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Free and Immobilized Laccases for *In-situ* and *Ex-situ* Polycyclic Aromatic Hydrocarbons Bioremediation

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Dedico este trabalho a minha querida esposa, pois sem a compreensão e o incentivo dela, nada do que foi alcançado seria possível.

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RESUMO

Há uma preocupação crescente com o aumento dos níveis tóxicos dos hidrocarbonetos policíclicos aromáticos derivados do petróleo no meio ambiente. Assim, é necessário desenvolver tecnologias eficientes de tratamento destes compostos, sendo a aplicação de lacase (enzima), um dos tratamentos mais promissores. Neste contexto, as condições otimizadas para a aplicação de lacases são frequentemente investigadas, com trabalho em condições ótimas de pH e temperatura, com mediadores de elevado custo (> R$ 360,00 g^{-1}) e enzimas para obtenção de elevados rendimentos. Mas devido ao uso de reagentes de alto custo em condições controladas, estes não são ainda capazes de resolver problemas de remediação de áreas contaminadas. Para o desenvolvimento de uma técnica de bioremediação economicamente viável e eficiente, a degradação dos hidrocarbonetos policíclicos aromáticos foi avaliada por lacases livre e imobilizada em espuma de poliuretano, material de baixo custo, para biorremediação em modo descontínuo em águas subterrâneas na ausência de mediadores. Os experimentos com a lacase livre apresentaram rendimento máximo de degradação de 94,37% do antraceno, 72,08% de naftaleno e 30,43% de benzo(a)pireno. O destque foi o antraceno que atingiu a concentração final de 0,70 mg L^{-1}, menor do que estabelecidopelas políticas internacionais de meio ambiente e foi detectada antraquinona, conhecido por ser menos tóxica, como produto da degradação. Por outro lado, a lacase imobilizada em espuma de poliuretano removeu 92,35% de antraceno e 97% de benzo(a)pireno. Uma sensível melhora no rendimento de remoção de outros 8 hidrocarbonetos policíclicos aromáticos testados em concentração mais baixa (μg L^{-1}), foi observado pela enzima imobilizada em comparação com os resultados da lacase livre. Lacase imobilizada removeu 77,24% de criseno até 34μg L^{-1}, 32,07% de pireno até 98 μg L^{-1},que em conjunto dos 0,95 mg L^{-1} finais de antraceno, foram os compostos cujas concentrações finais atenderam os limites de intervenção das políticas de proteção ambiental. Após a degradação foram identificados os seguintes produtos de biodegradação do antraceno e benzo(a)pireno: di-isoctil ftalato e tetradecano.A partir destes foram propostas vias de degradação, incluindo processos de oxidação e fissão de anel aromático que levaram à formação de quinona e de dietilftalato. Deste modo, há grande potencial em duas estratégias propostas de biorremediação enzimática: (I) aplicação da lacase livre, diretamente, nas águas subterrâneas para biorremediar os

hidrocarbonetos policíclicos aromáticos *in-situ*, e (II) utilizando biorreatores com lacase imobilizada em espuma de poliuretano para tratamento *ex-situ*.

Palavras-chave: Biodegradação de hidrocarbonetos policíclicos aromáticos. Lacase imobilizada. Espuma de poliuretano. Estratégias de biorremediação.

RESUMO EXPANDIDO

Introdução

Nos últimos anos têm aumentado a preocupação com a poluição de solos e aquíferos por compostos tóxicos derivados do petróleo, dentre eles os Hidrocarbonetos Policíclicos Aromáticos (HPAs). Para eliminar estas contaminações, existem diversas técnicas de remediação que podem atuar no local (*in-situ*) da contaminação ou fora dele (*ex-situ*). O uso de técnicas de bioremediação por micro-organismos é considerada uma estratégia de baixo custo com potenciais rendimentos. Por outro lado, o uso direto de enzimas é considerada uma estratégia promissora de biorremediação que ainda está em estudo. Dentre a classe de enzimas oxidativas, a enzima lacase é conhecida pelo seu potencial em degradar HPAs e compostos fenólicos. A ação catalítica desta enzima é extremamente eficiente e seletiva, com maior capacidade de catalisar a degradação de HPAs em menor tempo, quando comparado com micro-organismos. Apesar disto, esta reação oxidativa têm sido avaliada na literatura em condições controladas de pH e temperatura, na presença de outros compostos que atuam como mediadores de transferência de elétrons. Nestes estudos, elevados percentuais de degradação têm sido obtidos e os produtos iniciais identificados da degradação de HPAs por lacases são quinonas, que são menos tóxicas. No entanto, a aplicação em campo destes processos ainda não pode ser viabilizada devido ao custo elevado dos reagentes para manter a reação em condição controlada e mediar a transferência de elétrons. Nesta direção, a estabilidade ao pH e temperatura das enzimas têm sido melhorada por sua imobilização em suporte, visando manter eficiência de degradação em condições não controladas na presença de mediadores. O uso de caulinita e sílica, inorgânicos, como suportes já foram testados para imobilizar lacase para degradar HPAs, porém houve perda da eficiência de degradação apesar da melhoria da estabilidade. Por outro lado, polímeros orgânicos hidrofóbicos como poliglicidil metacrilato, poliacrilonitrila e poliuretano foram testados como suporte para imobilizar lacase e apresentaram melhoria da estabilidade e aumento de eficiência de remoção de determinados compostos. Daronch (2020) desenvolveu processo de imobilização *in-situ* de lacase em espuma de poliuretano (EPU) com uso de reagentes comerciais, de baixo custo. Há potencial neste bioprocesso para degradação eficiente de HPAs com uso de reagentes de baixo custo, em condições não ideais e sem o uso de mediatores. Assim sendo, este trabalho visa preencher as lacunas inexploradas da literatura, avaliando o uso de lacase livre e imobilizada em EPU para bioremediação *in-situ* e *ex-situ* de HPAs.

Objetivos

Neste contexto, o objetivo principal deste trabalho foi avaliar a viabilidade da biodegradação de HPAs por lacase livre e imobilizada em espuma de poliuretano. Inicialmente, sugeriu-se determinar os efeitos do pH e do uso do mediador ABTS na estabilidade e degradação de HPAs, respectivamente. Deste modo, sem uso de mediadores com adoção de pH neutro (não ideal), este trabalho visa avaliar a biodegradação de antraceno, benzo(a)pireno e naftaleno por lacase livre em amostras naturais de águas subterrâneas visando aplicação direta *in-situ*. Além de, nas mesmas condições similares à de uma área contaminada, determinar rendimentos de biodegradação de HPAs por lacase livre e imobilizada em EPU. Assim, identificar os produtos de degradação de antraceno e benzo(a)pireno, e por fim, propor duas potenciais estratégias de bioremediação considerando o custo, eficiência e políticas ambientais.

Metodologia

A metodologia, assim como os resultados, separou-se em duas seções quanto a biodegradação de HPAs: (i) por lacase livre; (ii) por lacase imobilizada em EPU. Em todas as bateladas de experimentos utilizou-se erlenmeyers de 125 mL e orbital shaker. No primeiro item (i), foi proposta uma estratégia de bioremediação *in-situ* de HPAs de solos e aquíferos contaminados pela aplicação direta de lacase livre. A metodologia deste item (i) seguiu as etapas: a) caracterização da estabilidade da enzima livre frente ao pH; b) avaliação do efeito do pH e do uso de mediator ABTS na biodegradação de HPAs por lacase livre em solução modelo; c) determinação dos rendimentos de biodegradação de antraceno, benzo(a)pireno e naftaleno por lacase livre em amostras naturais de águas subterrâneas; d) identificação do produto formado pela biodegradação de antraceno por análise qualitativa em cromatografia gasosa-espectroscopia de massa (CG-EM). Quanto ao segundo item (ii), outra estratégia de bioremediação (*ex-situ*) foi proposta com uso de enzima imobilizada, onde: a)

aplicou-se a técnica de imobilização de lacase em EPU, com uso de reagentes comerciais, previamente desenvolvida pelo grupo de pesquisa (DARONCH, 2020), b) utilizou-se uma combinação de 16 HPAs (em mg L^{-1} e μg L^{-1}) como contaminantes modelo nos ensaios de degradação por lacase livre e imobilizada em EPU; c) foi determinado o percentual de degradação dos HPAs; d) foram identificados os produtos formados pela biodegradação de antraceno e benzo(a)pireno por CG-EM e teste de combinação de qualidade do espectro de massa, e foi proposto o mecanismo de degradação.

