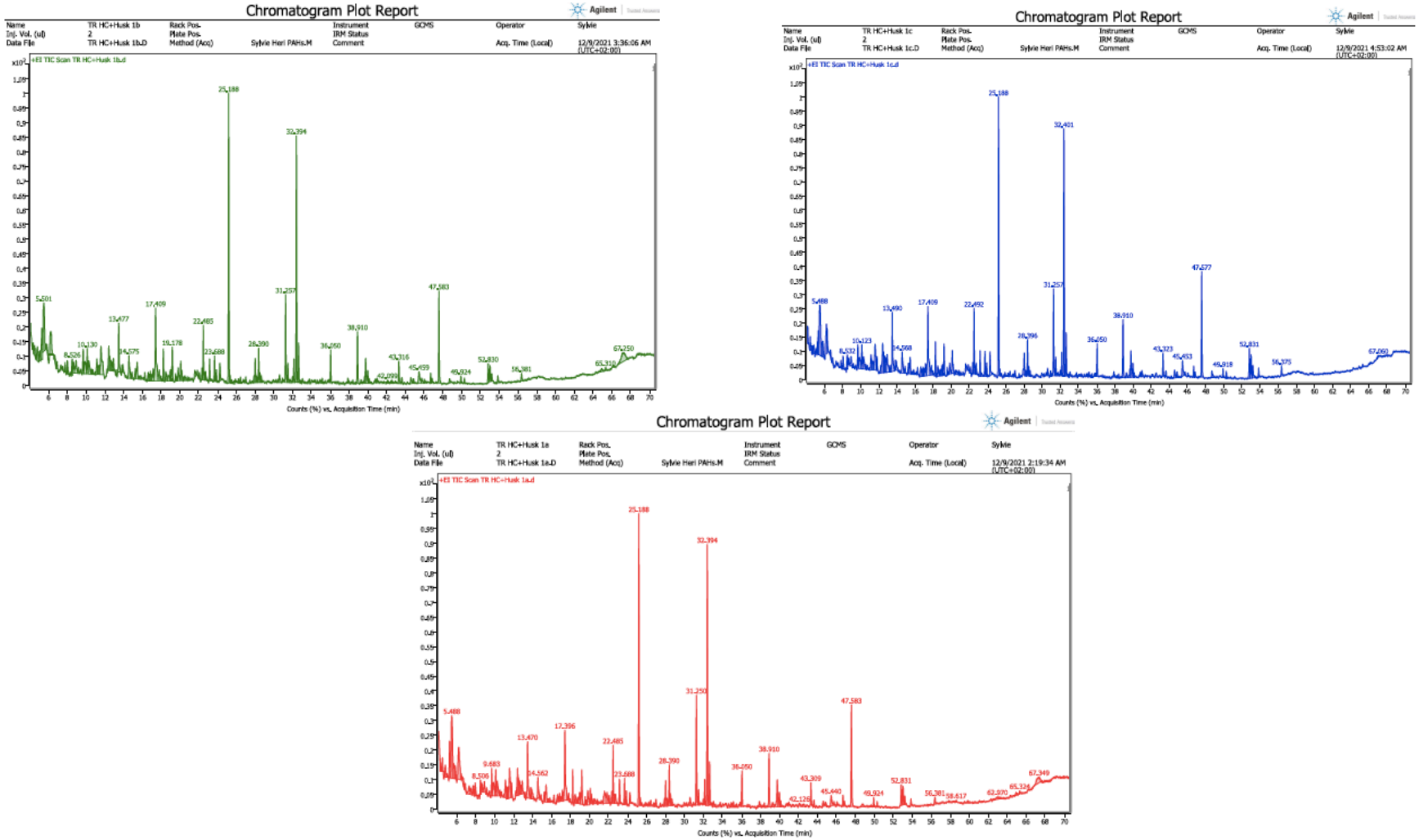

Figure E 1: GC-MS chromatograms of control PAHs without fungal remediation (done in triplicate to confirm results' replicability)

Appendix F: Research article derived from thesis

Applied Microbiology and Biotechnology

Comparative evaluation of chromogenic reagents assay methods for lignin modifying enzymes (LMEs) activity in standardized quantitative UV-Spectrophotometry

--Manuscript Draft--

Manuscript Number:	
Full Title:	Comparative evaluation of chromogenic reagents assay methods for lignin modifying enzymes (LMEs) activity in standardized quantitative UV-Spectrophotometry
Article Type:	Original Article
Section/Category:	Biotechnologically relevant enzymes and proteins
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Abstract:	The study reviewed and evaluated assay methods that used different substrates to analyse economically significant lignin modifying enzymes (LMEs; laccase, MnP, and LiP) produced from wild fungal isolates autochthonous to Gauteng Province, South Africa. Qualitative screening for LME activity using guaiacol and azure B agar plate assay identified two basidiomycotic white rot fungi, Trametes hirsuta (TR) and Schizophyllum sp. (SC), as high producers of LMEs compared to ascomycotic isolates Myrmaecium rubricosum (MR), Trichoderma harzianum (TH), Fusarium fujikuroi (FU), Curvularia coatesiae (CC), Penicillium oxalicum (PE), and Aspergillus niger (AS). Isolates TR and SC produced LMEs at high levels using corn husk lignin as a carbon source. DMP and Mn-malonate assays showed no significant differences in crude enzyme extracts or pure MnP, but LiP and laccase activities depended on the assay type. Commercial laccases and crude enzyme extracts had lower sensitivity and catalytic efficiency for the guaiacol assay than the ABTS assay, while veratryl alcohol gave significantly higher LiP activity values, compared to the azure B assay. This study shows that LME activity assay sensitivity depends on substrate efficiency. The study also found two novel fungal isolates that could produce LMEs for industrial enzymes used in biotechnological applications.
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