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Bioremediation and biodegradation of selected polyaromatic hydrocarbons by fungi
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Dissertation for PhD degree in Chemical and Biological Engineering

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January 2021

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“There is no real ending. It’s just the place where you stop the story.”

-Frank Herbert

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STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledge the Code of Ethical Conduct of the University of Minho.

Bioremediation and biodegradation of selected polyaromatic hydrocarbons by fungi

Abstract

Despite the amount of pollutants in the ecosphere has been increasing for a long time, soil contaminated with polyaromatic hydrocarbons (PAH) still remains one of the most important environmental problems worldwide. PAH are universal pollutants with a big variation in their concentration levels in different regions. PAH tend to bind to clays as these have high surface area, which allows more binding and adsorption of PAH on finer soil fractions. However, clay particles reduce in size as time goes by, diminishing porosity and transferable species in soil, and with aging of PAH also lose bioavailability, becoming persistent. Despite the different reported bioremediation techniques, this work focus on the biological degradation of PAH with fungi and their enzymes, mainly laccase and laccase-mediator systems. Laccases are receiving attention from different researchers due to their specific nature. They may be used in different industrial applications as they present the ability to oxidize a broad spectrum of phenolic and nonphenolic compounds. Moreover, processes with laccases are quite competitive with conventional chemical processes in different industries. However, the main limiting factor to their application still remains – large-scale production of laccase, mainly of highly active and stable laccase, is still to be attained.

The work is globally divided into two parts, depending on the soil used: artificially contaminated or naturally contaminated soils. In the first part of this work the effect of soil pH and of different supports was evaluated, and the enzyme activities during the PAH degradation in soil were measured. Moreover, laccase degradation was set up, produced by fungal co- cultivation, using kiwi peels as substrate. The produced laccase was applied to PAH contaminated soil to evaluate its efficiency on enzymatic bioremediation. In a second part, heavily PAH and metals polluted soil obtained from Lagos, Nigeria, was used. *Trametes versicolor* was isolated from this soil and used in degradation experiments, with plantain peels as support. Laccase degradation assays were also set up, in batch and in PBR, with and without mediators. The mediators used were ferulic acid, coumaric acid and ABTS.

It was observed that mediators as ABTS and ferulic acid were more successful enhancing PAH degradation.

Key words: soil degradation, PAH, fungi, laccase, laccase-mediator system

Biorremediação e biodegradação de selecionados hidrocarbonetos poliaromáticos por fungos

Sumário

Apesar da crescente poluição da ecosfera que se verifica desde há muito tempo, a contaminação dos solos com hidrocarbonetos poliaromáticos (PAH) mantem-se como um importante problema ambiental a nível mundial. Os PAH são poluentes universais com uma enorme variação dos seus níveis de concentração em diferentes regiões. Os PAH tendem a ligar-se a argilas graças à elevada área superficial destas últimas, favorecendo a sua ligação e adsorção em frações mais finas de solo. No entanto, com a passagem do tempo e os efeitos de erosão, as partículas de argila vão diminuindo de tamanho, reduzindo a porosidade e a transferência de espécies no solo, assim como os PAH vão perdendo biodisponibilidade, tornando-se mais persistentes. Apesar de diferentes técnicas de biorremediação já terem sido publicadas e exploradas, este trabalho foca-se na degradação biológica de PAH com fungos e suas enzimas, em especial com lacases e sistemas lacases-mediadores. As lacases têm recebido especial atenção por parte dos investigadores graças à sua natureza específica. Elas podem ser usadas em diferentes aplicações industriais já que têm a capacidade de oxidar um largo espetro de compostos fenólicos e não fenólicos. Além disso, processos que usam lacases são bastante competitivos com processos químicos convencionais usados em indústrias distintas. No entanto, o principal fator limitante da sua aplicação ainda permanece – a produção de lacase em larga escala, sobretudo de lacase de elevadas atividade e estabilidade, ainda não foi conseguida

Este trabalho está dividido em duas partes gerais, dependendo do solo usado: solo artificialmente contaminado ou solo naturalmente contaminado. Na primeira parte deste manuscrito, o efeito do pH do solo e de diferentes suportes foi avaliado, assim como foram medidas as atividades enzimáticas durante a degradação de PAH no solo. A degradação por lacase foi avaliada por co-cultivo de fungos em substrato de cascas de kiwi. A lacase produzida foi aplicada em solo contaminado por PAH para avaliação da eficiência da biorremediação enzimática. Na segunda parte do trabalho usou-se um solo muito poluído em PAH e metais, obtido em Lagos, na Nigéria. A partir desse solo, isolou-se o *Trametes versicolor* que foi usado nos ensaios de degradação, com cascas de banana como suporte. Realizaram-se ensaios em batch e em contínuo de degradação de lacase, sem e com mediadores. Os mediadores usados foram o ácido ferúlico, o ácido cumárico e o ABTS.

Observou-se que os mediadores ABTS e ácido ferúlico implementam com mais sucesso a degradação dos PAH.

Palavras-chave: degradação do solo, PAH, fungos, lacase, sistema mediador de lacase

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List of Abbreviations and Acronyms

ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

EU - European Union

FC - ferulic acid

DMAB - 3-dimethylaminobenzoic acid

K_{ow} - octanol–water partitioning coefficient

LDF - Litter-decomposing fungi

LiP - Lignin peroxidase

LME - Lignin-modifying enzymes

LMS - Laccase mediator system

MBTH - 3-methyl-2-benzthaiazolinone hydrazone hydrochloride

MnP - Manganese peroxidase

PAH - Polyaromatic hydrocarbons

PBR - packed-bed reactor

PC - p-coumaric acid

SCF - EU Scientific Committee for Food

SOM - Soil organic matter

SSF - Solid-state fermentation

VP - Versatile peroxidase

WRF - White-rot fungi

USEPA – United States environmental protection agency

Publications within the thesis

Papers in peer reviewed journals

Vipotnik, Z., Michelin, M., Tavares, T. Development of a packed bed reactor for the removal of aromatic hydrocarbons from soil using laccase/mediator feeding system. 2021. Microbiological Research. https://doi.org/10.1016/j.micres.2020.126687

Vipotnik, Z., Michelin, M., Tavares, T. Ligninolytic enzymes production during polycyclic aromatic hydrocarbons degradation: effect of soil pH, soil amendments and fungal co-cultivation. 2021. Biodegradation. https://doi.org/10.1007/s10532-021-09933-2

Vipotnik, Z., Michelin, M., Tavares, T. Biodegradation of chrysene and benzo[a]pyrene and removal of metals from naturally contaminated soil by *Trametes versicolor* strain and laccase produced thereof. (*submitted*)

Vipotnik, Z., Michelin, M., Tavares, T. Rehabilitation of a historically contaminated soil by different laccases and laccase mediator system. (*submitted*)

Oral communications

Vipotnik, Z., Michelin, M., Tavares, T. Degradation of Selected PAH's By Laccase-Mediator System in Soil. 5th World Congress on New Technologies (NewTech'19), 2019, Lisbon, Portugal

Posters in conferences

Vipotnik, Z., Michelin, M., Tavares, T. Chrysene, and benzo[a]pyrene degradation in soil packed bed reactor by laccase mediator system. The First FEMS Conference on Microbiology 2020 in Belgrade - FEMS Online Conference on Microbiology 2020

Vipotnik, Z., Michelin, M., Tavares, T. Optimization of laccase production by co-cultivation of fungi. the 43rd The FEBS Congress, 2018, Prague, Czech Republic

CHAPTER I –

General introduction

1.1 Framework

The intense industrial activity in the 20th century has been deleterious for environment, resulting in a large number of contaminated sites. Despite the overall amount of pollutants is increasing, soil contaminated with polyaromatic hydrocarbons (PAH) is still one of the most important environmental problems worldwide with innumerable places still polluted with PAH. In a study of Nam and co-authors [1] it was found that the average background PAH concentration in different soils worldwide followed the order Europe > North America > Asia > Oceania > Africa > South America. However, the concentration of different PAH varies widely, depending on the level of industrial development, production source and transport network. Kanaly and Harayama [2] reported that PAH concentrations in soils and sediments vary from 1mg/kg to over 300 g/kg. The average concentration in forest soil ranges from 5 μg to 100 μg/kg. Rural soil contains carcinogenic PAH at levels of 10–100 μg/kg, mainly from atmospheric fallout. However, it can also be found exceptionally up to 1000 μg/kg of PAH. Metropolitan areas have higher PAH concentrations, mainly because of the many sources of fossil fuel combustion. The majority of PAH concentrations in urban soil falls in the 600– 3000 μg/kg range. Higher values are probable near areas of heavy transportation and industrialization. Values in the order of 1000–3000 μg/kg are regarded as being in the upper range and nevertheless levels of 8 000–336 000 μg/kg have been reported for road dust [3,4].

Different soil remediation techniques have been reported during the past 20 years, but only a few have been accepted by some industries. Certain hazardous compounds such as pentachlorophenols (PCP), polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT), benzene, toluene, ethylbenzene and xylene (BTEX) as well as trinitrotoluene (TNT) are persistent in the environment and known to have carcinogenic and/or mutagenic effects.

Rehabilitation of PAH contaminated soils is a global concern and remediation of PAH-polluted sites is essential. This can be done by different methods which involve removal/isolation or alteration of the contaminant. The biological processes are green, low cost, implemented in situ, with lower potential risk factors, despite they take a longer time to achieve acceptable results.

1.2 Aim of the thesis

Currently, there is a lack of research that attempts to link laccase degradation studies to real

contaminated soil. In fact, there are several studies with laccase and LMS, most of them performed with buffers and specific media, and therefore homogeneity of PAH dissolved in buffers hardly compare with naturally aged, historically contaminated soil. Moreover, there are several properties of soil that also need to be considered such as physical (texture, composition and temperature), chemical (organic matter content, black carbon, pH, redox) and biological ones.

The main focus of this work was to assess the degradation of PAH in soil by fungi, as well as the laccases produced by them, in the presence and absence of mediators. LMS were investigated with synthetic (ABTS) and natural (p-CA, FA) mediators.

Specifically, this thesis intended to:

- Evaluate the effect of soil pH and substrate support on PAH degradation by fungi and enzyme production during degradation;
- Study the fungal cocultivation in improving PAH degradation in soil;
- Evaluate the production ligninolytic enzymes, namely laccase, manganese peroxidase and lignin, by fungi during the PAH degradation in soil;
- Optimize the laccase production and evaluate the PAH degradation by laccase/LMS on artificially contaminated soil, in batch and feed batch with continuous flow of laccase/mediator in PBR;
- Isolate PAH degrading fungi from heavily contaminated soil and reuse them for the biostimulation – bioagumentation of this soil;
- Evaluate LMS for PAH degradation and metals removal from naturally and heavily contaminated soil in PBR;
- Compare the potential of fungal laccases from several sources (produced "*in situ*" *vs* commercial) on rehabilitation of a historically contaminated soil.

1.3 Outline of the thesis

The thesis here presented reports the main results obtained from research undertaken at Centre of Biological Engineering (CEB), University of Minho, Braga, Portugal, and is organized into the following six chapters:

Chapter 1 introduces general aspects such as the background and scope of this work, as well as the

motivation which led to the proposal of the theme and attempts to integrate the various aspects developed throughout the thesis. The chapter is completed by the presentation of the aims of the work.

Chapter 2 is a Literature Review that aims to introduce the theoretical fundamentals and state of the art of the aspects more causally related to the theme. The span of the thesis is presented in this chapter,

Chapter 3 describes the materials and methods used to perform this work. This chapter is divided in sections corresponding to the two major themes: degradation of PAH in artificially polluted soil and in aged naturally polluted soil, by fungi and laccase-mediator systems.

Chapter 4 presents and discusses the main results obtained in two different aspects of the biological degradation of PAH in soil. This Chapter is divided into two main part, depends on soil used, as the previous one: two different polluted soils rehabilitated by two distinct entities, i.e. fungi and LMS.

Chapter 5 summarizes the main conclusions withdrawn from the work and exposes perspectives and suggestions for future work.

Chapter 6 encloses the references used to support these studies.

CHAPTER II

Literature review

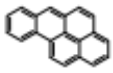
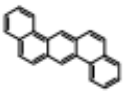
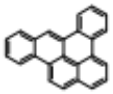
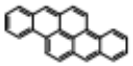
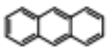
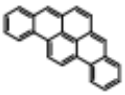
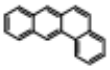
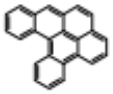
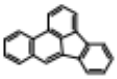
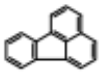
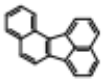
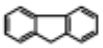
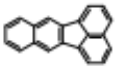
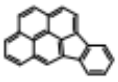
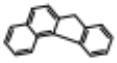
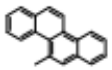
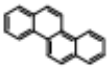
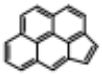
2.1 Polyaromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are some of the main pollutants typically present in contaminated soils, usually in combination with heterocyclic aromatic rings in which carbon atoms are substituted by nitrogen, sulphur or oxygen atoms. PAH substituted by alkyl groups are also common co-pollutants [5], together with heavy metals, defining complex mixtures. PAH are composed of two or more aromatic rings and there are more than one hundred PAH widely distributed and ubiquitously found in the environment. are on United States Environmental Protection Agency (USEPA) priority list (Table 2.1) as they are potentially toxic and seven of them are carcinogenic [6], although not all are being monitored by EU Scientific Committee for Food (SCF) and European Union (EU) (Table 2.1). PAH are mostly colourless, white, or pale-yellow solids, with low solubility in water, high melting and boiling points and low vapour pressure. Due to their complex structure, they are chemically stable and resistant to biodegradation and therefore they accumulate in environment, leading to a growing toxicity. Moreover, when those hazardous contaminants are in contact with soil, they may accumulate and affect the agricultural production and most of the food sector [7].

PAH can be released into environment by several anthropogenic and natural processes. The natural factors of the formation PAH include the cosmic, the endogenic geological and biological factors. Most of the PAH found in the environment belong to pyrogenic PAH with volcanism, the combustion of oil and oil products and the combustion of plant materials (wood, herbaceous plants, peat and coal) [8,9] as the main sources. Moreover, the content and composition of the PAH depend on temperature, heating duration and source of supply. A significant part of the PAH present in the environment is formed at temperatures higher than 500°C, yet the presence of compounds with different molecular weights in fossil fuels can confirm that PAH may also be formed at relatively low temperatures (100–150 °C) [8].

The refinery and petrochemical industries release PAH into the environment. The qualitative composition of the emissions from refineries depends on the initial hydrocarbon components of the oil and gas. The combustion of coal is a significant source of PAH in landscapes. Coals initially contain some amounts of aromatic compounds, but their content depends on the type of coal, its degree of metamorphization and its paleotemperature formation [11]. The combustion of oil products (benzene, diesel fuel) in internal combustion engines also results in the formation of PAH. Emissions from motor transport make the largest contribution to the pollution of air with polyarenes [9].

Table 2.1 Identification and chemical structure of the PAH frequently monitored according to recommendations of SCF, EU and USEPA [10].

EPA, SCF, EU	Benzo[*a*] pyrene		EPA, SCF, EU	Dibenz[*a,h*] anthracene	
EPA	Acenaphthene		EU+SCF	Dibenzo [*a,e*]pyrene	
EPA	Acenaphthylene		EU+SCF	Dibenzo [*a,h*]pyrene	
EPA	Anthracene		EU+SCF	Dibenzo [*a,i*]pyrene	
EPA, SCF, EU	Benz[*a*] anthracene		EU+SCF	Dibenzo [*a,l*]pyrene	
EPA, SCF, EU	Benzo[*b*] fluoranthene		EPA	Fluoranthene	
SCF, EU	Benzo[*j*] fluoranthene		EPA	Fluorene	
EPA, SCF, EU	Benzo[*k*] fluoranthene		EPA, SCF, EU	Indeno[1,2,3-*cd*]pyrene	
EU	Benzo[*c*]fluorene		EU+SCF	5-Methyl chrysene	
EPA, SCF, EU	Benzo[*ghi*] perylene		EPA	Naphthalene	
EPA, SCF, EU	Chrysene		EPA	Phenanthrene	
SCF, EU	Cyclopenta [*cd*]pyrene		EPA	Pyrene	

PAH are brought into the open environment predominantly due to incomplete combustion of wood, petrol, oil, and coal. Some originates from petroleum or coal deposits and volcanic events and are released into environment by various anthropogenic activities like residential heating, coal gasification and liquefaction plants, asphalt and carbon black production, motor vehicles exhaust, oil spillages, coke and aluminum productions, etc. [12,13]. Based on their molecular structure, arrangement, and number of benzene rings, PAH are classified into high molecular weight (HMW) and low molecular weight (LMW). Since all PAH are made up of benzene or its derivatives, they are thermodynamically very stable in the

environment, generally resisting degradation by microorganisms and thereby proving to be toxic, mutagenic and carcinogenic to living organisms [14]. LMW PAH, those with two and three rings, such as phenanthrene, fluorene, acenaphthylene, acenaphthene, anthracene and fluoranthene are the PAH prevalent in the gaseous phase while solid particles (volatile ash and soot) are enriched with polynuclear compounds. HMW PAH, with four and more rings, appear by the alternation of combustion and smoldering. LMW PAH are relatively volatile, soluble and more degradable than HMW PAH, which sorb strongly on soils, and are more hydrophobic, toxic and persistent to microbial degradation [15].

Chrysene is a crystalline, whitish HMW PAH, symmetrically composed of four fused benzene rings produced during the incomplete combustion of organic materials (like petroleum products, wood) and classified as a priority pollutant by USEPA [14,16,17]. It is released in open environment through various anthropogenic industrial activities, oil spillages, natural forest fires, etc. and reported as being potentially toxic and carcinogenic to human [16]. Like other PAH, chrysene undergoes volatilization, photolysis, adsorption, chemical oxidation and bioaccumulation in soils and aquifers [14,16]. Benzo[a]pyrene, a HMW five-ring PAH, is one of the most recalcitrant and toxic PAH [18], with very low water solubility, vapor pressure and a high octanol/water partitioning coefficient, suggesting a preference for non-aqueous phases and low microbial bioavailability [19]. Benzo[a]pyrene is naturally produced through forest fires, volcanic eruptions, cigarette smoke and burnt food as coffee, and belongs to one of the most toxic PAH. Benzo[a]pyren mechanism involve intercalation into DNA leading to hamper of transcription, which cause mutagenic of DNA [20]. Pyrene consists of four fused benzene rings, obtained during the combustion of organic compounds and it is used to make dyes, plastics and pesticides [13]. Most PAH are used in agricultural products, dyes, intermediaries in pharmaceuticals, photographic products, thermosetting plastics, lubricating materials, and other chemical industry products [13]. Pyrene, anthracene, acenaphthene and fluoranthene are used in the manufacture of pigments. Acenaphthene, fluoranthene, fluorene and phenanthrene are used in pesticides, resins and pharmaceuticals manufacture. Anthracene is used as diluent for wood preservatives [13].

2.2 Polyaromatic hydrocarbons in soil / soil as working environment

The standard total PAH content in soil ranges from 0.2 to 0.6 mg kg^{-1}. Depending on the amount of total PAH content, that is, 0.6-1, 1–5, 5–10 and over 10 mg kg^{-1} the soil is regarded as slightly polluted, polluted, heavily polluted and very heavily polluted, respectively [21]. PAH are universal pollutants with a

high degree of variation in their concentration levels in different regions, and the source of such contamination varies from country to country. Globally, HMW PAH are found to represent the majority of PAH contamination (80–90 %) at evaluated sites [22]. This clearly means that the remediation of most PAH contaminated sites is difficult due to the dominance of distinct HMW PAH mixtures that are less bioavailable and very much resistant to degradation. However, most abandoned industrial sites are still not easily accessible to researchers due to legal and liability issues associated with the transport and storage of contaminated soils and therefore a large number of contaminated sites are still to be intervened [22].

Soil, the major sink for many chemicals, may be affected physically, chemically, and biologically in its properties by these spillages. Soil properties are affected differently and a longer exposure to contaminants may affect geotechnical properties such as Atterberg limits, permeability, hydraulic conductivity, strength parameters, consolidation, compaction and shear strength as well as the biological properties (biomass, microbial, and enzyme activity) of soils [23]. Despite microorganisms have ability to degrade hazardous contaminants and to enhance a self-purification process, this only works with lighter pollution. With longer exposure to contaminants, microbial activity decreases [24] and soil also changes due to partial or full replacement of the pore liquid with PAH, chocking the soil pores, reducing the soil aeration and the water infiltration. A study of Magi and co-authors [25] confirmed PAH bind preferably to silt and clay, as clay has a high surface area, which allows more bonding and adsorption of PAH on finer soil fractions. In these, the inter-particle spaces are reduced, affecting the transfer of HMW PAH from one location to another due to their hydrophobic nature, leading to persistent toxicity and long-term effect [23]. In fact, soil has a particulate structure composed of organic and inorganic components and separated by pores containing water or/and air. Organic matter is very important for the behaviour of organic contaminants in soil and it can be divided into soft carbon, a flexible structure with humic and fulvic acids with reversible sorption, and hard carbon, a rigid and condensed structure, which involves humin, kerogen and pyrogenic carbon, associated to irreversible sequestration [26]. The octanol–water partitioning coefficient (K_{ow}) is related to the solubility of an organic compound in water, and it is also important in determining the sorption of PAH to soils. With increasing K_{ow}, the aqueous solubility decreases and the tendency of sorption to soil increases. Moreover, the mobility of PAH may be affected by their solubility and K_{ow} [13,27].

The rate and degree to which PAH are degraded or retained in soil are controlled by several factors: (1) soil properties, such as soil type, soil temperature, moisture, redox potential, nutrients, presence and

activity of naturally present microorganisms, and (2) physico-chemical properties of individual PAH such as molecular mass, bioavailability, and toxicity. As example, LMW PAH with low $K_{ow,}$ high water solubility and volatility, are rapidly dispersed and become more mobile and degradable, and on the other hand, HMW PAH are more resistant and persistent to degradation [22]. Moreover, PAH bioavailability in soil is reduced by aging, as they also become more persistent.

2.3 Remediation treatments for contaminated sites

PAH are very stable contaminants with a resistant nature in soil in comparison to other organic pollutants. Rehabilitation of PAH contaminated soil area becomes imperative, considering their toxic properties and persistence in soil. There are three main processes of degradation and remediation of contaminated soils: physical, chemical and biological. Selected treatment depends on the contamination type, the soil nature and properties and the risk associated with selected techniques. In general, the cost and adverse environmental effects caused by these methods discourage their widespread practice. As physico-chemical treatments have several limitations besides their high price, biological methods are gaining popularity due to eco-friendly nature and the effective bioconversion of toxic pollutants into less harmful and/or nontoxic forms. Usually, the biological option is effective and efficient, competitive and not chemically invasive. Nevertheless, during PAH degradation some epoxides and dihydrodiols are formed which are more toxic then PAH [28].

PAH biodegradation mechanisms usually start with the oxidation step, with two atoms of oxygen being incorporated into each aromatic ring to form cis-dihydrodiol. Monooxygenases enzymes are involved with this first initial oxidation to form trans-dihydrodiols. PAH bioremediation can be performed in two ways: *ex-situ* that is held off the contaminated site requiring excavation and site conditioning, and *in-situ* when soil decontamination is performed in its original location, without removing it from that area [29,30]. A comparison between the most known technique*s* suitable to remove PAH, metals and organic compounds from contaminated sites is presented in Table 2.2.

Table 2.1 Techniques suitable to remove PAH, metals, organic compounds from contaminated sites (adapted from [29–32])

Technique	Purpose	Benefits/Strength	Problems/Weakness	Cost
Solvent extraction / soil washing	Treatment with two or more solvents, either alone or in mixtures to extract PAH.	Easy and quick implementation, volume reduction of contaminants.	Contaminant transference, large amount of solvents is required, high cost. Not effective in treatment of clay and silty soils, solvents can be adsorbed on the soil.	Moderate
Chemical oxidation	Fenton's reagent, ozone, potassium permanganate, hydrogen peroxide used to oxidize PAH.	Oxidation of the organic contaminants simple and efficient, not dependent on toxicity of the contaminant.	High cost, excavation, pH dependency, natural microorganisms destroyed.	High
Photocatalytic degradation	Photocatalytic oxidation reaction in presence of UV light used to destroy PAH.	Pollutant removal at very low concentration, complete degradation of organic contaminants within few hours.	High cost, highly dependent on pH, temperature, amount and nature of photocatalyst, intensity and time of radiation. Not suitable for commercial application for volatile contaminants.	High
Electrokinetic remediation	Treatment of soils with low permeability and contaminated with heavy metals or with organic compounds.	Safe, suitable for clays with low permeability and heterogeneous soil, used for *in-situ* or *ex-situ* treatment, cost-effective, low electrical energy demand. Minimal equipment required.	Time-consuming, not efficient in low contaminant concentrations. Interaction between electrokinetic flux and contaminants.	Moderate
Thermal technology	Destruction or volatilization of PAH by high temperature.	High content removal, excavation is not required for *in-situ* treatment.	Cost-ineffective for large volumes of contaminated soils. Volatilization and release of contaminants from the soil into the air at high temperatures, less efficient at low temperature. Limited to volatile contaminants.	High
Phytoremediation	Plants are used to extract heavy metals from contaminated soil. It may take advantage from the synergistic interaction between plants and microorganisms.	Low cost, environment friendly, large-scale operation, soil structure preservation, safe, plants can be easy monitored.	Time-consuming, slow growth of plants, dependent on contaminant toxicity and bioavailability. Plant growth might require special conditions (moisture, temperature).	Low
Biological remediation	Biotransformation or mineralization of toxic organic compounds by microorganisms or their enzymes.	Natural process, environment friendly and cheap, contaminants are transformed into less or non-hazardous molecules.	Time-consuming, investigation on soil conditions to select effective microorganisms and nutrients.	Low

2.3.1 Biological treatments

Bioremediation performed by enzymes or by the whole fungal cells is a green process, successfully employed for the treatment of overgrowing various pollutants. Fungal bioremediation has been applied to 15 - 35 % of contaminated soil worldwide in order to control the environmental issues [32]. Soils can be exposed to physical, chemical, or biological rehabilitation having an effect on the diversity of microbial communities. Despite microorganisms are capable of adapting to different conditions, they are still favoured by the optimum conditions defined in laboratory as temperature, nutrients or pH. Microorganisms isolated from contaminated sites tend to show more degradation ability, as they are already adapted to the polluted environment and can survive in presence of the pollutants.

As mentioned before for general techniques, bioremediation can be conducted in two ways: *ex-situ*, held off the contaminated site, requiring excavation and site conditioning, and *in-situ*, performed without removing the soil from the area. Most of the actual installations are *in-situ* and they may include biostimulation, bioaugmentation and/or bioattenuation.

2.3.1.1 Biostimulation

Biostimulation is defined by the addition of nutrients to a contaminated soil to encourage the growth of naturally present microorganisms to degrade pollutants. Inorganic macronutrients (as N, P, K) or micronutrients (as Mg, S, Fe, Cl, Zn, Mn, Cu, Na) can be added to recover depleted soils in order to improve the degradation activity of microorganisms.

2.3.1.2 Bioagumentation

This procedure aims the improvement of self-healing capacity of contaminated areas by increasing the concentration of specific competent strains or consortia of microorganisms that are originally present in the site.

2.3.1.3 Bioattenuation

Bioattenuation includes a variety of chemical, physical and biological processes that reduce the toxicity, mass, volume or concentration of contaminants. These processes include aerobic or anaerobic biodegradation, sorption, volatilization, chemical or biological stabilization and a general transformation

of contaminants. With this method contaminants are transformed by the metabolic processes of microorganisms. The process is slow and usually is applied on sites with low contamination.

2.3.2 Limiting factors in successful biological degradation

Biotic factors as temperature, pH, humidity, aeration, nutrient concentration, redox potential and soil type may influence bioremediation. Moreover, specific biotic factors such as microorganisms competition, predation or synergism play a major role in the success of the biological remediation [31]. For most soils, the bioremediation efficiency depends on pH performance, in the range of 5 to 8, and on the temperature profile, in the interval of 5 °C to 30 °C, which means that during wintertime bioremediation is not effective. Organic matter is one of the most important soil parameters influencing the effectiveness of bioaugmentation as it plays a crucial role on the bioavailability of pollutants and supports the survival of the inoculated strains. The introduction of a microorganism in an eco-system may also constitute *per se* a limiting factor and even its permanence may be only temporary, since the microorganisms need to adapt to environmental conditions. For that reason the following criteria must be considered to promote bioaugmentation: contaminant availability for microorganisms, microbial activity, survival of microorganisms in the foreign environment and environmental conditions such as nutrient availability, water content and pore size of the aggregates [30,31]. Indirectly, also soil humidity impacts on the soil aeration. Moreover, hydraulic properties of soil also need to be considered together with soil texture, as all of them can limit microbial dispersion.

Bento et al. [33] concluded that the best approach to soil rehabilitation is the inoculation of preselected microorganisms isolated from their own environment. Following their isolation from environmental sites, these microorganisms (pure cultures, natural or artificial consortia) are grown under laboratory conditions and returned to the same soil [31,33]. In order to understand the complex physico-chemical-biological interactions between the microbial community and its environment, a proper evaluation of the role of indigenous or allochthonous microbial consortia, agricultural management systems, the quantity and quality of nutrients and the diversity of microbial communities in the contaminated soils must be envisaged and detailed. The addition of organic residues combined with a specialized microbial consortium has the potential to enhance the degradation of the contaminants under study and may become a promising technology. However, a combination of bioremediation technologies may reduce the competitiveness of the biological process and the ratio efficiency/costs has to be properly

evaluated [30].

2.4 Fungi

Fungi are a diverse group of organisms including mushrooms, moulds and yeasts. Currently, the kingdom of fungi is divided into seven phyla: Microsporidia, Blastocladiomycota, Neocallimastigomycota, Chytridiomycota, Glomeromycota, Ascomycota and Basidiomycota. Many moulds and yeasts belong to the phylum Ascomycota and typical mushrooms belong to the phylum Basidiomycota and further to the class Agaricomycetes [34]. There are 17 orders within the class Agaricomycetes, which can be divided into three ecophysiological groups, based on their habitat and role in nature: (1) mycorrhizal fungi, (2) wood-degrading fungi and (3) litter-decomposing fungi.

Wood-degrading fungi normally grow on dead trees. However, the fungi genera *Phanerochaete*, *Pleurotus* and *Trametes* may also survive in soil if suitable substrates are available [35]. They are divided into white-rot fungi (WRF) that represent over 90 % of all wood rotting basidiomycetes, and brown-rot fungi that represent 7 % of the same basidiomycetes. WRF are degraders of all components in wood (lignin, hemicellulose and cellulose). Litter-decomposing fungi (LDF) are found in top layer of soil where they degrade plant litter material. WRF and LDF are the only organisms capable of mineralizing lignin efficiently and for that they are called ligninolytic fungi, but LDF are not so efficient lignin degraders as WRF. Yet they are more suitable for bioremediation as they grow better in the soil than WRF [35,36].

Ascomycetes, basidiomycetes and deuteromycetes fungi can produce laccases and, among them, white-rot basidiomycetes are the most efficient lignin degraders and laccase producers [37]. White-rot fungi (WRF) as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Cirnipellis stipitaria* and *Pleurotus ostreatus* perform efficient degradation of most PAH, when these work as the solely carbon source. Enzymatic mechanisms for PAH degradation by WRF have been widely discussed and it has been recognized that WRF degrade PAH by the synthesis of lignin modifying enzymes [38], but the specific type of enzymes secreted may differ between fungi [37].

Laccases are a family of copper-containing oxidases found in a variety of bacteria, fungi, insects and plants [39]. They are capable of oxidizing a wide range of compounds such as mono-, di-, poly- and methoxy-phenols, aromatic and aliphatic amines, hydroxyindoles, benzenethiols, carbohydrates and inorganic/organic metal compounds [37,40]. The non-specific nature of their activity on a variety of substrates makes them ideal catalysts for different industrial applications, that are efficient, sustainable

and environment friendly. Nonetheless, large-scale applications of laccases are limited by the economical factor and the efficiency of the enzymes [37]. Regarding peroxidases, depending on the source of activity, they are classified as lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). These enzymes usually catalyze the first attack to the PAH molecule [41]. MnP and LiP are heme peroxidases, mostly reported for the degradation of toxic compounds by basidiomycetes, including WRF. They also require the presence of hydrogen peroxidase and manganese peroxidase for activity. VP enzymes are capable of oxidizing both phenolic and non-phenolic compounds [42,43].

Diverse fungi are known for their capability to degrade PAH and their success in the remediation of contaminated soils has been reported in several studies in the past two decades, still with increasing attention [44–47]. They are tolerant to high concentrations of organic compounds and able to grow in extreme conditions, as low pH and high temperatures. Most studies on the biodegradation of PAH by fungi focused on WRF, but microorganisms isolated from contaminated environments are capable of degrading PAH due to their increased cell affinity to hydrophobic substances, which enable them to absorb and use these molecules [29,48,49]. Although most of these studies have focused on the biodegradation of PAH by WRF, but Boyle and co-authors [50] found that white-rot fungi growing in soil could not breakdown significant amounts of PAH, despite the fact that in liquid medium they were capable to degrade most of the USEPA PAH. Similar trend was confirmed by Borras et al. [51] and by Rama et al. [52], who used *T. versicolor* in soil, but did not achieve comparable results as in the liquid medium. Moreover, the fungi growth was affected by bacteria present in soil. Lignin is efficiently degraded by white-rot Basidiomycetes fungi, while Ascomycetes and Deuteromycetes oxidize phenolic compounds to phenoxy radicals and quinines [53].

Species belonging to the genera *Trichoderma*, *Fusarium*, *Penicillium*, *Stachybotrys*, *Aspergillus*, *Cladosporium*, *Mortierella*, *Beauveri*a, *Engyodontium* are examples of fungi that are common in polluted soil [54]. These fungi tend to defend the resource against potential invaders, either by sequestering critically limiting nutrients or by producing inhibitory metabolites [54]. Moreover, they are generally characterized by high tolerance to environmental stresses such as the presence of pollutants, and are identified as potential bioremediation agents of soil [55]. Independently of their degradation capabilities, these fungi are capable to decrease the concentration of organic pollutants such as PAH, PCB, chlorobenzoic acids (CBA) and endosulfan by accumulating them in the intracellular lipid vesicles. Moreover, these vesicles may have a role in the biodegradation process too [54,56].

Trichoderma species belong to the group of filamentous fungi classified as Ascomycetes (Order Hypocreales). *Trichoderma* spp. are free-living fungi that are common in soil and root ecosystems and genetically they are remarkably diverse with wide range of capabilities among different strains of agricultural and industrial significance [57–59]. Several researchers studied the potential of *Trichoderma* spp. to tolerate and detoxify environmental contaminants including heavy metal compounds, pesticides and some PAH [56,57,59–62].

2.5 Laccase and laccase mediator systems

Laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) belongs to a family of multicopper oxidases that can oxidize several phenolic compounds by transferring one electron with the concomitant reduction of oxygen to water. It was first discovered in the Japanese lacquer tree *Rhus vernicifera,* in 19th century. It was found in plants, fungi, bacteria and some insects. Up to now more than 100 laccases have been isolated and characterized. They are glycoproteins, with molecular weights ranging from 50 to130 kDa. The fungal laccases contain a carbohydrate portion of 10 to 30 % of the molecular weight, while in plant laccases the portion of carbohydrates is up to 45 % [63,64]. Laccases contain 4 cooper ions that are classified into three types: T1, T2 and T3 depending to their spectroscopic and ESR parameters [64,65]. T1 potential has been determined for many laccases and it varies between 430 - 780 mV. The potential of T2 is known only for the low potential plant laccase from *R. vernisifera* (390 mV) and high-potential fungal laccase from *T. hirsuta* (400 mV). T3 laccase potential from *R. vernisifera* and *T. versicolor* are 460 and 785 mV, respectively [64]. On the grounds of the potential of T1, all copper containing oxidases are divided into three groups: high-, medium- and low-potential enzymes [64,66]. Low potential laccases include enzymes with T1 potentials below 430 mV, medium potential ranges from 470 to 710 mV and T1 potential is considered high above 710 mV. Laccases only directly oxidize compounds whose ionization potentials do not exceed (or insignificantly exceed) the redox potential of the T1 copper ion [67].

Laccases are nonspecific, i.e. they can catalyse oxidation of various organic and inorganic substrates such as *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, methoxy phenols, lignins, aryl diamines, $Mo(CN)_8^{4-}$, $Fe(CN)_6^{4-}$, $Os(CN)_6^{4-}$, $W(CN)_8^{4-}$ and some inorganic ions with simultaneous direct reduction of dioxygen to water, without intermediate production of hydrogen peroxide [1, 32, 46–48]. They oxidize Mn^{2+} in the presence of chelating agents [50–52]. Most fungal laccases are extracellular,

with isoelectric points (pI) between 3 and 7 and with optimum pH of 3 to 6. However, their properties depends on the source and the roles to play by the laccase [68]. The optimal temperature of laccase production differs from strain to strain. Generally, 25 °C is the optimal temperature in presence of light and 30 °C in dark [69,70]. pH is also one of the limiting factors in laccase production. The optimum value of pH varies according to the substrate because different substrates cause different reactions for stability and activity of laccases. At high pH the potential difference between the phenolic substrate and the T1 copper can increase the substrate oxidation while the hydroxide anion (OH^-) binds to the T2/T3 copper centre. Most studies show that the optimal is pH between 3.5 and 6, with the optimum pH for L1 (isozyme of laccase) being 4.0 whereas the optimum pH for L2 is 5.0 [70,71]. SSF is very suitable for enzyme production using natural substrates as agricultural residues, as they mimic the natural growth conditions of fungi. Moreover, lignocellulosic materials promote fungal growth and make processes more economical [72]. However, mass and heat transfer are still limiting factors and therefore, reactors need to be optimized, although large production is still questionable. Until now different bioreactor configurations have been studied for laccase production such as immersion configuration, expanded bed, tray, in which tray configuration has given the best response [73]. Moreover laccase production was also studied with inert (nylon) and non-inert supports (barley bran) [73].

In 1990, Bourbonnais and Paice [74] found that the diammonium salt of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) serve as laccase substrate mediator, increasing the conversion by laccase. Redox mediators are low molecular weight compounds, which act as electron shutters between laccase and substrates that the laccases are not able to oxidise by themselves. Once mediators are oxidised by the laccase and released from the active site, they can perform oxidative reactions on the substrates (Figure 2.2). Therefore, mediators can expand the substrate scope of laccases against recalcitrant compounds [75].

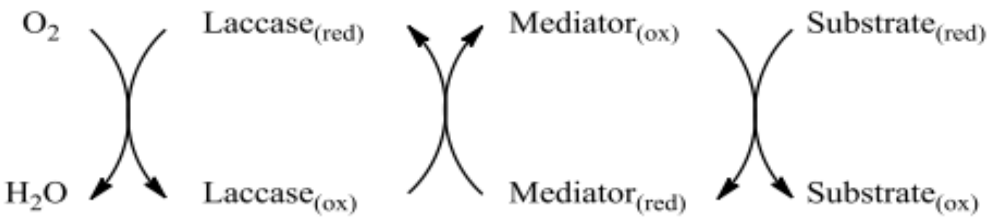


Figure 2.1 The role of a mediator in the catalytic cycle of laccases. Oxidised laccase is reduced by the four-electron oxidation of four mediator molecules. The oxidised mediators are capable of oxidising substrates that are not oxidised by laccase.

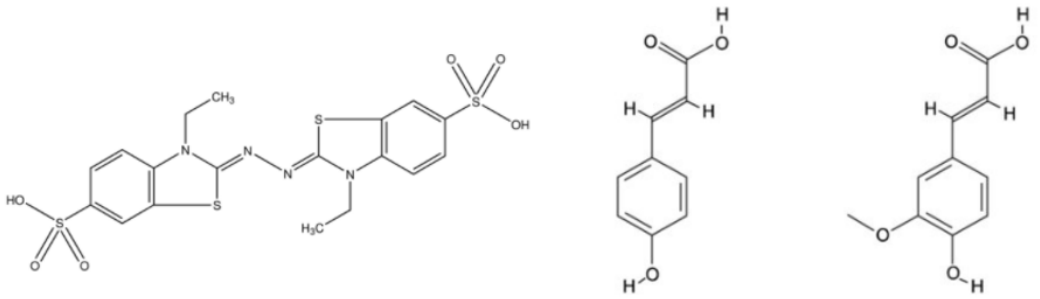
Laccase has strong stability and very low substrate specificity that makes it suitable for PAH degradation [76]. Comparing with microorganism degradation, enzyme-catalyzed reactions are faster and have higher efficiencies, lower toxicity, a wider application range and constitute a simple operation. The relatively low redox potential of laccase is its main limitation, which has been overcome by using redox mediators like ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) or 1-hydroxybenzotriazole (HBT) that allow the oxidation of non-phenolic compounds with higher redox potentials [77]. Laccase mediator system (LMS) was first applied to the biodegradation of lignin. Because of its vast reaction capabilities as well as the broad substrate specificity, it also possesses great biotechnological potential [71]. Despite ABTS and HBT are commonly used mediators, their industrial-scale application still involves high costs and therefore, natural redox mediators may be a better option, as they exist in nature and are involved in the natural degradation of lignin by laccase producing fungi [78]. Natural mediators in comparison with artificial ones, can be eco-friendly and also cost effective [79]. Some fungal metabolites such as phenol, aniline, 4-hydroxy- benzoic acid and 4-hydroxybenzyl alcohol can act as laccase mediators [78].

Different mediators follow different catalytic mechanisms. ABTS as mediator of substrate oxidation proceeds via an electron transfer route. ABTS is first oxidized to its radical cation ($ABTS^{\cdot+}$) and then to the di-cation ($ABTS^{2+}$) with redox potentials of 472 and 885 mV, respectively. However, HBT and violuric acid (N-OH type mediators) form the N-oxy radical upon laccase oxidation and subsequent deprotonation. Phenolic mediators follow a radical hydrogen abstraction mechanism, but with a phenoxy radical as the intermediate [80,81].