Resultados e Discussão

Na (i) lacase livre obteve boa estabilidade em pH 7, de aproximadamente 80% de atividade enzimática relativa. Apesar de confirmada a influência do mediador ABTS, nos experimentos conduzidos em pH 7 sem o uso do mediador foram obtidas biodegradações de 50,59% de antraceno e 42,15% de benzo(a)pireno. Quando se diz respeito da atuação da lacase livre nas amostras de águas subterrâneas contaminadas, diferentes comportamentos foram observados. Provavelmente a variação das características físico-químicas e microbiológicas destas amostras podem ter influenciado a estabilidade da enzima e consequentemente, as taxas de oxidação de HPAs. Após 5 dias de incubação dos experimentos, a amostra de água subterrânea 2 foi a que apresentou os melhores resultados, 94,37% de oxidação do antraceno e 30,43% de benzo(a)pireno. Antraquinona, que é conhecida por ser menos tóxica, foi identificada como produto de degradação do antraceno no tempo de retenção de 7,61 min. A concentração final obtida de 0,70 mg L^{-1} de antraceno foi menor do que os valores limites de legislações internacionais de gerenciamento de áreas contaminadas. Considerando o cenário de exposição destas legislações, a concentração final de antraceno teria atingido níveis aceitáveis não havendo riscos para a saúde humana. Os resultados demonstram o potencial da aplicação direta de lacase para bioremediação *in-situ* de HPAs.

Lacase imobilizada em EPU foi avaliada em (ii) para biodegradação de todos os 16 HPAs listados como poluentes prioritários em condições não ideais de pH e temperatura, na ausência de mediadores, condições estas próximas a de uma área contaminada. Os resultados de biodegradação dos HPAs testados em mg L^{-1} com lacase em EPU alcançaram 92,35% de remoção de antraceno e 97% de remoção de benzo(a)pireno. Análise qualitativa por CG-EM e análise do espectro de massa levaram a identificação de di-isocotil ftalato e tetradecano como produtos de degradação de antraceno e benzo(a)pireno. O mecanismo de degradação destes compostos foi proposto, com a ocorrência de processos de oxidação dos HPAs e abertura de anéis aromáticos, levaram a formação de quinona e dietil ftalato. Em seguida, por meio dos últimos processos, a polimerização e metilação, levaram às formações dos produtos de degradação identificados. Para os demais HPAs testados em μg L^{-1}, lacase imobilizada em EPU melhorou a remoção em 8 deles, havendo aproximadamente 77% de remoção de criseno e 32% de pireno, até as concentrações finais de 38 e 98 μg L^{-1}, respectivamente. Assim sendo, as concentrações finais de antraceno, pireno e criseno atingiam concentrações finais abaixo dos limites legais após a biodegradação por lacase em EPU.

Considerações Finais

Lacases livre e imobilizada em EPU foram testadas para biodegradação de HPAs em condições não ideais na ausência do usual mediador ABTS, de alto custo (> R$ 360,00 g^{-1} – Sigma Aldrich). A remoção de antraceno e benzo(a)pireno testados em mg L^{-1} e outros 8 HPAs em μg L^{-1} foram melhoradas por lacase imobilizada em EPU. Foram alcançadas concentrações de antraceno, pireno e criseno em níveis inferiores aos limites legais das políticas ambientais com uso de lacase em EPU. Este tratamento foi desenvolvido utilizando suporte de baixo custo para imobilização enzimática, eliminando o uso de mediadores e produtos químicos de alto custo para manter condições controladas. Para a aplicação em campo, o último desafio é substituir por lacases comerciais, visando obter resultados de degradação semelhantes, para consagrar a redução de custo proposta no processo. Assim, há potencial em duas estratégias para biorremediação de hidrocarbonetos policíclicos aromáticos por lacases: (I) aplicação da lacase livre, diretamente, em águas subterrâneas para biorremediação de hidrocarbonetos aromáticos policíclicos *in-situ*, e (II) utilizando lacase imobilizada em EPU em biorreatores por tratamento *ex-situ*.

Palavras-chave: Biodegradação de hidrocarbonetos policíclicos aromáticos. Lacase imobilizada. Espuma de poliuretano. Estratégias de biorremediação.

ABSTRACT

There is a growing concern with the increase in the levels of toxic polycyclic aromatic hydrocarbons derived from petroleum in the environment. Thus, it is necessary to develop efficient technologies for the treatment of these compounds, with the application of laccase (enzyme), one of the most promising treatments. In this context, the optimized conditions for the application of laccases are frequently investigated, working on optimal pH and temperature conditions with high-cost mediators (>$ 60,00 g^{-1}) and enzymes to achieve high degradation yields. But due to use of expensive reagents and controlled conditions they are not yet capable of solving practice cleanup PAH polluted sites. Towards development an economically viable and efficient bioremediation technique, degradation polycyclic aromatic hydrocarbons were evaluated by free and immobilized laccase on polyurethane foam - a low-cost material - in batch mode in groundwater without mediators. Experiments with free laccase showed a maximum yield of 94.37% of anthracene, 72.08% of naphthalene and 30.43% benzo(a)pyrene. The highlight was anthracene, which achieved (0.70 mg L^{-1}) final concentration, less than established by international environmental policies, and the knew less toxic anthraquinone was detected as a degradation product. On the other hand, the laccase immobilized on polyurethane foam removed 92.35% anthracene and 97% benzo(a)pyrene. A noticeable improvement in the removal yield of others 8 polycyclic aromatic hydrocarbons tested in lower concentration (μg L^{-1}), was observed by the enzyme immobilized in comparison with the results of the free laccase. Immobilized laccase removed 77.24% of chrysene up to 34 μg L^{-1}, 32.07% of pyrene up to 98 μg L^{-1}, which, together with the final 0.95 mg L^{-1} of anthracene, were the compounds whose final concentrations met the intervention limits of environmental protection policies. After degradation, the biodegradation products of anthracene and benzo(a)pyrene were identified: diisooctyl phthalate and tetradecane. From which degradation pathways have been proposed, including processes of oxidation and aromatic ring fission that led to the formation of quinone and diethyl phthalate. Thus, there is great potential in two proposed enzymatic bioremediation strategies on non-optimal conditions with reagents cost reduction: (I) application of the free laccase,

directly, in groundwater to bioremediate polycyclic aromatic hydrocarbons *in-situ*, and (II) using laccase immobilized on PUF in bioreactors by *ex-situ* treatment.

Keywords: Polycyclic aromatic hydrocarbons biodegradation. Immobilized laccase. Polyurethane foam. Bioremediation strategies.

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ABTS	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
Ace	Acenaphthene
Acy	Acenaphthylene
ANQ	9,10-anthraquinone
Ant	Anthracene
BaA	Benzo(a)anthracene
BaP	Benzo(a)pyrene
BbF	Benzo(b)fluoranthene
BgP	Benzo(g;h;i)perylene
BkF	Benzo(k)fluoranthene
CETESB	Companhia Ambiental do Estado de São Paulo
Chr	Chrysene
DaA	Dibenzo(a;h)anthracene
DTIV	Dutch Target and Intervention Values
EPA	Enviromental Protection Agency
Fla	Fluoranthrene
Flu	Fluorene
GC-MS	Gas chromatography mass spectrometry
HBT	1-hidroxybenzothyazole
HPLC	High performance liquid chromatography
IcP	Indeno(1,2,3-cd)pyrene
ISCO	*In-situ* chemical oxidation
m/z	Mass spectra
mM	Mili molar
Nap	Naphthalene
NAPL	Non-aqueous phase liquid
NCO	Isocyanate
NIST	National Institute of Standards and Technology
NTU	Nephelometric turbidity units
NZME	New Zeland Ministry of Environment

ORP	Oxidation-reduction potential
PAHs	Polycyclic aromatic hydrocarbons
Phe	Phenanthrene
PT	Proficiency test
PUF	Polyurethane foam
Pyr	Pyrene
SEBR	Surfactant enhanced bioremediation
SER	Surfactant-enhanced remediation
S-ISCO	Surfactant enhanced *in-situ* chemical oxidation
USEPA	United States Environmental Protection Agency

SUMMARY

CONCEPTUAL DIAGRAM

Free and Immobilized Laccases for *In-situ* and *Ex-situ* Polycyclic Aromatic Hydrocarbons Bioremediation

What?
Biodegradation of polycyclic aromatic hydrocarbons using free and immobilized laccase.
Why?
• There is a growing interest in using the enzyme laccase for degradation of polycyclic aromatic hydrocarbons. • The enzymatic catalysis of oxidation reactions of polycyclic aromatic hydrocarbons by *Trametes versicolor* laccase has been essentially related to well-controlled systems. • The low-cost commercial polyurethane foams have been used successfully as support material for laccase immobilization.
Hypotheses
• Is it necessary to use mediator to degrade efficiently polycyclic aromatic hydrocarbons by free and immobilized laccase on polyurethane foams? • Do free and immobilized laccase on polyurethane foams lead to enhanced polycyclic aromatic hydrocarbons removal yields under non-optimal conditions, in *in-situ* and *ex-situ*, respectively?