Moreover, the effect of a mediator also depends on the laccase, the substrate, the recyclability of the mediator and the stability of the laccase in the presence of the mediator [64,75,81,82]. Mediators might cause toxicity or inactivation of the laccase [83,84]. An ideal redox mediator must be a good laccase substrate; its oxidized and reduced forms must be stable and yet must not inhibit the enzymatic reaction or be toxic to the environment in longer terms.

p-Hydroxycinnamic acids are one of the group of mediators, mostly found in lignin in vascular plants [85].They are found in plant food, mostly seeds and fruits, where they act as antioxidants. 4-hydroxycinnamic acid (*p*-Coumaric acid – PCA) and 3-methoxy-4-hydroxycinnamic (ferulic acid – FA) are abundant in soil, they incorporate into the humic substrates [86]. Laccase mediated by *p*-hydroxycinnamic acids leads to a variety of oxidations and coupling products depending on their aromatic

rings and reaction conditions [79,87].



ABTS *p*-Coumaric acid Ferulic acid

Figure 2.2 Mediators used in the present study (synthetic ABTS and natural *p*-coumaric and ferulic acids)

Many research efforts have been made to find a suitable process for the remediation of PAH contaminated soil (Table 2). However, most works using laccase were focused on a liquid system (without soil) and on a synthetic mediator such as ABTS or HBT [88–92]. Additionally, it has been reported that the low PAH degradation rates in the soil are due to the low activity of the laccase-mediator system in an environment lacking water [93].

Table 2.3 Laccase and/or LMS in degradation of PAH

Laccase source	Reactor	Conditions	Time	Mediator	PAH	Degradation yield	Degradation in soil	Reference
Pycnoporus cinnabarinus	4 mL vials	2 U/mL laccase, 100 mM sodium citrate buffer, pH 5 (containing 1 % Tween 20), 30 °C, in dark	24 h	/	benzo[a]pyrene (50 µM) pyrene (50 µM) anthracene (50 µM)	12% 8% 60%	no	Cañas et al., 2007
				1 mM p-coumaric acid	benzo[a]pyrene (50 µM) pyrene (50 µM) anthracene (50 µM)	92% 62% 95%		
Leucoagaricus gongylophorus strain FF-2006	5 mL vials	50 U laccase, 20 mM sodium citrate buffer, pH 6, with 0.25 % v/v Tween 20, 100 rpm	24 h	/	anthracene (10 ppm)	64.9%	no	Ike et al., 2019
				1mM ABTS	anthracene (10 ppm) fluorene (100 ppm)	71.8% 41.7%		
Trametes versicolor 951022	2.5 mL Eppendorf tube	5 U/mL enzyme solution, 0.1 M sodium acetate buffer, pH 5, 40 °C, in horizontal shaker	2 h	5mM ABTS	phenanthrene (20 mg/L)	40%	no	Han et al., 2004
				5mM HBT		30%		
Trametes versicolor (maleic anhydride modified laccase)	HiTrap DEAE FF	1.5ml of enzyme solution 0.1g/l dissolved in 20 mM di-sodium hydrogen phosphate-citric acid buffer pH 4.5, 30 °C	72 h	/	acenaphthylene anthracene	50% 36%	no	Liu and Hua, 2014
				1mM ABTS	acenaphthylene anthracene	83% 94%		
Trametes versicolor	HiTrap DEAE FF	1.5ml of enzyme solution 0.1g/l dissolved in 20 mM di-sodium hydrogen phosphate-citric acid buffer pH 4.5, 30 °C	72 h	/	acenaphthylene anthracene	35% 7%	no	Liu and Hua, 2014
				1 mM ABTS	acenaphthylene anthracene	70% 97%		
Pycnoporus sanguineus KUM60954	Erlenmeyer flask	30 U/mL laccase, 50 mM sodium citrate buffer, 40 °C, 80 rpm	24 h	/	10 ppm mixture: anthracene phenanthrene pyrene	 89.8% 36.8% 95.7%	no	Munusamy et al. 2008

Trametes versicolor	15 mL glass tubes	30 U laccase, 0.1 M Britton-Robinson buffer, pH 4, 40 °C	48 h	/	1 mg/L benzo[a]pyrene	60%	no	Li et al. 2010
				20 mM ABTS		98%		
Trametes versicolor	15 mL glass tubes	100 U laccase, 10 g aged, contaminated soil, water content adjusts to 70 % moisture and maintain with ultra-pure water daily	10 days	/	phenanthrene anthracene pyrene benzo[a]pyrene	15.6% 52.7% 2.8% 40.8%	yes	Li et al. 2010
				1 mmol/kg ABTS	phenanthrene anthracene pyrene benzo[a]pyrene	32.4% 74.6% 7.5% 56.7%		
Cocultivation of *Trichoderma viride* and *Penicillium chrysogenum*	PBR	2 U/mL laccase in a continuous flow of 0.015 mL/min into 100 g of spiked soil	25 days	/	300 ppm mixture: phenanthrene anthracene pyrene benzo[a]pyrene chrysene fluorene	 60% 49% 68% 50% 55% 55%	yes	This work, chapter 4.2
				1mM ABTS	300 ppm mixture: phenanthrene anthracene pyrene benzo[a]pyrene chrysene fluorene	 60% 72% 83.5% 96% 81.8% 75%		
				1mM p-coumaric acid	300 ppm mixture: phenanthrene anthracene pyrene benzo[a]pyrene chrysene flourene	 48% 72% 83.5% 96% 81.8% 75%		
				1mM ferulic acid	300 ppm mixture: phenanthrene anthracene	 72% 60.8%		

					pyrene benzo[a]pyrene chrysene fluorene	87% 91.3% 77.5% 68.5%		
Trametes versicolor	PBR	2 U/mL laccase in a continuous flow of 15 mL/min into 300 g of naturally aged, contaminated soil	35 days	/	benzo[a]pyrene chrysene	35.6 % 75.9 %	yes	This work, chapter 4.3
				1mM ABTS	benzo[a]pyrene chrysene	36.5 % 87.9 %		
				1mM p-coumaric acid	benzo[a]pyrene chrysene	37.9 % 76.2 %		
				1mM ferulic acid	benzo[a]pyrene chrysene	38.8 % 81.0 %		
Commercial Trametes versicolor	100 mL amber flask	20 mL of 10 U/mL of laccase mixed with 20 g of naturally polluted soil	12 days		Total USEPA PAH		yes	This work, chapter 4.4
				Laccase 1 mM ABTS 1 mM coumaric acid 1 mM ferulic acid		43.5 % 57.8 % 47.2 % 54.1 %		
Trametes versicolor				Laccase 1 mM ABTS 1 mM coumaric acid 1 mM ferulic acid		55.2 % 65.8 % 46.1 % 58.8 %		
A. aegerita				Laccase 1 mM ABTS 1 mM coumaric acid 1 mM ferulic acid		62.2 % 56.2 % 51.1 % 54.0 %		
Aspergillus sp. Novozym 51003				Laccase 1 mM ABTS 1 mM coumaric acid 1 mM ferulic acid		47.2 % 52.6 % 39.9 % 54.6 %		

CHAPTER III

Material and methods

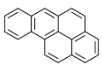
3.1 Experimental soil

Two types of soil were used in this work: (1) sterile and artificially contaminated spiked soil (chapter 4.2.1 and 4.2.2) and (2) non-sterile naturally contaminated soil (chapter 4.2.3 and 4.2.4), whose assays give an extra challenge as contaminants are less bio-accessible and often there are other inorganic and organic contaminants. Moreover, in non-sterile soil there is also some competition between microorganisms.

Naturaduba Agro soil (Gintegral – Gestão Ambiental, S.A.) was used in chapter 4.2.1 and 4.2.~~1~~2, presenting 49.1 % of organic material, 0.99 % N, 0.20 % P_2O_5, 0.49 % K_2O, 2.17 % Ca, 0.26 % Mg, 0.17 % S, 32.2 % humidity. The soil was first heated for two days at 60 °C. The initial soil pH was 7.2 and adjusted to 5.0 with HCl, as specified in the experiments. Six USEPA priority PAH were used (Table 3.1). They exist as crystalline solids at room temperature. An amount of 1000 mg of each PAH was added to 1000 mL of acetonitrile and used to contaminate soil, giving a desired final concentration of 300 ppm (50 ppm each PAH). After acetonitrile evaporation, the soil was left at 4 °C for 60 days.
Soil used in chapter 4.3 was collected in Lagos (southwest Nigeria) 6°32'46.1"N 3°16'09.0"E, in a condensed neighborhood. It was collected in late November (dry season), with an average air temperature of 32 °C. Samples were collected from the soil surface (0–30 cm depth) and kept into polyethylene bags at 4 °C until use.

Naturally contaminated soil, used in chapter 4.4 was also collected in Lagos, nearby a dumping site, 06°34'27.0 N 003°15'101E. It was collected in early December (dry season), with average air temperature of 30 °C. Samples were collected from 15 - 30 cm depth and kept into polyethylene bags at 4 °C until use.

Table 3.1 Structure, molecular weight and ionization potential (IP) value of selected polycyclic aromatic hydrocarbons used in artificially contaminated soil.

Compound	Structure	Molecular weight (g/mol)	IP value (eV)*	Purity	Supplier/CAS
Fluorene		166.219	7.89	98 %	Acros Organics, CAS 86-73-7
Phenanthrene		178.229	7.91	97 %	Acros Organics, CAS 85-01-8
Anthracene		178.229	7.43	99 %	Acros Organics, CAS 120-12-7
Pyrene		202.251	7.43	98 %	Acros Organics, CAS 129-00-0
Chrysene		228.288	7.59	95 %	Sigma-Aldrich, CAS 218-01-9
Benzo[a]pyrene		252.309	7.12	96 %	Sigma-Aldrich, CAS 50-32-8

*[94]

3.2 Fungi and cultivation

3.2.1 Fungal strains

Fungal strains used in this work were: *Trichoderma viride* (EXF8977), *Penicillium chrysogenum* (EXF1818), *Agrocybe aegerita* (EXF3253), *Trametes versicolor* (isolated from contaminated soil) and *Irpex lacteus* (MUM 04.98). EXF strains were obtained from the Infrastructural Centre Mycosmo, MRIC UL, Slovenia and MUM strain came from the Fungal Culture Collection - Micoteca da Universidade do Minho, Portugal. Stock cultures were maintained on malt extract agar plates, first at 4 °C and then at -20 °C.

For conservation at -20 °C, fungi were grown in 6 cm diameter Petri dishes containing 10 mL of MEA and incubated at 27 °C for 7 days. 1.5 mL of 20 % glycerol [v/v] were dropped on the colony and the spores were suspended by reflux with a plastic Pasteur pipette. This suspension was transferred to a 2.0 mL cryovial and left for 1 hour at room temperature and then at 4 °C overnight to allow glycerol to

diffuse into the cells. The cryovials were then stored at -20 °C.

The liquid inoculum was prepared by cutting four agar plugs (5 mm x 5mm) from malt extract agar plates. Those were extruded through a syringe into 500 mL Erlenmeyer flasks containing 200 mL of sterile malt extract (2 % w/v). Fungi were cultivated at room temperature, with continuous agitation at 120 rpm for 3 days for *T. viride*, *T. versicolor* and A. *aegerita* for 5 days, and 6 days for *P. chrysogenum* and *I. lacteus,* respectively.

3.2.2 Fungal collection and isolation

The fungi were collected from soil samples (6°32'46.1"N 3°16'09.0"E - Lagos, Nigeria). Fungal strains were isolated by the serial dilution technique, prepared by mixing in vortex 1g of soil with 10 mL of distilled water and further diluted to 10^{6}. A volume of 0.1 mL was pipetted onto plates with Rose Bengal agar (Sigma) and Sabouraud agar (40 g/L dextrose, 10 g/L peptone, 15 g/L agar, 1000 mL dH_2O, pH 5.6 ±0.2), incubated at 28 °C. The pure culture obtained was transferred to MEA and kept at 4 °C for further use.

3.2.2.1 Laccase screening of isolated fungi and identification

For a preliminary screening of laccase, 20 dried ABTS-impregnated discs were placed into an empty standard flat-bottom 96-well microplate [95]. The screening of laccase activity was started by the addition of 10 µl aliquots of each sample (in this work, 48 h of old fungi biomass from malt broth) to the discs and left for 10 min at 30 °C. Samples with laccase activity developed a green bluish color and were further tested on laccase plate assay, where a diameter of 1 cm of mycelium from each isolated strain was inoculated into MEA, containing 10 mL of 20 mM ABTS and 1 mL of 100 mM $CuSO_4$ per 1 L medium. The formation of a halo in the plates supplemented with ABTS indicated a positive laccase secretion. The diameters of the halo zones and of the mycelium were measured at regular intervals of time for 5 days, after the organisms were selected for benzo[a]pyrene and chrysene degradation.

The identification of the selected fungus was performed molecularly by DNA amplification and sequencing. Fungus was grown on potato dextrose agar for 5 days at 28 °C and genomic DNA extraction was performed as described Rodrigues et al. [96]. PCR amplification was achieved with ITS1/ITS4 primers and PCR reactions were carried out in a thermal cycler BioRad Mycycler, in a final volume of 50

μL containing 10 μL of 5x Go-Flexi Taq $MgCl_2$-free reaction buffer (Promega), 1.5 mM $MgCl_2$, 1.25 U of Go-Flexi Taq polymerase (Promega), 200 μM of each primer, 1 μL dNTP (Bioron) and 2 μL of genomic DNA. Amplifications were carried out in a Bio-Rad MYCYCLER thermal cycler using a temperature gradient protocol as follows: initial denaturation of DNA for 4 min at 95 °C, 34 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 50 s with extension at 72 °C for 1 min and a final extension cycle at 72 °C for 7 min. PCR products were purified with the commercial kit GF-1 PCR CleanUp Kit (Vivantis), according to manufacturer's instructions. The sequencing was conducted by Microsynth (Switzerland) and manually adjusted by chromatogram comparison and then aligned with the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm.

3.3 SSF enzymes production

3.3.1 Substrate support preparation

Kiwi peels and plantains peels (obtained on the market in Lagos) were cut to 1 cm^2 and pretreated with 83 mM of KOH at room temperature (≈ 26°C) for 20 min, in a ratio of 1:3, to neutralise organic acids [97]. Peanut shells, pine bark and cork were minced to size of 6 mm. After that, they were washed twice with deionised water, dried at 60 °C and autoclaved at 121°C for 15 min prior to use.

3.3.2 SSF in Erlenmeyer flasks

The 500 mL Erlenmeyer flasks were filled with 200 g of substrate, autoclaved at 121 °C for 15 min and inoculated with 5 mL of fungal biomass per flask and left at room temperature for 3 weeks. After that, the mixture was suspended in 400 mL of 50 mM sodium acetate buffer (pH 4.5) and mixed continuously (100 rpm) for 1 hour at room temperature. This suspension was filtered through a nylon cloth and centrifuged at 7500 *g* for 15 min. The recovered supernatant was used for enzymatic assays. SSF was performed in duplicate.

3.3.3 SSF in trays

Aluminum trays (17×10×7 cm) were filled with 200 g of the substrate, covered with aluminum foil with small holes for aeration, autoclaved at 121 °C for 15 min, inoculated with 5 mL of fungal biomass

and left at room temperature for 3 weeks. Samples were taken once per week. After that, the mixture was suspended in 400 mL of 50 mM sodium acetate buffer (pH 5) and mixed continuously (100 rpm) for 1 hour at room temperature. This suspension was filtered through a nylon cloth and the filtrate was centrifuged at 7500 *g* for 15 min and used for enzymatic assays. SSF was performed in duplicate.

3.3.4 SSF in a packed-bed bioreactor

The horizontal packed-bed bioreactor (PBB) (Figure 3.1) consisted of a jacketed glass column (34 cm length and 3 cm internal diameter) connected to a filtered-air supply. The air was passed through a 0.45 μm cellulose filter and bubbled into distilled water before entering the column. The airflow was measured and controlled by a flowmeter (Aalborg Instruments & Controls, Inc., USA). The bioreactor and 50 g of kiwi peels as substrate were previously sterilized separately. The substrate was inoculated with 2 mL of fungal inoculum suspension and mixed. After that, the column was filled with the inoculated substrate and maintained at room temperature for 3 weeks. SSF were performed in duplicate, at different aeration rates of 0.05 L min^{-1}, 0.1 L min^{-1} and 0.2 L min^{-1}. After SSF, the fermented substrate was transferred to 500 mL Erlenmeyer flasks to proceed with the enzyme extraction, as described previously.

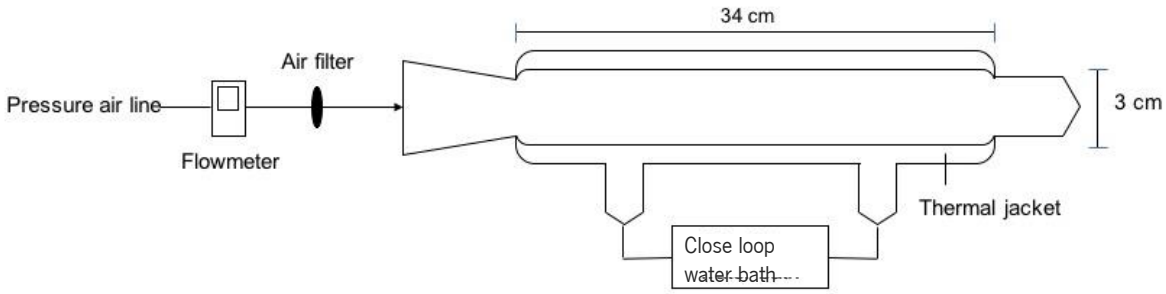


Figure 3.1 Horizontal packed-bed bioreactor design for laccase production.

3.4 Soil rehabilitation

3.4.1 Fungi rehabilitation of artificially contaminated soil

The degradation assays were performed in 500 mL flasks with 180 g of previously spiked soil, pH adjustment, 20 g of substrate and a total of 10 mL of fungal inoculum. The degradation time was set to

8 weeks with samples collection once a week. Assays were done in duplicate, at room temperature and in the dark.

3.4.2 Laccase and LMS rehabilitation of artificially contaminated soil

PAH degradation was evaluated using laccase enzyme and LMS in amber laboratory bottle, named laccase-batch degradation, and in packed bed reactor (PBR), named laccase-fed degradation.

Laccase batch degradation was set up into 100 mL amber laboratory bottle with 20 g of soil at pH 5.0, containing 2 mL of the PAH mixture appropriately diluted to obtain the concentrations of 100, 300 and 600 ppm of a total mixture of the compound. A laccase load of 2 U/mL (4 mL) was used for the PAH degradation. The addition of 1 mM (2 mL) of ABTS, coumaric acid and ferulic acid mediators was also evaluated. The degradation was performed in duplicate at 30 °C, in the dark, for 5 days. Samples were taken on days 0, 3 and 5 for evaluation of PAH concentration by UHPLC. The boiled enzyme was used as a control.

Laccase-fed degradation was set up in PBR (Figure 3.2), consisting of a vertical glass column (25 cm length and 3 cm Ø) filled with contaminated soil (100 g soil spiked with 50 mL of the PAH mixture at 300 ppm of each compound). A load of 2 U/mL of laccase and 1 mM of the mediator was added, in a continuous flow of 0.015 mL/min, using a laboratory peristaltic pump (Masterflex, Cole-Parmer). The laccase flow was refreshed each two days. In order to avoid heterogeneous degradation, laccase solution was equally distributed over the whole contaminated soil through an inner tube with small holes in the middle of the column. This procedure was done in duplicate and performed for 25 days at room temperature. Soil samples were taken each 5 days for PAH concentration measurement by UHPLC and the assessment of removal efficiency.

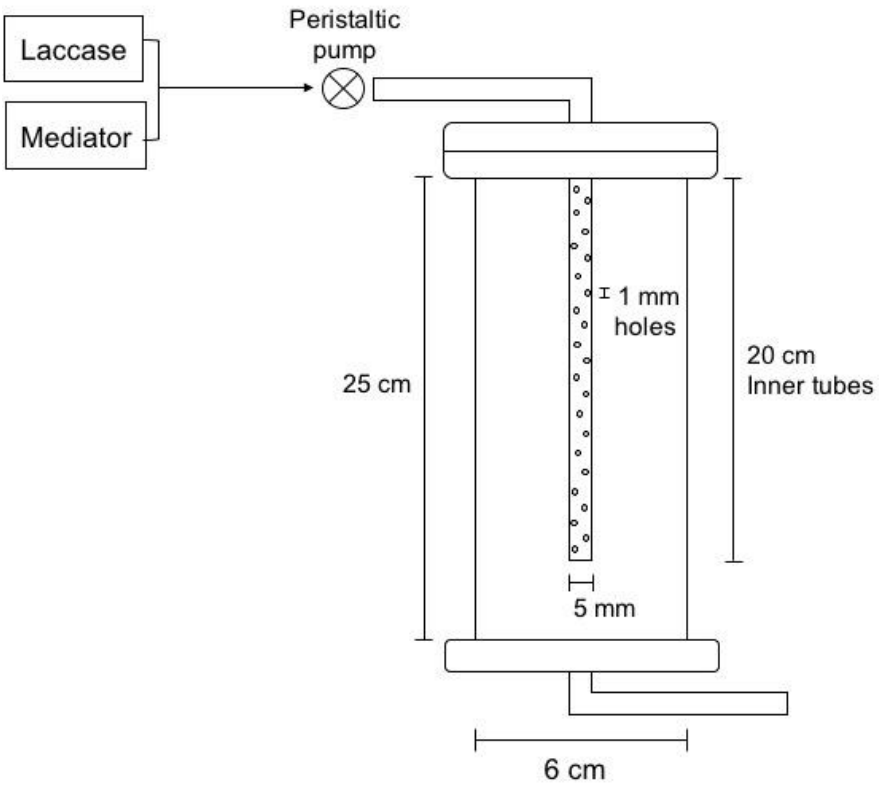


Figure 3.2 Packed-bed reactor design for PAH degradation, fed by an inner hollow tube.

3.4.3 PAH degradation and metals removal by fungi and their laccase in contaminated soil

PAH degradation and metals removal were evaluated in soil using two strategies: degradation by the isolated fungus, namely *Trametes versicolor,* in amber laboratory bottle, and enzymatic degradation by laccase produced from *T. versicolor* in packed bed reactor. Laccase mediator system (LMS) was evaluated in the second strategy.

3.4.3.1 Soil rehabilitation by Trametes versicolor

Fungal batch assay was set up in 100 mL amber laboratory bottles with 50 g of naturally contaminated soil, 10 g of plantain peels and 1 ml of fungal inoculum. Experiment was set up for 8 weeks at room temperature, with samples collection once a week and, at the same time, each bottle was sprayed with air, at a flow rate of 0.02 ml/min, for 1 minute. Experiments were performed in the dark and in triplicate.

3.4.3.2 Soil rehabilitation by laccase

Laccase batch assay was set up into 100 mL amber laboratory bottles with 20 g of contaminated soil, 10 mL of laccase load at 2 U/g and 6 U/g, and mediators (ABTS, coumaric acid, ferulic acid) at concentrations of 0.5, 1 and 2 mM. Experiments were set up for 7 days in triplicate, at 30 °C and in the dark. Boiled enzyme was used as control.

Laccase-fed degradation was set up in a packed-bed bioreactor (PBR) consisting of a vertical glass column (25 cm length, 3 cm Ø, and 3.5 cm width) filled with 300 g of contaminated soil (Figure 3.2). A load of 2 U/g of laccase and 1 mM of mediator was used in a continuous flow of 0.015 mL/min, using a laboratory peristaltic pump (Masterflex, Cole-Parmer). The laccase flow was refreshed every five days. This procedure was done in duplicate and performed during 35 days at room temperature (28 °C), in the dark. Soil samples were taken every 5 days for assessment of the removal efficiency of the pollutants.

3.4.4 PAH degradation in contaminated soil by four different laccases

Four different laccases were used in soil rehabilitation assays: commercial laccase from *Trametes versicolor* and from *Aspergillus sp*. Novozym 51003 (purchased at Sigma), and laccases produced thought SSF by *Trametes versicolor* and *Agrocybe aegerita.*

Laccase degradation assay was set up into 100 mL amber laboratory bottles with 20 g of aged, contaminated soil and 20 mL of 10 U/g laccase with 1mM of mediators (ABTS, coumaric acid, ferulic acid). Experiment was set up for 12 days in triplicate (until laccase was no longer detected), at 30 °C in the dark. Boiled enzyme was used as control. Samples for measuring PAH degradation were taken on days 0, 1, 3, 6, 9 and 12.

3.5 Analyses methods

3.5.1 Soil characterization

The soil samples were dried for 2 days at 60 °C and sieved in a 2.0 mm mesh. The pH of the samples was measured in a soil / H_2O ratio of 1:2. The moisture content of the sample was assessed in a hot air incubator at 105 °C and organic matter was determined as the percentage loss on ignition of 2.0 g of soil in an oven at 450 °C for 4 h.

Metals were extracted by digesting 1 g of each sample with 10 mL of aqua regia (concentrated

hydrogen chloride and nitric acid, ratio 3:1) (US EPA method 3050b) [98]. The digestion tubes were covered, heated in a water bath for 2 h up to 100 °C with intermittent shaking, then cooled to room temperature and filtered with 110 mm pore size filter paper, followed by dilution. Metals extraction used in assay was done by filtration, in briefly, 2.5 g of soil was mixed with 2.5 ml HNO_3 and dH_2O, filtered after 24 h and diluted [99]. PAH extraction was done by adding 1.5 mL of acetonitrile to 0.5 g of soil and extraction was carried out using a rotating shaker at 160 rpm for 30 minutes and a 40kHz Sonicator for another 10 minutes. The samples were centrifuged at 8000 *g* and the supernatant was transferred to 2 mL vials.

Extraction efficiencies of the selected method for the six PAH under study, in Naturaduba Agro soil, were 78.12 % for phenanthrene, 72.46 % for anthracene, 83.21 % for fluorene, 91.42 % for pyrene, 69.98 % for benzo[a]pyrene and 86.21 % for chrysene. Selected PAH were always used in mixture.

3.5.2 Enzyme extraction and characterization

3.5.2.1 Soil enzyme extraction

Each week, 18 g of soil were taken from each of 500 mL Pyrex™ borosilicate glass reagent bottles of which 3 g were placed into 15 mL centrifuge tubes with 8 mL of buffer, according to the extracted enzyme. Laccase extraction buffer contained 100 mM sodium acetate, at pH 5 and pH 7, 5 mM $CaCl_2$, 0.05 % tween 80 and 1 % polyvinylpolypyrrolidone. For manganese peroxidase (MnP) extraction, 50 mM phosphate buffer adjusted to pH 5 and to pH 7 was used and for lignin peroxidase (LiP) extraction 50 mM sodium acetate buffer, pH 5 and pH 7 with 1 % polyvinylpolypyrrolidone was used. Samples were placed in a rotary shaker (100 rpm at 25 °C) for an hour and centrifuged at 7500 *g* for 15 min. Supernatant was transfer to fresh tubes and analysed for the activity of each enzyme [100].

3.5.2.2 Enzyme characterization

Laccase activity was measured by the oxidation of 2,2-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS) in 100 mM sodium acetate buffer, pH 5 or 7, according to the extraction buffer, at 30 °C [74]. MnP reaction mixture contained 100 μL of succinate buffer (adjusted at pH 5 or 7) with 0.1 μM of 3-methyl-2-benzthaiazolinone hydrazone hydrochloride (MBTH), 5 μL of 3-dimethylaminobenzoic acid (DMAB), 0.2 μM of $MnSO_4$ and 0.1 μM H_2O_2. LiP assay was performed with similar procedure as for MnP assay except that H_2O_2 was omitted [100,101]. Enzyme activities were measured using a spectrophotometer (BioTek

Synergy HT) at 420 nm (laccase), 590 nm (MnP) and 401 nm (LiP). One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of product per min under the assay conditions.

3.5.3 UHPLC and ICP

The quantification of PAH was performed by ultra-high-performance liquid chromatography, using a Shimadzu Nexera X2 (Shimadzu, USA) with one multi-channel pump (LC-30AD), an autosampler (SIL30AC), an oven (CTO-20AC), a diode array detector (M-20A) and a system controller (CBM-20A) with built-in software (LabSolutions). For the PAH quantification, a Kinetex PAH C18 column (Phenomenex, Inc. CA, USA) was used. The mobile phase was ultrapure water (pump A) and acetonitrile (pump B).
For chapter 4.1.1 -4.1.3 the following protocol was used: starting mobile phase composition was 51 % A, decreased to 4.5 % A in 12.03 min, remaining in this percentage until 16.3 min, increased again to 51 % (17.25 min) and remaining in this percentage for 2.35 min. The flow rate was 0.6 mL/min, samples were monitored by a diode array detector from 190 to 400 nm and chromatograms were extracted at 252 nm. Column oven was set at 25 ° C and the injection volume was 15 μL. Standards were prepared by mixing 6 selected PAH (Figure3.3).

The quantification of the EPA 16 PAH in chapter 4.1.4 followed the protocol: starting mobile phase composition was 30 % A, decreased to 0 % A in 10 min, started increasing to 30% A until 12.5 min and remained in this percentage until 15 min. The flow rate was 1.5 mL/min, samples were monitored by a diode array detector from 190 to 400 nm and chromatograms were extracted at 252 nm. Column oven was set at 30 ° C, and the injection volume was 10 μL. PAH Calibration Mix in acetonitrile (Sigma) was used as standard (Figure 3.4).

Metals concentrations were measured by an ICP-OES (Optima 8000, PerkinElmer), with detection of iron at 238.204 nm, aluminum at 396.153 nm, lead at 220.353 nm and magnesium at 285.213 nm. The operating conditions were as stated: 1300 W RF power, 8 L/min argon plasma flow, 0.2 L/min auxiliary gas flow and 0.5 L/min nebulizer gas flow. ICP multi-element standard solution IV (Sigma) was used as standard for calibration curve.

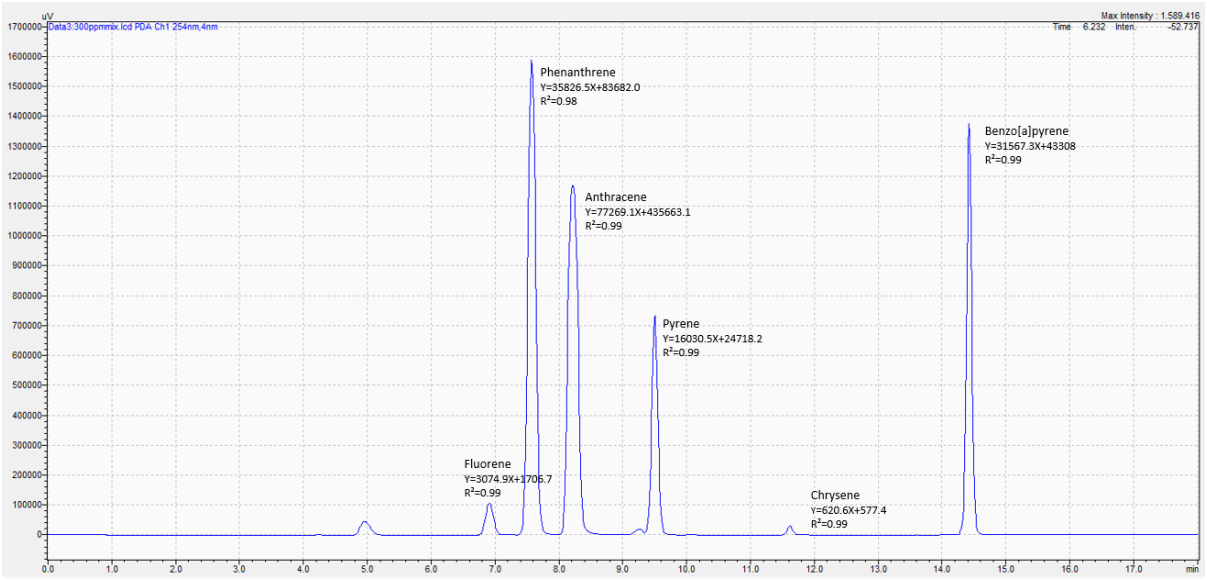


Figure 3.3 Standard chromatogram for UHPLC analysis of six PAH in total 300 ppm mix (50 ppm each).

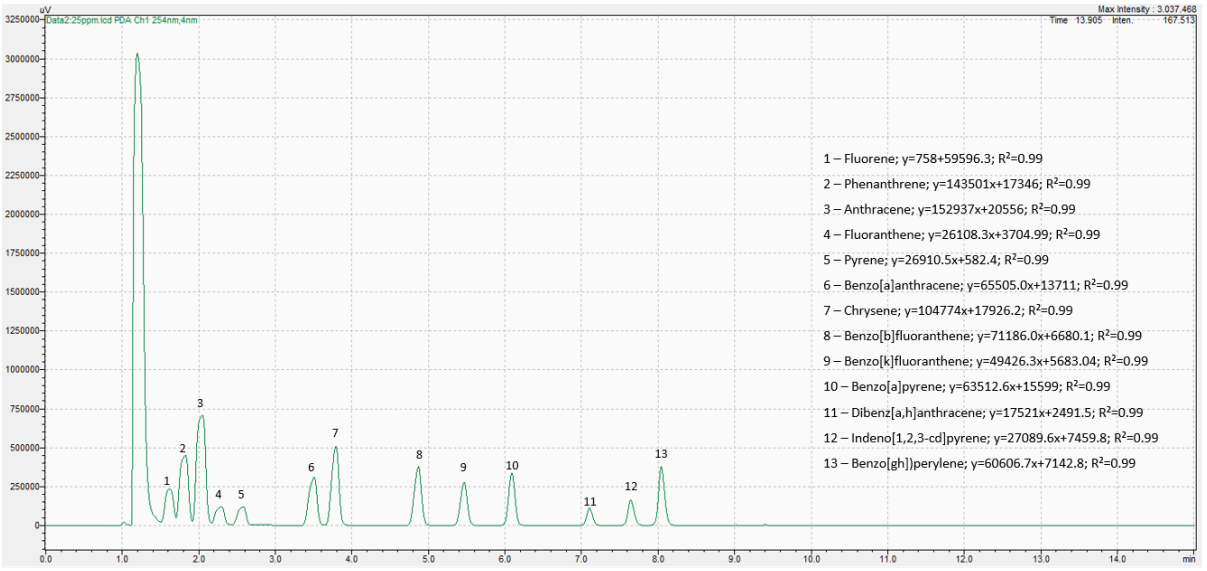


Figure 3.4 Standard chromatogram for UHPLC analysis of USEPA 16 PAH mixture (25 ppm each).

CHAPTER IV

Results and discussion

4.1 Ligninolytic enzymes production during polycyclic aromatic hydrocarbons degradation: effect of soil pH, soil amendments and fungal co-cultivation

4.1.1 Evaluation of SSF enzyme production

SSF mimics the conditions under which fungi grow naturally [44], as lignocellulosic residues promote a good fungal growth due to the polysaccharides and lignin present in these materials [72]. A screening of fungi that produce lignin-modifying enzymes was performed, aiming to evaluate the production of laccase, lignin peroxidase and manganese peroxidase (Tables 4.1–4.3). For that, three fungal strains were evaluated through SSF, using kiwi peels and peanut shells as substrates at pH 5 and 7. *Penicillium chrysogenum* and *Trichoderma viride* were selected for this study as they are normally present in soil with different organic pollutants, despite not reaching such high production of enzymes as *A. aegerita*, which belong to the litter decomposing fungi. The highest enzymatic production was obtained for *A. aegerita* grown in kiwi peels, with laccase levels of 7.43 U/g at pH 7, with 2 weeks fermentation (Table 4.1). For MnP, 7.21 U/g was achieved at pH 7 with 4 weeks fermentation (Table 4.2), while for LiP 2.24 U/g was obtained at pH 5, with 5 weeks fermentation (Table 4.3), all of them for the fungal strain grown on kiwi peels. When peanut shells was used as substrate, the maximum production was reached at the same pH and fermentation time, being 4.80 U/g of laccase and 6.88 U/g of MnP, while for LiP 2.08 U/g was verified at pH 7, after 5 weeks fermentation.

Penicillium chrysogenum and Trichoderma viride also achieved better laccase production at pH 7, with 2.18 U/g at 2 weeks fermentation on peanut shells for *T. viride,* and 1.89 U/g at 5 weeks fermentation on kiwi peels for *P. chrysogenum*. MnP activity was not detected on *P. chrysogenum* extracts, but *T. viride* was able to produce 3.58 U/g at pH 5, after 4 weeks fermentation on peanut shells. *T. viride* was not able to produce LiP, while traces of its activity were detected in the extracts of *P. chrysogenum*. Therefore, both organic residues were effective for the enzymes production, which could be related to their chemical composition.

Table 4.1 Laccase production by SSF on kiwi peels and peanut shells by *T. viride, P. chrysogenum, A. aegerita* and their co-cultures in U/g

Fungi + substrate	SSF at pH 5 (weeks)						SSF at pH 7 (weeks)					
	1	2	3	4	5	6	1	2	3	4	5	6
T. viride (KP)	0.89±0.01	0.71±0.02	1.45±0.01	**1.78±0.06**	0.93±0.01	0.18±0.07	0.27±0.02	0.22±0.05	0.77±0.05	0.52±0.02	**1.24±0.05**	0.74±0.02
T. viride (PS)	0.20±0.06	1.14±0.09	1.52±0.05	**1.78±0.06**	0.81±0.03	0.17±0.02	0.56±0.01	**2.18±0.07**	2.15±0.02	1.93±0.05	1.04±0.03	0.60±0.06
P. chrysogenum (KP)	0.42±0.08	0.71±0.02	**1.39±0.02**	0.64±0.01	0.53±0.01	0.18±0.07	n.d.	n.d.	0.48±0.03	0.58±0.05	**1.89±0.09**	0.89±0.03
P. chrysogenum (PS)	0.20±0.02	0.47±0.02	**0.82±0.01**	0.21±0.09	0.25±0.02	0.07±0.07	n.d.	n.d.	0.98±0.04	1.16±0.02	**1.17±0.01**	1.19±0.05
A. aegerita (KP)	0.51±0.09	1.38±0.04	**6.69±0.04**	6.20±0.05	5.99±0.01	2.39±0.07	2.15±0.02	**7.43±0.06**	6.27±0.06	5.80±0.05	4.66±0.07	4.36±0.07
A. aegerita (PS)	0.28±0.01	1.26±0.01	**4.71±0.09**	3.75±0.03	2.22±0.02	1.80±0.06	2.33±0.07	**4.80±0.05**	3.30±0.01	3.66±0.07	3.99±0.04	2.92±0.01
AA + TV (KP)	0.21±0.04	1.86±0.03	3.38±0.02	3.61±0.07	**3.92±0.07**	2.39±0.07	1.45±0.05	**4.49±0.02**	3.11±0.04	3.75±0.01	2.99±0.01	2.42±0.03
AA + TV (PS)	0.18±0.01	1.72±0.05	**3.97±0.05**	3.24±0.07	3.75±0.03	2.22±0.02	0.37±0.05	1.21±0.05	2.62±0.03	**3.65±0.02**	3.56±0.04	2.68±0.03
TV + PC (KP)	0.89±0.05	**1.48±0.01**	1.39±0.02	0.97±0.02	0.57±0.09	0.54±0.01	0.53±0.02	0.54±0.01	1.01±0.09	**1.06±0.02**	0.98±0.01	0.97±0.05
TV + PC (PS)	1.03±0.07	0.33±0.07	**2.26±0.03**	1.20±0.03	0.59±0.03	0.52±0.01	0.75±0.03	1.15±0.08	**2.06±0.03**	1.44±0.07	0.96±0.06	0.96±0.02
AA + PC (KP)	1.45±0.02	2.55±0.06	**2.68±0.01**	2.67±0.08	2.63±0.02	2.48±0.08	1.81±0.04	3.56±0.04	**3.68±0.03**	4.20±0.08	3.83±0.02	3.48±0.08
AC + PC (PS)	1.46±0.07	2.77±0.09	**2.93±0.03**	2.65±0.08	2.46±0.01	2.28±0.09	1.80±0.05	3.49±0.05	3.59±0.01	**3.87±0.06**	3.86±0.01	3.16±0.09

TV: T. viride; PC: P. chrysogenum; AA: A. aegerita; KP: Kiwi peels; PS: Peanut shells; n.d.: not detected.

Table 4.2 Manganese peroxidase production by SSF on kiwi peels and peanut shells by *T. viride, P. chrysogenum, A. aegerita* and their co-cultures in U/g

Fungi + substrate	SSF at pH 5 (weeks)						SSF at pH 7 (weeks)					
	1	2	3	4	5	6	1	2	3	4	5	6
T. viride (KP)	0.07±0.07	0.14±.03	0.60±0.04	**3.40±0.03**	2.96±0.02	2.80±0.03	0.21±0.17	0.41±0.06	0.66±0.08	**0.96±0.01**	0.80±0.03	0.61±0.02
T. viride (PS)	0.07±0.07	0.27±0.05	0.66±0.09	**3.58±0.03**	3.26±0.04	2.92±0.08	0.29±0.19	0.45±0.07	0.90±0.02	**1.02±0.09**	0.98±0.07	0.70±0.06
P. chrysogenum (KP)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P. chrysogenum (PS)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A. aegerita (KP)	0.66±0.11	1.71±0.04	3.46±0.04	**3.58±0.03**	3.00±0.04	2.73±0.02	0.77±0.11	3.02±.02	5.78±0.04	**7.21±0.02**	6.71±0.01	6.12±0.08
A. aegerita (PS)	0.62±0.09	1.70±0.05	3.39±0.01	**3.49±0.05**	2.97±0.06	2.93±0.01	0.66±0.03	2.83±0.03	5.61±0.07	**6.88±0.08**	5.45±0.01	5.03±0.06
AA + TV (KP)	0.63±0.01	1.92±0.08	3.51±0.01	**3.58±0.03**	3.26±0.04	2.92±0.08	0.63±0.14	1.92±0.08	3.51±0.11	**3.58±0.03**	3.26±0.04	2.92±0.08
AA + TV (PS)	0.62±0.00	1.90±0.11	**3.46±0.04**	3.39±0.04	3.08±0.04	2.90±0.01	0.62±0.02	1.90±0.11	**3.46±0.04**	3.39±0.06	3.08±0.04	2.90±0.01
TV + PC (KP)	0.28±0.04	0.33±0.02	0.65±0.08	**1.29±0.01**	0.74±0.03	0.60±0.07	0.25±0.07	0.39±0.02	0.81±0.01	**1.04±0.04**	0.90±0.01	0.68±0.06
TV + PC (PS)	0.28±0.07	0.55±0.06	0.72±0.06	1.10±0.07	**1.11±0.03**	1.02±0.04	0.65±0.03	0.38±0.01	0.72±0.08	**1.00±0.04**	0.96±0.07	0.85±0.06
AA + PC (KP)	0.44±0.03	0.36±0.08	0.72±0.06	**1.29±0.04**	1.19±0.09	0.89±0.02	0.71±0.04	1.72±0.02	2.17±0.03	**2.22±0.04**	2.17±0.01	1.71±0.02
AC + PC (PS)	0.31±0.11	0.55±0.04	1.19±0.09	**1.59±0.04**	1.35±0.02	0.76±0.04	0.63±0.04	1.33±0.01	2.06±0.01	**2.08±0.01**	1.85±0.01	1.53±0.02

TV: T. viride; PC: P. chrysogenum; AA: A. aegerita; KP: Kiwi peels; PS: Peanut shells; n.d.: not detected

Table 4.3 Lignin peroxidase production by SSF on kiwi peels and peanut shells by *T. viride, P. chrysogenum, A. aegerita* and their co-cultures in U/g

Fungi + substrate	SSF at pH 5 (weeks)						SSF at pH 7 (weeks)					
	1	2	3	4	5	6	1	2	3	4	5	6
T. viride (KP)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
T. viride (PS)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P. chrysogenum (KP)	0.07±0.07	0.15±0.06	0.49±0.03	**0.66±0.09**	0.57±0.07	0.49±0.04	0.06±0.06	0.28±0.04	0.48±0.02	0.54±0.07	**0.64±0.04**	0.54±0.08
P. chrysogenum (PS)	0.06±0.06	0.13±0.03	0.59±0.04	**0.62±0.05**	0.51±0.03	0.49±0.03	0.06±0.06	0.42±0.03	0.55±0.06	**0.72±0.06**	0.65±0.03	0.65±0.03
A. aegerita (KP)	0.69±0.02	1.38±0.02	1.61±0.07	2.16±0.09	**2.24±0.09**	2.13±0.08	0.06±0.06	0.36±0.04	0.54±0.07	**0.69±0.02**	0.51±0.04	0.51±0.04
A. aegerita (PS)	0.45±0.09	0.72±0.06	0.99±0.05	1.24±0.07	**1.75±0.04**	1.52±0.02	0.44±0.02	0.69±0.02	0.72±0.06	2.01±0.04	**2.08±0.04**	1.69±0.09
AA + TV (KP)	0.70±0.06	1.10±0.08	**1.17±0.06**	0.98±0.07	0.77±0.05	0.62±0.08	0.69±0.02	0.99±0.05	1.09±0.07	**1.79±0.05**	1.39±0.01	2.64±0.01
AA + TV (PS)	0.63±0.04	1.02±0.09	**1.07±0.05**	0.88±0.01	0.65±0.03	0.52±0.06	0.69±0.02	0.72±0.06	0.95±0.01	**1.70±0.05**	1.38±0.02	0.94±0.05
TV + PC (KP)	0.63±0.04	0.98±0.03	**1.16±0.02**	1.13±0.02	1.10±0.07	1.80±0.07	0.54±0.07	0.62±0.08	0.89±0.02	**1.16±0.02**	1.04±0.01	0.83±0.06
TV + PC (PS)	0.62±0.04	0.98±0.07	**1.11±0.01**	1.02±0.02	1.12±0.02	1.73±0.07	0.28±0.04	0.48±0.02	0.64±0.04	**0.90±0.03**	0.60±0.07	0.60±0.07
AA + PC (KP)	0.63±0.04	0.98±0.03	**2.10±0.08**	1.98±0.07	1.78±0.07	1.65±0.05	0.42±0.03	0.55±0.06	0.72±0.06	**1.43±0.09**	0.89±0.02	0.62±0.08
AC + PC (PS)	0.62±0.01	0.96±0.05	**2.11±0.01**	1.99±0.02	1.83±0.06	1.79±0.09	0.36±0.04	0.54±0.07	0.69±0.02	**1.09±0.02**	0.76±0.04	0.52±0.06

TV: T. viride; PC: P. chrysogenum; AA: A. aegerita; KP: Kiwi peels; PS: Peanut shells; n.d.: not detected

Kiwi peels contain 53.73 % of carbohydrates, 3.84 % of protein, 6.93 % of soluble and 18.92 % of insoluble dietary fibres [102], 25.26 % of lignin [103], free sugars and fatty acids [104], besides higher mineral content than their respective edible parts [102]. Peanut shells are rich in many functional compounds as cellulose, hemicellulose and lignin [105], in which lignin can represent up to 41.3 % of the lignocellulose, being this value 2 to 4 times higher than for most common agricultural residues, such as rice straw, wheat straw, sugarcane bagasse and corn cob/stover [106].

Co-cultivation of the studied fungi was also investigated in order to improve the production of ligninolytic enzymes. Usually, in lab conditions there are no interactions as competition, symbiotic or antagonistic effects with just one species cultivation, while co-cultivation of microorganisms might be a better approach to induce diversity in enzymatic production, as co-cultivations frequently allow exposure to other chemical signals, which could be overlooked in mono species cultivation. Moreover, genomic sequencing analyses even show that some secondary metabolism is not triggered or/and expressed when species are exposed to laboratory conditions [107]. Thus, co-cultivation may promote the activation of genes that are not detected in single cultivation, which enables a better understanding of cultures in natural environment [108]. In general, no synergetic effect was observed when the microorganisms were co-cultivated, except for LiP activity, for which the enzyme levels detected in the co-culture of *P. chrysogenum* and *T. viride* were higher than those measured for each species grown separately. However, and regardless of that, the levels of enzyme activity were always higher for *A. aegerita*.

Many studies have reported the study of several fungi and lignocellulosic residues for the production of ligninolytic enzymes, which resulted in different levels of enzyme production. For example, Balaji et al. [109] isolated 21 potential PAH-degrading fungi from petroleum hydrocarbons contaminated soil samples, with *Trichoderma viride* and *Penicillium chrysogenum* were among them. *T. viride* was able to produce 10 ± 0.02 U/mL of lipase and 23 ± 0.05 U/mL of laccase, and *P. chrysogenum* produced 112 ± 0.50 U/mL of lipase, 79 ± 0.60 U/mL of laccase and 47 ± 0.01 U/mL of peroxidase [109].

Other authors have reported the production of lignin-degrading enzymes by these microorganisms. For example, Cabrera and coworkers [110] obtained a maximum production of laccase by *Trichoderma viride* M5-2 after 48 h of submerged fermentation woth wheat bran, 0.22 U/mL. The maximum laccase production by *Trichoderma viride*, isolated from coconut husk, was 0.65 U/mL after 96 h of incubation in medium with guaiacol [111]. Senthivelan and his team [112] obtained an optimal laccase production (7.9 U/mL) at the 5th day of a 7-day assay with *Penicillium chrysogenum* in an optimized medium. Other fungi have also been reported regarding the production of these enzymes, as well as the use of

lignocellulosic residues as peanut shells for such purpose. The fungus *Pycnoporus* SYBC-L3 reached the highest activity of ~5 U/g on peanut shells within 25 days of assays [113]. *Funalia trogii* IFP0027 grown on peanut shells was able to produce 11.9 U/mL of laccase after144 h of fermentation [114]. *Ganoderma. lucidum* cultivated on peanuts shells produced at the 4th day a maximum amount of 332± 6.66 U/mL of MnP, and with 70 % (w/w) of moisture the MnP production increased to 545 ± 3.55 U/mL in 5 days fermentation. At the same time, the highest production of LiP (95±1.52 U/mL) with 9 days and laccase (44 ±1.32 U/mL) with 5 days of fermentation were registered [115].

In liquid basal medium *A. aegerita* was capable of producing 30.8 ± 0.51 U/L of laccase, 52.29 ± 0.11 U/L of MnP and 18.63 ± 0.05 U/L of LiP after 14 days cultivation at 27 °C [116]. A different combination of solid waste, wheat straw and milled grain was used as cultivation substrate for *A. aegerita* 156 [117]. Laccase production varied between 0.1 and 6.2 U g^{-1} and MnP activity ranged between 0.3 and 0.9 U g^{-1}. However, after fruiting (beyond 78 days), laccase and peroxidases activity decreased.

4.1.2 PAH degradation

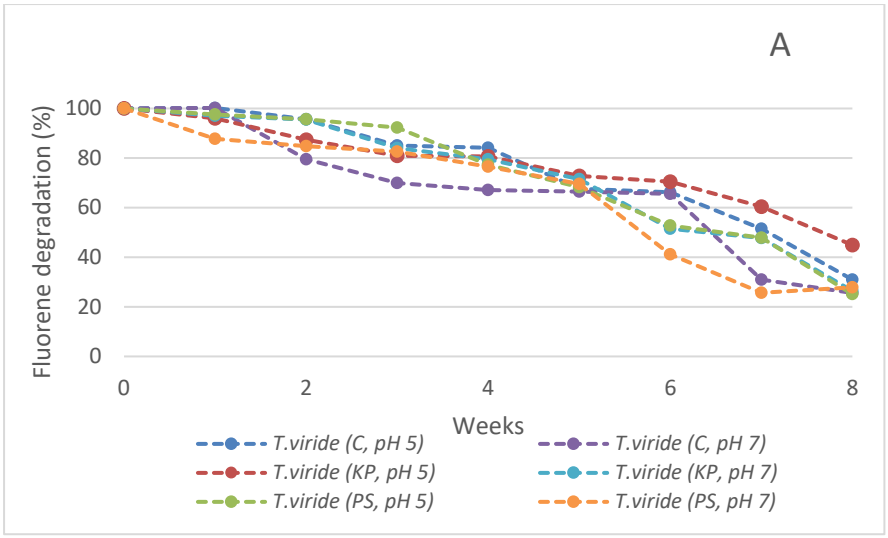
The distribution of PAH in soil depends mainly on the PAH hydrophobicity and their affinity towards microcompartments of the aggregates [118]. Thus PAH molecules with higher hydrophobicity and more aromatic rings present stronger sorption affinity [119]. In the current work, artificially contaminated soil was used. So, the rehabilitation rate of PAH in soil is expected to be higher than naturally contaminated soil, as observed by Huesemann and co-workers [120] for anthracene degradation in freshly spiked soil (72 % degradation) and aged soil (34 % degradation).

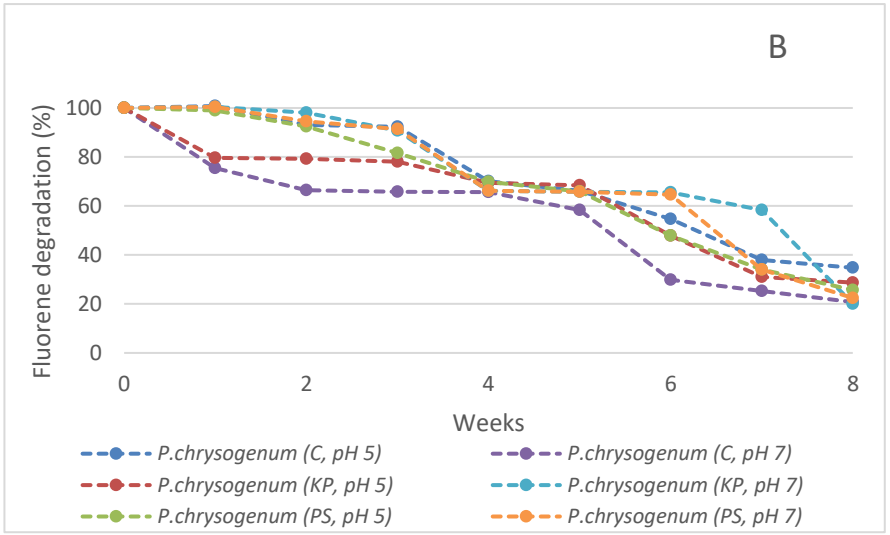
The potential of the studied fungi, as well as the influence of the cultivation strategy (axenic culture or co-culture), of soil amendment (kiwi peels and peanut shells), and of soil pH (5 and 7) in the degradation of PAH in soil was evaluated, Figs. 4.1 to 4.4. In general, *A. aegerita* was the most promising fungus for PAH degradation and the co-cultivation did not improved the degradation of the pollutants, with exception of chrysene. Also, pH has a stronger influence on PAH degradation than either amendment.

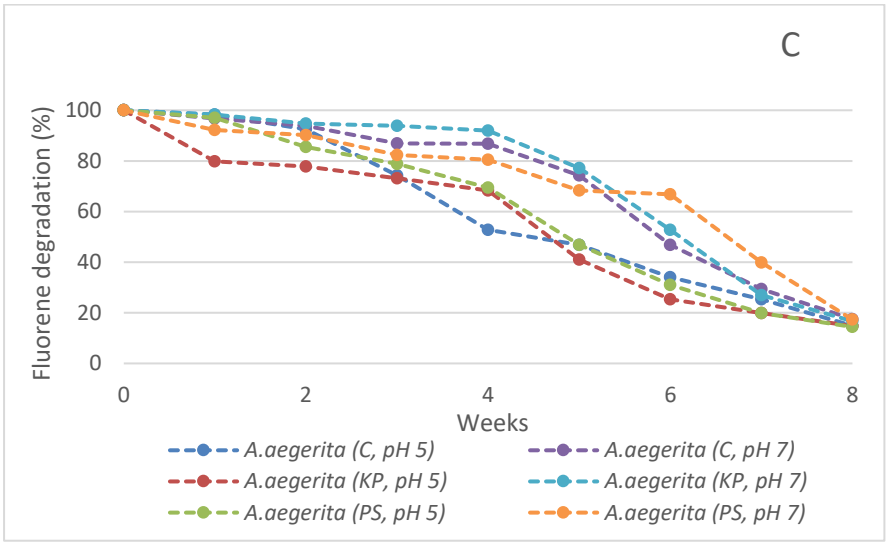
Fluorene (Fig. 4.1 A) was more degradable in soil at pH 5 as *A. aegerita* was able to remove around 85.0 % of this pollutant after 8 weeks degradation, using both peanut shells and kiwi peels as soil amendment. Similar degradation yields were achieved with the cocultivation of *A. aegerita* with *P.*

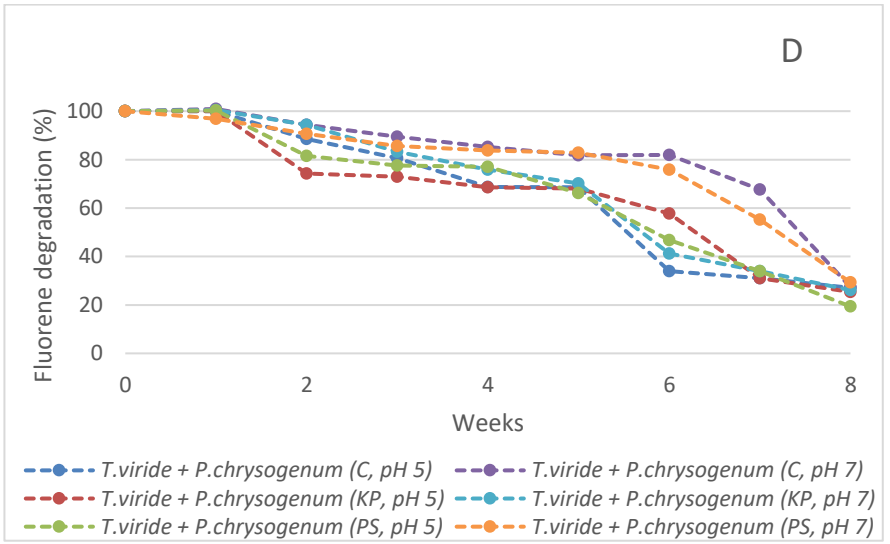
chrysogenum (86.1 %) and *A. aegerita* with *T. viride* (85.8 %). Degradation of pyrene (Fig. 4.1 B) after 8 weeks of assay ranged between 61 and 87 %. In all combinations better degradation results were obtained at soil pH 5, with highest degradation by co-cultivation of *A. aegerita* with *T. viride* and *A. aegerita* with *P. chrysogenum* on kiwi peels, 87.0 % for both situations. Axenic culture of *A. aegerita* was able to degrade 85.2 % of pyrene.

There was less remaining chrysene (Fig. 4.3) at soil pH 7, with the highest degradation obtained by cocultivation of *A. aegerita* with *T. viride* (Fig. 4.3 E), and *A. aegerita* with *P. chrysogenum* (Fig. 4.3 F) on peanuts shells, 89 %, while a 78 % degradation of chrysene was obtained by *A. aegerita* (Fig. 4.3 C). A different pattern of degradation was observed with benzo[a]pyrene (Fig. 4.4), as being one of the most persistent PAH. *Tricoderma viride* was the least efficient, just with 6 % of the benzo[a]pyrene degradation, being capable of degrading it only between 4 % and 31 %, (Fig. 4.4 A). *Agrocybe aegerita* alone achieved the highest degradation at soil pH 5, 87 %, without soil amendment (Fig. 4.4 C). With kiwi peels as support, 87 % degradation was observed in comparison to 82 % reached with peanut shells as support. *Penicillum chrysogenum* grew better at soil pH 5, with a degradation ranging between 58 % and 72 %, (Fig. 4.4 B).


A
Fluorene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
T.viride (C, pH 5)
T.viride (C, pH 7)
T.viride (KP, pH 5)
T.viride (KP, pH 7)
T.viride (PS, pH 5)
T.viride (PS, pH 7)


B
Fluorene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
P.chrysogenum (C, pH 5)
P.chrysogenum (C, pH 7)
P.chrysogenum (KP, pH 5)
P.chrysogenum (KP, pH 7)
P.chrysogenum (PS, pH 5)
P.chrysogenum (PS, pH 7)


C
Fluorene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
A.aegerita (C, pH 5)
A.aegerita (C, pH 7)
A.aegerita (KP, pH 5)
A.aegerita (KP, pH 7)
A.aegerita (PS, pH 5)
A.aegerita (PS, pH 7)


D
Fluorene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
T.viride + P.chrysogenum (C, pH 5)
T.viride + P.chrysogenum (C, pH 7)
T.viride + P.chrysogenum (KP, pH 5)
T.viride + P.chrysogenum (KP, pH 7)
T.viride + P.chrysogenum (PS, pH 5)
T.viride + P.chrysogenum (PS, pH 7)

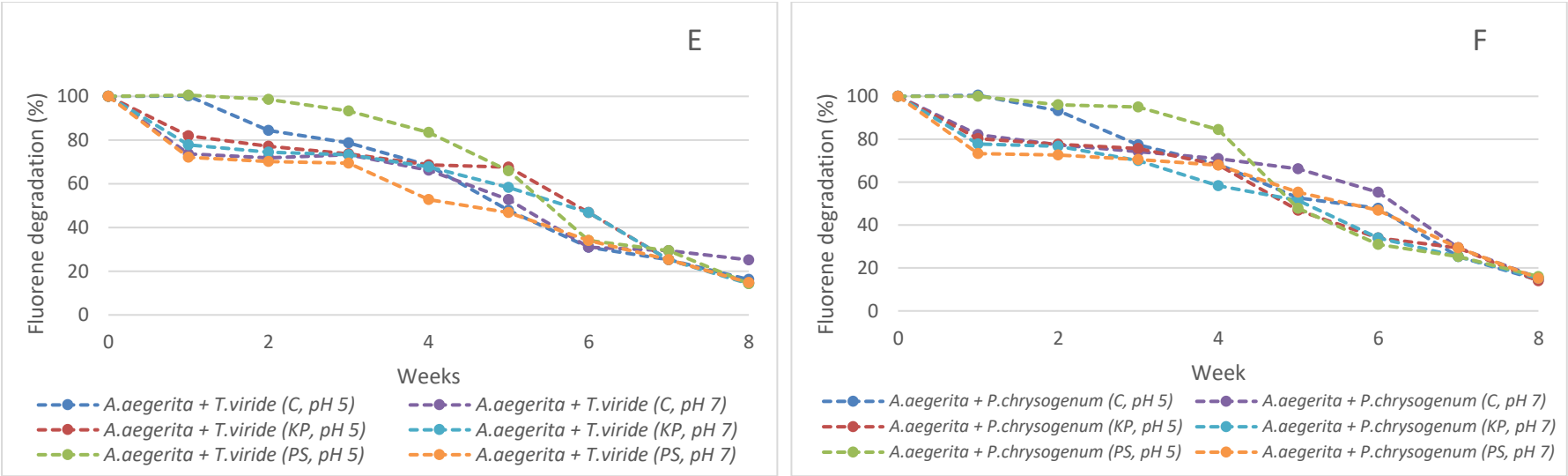
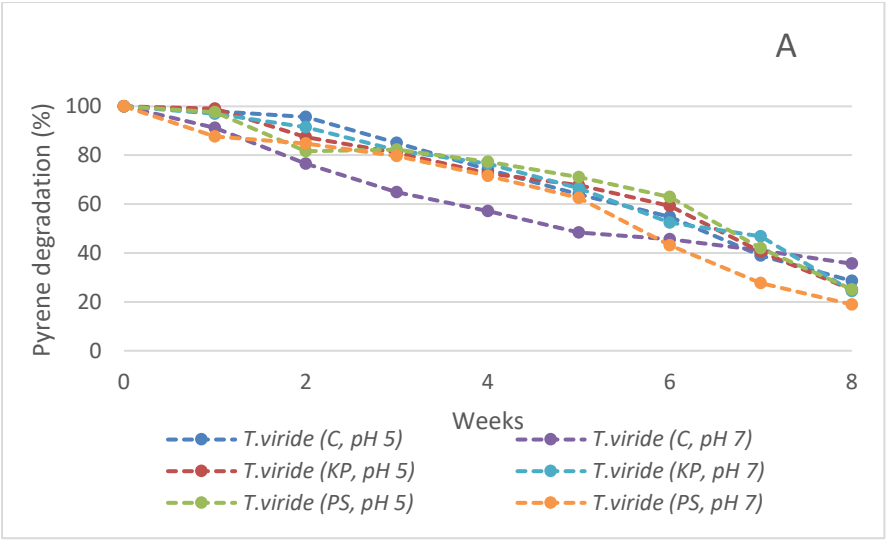
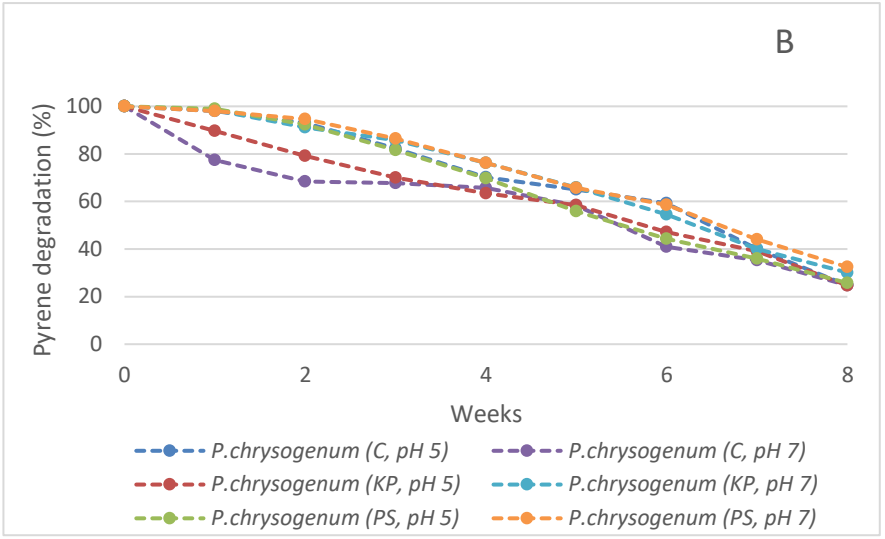
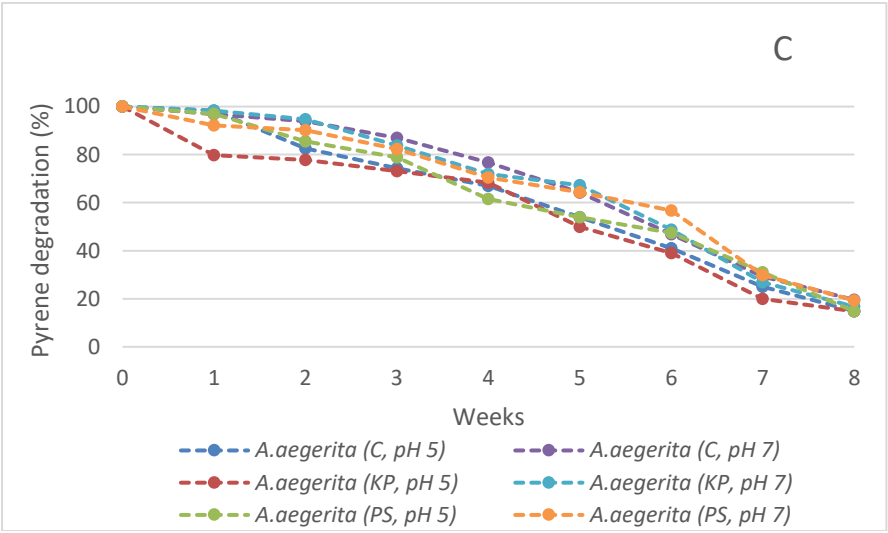
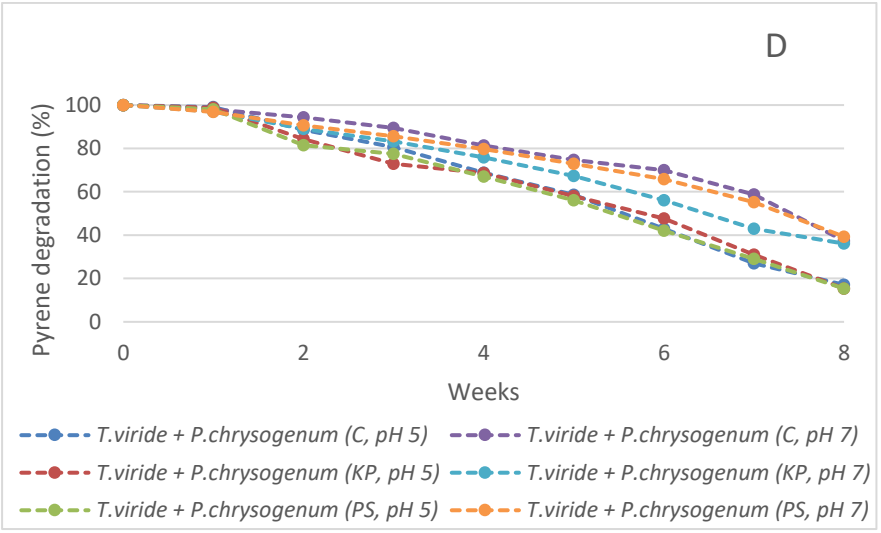


Figure 4.1 Fluorene degradation by the fungal strains: *T. viride* (A), *P. chrysogenum* (B), *A. aegerita* (C), and their co-culture: *T. viride + P. chrysogenum* (D), *A. aegerita + T. viride* (E) and *A. aegerita + P. chrysogenum* (F), in soil with a mixture of selected PAH


A
Pyrene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
T.viride (C, pH 5)
T.viride (C, pH 7)
T.viride (KP, pH 5)
T.viride (KP, pH 7)
T.viride (PS, pH 5)
T.viride (PS, pH 7)


B
Pyrene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
P.chrysogenum (C, pH 5)
P.chrysogenum (C, pH 7)
P.chrysogenum (KP, pH 5)
P.chrysogenum (KP, pH 7)
P.chrysogenum (PS, pH 5)
P.chrysogenum (PS, pH 7)


C
Pyrene degradation (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Weeks
A.aegerita (C, pH 5)
A.aegerita (C, pH 7)
A.aegerita (KP, pH 5)
A.aegerita (KP, pH 7)
A.aegerita (PS, pH 5)
A.aegerita (PS, pH 7)


D
Pyrene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
T.viride + P.chrysogenum (C, pH 5)
T.viride + P.chrysogenum (C, pH 7)
T.viride + P.chrysogenum (KP, pH 5)
T.viride + P.chrysogenum (KP, pH 7)
T.viride + P.chrysogenum (PS, pH 5)
T.viride + P.chrysogenum (PS, pH 7)

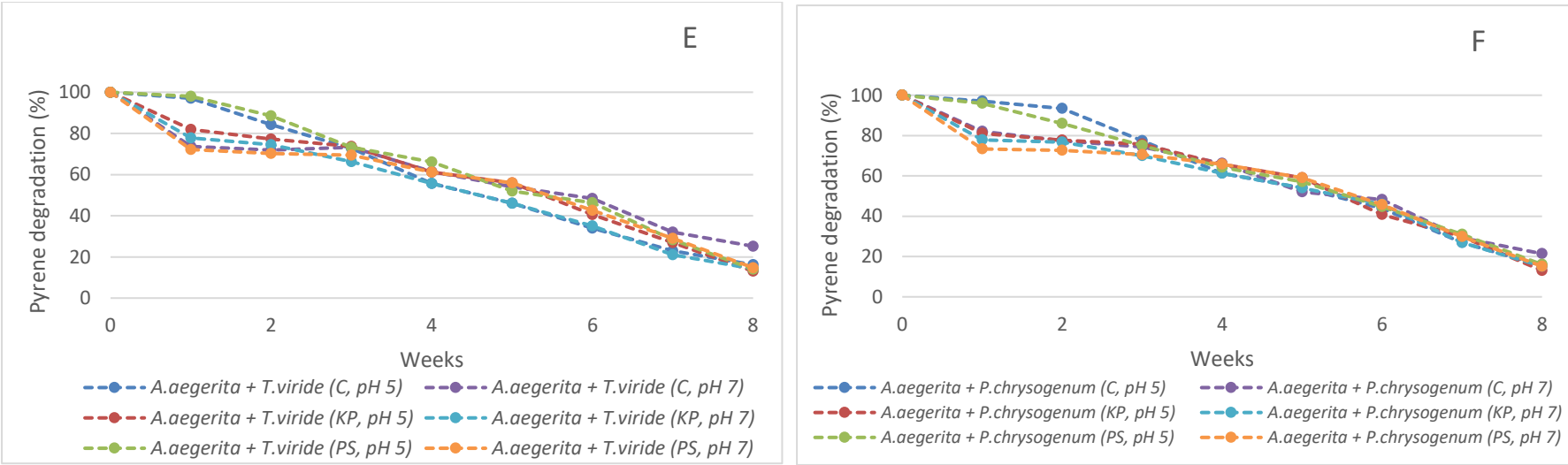
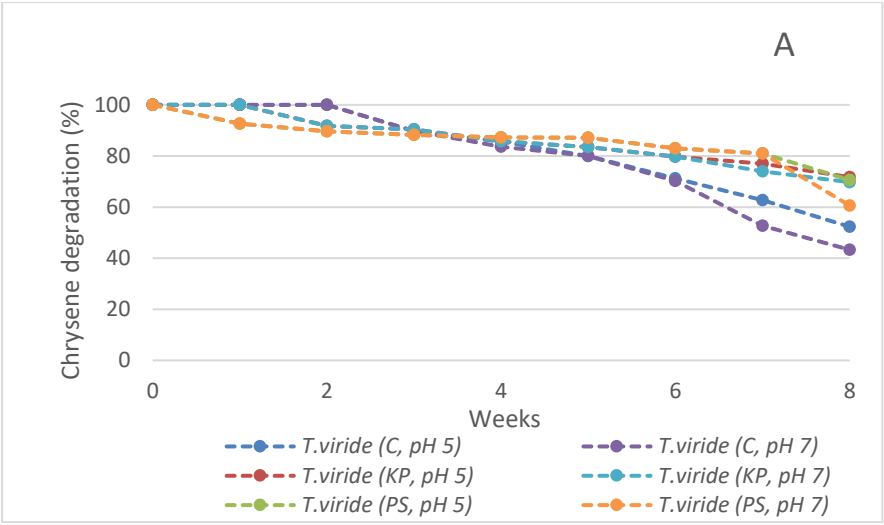
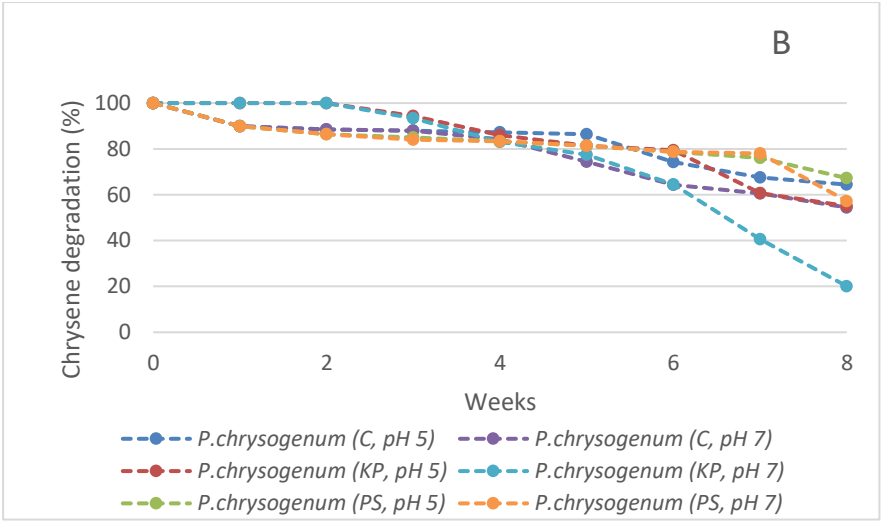
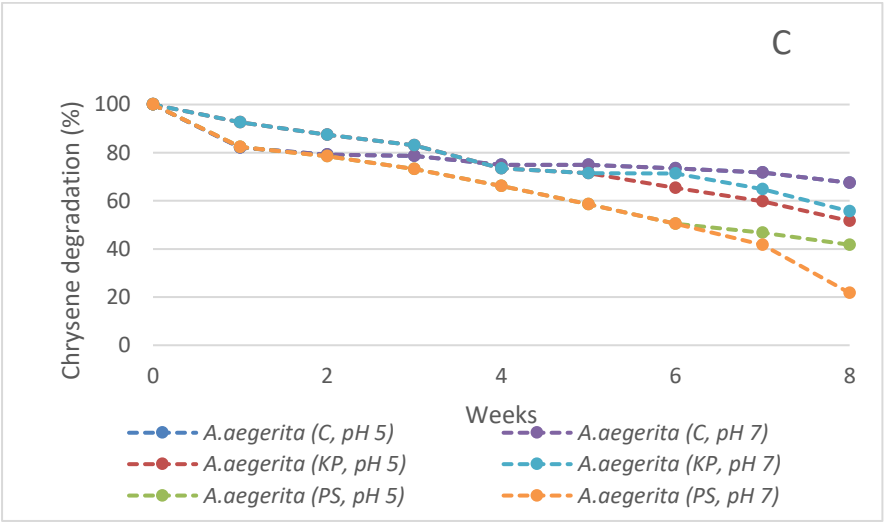
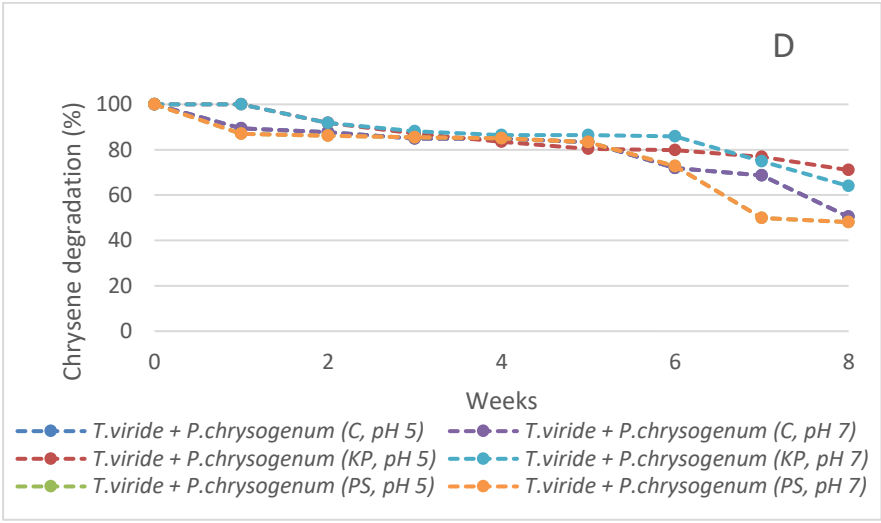


Figure 4.2 Pyrene degradation by the fungal strains: *T. viride* (A), *P. chrysogenum* (B), *A. aegerita* (C), and their co-culture: *T. viride* + *P. chrysogenum* (D), *A. aegerita* + *T. viride* (E) and *A. aegerita* + *P. chrysogenum* (F), in soil with a mixture of selected PAH


A
Chrysene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
T.viride (C, pH 5)
T.viride (C, pH 7)
T.viride (KP, pH 5)
T.viride (KP, pH 7)
T.viride (PS, pH 5)
T.viride (PS, pH 7)


B
Chrysene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
P.chrysogenum (C, pH 5)
P.chrysogenum (C, pH 7)
P.chrysogenum (KP, pH 5)
P.chrysogenum (KP, pH 7)
P.chrysogenum (PS, pH 5)
P.chrysogenum (PS, pH 7)


C
Chrysene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
A.aegerita (C, pH 5)
A.aegerita (C, pH 7)
A.aegerita (KP, pH 5)
A.aegerita (KP, pH 7)
A.aegerita (PS, pH 5)
A.aegerita (PS, pH 7)


D
Chrysene degradation (%)
100
80
60
40
20
0
0
2
4
6
8
Weeks
T.viride + P.chrysogenum (C, pH 5)
T.viride + P.chrysogenum (C, pH 7)
T.viride + P.chrysogenum (KP, pH 5)
T.viride + P.chrysogenum (KP, pH 7)
T.viride + P.chrysogenum (PS, pH 5)
T.viride + P.chrysogenum (PS, pH 7)