1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are toxic chemical compounds present in products of petrochemical origin (NACCI *et al*., 2002; DODOR *et al.*, 2004; RENGARAJAN *et al.*, 2015; YUAN *et al.*, 2017). Improper management and accidents involving the direct spillage of these products and derivatives in soils and surface water, such as lakes, rivers, and oceans, and indirectly in groundwater contribute to increasing PAHs levels in the environmental (ADENIJI; OKOH; OKOH, 2019; KARIM; HUSAIN, 2010, LI; LI; LIU, 2017). High potential for PAHs contamination has been founded in combustible stations due to underground combustible tanks, pipes, lubricant ramp, and oil-water separation systems (ABDEL-SHAY; MANSOUR, 2016; MARIANO *et al.*, 2007). It is common to observe in PAHs contaminated sites the occurrence of leaks, spills, flor, and soil infiltration (primary mechanisms), and release of contaminants, through infiltration and dispersion in the soil, down to lower layers of soil and groundwater (secondary mechanisms) (KUPPUSAMY *et al.*, 2017; LIANG, *et al* 2017; SUN, *et al.*, 2019).

The permitted PAHs concentration levels in soils and groundwater are provided by environmental protection and cleanup policies (CETESB, 2014; DTIV, 2000; EPA, 2003; NZME, 1997). In Brazil, CETESB (2014) guides principles that set contaminant limits to quantify the contamination of soil and groundwater. These policies consider the migration scenario of contaminants to the waters and groundwater, and provide necessary actions for pollution prevention and control. For example, combustible stations need to collect groundwater samples from wells periodically, to monitor changes in groundwater quality in the aquifer. If PAHs or other hydrocarbons are detected above the limits, the response from the combustible station will proceed to site investigation and simulate risk analyzes to human health, where targets for reducing contaminant concentration will be determined. After that, once know the contaminated site extension, the next phase is applying the remediation technique.

Site remediation can be directly in the contamination source (*in-situ*) or outside (*ex-situ*) (OSSAI, *et al.*, 2020). Currently integrated technologies have been developing against soil excavation (KUPPUSAMY *et al.*, 2017; LIANG, *et al* 2017), such as *in-situ* surfactant flushing and soil washing, where PAHs are transferred from soils to the aqueous phase in groundwater (LIANG *et al.*, 2017; PERINI, *et al.*, 2020). Once

discharged into groundwater, PAHs can be degraded through other treatment technologies (LAMICHHANE; KRISHNA; SARUKKALIGE, 2017; MOHAN *et al.*, 2006; PERINI, *et al.*, 2020). The bioremediation by microorganisms is an effective and low-cost way to manage contaminated sites. Since it is difficult to control microorganisms, the researchers started to evaluate the direct use of enzymes as emerging techniques for removing PAHs (KUPPUSAMY *et al.*, 2017; MOHAN *et al.*, 2006; RAO *et al.*, 2014).

The laccases are the enzymes among the oxiredutases, which has knew potential to PAHs degradation. Laccases from *Trametes versicolor* has been reported as an biocatalyst for removal of anthracene, benzo(a)pyrene, and others PAHs (COLLINS *et al.*, 1996; LI *et al.*, 2014; RANGELOV & NICELL, 2015; WU *et al.*, 2008). The first step of PAHs biotransformation by laccases is an oxidation that, form quinones that are less toxic. It is worth to mention that use of controlled reaction conditions and expensive mediators as ABTS and HBT are often used to enhance PAHs bioremediation yields (HU *et al.*, 2009; ARCA-RAMOS *et al.*, 2014; LI *et al.*, 2014). However, it is expected to make a current trend possible to field application, when cost reduction is associated to degradation efficiency. In this sense, there is an empty space on literature that must be explored, evaluating PAHs degradation by laccases without high-cost mediator and in non-optimal pH experiment conditions.

The optimal pH range of laccases is between 4 and 5, which also is the condition where laccase has high stability (COLLINS *et al.*, 1996; MAJCHERCZYK *et al.*, 1998). However, the observed pH range in groundwater detected in other studies usually is a more neutral range (pH 6 to 7) (ADENIJI; OKOH; OKOH, 2019; LI; LI; LIU, 2017). Thus, to increase the enzyme stability in non-ideal conditions of pH and temperature, laccase can be immobilized on different supports targering PAHs degradation. When immobilized in kaolinite improvements were observed on the laccase stability at temperature, pH, inhibitors, and storage (DODOR *et al.*, 2004). Immobilized on silica, it was detected better stability on pH and temperature than free laccase, however degradation yields were slightly lower than free laccase (BAUTISTA; MORALEZ; SANZ, 2015; HU *et al.*, 2009). Nevertheless, those inorganics supports, kaolinite, and silica, did not improve PAHs degradation yields compared with free laccase. On the other hand, hydrophobic supports for laccase based on organics polymers have been shown promising results in phenolic compounds removal than free

enzyme. Laccase immobilized on poly(glycidyl methacrylate was capable to remove 90% of bisphenol A and 100% of Congo Red dye in batch experiments (BAYRAMOGLU; KARAGOZ; ARICA, 2018). Polyacrylonitrile beads were used as support to immobilized laccase, showing complete degradation of nonylphenol and octylphenol (CAPATANE *et al.*, 2013). Laccase has been also immobilized on commercial polyurethane foam (PUF) (DARONCH, 2020; STENHOLM *et al.*, 2020). Results showed high removal capability of nonylphenol polyethoxylates by laccase from *T. versicolor* immobilized on PUF, due to biodegradation and adsorption on the support (STENHOLM *et al.*, 2020). In this sense, the use of PUF - a low-cost material – as support for laccase has potential to make this bioprocess efficient to PAHs degradation.

Giving this context, this work milestone intends to tackle these gaps, evaluating free and immobilized laccase on PUF for PAHs *in-situ* and *ex-situ* bioremediation.

1.1 OBJECTIVES

1.1.1 General Objective

To assess the feasibility of the biodegradation of PAHs by free and immobilized laccase on polyurethane.

1.1.2 Specific Objectives

- To determine the laccase stability under pH range closer to a contaminated site;
- To assess the effect on PAHs biodegradation by free laccase of the mediator ABTS and the feasibility of work without it;
- To evaluate the biodegradation of anthracene, benzo(a)pyrene, and naphthalene by free laccase under natural groundwater samples, without mediator aiming to *in-situ* directly application;
- To determine biodegradation yields of PAHs by free and immobilized laccase on PUF under non-optimal conditions similar to a contaminated site;
- To identify by GC-MS resulting products of anthracene and benzo(a)pyrene removal by free and immobilized laccase on PUF treatment to confirm biodegradation.
- To propose two enzymatic bioremediation strategies considering its application potential under reagents cost and removal efficiency until safe limits of environmental protection policies.

1.2 CHAPTERS OVERVIEW

In chapter 1, the introduction of this work is presented, and its general and specific objectives are described below. An overview is also presented, containing a brief description of all work, chapter by chapter. The state of art and the potential of PAHs biodegradation by free and immobilized laccase were described in chapter 2.

In chapter 3, polycyclic aromatic hydrocarbons biodegradation by free laccase were evaluated under non-optimal conditions without expensive mediators, making a potential bioremediation strategy. The homogeneous biocatalytic process consists of application of the free laccase, directly, in groundwater to bioremediate polycyclic aromatic hydrocarbons *in-situ*. The methods and results of PAHs degradation by free laccase were carefully described. Batch experiments were performed in the model and 4 groundwater samples. The pH and mediator enhancers influence on laccase stability, PAHs degradation rates, enzyme activity, and oxidation products were also determined using anthracene, benzo(a)pyrene, and naphthalene. Results associated with all presented literature review based the proposed strategy to remediate polluted sites based on surfactant-enhanced *in-situ* enzymatic oxidation. This chapter were published in the Journal of Environmental Chemical Engineering (PERINI *et al*., 2020).