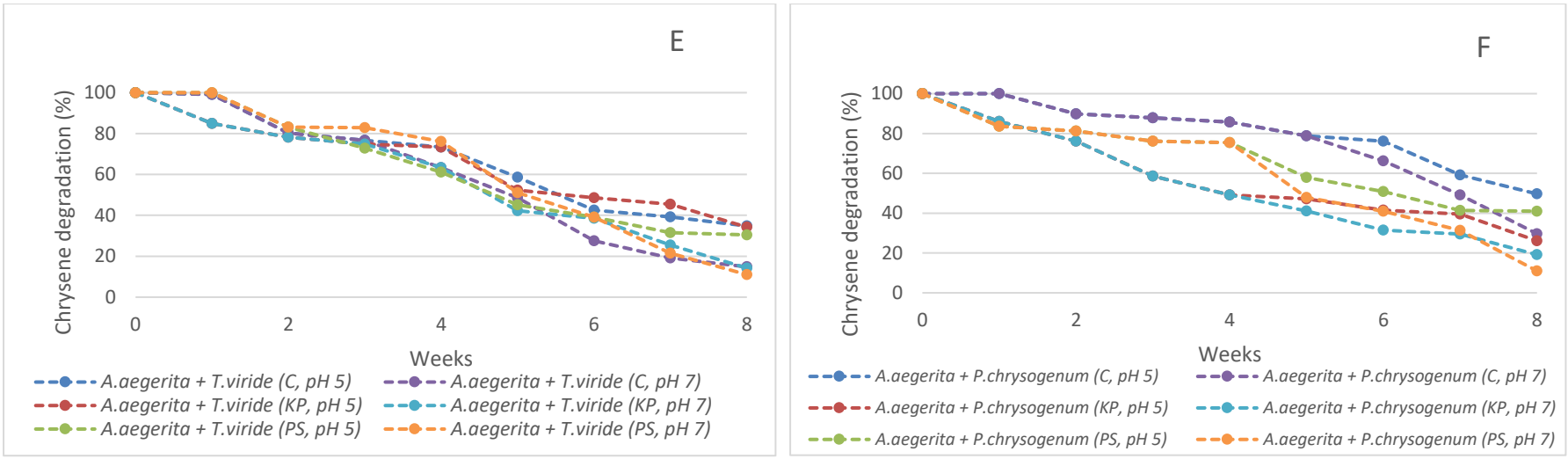
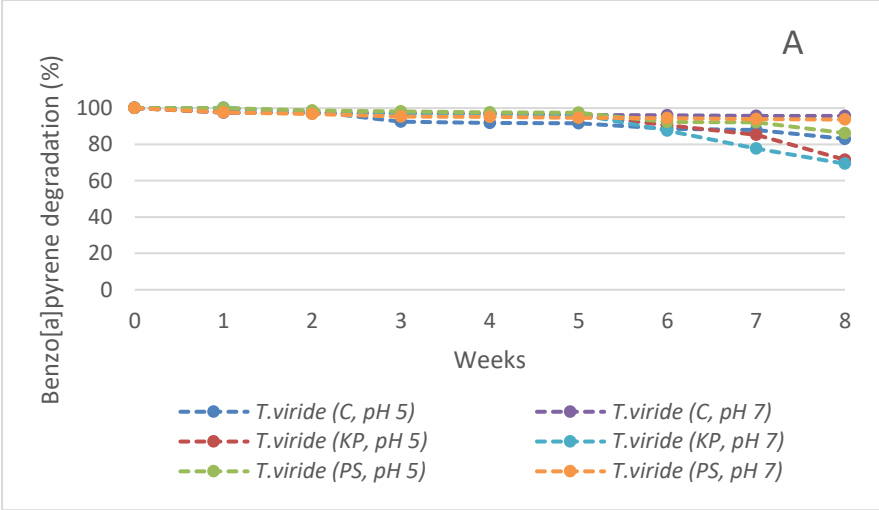
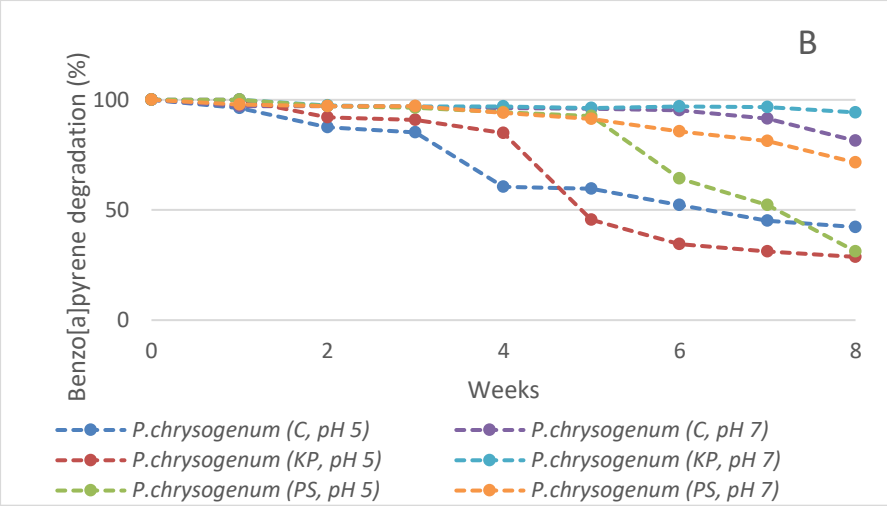
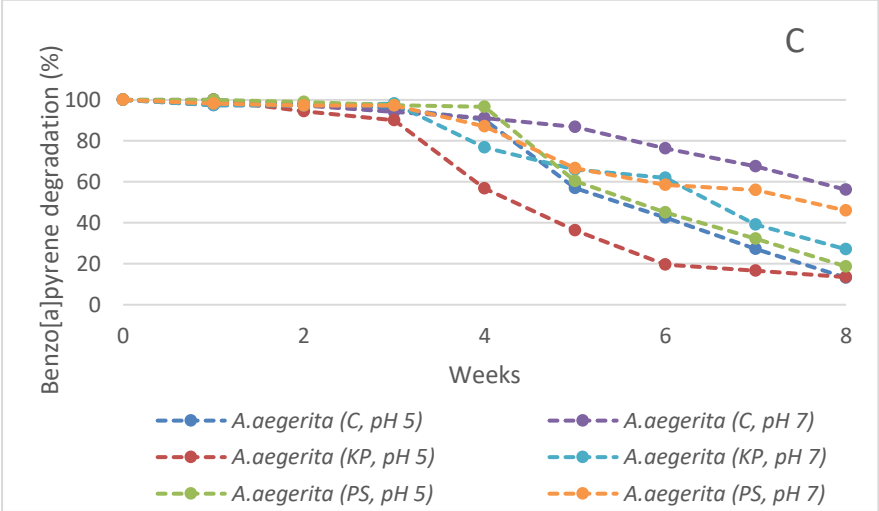
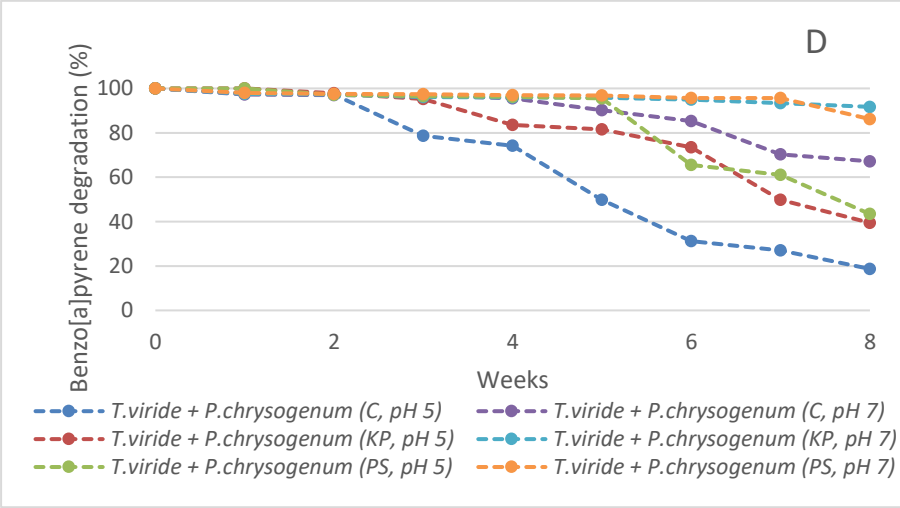


Figure 4.3 Chrysene degradation by the fungal strains: *T. viride* (A), *P. chrysogenum* (B), *A. aegerita* (C), and their co-culture: *T. viride* + *P. chrysogenum* (D), *A. aegerita* + *T. viride* (E) and *A. aegerita* + *P. chrysogenum* (F), in soil with a mixture of selected PAH


A
Benzo[a]pyrene degradation (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8
Weeks
T.viride (C, pH 5)
T.viride (C, pH 7)
T.viride (KP, pH 5)
T.viride (KP, pH 7)
T.viride (PS, pH 5)
T.viride (PS, pH 7)


B
Benzo[a]pyrene degradation (%)
100
50
0
0 2 4 6 8
Weeks
P.chrysogenum (C, pH 5)
P.chrysogenum (C, pH 7)
P.chrysogenum (KP, pH 5)
P.chrysogenum (KP, pH 7)
P.chrysogenum (PS, pH 5)
P.chrysogenum (PS, pH 7)


C
Benzo[a]pyrene degradation (%)
100
80
60
40
20
0
0 2 4 6 8
Weeks
A.aegerita (C, pH 5)
A.aegerita (C, pH 7)
A.aegerita (KP, pH 5)
A.aegerita (KP, pH 7)
A.aegerita (PS, pH 5)
A.aegerita (PS, pH 7)


D
Benzo[a]pyrene degradation (%)
100
80
60
40
20
0
0 2 4 6 8
Weeks
T.viride + P.chrysogenum (C, pH 5)
T.viride + P.chrysogenum (C, pH 7)
T.viride + P.chrysogenum (KP, pH 5)
T.viride + P.chrysogenum (KP, pH 7)
T.viride + P.chrysogenum (PS, pH 5)
T.viride + P.chrysogenum (PS, pH 7)

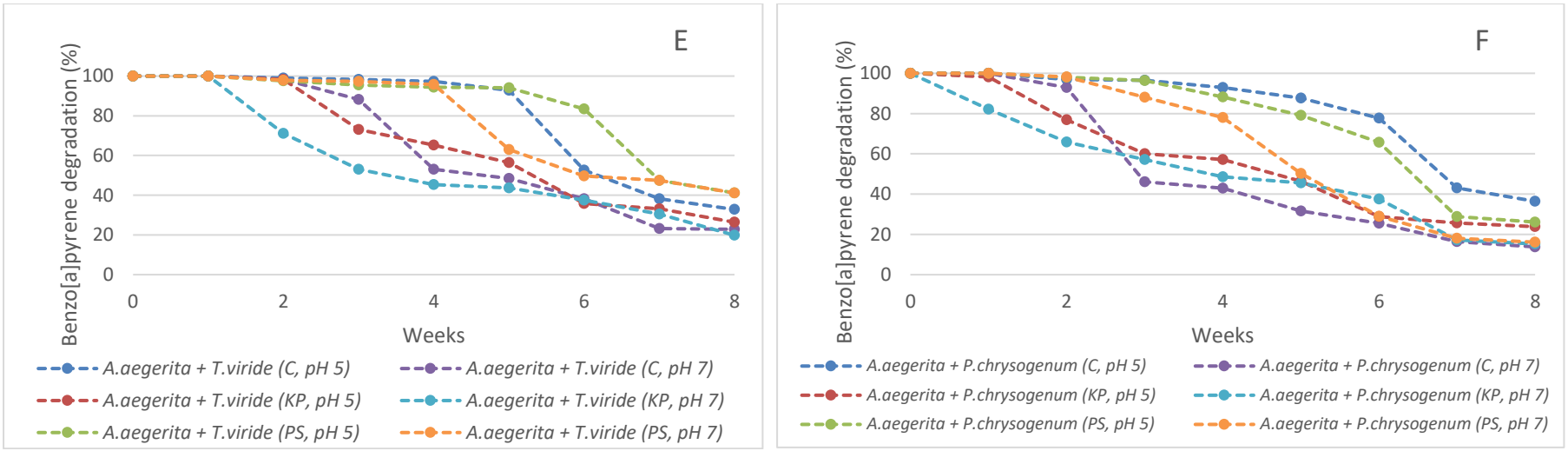


Figure 4.4 Benzo[a]pyrene degradation by the fungal strains: *T. viride* (A), *P. chrysogenum* (B), *A. aegerita* (C), and their co-culture: *T. viride* + *P. chrysogenum* (D), *A. aegerita* + *T. viride* (E) and *A. aegerita* + *P. chrysogenum* (F), in soil with a mixture of select PAH

In this study a higher degradation rate was observed at soil pH 5 for fluorene, pyrene and benzo[a]pyrene, and in the case of chrysene, a higher degradation was observed at soil pH 7. Pawar [101] observed a higher and faster degradation by a consortium of bacterial population, at pH 7.5, at which the nutrient availability is greater. However, fungi grew actively at acidic pH 5 and 5.5, which also indicates that fungi are more tolerant to acidic soil than bacteria.

The distribution of PAH in soil depends mainly on the PAH hydrophobicity and their affinity towards microcompartments of the aggregates [118]. *In situ* degradation of PAH is often slow, as PAH get more and more persistent the longer, they stay in soil. Their persistence may be affected also by nutrients (more availability at neutral pH), by their bioavailability (sorption on particles), temperature, oxygen, pH and by the presence of PAH-degrading microorganisms. According to Pawar [101], there are different dominant fungal species depending on the soil pH. *Penicillium* species predominate at acidic soil pH and with fewer *Aspergillus* populations, whereas at alkaline conditions (pH 8.0 and 8.5) *Aspergillus* is predominant and *Penicillium* is not detected. However, that study indicated that fungi play an active role in the degradation at acidic soil and bacteria play a role in the degradation at neutral soil pH. Chupungars and co-workers [121] reported that *Agrocybe* sp. CU-43, isolated from Thailand soil, was able to degrade 100 ppm of fluorene in N-limiting medium (pH 4.5, 130 rpm at 28 °C) in 6 days. By increasing the concentration to 250 and to 500 ppm, the time needed to complete degradation increased to 3 and to 4 weeks, respectively.

4.1.3 Evaluation of enzyme production during PAH degradation

Enzymatic activities in the soil were assessed considering that they depend on environmental factors and on co-existing chemicals, as reported by Bonomo and colleagues [122], supporting that enzyme activities are affected by soil pH. The obtained results of the current study are presented in Tables 4.4 to 4.6. The highest laccase levels were detected in the extracts of *A. aegerita* (Table 4.4), being higher in soil at neutral pH than at pH 5. During PAH degradation by *A. aegerita* with kiwi peels as soil amendment, 2.15 U/g of laccase were detected in the 1st week, increasing to 6.43 U/g in the 2nd week, which was the highest enzymatic level reached and then it started slowly to decrease. At the same time, *A. aegerita* was also capable of producing MnP (Table 4.5), with 3.02 U/g in the 2nd week, increasing up to 7.21 U/g in 4th week and decreasing very slowly to 5.88 U/g at the end of the process (8 weeks). With peanut shells as soil amendment, the obtained results were lower, with a maximum laccase production

of 4.80 U/g and 6.88 U/g of MnP. LiP activity was also detected in the extracts of *A. aegerita*, being 3.39 U/g with kiwi peels as soil amendment and 2.57 U/g on peanut shells, both in soil at pH 7, (Table 4.6).

On the other hand, laccase and MnP levels reached with *T. viride* were higher at soil pH 5, with 1.78 U/g at the 5th week and 1.17 U/g at the 4th week, respectively, both with kiwi peels. Around 1.69 U/g of laccase were detected on *P. chrysogenum* extracts from soil at pH 5 with kiwi peels (4th week), while no MnP activity was observed. Despite the highest PAH degradation potential was observed in general in soil at pH 5 by *A. aegerita*, simultaneous production of enzymes was more modest at this pH, being maximal in soil at pH 7. However, the highest levels of enzymes produced by *T. viride* and *P. chrysogenum* were detected in soil at pH 5, showing in these cases that PAH degradation appears to be correlate with enzyme production.

In natural soil, the enzyme activities are impaired due to absorption and immobilization between soil particles and organic matter depending on soil type [123]. In the study of Ting and co-workers [124] the laccase production by *G. lucidum* was enhanced when the PAH were present. Laccase activity peaked on the 6th day and then slowly dropped in the cultures. The maximum mean laccase activity, 670 U/L, was detected in the cultures with 20 mg/L of PAH (phenanthrene and pyrene). When the concentrations of PAH in cultures were elevated to 50 and to 100 mg/L, laccase activities were reduced to 450 and to 220 U/L, respectively. At the same time, the highest PAH degradation and laccase production with *G. lucidum* in liquid medium was observed at pH 4, on the 6th day with 812 U/L [124].

Table 4.4 Laccase activity during PAH degradation in soil at pH 5 and pH 7 by *T. viride*, *P. chrysogenum*, *A. aegerita* and their co-culture in U/g

Fungi + substrate	SSF at pH 5 (weeks)								SSF at pH 7 (weeks)							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
T. viride (C)	0.07±0.07	0.89±0.01	0.7±0.02	1.45±0.04	**1.61±0.02**	0.70±0.07	0.37±0.05	n.d	0.07±0.07	0.21±0.02	0.60±0.04	0.66±0.09	**0.70±0.07**	0.59±0.08	0.48±0.09	0.21±0.02
T. viride (KP)	0.07±0.07	1.20±0.005	1.14±0.09	1.52±0.05	**1.78±0.06**	0.93±0.01	0.18±0.07	n.d	0.07±0.07	0.46±0.05	0.73±003	0.87±0.02	**0.93±0.01**	0.56±0.05	0.56±0.01	0.55±0.01
T. viride (PS)	0.07±0.07	0.42±0.08	0.71±0.02	**1.39±0.02**	0.64±0.012	0.25±0.02	0.07±0.07	n.d	0.07±0.07	0.32±0.06	0.72±0.06	0.79±0.01	**0.96±0.06**	0.96±0.02	0.74±0.02	0.60±0.06
P. chrysogenum (C)	0.06±0.06	0.20±0.05	0.47±0.05	**0.82±0.01**	0.21±0.09	n.d	n.d	n.d	n.d	0.06±0.06	0.55±0.03	**0.67±0.05**	0.53±0.02	n.d.	n.d	n.d.
P. chrysogenum (KP)	0.06±0.06	0.51±0.09	0.93±0.08	**1.69±0.04**	0.20±0.05	n.d	n.d	n.d	n.d	0.06±0.06	0.66±0.01	**0.75±0.04**	0.69±0.02	0.60±0.07	0.43±0.04	n.d.
P. chrysogenum (PS)	0.06±0.06	0.28±0.01	0.61±0.02	**0.95±0.04**	0.54±0.01	n.d	n.d	n.d	n.d	0.06±0.06	0.65±0.08	**0.69±0.07**	0.61±0.09	0.53±0.02	0.41±0.04	n.d.
TV + PC (C)	0.12±0.03	0.11±0.025	0.20±0.07	**0.68±0.01**	0.58±0.1	0.55±0.03	n.d	n.d	0.12±0.03	0.23±0.02	0.62±0.04	0.68±0.09	**0.71±0.07**	0.69±0.02	0.59±0.08	0.48±0.09
TV + PC (KP)	0.12±0.03	1.04±0.01	**1.48±0.01**	1.39±0.03	0.97±0.02	0.57±0.09	0.54±0.01	n.d	0.12±0.03	0.47±0.05	0.79±003	0.91±0.02	**0.98±0.01**	0.97±0.05	81±0.01	0.69±0.07
TV + PC (PS)	0.12±0.03	1.03±0.07	1.33±0.07	**2.26±0.03**	1.20±0.03	0.59±0.03	0.52±0.01	n.d	0.12±0.03	0.31±0.06	0.78±0.06	0.86±0.01	**0.96±0.06**	0.96±0.02	0.84±0.02	0.77±0.02
A. aegerita (C)	0.17±0.02	1.75±0.01	2.31±0.03	2.63±0.09	**2.69±0.04**	2.34±0.06	1.72±0.05	1.53±0.02	2.05±0.04	4.31±0.03	4.63±0.09	**4.69±0.04**	2.34±0.06	1.82±0.05	1.03±0.08	0.93±0.02
A. aegerita (KP)	0.21±0.04	1.86±0.02	3.38±0.02	3.61±0.07	**3.92±0.07**	2.39±0.07	1.83±0.06	1±0.01	2.15±0.02	**6.43±0.06**	6.27±0.06	5.80±0.05	3.99±0.04	2.92±0.01	1.94±0.08	1.38±0.07
A. aegerita (PS)	0.18±0.01	1.72±0.05	**3.97±0.05**	3.24±0.07	**3.75±0.03**	2.22±0.02	1.80±0.06	1±0.09	2.33±0.07	**4.80±0.05**	4.30±0.15	4.66±0.07	2.80±0.02	1.93±0.08	1.82±0.01	1.03±0.08
AA + TV (C)	0.25±0.01	0.75±0.06	0.97±0.05	1.21±0.0	**1.11±0.03**	1.02±0.04	1.00±0.01	0.92±0.04	0.55±0.07	1.73±0.03	2.99±0.01	**3.42±0.03**	2.86±0.01	2.80±0.01	2.00±0.04	2.00±0.01
AA + TV (KP)	0.35±0.02	0.55±0.03	0.84±0.03	1.63±0.02	**2.05±0.06**	1.58±0.05	1.52±0.07	1.50±0.02	0.76±0.01	1.81±0.04	3.56±0.04	**3.68±0.03**	3.20±0.08	2.83±0.02	2.48±0.08	2.26±0.04
AA + TV (PS)	0.35±0.02	0.43±0.02	0.82±0.03	**1.11±0.03**	1.02±0.04	0.70±0.04	0.61±0.01	0.57±0.01	0.72±0.09	1.80±0.05	3.49±0.05	**3.59±0.01**	2.87±0.06	2.86±0.01	2.16±0.09	2.05±0.03
AA + PC (C)	0.11±0.03	0.12±0.01	0.37±0.02	**1.29±0.01**	1.02±0.04	0.82±0.04	0.80±0.04	0.78±0.01	0.55±0.07	1.73±0.03	2.99±0.01	**3.42±0.03**	2.86±0.01	2.80±0.01	2.00±0.04	2.00±0.01
AA + PC (KP)	0.17±0.01	0.27±0.05	0.48±0.05	**2.24±0.09**	1.29±0.04	1.35±0.12	1.15±0.12	1.11±0.01	0.44±0.01	0.99±0.05	1.09±0.07	1.72±0.01	**1.93±0.08**	1.03±0.08	1.00±0.06	0.85±0.09
AA + PC (PS)	0.12±0.03	0.25±0.02	0.43±0.01	**1.75±0.04**	1.59±0.04	1.19±0.09	1.09±0.09	1.28±0.02	0.31±0.01	0.72±0.08	0.95±0.01	**1.82±0.01**	1.43±0.09	1.11±0.02	1.03±0.08	0.86±0.05

TV: T. viride; PC: P. chrysogenum; AA: A. aegerita; KP: Kiwi peels; PS: Peanut shells; n.d.: not detected

Table 4.5 MnP activity during PAH degradation in soil at pH 5 and pH 7 by *T. viride*, *P. chrysogenum*, *A. aegerita* and their co-culture in U/g

Fungi + substrate	SSF at pH 5 (weeks)								SSF at pH 7 (weeks)							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
T. viride (C)	0.06±0.06	0.41±0.06	0.63±0.04	**0.93±0.09**	0.83±0.09	0.64±0.05	0.45±0.03	0.44±0.03	0.21±0.07	0.41±0.06	0.66±0.08	**0.96±0.01**	0.80±0.03	0.61±0.02	0.56±0.01	0.31±0.08
T. viride (KP)	0.06±0.06	0.45±0.07	1.10±0.08	**1.17±0.06**	0.98±0.07	0.77±0.05	0.51±0.04	0.48±0.02	0.29±0.09	0.45±0.07	0.90±0.02	**1.02±0.09**	0.98±0.07	0.70±0.06	0.61±0.02	0.40±0.03
T. viride (PS)	0.06±0.06	0.43±0.04	1.02±0.09	**1.17±0.05**	0.88±0.01	0.65±0.03	0.48±0.06	0.44±0.03	0.27±0.01	0.43±0.04	0.71±0.02	**0.88±0.01**	0.73±0.07	0.65±0.05	0.59±0.05	0.38±0.09
P. chrysogenum (C)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P. chrysogenum (KP)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P. chrysogenum (PS)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TV + PC (C)	0.70±0.06	0.96±0.01	1.71±0.01	**1.90±0.06**	1.87±0.01	0.95±0.03	0.44±0.05	0.63±0.02	0.25±0.04	0.77±0.01	**1.08±0.06**	1.07±0.02	0.96±0.01	0.74±0.07	0.61±0.05	0.52±0.01
TV + PC (KP)	0.25±0.07	0.98±0.03	**1.16±0.02**	1.13±0.02	1.10±0.07	0.80±0.07	0.71±0.09	0.58±0.04	0.25±0.07	0.79±0.01	1.05±0.08	**1.15±0.02**	0.92±0.08	0.71±0.09	0.45±0.07	0.32±0.06
TV + PC (PS)	0.25±0.07	0.98±0.07	**1.02±0.09**	1.02±0.02	0.92±0.02	0.73±0.07	0.68±0.06	0.56±0.03	0.25±0.07	0.79±0.02	0.81±0.01	**1.04±0.04**	0.90±0.01	0.68±0.06	0.41±0.06	0.32±0.05
A. aegerita (C)	0.71±0.05	1.05±0.03	**2.17±0.01**	2.17±0.11	2.14±0.02	2.10±0.07	2.00±0.09	1.75±0.01	0.65±0.03	2.79±0.01	4.12±0.08	**5.04±0.04**	4.96±0.07	4.85±0.06	3.79±0.09	3.69±0.05
A. aegerita (KP)	0.93±0.09	1.12±0.08	3.21±0.02	**3.72±0.08**	2.59±0.02	2.20±0.01	2.17±0.01	1.82±0.06	0.77±0.01	3.02±0.02	5.78±0.01	**7.21±0.02**	6.71±0.01	6.12±0.08	5.93±0.03	5.88±0.07
A. aegerita (PS)	0.77±0.01	1.08±0.06	3.41±0.01	**3.61±0.07**	2.72±0.04	2.35±0.09	2.23±0.09	1.97±0.02	0.66±0.03	2.83±0.03	5.61±0.07	**6.88±0.08**	5.45±0.01	5.03±0.06	4.80±0.08	4.61±0.07
AA + TV (C)	0.70±0.06	0.96±0.01	1.21±0.01	**2.90±0.06**	2.87±0.05	1.63±0.02	1.43±0.02	1.02±0.02	0.58±0.04	1.74±0.01	3.38±0.08	**3.40±0.03**	2.96±0.01	2.80±0.03	2.63±0.04	2.53±0.09
AA + TV (KP)	0.63±0.04	0.98±0.03	1.16±0.02	**2.13±0.02**	2.10±0.07	1.80±0.07	1.71±0.09	1.58±0.04	0.63±0.04	1.92±0.08	3.51±0.01	**3.58±0.03**	3.26±0.04	2.92±0.08	2.71±0.04	2.66±0.01
AA + TV (PS)	0.62±0.04	0.98±0.07	1.71±0.01	**2.02±0.02**	1.92±0.02	1.73±0.0.7	1.68±0.06	1.56±0.03	0.62±0.04	1.90±0.01	**3.46±0.04**	3.39±0.02	3.08±0.04	2.90±0.01	2.70±0.05	2.62±0.09
AA + PC (C)	0.41±0.01	0.66±0.01	**1.98±0.03**	1.90±0.02	1.71±0.08	1.60±0.02	1.52±0.03	1.40±0.03	0.45±0.07	1.63±0.03	2.96±0.01	**3.32±0.03**	2.96±0.01	2.70±0.01	2.03±0.04	2.05±0.01
AA + PC (KP)	0.63±0.04	0.98±0.03	**2.10±0.08**	1.98±0.07	1.78±0.07	1.65±0.05	1.65±0.05	1.48±0.05	0.66±0.01	1.71±0.04	3.46±0.04	**3.58±0.03**	3.00±0.08	2.73±0.02	2.38±0.08	2.16±0.04
AA + PC (PS)	0.62±0.01	0.96±0.05	**2.11±0.01**	1.99±0.02	1.83±0.06	1.79±0.09	1.64±0.05	1.44±0.03	0.62±0.09	1.70±0.05	3.39±0.05	**3.49±0.01**	2.97±0.06	2.93±0.01	2.06±0.09	2.05±0.03

TV: T. viride; PC: P. chrysogenum; AA: A. aegerita; KP: Kiwi peels; PS: Peanut shells; n.d.: not detected

Table 4.6 LiP activity during PAH degradation in soil at pH 5 and pH 7 by *T. viride*, *P. chrysogenum*, *A. aegerita* and their co-culture in U/g

Fungi + substrate	SSF at pH 5 (weeks)								SSF at pH 7 (weeks)							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
T. viride (C)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
T. viride (KP)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
T. viride (PS)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P. chrysogenum (C)	0.06±0.06	0.13±0.03	0.59±0.04	**0.63±0.01**	0.51±0.03	0.49±0.03	0.25±0.07	0.14±0.02	0.06±0.06	0.28±0.04	0.48±0.02	0.54±0.07	**0.64±0.04**	0.54±0.08	0.41±0.02	0.24±0.06
P. chrysogenum (KP)	0.06±0.06	0.18±0.01	0.51±0.04	**0.74±0.02**	0.57±0.05	0.51±0.03	0.32±0.06	0.32±0.02	0.06±0.06	0.42±0.03	0.55±0.06	**0.72±0.06**	0.65±0.03	0.57±0.07	0.46±0.08	0.27±0.06
P. chrysogenum (PS)	0.06±0.06	0.19±0.02	0.49±0.01	**0.60±0.09**	0.58±0.03	0.56±0.03	0.31±0.05	0.30±0.08	0.06±0.06	0.36±0.04	0.54±0.07	**0.69±0.02**	0.51±0.04	0.50±0.01	0.44±0.04	0.24±0.09
TV + PC (C)	0.12±0.03	0.24±0.07	0.48±0.03	**0.60±0.05**	0.56±0.04	0.54±0.08	0.41±0.02	0.24±0.06	0.12±0.03	0.21±0.06	0.48±0.02	0.57±0.06	**0.60±0.03**	0.57±0.08	0.55±0.01	0.32±0.06
TV + PC (KP)	0.12±0.03	0.20±0.01	0.42±0.06	0.72±0.06	**1.25±0.08**	0.90±0.04	0.81±0.04	0.67±0.01	0.12±0.03	0.29±0.03	0.54±0.08	0.70±0.02	**0.71±0.01**	0.63±0.02	0.42±0.05	0.31±0.05
TV + PC (PS)	0.12±0.03	0.17±0.09	0.27±0.05	0.57±0.06	**0.70±0.04**	0.66±0.09	0.59±0.03	0.29±0.08	0.12±0.03	0.24±0.07	0.53±0.05	**0.66±0.04**	0.65±0.07	0.54±0.09	0.50±0.01	0.30±0.04
A. aegerita (C)	0.65±0.08	0.72±0.06	1.31±0.03	1.63±0.09	**2.13±0.08**	1.93±0.08	1.69±0.04	0.72±0.05	0.44±0.07	0.69±0.02	0.72±0.06	2.01±0.04	**2.08±0.04**	1.69±0.09	1.09±0.07	0.60±0.07
A. aegerita (KP)	0.69±0.02	1.38±0.02	1.61±0.07	2.16±0.09	**2.24±0.09**	2.13±0.08	1.73±0.06	1.41±0.01	0.69±0.01	0.99±0.05	1.09±0.07	2.79±0.05	**3.39±0.01**	2.64±0.01	1.18±0.04	0.76±0.04
A. aegerita (PS)	0.45±0.09	0.72±0.06	0.99±0.05	1.24±0.01	**1.75±0.04**	1.52±0.02	1.10±0.06	0.83±0.09	0.54±0.07	0.72±0.08	0.95±0.01	**2.57±0.05**	2.41±0.06	1.76±0.01	1.13±0.06	0.83±0.06
AA + TV (C)	0.28±0.07	0.55±0.06	0.72±0.06	1.10±0.07	**1.11±0.03**	1.02±0.04	0.70±0.04	0.61±0.01	0.44±0.07	0.52±0.06	0.74±0.02	**1.59±0.07**	1.13±0.07	0.86±0.05	0.69±0.09	0.37±0.05
AA + TV (KP)	0.41±0.01	0.65±0.08	1.02±0.04	1.63±0.02	**2.05±0.06**	1.58±0.05	1.17±0.04	0.74±0.03	0.69±0.02	0.77±0.0	0.95±0.01	**1.70±0.05**	1.38±0.02	0.94±0.05	0.66±0.07	0.46±0.09
AA + TV (PS)	0.34±0.02	0.54±0.07	0.99±0.05	**1.19±0.09**	0.80±0.04	0.57±0.06	0.55±0.04	0.36±0.08	0.54±0.07	0.62±0.08	0.89±0.02	**1.60±0.02**	1.24±0.01	0.83±0.06	0.50±0.09	0.42±0.06
AA + PC (C)	0.28±0.04	0.33±0.02	0.65±0.08	**1.29±0.01**	0.74±0.01	0.60±0.07	0.34±0.07	0.28±0.09	0.28±0.04	0.48±0.02	0.64±0.04	**0.90±0.03**	0.60±0.07	0.42±0.06	0.34±0.07	0.22±0.08
AA + PC (KP)	0.44±0.01	0.36±0.08	0.72±0.06	**1.29±0.04**	1.19±0.09	0.89±0.02	0.42±0.06	0.30±0.08	0.42±0.03	0.55±0.06	0.72±0.06	**1.43±0.09**	0.89±0.02	0.62±0.08	0.42±0.06	0.32±0.08
AA + PC (PS)	0.31±0.01	0.55±0.04	1.18±0.09	**1.59±0.04**	1.35±0.12	0.76±0.04	0.43±0.08	0.37±0.05	0.36±0.04	0.54±0.07	0.69±0.02	**1.09±0.02**	0.76±0.04	0.52±0.06	0.43±0.08	0.31±0.01

TV: T. viride; PC: P. chrysogenum; AA: A. aegerita; KP: Kiwi peels; PS: Peanut shells; n.d.: not detected

In Cao and co-workers' study [19], during the benzo[a]pyrene degradation in liquid culture by *L. theobromae,*123 U/L of LiP and 424.9 U/L of laccase were detected at the 4th day. The LiP and laccase activities increased during the initial period and started decreasing after the peak on the 4th day, when benzo[a]pyrene degradation starts. The changing enzyme activities agreed with the benzo[a]pyrene degrading rates, which suggested that those enzymes were involved in its degradation [19]. Three fungal strains were evaluated in this study. All of them were able to degrade fluorene, pyrene, chrysene and benzo[a]pyrene. The degradation potential was affected by pH, cocultivation and soil amendment. Agricultural residues used as a substrate support for fungi growth is known to induce or enhance the activities of ligninolytic enzymes such as laccase, MnP and LiP, and increase the degradation yields of PAH by fungi. Results obtained suggest the involvement of ligninolytic enzymes in PAH degradation. Moreover, the results indicated the importance of considering fungi, which are naturally present in soil, for degradation purpose of persistent pollutant.

4.2 Development of a packed bed reactor for the removal of aromatic hydrocarbons from soil using laccase/mediator feeding system

4.2.1. Laccase screening

A screening of laccase producing fungi was performed, aiming to produce high levels of laccase to be applied in PAH degradation. However, at same time priority was given to fungi typically found at contaminated site and not belonging to white rot or litter decomposing fungi, whose are known for higher laccase production. For that, three fungal strains were evaluated through SSF, using kiwi peels as substrate at pH 4.5 and 7.0. Lignin present and the polysaccharides in these materials act as growth and induction substrates, promoting a good fungal growth [72] and the production of extracellular enzymes [125], including laccase [126], while making the process more competitive [72].

Kiwi peels are an interesting residue from food processing industry due to their high availability and low cost [127]. Portugal producing 1.8 % (35,410 tons) of total global kiwi production per year. However, up to 30 % of that amount represent peels. Lignin content contributes to the insoluble dietary, inducing laccase production by the fungal species.

The higher enzymatic levels were obtained for *Trichoderma viride* (1.45 U/mL) at pH 4.5 for 3 weeks (Table 4.7). However, the co-cultivation of *Trichoderma viridae* with *Penicillium chrysogenum*

showed a similar production at pH 4.5 at 2nd and 3rd weeks, besides a better growth (see protein data). The co-cultivation of microorganisms is interesting due to the wide diversity of laccase that can be produced, with different properties and mode of action, which could be an advantage in the degradation of different PAH. Moreover, higher enzyme levels can be achieved with this approach [128]. Meehnian and team [129] also explored the co-cultivation strategy, using the white-rot fungi *Daedalea flavida* and *Phlebia radiata,* to enhance the production of ligninolytic enzymes, including laccase, during pretreatment of cotton stalks in SSF. Laccase production increased to 14.19 ± 0.85 IU g^{-1} with the co-culture, which was three- and eight-folds higher than the obtained by the monocultures of *D. flavida* and *P. radiata*, respectively.

Table 4.7 Laccase production by fungi

		Laccase activity (U/mL)			Protein (mg/mL)		
Strain	**pH**	**1 week**	**2 weeks**	**3 weeks**	**1 week**	**2 weeks**	**3 weeks**
IL	4.5	0.60±0.01	0.58±0.02	0.93±0.18	1.43±0.04	1.29±0.02	0.87±0.31
IL	7.0	0.53±0.09	0.41±0.07	0.40±0.09	2.08±0.12	1.12±0.09	2.18±0.35
IL+TV	4.5	0.46±0.08	0.42±0.09	0.94±0.03	1.58±0.11	0.88±0.03	1.59±0.17
IL+TV	7.0	0.42±0.22	0.58±0.06	1.10±0.15	2.90±0.03	0.70±0.18	0.79±0.29
PC	4.5	0.45±0.02	0.47±0.01	0.70±0.06	0.70±0.01	2.17±0.12	1.84±0.29
PC	7.0	0.98±0.09	1.16±0.04	1.17±0.09	3.48±0.04	2.34±0.01	1.19±0.20
PC+TV	4.5	1.04±0.07	1.48±0.07	1.39±0.03	3.72±0.17	1.78±0.04	2.80±0.36
PC+TV	7.0	0.53±0.08	0.54±0.03	1.06±0.11	0.87±0.20	1.91±0.03	2.40±0.42
TV	4.5	0.89±0.04	0.70±0.05	1.45±0.01	0.76±0.29	1.19±0.09	1.63±0.51
TV	7.0	0.54±0.01	0.27±0.04	0.52±0.05	2.37±0.36	3.49±0.11	4.08±0.22

IL: *Irpex lacteus;* TV: *Trichoderma viride;* PC: *Penicillium chrysogenum;* IL+TV: cocultivation of *I. lacteus* and *T. viride;* PC+TV: cocultivation of *P. chrysogenum* and *T. viride. The fungi were not able to grow in the IL+PC cocultivation.*

Although the pH is not controlled during most cultivations, the initial pH is usually set between 4 and 6 prior to inocula [130]. Thurston [131] reported higher laccase production when fungi were grown in a medium with pH 5.0. Optimal laccase production from *Trametes pubescens* was obtained between pH 3.0 and 4.5 [132]. Thus, the co-cultivation of the selected species (*Trichoderma viridae* and *Penicillium chrysogenum*) was used in this work at pH 4.5.

4.2.2 Laccase production

Laccase production was performed by SSF using Erlenmeyer flasks and PBR in different aeration rates (Table 4.8). There are several bioreactor designs used for SSF, but their major limits are heat and mass transfer in solid media [130]. In this work, a horizontal PBR with a jacketed glass column was used, where laccase production was performed with a constant air supply.

Table 4.8 Laccase production by co-cultivation of *Trichoderma viride* and *Penicillium chrysogenum* on kiwi-peels

SSF in lab flasks		SSF in packed bed bioreactor*	
Temperature (°C)	Laccase activity (U/mL)	Airflow (L min^{-1})	Laccase activity (U/mL)
RT	2.21±0,3	0.05	3.79±0.75
30	2.22±0.3	0.1	5.45±0.06
40	2.21±0.8	0.2	6.32±0.71

*SSF in packed bed bioreactor were performed at room temperature (≈ 26 °C).

The highest value of laccase production (6.32 U/mL) was obtained in PBR with an aeration rate of 0.2 mL/min, followed by 0.1 and 0.05 mL/min with a laccase production of 5.45 and 3.79 U/mL, respectively (Table 4.8). SSF in Erlenmeyer flasks were performed at different temperatures (room temperature, 30 °C and 40 °C), presenting no significant differences among the results of laccase production (2.21 U/mL). These assays aimed to assess the overall effect of temperature, as well as the overall effect of aeration on laccase production by SSF.

Oxygen supply showed to be one limiting factor for microorganism growth (data not shown), as well as for laccase production, another limiting factor in laccase production is temperature, being that the optimal temperature of laccase production diverse from one to another fungal strain. In the presence of light, a temperature of 25 °C is generally accepted as the optimum, but in dark conditions, the optimal temperature generally is 30 °C [12,131], reducing the production when fungi are cultivated at temperatures higher than 30 °C [133].

Rodríguez Couto et al. [134] tested several bioreactor configurations such as immersion, expanded-bed and trays, with nylon sponge and barley bran as support. The first two were supplied with humidified air in a continuous flow at 0.5 vvm and the tray reactor was kept at 90 % environmental humidity and passive aeration. Maximum laccase production by *T. versicolor* was reached between the 18th and the

20th day of cultivation, with 0.23, 0.13 and 0.34 U/mL, respectively. In another work, Rodríguez Couto et al. [135] achieved 6 U/mL of laccase by *T. pubescens* under SSF in temporary immersion bioreactor, using sunflower-seed shells as substrate and 60 L/h air- flowrate. Rosales et al. [136] tested laccase production in a fixed-bed reactor (FBR) and tray reactor on KOH pretreated orange peels by *T. hirsute.* In FBR laccase production reached 3 U/mL on 11th day and in the tray reactor, 12.7 U/mL were reached on the 23rd day. Rosales et al. [127] also reported laccase production on pretreated kiwi peels and ammonium supplementation by *Trametes hirsute,* attaining a value of 5.4 U/mL on the 13th day. Böhmer and co-workers [137] have reported laccase production by *T. hirsuta* in a modular bioreactor, under SSF on a mixture of pine wood chips and orange peels, achieving circa 0.7 U/mL and 1.1 U/mL in media supplemented with copper sulfate and xylidine, respectively. Another work has also reported the laccase production by white-rot fungi in a bioreactor under submerged fermentation [138].

Dependence of laccase activity on temperature and pH is presented in Figure 4.5. Maximal activity was reached at pH 5.0 and 30 °C. Similar optimal conditions were reported by Patel and Gupte [139] and Chenthamarakshan et al. [140] for laccase from *Tricholoma giganteum* and from *Marasmiellus palmivorus,* respectively, both produced under SSF. Regarding pH stability, the enzyme showed higher activity in more acid pH, i.e. 3.5–5.0, being significantly reduced at pH 5.5 and 6.0. Regarding temperature, activity decreased gradually for temperatures higher than 30 °C.

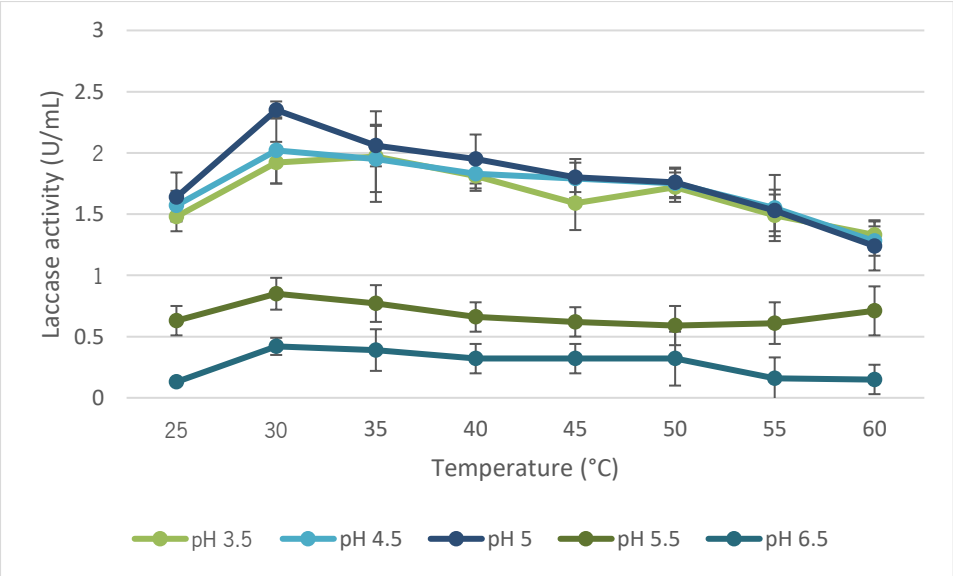


Figure 4.5 Laccase activity at different temperatures and pH.