In chapter 4, immobilized laccase on commercial polyurethane foams was investigated to biodegrade PAHs, under non-optimal conditions without expensive mediators. The heterogeneous biocatalytic process consists of application of laccase immobilized on PUF in bioreactors by *ex-situ* treatment. The state of the art of PAH degradation by laccases, including remediation techniques and protection cleanup policies were carefully described. PAHs degradation rates were showed for 16 PAHs and final concentration were compared to legal limits of protection policies. Oxidation products of anthracene and benzo(a)pyrene were identified by GC-MS and the degradation pathway was showed. The bioremediation strategy to remedy polluted sites based on surfactant-enhanced and pump-and-treat was proposed as an *ex-situ* treatment. The results presented in this chapter were submitted (Under Review status) to the Journal of Polymers and the Environment in August 2020.

The final chapter describes the conclusions of this work. Then, some insights on PAHs contamination scenarios, in particular for two proposed enzymatic treatment in practical application were detailed and the suggestions for future works.

2 LITERATURE REVIEW

This chapter describes the state of the art about the enzymatic oxidation of anthracene, benzo(a)pyrene and others PAHs by laccase, in particular on remediation of contaminated sites, the PAHs effects on human health, contamination mechanisms and environmental protection policies. Subsequently, methods of remediation of PAHs contaminated sites, use of oxidative enzymes, and the laccase enzyme were detailed. The use of techniques of enzymatic immobilization with a focus on the degradation of the compounds under study was also showed. Moreover, special emphasis will be given to the enzymatic oxidation of anthracene, benzo(a)pyrene and and others PAHs by laccase from *Trametes versicolor*, presenting relevant results from the literature about this topic, the main focus of the present work.

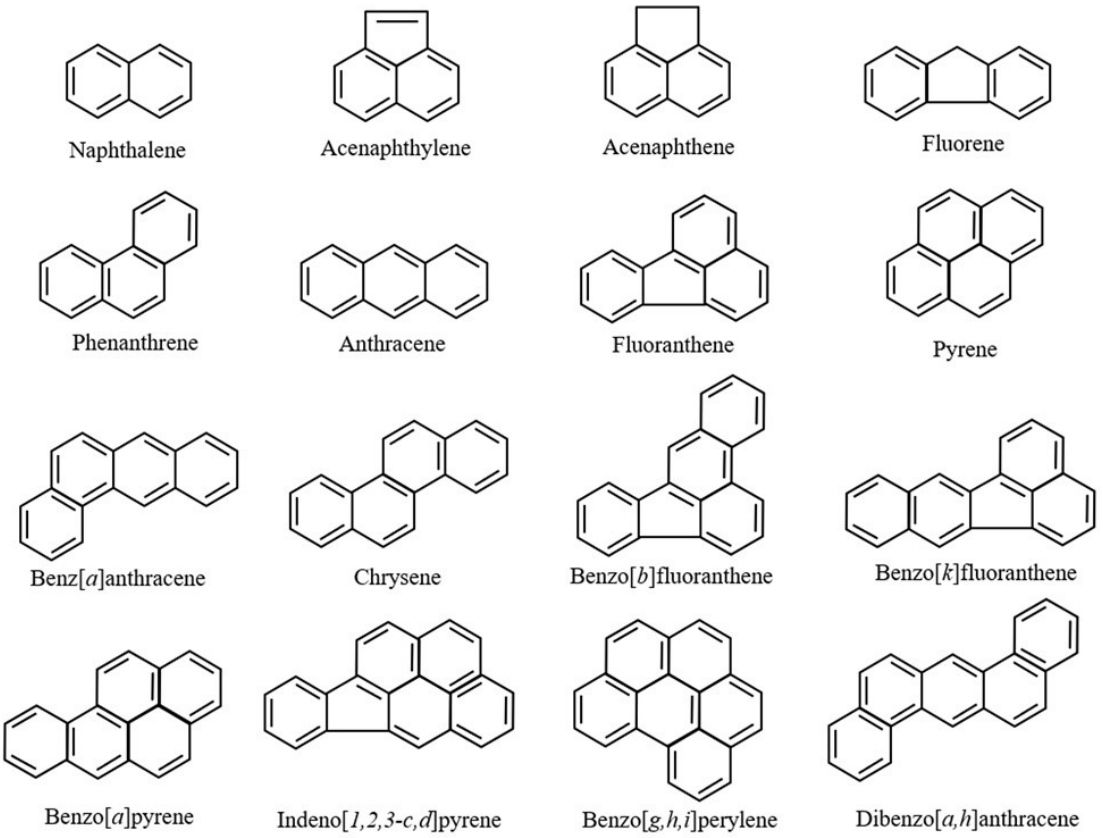
2.1 POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons are considered pollutants that may be widespread in soils and waters. These compounds consist of aromatic hydrocarbons with two or more fused benzene rings that are formed during the thermal decomposition of organic molecules and their subsequent recombination (HARITASH; KAUSHIK, 2009; AYDIN *et al.*, 2017). They are semi-volatile organic compounds that can be formed at high-temperature combustion processes, which the main emission source is related to incomplete combustion of organic materials (JUNG, *et al.* 2010, TIMONEY; LEE, 2011). Petrochemical activities such as process and refining in oil production, as well as accidents involving the direct spillage of its products and derived in soils and in receiving bodies, such as lakes, rivers, and oceans, and indirectly in groundwater also considerably raises environmental levels of PAHs (ADENIJI; OKOH; OKOH, 2019; KARIM; HUSAIN, 2010, LI; LI; LIU, 2017). In urban areas, processes that use fossil oil and fuels are the potentially polluting activities, such as gas stations, steel making, or coking plants of petroleum drilling and other activities (KUPPUSAMY *et al.*, 2017).

There are 16 PAHs listed as priority pollutants by the Protection Agency Environmental Protection of the United States of America (USEPA), for presenting toxicity, carcinogenic and mutagenic. They are: acenaphthene, acenaphtylene,

anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)pyrene, chrysene, dibenzo(a,h)anthracene, phenanthrene, fluoranthene, fluorene, indene(1,2,3-c,d)pyrene, naphthalene and pyrene, whose structures are shown in Figure 1 (AYDIN *et al.*, 2017; HONDA; SUZUKI, 2020; HJUNG *et al.*, 2010; LLOBET *et al.*, 2006).

Figure 1- Structure of the 16 Polycyclic Aromatic Hydrocarbons listed by USEPA as priority pollutants.



Source: Honda and Suzuki, 2020.

2.1.1 Effects on human health

PAHs can promote genetic defects in humans and have impacts on flora and fauna of the affected habitats through absorption and accumulation in food chains (NACCI *et al.*, 2002; DODOR *et al.*, 2004; RENGARAJAN *et al.*, 2015; YUAN *et al.*, 2017).

The main PAHs exposure routes are dermal, oral, and inhalation. While dermal exposure was accentuated in people working in activities related to oil and

petrochemicals, the use of contaminated groundwater, for ingestion or cooking, it is considered another important source of exposure. The amount absorbed by inhalation depends on the degree of atmospheric contamination related to industrialization, vehicle traffic, and urbanization (RENGARAJAN *et al.*, 2015; FRANKEN *et al.*, 2017).

Due to their hydrophobicity (chemical structure), the high liposolubility of PAHs facilitates rapid absorption in the intestines, with subsequent accumulation in adipose tissues. According to Zhang; Xue; Dai, (2010), PAHs rapid absorption occurs by the respiratory tract.

Since some of these PAHs are carcinogenic, there is a great interest in these compounds as environmental contaminants. Studies have indicated that two- and three-ring HPAs are extremely toxic compounds, while high molecular weight can cause cancer, congenital disabilities and reproductive abnormalities (RENGARAJAN *et al.*, 2015; YUAN *et al.*, 2017). Table 1 shows, in a compiled form, the main toxic and carcinogenic effects associated with PAHs.

Table 1**:** Polycyclic Aromatic Hydrocarbons with toxic and carcinogenic effects.