The effect of temperature on laccase stability is showed in Table 4.9. The stability was higher at temperatures from 25 °C to 30 °C, remaining more than 70 % of the initial activity, after 5 days of incubation. At 35 °C, around 64.4 % and 42.6 % of the laccase activity were verified after 3 and 5 days of incubation, respectively. The enzyme showed low stability at 40 °C, presenting 35.2 % and 10.5 % of residual activity after 3 and 5 days of incubation, respectively. Laccase from *T. modesta* was stable at 40 °C for 180 min, with maximum stability at 50 °C (stable for 6 hours), but at higher temperatures (60 °C) activity decreases [141]. Laccase from wild and mutant *Shiraia sp.* was utterly stable at 35 °C for 30 h, but at 50 °C the stability gradually decreased (Du et al., 2015). It is important to highlight that in the current work, the stability was evaluated for longer incubation periods since the PAH degradation studies required a prolonged time of incubation.

Table 4.9 Effect of temperature on laccase stability at pH 5.0

Temperature	**Relative laccase activity (%)**		
(°C)	**0 days**	**3 days**	**5 days**
25	100	93.25±3.4	72.60±4.6
30	100	89.85±4.6	77.54±3.8
35	100	64.44±4.8	42.59±3.7
40	100	35.15±2.7	10.50±2.4

Regarding the pH effect, laccase showed high stability at pH 5.0, remaining with almost 80 % of the initial activity after 5 days of incubation (Table 4.10). At pH 4.0 and 6.0 the enzyme still presented ≈ 70 % of activity, while in pH 7.0, around 52 % of the initial activity was verified after 5 days of incubation. However, at pH 3.0 the relative laccase activity was significantly reduced to 27.1 % at the 5th day. Góralczyk-Bińkowska and coworkers [142] reported more than 80 % stability for laccase from *Nectriella pironii* at pH from 4.0 to 9.0, however, the stability was evaluated for only 3 h. The pH could affect the enzymatic activity by changing the electrostatic properties of the protein surface and reaction centre or by influencing the stability of the enzyme [122]. Considering the removal of PAH in contaminated soil, the high stability of the enzyme at room temperature at pH ranging from 4.0 to 7.0, is very important for its application in this proposal.

Table 4.10 Effect of pH on the laccase stability at 30 °C

	Relative laccase activity (%)		
pH	**0 day**	**3 days**	**5 days**
3.0	100	91.1±0.4	27.1±0.6
4.0	100	87.5±1.2	66.3±2.2
5.0	100	89.65±0.4	78.5±1.3
6.0	100	72.7±5.6	68.8±6.7
7.0	100	71.1±3.3	52.4±4.5

4.2.3 PAH degradation in soil

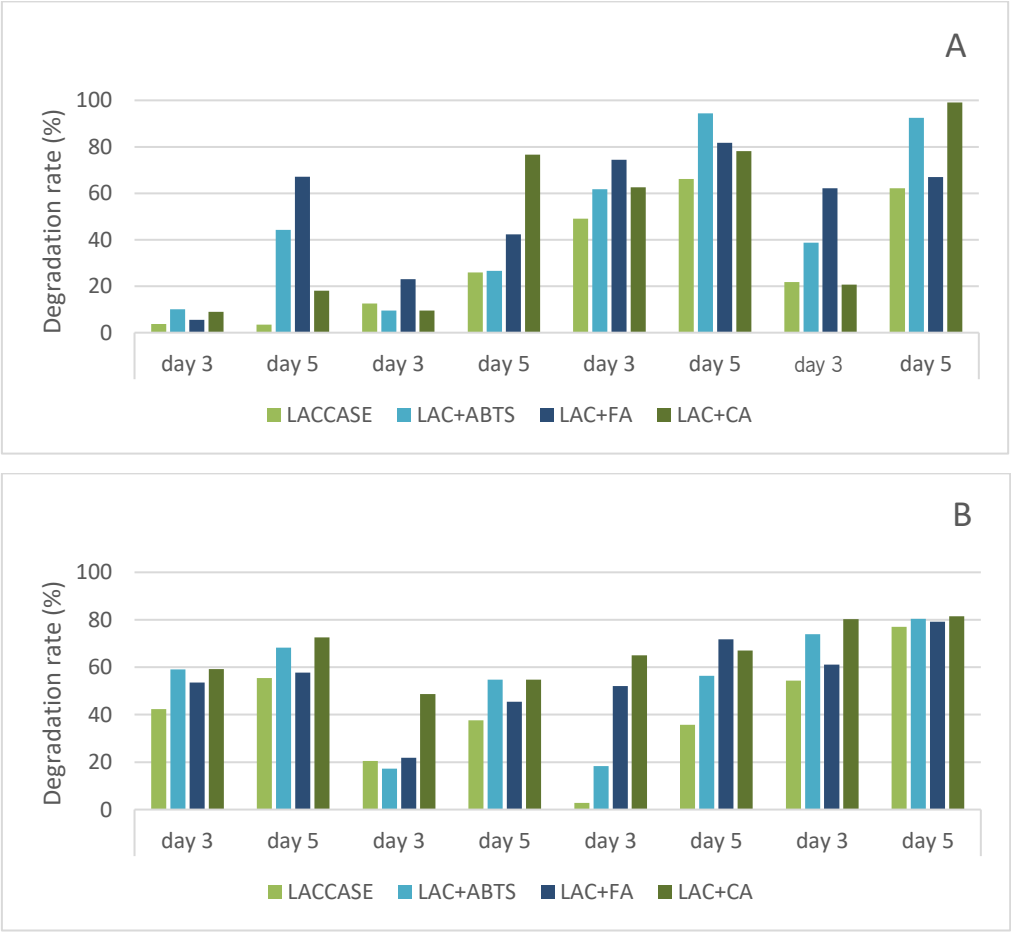
The role of different mediators in the degradation of PAH in soil by laccase was evaluated in batch (Erlenmeyer flasks) and in feed-batch in PBR. Laccase was capable of degrading the selected PAH and the degradation increased with the addition of a mediator. Results showed that the laccase obtained from co-cultivation of *Penicillium chrysogenum* and *Trichoderma viride* on kiwi peels with an appropriate mediator promotes the degradation of fluorene, anthracene, pyrene, chrysene and benzo[a]pyrene, in concentrations ranging from 100 ppm till 600 ppm, in soil.

Some of the relevant parameters determining laccase activity in PAH degradation is the mediator and pH, as well as the incubation temperature for maximal laccase activity [143]. In general, the mediators improve the degradation of PAH. Regarding temperature, a higher temperature is preferable for laccase catalysis, but it also leads to a faster loss of activity [144,145]. Thus, the use of room temperature may be advisable, mainly when applied to naturally contaminated environments. In this work, a higher degradation yield of PAH in soil was achieved at 30 °C when compared to 40 °C (data not shown). PAH degradation was assessed for 3 and 5 days in Erlenmeyer flasks, being the maximum reached within 5 days for all compounds (Fig. 4.6).

PAH molecules with higher hydrophobicity and more aromatic rings (see Table 3.1) present stronger sorption affinity [119]. Thus, these molecules diffuse into hydrophobic parts over time (ageing), decreasing their bioavailability to be degraded by microorganisms and/or their enzymes, and consequently hindering their reduction [146]. In the current work artificially, contaminated soil was used. So, the degradation rate of PAH in soil is expected to be higher than naturally contaminated soil, as observed by Huesemann and coworkers [120] for anthracene degradation in freshly spiked soil (72 %

degradation) and aged soil (34 % degradation).

In the current work, pyrene was the most degraded compound, achieving 99 % degradation with laccase mediated by *p*-coumaric acid, in the assay starting from 600 ppm of the compound (maximal degradation rate). Regarding the other mediators, 92 % of the compound was degraded with ABTS, 67 % with ferulic acid, and 62 % with just laccase (without mediator). Thus, LMS allowed an increment of more than 35 % in the degradation of 600 ppm of the compound. However, for ABTS and ferulic acid as mediators, the maximal degradation was achieved in the assays containing 300 ppm of the compound, with 94 % and 82 % degradation, respectively. Laccase (without mediator) was able to degrade 66 % of pyrene at 300 ppm in soil (Fig. 4.6 A).



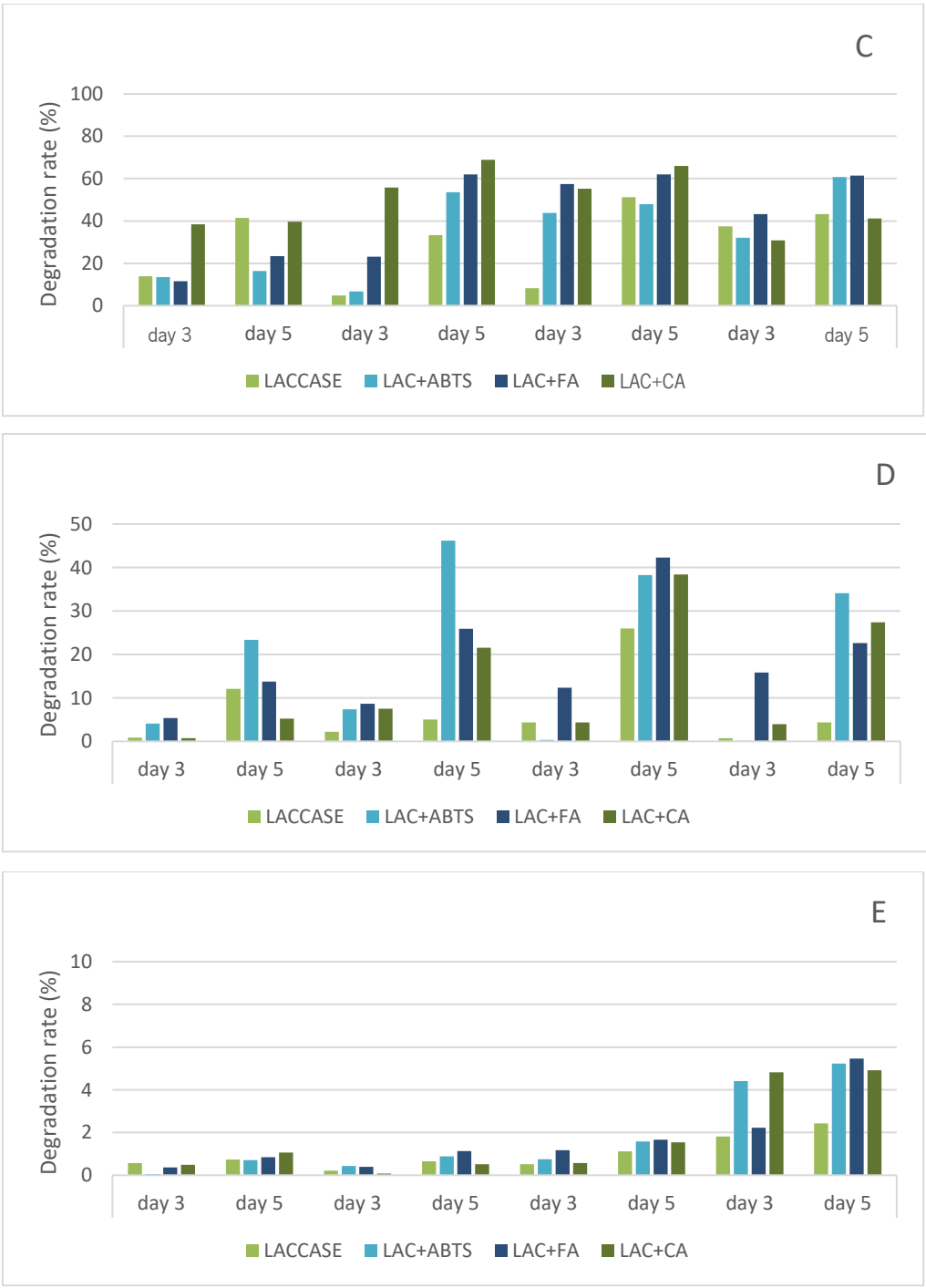


Figure 4.6 PAH degradation in batch by laccase with different concentrations (A) Pyrene, (B) Fluorene, (C) Chrysene, (D) Anthracene, (E) Benzo(a)pyrene. Degradation was performed at 30 °C in the dark, using an Erlenmeyer flask. The graphs are presented in different scales.

All mediators were effective for fluorene degradation, being the degradation slightly more effective with *p*-coumaric as mediator. Additionally, maximal degradation was observed in the assay with 600 ppm fluorene, in which the degradation reached 82 % with *p*-coumaric, 80 % with ABTS and 79 % with ferulic acid. Laccase (without mediator) was able to degrade 77 % of the pollutant (Fig. 4.6 B). The degradation

of chrysene was maximal with laccase mediated by *p*-coumaric, at 100 ppm of the compound, achieving 69 % degradation. The degradation decreased at higher concentrations, with maximal removal of 41 % at 600 ppm. Laccase mediated with ferulic acid was able to degrade 62 % of chrysene in all concentrations assayed. Maximal degradation with laccase plus ABTS (≈ 61 %) was observed at 600 ppm of the compound, while laccase without mediator degraded 51 % at 300 ppm (Fig. 4.6 C).

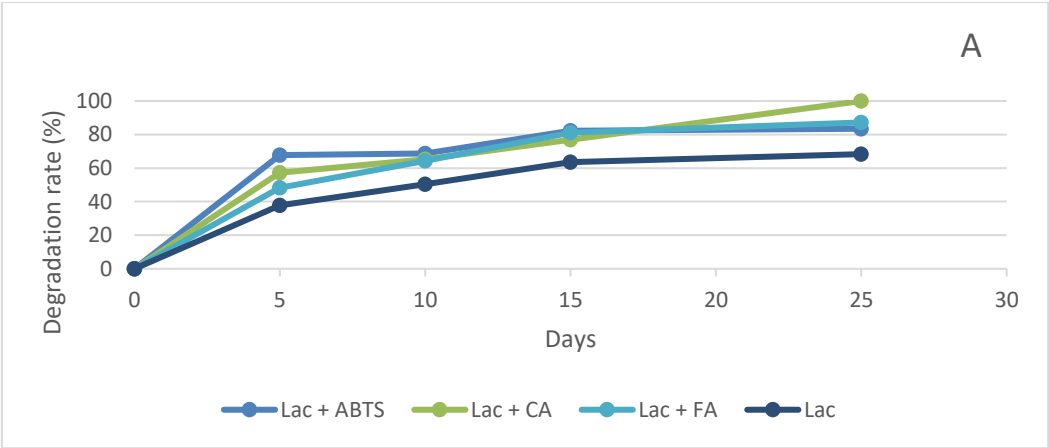
ABTS was the best mediator for anthracene degradation, with 46 % degradation at 100 ppm, while with ferulic acid and *p*-coumaric, 42 % and 38 % degradation, respectively, were achieved at 300 ppm of the compound. Only 26 % of anthracene was degraded by laccase in the absence of mediator, at 300 ppm. At 600 ppm, the degradation rate decreased significantly for laccase, as well as for most of LMS (Fig. 4.6 D). The lowest degradation was observed with benzo[a]pyrene. Only 5.4 % degradation was achieved with LMS, while laccase was able to degrade 2.4 % of the compound (Fig. 4.6 E). Li et al. [93] studied PAH degradation using laccase from *T. versicolor* in batch and verified higher degradation rates for anthracene (74.6 %) and benzo[a]pyrene (56.7 %) mediated with ABTS, except for pyrene in which only 7.5 % degradation was achieved. It is important to highlight that in this work, the authors used a high enzyme load (100 U laccase) and maintained a high humidity in the medium (70 %).

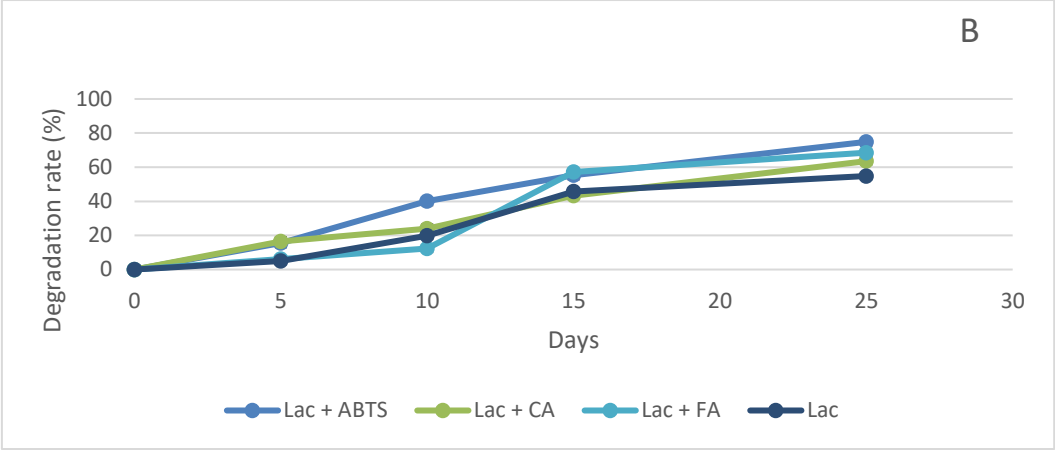
Regarding PBR trials, a laccase/mediator feeding was kept for 25 days and refreshed every 48 h, aiming to keep laccase activity levels throughout the process, in an attempt to improve the degradation. In general, the degradation of PAH reached in PBR was higher than in Erlenmeyer flasks, mainly for benzo[a]pyrene, except for fluorene, where a slightly lower degradation (≈ 7 %) was observed (Fig. 4.7). This result could be related to the heterogeneity of soil, as commented previously. However, it should be noticed that the higher moisture content of the soil at the end of the PBR trials (86 % in relation to 27 % at the beginning of assay) due to the constant and slow laccase feeding may have helped to improve the degradation processes. PBR has been successfully used for the degradation of other compounds such as dyes (Mosleh et al., 2018, 2017, 2016). For example, Mosleh et al. [147] used a rotating PBR for the degradation of pollutant dyes using photocatalyst compounds, as Cu-based nanoparticles, achieving over 90 % of degradation. They reported some benefits of using rotating PBR compared to conventional reactors such as high interfacial surface area caused by the rotational movement, which enhances mass transfer and mixing processes. In the current work, a PBR with a microperforated inner tube was used to allow a homogeneous and efficient degradation process.

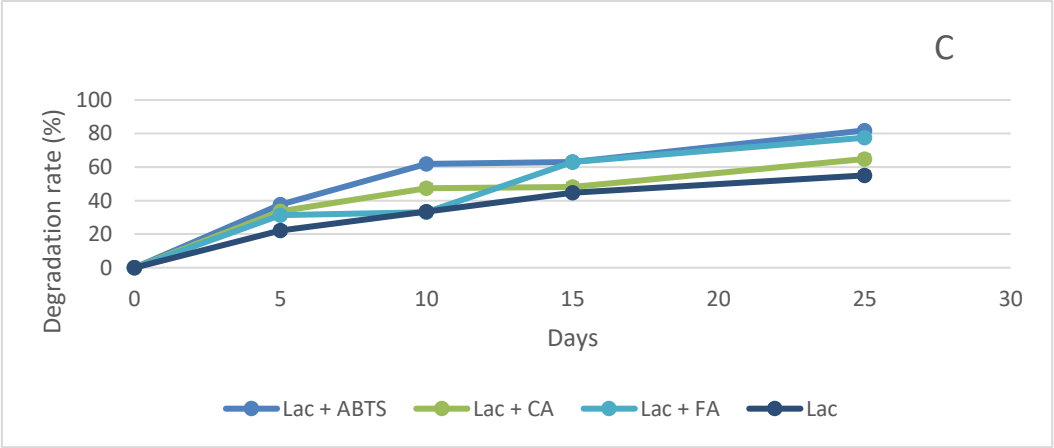
The most degraded compound was pyrene, 100 % degradation with *p*-coumaric acid as a mediator

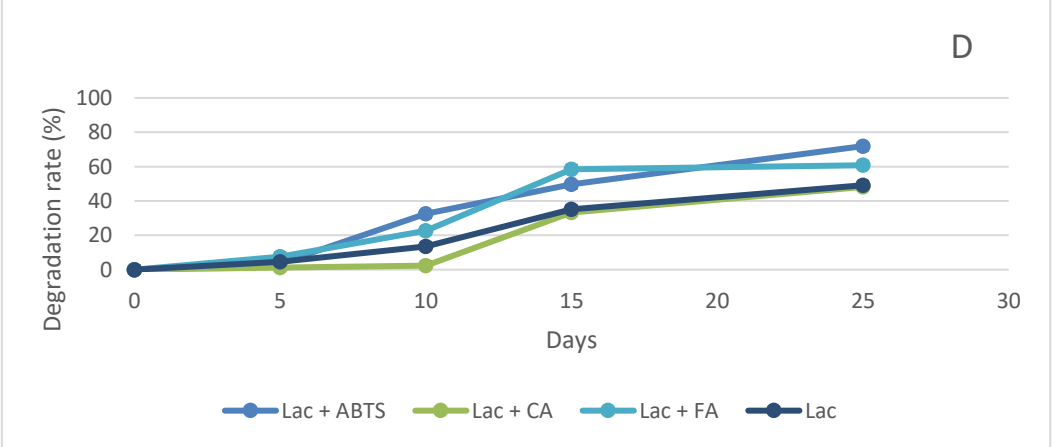
and laccase mediated by ferulic acid and ABTS reached 87 % and 84 % degradation, respectively (Fig. 4.7 A). Surprisingly, benzo[a]pyrene, the most carcinogenic, mutagenic and persistent PAH, was almost totally degraded by laccase mediated by ABTS (96 %) in the PBR system, although laccase with ferulic acid was not far behind, with 6% lower degradation rate (Fig. 4.7 E). This represents an increase of more than 90 % compared to the batch degradation of benzo[a]pyrene. Crucial factors affecting PAH degradation are their molecular weight and their bioavailability [149]. PAH with high molecular weight such as benzo[a]pyrene (see Table 3.1) was less degradable in batch probably due to the higher heterogeneity of the soil which is related to the lack of moisture. Li et al. [93] reported that LMS works actively in water environment or in soil with a high capacity of water retention as in slurry. The authors achieved 56.7 % of benzo[a]pyrene degradation in soil with laccase plus ABTS, as commented previously. They suggested that the relatively low degradation rate is probably related to the environment lacking water. Also Wu et al. [47] achieved 60 % of benzo[a]pyrene degradation in soil with laccase.

The degradation rate achieved for chrysene was 81.8 % with ABTS and 77.5 % with ferulic acid (Fig. 4.7 C). Similar results were achieved for fluorene, phenanthrene and anthracene. Decomposition of fluorene was constant over the entire period of the assay with laccase plus ABTS, achieving 75 % degradation. Degradation rates of 68.5% and 63.5 % were reached with laccase mediated by ferulic acid and *p*-coumaric acid, respectively, at the end of the process (Fig. 4.7 B). Anthracene started degrading after 5 days with ABTS or ferulic acid as mediators, but only started degrading after 10 days with *p*-coumaric acid, in parallel with phenanthrene, the less degradable pollutant in 25 days of the experiment. These molecules reached 72 % degradation in processes mediated by ABTS (Fig. 4.7 D). Phenanthrene also reached 72 % removal when mediated by ferulic acid, followed by ABTS and by *p*-coumaric acid with 60 % and 48 % degradation, respectively (Fig. 4.7 F).


A
Degradation rate (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Days
Lac + ABTS
Lac + CA
Lac + FA
Lac


B
Degradation rate (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Days
Lac + ABTS
Lac + CA
Lac + FA
Lac


C
Degradation rate (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Days
Lac + ABTS
Lac + CA
Lac + FA
Lac


D
Degradation rate (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Days
Lac + ABTS
Lac + CA
Lac + FA
Lac

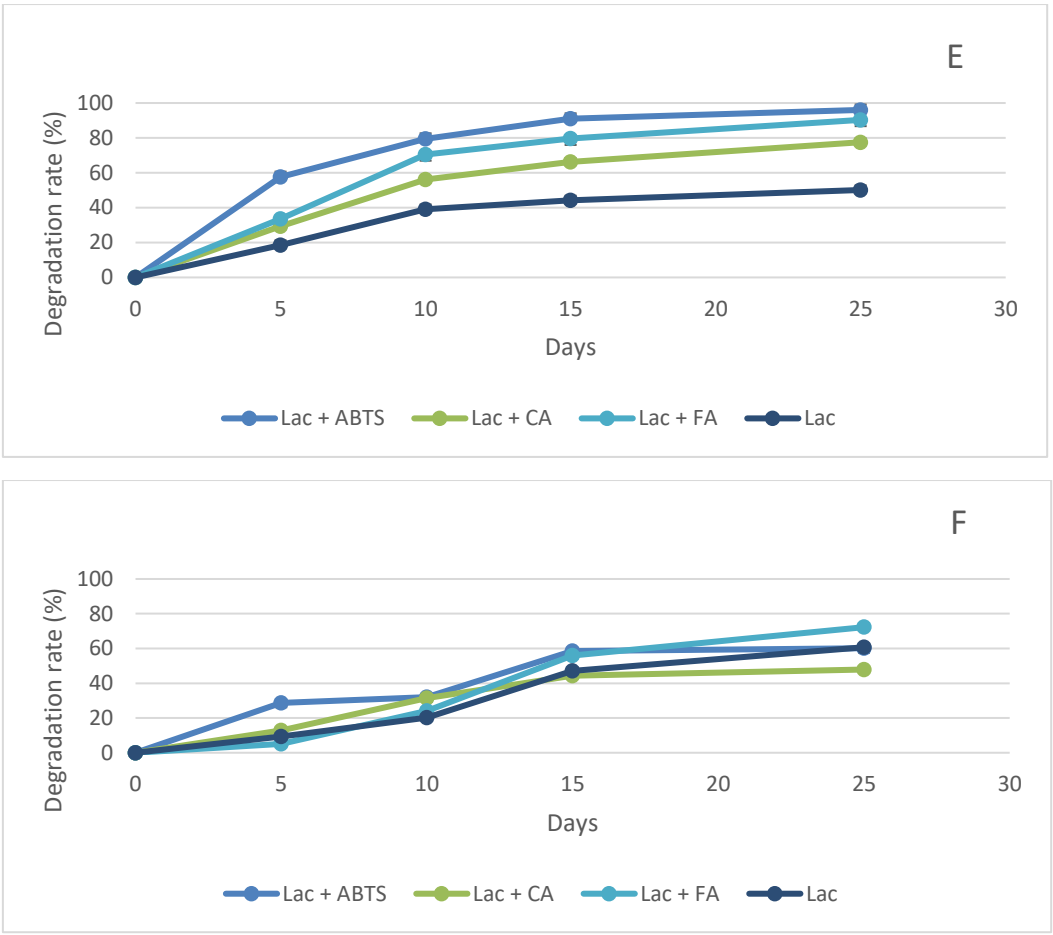


Figure 4.7 PAH degradation in soil in a packed-bed reactor by LMS. (A) Pyrene, (B) Fluorene (C) Chrysene, (D) Anthracene, (E) Benzo[a]pyrene, (F) Phenanthrene. Degradation was performed at room temperature in the dark for 25 days, using 300 ppm of total PAH.

Therefore, all mediators were effective for PAH degradation, achieving at least 72 % degradation. ABTS was found to be the best mediator for benzo[a]pyrene, chrysene, fluorene and anthracene with degradation of 96 %, 82 %, 75 % and 72 %, respectively. About 72 % of phenanthrene were degraded with laccase mediated by ferulic acid and in the case of pyrene 100 % degradation was reached with *p*-coumaric acid as mediator. Few works have explored the use of natural mediators for PAH degradation [150,151] and they were performed in liquid media instead of soil. Table 2.3 presents similar attempts in the area of PAH degradation by laccases and LMS compared to the results obtained in this study. It is also important to note that these degradation yields were achieved within 25 days (maximal degradation time considered). So, a higher degradation rate could be achieved with extended time or possibly with higher laccase loading and mediator.

It has been reported that the specific laccase catalysis is related to the ionization potentials (IPs) of the substrates; therefore, laccase can only oxidize compounds with IP less than 7.45 eV [152]. Thus, the most degradable compounds should be benzo[a]pyrene > pyrene ≥ anthracene > chrysene > fluorene > phenanthrene. In our work, the degradation yields were ordered as pyrene > benzo[a]pyrene > chrysene > fluorene > phenanthrene > anthracene in PBR. Not only IP values but also the solubility among PAH may have an impact on their degradation [152]. The solubility of PAH increase with higher temperatures, which in turn influences the bioavailability of PAH molecules [149]. The longer PAH stay in contact with soil, the stronger the sorption and the lesser the chemical and biological extractability of the contaminants becomes [149,153,154]. Li et al. [93] also reported that the theory of IP only partially justifies the observations. IP of each top degradable PAH (anthracene, benzo[a]pyrene and benzo[a]anthracene) was below 7.45 eV, but the remaining molecules were in a different order. More, pyrene with 7.43 eV was poorly degraded. Therefore, further studies were suggested for full elucidation of the catalytic mechanism of laccase. Han et al. [155] reported that phenanthrene was degraded only in the presence of 5 mM of ABTS or HBT as mediators for 5 U/mL of laccase purified from *T. versicolor* 951022, which was able to oxidize, respectively, 40 % and 30 % of 20 mg/L of phenanthrene in 2 h,.

Li et al. [93] observed the highest degradation rate of 1mg/L benzo[a]pyrene with laccase plus ABTS after 48 hours incubation, despite there was no significant difference between 24 and 48 hours. The experiment was carried out at 40 °C, pH 4.0 and 30 U of laccase. At the same time, the degradation in soil with 70 % moisture was tested. In 10 days, they observed 40.8 % of degradation with 10 U/g laccase and 56.7 % degradation by adding 1mmol/kg ABTS to the enzyme. Wu et al. [47] studied the degradation of PAH in contaminated soil, with different concentrations of laccase, i.e. 1, 3 and 10 U/mL, without any mediator. Regardless of the initial amount of enzyme, its activity gradually declined throughout incubation time till there was no enzyme activity left in any of the soil microcosm, i.e. after 14 days. Laccase transformed several PAH efficiently after 24 hours of incubation, being anthracene and benzo[a]pyrene the most degradable. The total PAH removal by enzyme bioremediation in soil microcosm, after 14 days, ranged between 67.6 % and 82.4 % [47]. In the current work, PAH degradation varied between 72 % and 100 % after 25 days of assay, with 2 U/mL of laccase and 1 mM of the mediator. The constant laccase/mediator feed in the PBR assays throughout the process allowed to work with constant activity levels, improving the degradation rates of PAH in soil. Data from this study confirm previous observations suggesting the importance of mediators in the oxidation of PAH. The most commonly used mediator, ABTS, is too expensive for practical soil remediation, and the toxic effects from using it for long periods are unknown for now. Fortunately, natural mediators are capable of competing

with the synthetic one, and in some cases good results can be obtained without a mediator, which suggests the possibility of other compounds present in the soil to enhance enzymatic oxidation.

4.3 Biodegradation of chrysene and benzo[a]pyrene and removal of metals in naturally contaminated soil by a *Trametes versicolor* strain and laccase produced thereof

4.3.1 Soil characterization and fungal screening

With the rapid industrialization and demand for crude oil in developing countries, toxic chemicals and metals are spreading and becoming a threat to the environment and the food chain. Most of the times, organic pollutants are present mixed with heavy metals. In Nigeria the negative effects of oil include pipeline leakages, indiscriminate dumping of hydrocarbon wastes, leakages from transporting vessels/vehicles moving all over the country, with different effects on environment and impacts on health [156]. Another contaminating factor is the open low temperature wastes burning on dumping sites, creating anthropogenic contamination from petrogenic and pyrogenic sources.

The physicochemical characteristics of the Nigerian soil under study are shown in Table 4.11. The major pollutants considered in the present study were HMW-PAH, mainly chrysene and benzo[a]pyrene, with 367.94 mg/kg and 11.74 mg/kg in samples, respectively. This soil is classified as sandy loam with 64 % of sand, 16.2 % of silt and 19.8 % of clay. The pH value of 8.3 ± 0.2 indicates that the soil is alkaline in the studied area, with 18 % of moisture at the beginning of the experiments. As expected, there were also 625.37 mg/kg of iron and 238.33 mg/kg of aluminum. The main reason for such high amounts is the location where the samples were collected, with high PAH and metal concentrations as a result of their accumulation from surface run-off, municipal and industrial waste discharges and aerial deposition from industrial pipes, probably with evident fluctuations depending on the dry and wet season cycles.

Oketola & Akpotu and Adeyi & Oyeleke research reported the formation of leachates from municipal solid waste dumpsites in Nigeria, containing high concentrations of metals, PAH and PCB, which are further distributed into soil, water and sea, and from there entering into food chain [157,158]. The improper handling of different residues contributes to the addition of metals and PAH to the top layer of soil. The efficiency of the degradation of polluting compounds in the soil differ randomly and is more complex than in liquid media, mainly due to the low bioavailability of substrates, and so there are several factors that need to be considered in the bioremediation approach. Adeyi and Oyeleke observed the migration of metals to the topsoil. In most cases, metal concentrations were higher in the topsoil, which

is evidence of recent anthropogenic contamination, with limited evidence of migration to the subsoil, which also indicates that there is little risk of groundwater contamination. At same time, it was also observed that concentrations of individual PAH were higher in soil at the 0–15 cm level compared to soil at the 15–30 cm level [157].

Table 4.11 Physicochemical characteristics of soil

Site	Value
pH	8.3 ± 0.2
MC, %	18 ± 0.013
TOC, %	0.86 ± 0.09
Total nitrogen, g/kg	0.043
Total phosphor, g/kg	0.03
Chrysene concentration, mg/kg	367.94 ± 2.45
Benzo[a]pyrene concentration, mg/kg	11.74 ± 0.68
Iron concentration, mg/kg	625.37 ± 4.17
Aluminum concentration, mg/kg	238.33 ± 1.55

One of the aspects of the bioremediation of soils contaminated by oil derivatives with autochthonous microorganisms is the isolation and identification of fungal strains from polluted soil in order to select the most active to degrade or remove the pollutants. In this sense, seventeen pure fungal cultures were isolated from the collected soil samples, and further screened for laccase production in 96-well microplate (Fig. 4.8 A). Among them, two rapidly showed greenish blue color (fungal strain 3 and 14), indicating laccase activity, and therefore they were selected for further laccase screening in plate (Fig. 4.8 B). The one with the highest laccase production (4.5±0.27 cm – determined through halo formation) was selected for the present study.

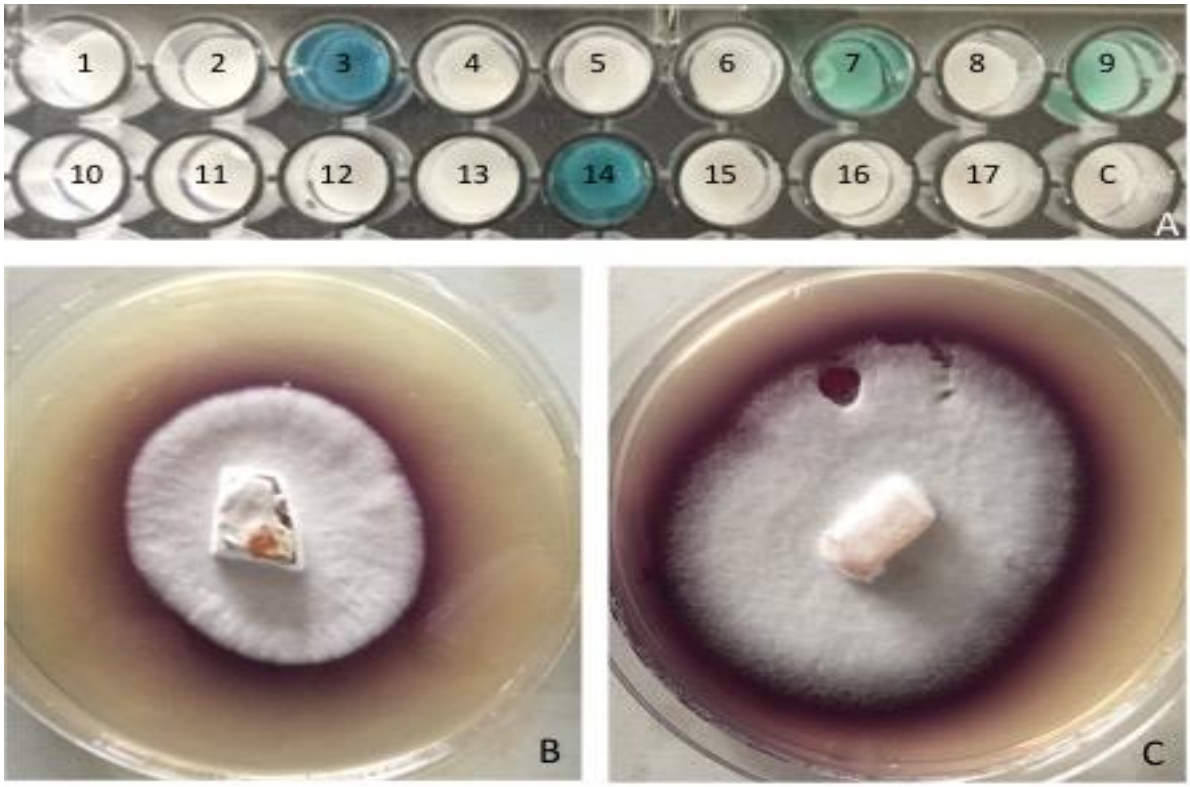


Figure 4.8 Laccase screening of fungi isolated from Nigerian soil. ABTS-impregnated discs for 17 isolated fungi (A), laccase plate assay on MEA with 20 mM ABTS and 100 mM $CuSO_4$ for fungal strains n° 3 (B) and n° 14 (C) that showed the highest laccase oxidation. Fungus n°14 was identified as *Trametes versicolor.*

4.3.2 Molecular identification of fungus

Molecular identification revealed 99.31 % sequence similarity of the fungus selected for this work with *Trametes versicolor* (Table 4.12). *Trametes versicolor* is well known by its ability to degrade different organic pollutants, as well as other white-rot fungi as *Phanerochaete chrysosporium*, *Cirnipellis stipitaria* and *Pleurotus ostreatus*. They have the ability to efficiently degrade most PAH using them as an exclusive carbon source [16,159]. It has been recognized that white-rot fungi degrade PAH by the synthesis of lignin modifying enzymes, as laccases. *Trametes versicolor* can secrete high levels of laccase and due to its oxireductive nature, this enzyme can oxidize various types of toxic chemical compounds into nontoxic ones [130], making *Trametes versicolor* an important contributor to bioremediation research. These enzymes usually catalyse the first step of PAH molecules degradation [41] *T. versicolor* has also been reported as relevant on biosorption studies of heavy metals [160,161].

Table 4.12 Basidiomycetes fungal strains showing similarity with isolated *Trametes versicolor*, on their 18S rRNA sequence.

Nº	Strain	Accession No.	Similarity (%)
1	*Trametes versicolor* CFMR FP-135156	NR_154494.1	99.31
2	*Megasporoporia bannaensis* BJFC Dai 12306	NR_120300.1	87.62
3	*Ganoderma sandunense* GACP 18012501	NR_164049.1	86.96
4	*Ganoderma meredithae* CBS 271.88	NR_164435.1	86.71
5	*Theleporus membranaceus* IFP Dai 12075	NR_119985.1	86.64
6	*Ganoderma destructans* CBS 139793	NR_132919.1	86.16
7	*Ganoderma weixiensis* HKAS 100649	NR_166271.1	85.84
8	*Ganoderma nasalanense* GACP 17060211	NR_164048.1	85.64
9	*Perenniporia macropora* IFP Zhou 407	NR_158795.1	85.54
10	*Mycoleptodonoides sharmae* BSHC KD-11-122	NR_164221.1	85.24

4.3.3 Evaluation of laccase production

Laccase production was performed by SSF using Erlenmeyer flasks, at room temperature, for 6 weeks and assessed by weekly sampling. The highest amount of laccase detected on plantain peels was 38.8 U/g after 3 weeks of fermentation (Figure 4.9). Plantain peels represent a local agricultural waste in Nigeria and such residue contains polysaccharides and phenolic compounds that can stimulate both the fungal growth and subsequent laccase production. Each kilogram of dry plantain peels contains 6 – 10 % of protein, 6 – 12 % of ash, 2 – 6 % of lipids, 11 – 39 % of starch and 33 – 43 % of total dietary fibre [162]. It also has high content of cellulose (131.7 g/kg), hemicellulose (46.1 g/kg) and lignin (17.1 g/kg), with the main individual components being mannose (293 g/kg) and uronic acid (172 g/kg) [163]. Osma and co-workers achieved 63 U/L of laccase within 3 days of fermentation and 1570 U/L within 20 days, by cultivation of *Trametes pubescens* on banana peels (7 g of substrate with 20 ml of culture medium) [164].

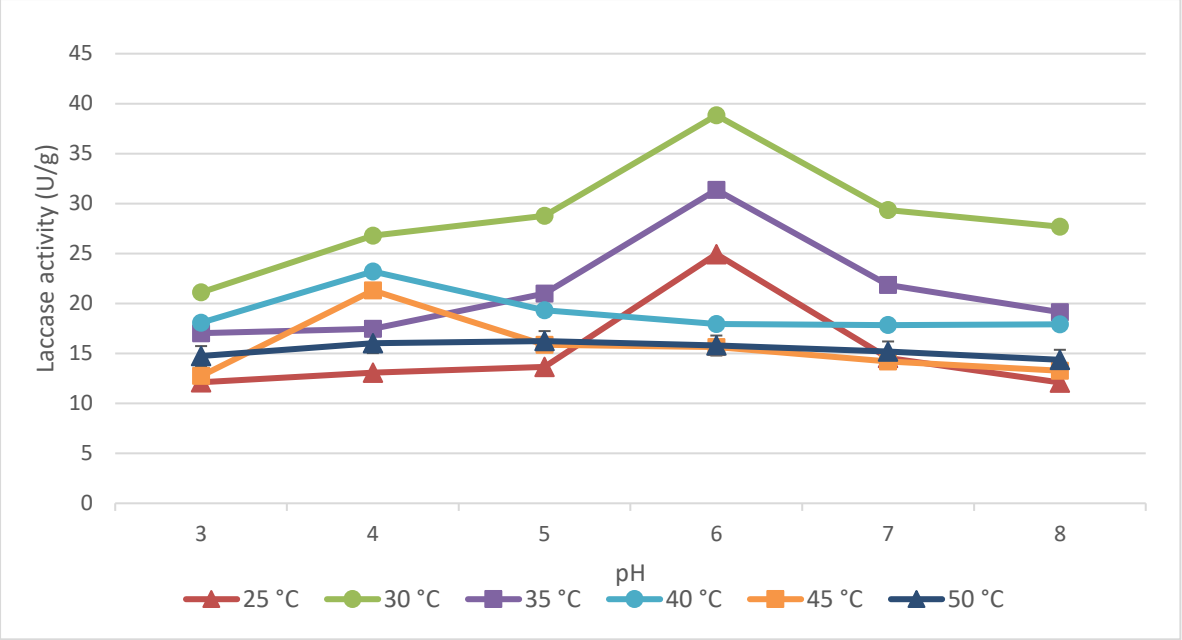
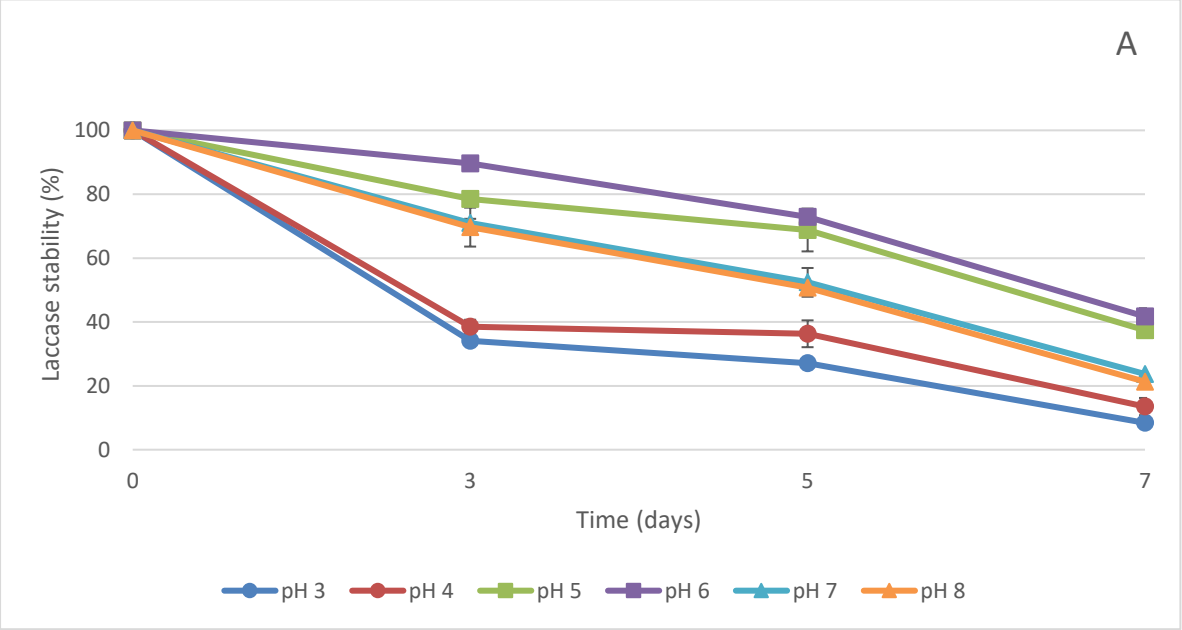


Figure 4.9 Laccase activity at different temperatures and pH. *Trametes versicolor* was cultivated in SSF for 21 days, using plantain peels as substrate.

The dependence of laccase activity on temperature and pH is shown in Figure 4.9. Maximal laccase activity was reached at pH 6 and 30 °C. A number of reports have indicated that the optimal pH for fungal laccase activities varies from 3 to 7, depending on the fungal species, implying that laccase remediation is unsuitable for alkali soil [93,165]. However, it is important to highlight that the microorganisms isolated from contaminated environments are capable of degrading PAH due to their increased cell affinity to hydrophobic substances that enable them to absorb and utilize the PAH and accumulate heavy metals. They are also capable of producing a variety of enzymes, including laccase, lignin peroxidase and manganese peroxidase, which transform PAH and heavy metals into less harmful and simpler forms [79,166–169].

An interesting observation was that the produced laccase reached its maximal activity at pH 6 for temperatures between 25 °C and 35 °C, but at higher temperatures the optimum pH was 4. Enzyme showed higher activity at lower temperatures at less acid pH, but at higher temperatures it preferred a more acid pH. One of the limiting factors in laccase production is temperature. In the presence of light, the temperature of 25 °C is generally accepted as optimal, but in dark conditions the best temperature is generally 30 °C [12,38,131], reducing the production when fungi are cultivated at temperatures higher than 30 °C [133]. However, the optimal temperature and pH for laccase production diverse from one to another fungal strain.

The effect of temperature and pH on laccase stability was observed for 7 days (Figure 4.10), since pollutant degradation assays by laccase were performed in such a period of time. Regarding the enzyme stability as a function of temperature, it was higher at 30 °C, with 54 % of activity remaining after 7 days, followed by 25 °C and 35 °C with 39.4 % and 42.3 % of activity, respectively, after 7 days. With increasing temperature, stability decreases, presenting 23.1 %, 7.2 % and 4.9 % of laccase activity at 40 °C, 45 °C and 50 °C ,respectively, after 7 days. Laccase showed the highest stability at pH 6, followed by pH 5. The stability decreased during the incubation time. After 7 days there were still 41.7 % and 37.4 % of residual activity at pH 6 and pH 5, respectively. The highest reduction was observed at pH 3 and 4, with 8.4 % and 13.5 % of residual activity, followed by pH 7 and 8 with 23.7 % and 21.3 % of activity, respectively. Considering the removal of PAH and heavy metals in contaminated soil, the high stability of the enzyme at room temperature, as well as in pH from 6.0 to alkaline, is particularly important for its application herein foreseen, as soil pH was 8.3 ± 0.2.



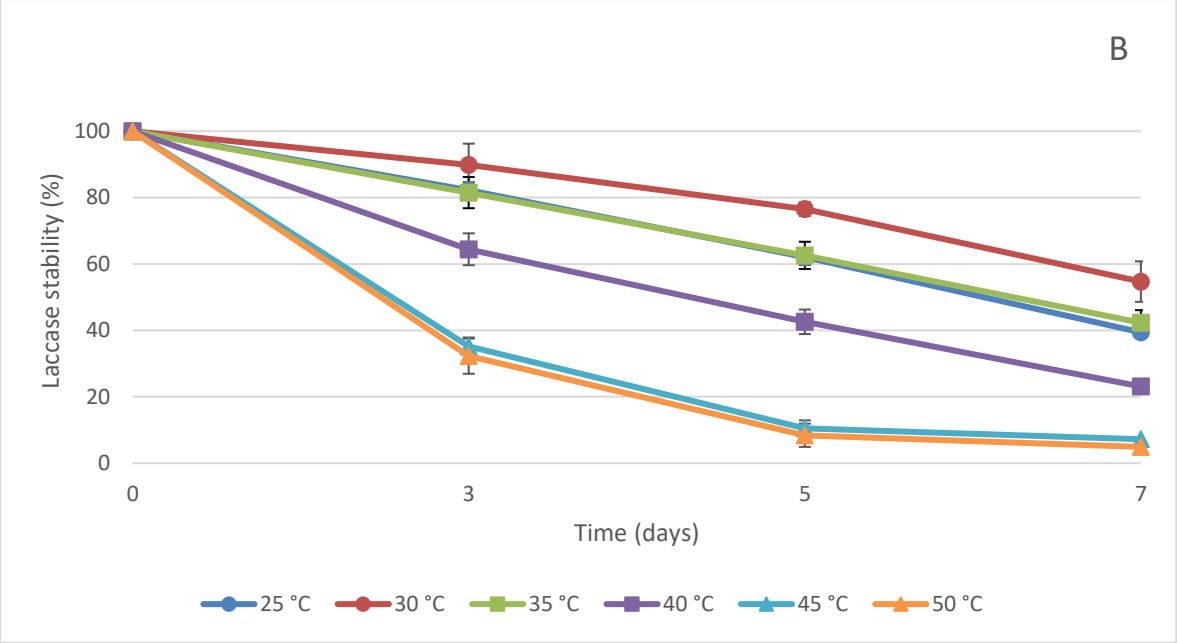


Figure 4.10 Effect of pH (A) and temperature (B) on the relative laccase activity (%). The temperature and pH stabilities were performed at 30 ℃ and pH 6, respectively.

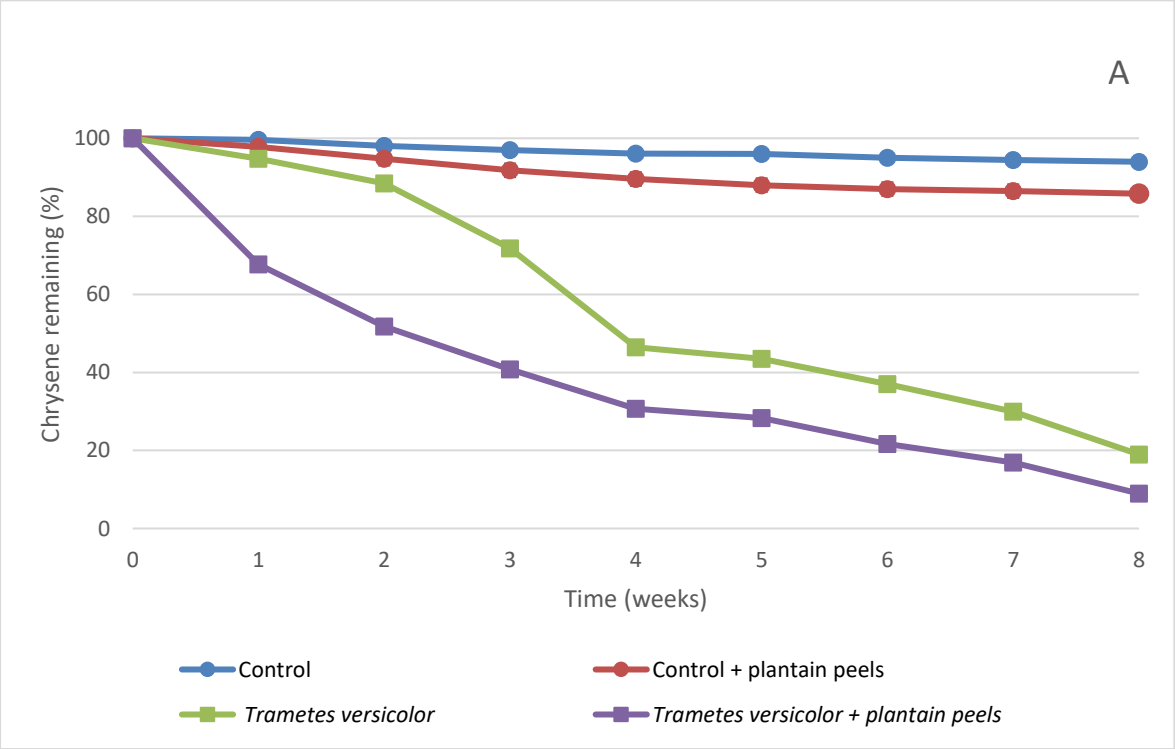
4.3.4 PAH degradation and metals reduction

4.3.4.1 Fungal treatment

The longer PAH remain in contact with the soil, the stronger is their degradation and the lower is the chemical and biological extractability of the contaminants [149,153,154]. Biodegradation of PAH using microorganisms has been proven to be an efficient way to degrade PAH into less toxic forms. This method is relatively cheap, easily managed, and eco-friendly. However, the presence of organic and inorganic contaminants on the same site can affect the efficiency of bioremediation.

Figure 4.11 presents the degradation of chrysene (A) and benzo[a]pyrene (B) in nonsterile soil by *Trametes versicolor*, with and without plantain peels as support. Around 81 % of chrysene was degraded only by *T. versicolor,* and using plantain peels as support, the degradation increased to 91.0 % in 8 weeks assays (Fig. 4.11 A). Benzo[a]pyrene was less degradable. In 8 weeks, 38.0 % degradation was reached only with *T. versicolor* and 49.1 % with the support (Fig. 4.11 B). The degradation rates by *T. versicolor* over time showed that the fungus was capable to start degrading PAH from the first week when plantain peels were used as support, mainly because of support-induced laccase production. When no support was used, the fungus took 2 weeks to start degrading the compounds. Biache et al. [170] also reported

a higher degradation rate for chrysene than for benzo[a]pyrene by the microbial community, which is probably related with its lower molecular weight. Borràs et al. [51] observed similar degradation rates with *T. versicolor*, which was capable of a faster and more extensive removal of PAH in artificially spiked soil, despite its weaker growth. The removal of a total of 16 priority PAH from USEPA by *T. versicolor* was 49 % in 10 weeks. Rama et al. [52] obtained similar results with degradation of 16 priority PAHs from USEPA, which was 38 % in 20 weeks. However, their study was carried out with industrially contaminated soil, using peels of agricultural residues for cultivation. On the other hand, Baltrons et al. [171] have reported that the biodegradation of PAH of 3 and 4 rings (phenanthrene, fluoranthene and pyrene) was lower, as the concentration of metals increased, but no important effect on the biodegradation of HMW-PAH (benzo[b]fluoranthene and benzo[a]pyrene) was observed at the different concentrations of metals studied.



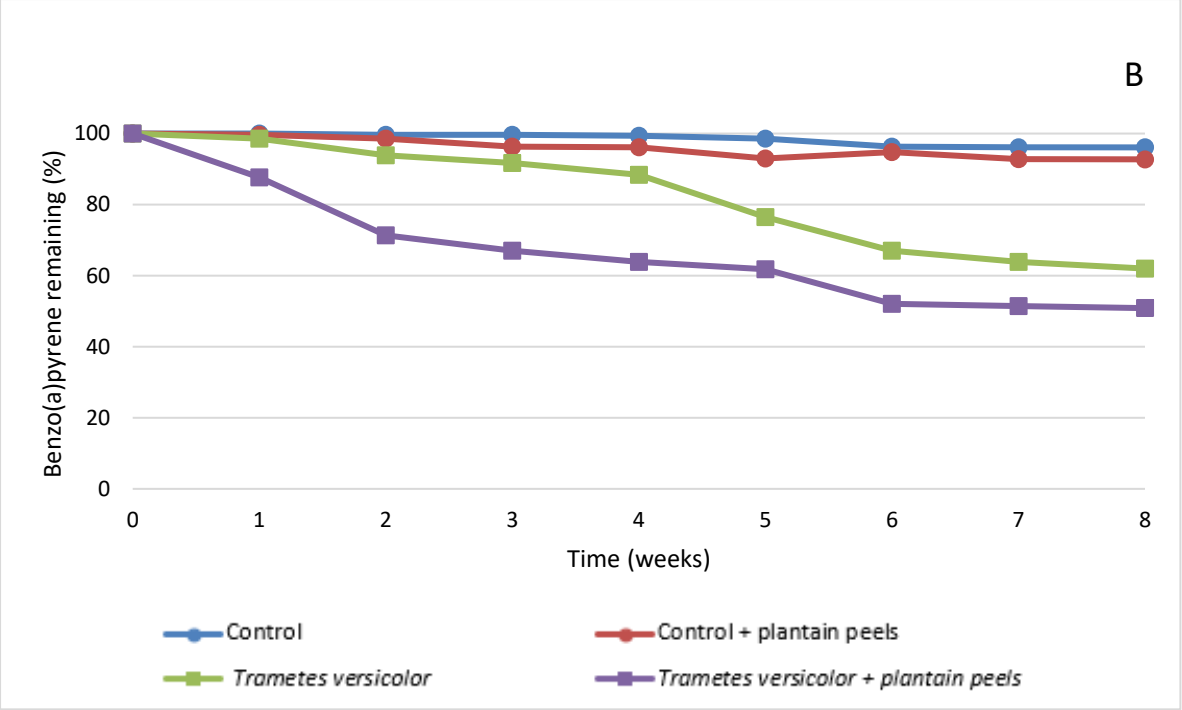


Figure 4.11 Degradation of chrysene (A) and benzo[a]pyrene (B) in batch by *Trametes versicolor* with and without plantain peels as support at room temperature for 8 weeks

Aluminium and iron load sorption by *T. versicolor* are presented in Figure 4.12. In 8 weeks, it was capable of accumulate 46.1 % of aluminium and 57.22 % of iron. By adding plantain peels, these amounts increased 2.04 % and 4.61 %, respectively.

Bamforth and Singelton [12] reported that some metals may be too toxic for white-rot fungi and may have a negative effect on the activity of their ligninolytic enzymes. However, many of these metals naturally exist in soil in trace concentrations. *T. pubescence* was able to withstand 1000 mg/L of Pb and Ni, removing 99 % of Pb and 8.6 % of Ni [169], while *T. versicolor* was able to adsorb almost 0.300 mg/g of Cd from contaminated effluent [160]. Biosorption of Cu, Pb and Zn by immobilized *T. versicolor* was also reported by Bayramoğlu and co-authors [161].

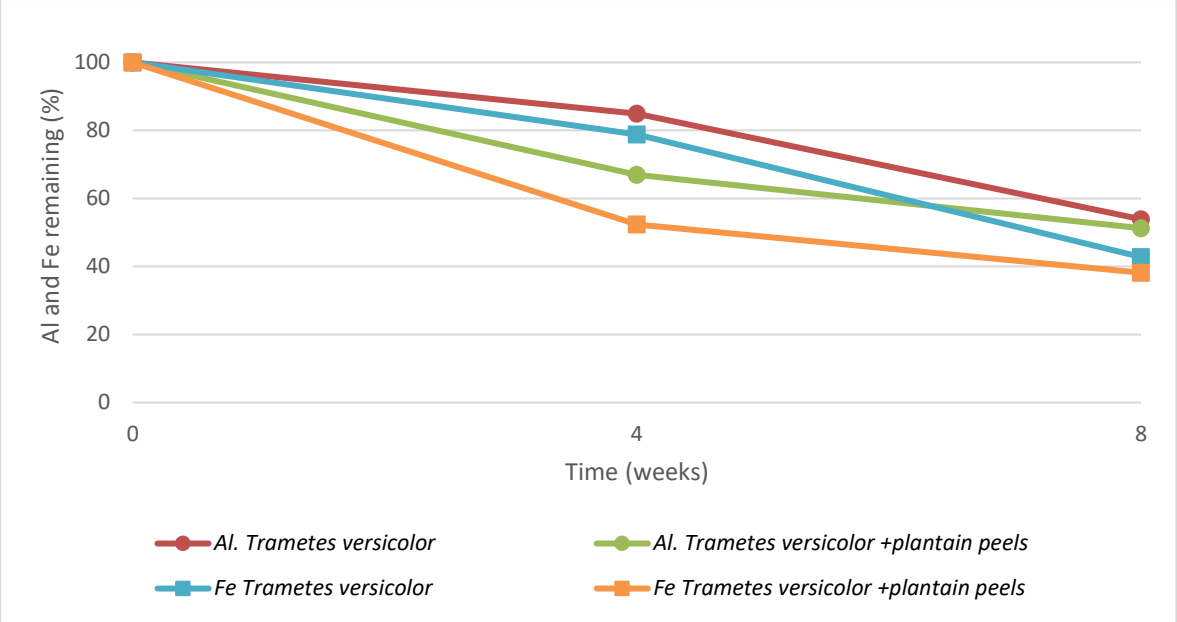


Figure 4.12 Iron and aluminium sorption in batch by *T. versicolor* with and without plantain peels as support at room temperature for 8 weeks

4.3.4.2 Laccase treatment in batch

Enzymatic treatment of contaminated soil may be considered as an alternative and/or as a supplement to microbial bioremediation. Its main advantages include high reaction activity, low sensitivity to high pollutant concentration, coverage of a wide range of physicochemical gradients in the environmental matrix, therefore being easy to control. PAH degradation by ligninolytic enzymes produced by white-rot fungi such as laccases has been reported by other authors [89,90,172,173]. However, there is still a limited number of published reports dealing with enzymatic remediation of soil, mainly due to the high cost of large-scale production of commercial laccase and to the high amount needed. Utilizing bio-wastes to produce an enzyme can reduce the production costs while generating high concentrations of products [174]. Agricultural wastes contain polysaccharides and phenolic compounds that might stimulate fungal growth and enzyme production.

The efficiency of laccase action was evaluated in batch using two laccase loads (Figure 4.13). After 7 days, the highest chrysene degradation rate was obtained with 6 U/g of laccase with 57.98 % of degradation, which turns to 55.6 % when using 2 U/g (Fig. 4.13 A). The most efficient benzo[a]pyrene degradation was achieved with 2 U/g with 8.7 % of degradation, followed by 6.5 % with 6 U/g (Fig. 4.13 B).

Wu et al. [47] studied the effect of 3 different initial concentrations of laccase DAIWA Y120 (from *Trametes*, obtained by Amano Enzyme Inc) (1, 3 and 10 U/g) on the degradation of 15 PAH in soil with initial total concentration of 10,834.65 μg/kg, during 14 days. After 1 day of incubation, anthracene and benzo[a]pyrene were the most degradable of the 15 PAH. The benzo[a]pyrene with 1, 3 and 10 U/g of laccase was degradable in 49 %, 57 % and 60 %, respectively. After 14 days, laccase activity was not detected in the soil samples and during this time the degradation of total PAH was 17.6 % with 3 U/g, 32.4% with 1 U/g and 31 % with 10 U/g.

The obtained data indicate that laccase transforms PAH efficiently with low initial laccase load, thus the PAH degradation was evaluated using 2 U/g of laccase in combination with 0.5, 1 and 2 mM of mediators (Table 4.13). The highest degradation was observed with 0.5 mM of ABTS and ferulic acid, and with 1mM of coumaric acid. After 7 days, the highest degradation rate achieved for chrysene was 67.4 % using ABTS as a mediator. It was 11.8 % higher than using only laccase, being this improvement on degradation of 9.6 % and 9.8 % with ferulic acid and coumaric acid, respectively.

Benzo[a]pyrene was degraded in 8.7 % with only laccase and by adding 0.5 mM of ABTS and ferulic acid, its degradation increased by 4.9 % and 1.6 %, respectively. However, with the increase in the molarity of these two mediators, the degradation was lower than with laccase alone. For coumaric acid, the lowest degradation ratio was observed with 0.5 mM of this mediator, yet with 1 and 2 mM there was no significant difference in the benzo[a]pyrene degradation, this being 2.1 % and 1.9 % higher than when using only laccase.

Table 4.13 Removal (%) of chrysene and benzo[a]pyrene with 2 U/g laccase and different concentrations of mediator

	Chrysene			Benzo[a]pyrene		
	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7
Laccase	33.73 ± 0.35	43.90 ± 0.40	55.58 ± 0.35	5.41 ± 0.42	6.40 ± 0.67	8.65 ± 0.64
L+ 0.5mM ABTS	37.42 ± 0.43	46.89 ± 0.33	67.41 ± 0.14	4.68 ± 0.84	10.37 ± 0.46	13.60 ± 0.15
L+ 1mM ABTS	26.32 ± 0.13	34.68 ± 0.04	49.94 ± 0.03	2.15 ±0.19	4.73 ± 0.52	8.38 ± 0.42
L+ 2mM ABTS	19.66 ± 0.4	50.84 ± 0.16	53.35 ± 0.02	3.91 ± 0.45	5.20 ± 0.51	6.23 ± 0.14
L+ 0.5mM FA	32.69 ± 0.02	53.90 ± 0.03	65.15 ± 0.03	8.16 ± 0.62	8.59 ± 0.19	10.20 ± 0.52
L+ 1mM FA	16.33 ± 0.23	31.94 ± 0.21	37.00 ± 0.23	4.32 ± 0.25	5.12 ± 0.09	6.21 ± 0.31
L+ 2mM FA	14.55 ± 0.04	19.58 ± 0.42	26.79 ± 0.03	2.91 ± 0.16	3.50 ± 0.19	6.87 ± 0.41
L+ 0.5mM CA	9.23 ± 0.53	17.95 ± 0.04	24.23 ± 0.12	0.57 ± 0.32	2.23 ± 0.32	3.94 ± 0.52
L+ 1mM CA	26.81 ± 0.44	53.34 ± 0.402	65.36 ± 0.03	3.12 ± 0.31	8.43 ± 0.21	10.75 ± 0.74
L+ 2mM CA	51.07 ± 0.42	57.20 ± 0.42	58.60 ± 0.03	7.11 ± 0.71	8.04 ± 0.41	10.60 ± 0.71

Some of the relevant parameters determining laccase activity in PAH degradation are the mediator and pH, as well as the incubation temperature for maximal laccase activity [143]. A higher temperature is preferable for laccase catalysis, but it also leads to a faster loss of activity [144,145]. In general, mediators improve the degradation rates of PAH. However, the most commonly used synthetic mediators such as ABTS and hydroxybenzotriazole (HBT) are expensive and after a long-term usage, toxic effects might occur. Li et al. [93] also reported that LMS works actively in water environment or in soil with high content in water as in slurry. Experiments were carried out with 30 U/g of laccase plus ABTS at 40 °C, pH 4.0 and the degradation of 1 mg/L benzo[a]pyrene after 24 hours was approximately 60 % of the original value. At the same time, the degradation of such compound in soil with 70 % moisture and 10 U/g of laccase was assessed. After 10 days, 40.8 % degradation was confirmed, which increased to 56.7% by adding 1 mmol/kg ABTS.

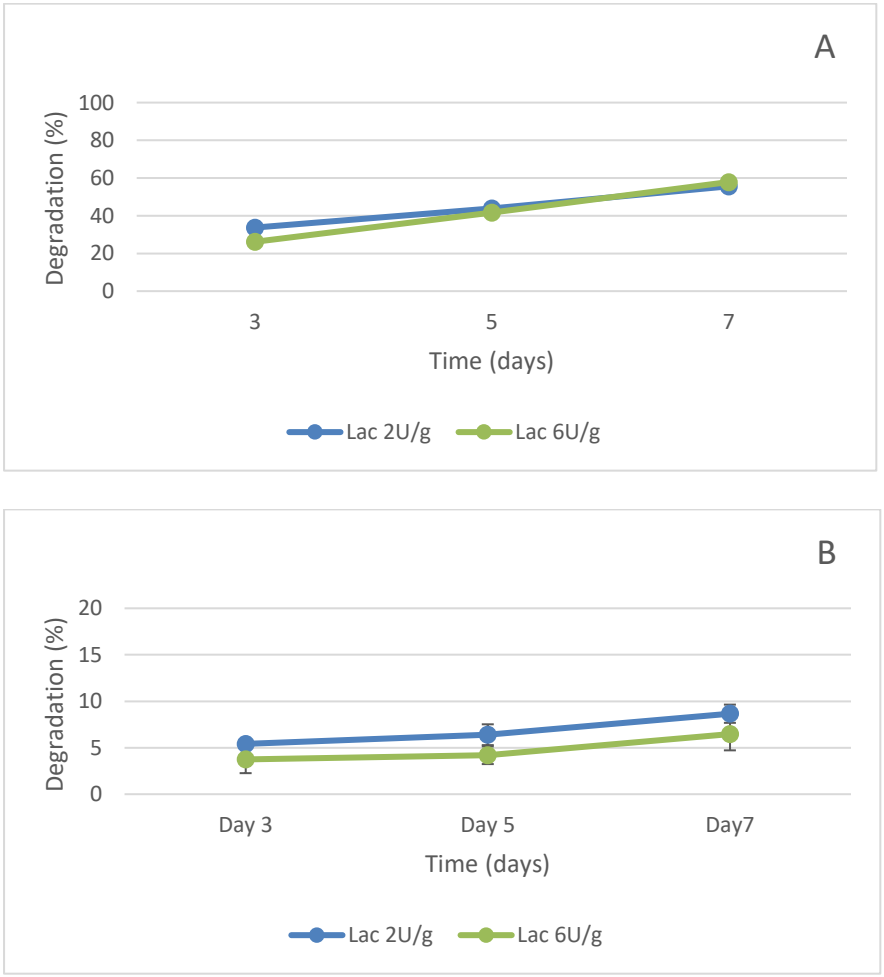


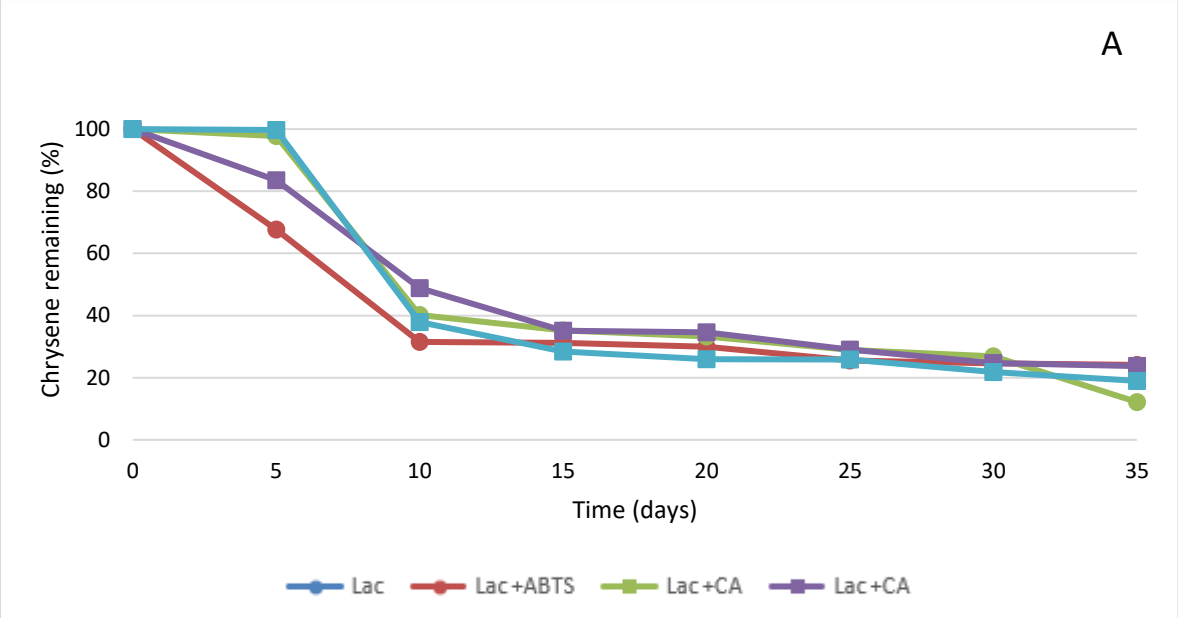
Figure 4.13 Degradation of chrysene (A) and benzo[a]pyrene (B) with different initial laccase loads.

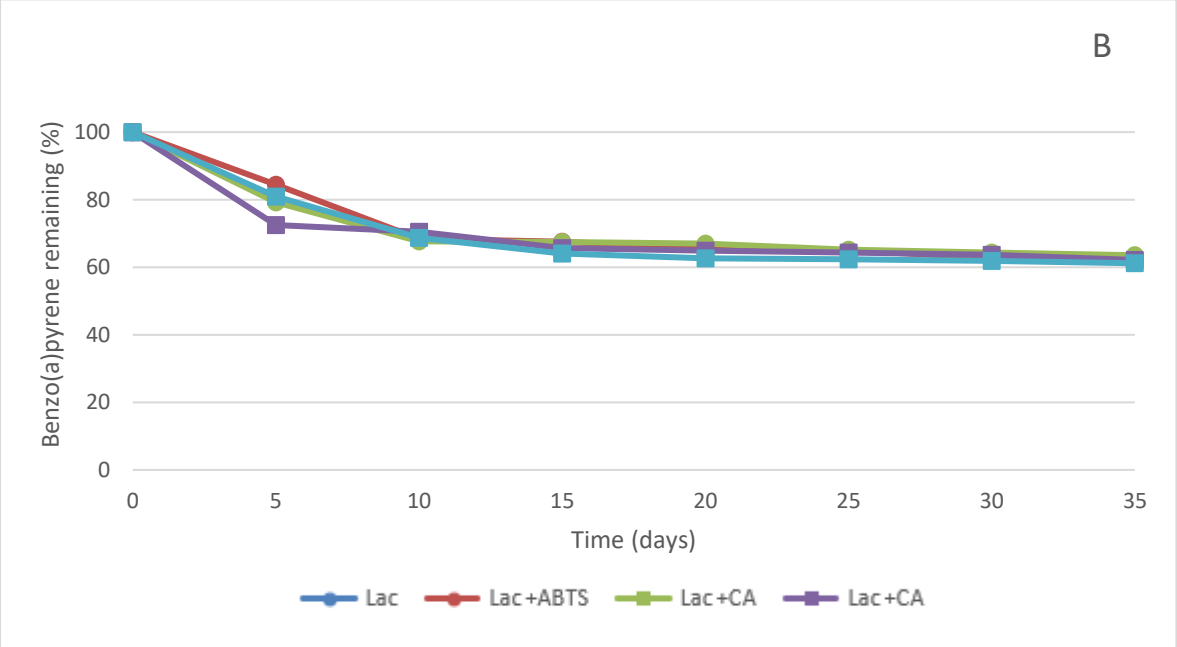
4.3.4.3 Laccase treatment in PBR (fed-batch)

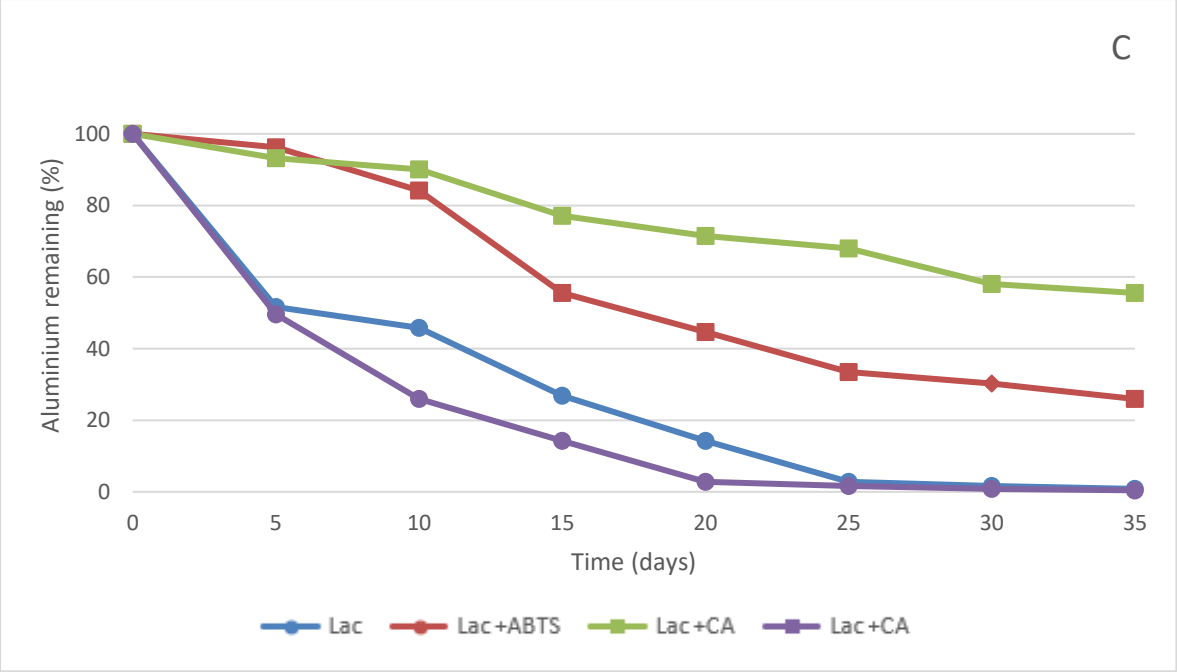
As in batch set-up, also in PBR laccase starts transforming PAH immediately after entering in contact with the soil (Figure 4.14), even if the mixture of soil and laccase differs from the first approach. It is possible to prepare a slurry in batch, but column assays aim to mimic a microcosm under field conditions. Therefore, 300 g of soil in a fixed bed column were daily fed with 21.6 mL of crude laccase, with and without mediator. Degradation was rapid in the first 10 days and then started to slow down, despite fresh laccase was used every 5 days.

Laccase removed 68.5 % of chrysene in the first 10 days and, in total of 35 days, 75.9 % till the end of the experiment. Despite the fact that laccase with ABTS was capable of removing more chrysene, 87.9 %, by the end of the assay, the process was slower during the whole period. By adding ferulic acid, 81.0 % of chrysene was removed and with coumaric acid 76.3 % removal was reached (Fig. 4.14 A). The degradation of benzo[a]pyrene was similar with and without mediator. Laccase degraded 35.6 % of this molecule, while with ferulic acid, coumaric acid and ABTS as mediators the degradation changed to 38.8 %, 37.9 % and 36.5 %, respectively (Fig. 4.14 B), showing a limitation in benzo[a]pyrene degradation by laccase and LMS, one of the most recalcitrant and toxic PAH. Moreover, soil used in this assay was heavily contaminated, so it is possible that other compounds might compete in the degradation pathway or even inhibit the activity of laccase.

Laccase achieved, per se, the highest removal rates for iron. In 35 days, 99.2 % of iron was removed by laccase, reducing to 90.2 % when mediated with ABTS, 76.1 % with coumaric acid and 74.2 % with ferulic acid (Fig. 4.14 C). Regarding aluminum, the remaining concentration in soil with the laccase treatment was 0.82 % and 0.41 % when the treatment was mediated with ABTS. Laccase with ferulic acid removed 74.06 % aluminum and with coumaric acid 44.46 % removal was achieved (Fig. 4.14 D).


A
Chrysene remaining (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Time (days)
Lac
Lac+ABTS
Lac+CA
Lac+CA


B
Benzo(a)pyrene remaining (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Time (days)
Lac
Lac+ABTS
Lac+CA
Lac+CA



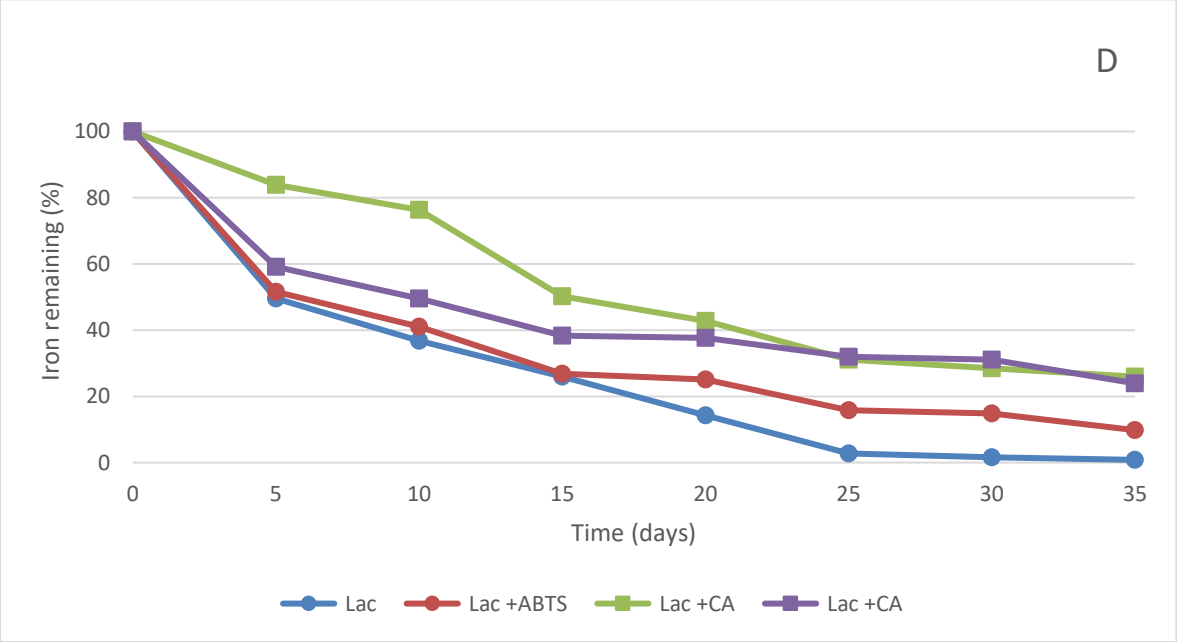


Figure 4.14 Degradation of chrysene (A), benzo[a]pyrene (B), and removal of aluminium (C) and iron (D) in soil by laccase and laccase-mediator system in PBR.

In a previous study, a commercial soil was spiked with 300 ppm of 6 PAH, and within 25 days, 81.8 % of chrysene and 96 % of benzo[a]pyrene were removed by a load of 2 U/mL of laccase from cocultivation of *Penicillium chrysogenum* and *Trichoderma viride* with 1 mM ABTS, while the moisture content of soil increased to 59 % by the end of experiment, indicating that the LMS works effectively in a moisturized environment. In the current study, moisture content at the end of the assay was 31±0.1 %.

However, the different incubation conditions and compositions of the reaction mixtures make it difficult to compare the ability of laccases from different fungal species to degrade PAH. Jones et al. [175] reported an inhibition effect on PAH degradation when a soil contains a mixture of contaminants and more than one substrate is metabolized by the same enzymes. Although metal ions such as copper, iron and nickel can act as cofactors to assist cell growth in trace levels, they can become toxic when in excess to most living systems [169,176]. In the present study, an efficient degradation of PAH in soil was achieved without redox mediators, which indicates that some compounds present in the soil may have acted as mediators in the enzymatic oxidation. Enzymatic treatment of contaminated soil reveals to be an alternative or a supplement to microbial bioremediation.