Effect	PAHs
Toxic	Anthracene, acenaphene, acenaphthene, fluoranthene, fluorene, naphthalene, pyrene and phenanthrene.
Toxic and carcinogenic	Benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)pyrene, chrysene, dibenzo(a,h)anthracene, indene(1,2,3-c,d)pyrene,

Source: Honda and Suzuki, 2020.

This carcinogenic action of PAHs occurs after these contaminants pass by certain body transformations, through the metabolic reactions that generate epoxides, which are species with carcinogenic and mutagenic properties (LIAMIN *et al.*, 2017; YUAN *et al.*, 2017).

2.1.2 Forms of contamination

Urban centers are the places with the highest potential for PAHs contamination.Several establishments exist where there is the handling of petroleum products, such as machine shops, vehicle washing, garages cars, and fuel resale stations.

Among these establishments, undoubtedly, the highest contamination potential is observed at the fuel retails, whose release of PAHs can directly reach the air and soil, and indirectly, groundwater. High potential for PAHs contamination has been founded in combustible stations due to underground combustible tanks, pipes, lubricant ramp, and oil-water separation systems (ABDEL-SHAY; MANSOUR, 2016; MARIANO *et al.*, 2007). Regarding PAHs contaminated site, it occurs by two sequential mechanisms, leaks, spills, floor, and soil infiltration (primary mechanisms), and release of contaminants, through infiltration and dispersion in the soil, down to lower layers of soil and groundwater (secondary mechanisms) (KUPPUSAMY *et al.*, 2017; LIANG, et al 2017; SUN, *et al.*, 2019).

Due to the physicochemical properties of hydrocarbons, the process is the dissolution of the contaminant on non-aqueous liquid phase (NAPL) (BESHA *et al.*, 2018) in groundwater, whose transfer rates are limited by dissolution kinetics and the solubility of contaminants in water. When the concentration of the contaminating substance exceeds the solubility limit, there is the formation of a heterogeneous phase (immiscible) in the aquifer, which varies with the density of these compounds.

In groundwater, the mass of contaminants in the plume is affected by the transfer of pollutants from the source of contamination. In the case of PAHs, due their tendency to be sorbed to the soil, it is difficult to form a free phase in the aquifer (SUN, *et al.*, 2019). However, the most common occurrence of these compounds is in the form of dissolved plumes, without heterogeneous phase.

The rapid identification and control of contamination sources is a factor that contributes significantly to do not occurrence saturation and, consequently, the heterogeneous phase in the aquifer. Thus, eliminating the contaminant transport by diffusion, which is a dependent function of the concentration gradient.

2.1.3 Legislation

Due to the inevitable and frequent potential of contamination by petroleum hydrocarbons, including PAHs, and their consequent effects in human health, the most of countries have intensified environmental inspection and monitoring from protection and cleanup policies.

In Brazil, the pioneer policies about the subject have been regulated by the São Paulo State environmental agency, CETESB. Brazilian national policies and technical norms were developed based on CETESB publications. These policies provide for pollution prevention, and control and establishes guidelines for the environmental licensing of fuel stations. The law forces the maintenance of environmental control systems, such as groundwater monitoring system by wells. Groundwater samples must be periodically collected from wells, and analysis of the PAHs and other hydrocarbons, to monitor changes in groundwater quality in the aquifer.

Board Decision 045 (CETESB, 2014) guides principles that defines the contaminant limits of soil and groundwater. The guiding values of soil (mg kg^{-1}) and groundwater (µg L^{-1}) were defined in values of quality reference, prevention, and intervention. Intervention value is the concentration above which there are potential risks on human health, considered in a generic standardized exposure scenario. The limit values were defined according to future use of the area, considering agricultural, residential, and industrial, similar to how it happens in other country's policies. Concentration limits of anthracene, pyrene, chrysene and benzo(a)pyrene was showed in Table 2 for soil and groundwater mediums according some international environmental protection policies.

Table 2: Concentration limits in soil and groundwater of some PAHs according international environmental protection policies

Medium	Unit	Ant	Pyr	Chr	BaP	Protection Policy
Soil	mg kg^{-1}	4,600	-	600	0.8	CETESB, 2014
Soil	mg kg^{-1}	10,000	9,400	-	8.5	NZME, 1997
Groundwater	µg L^{-1}	5	-	0.2	0.05	DTIV, 2000
Groundwater	µg L^{-1}	900	-	41	0.7	CETESB, 2014
Groundwater	µg L^{-1}	1,000	100	-	0.7	NZME, 1997
Groundwater	µg L^{-1}	10,000	-	-	1.0	EPA, 2003

Considering the migration scenario of contaminants to the groundwater, based on the concept of soil multi-functionality, the Dutch policy (DTIV, 2000) established the guiding values of soil and groundwater quality. The most of Dutch values for groundwater is more restrictive comparing to Brazilian values for intervention purposes.

For example, the limit values of Dutch policy are 5 and 0.05 μg L^{-1}, for anthracene and benzo(a)pyrene, respectively.

Despite this, while for anthracene, the intervention value of Brazilian policy is 900 μg L^{-1}, the interim value of Irish policy (EPA, 2003) is 10,000 μg L^{-1}, and 1,000 μg L^{-1} in New Zealand law (NZME, 1997). On the other side, Canadian (CCME, 2010), and also North American (USEPA, 1996), policies do not have limits for groundwater, which must be calculated in the case by case, after site investigations. Finally, each country has its criteria in the establishment of contaminant limits in protection and cleanup policies.

2.2 REMEDIATION AND BIOREMEDIATION TECHNIQUES

Remediation is a necessary action for the rehabilitation of a contaminated site, which consists of applying techniques to remove, contain, or reduce the concentration of contaminants. The aim of remediation is to achieve a level of risk tolerable for the future use of the area, in terms of contaminant concentration (CETESB, 2014). The places where remediation performed can be classified as acting on the spot (*in-situ*), and acting outside the place of origin (*ex-situ*), where contamination (OSSAI, *et al.*, 2020).

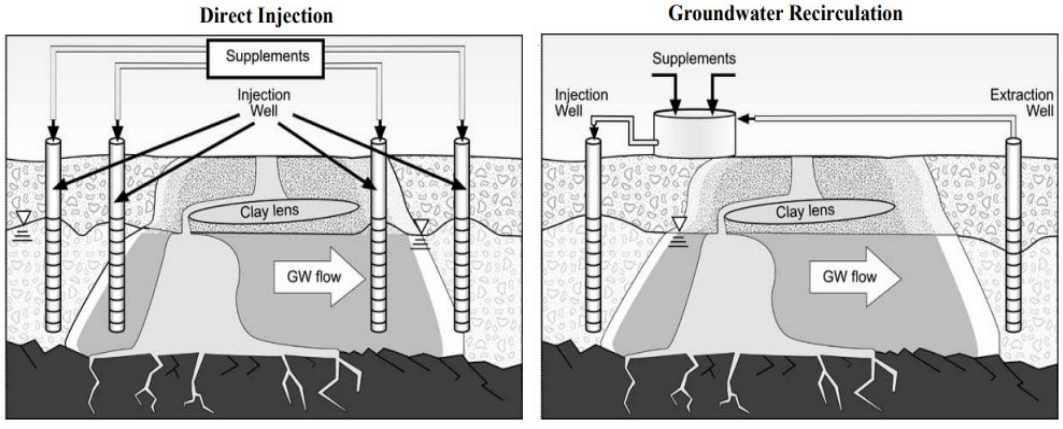
A wide variety of technologies used in the remediation of contaminated sites by petroleum-derived compounds is known. Dispersion by surfactants' action (LAMICHHANE; KRISHNA; SARUKKALIGE, 2017; LIANG, *et al.*, 2017; VALDERRAMA, *et al.*, 2008), chemical oxidation (BESHA, *et al.*, 2018; CUNHA; BERTOLO, 2012; HUANG; COUTTENEYE; HOAG, 2005; HULING; PIVETZ, 2006; LEMAIRE, *et al.*, 2019) or bioremediation (BALDANTONI, *et al.* 2017; MARIANO, *et al.*, 2007; MOHAN, *et al.*, 2006) can be used in the soil and groundwater dissolved plume of the contaminant.

In-situ chemical oxidation (ISCO) is a remediation method that has been used for contaminated groundwater, because it presents good immediate yields (LEMAIRE, *et al.*, 2019). In the chemical oxidation oxidizing agent solution is injected into the aquifer or the water is pumped and treated in an external reactor (pump-and-treat). Several injection campaigns for the application of the oxidizing agent at the remediation are necessary in ISCO, until contaminant concentrations are reduced to acceptable risk levels.