4.4 Evaluation of the degradation of polyaromatic hydrocarbons of an aged, contaminated soil by different laccases

4.4.1 Soil characteristics

Soil used in this research was collected nearby a dumping site in Lagos (06°34'27.0 N 003°15'101E). The physicochemical characteristics of soil studied are shown in Table 4.14. Soil was classified as sandy loam, with 63.3 % of sand, 15.4 % of silt and 21.3 % of clay. The pH value of 8.1 ± 0.4 indicates that the soil is alkaline in the studied area, with 19 % of moisture at the beginning of the experiments. Total concentration of USEPA priority PAH detected was 74.9 ppm, yet volatile PAH were not detected at UHPLC (Table 4.15). Also, all 23 metals were detected, but only 4 (lead, magnesium, iron, and aluminum) (Table 4.15) showed a relevant presence, while the remaining were only detected in trace concentrations, so they might be naturally present in soil at the sampling site. When pollutants are detected in the topsoil (0 – 15 cm depth), usually there is evidence of recent anthropogenic contamination followed by migration during the wet season. In this soil under study, the samples were taken from the 15 – 30 cm layer, which indicates that the pollutants have been there for long, making them less bioavailable and more persistent.

Table 4.14 Physicochemical characteristics of soil

Site	Value
pH	8.1 ± 0.4
MC, %	19 ± 0.001
TOC, %	0.87 ± 0.07
Total nitrogen, g/kg	0.033
Total phosphor, g/kg	0.03
Soil composition (sandy loam):	
Sand	63.3%
Silt	15.4%
Clay	21.3%

Table 4.15 Concentration of PAH and metals in soil

	ppm
Total PAH	74.87
Fluorene	5.951
Phenanthrene	6.044
Anthracene	3.319
Fluoranthene	23.013
Pyrene	13.066
Benzo[a]anthracene	1.776
Chrysene	1.789
Benzo[b]fluoranthene	3.508
Benzo[k]fluoranthene	3.034
Benzo[a]pyrene	5.243
Dibenz[a,h]anthracene	3.402
Indeno[1,2,3-cd]pyrene	3.034
Benzo[ghi]perylene	1.691
Metals	
Lead	1.035
Aluminum	41.69
Magnesium	60.43
Iron	132.1

Several researchers reported that improper handling of different residues is contributing to the

addition of metals and PAH to the atmosphere and mainly in the top layer of soil. Moreover, leachate from dumpsites with high concentrations of metals, PAH and PCB contribute to a further distribution of these pollutants into soil, water and sea, and from there reaching food chains [157,158].

4.4.2 Laccase production by *T. versicolor* and *A. aegerita*

Different microorganisms produce different amounts of enzymes depending on the substrate. Solid state fermentation is considered one of best methods for the culture of filamentous fungi and for the production of ligninolytic enzymes because they are grown under conditions that emulate their natural habitat, and as a result, they are able to produce certain enzymes at high levels. Therefore, appropriate substrate is important for the success of the enzyme production under SSF, and its optimization in lab conditions is a crucial and vital approach to achieve high and cost-effective yields of laccase. Laccase production was performed by SSF using Erlenmeyer flasks and trays, at room temperature (26 – 28 °C), for 3 weeks and assessed by sampling once a week. The chosen fungi for this study were *T. versicolor* (previously isolated from contaminated soil of Lagos) and *A. aegerita* from Mycosmo culture collection.

Kiwi peels, pine bark, peanut shells and cork were selected as SSF growth substrates as they all are agro-industrial wastes and/or by-products. The polysaccharides and lignin present in these materials act as growth and induction sources, promoting a good fungal growth and the production of extracellular enzymes. Fig. 4.15 shows that among the substrates tested, pine bark supported the highest production of laccase by both fungi. For *T. versicolor* there was a production of 11.74 U/g of laccase in the 2nd week in Erlenmeyer flasks and by *A. aegerita* 11.24 U/g in Erlenmeyer flasks and 11.48 U/g in trays, both in the 3rd week of production.

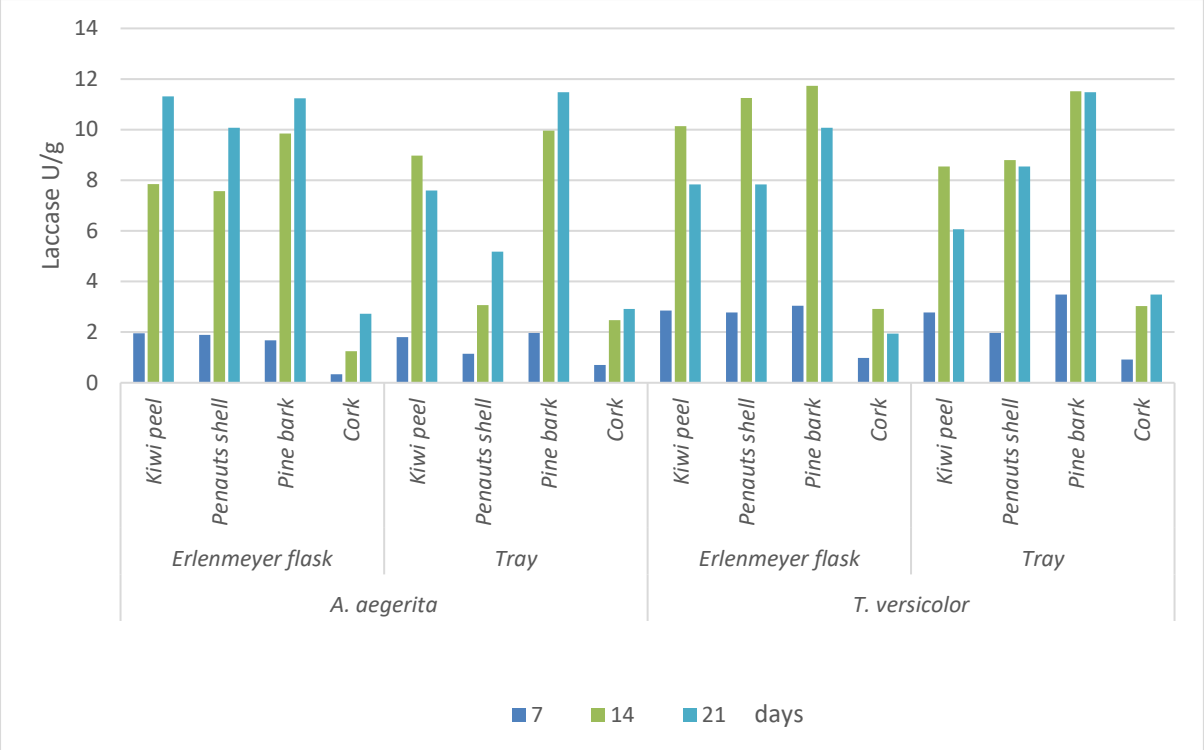


Figure 4.15 Laccase production by *T. versicolor* and *A. aegerita* on 4 different substrates in Erlenmeyer flasks and aluminium tray

With kiwi peels as support, the highest production obtained by *A. aegerita* was 11.31 U/g in the 3rd week in Erlenmeyer flask, and 8.98 U/g in the 2nd week in trays, while the highest production obtained with *T. versicolor* was 10.13 U/g in flask in the 2nd week and 8.55 U/g in trays. Using peanuts shells, *T. versicolor* reached 11.25 U/g in flask and 8.55 U/g in trays. Cork was the least appropriate substrate selected, with the highest production of 3.49 U/g obtained in tray by *T. versicolor*. Overall, laccase production by *A. aegerita* was slightly higher in trays, while by *T. versicolor* it was better in Erlenmeyer flasks, much depending on the substrate.

In this context, Rodríguez Couto et al. [134] tested several bioreactor configurations such as immersion, expanded-bed and tray, with nylon sponge and barley bran as support. The first two were supplied with humidified air in a continuous flow at 0.5 vvm, and the tray reactor was kept at 90 % environmental humidity and passive aeration. Maximum laccase production by *T. versicolor* was reached, for each configuration, between the 18th and the 20th days of cultivation, with 0.23, 0.13 and 0.34 U/mL, respectively. In another work, Rodríguez Couto [135] achieved 6 U/mL of laccase by *T. pubescens* under SSF in temporary immersion bioreactor, using sunflower-seed shells as substrate and 60 L/h air-flowrate. Rosales and co-workers [136] tested laccase production in a tray reactor on orange peels by *T. hirsute*

and reached maximum production on the 23rd day, 12.7 U/mL. Pinheiro and colleagues [177] also studied laccase production by *T. versicolor* on vinasse and cotton gin wastes, achieving 5005.55 U/L on the 12th day in BioFlo 310 bioreactor, 816.66 U/L on the 7th day in Erlenmeyer flask and 235.89 U/L on the 12th day in an aluminum tray.

4.4.3 Laccase stability

It is known that several metals can inhibit the enzyme activity [169,178,179] Thus, the laccase activity was followed during the PAH degradation due to the possible effect of the metals in inhibiting its activity. As shown in Figure 4.16, the activity of each laccase declined smoothly over the whole incubation time, which could be related to the presence of the metals in the soil. The activity of the four studied laccases increased slightly on the 2nd day, which might be due to the effect of some soil factors, or the presence of other phenolic compounds in soil. At 12th day, the activity of the commercial *Aspergillus sp.* laccase was not detected anymore, therefore the assay was finished. Similar behaviour was observed in the work of Wu and collaborators [47].

Enayatizamir and co-workers [169] tested the effect of different concentrations of Pb on the laccase activity. It was observed that a 100 ppm Pb concentration decreased the laccase stability, while it kept increasing at 200, 400 and 600 ppm of Pb due to its precipitation. Further increase in Pb concentration would lead to a constant decrease of the laccase activity. The negative effect of Pb is mainly due to the inactivation of the enzyme by the metal [169]. Lorenzo and co-workers [178] tested metals inhibition on laccase produced by SmF of *T. versicolor* on barley bran. It was observed that Lac II with syringaldazine as substrate mediator was strongly inhibit by Cd^{2+}, and Zn^{2+} in the range 0.5 to 80 mM did not affect laccase. On the other hand, the addition of Cu^{2+} stimulated laccase activity and even increased it by 17 %. However, when the presence of Cu^{2+} was higher than 2 mM, laccase was significant inhibited. In Baldrian and Gabriel [180] and Baldrian [181] studies, 1 mM Mn^{2+} caused 91 % of inhibition on laccase from *Daedalea quercina.* Moreover, they also reported that Zn^{2+} caused inhibition and decreased the activity of the laccase produced by *Pleurous ostreatus.*

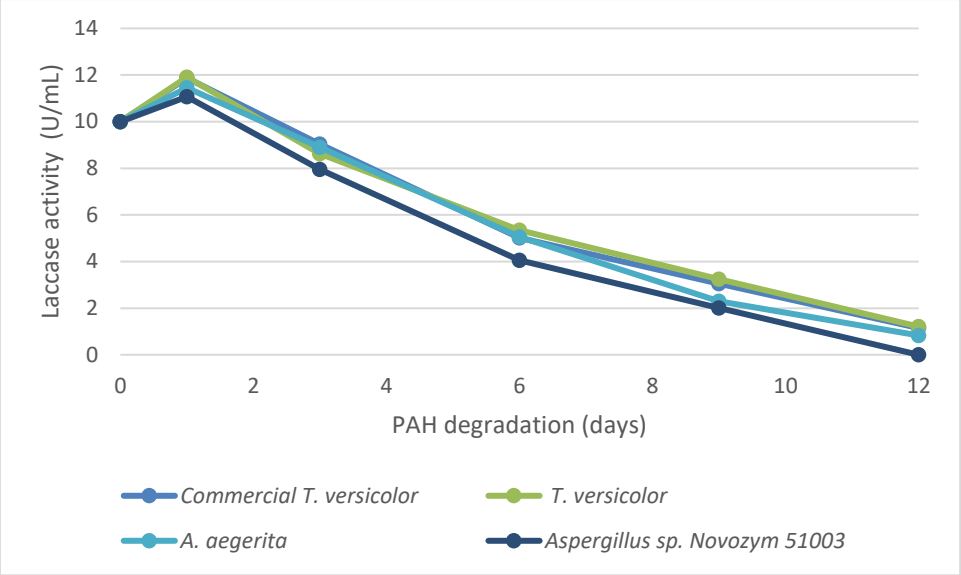


Figure 4.16 Laccases stability during 12 days PAH degradation in soil

Zhou and co-workers [179] studied the effect of metals on commercial laccase from *T. versicolor* in buffer. Metal cations as K^{+}, Na^{+}, Mg^{2+}, Ca^{2+} and Cu^{2+} and the anion SO_4^{2-} had almost no effect on laccase activity during the initial stage of the catalytic reactions but an inhibitory effect was shown at 30 mM of each compound. High concentrations of Mn^{2+} only showed a week inhibition on laccase and Fe^{2+} had no direct effect on the binding of laccase to its substrate, but strongly retarded the progress of the catalytic reaction by reducing the intermediate free radicals. Moreover, it also confirmed the results of Lorenzo's study, in which laccase stability can be activated during enzymatic reaction with appropriate concentration of Cu^{2+} [178,179]. It should be noticed that all those results were obtained from buffers with one organic or inorganic compound and optimal pH conditions, and not with a mixture of compounds or in soil.

On the other hand, the results of some studies suggest that zero-valent iron (Fe^{0}) can enhance pollutant transformation in soil [182]. The results published by the team of Niladevi [183] showed that Fe^{2+} and Fe^{3+} strongly inhibit the activity of laccase. However, Zhao and colleagues [184] defended that there was none evidence of a correlation between the degradation of DDT by laccase and the iron content of soil.

4.4.4 Soil rehabilitation

The main advantages of enzyme degradation include low sensitivity to high pollutant concentrations in comparison to fungi and bacteria, high reaction activity and ability to cover a huge

range of physicochemical gradients in the environmental matrix. Moreover, enzyme degradation is easy to control. Some of the relevant parameters determining laccase activity in PAH degradation are the mediator and pH, as well as the incubation temperature for maximal laccase activity. Despite a higher temperature is preferable for laccase catalysis, at the same time it also leads to a faster loss of activity [144,145].

Degradation of each PAH is presented on Table 4.16. Overall, most of the considered PAH were degraded. The laccases of the studied fungi were able to degrade more PAH than commercial laccases, which may be related to enzymatic activities other than laccases, since the laccases of *T. versicolor* and *A. aegerita* were not purified. Both of them are also capable of producing manganese peroxidase and *A. aegerita* also produces a versatile peroxidase (data not shown). Commercial laccase from *T. versicolor* degraded 43.6 % of total PAH, while laccase with coumaric acid, ferulic acid and ABTS mediators degraded 47.4 %, 54.2 % and 57.9 %, respectively, thus achieving higher degradation rates than just laccase. On the other hand, laccases from *T. versicolor*, *A. aegerita* and *Aspergillus sp.* with coumaric acid as mediator led to 46.2 %, 51.3 %, and 46.6 % of degradation, respectively, which were less than the values reached by laccase alone, able to degrade 55.4 %, 62.3 % and 47.3 %, depending on its origin, in the same order as before. Laccase from *A. aegerita* degraded 54.1 % and 56.2 % of total PAH with ferulic acid and ABTS as mediator, respectively, while with *Aspergillus sp.* laccase the degradation increased to 54.7 % with ferulic acid and with *T. versicolor* laccase to 58.9 % with ferulic acid and 66.1 % with ABTS.

After 24 h of incubation, anthracene, chrysene, benzo[b]fluoranthene and dibenz[a,h]anthracene were the most affected from all detected PAH. Removal yield of anthracene was 65.4 % by commercial *T. versicolor* laccase, 35.4 % by laccase from *T. versicolor*, 42.5 % by *A. aegerita* laccase and 38.7 % by commercial laccase from *Aspergillus sp*. Dibenz[a,h]anthracene was reduced by commercial *T. versicolor* laccase*, T. versicolor*, *A. aegerita* and *Aspergillus sp.* for 51.8 %, 52.4 %, 56.2 % and 63.4 %, respectively. Removal of chrysene was 85.1 %, 58.2 %, 78.7 % and 80.6 % by commercial *T. versicolor* laccase, *T. versicolor*, *A. aegerita* and *Aspergillus sp.* laccase Removal of benzo[b]fluoranthene was 97.5 % by commercial *T. versicolor* laccase, 56.7 % by *T. versicolor* laccase, 72.8 % by *A. aegerita* laccase and 65.4 % by *Aspergillus sp*. laccases.

Wu and team [47] studied the effect of 3 different initial concentrations of laccase DAIWA Y120 (from *Trametes*, obtained by Amano Enzyme Inc), i.e. 1, 3 and 10 U/g, on the degradation of 15 PAH in soil, with concentration of 10.835 mg/kg, during 14 days. After 1 day of incubation, anthracene and

benzo[a]pyrene were the most affected of the 15 PAH. The benzo[a]pyrene with 1, 3 and 10 U/g of laccase was reduced in 49 %, 57 % and 60 %, respectively. After 14 days, laccase activity was not detected in the soil samples and at this moment the degradation of total PAH was 17.6 % with 3 U/g, 32.4 % with 1 U/g and 31 % with 10 U/g. Li and co-workers [93] observed the highest degradation rate of 1 mg/L of benzo[a]pyrene with laccase plus 1 mmol/L of ABTS after 48 hours incubation, despite there was no significant difference between 24 and 48 hours. Degradation yield was 60 % with laccase and 100 % with laccase and ABTS. The experiment was carried out at 40 °C, pH 4.0, with 30 U of laccase. On the other hand, the degradation in soil with 70 % moisture was tested, achieving 40.8 % of degradation with 10 U/g of laccase in 10 days, and increasing to 56.7 % with the addition of 1 mmol/kg of ABTS [93].

In the study of Lorenzo and co-workers [178] different influence of organic compounds on laccase were observed, however it was also depended on the mediator used. With ABTS as a mediator, the laccase activity was inhibited only by hydroxylammonium chloride, but with DMP and syringaldazine as mediators, all organic compounds tested caused inhibition in laccase activity.

Table 4.16 PAH degradation in soil by different laccases and LMS

		C. *T. versicolor*				*T. versicolor*				*A. aegerita*				*Aspergillus sp.* - Novozym 51003			
PAH	ppm	Lac	Lac + ABTS	Lac + FA	Lac + CA	Lac	Lac + ABTS	Lac + FA	Lac + CA	Lac	Lac + ABTS	Lac + FA	Lac + CA	Lac	Lac + ABTS	Lac + FA	Lac + CA
Fluorene	5.951	3.951	1.988	3.746	3.313	2.803	2.165	2.58	3.221	2.509	2.853	3.747	2.915	3.245	3.083	2.753	2.798
Phenanthrene	6.044	4.123	2.654	3.047	3.522	3.037	2.419	2.928	3.335	2.823	3.052	3.036	3.196	3.531	3.395	2.971	3.853
Anthracene	3.319	0.881	0.23	0.18	0.449	0.093	0.061	0.01	0.58	0.073	0.133	0.141	0.277	0.57	0.388	0.254	0.513
Fluoranthene	23.013	15.81	10.58	9.736	11.925	9.693	6.936	9.017	12.919	8.576	10.288	10.016	11.061	12.272	12.441	10.678	12.634
Pyrene	13.066	8.275	4.967	5.426	6.136	4.969	3.649	4.61	6.594	4.375	5.354	5.099	5.715	6.852	6.645	5.429	6.611
Benzo(a)anthracene	1.776	1.106	0.807	0.83	1.052	0.735	0.509	0.657	1.015	0.612	0.778	0.747	0.872	0.979	0.921	0.891	1.028
Chrysene	1.789	0.85	0.69	0.642	0.804	0.649	0.453	0.582	0.829	0.537	0.61	0.661	0.768	0.79	0.819	0.634	0.907
Benzo(b)fluoranthene	3.508	1.309	1.913	1.764	2.149	2.149	1.412	1.647	2.286	1.556	1.686	1.794	2.143	2.14	2.2	1.885	2.457
Benzo-k-fluoranthene	3.034	0.614	1.422	1.337	1.542	1.818	1.007	1.185	1.065	1.148	1.177	1.35	1.546	1.606	1.605	1.366	1.881
Benzo(a)pyrene	5.243	3.012	3.575	3.664	3.768	3.52	3.43	3.526	3.801	3.371	3.416	3.642	3.699	3.8	3.856	3.545	3.879
dibenz(a,h)anthracene	3.402	1.4678	1.506	1.44	1.836	1.095	1.223	1.334	1.343	1.235	0.967	1.208	1.198	1.183	1.155	0.98	1.208
Indeno(1,2,3-cd)pyrene	3.034	0.568	0.725	2.025	2.186	2.091	1.669	1.871	2.384	0.806	1.856	1.998	2.347	2.161	2.197	2.071	1.841
Benzo(g,h,i)perylene	1.691	0.31	0.549	0.519	0.797	0.818	0.531	0.842	0.946	0.631	0.677	0.993	0.835	0.36	0.497	0.549	0.363
Total PAH	74.87	42.2768	31.606	34.356	39.479	33.47	25.464	30.789	40.318	28.252	32.847	34.432	36.572	39.489	39.202	34.006	39.973

There might also exist other phenolic compounds, naturally present in soil, which can bond with laccase. When a comparison was made with the same soil (Table 4.17) previously sterilized at 121 °C for 60 min to eliminate all microorganisms from it, PAH degradation increased to 65.7 % with commercial laccase from *T. versicolor*, to 66.0 % with *T. versicolor* laccase, to 74.9 % with *A. aegerita* laccase, and to 56.1 % with commercial *Aspergillus sp*. laccase, representing an increase of 9 to 22 %. Also, after sterilization, degradation with laccase and coumaric acid as mediators increased to 60.8 %, 77.9 %, 68.5 % and 57.9 %, respectively for commercial *T. versicolor*, *T. versicolor*, *A. aegerita* and *Aspergillus sp*. laccases, representing an increase of 32 % for *T. versicolor* laccase. For ferulic acid, an improvement in degradation after soil sterilization was observed only for the laccases of *A. aegerita* and *Aspergillus sp*., and for ABTS only for the laccase of *A. aegerita.* However, further assays are needed before an explanation is forwarded, as these observations may result from soil sterilization and consequent changes in its condition and composition.

Table 4.17 Remaining PAH (in ppm) after laccase degradation. Comparison of sterile and nonsterile soil

	commercial *T. versicolor*				*T. versicolor*			
	Lac	Lac + ABTS	Lac + FA	Lac + CA	Lac	Lac + ABTS	Lac + FA	Lac + CA
Nonsterile soil	42.28	31.61	34.36	39.48	33.47	25.46	30.79	40.32
Sterile soil	25.72	32.16	38.84	29.33	25.49	29.99	32.62	16.56
	A. aegerita				Commercial *Aspergillus sp* - Novozym 51003			
	Lac	Lac + ABTS	Lac + FA	Lac + CA	Lac	Lac + ABTS	Lac + FA	Lac + CA
Nonsterile soil	28.25	32.85	34.43	36.57	39.49	39.20	34.01	39.97
Sterile soil	18.82	17.89	24.94	23.56	32.84	44.46	26.70	31.55

According to the presented results, different laccases and LMS may degrade or transform different pollutants in real environment. Despite the high price of laccase production, it works very efficiently in a first approach to the rehabilitation of soils contaminated with persistent pollutants, although tests on site need yet to be performed. At present, it is difficult to select only one strategy to treat a real contaminated soil and a combination of different approaches, including degradation by laccase, may enhance better results.

CHAPTER V

Conclusion and future perspectives

PAH degradation has been studied for almost three decades and yet many questions still remain, mainly as almost 70 % of all research was done in buffer/media at optimal conditions, where PAH act differently as they are distributed homogenously and also do not interfere with inter-medium pore space of soil. With fast urbanization, their concentration has become approximately 2–10 times higher in urban areas, where they are adsorbed and accumulate in the upper surface layer of the soil, finding their way into the ecosphere. The soil appears to act as a long-term storage area for PAH as they are deposited there. PAH in environment are present as mixtures, usually with other toxic compounds, and therefore co-metabolism plays a very crucial role in bioremediation of PAH contaminated sites. Bioremediation is a low-cost but slow process, in which microorganisms degrade or transform contaminants into less hazardous or non-hazardous substances. Many microbial strains are known to be effective as bio-remediators, but only under controlled and/or optimal laboratory conditions. Ascomycetes, basidiomycetes and deuteromycetes fungi can produce laccases and among them white-rot basidiomycetes are the most efficient lignin degraders and laccase producers. However, white-rot fungi need lignocellulosic material to grow and produce enzymes. On the other side, species belonging to the genera *Trichoderma*, *Fusarium*, *Penicillium*, *Stachybotrys*, *Aspergillus*, *Cladosporium*, *Mortierella*, *Beauveri*a and *Engyodontium* are commonly found in polluted soils. Moreover, these fungi tend to defend their resources against potential invaders, either by sequestering critically limiting nutrients or by producing inhibitory metabolites. They are generally characterized by high tolerance to environmental stresses such as the presence of pollutants and are indicated as potential bioremediation agents in soil.

A biotechnological approach to remove PAH from soil was evaluated in this work using fungi, laccase and laccase mediator systems. To make this degradation process more competitive and to reduce costs, laccase was produced from agricultural waste (kiwi and plantain peels, as well as peanut shells, pine bark and cork) and crude laccase was used in all assays, resulting in an efficient degradation, as well as a faster and simpler process when compared to degradation by microorganisms. Many research efforts have been made to find a suitable process for the remediation of PAH contaminated soil. However, most works using laccase were focused on a liquid system and using a synthetic mediator such as ABTS or HBT. On the other side, natural compounds such as phenolic ones derived from lignin degradation (acetosyringone, syringaldehyde, vanillin, acetovanillone, ferulic acid and *p*-coumaric acid) can be obtained at low cost or for free because they exist abundantly in nature and are not toxic to the environment. As concluded from the obtained data, natural mediators are capable of competing with the synthetic ones and in some cases very good results are obtained without a mediator, which suggests the possibility of other compounds present in the soil or crude enzymes extracts, to enhance enzymatic oxidation. All work presented here was done with soil, first artificially contaminated soil to study the efficiency of laccase on PAH degradation, and later naturally contaminated soil to test the presented theory.

In a first part, PAH degradation by three fungal strains (*Trichoderma viride, Penicillium chrysogenum* and *Agrocybe aegerita)* was evaluated with spiked soil. The effect of soil pH and of different soil amendment was assessed, as well as the enzyme activities during the PAH degradation in soil. In this study, all fungi combinations were able to degrade fluorene, pyrene, chrysene and benzo[a]pyrene. The degradation potential was affected by pH, cocultivation and substrate support. Agricultural residues used as substrate supports for the fungi growth are known to induce or enhance the activities of ligninolytic enzymes such as laccase, MnP and LiP, and to increase the degradation rates of PAH. Results obtained suggest the involvement of ligninolytic enzymes in PAH degradation. Biodegradation assays with a mixture of 200 ppm of PAH were evaluated for 8 weeks. The maximum laccase activity by solid state fermentation SSF (7.43 U/g) was obtained for *A. aegerita* on kiwi peels in 2 weeks' time and the highest MnP activity (7.21 U/g) was reached in 4 weeks, both assays at pH 7. Fluorene, pyrene, and benzo[a]pyrene reached higher degradation rates in soil at pH 5, while chrysene was more degradable at pH 7. About 85 % to 90 % of the pollutants were degraded by fungal remediation. Regarding benzo[a]pyrene, 13 % remained in soil after treatment with *A. aegerita.* Despite the increase in the degradation rate of some PAH with cocultivation, a higher enzyme production during degradation was observed when fungi were cultivated alone.

In the following section the optimization of the laccase production and evaluation of the PAH degradation by laccase/LMS on artificially contaminated soil, in batch and fed-batch with continuous flow of laccase/mediator in PBR was studied. Collected data confirm previous observations suggesting the importance of mediators in the oxidation of PAH. The most commonly used mediator, ABTS, is too expensive for practical soil remediation and its long-term toxic effects are unknown for now. Fortunately, natural mediators are capable to compete with the synthetic one. Results showed that laccase mediator system was effective in the degradation of pyrene, fluorene, chrysene and, at a lower extension, anthracene. Mediators improved PAH degradation and natural mediators (ferulic acid and *p*-coumaric acid) were as effective as the synthetic one, ABTS. However, the process was not effective in the case of the benzo[a]pyrene degradation, one of the most recalcitrant and toxic PAH. This weak degradation could be related to the low activity of the laccase mediator system in an environment lacking water. To overcome this issue, a PAH contaminated soil degradation system was developed in packed bed reactor (PBR) fed with laccase/mediator. Continuous flow of laccase/mediator improved PAH degradation, achieving 74.8 %, 71.9 %, 72.2 %, 81.8 % and 100 % degradation for fluorene, anthracene, phenanthrene, chrysene and pyrene, respectively. This system was able to degrade 96 % benzo[a]pyrene, which was 90 % higher than the degradation in batch system.

In a second part, assays were performed with naturally contaminated soil obtained from Lagos, Nigeria. The collection of soil samples was from the upper layer (0–30 cm). *Trametes versicolor* was isolated from this soil and used in degradation experiments, with plantain peels as support.

The objective of this study was the assessment of the degradation rates of chrysene and benzo[a]pyrene, as well as the removal of aluminum and iron from contaminated soil. Two different strategies were applied in this study: rehabilitation by *T. versicolor* (strategy I) and by laccase/LMS, in fed-batch mode in PBR (strategy II). After 8 weeks of rehabilitation by *T. versicolor* chrysene degradation reached 82.0 %, which increased to 91.0 % with the support. Benzo[a]pyrene was less degradable, with 38.0 % and 49.1 % of degradation, with and without support, respectively. *Trametes versicolor* was capable of accumulate 46.1 % of aluminums and 57.2 % of iron. By adding plantain peels, these amounts increased for 2.1 % and 4,6 %, respectively. At the same time, laccase was produced by *Trametes versicolor* on plantain peels, achieving 37.8 U/g of crude laccase within 3 weeks in Erlenmeyer flasks during SSF at 30 °C. Laccase degradation experiments were set up in PBR, with a constant feed of 21.6 mL/day of laccase, with and without mediators. 75.9 % degradation of chrysene was achieved by laccase in 35 days. The highest degradation was observed with ABTS as mediator, 87.9%. Benzo[a]pyrene degradation with laccase reached 35.6 %, raising to 38.8 % with ferulic acid as mediator. In the study with naturally contaminated soil, an efficient degradation of PAH was achieved without redox mediators, which indicates that some compounds present in the soil, or even in the enzymatic extract may have acted as mediators in the enzymatic oxidation.

The last assays used a contaminated soil collected at a dumping site in Lagos, Nigeria, at 15 – 30 cm depth and with a total concentration of USEPA PAH of 74.87 ppm. Rehabilitation of PAH contaminated soil was evaluated using laccase, and for this purpose four different laccases were used: commercial laccases from *T. versicolor* and from *Aspergillus sp.* - Novozym 51003, and laccase produced through solid state fermentation (SSF) on agricultural residues by *T. versicolor* and *A. aegerita.* After 12 days of treatment with laccases and mediators, the total PAH in soil was reduced to 42.2 mg/kg by the action of commercial *T. versicolor* laccase, to 33.4 mg/kg by laccase of *T. versicolor* strain, to 28.2 mg/kg by *A. aegerita* laccase and to 39.4 mg/kg by the action of *Aspergillus sp.* laccase. Despite mediators such as ABTS, ferulic acid and coumaric acid were also used, it was observed that laccase alone could achieve a high degradation of the contaminants load.

As final remarks, enzymatic treatment of contaminated soil revealed to be an alternative or a supplement to microbial bioremediation. According to all these results, different laccases and LMS may degrade or transform different pollutants in real environment. Despite the high price of the commercial laccase, it can be

reduced through its production on agricultural residues. But large-scale operation still needs to be optimized as well as the study of the enzyme stability and usage in field.

Moreover, the combination of different approaches may produce better results, especially as microbes usually require special conditions, where the toxicity of PAH metabolites is of particular concern, and on the other side, enzyme-catalysed reactions are faster and have high efficiencies, lower overall toxicity, a wide application range and constitute a simple operation. Therefore, they are a good option for a first attack to the pollutant.

Few points that should be considered in future research:

- Analyzing degradation pathway of PAH by laccase in mixture with and without presence of other organic and inorganic pollutant.
- Testing effect of different soil structure and compositions on laccase activity
- Upscaling and optimizing PBR to large scale and implementing it in field. Moreover, checking ability of laccase in larger scale operations and under different weather conditions and temperature.
- Testing laccase immobilization and its reusing in soil contamination
- Testing effect of naturally presence microorganism in soil on laccase activity

CHAPTER VI

References

[1] J.J. Nam, A.J. Sweetman, K.C. Jones, Polynuclear aromatic hydrocarbons (PAHs) in global background soils, J. Environ. Monit. 11 (2009) 45–48. https://doi.org/10.1039/b813841a.
[2] R.A. Kanaly, S. Harayama, Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria, J. Bacteriol. 182 (2000) 2059–2067. https://doi.org/10.1128/JB.182.8.2059-2067.2000.
[3] WHO, Air Quality Guidelines : Polycyclic aromatic hydrocarbons, Air Qual. Guidel. Polycycl. Aromat. Hydrocarb. 1 (2000) 1–24. http://www.euro.who.int/__data/assets/pdf_file/0015/123063/AQG2ndEd_5_9PAH.pdf.
[4] C.A. Menzie, B.B. Potocki, J. Santodonato, Exposure to carcinogenic PAHs in the environment, Environ. Sci. Technol. 26 (1992) 1278–1284. https://doi.org/10.1021/es00031a002.
[5] O. Idowu, K.T. Semple, K. Ramadass, W. O'Connor, P. Hansbro, P. Thavamani, Beyond the obvious: Environmental health implications of polar polycyclic aromatic hydrocarbons, Environ. Int. 123 (2019) 543–557. https://doi.org/10.1016/j.envint.2018.12.051.
[6] H. Cao, S. Chao, L. Qiao, Y. Jiang, X. Zeng, X. Fan, Urbanization-related changes in soil PAHs and potential health risks of emission sources in a township in Southern Jiangsu, China, Sci. Total Environ. 575 (2017) 692–700. https://doi.org/10.1016/j.scitotenv.2016.09.106.
[7] I.A. Mirsal, Soil Pollution. Origin, Monitoring and Remediation. 2nd Edition, 2004.
[8] A.S. Tsibart, A.N. Gennadiev, Polycyclic aromatic hydrocarbons in soils: Sources, behavior, and indication significance (a review), Eurasian Soil Sci. 46 (2013) 728–741. https://doi.org/10.1134/S1064229313070090.
[9] C. Belis, I. Offenthaler, P. Weiss, PAHS_IN_FORESTS.pdf, (2011).
[10] D. Lerda, Polycyclic Aromatic Hydrocarbons (PAHs) Factsheet, JRC Tech. Notes. 3 (2010) 1–25.
[11] G. Liu, Z. Niu, D. Niekerk, J. Xue, L. Zheng, Reviews of Environmental Contamination and Toxicology, 2006.
[12] S.M. Bamforth, I. Singleton, Bioremediation of polycyclic aromatic hydrocarbons: Current knowledge and future directions, J. Chem. Technol. Biotechnol. 80 (2005) 723–736. https://doi.org/10.1002/jctb.1276.
[13] H.I. Abdel-Shafy, M.S.M. Mansour, A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation, Egypt. J. Pet. 25 (2016) 107–123. https://doi.org/10.1016/j.ejpe.2015.03.011.
[14] S. Vaidya, N. Devpura, K. Jain, D. Madamwar, Degradation of chrysene by enriched bacterial consortium, Front. Microbiol. 9 (2018) 1–14. https://doi.org/10.3389/fmicb.2018.01333.
[15] J. Sikkema, J.A.M. De Bont, B. Poolman, Mechanisms of Membrane Toxicity of Hydrocarbons, Microbiol. Rev. 59 (1995) 201–222.
[16] T. Hadibarata, S. Tachibana, K. Itoh, Biodegradation of chrysene, an aromatic hydrocarbon by Polyporus sp. S133 in liquid medium, J. Hazard. Mater. 164 (2009) 911–917. https://doi.org/10.1016/j.jhazmat.2008.08.081.
[17] A.S. Nayak, S. Sanjeev Kumar, M. Santosh Kumar, O. Anjaneya, T.B. Karegoudar, A catabolic pathway for the degradation of chrysene by *Pseudoxanthomonas sp.* PNK-04, FEMS Microbiol. Lett. 320 (2011) 128–134. https://doi.org/10.1111/j.1574-6968.2011.02301.x.
[18] X. Li, X. Lin, R. Yin, Y. Wu, H. Chu, J. Zeng, T. Yang, Optimization of laccase-mediated benzo[a]pyrene oxidation and the bioremedial application in aged polycyclic aromatic hydrocarbons-contaminated soil, J. Heal. Sci. 56 (2010) 534–540. https://doi.org/10.1248/jhs.56.534.
[19] H. Cao, C. Wang, H. Liu, W. Jia, H. Sun, Enzyme activities during Benzo[a]pyrene degradation by the fungus *Lasiodiplodia theobromae* isolated from a polluted soil, Sci. Rep. 10 (2020) 1–11. https://doi.org/10.1038/s41598-020-57692-6.
[20] P.S. Phale, A. Sharma, K. Gautam, Microbial degradation of xenobiotics like aromatic pollutants from the terrestrial environments, Elsevier Inc., 2019. https://doi.org/10.1016/B978-0-12-816189-

0.00011-1.
[21] M. Malawska, B. Wiołkomirski, An analysis of soil and plant (*Taraxacum officinale*) contamination with heavy metals and polycyclic aromatic hydrocarbons (PAHs) in the area of the railway junction ilawa glówna, Poland, Water. Air. Soil Pollut. 127 (2001) 339–349. https://doi.org/10.1023/A:1005236016074.
[22] S. Kuppusamy, P. Thavamani, K. Venkateswarlu, Y.B. Lee, R. Naidu, M. Megharaj, Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: Technological constraints, emerging trends and future directions, Chemosphere. 168 (2017) 944–968. https://doi.org/10.1016/j.chemosphere.2016.10.115.
[23] Sakshi, S.K. Singh, A.K. Haritash, Polycyclic aromatic hydrocarbons: soil pollution and remediation, Int. J. Environ. Sci. Technol. 16 (2019) 6489–6512. https://doi.org/10.1007/s13762-019-02414-3.
[24] M. Hreniuc, M. Coman, B. Cioruţa, Considerations regarding the soil pollution with oil products in Săcel-Maramureş, Int. Conf. Sci. Pap. AFASES. Brasov. (2015) 28–30. https://www.researchgate.net/publication/305918892.
[25] E. Magi, R. Bianco, C. Ianni, M. Di Carro, Distribution of polycyclic aromatic hydrocarbons in the sediments of the Adriatic Sea, Environ. Pollut. 119 (2002) 91–98. https://doi.org/10.1016/S0269-7491(01)00321-9.
[26] M. Chen, P. Xu, G. Zeng, C. Yang, D. Huang, J. Zhang, Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs, Biotechnol. Adv. 33 (2015) 745–755. https://doi.org/10.1016/j.biotechadv.2015.05.003.
[27] U. Munusamy, V. Sabaratnam, S. Muniandy, N. Abdullah, A. Pandey, E.B.G. Jones, Biodegradation of polycyclic aromatic hydrocarbons by laccase of *Pycnoporus sanguineus* and toxicity evaluation of treated PAH, Biotechnology. 7 (2008) 669–677. https://doi.org/10.3923/biotech.2008.669.677.
[28] C.E. Cerniglia, J.B. Sutherland, Degradation of Polycyclic Aromatic Hydrocarbons by Fungi, in: K.N. Timmis (Ed.), Handb. Hydrocarb. Lipid Microbiol., Springer Berlin Heidelberg, Berlin, Heidelberg, 2010: pp. 2079–2110. https://doi.org/10.1007/978-3-540-77587-4_151.
[29] F. Fernández-Luqueño, C. Valenzuela-Encinas, R. Marsch, C. Martínez-Suárez, E. Vázquez-Núñez, L. Dendooven, Microbial communities to mitigate contamination of PAHs in soil-possibilities and challenges: A review, Environ. Sci. Pollut. Res. 18 (2011) 12–30. https://doi.org/10.1007/s11356-010-0371-6.
[30] M.S. Vásquez-Murrieta, O.J. Hernández-Hernández, J.A. Cruz-Maya, J.C. Cancino-Díaz, J. Jan-Roblero, Approaches for Removal of PAHs in Soils: Bioaugmentation, Biostimulation and Bioattenuation, in: 2016: pp. 141–157. https://doi.org/http://dx.doi.org/10.5772/64682.
[31] T. Lebeau, Bioaugmentation for In Situ Soil Remediation: How to Ensure the Success of Such a Process, 2011. https://doi.org/10.1007/978-3-642-19769-7_7.
[32] M.L. Verma, M. Thakur, J.S. Randhawa, D. Sharma, A. Thakur, H. Meehnian, A.K. Jana, Biotechnological Applications of Fungal Enzymes with Special Reference to Bioremediation, (2020) 221–247. https://doi.org/10.1007/978-3-030-38196-7_10.
[33] F.M. Bento, F. Camargo, B. Okeke, W.T. Frankenberger-Júnior, Bioremediation of soil contaminated by diesel oil, Brazilian J. Microbiol. 34 (2003) 2411–2418.
[34] D.S. Hibbett, M. Binder, J.F. Bischoff, M. Blackwell, P.F. Cannon, O.E. Eriksson, S. Huhndorf, T. James, P.M. Kirk, R. Lücking, H. Thorsten Lumbsch, F. Lutzoni, P.B. Matheny, D.J. McLaughlin, M.J. Powell, S. Redhead, C.L. Schoch, J.W. Spatafora, J.A. Stalpers, R. Vilgalys, M.C. Aime, A. Aptroot, R. Bauer, D. Begerow, G.L. Benny, L.A. Castlebury, P.W. Crous, Y.C. Dai, W. Gams, D.M. Geiser, G.W. Griffith, C. Gueidan, D.L. Hawksworth, G. Hestmark, K. Hosaka, R.A. Humber, K.D. Hyde, J.E. Ironside, U. Kõljalg, C.P. Kurtzman, K.H. Larsson, R. Lichtwardt, J. Longcore, J. Miadlikowska, A. Miller, J.M. Moncalvo, S. Mozley-Standridge, F. Oberwinkler, E. Parmasto, V. Reeb, J.D. Rogers, C. Roux, L. Ryvarden, J.P. Sampaio, A. Schüßler, J. Sugiyama, R.G. Thorn, L. Tibell, W.A. Untereiner, C. Walker,

Z. Wang, A. Weir, M. Weiss, M.M. White, K. Winka, Y.J. Yao, N. Zhang, A higher-level phylogenetic classification of the Fungi, Mycol. Res. 111 (2007) 509–547. https://doi.org/10.1016/j.mycres.2007.03.004.
[35] P. Baldrian, Wood-inhabiting ligninolytic basidiomycetes in soils: Ecology and constraints for applicability in bioremediation, Fungal Ecol. 1 (2008) 4–12. https://doi.org/10.1016/j.funeco.2008.02.001.
[36] E. Winquist, K. Björklöf, E. Schultz, M. Räsänen, K. Salonen, F. Anasonye, T. Cajthaml, K.T. Steffen, K.S. Jørgensen, M. Tuomela, Bioremediation of PAH-contaminated soil with fungi - From laboratory to field scale, Int. Biodeterior. Biodegrad. (2014). https://doi.org/10.1016/j.ibiod.2013.09.012.
[37] J. Yang, W. Li, T. Bun Ng, X. Deng, J. Lin, X. Ye, Laccases: Production, expression regulation, and applications in pharmaceutical biodegradation, Front. Microbiol. 8 (2017). https://doi.org/10.3389/fmicb.2017.00832.
[38] S.B. Pointing, Feasibility of bioremediation by white-rot fungi, Appl. Microbiol. Biotechnol. 57 (2001) 20–33. https://doi.org/10.1007/s002530100745.
[39] H. Forootanfar, M.A. Faramarzi, Insights into laccase producing organisms, fermentation states, purification strategies, and biotechnological applications, Biotechnol. Prog. 31 (2015) 1443–1463. https://doi.org/10.1002/btpr.2173.
[40] J.R. Jeon, P. Baldrian, K. Murugesan, Y.S. Chang, Laccase-catalysed oxidations of naturally occurring phenols: From in vivo biosynthetic pathways to green synthetic applications, Microb. Biotechnol. 5 (2012) 318–332. https://doi.org/10.1111/j.1751-7915.2011.00273.x.
[41] K.T. Steffen, A. Hatakka, M. Hofrichter, Degradation of Benzo[a]pyrene by the Litter-Decomposing Basidiomycete, Appl. Environ. Microbiol. 69 (2003) 3957–3964. https://doi.org/10.1128/AEM.69.7.3957.
[42] R. Deshmukh, A.A. Khardenavis, H.J. Purohit, Diverse Metabolic Capacities of Fungi for Bioremediation, Indian J. Microbiol. 56 (2016) 247–264. https://doi.org/10.1007/s12088-016-0584-6.
[43] T. Kadri, T. Rouissi, S. Kaur Brar, M. Cledon, S. Sarma, M. Verma, Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by fungal enzymes: A review, J. Environ. Sci. (China). (2017). https://doi.org/10.1016/j.jes.2016.08.023.
[44] P. Singh Nee Nigam, A. Pandey, Biotechnology for agro-industrial residues utilisation: Utilisation of agro-residues, Springer Netherlands, 2009. https://doi.org/10.1007/978-1-4020-9942-7.
[45] H. Wu, B. Sun, J. Li, Polycyclic aromatic hydrocarbons in sediments/soils of the rapidly urbanized lower reaches of the river Chaohu, China, Int. J. Environ. Res. Public Health. 16 (2019). https://doi.org/10.3390/ijerph16132302.
[46] O. Potin, E. Veignie, C. Rafin, Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by Cladosporium sphaerospermum isolated from an aged PAH contaminated soil, FEMS Microbiol. Ecol. 51 (2004) 71–78. https://doi.org/10.1016/j.femsec.2004.07.013.
[47] Y. Wu, Y. Teng, Z. Li, X. Liao, Y. Luo, Potential role of polycyclic aromatic hydrocarbons (PAHs) oxidation by fungal laccase in the remediation of an aged contaminated soil, Soil Biol. Biochem. 40 (2008) 789–796. https://doi.org/10.1016/j.soilbio.2007.10.013.
[48] R.H. Peng, A.S. Xiong, Y. Xue, X.Y. Fu, F. Gao, W. Zhao, Y.S. Tian, Q.H. Yao, Microbial biodegradation of polyaromatic hydrocarbons, FEMS Microbiol. Rev. 32 (2008) 927–955. https://doi.org/10.1111/j.1574-6976.2008.00127.x.
[49] K.T. Steffen, S. Schubert, M. Tuomela, A. Hatakka, M. Hofrichter, Enhancement of bioconversion of high-molecular mass polycyclic aromatic hydrocarbons in contaminated non-sterile soil by litter-decomposing fungi, Biodegradation. 18 (2007) 359–369. https://doi.org/10.1007/s10532-006-9070-x.
[50] D. Boyle, C. Wiesner, A. Richardson, Factors affecting the degradation of polyaromatic hydrocarbons in soil by white-rot fungi, Soil Biol. Biochem. 30 (1998) 873–882. https://doi.org/10.1016/S0038-

0717(97)00219-8.
[51] E. Borràs, G. Caminal, M. Sarrà, Č. Novotný, Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes versicolor* and *Irpex lacteus* from contaminated soil, Soil Biol. Biochem. 42 (2010) 2087–2093. https://doi.org/10.1016/j.soilbio.2010.08.003.
[52] R. Rama, J.C. Sigoillot, V. Chaplain, M. Asther, C. Jolivalt, C. Mougin, Inoculation of filamentous fungi in manufactured gas plant site soils and pah transformation, Polycycl. Aromat. Compd. 18 (2001) 397–414. https://doi.org/10.1080/10406630108233817.
[53] C. Eggert, U. Temp, J.F.D. Dean, K.E.L. Eriksson, A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase, FEBS Lett. (1996). https://doi.org/10.1016/0014-5793(96)00719-3.
[54] A. Anastasi, V. Tigini, G.C. Varese, The Bioremediation Potential of Different Ecophysiological Groups of Fungi, (2013) 29–49. https://doi.org/10.1007/978-3-642-33811-3_2.
[55] V. Tigini, V. Prigione, S. Di Toro, F. Fava, G.C. Varese, Isolation and characterisation of polychlorinated biphenyl (PCB) degrading fungi from a historically contaminated soil, Microb. Cell Fact. 8 (2009) 5. https://doi.org/10.1186/1475-2859-8-5.
[56] A. Verdin, A.L.H. Sahraoui, R. Durand, Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes, Int. Biodeterior. Biodegrad. 53 (2004) 65–70. https://doi.org/10.1016/j.ibiod.2003.12.001.
[57] L. Yao, Y. Teng, Y. Luo, P. Christie, W. Ma, F. Liu, Y. Wu, Y. Luo, Z. Li, Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *trichoderma reesei* FS10-C and effect of bioaugmentation on an aged PAH-contaminated soil, Bioremediat. J. 19 (2015) 9–17. https://doi.org/10.1080/10889868.2014.939137.
[58] G.E. Harman, C.R. Howell, A. Viterbo, I. Chet, M. Lorito, Trichoderma species — opportunistic, avirulent plant symbionts, Nat. Rev. Microbiol. 2 (2004) 43–56. https://doi.org/10.1038/nrmicro797.
[59] P. Tripathi, P.C. Singh, A. Mishra, P.S. Chauhan, S. Dwivedi, R.T. Bais, R.D. Tripathi, Trichoderma: A potential bioremediator for environmental clean up, Clean Technol. Environ. Policy. 15 (2013) 541–550. https://doi.org/10.1007/s10098-012-0553-7.
[60] C. Ravelet, S. Krivobok, L. Sage, R. Steiman, Biodegradation of pyrene by sediment fungi, Chemosphere. 40 (2000) 557–563. https://doi.org/10.1016/S0045-6535(99)00320-3.
[61] A. Saraswathy, R. Hallberg, Degradation of pyrene by indigenous fungi from a former gasworks site, FEMS Microbiol. Lett. 210 (2002) 227–232. https://doi.org/10.1016/S0378-1097(02)00606-7.
[62] C. Machín-Ramírez, D. Morales, F. Martínez-Morales, A.I. Okoh, M.R. Trejo-Hernández, Benzo[a]pyrene removal by axenic- and co-cultures of some bacterial and fungal strains, Int. Biodeterior. Biodegrad. 64 (2010) 538–544. https://doi.org/10.1016/j.ibiod.2010.05.006.
[63] P. Baldrian, Fungal laccases-occurrence and properties, FEMS Microbiol. Rev. 30 (2006) 215–242. https://doi.org/10.1111/j.1574-4976.2005.00010.x.
[64] O. V. Morozova, G.P. Shumakovich, S. V. Shleev, Y.I. Yaropolov, Laccase-mediator systems and their applications: A review, Appl. Biochem. Microbiol. 43 (2007) 523–535. https://doi.org/10.1134/S0003683807050055.
[65] E.M. Ko, Y.E. Leem, H.T. Choi, Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*, Appl. Microbiol. Biotechnol. 57 (2001) 98–102. https://doi.org/10.1007/s002530100727.
[66] S. Shleev, A. Christenson, V. Serezhenkov, D. Burbaev, A. Yaropolov, L. Gorton, T. Ruzgas, Electrochemical redox transformations of T1 and T2 copper sites in native Trametes hirsuta laccase at gold electrode, Biochem. J. 385 (2005) 745–754. https://doi.org/10.1042/BJ20041015.
[67] F. Xu, Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases, J. Biol. Chem. 272 (1997) 924–928. https://doi.org/10.1074/jbc.272.2.924.
[68] V. Madhavi, S.S. Lele, Laccases-properties and applications, Bioresources. 4 (2009) 1674–1717. https://doi.org/10.1016/B978-0-12-805419-2.00007-1.

[69] C.F. Thurston, The structure and function of fungal laccases, Microbiology. 1 (1994) 19–26.
[70] S.B. Pointing, Feasibility of bioremediation by white-rot fungi, Appl. Microbiol. Biotechnol. 57 (2001) 20–33. https://doi.org/10.1007/s002530100745.
[71] A. Kunamneni, F.J. Plou, A. Ballesteros, M. Alcalde, Laccases and their applications: a patent review., Recent Pat. Biotechnol. 2 (2008) 10–24. http://www.ncbi.nlm.nih.gov/pubmed/19075849.
[72] S.R. Couto, J.L. Toca-Herrera, Laccase production at reactor scale by filamentous fungi, Biotechnol. Adv. 25 (2007) 558–569. https://doi.org/10.1016/J.BIOTECHADV.2007.07.002.
[73] A. Pandey, P. Selvakumar, C.R. Soccol, P. Nigam, Solid state fermentation for the production of industrial enzymes, Curr. Sci. 77 (1999) 149–162. http://www.jstor.org/stable/24102923.
[74] R. Bourbonnais, M.G. Paice, Oxidation of non-phenolic substrates An expanded role for lactase in lignin biodegradation, 1990.
[75] A.I. Cañas, S. Camarero, Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes, Biotechnol. Adv. 28 (2010) 694–705. https://doi.org/10.1016/j.biotechadv.2010.05.002.
[76] M. Fernández-Fernández, M.Á. Sanromán, D. Moldes, Recent developments and applications of immobilized laccase, Biotechnol. Adv. 31 (2013) 1808–1825. https://doi.org/10.1016/J.BIOTECHADV.2012.02.013.
[77] P. Upadhyay, R. Shrivastava, P.K. Agrawal, Bioprospecting and biotechnological applications of fungal laccase, 3 Biotech. 6 (2016) 1–12. https://doi.org/10.1007/s13205-015-0316-3.
[78] C. Johannes, A. Majcherczyk, Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems, Appl. Environ. Microbiol. 66 (2000) 524–528. https://doi.org/10.1128/AEM.66.2.524-528.2000.
[79] S. Camarero, A.I. Cañas, P. Nousiainen, E. Record, A. Lomascolo, M.J. Martínez, Á.T. Martínez, p-hydroxycinnamic acids as natural mediators for laccase oxidation of recalcitrant compounds, Environ. Sci. Technol. 42 (2008) 6703–6709. https://doi.org/10.1021/es8008979.
[80] M.R. Hu, Y.P. Chao, G.Q. Zhang, Z.Q. Xue, S. Qian, Laccase-mediator system in the decolorization of different types of recalcitrant dyes, J. Ind. Microbiol. Biotechnol. 36 (2009) 45–51. https://doi.org/10.1007/s10295-008-0471-1.
[81] D.W.S. Wong, Structure and action mechanism of ligninolytic enzymes, 2009. https://doi.org/10.1007/s12010-008-8279-z.
[82] R. Pogni, M.C. Baratto, A. Sinicropi, R. Basosi, Spectroscopic and computational characterization of laccases and their substrate radical intermediates, Cell. Mol. Life Sci. 72 (2015) 885–896. https://doi.org/10.1007/s00018-014-1825-7.
[83] S.-S. Weng, S.-M. Liu, H.-T. Lai, Application parameters of laccase–mediator systems for treatment of sulfonamide antibiotics, Bioresour. Technol. 141 (2013) 152–159. https://doi.org/10.1016/J.BIORTECH.2013.02.093.
[84] D. Becker, S. Varela Della Giustina, S. Rodriguez-Mozaz, R. Schoevaart, D. Barceló, M. de Cazes, M.P. Belleville, J. Sanchez-Marcano, J. de Gunzburg, O. Couillerot, J. Völker, J. Oehlmann, M. Wagner, Removal of antibiotics in wastewater by enzymatic treatment with fungal laccase – Degradation of compounds does not always eliminate toxicity, Bioresour. Technol. 219 (2016) 500–509. https://doi.org/10.1016/j.biortech.2016.08.004.
[85] T. Bach Tuyet Lam, K. Iiyama, B.A. Stone, Determination of etherified hydroxycinnamic acids in cell walls of grasses, Phytochemistry. 36 (1994) 773–775. https://doi.org/10.1016/S0031-9422(00)89815-5.
[86] K. Suominen, V. Kitunen, A. Smolander, Characteristics of dissolved organic matter and phenolic compounds in forest soils under silver birch (*Betula pendula*), Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), Eur. J. Soil Sci. 54 (2003) 287–293. https://doi.org/10.1046/j.1365-2389.2003.00524.x.
[87] F. Carunchio, C. Crescenzi, A. Maria, A. Messina, A. Maria, EndNoteQuestion8, 55 (2001) 189–200.

[88] Liu Yulong, Hua Xiufu, Degradation of acenaphthylene and anthracene by chemically modified laccase from *Trametes versicolor*, RSC Adv. 4 (2014) 31120–31122. https://doi.org/10.1039/C4RA02807D.
[89] P.T.L. Ike, W.G. Birolli, D.M. dos Santos, A.L.M. Porto, D.H.F. Souza, Biodegradation of anthracene and different PAHs by a yellow laccase from *Leucoagaricus gongylophorus*, Environ. Sci. Pollut. Res. 26 (2019) 8675–8684. https://doi.org/10.1007/s11356-019-04197-z.
[90] X. Li, G. La, Q. Cheng, F. Wang, F. Feng, B. Zhang, Z. Zhang, Profile of natural redox mediators production of laccase-producing fungus *Pleurotus ostreatus.*, Bull. Environ. Contam. Toxicol. 93 (2014) 478–482. https://doi.org/10.1007/s00128-014-1340-4.
[91] Y. Li, J. Shao, X. Wang, Y. Deng, H. Yang, H. Chen, Characterization of modified biochars derived from bamboo pyrolysis and their utilization for target component (furfural) adsorption, Energy and Fuels. (2014). https://doi.org/10.1021/ef500725c.
[92] H. Punnapayak, S. Prasongsuk, K. Messner, K. Danmek, P. Lotrakul, Polycyclic aromatic hydrocarbons (PAHs) degradation by laccase from a tropical white rot fungus *Ganoderma lucidum*, African J. Biotechnol. 8 (2009) 5897–5900.
[93] X. Li, X. Lin, R. Yin, Y. Wu, H. Chu, J. Zeng, T. Yang, Optimization of Laccase-mediated Benzo[a]pyrene Oxidation and the Bioremedial Application in Aged Polycyclic Aromatic Hydrocarbons-contaminated Soil, 2010.
[94] R. Levin, S. Lias, Ionization potential and appearance potential measurments, (1983).
[95] A.A. Dias, A.J.S. Matos, I. Fraga, A. Sampaio, R.M.F. Bezerra, An Easy Method for Screening and Detection of Laccase Activity, Open Biotechnol. J. 11 (2017) 89–93. https://doi.org/10.2174/1874070701711010089.
[96] P. Rodrigues, A. Venâncio, Z. Kozakiewicz, N. Lima, A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of Aspergillus Section Flavi isolated from Portuguese almonds, Int. J. Food Microbiol. 129 (2009) 187–193. https://doi.org/10.1016/j.ijfoodmicro.2008.11.023.
[97] M. Stredansky, E. Conti, Xanthan production by solid state fermentation, Process Biochem. (1999). https://doi.org/10.1016/S0032-9592(98)00131-9.
[98] US EPA, Selected Analytical Methods for Environmental Remediation and Recovery (SAM) – 2012, (2012).
[99] A. Kucak, M. Blanusa, Comparison of two extraction procedures for determination of trace metals in soil by atomic absorption spectrometry, Arh. Hig. Rada Toksikol. 49 (1998) 327–334.
[100] A. D'Annibale, F. Rosetto, V. Leonardi, F. Federici, M. Petruccioli, Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons, Appl. Environ. Microbiol. (2006). https://doi.org/10.1128/AEM.72.1.28-36.2006.
[101] R.M. Pawar, The Effect of Soil pH on Bioremediation of Polycyclic Aromatic Hydrocarbons (PAHS), J. Bioremediation Biodegrad. 06 (2015). https://doi.org/10.4172/2155-6199.1000291.
[102] M.B. Soquetta, F.S. Stefanello, K.D.M. Huerta, S.S. Monteiro, C.S. Da Rosa, N.N. Terra, Characterization of physiochemical and microbiological properties, and bioactive compounds, of flour made from the skin and bagasse of kiwi fruit (*Actinidia deliciosa*), Food Chem. 199 (2016) 471–478. https://doi.org/10.1016/j.foodchem.2015.12.022.
[103] A. Gençer, The utilization of kiwi (actinidia deliciosa) pruning waste for kraft paper production and the effect of the bark on paper properties, Drewno. 58 (2015) 103–113. https://doi.org/10.12841/wood.1644-3985.084.08.
[104] M. Dias, C. Caleja, C. Pereira, R.C. Calhelha, M. Kostic, M. Sokovic, D. Tavares, I.J. Baraldi, L. Barros, I.C.F.R. Ferreira, Chemical composition and bioactive properties of byproducts from two different kiwi varieties, Food Res. Int. 127 (2020) 108753. https://doi.org/10.1016/j.foodres.2019.108753.
[105] P.A. Duc, P. Dharanipriya, B.K. Velmurugan, M. Shanmugavadivu, Groundnut shell -a beneficial bio-waste, Biocatal. Agric. Biotechnol. 20 (2019) 101206.

https://doi.org/10.1016/j.bcab.2019.101206.
[106] F. Anike, M. Yusuf, O. Isikhuemhen, Co-Substrating of Peanut Shells with Cornstalks Enhances Biodegradation by *Pleurotus ostreatus*, J. Bioremediation Biodegrad. 07 (2016). https://doi.org/10.4172/2155-6199.1000327.
[107] P.J. Rutledge, G.L. Challis, Discovery of microbial natural products by activation of silent biosynthetic gene clusters, Nat. Rev. Microbiol. 13 (2015) 509–523. https://doi.org/10.1038/nrmicro3496.
[108] A. Marmann, A.H. Aly, W. Lin, B. Wang, P. Proksch, Co-cultivation - A powerful emerging tool for enhancing the chemical diversity of microorganisms, Mar. Drugs. 12 (2014) 1043–1065. https://doi.org/10.3390/md12021043.
[109] V. Balaji, P. Arulazhagan, P. Ebenezer, Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds, J. Environ. Biol. 35 (2014) 521–529.
[110] C. Cabrera, M.A. Vázquez, J.C.D. Mendoza, N.A. Dorta, Production of lignocellulases enzymes from Trichoderma viride M5-2 in wheat bran (*Triticum aestivum*) and purification of their laccases Producción de enzimas lignocelulasas de Trichoderma viride M5-2 en salvado de trigo (*Triticum aestivum*) y purificac, 54 (2020) 55–66.
[111] L.M. Divya, G.K. Prasanth, C. Sadasivan, Potential of the salt-tolerant laccase-producing strain *Trichoderma viride* Pers. NFCCI-2745 from an estuary in the bioremediation of phenol-polluted environments, J. Basic Microbiol. 54 (2014) 542–547. https://doi.org/10.1002/jobm.201200394.
[112] T. Senthivelan, J. Kanagaraj, R.C. Panda, T. Narayani, Screening and production of a potential extracellular fungal laccase from P*enicillium chrysogenum*: Media optimization by response surface methodology (RSM)and central composite rotatable design (CCRD), Biotechnol. Reports. 23 (2019) e00344. https://doi.org/10.1016/j.btre.2019.e00344.
[113] J. Liu, Z. Wang, H. Li, C. Hu, P. Raymer, Q. Huang, Effect of solid state fermentation of peanut shell on its dye adsorption performance, Bioresour. Technol. 249 (2018) 307–314. https://doi.org/10.1016/j.biortech.2017.10.010.
[114] G. Li, X. Liu, L. Yuan, Improved laccase production by *Funalia trogii* in absorbent fermentation with nutrient carrier, J. Biosci. Bioeng. 124 (2017) 381–385. https://doi.org/10.1016/j.jbiosc.2017.05.002.
[115] F.T. Nisa, M. Asgher, Z. Anwar, A. Afroz, N. Zeeshan, U. Rashid, M. Irshad, S. Mehmood, K. Sughra, Influence of culture conditions on ligninolytic enzymes production from *ganoderma lucidum* using agro-industrial residues, Fresenius Environ. Bull. 23 (2014) 3441–3447.
[116] H.M. Shantaveera, - Ramalingappa, Lignolytic Enzymes Production from Selected Mushrooms, Int. J. Appl. Sci. Biotechnol. 3 (2015) 308–313. https://doi.org/10.3126/ijasbt.v3i2.12732.
[117] O.S. Isikhuemhen, N.A. Mikiashvili, V. Kelkar, Application of solid waste from anaerobic digestion of poultry litter in *Agrocybe aegerita* cultivation: Mushroom production, lignocellulolytic enzymes activity and substrate utilization, Biodegradation. 20 (2009) 351–361. https://doi.org/10.1007/s10532-008-9226-y.
[118] B. Styrishave, E. Björklund, A. Johnsen, B. Halling-Sørensen, The spatial heterogeneity of polycyclic aromatic hydrocarbons in soil depends on their physico-chemical properties, Water. Air. Soil Pollut. 223 (2012) 969–977. https://doi.org/10.1007/s11270-011-0916-4.
[119] M. Zhang, M. Ahmad, S.S. Lee, L.H. Xu, Y.S. Ok, Sorption of polycyclic aromatic hydrocarbons (PAHs) to lignin: Effects of hydrophobicity and temperature, Bull. Environ. Contam. Toxicol. 93 (2014) 84–88. https://doi.org/10.1007/s00128-014-1290-x.
[120] M.H. Huesemann, T.S. Hausmann, T.J. Fortman, Does bioavailability limit biodegradation, Biodegradation. 15 (2004) 261–274.
[121] K. Chupungars, P. Rerngsamran, S. Thaniyavarn, Polycyclic aromatic hydrocarbons degradation by *Agrocybe sp*. CU-43 and its fluorene transformation, Int. Biodeterior. Biodegrad. 63 (2009) 93–99. https://doi.org/10.1016/j.ibiod.2008.06.006.

[122] R.. Bonomo, G. Cennamo, R. Purrello, A.. Santoro, R. Zappalà, Comparison of three fungal laccases from *Rigidoporus lignosus* and *Pleurotus ostreatus*: correlation between conformation changes and catalytic activity, J. Inorg. Biochem. 83 (2001) 67–75. https://doi.org/10.1016/S0162-0134(00)00130-6.

[123] D.C. Naseby, J.M. Lynch, Rhizosphere soil enzymes as indicators of perturbations caused by enzyme substrate addition and inoculation of a genetically modified strain of Pseudomonas fluorescens on wheat seed, Soil Biol. Biochem. 29 (1997) 1353–1362. https://doi.org/10.1016/S0038-0717(97)00061-8.

[124] W.T.E. Ting, S.Y. Yuan, S.D. Wu, B. V. Chang, Biodegradation of phenanthrene and pyrene by *Ganoderma lucidum,* Int. Biodeterior. Biodegrad. 65 (2011) 238–242. https://doi.org/10.1016/j.ibiod.2010.11.007.

[125] K. Brijwani, P. V. Vadlani, Cellulolytic enzymes production via solid-state fermentation: Effect of pretreatment methods on physicochemical characteristics of substrate, Enzyme Res. 2011 (2011). https://doi.org/10.4061/2011/860134.

[126] S. Rodríguez Couto, M.A. Sanromán, Application of solid-state fermentation to ligninolytic enzyme production, Biochem. Eng. J. 22 (2005) 211–219. https://doi.org/10.1016/J.BEJ.2004.09.013.

[127] E. Rosales, S.R. Couto, M.Á. Sanromán, Reutilisation of food processing wastes for production of relevant metabolites: Application to laccase production by Trametes *hirsuta,* J. Food Eng. 66 (2005) 419–423. https://doi.org/10.1016/j.jfoodeng.2004.04.010.

[128] H.L. Hu, J. van den Brink, B.S. Gruben, H.A.B. Wösten, J.-D. Gu, R.P. de Vries, Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* and with other fungi, Int. Biodeterior. Biodegradation. 65 (2011) 248–252. https://doi.org/10.1016/J.IBIOD.2010.11.008.

[129] H. Meehnian, A.K. Jana, M.M. Jana, Pretreatment of cotton stalks by synergistic interaction of *Daedalea flavida* and *Phlebia radiata* in co-culture for improvement in delignification and saccharification, Int. Biodeterior. Biodegrad. 117 (2017) 68–77. https://doi.org/10.1016/j.ibiod.2016.11.022.

[130] K. Brijwani, A. Rigdon, P. V. Vadlani, Fungal laccases: Production, function, and applications in food processing, Enzyme Res. 2010 (2010). https://doi.org/10.4061/2010/149748.

[131] C.F. Thurston, The structure and function of fungal laccases, Microbiology. 16 (1994) 19–26. www.microbiologyresearch.org.

[132] P.J. Strong, Improved laccase production by *trametes pubescens* MB89 in distillery wastewaters, Enzyme Res. 2011 (2011). https://doi.org/10.4061/2011/379176.

[133] E. Lang, A. Gonser, F. Zadrazil, Influence of incubation temperature on activity of ligninolytic enzymes in sterile soil by Pleurotus sp. and Dichomitus squalens, J. Basic Microbiol. 40 (2000) 33–39. https://doi.org/10.1002/(SICI)1521-4028(200002)40:1<33::AID-JOBM33>3.0.CO;2-Q.

[134] S. Rodríguez Couto, D. Moldes, A. Liébanas, A. Sanromán, Investigation of several bioreactor configurations for laccase production by *Trametes versicolor* operating in solid-state conditions, Biochem. Eng. J. 15 (2003) 21–26. https://doi.org/10.1016/S1369-703X(02)00180-8.

[135] S. Rodríguez-Couto, Production of laccase and decolouration of the textile dye Remazol Brilliant Blue R in temporary immersion bioreactors, J. Hazard. Mater. 194 (2011) 297–302. https://doi.org/10.1016/j.jhazmat.2011.07.098.

[136] E. Rosales, S. Rodríguez Couto, M.A. Sanromán, Increased laccase production by *Trametes hirsuta* grown on ground orange peelings, Enzyme Microb. Technol. 40 (2007) 1286–1290. https://doi.org/10.1016/J.ENZMICTEC.2006.09.015.

[137] U. Böhmer, S. Frömmel, T. Bley, M. Müller, K. Frankenfeld, P. Miethe, Solid-state fermentation of lignocellulotic materials for the production of enzymes by the white-rot fungus *Trametes hirsuta* in a modular bioreactor, Eng. Life Sci. 11 (2011) 395–401. https://doi.org/10.1002/elsc.201000162.

[138] W. Hailei, L. Ping, Y. Yuhua, L. Yufeng, Overproduction of laccase from a newly isolated *Ganoderma lucidum* using the municipal food waste as main carbon and nitrogen supplement, Bioprocess Biosyst.

Eng. 38 (2015) 957–966. https://doi.org/10.1007/s00449-014-1341-z.
[139] H. Patel, A. Gupte, Optimization of different culture conditions for enhanced laccase production and its purification from *Tricholoma giganteum* AGHP, Bioresour. Bioprocess. 3 (2016). https://doi.org/10.1186/s40643-016-0088-6.
[140] A. Chenthamarakshan, N. Parambayil, N. Miziriya, P.S. Soumya, M.S.K. Lakshmi, A. Ramgopal, A. Dileep, P. Nambisan, Optimization of laccase production from *Marasmiellus palmivorus* LA1 by Taguchi method of Design of experiments, BMC Biotechnol. 17 (2017) 1–10. https://doi.org/10.1186/s12896-017-0333-x.
[141] G.S. Nyanhongo, J. Gomes, G. Gübitz, R. Zvauya, J.S. Read, W. Steiner, Production of laccase by a newly isolated strain of *Trametes modesta*, Bioresour. Technol. 84 (2002) 259–263. https://doi.org/10.1016/S0960-8524(02)00044-5.
[142] A. Góralczyk-Binkowska, A. Jasinska, A. Dlugonski, P. Plocinski, J. Dlugonski, Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park, PLoS One. 15 (2020) 1–18. https://doi.org/10.1371/journal.pone.0231453.
[143] X. Jin, X. Yu, G. Zhu, Z. Zheng, F. Feng, Z. Zhang, Conditions Optimizing and Application of Laccase-mediator System (LMS) for the Laccase-catalyzed Pesticide Degradation, Sci. Rep. (2016). https://doi.org/10.1038/srep35787.
[144] J. Zhang, X. Liu, Z. Xu, H. Chen, Y. Yang, Degradation of chlorophenols catalyzed by laccase, Int. Biodeterior. Biodegradation. 61 (2008) 351–356. https://doi.org/10.1016/J.IBIOD.2007.06.015.
[145] N. Aktaş, A. Tanyolaç, Reaction conditions for laccase catalyzed polymerization of catechol, Bioresour. Technol. 87 (2003) 209–214. https://doi.org/10.1016/S0960-8524(02)00254-7.
[146] A.S. Allard, M. Remberger, A.H. Neilson, The negative impact of aging on the loss of PAH components in a creosote-contaminated soil, Int. Biodeterior. Biodegrad. 46 (2000) 43–49. https://doi.org/10.1016/S0964-8305(00)00050-0.
[147] S. Mosleh, M.R. Rahimi, M. Ghaedi, K. Dashtian, S. Hajati, Sonochemical-assisted synthesis of CuO/Cu2O/Cu nanoparticles as efficient photocatalyst for simultaneous degradation of pollutant dyes in rotating packed bed reactor: LED illumination and central composite design optimization, Ultrason. Sonochem. 40 (2018) 601–610. https://doi.org/10.1016/j.ultsonch.2017.08.007.
[148] S. Mosleh, M.R. Rahimi, M. Ghaedi, K. Dashtian, S. Hajati, S. Wang, Ag3PO4/AgBr/Ag-HKUST-1-MOF composites as novel blue LED light active photocatalyst for enhanced degradation of ternary mixture of dyes in a rotating packed bed reactor, Chem. Eng. Process. - Process Intensif. 114 (2017) 24–38. https://doi.org/10.1016/j.cep.2017.01.009.
[149] D. Ghosal, S. Ghosh, T.K. Dutta, Y. Ahn, Corrigendum to "Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): A review" [Front. Microbiol. 2016, 7:1369]. doi: 10.3389/fmicb.2016.01369, Front. Microbiol. 7 (2016). https://doi.org/10.3389/fmicb.2016.01837.
[150] A.I. Cañas, M. Alcalde, F. Plou, M.J. Martínez, Á.T. Martínez, S. Camarero, Transformation of polycyclic aromatic hydrocarbons by laccase is strongly enhanced by phenolic compounds present in soil, Environ. Sci. Technol. 41 (2007) 2964–2971. https://doi.org/10.1021/es062328j.
[151] C. Johannes, A. Majcherczyk, Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems, Appl. Environ. Microbiol. 66 (2000) 524–528. https://doi.org/10.1128/AEM.66.2.524-528.2000.
[152] N.N. Pozdnyakova, J. Rodakiewicz-Nowak, O. V. Turkovskaya, J. Haber, Oxidative degradation of polyaromatic hydrocarbons and their derivatives catalyzed directly by the yellow laccase from Pleurotus ostreatus D1, J. Mol. Catal. B Enzym. (2006). https://doi.org/10.1016/j.molcatb.2006.04.002.
[153] L. Luo, S. Lin, H. Huang, S. Zhang, Relationships between aging of PAHs and soil properties, Environ. Pollut. (2012). https://doi.org/10.1016/j.envpol.2012.07.003.
[154] A. Martin, Critical Review Aging, Bioavailability, and Overestimation of Risk from Environmental Pollutants, (2000). https://doi.org/10.1021/es001069.

[155] M.-J. Han, H.-T. Choi, H.-G. Song, Degradation of phenanthrene by *Trametes versicolor* and its laccase., J. Microbiol. 42 (2004) 94–8. http://www.ncbi.nlm.nih.gov/pubmed/15357301.
[156] E.S. Okonofua, J.O. Babatola, O.O. Ojuri, F IELD P ILOT S TUDY O N T HE A SSESSMENT O F S ELECTED, 10 (2019) 121–134.
[157] A.A. Adeyi, P. Oyeleke, Heavy metals and polycyclic aromatic hydrocarbons in soil from e-waste dumpsites in Lagos and Ibadan, Nigeria, J. Heal. Pollut. 7 (2017) 71–84. https://doi.org/10.5696/2156-9614-7.15.71.
[158] A.A. Oketola, S.O. Akpotu, Assessment of solid waste and dumpsite leachate and topsoil, Chem. Ecol. 31 (2015) 134–146. https://doi.org/10.1080/02757540.2014.907280.
[159] S. Bhattacharya, A. Das, K. Prashanthi, M. Palaniswamy, J. Angayarkanni, Mycoremediation of Benzo[a]pyrene by *Pleurotus ostreatus* in the presence of heavy metals and mediators, 3 Biotech. 4 (2014) 205–211. https://doi.org/10.1007/s13205-013-0148-y.
[160] A. Manna, E. Sundaram, C. Amutha, V.S. Vasantha, Efficient Removal of Cadmium Using Edible Fungus and Its Quantitative Fluorimetric Estimation Using (Z)-2-(4 H-1,2,4-Triazol-4-yl)iminomethylphenol, ACS Omega. 3 (2018) 6243–6250. https://doi.org/10.1021/acsomega.8b00342.
[161] G. Bayramoğlu, S. Bektaş, M.Y. Arica, Biosorption of heavy metal ions on immobilized white-rot fungus *Trametes versicolor*, J. Hazard. Mater. 101 (2003) 285–300. https://doi.org/10.1016/S0304-3894(03)00178-X.
[162] T. Happi Emaga, R.H. Andrianaivo, B. Wathelet, J.T. Tchango, M. Paquot, Effects of the stage of maturation and varieties on the chemical composition of banana and plantain peels, Food Chem. 103 (2007) 590–600. https://doi.org/10.1016/j.foodchem.2006.09.006.
[163] J.S.A. Cortez, B.I. Kharisov, T.E.S. Quezada, T.C.H. García, Micro- and nanoporous materials capable of absorbing solvents and oils reversibly: the state of the art, Pet. Sci. 14 (2017) 84–104. https://doi.org/10.1007/s12182-016-0143-0.
[164] J.F. Osma, J.L. Toca Herrera, S. Rodríguez Couto, Banana skin: A novel waste for laccase production by Trametes pubescens under solid-state conditions. Application to synthetic dye decolouration, Dye. Pigment. 75 (2007) 32–37. https://doi.org/10.1016/j.dyepig.2006.05.021.
[165] B. Vandelun Ado, A. Anthony Onilude, H.O. Apeh Oluma, D.M. Mabitine, Production of Fungal Laccase under Solid State Bioprocessing of Agroindustrial Waste and Its Application in Decolourization of Synthetic Dyes, J. Adv. Biol. Biotechnol. 21 (2019) 1–17. https://doi.org/10.9734/jabb/2019/v21i430100.
[166] X. Xu, W. Liu, S. Tian, W. Wang, Q. Qi, P. Jiang, X. Gao, F. Li, H. Li, H. Yu, Petroleum Hydrocarbon-Degrading Bacteria for the Remediation of Oil Pollution Under Aerobic Conditions: A Perspective Analysis, Front. Microbiol. 9 (2018) 1–11. https://doi.org/10.3389/fmicb.2018.02885.
[167] A.K. Haritash, C.P. Kaushik, Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review, J. Hazard. Mater. 169 (2009) 1–15. https://doi.org/10.1016/J.JHAZMAT.2009.03.137.
[168] A.K. Ani, F. Ezeugwu, E. Ochin, Growth and Optimization Processes of Mixed Microbial Population Degrading Chrysene, Gazi Univ. J. Sci. 31 (2018) 740–757.
[169] N. Enayatizamir, J. Liu, L. Wang, X. Lin, P. Fu, Coupling Laccase production from *Trametes pubescence* with heavy metal removal for Economic Waste Water Treatment, J. Water Process Eng. 37 (2020) 101357. https://doi.org/10.1016/j.jwpe.2020.101357.
[170] C. Biache, S. Ouali, A. Cébron, C. Lorgeoux, S. Colombano, P. Faure, Bioremediation of PAH-contamined soils: Consequences on formation and degradation of polar-polycyclic aromatic compounds and microbial community abundance, J. Hazard. Mater. 329 (2017) 1–10. https://doi.org/10.1016/j.jhazmat.2017.01.026.
[171] O. Baltrons, M. López-Mesas, M. Vilaseca, C. Gutiérrez-Bouzán, F. Le Derf, F. Portet-Koltalo, C. Palet, Influence of a mixture of metals on PAHs biodegradation processes in soils, Sci. Total Environ. 628–629 (2018) 150–158. https://doi.org/10.1016/j.scitotenv.2018.02.013.

[172] N. Agrawal, P. Verma, S.K. Shahi, Degradation of polycyclic aromatic hydrocarbons (phenanthrene and pyrene) by the ligninolytic fungi *Ganoderma lucidum* isolated from the hardwood stump, Bioresour. Bioprocess. 5 (2018). https://doi.org/10.1186/s40643-018-0197-5.
[173] N. Agrawal, S.K. Shahi, Degradation of polycyclic aromatic hydrocarbon (pyrene) using novel fungal strain *Coriolopsis byrsina* strain APC5, Int. Biodeterior. Biodegrad. 122 (2017) 69–81. https://doi.org/10.1016/j.ibiod.2017.04.024.
[174] S.K. Panda, S.S. Mishra, E. Kayitesi, R.C. Ray, Microbial-processing of fruit and vegetable wastes for production of vital enzymes and organic acids: Biotechnology and scopes, Environ. Res. 146 (2016) 161–172. https://doi.org/10.1016/j.envres.2015.12.035.
[175] M.D. Jones, E.A. Rodgers-Vieira, J. Hu, M.D. Aitken, Association of growth substrates and bacterial genera with benzo[a]pyrene mineralization in contaminated soil, Environ. Eng. Sci. 31 (2014) 689–697. https://doi.org/10.1089/ees.2014.0275.
[176] P. Baldrian, Interactions of heavy metals with white-rot fungi, Enzyme Microb. Technol. 32 (2003) 78–91. https://doi.org/10.1016/S0141-0229(02)00245-4.
[177] V.E. Pinheiro, M. Michelin, A.C. Vici, P.Z. de Almeida, M. de L. Teixeira de Moraes Polizeli, *Trametes versicolor* laccase production using agricultural wastes: a comparative study in Erlenmeyer flasks, bioreactor and tray, Bioprocess Biosyst. Eng. 43 (2020) 507–514. https://doi.org/10.1007/s00449-019-02245-z.
[178] M. Lorenzo, D. Moldes, S. Rodríguez Couto, M.A. Sanromán, Inhibition of laccase activity from Trametes versicolor by heavy metals and organic compounds, Chemosphere. 60 (2005) 1124–1128. https://doi.org/10.1016/j.chemosphere.2004.12.051.
[179] C. Zhou, A. Dong, Q. Wang, Y. Yu, X. Fan, Y. Cao, T. Li, Effect of Common Metal Ions and Anions on Laccase, BioResources. 12 (2017) 5102–5117.
[180] P. Baldrian, J. Gabriel, Copper and cadmium increase laccase activity in *Pleurotus ostreatus*, FEMS Microbiol. Lett. 206 (2002) 69–74. https://doi.org/10.1016/S0378-1097(01)00519-5.
[181] P. Baldrian, Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme, Appl. Microbiol. Biotechnol. 63 (2004) 560–563. https://doi.org/10.1007/s00253-003-1434-0.
[182] P.J. Shea, T.A. Machacek, S.D. Comfort, Accelerated remediation of pesticide-contaminated soil with zerovalent iron, Environ. Pollut. 132 (2004) 183–188. https://doi.org/10.1016/j.envpol.2004.05.003.
[183] K.N. Niladevi, N. Jacob, P. Prema, Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticus*: Purification and characterization, Process Biochem. 43 (2008) 654–660. https://doi.org/10.1016/j.procbio.2008.02.002.
[184] Y.C. Zhao, X.Y. Yi, M. Zhang, L. Liu, W.J. Ma, Fundamental study of degradation of dichlorodiphenyltrichloroethane in soil by laccase from white rot fungi, Int. J. Environ. Sci. Technol. 7 (2010) 359–366. https://doi.org/10.1007/BF03326145.