The most used substances in chemical oxidation processes of petroleum-based compounds are potassium permanganate, sodium, and hydrogen peroxide. Cunha and Bertolo (2012) observed the concentration reduction of 1,1-dichloroethene in groundwater for 22 months, from 200 to 24 μg L^{-1} due to injection of the potassium permanganate oxidant. Considering the time-consuming process of an ISCO remediation, associated with the high commercial value of oxidizing agents, this way of reducing the mass of contaminants in remediation processes, is considered relatively expensive (KUPPUSAMY *et al.*, 2017). Besides, the pump-and-treat chemical oxidation for *in-situ* treatment of dissolved plumes of PAHs proved to be ineffective in reducing the mass of contaminants in the aqueous phase to adequate levels (MATEAS *et al.*, 2017).

The bioremediation techniques are considered a strategy that can be adopted as an effective and low-cost way of managing contaminated sites, that is, making counterpoint to remediation. Figure 2 show *in-situ* bioremediation techniques applicable to dissolved plumes in groundwater by direct injection and circulation. One of them is based on nutrients and microorganisms injection. The other in the extraction of groundwater downstream to a reactor, more nutrients is added for subsequent injection upstream of the contamination plume (USEPA, 2000).

Figure 2 - Representation of *in-situ* bioremediation techniques by direct injection and groundwater recirculation.



Source: Modified from (USEPA, 2000).

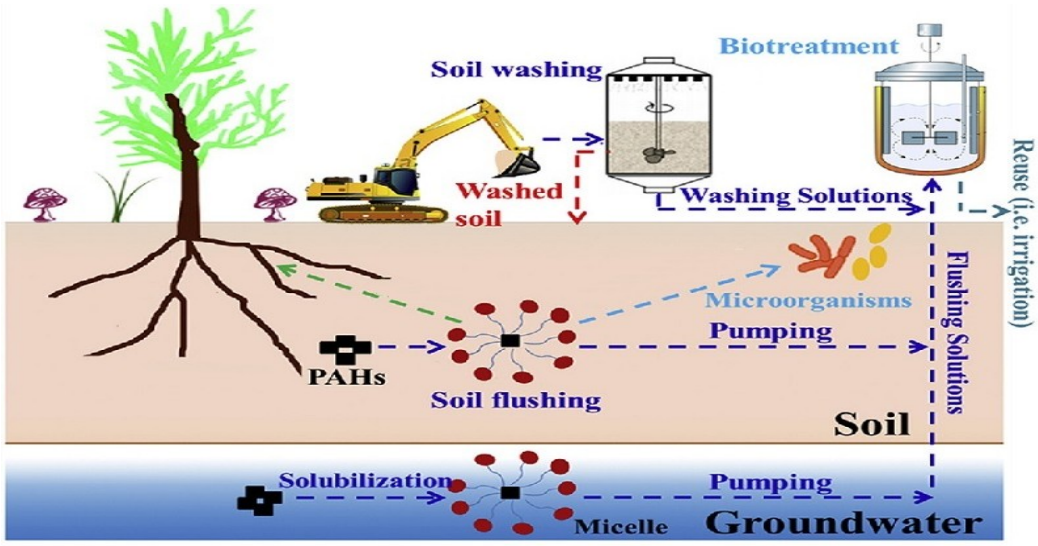
These techniques involve the use of naturally microorganisms occurring (native) or cultivated, to degrade contaminants in groundwater and soil. Generally,

bacteria, filamentous fungi and yeasts can be used, but bacteria are applied in the most of cases (HAZE; PRINCE; MAHMOUDI, 2016; MARIANO, *et al.*, 2007; MOHAN, *et al.*, 2006). Organic compounds (contaminants) can be metabolized by microorganisms by fermentation, aerobic and anaerobic respiration or co-metabolic processes, in which there is the need to maintain favorable pH and temperature conditions, and the supply of nutrients (C, N, P, K) and electron terminal acceptors (O_2, NO_3, CO_2, SO_4). Since in unfavorable conditions, the degradation of the contaminants could not happen.

This biodegradation process of petroleum-based compounds can occur passively and at a lower speed, through the process called "natural attenuation", and simultaneously involves the occurrence of other processes of natural origin such as volatilization, dispersion, dilution and adsorption in the subsurface of the soil (OSSAI, *et al.*, 2020). However, only biodegradation promotes the degradation of contaminants, while the other processes mentioned above only involve the transfer of contaminants from one location to another or the retention of the contaminant (MARIANO, *et al.*, 2007; MOHAN, *et al.*, 2006).

Higher contaminant solubility can be achieved by the use of surfactants. The integration of techniques is commonly used, as for example, the use of surfactants associated with other remediation processes, such as chemical oxidation or *in-situ* bioremediation (KUPPUSSAMY *et al.*, 2017; LIANG *et al.*, 2017; MATEAS *et al.*, 2017). These processes are shown in the Figure 3.

Figure 3 - Scheme of the integrated remediation process.



Source: Adapted from Liang, *et al.*, 2017.

A bioremediation system based on the use of surfactant has been proposed, as illustrated in Figure 3, for removing PAHs from contaminated soil and water by the use of soil washing, phytoremediation techniques, followed by extraction and reactor biodegradation, washing solutions and groundwater (LIANG *et al.*, 2017). The authors conclude that the performance of biodegradation treatment in the face of chemical oxidation, in this proposal of integrating techniques, is feasible when the cost of the operating system is considered a limiting factor, including reagent concentration and energy demand (LIANG *et al.*, 2017).

Certainly, the techniques and the supply of nutrients to microorganisms influence the rate of metabolization of these hydrocarbons derived from oil, but there are other related factors. Some are more easily biodegraded than others, due to different rate of degradation. In particular by dependence on the concentration of the contaminant and the number of species catalysts, such as enzymes produced *in-situ* by these microorganisms. Thus any factor affecting the concentration of the contaminant, the number of microorganisms, or the number of specific enzymes, can increase or decrease contaminant biodegradation (MOHAN, *et al.*, 2006).

In this sense, several studies have evaluated the processes of bioremediation of oil-derived hydrocarbons in soil and groundwater, considering the enzymatic production of microorganisms and the co-metabolic mechanism involved (UHNAKOVA *et al.*, 2009; HADIBARATA *et al.*, 2012; XIE *et al.*, 2015).

The degradation of aromatic phenolic compounds used in chemical industry such as flame retardants, in the production of polymers and pesticides, in submerged cultivation of *Trametes versicolor* was evaluated by Uhnaková *et al.* (2009). The levels of laccase production, which reached 63 U L^{-1}, were monitored in the experiments, in 4 days. The results were satisfactory, yielding 65 to 85% in the reduction in the concentration of compounds, associated with the high potential of the enzyme to degrade aromatic phenolic compounds.

Hadibarata *et al.* (2012) evaluated the degradation of HPA pyrene in liquid medium by the white oxidation fungus *Polyporus sp.* S133, associated with bioremediation co-metabolic by laccase at 25 ^{o}C and pH 3, and dioxigen enzymes at 50 ^{o}C and pH 5.

Another bioremediation model was evaluated by Xie *et al.* (2015) the degradation of anthracene contained in a soil sample by enzymes secreted in a

bioreactor containing mycelia in *Ganoderma lucidum* grains in corn cob, hydrophobically immobilized in calcium alginate modified by polycaprolactone. The results showed the removal of anthracene from the soil was 96.2 ± 2.0% after 20 days of incubation at pH 5.0 and 45 °C. The degradation was attributed to rapid secretion of laccase enzymes, which were stimulated by the lignocellulosic substances of the corn cob.

The ability of these aerobic and anaerobic fungi to metabolize aromatic compounds is evident. In this sense, the degradation of PAHs by these fungi was evaluated at the molecular level, and the degradation mechanisms have been proposed. It was possible to observe an important role of enzymes as catalysts of biodegradation reactions (AYDIN *et al.*, 2017).

Based on the principle that enzymes are produced *in-situ* by microorganisms and are responsible for biodegradation, some authors have evaluated direct use of enzymes for bioremediation. However, these are considered emerging techniques for PAH removal and are still under development (KUPPUSAMY *et al.*, 2017; MOHAN *et al.*, 2006; RAO *et al.*, 2014). The catalytic action of enzymes is extremely efficient and selective compared to chemical catalysts due to the fewer reaction conditions, higher reaction rates, greater specificity and ability to catalyze reactions at relatively low temperatures and a wide pH range (MOHAN *et al.*, 2006).

2.3 OXIDATIVE ENZYMES

The classes of degradative enzymes, which can mediate these transformations of polluting substances in biotransformation processes, are oxidoreductases, hydrolases, and lyases, whose sources as vegetal, bacteria and fungi. The hydrolases are enzymes capable of the transformation of effluents from the food industry, animal feed, textiles, paper, pesticide, among others. At the same time, lyases can metabolize nitriles and cyanides. The oxidoreductases can degrade compounds generated in the petrochemical, paper and cellulose, textile, and pharmaceutical industries (DEMARCHE *et al.*, 2012).

The oxidative enzymes oxidoreductases have a significant potential for biotechnological and environmental applications. However, the use of these catalysts in industrial processes is still limited, due to the complexity and, eventually, low catalytic activity (BERNHARDT, 2006).

Therefore, this potential of oxidative enzymes has been extensively studied in order to develop technologies to enable the practical application of these enzymes in the biodegradation of polluting compounds. The main oxidoreductase enzyme involved in the transformation of PAHs and phenolic compounds are chloroperoxidases, laccases, lignin-peroxidase, manganese-peroxidase, and tyrosinase (DEMARCHE *et al.*, 2012; RAO *et al.*, 2014; AYDIN *et al.*, 2017; KADRI *et al.*, 2017).

For phenolic and PAHs biodegradation purpose, fungal oxidoreductases are highlighted (HARITASH; KAUSHIK, 2009; PANG *et al.*, 2015, BALDANTONI *et al.*, 2017). They are produced from decomposing fungi white, which have an extracellular enzyme system capable of tolerating high concentrations of toxic pollutants. As an example, Baldantoni *et al.* (2017) evaluated the degradation of anthracene and benzo(a)pyrene in contaminated soils, which was incubated with a fungal consortium, the activities of laccase enzymes being monitored, diphenol oxidase and peroxidase, produced by cometabolic processes of fungi.

Enzymes such as tyrosinase, laccase, lignin peroxidase, and manganese peroxidase are produced by these fungi, such as *Trametes versicolor*, a large producer of laccase (NEVES *et al.*, 2013). This ability to degrade organic pollutants is associated with the lignin degradation system of these fungi (HARITASH; KAUSHIK, 2009).

The primary reaction carried out by oxidative enzymes is a reaction of oxidative coupling that results in the formation of products of increasing complexity, cross-coupling reactions may occur between substrates of different natures (KOBAYASHI; HIGASHIMURA, 2003). The use of peroxidase has the disadvantage that these enzymes require stoichiometric amounts of hydrogen peroxide as an oxidizer, whereas tyrosinase and laccase require only molecular oxygen for the activity (HARITASH; KAUSHIK, 2009; BAYRAMOGLU; AKBULUT; ARICA, 2013).

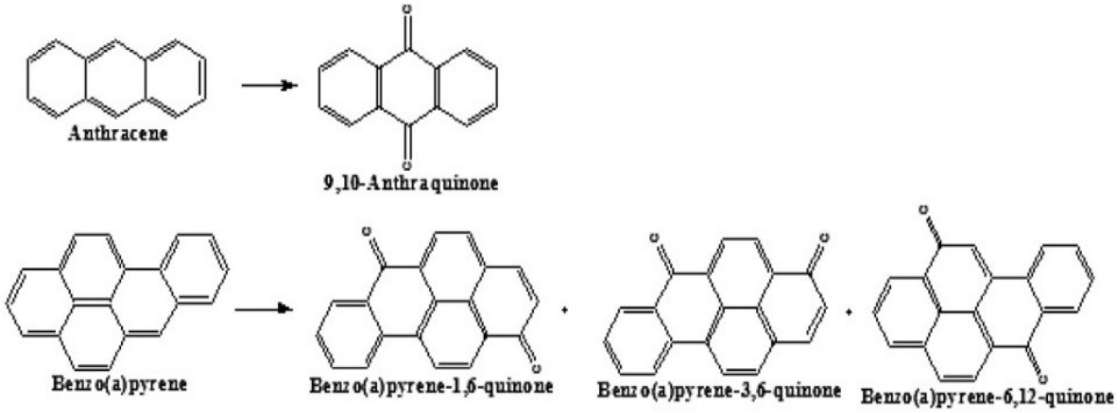
Despite this, the enzyme manganese peroxidase has been studied in processes of biodegradation of PAHs, and promising results were obtained, according to studies of carried out by Eibes *et al.* (2006). In this study, anthracene degradation was evaluated, pyrene, and dibenzothiophene by manganese peroxidase from the fungal origin (*Bjerkandera* sp.). A higher degradation efficiency for anthracene was observed, which was degraded to anthroquinone.

Anthroquinone was the identified biodegradation product of anthracene biodegradation, according observed by Hu *et al.* (2009), using *T. versicolor* laccase, and

other authors with peroxidases (HERNANDEZ; WERBERICH; D'ELIA, 2008; KARIM; HUSAIN, 2010).

Benzo(a)pyrene, when degraded by a ligninolytic enzyme of fungal origin, is converted to the benzo(a)pyrene-1-6-quinone, benzo(a)pyrene-3-6-quinone isomers and benzo(a)pyrene-6-12-quinone (KADRI *et al.*, 2017). Figure 4 illustrates the reaction of enzymatic oxidation of anthracene and benzo(a)pyrene to quinones, which are the first biodegradation products considered less toxic substances than PAHs (HU *et al.*, 2009; LI *et al.*, 2014).

Figure 4 - Reaction of enzymatic oxidation of anthracene and benzo(a)pyrene by ligninolytic enzymes. .



Source: Modified from Kadri *et al.* (2017).

Among the reductases, laccase has been chosen in most studies, and for direct application to PAHs enzymatic degradation, due to the lower complexity and higher availability.

2.3.1 Laccases

Laccases, benzenodiol oxygen oxidoreductase (EC 1.10.3.2), are molecules of dimeric or tetrameric glycoprotein, which usually contain four atoms of copper per monomer, distributed in three redox sites (GIANFREDA; XU; BOLLAG, 1999). As they work with molecular oxygen at room temperature, they produce water as a resulting product. They do not require the presence of NADH or NADPH in mechanisms, like many other oxidoreductases (KOSCHRRECK *et al.*, 2008).

Due to their high specificity, laccases have the potential to catalyze the oxidation of aromatic micropollutants in wastewater (SPINA *et al.*, 2015). They are considered very versatile enzymes in the biodegradation of phenolic compounds and PAHs (RAO *et al.*, 2014).

The influence of enzymatic kinetic, together with enzyme stability factors, has been evaluated and optimized in factorial designs. In this way, higher degradation yields of polluting compounds by laccase have been determined, known as the optimal condition. In a work carried out by Margot *et al.* (2013), based on a factorial design, enzymatic oxidation of dichlorophenac, mefenamic acid, triclosan, and bisphenol A was studied from *T. versicolor* laccase. The authors determined yields on biodegradation in different conditions of pH, temperature, reaction time, from the mixture of compounds as substrate and the pure compounds. Removal rates for compounds were different in the mixture of pollutants when compared with solutions of a single compound, being observed that, in general (MARGOT *et al.*, 2013).

The complexity of the substrate incubation mix was assessed using a municipal wastewater sample, which was collected from a sewage treatment plant. The sample contained at least nine micro-polluting xenobiotic compounds such as drugs, pesticides, plasticizers, and personal care products, and was submitted to enzymatic oxidation by free laccase, from *T. versicolor*. The severe chemical and biological wastewater conditions had influence on the stability of the enzyme. Despite this, the enzymatic degradation in the effluent with 100 U L^{-1} of laccase in 24 hours, occurred with the conversion of more than 70% for most of the analytes present in the effluent sample (SPINA *et al.*, 2015).

The excellent stability of laccase when in the presence of other substances was also found in experiments with other soluble compounds, supposedly considered inhibitors, dithiothreitol, thioglycolic acid, cysteine, di-ethyldithiocarbamic acid (JOHANNES; MAJCHERCZYK, 2000), and potassium dichromate, EDTA and potassium ferricyanide (PACHECO; SOARES, 2014). However, in these studies, when in the presence of sodium azide (NaN3), the enzyme lost its activity.

In addition to the situations reported above, it should be noted that the capacity of the laccase to oxidize a compound is related to the redox potential of the enzyme. As the oxidoreduction potential of laccases is lower than that of non-phenolic compounds, these enzymes have deficiency in oxidizing these substances. However, the presence of

molecules capable of acting as electron transfer mediators provide laccases with the ability to oxidize non-phenolic structures, such as aromatic alcohols (FABRINNI; GALLI; GENTILLI, 2002), PAHs (KOSCHRRECK *et al.*, 2008; ARCA-RAMOS *et al.*, 2014), indoles (AZIMI *et al.*, 2016), thus expanding the range of compounds that can be oxidized by these enzymes.

The three compounds that are commonly used as mediators are TEMPO (n-oxyl-2,2,6,6-tetramethylpiperidine), ABTS (acid 2 , 2 '- azino - bis (3 - ethylbenzothiazoline - 6 -sulfonic) and HBT (1 - Hydroxybenzotriazole), which act by different mechanisms, ionic, electron transfer and radical, respectively (FABRINNI; GALLI;GENTILLI, 2002). Usually, ABTS and HBT mediators are used in trials of enzymatic oxidation of PAHs catalyzed by *T. versicolor* laccase (ARCA-RAMOS *et al.*, 2014; BAUTISTA; MORALES; SANZ, 2015; COLLINS *et al.*, 1996; DODOR *et al.*, 2004; HU *et al.*, 2009; JOHANNES; MAJCHERCZYK; HÜTTERMANN, 1996, WU *et al.*, 2008). These studies compared the performance in the oxidation of PAHs, in tests in the presence and absence of mediators, and concluded that the highest returns occur with the use of a mediator. The presence of mediator compounds expanded the substrate spectrum of laccases to PAHs compounds, thus creating a novel catalytic action of this enzyme.

The laccase activity is also influenced by the ionization potential of the substrate. For example, the high ionization potential of naphthalene hinders its enzimatic degradation by laccases (GIANFREDA; XU; BOLLAG, 1999). Isolated laccase from *Marasmius quercophilusstrain* 19 was tested on the degradation of 4 PAHs, anthracene, benzo (a) pyrene, naphthalene, and phenanthrene. It was observed that the potential of ionization of PAHs affected the enzymatic transformation. The enzyme was able to oxidize only anthracene and benzo(a)pyrene, which have ionization potentials lower than 7.55 eV (COLLINS *et al.*, 1996; FARNET *et al.*, 2009). The same can be observed in other assays of enzymatic oxidation of 14 PAHs by *Trametes versicolor* free laccase in the presence ABTS mediator, which also resulted only in the oxidation of anthracene and benzo(a)pyrene (LI *et al.*, 2014).

In contrast, Bautista *et al.* (2015) and Cho *et al.* (2002) concluded that there was no influence of the PAH ionization potentials in the degradation capacity. In addition to anthracene and benzo(a)pyrene, the laccase is able to degrade fluoranthene, phenanthrene, and naphthalene PAHs, which have higher ionization potentials, equal to

7.95; 7.91 and 8.13 (MAJCHERCZYK; JOHANNES; HÜTTERMANN, 1998); respectively.

Other factors linked to enzymatic activity and, consequently on the stability of these enzymes, such as temperature and pH, are considered to limit the degradation of PAHs. As an example, it is worth mentioning that free laccase from *Trametes versicolor* was evaluated in 0.1 mol L^{-1} sodium acetate buffer (pH 5) at 30 ^{o}C, with the relative enzymatic activity of 55% (ARCA-RAMOS *et al.*, 2012). In contrast, the determination of the optimal pH of laccase free, from *Trametes versicolor*, was evaluated at 25 ^{o}C in 0.1 mol L^{-1} tartrate buffer (pH 3.0 to 5.5) and the results indicated pH 4 as ideal, while the enzymatic activity relative value remained at 100% (PACHECO; SOARES, 2014).

Due to the limitation of laccas stability only in acid pH, several studies have searched the use of alternative techniques to promote the stability of this enzyme in more neutral and alkaline pH. The goal has been to turn the use of the enzyme viable for the treatment of contaminated waters, wastewaters, and soils. However, enzyme biodegradation assays of PAHsare commonly carried out at pH and temperature conditions different from observed in wastewater, soil, and groundwater. In this sense, enzyme immobilization is one of the most promising strategies.

2.4 LACCASE IMMOBILIZATION

Free laccases have limited useful life during use or storage because they are subject to chemical, physical and biological factors (PACHECO; SOARES, 2014).. These undesirable characteristics can be minimized with the process of immobilization of the enzyme in solid materials, a fact that favors the biocatalytic development process and allows for use on an industrial scale (ADDORISIO *et al.*, 2013; BAUTISTA; MORALES; SANZ, 2015). Although immobilized enzymes, when compared to free enzymes, have reduced enzyme activity due to diffusion limitation and Michaelis Menten constant increased, the enzymatic immobilization technique offers some advantages in bioremediation applications (PANG *et al.*, 2015). In reactions catalyzed by free enzymes, there is difficulty in separating the biocatalyst at the end of the reaction. Thus an alternative for maintaining stability and favoring the recovery of biocatalyst is the immobilization of the enzyme (ADDORISIO *et al.*, 2013).

Immobilization is usually achieved by contacting a material used for immobilization, named support, with the enzyme that is intended to be immobilized under controlled environmental conditions. Many solid supports were already investigated for laccases immobilization such as kaolinite (DODOR *et al.*, 2004), aldehyde sepharose (ADDORISIO *et al.*, 2013), chitosan (PACHECO; SOARES, 2014), ceramic and porous glass (PLAGERMANN; LANGERMANN; KRAGL, 2014), nanostructured silica SBA-15 (HU *et al.*, 2009; BAUTISTA; MORALES; SANZ, 2010; BAUTISTA; MORALES; SANZ, 2015), and nanoparticles of carbon (PANG *et al.*, 2015).

On the other hand, the use of immobilized laccase on synthetic organic polymers has also been evaluated for phenol compounds oxidation (BAYRAMOGLU; KARAGOZ; ARICA, 2018; CAPATANE *et al.*, 2013). These synthetic organic polymers used as support to immobilize laccase have hydrophobic character, and high standard activities have been shown in the biodegradation of organic compounds.

Polyacrylonitrile beads were used as support to immobilize laccase, showing complete degradation of nonylphenol and octylphenol, with slightly Km increased (CAPATANE *et al.*, 2013). One novel synthetic polymer, poly(glycidyl methacrylate), was developed as laccase support, and 90% of bisphenol A and 100% of Congo Red dye was removed in batch experiments (BAYRAMOGLU; KARAGOZ; ARICA, 2018).

Commercial polyurethane foam (PUF) has been shown as efficient support of other enzymes, for instance lipases (higher pH and temperature stabilities) (BRESOLIN *et al.*, 2019; NYARI *et al.*, 2016). Daronch (2020) used this low-cost support to stabilize laccase and demonstrated the one-step laccase immobilization process onto PUF. Another study showed high removal capability of nonylphenol polyethoxylates by laccase from *T. versicolor* immobilized on PUF. The yields of laccase on PUF were related to the compounds degrade capability associated with the support's adsorption (STENHOLM *et al.*, 2020).

There are no studies in the literature that report the use of laccase immobilized on PUF as biocatalysis for PAHs biodegradation. In contrast, recent work from this group of researchers evaluated the immobilization of laccase from *T. versicolor* on PUF (DARONCH, 2020). Thus, the application of laccases immobilized on PUF was an opportunity explored in this study.

2.5 BIODEGRADATION OF PAHs BY LACCASES

The enzymatic oxidation reaction of anthracene and benzo(a)pyrene by laccase of *T. versicolor* are usually evaluated on literature due to their ionization potential. However, biodegradation by laccase of other PAHs has also been observed. These studies have been proposed to make this biodegradation process viable for the bioremediation of contaminated sites. Considering this purpose, it is important to connect studies that are still under study to environmental protection policies. Certainly, it brings more possibility to apply these processes on the field. In this direction, Table 3 lists information about the ten more degradable PAHs by laccase, of 16 PAHs listed as priority pollutants by USEPA, the state of the art of this PAHs oxidation by free and immobilized laccases.

The first studies that evaluated the degradation PAHs by free laccases of *T. versicolor* in aqueous medium used the mediators ABTS and HBT for enzymatic catalysis (COLLINS *et al.*, 1996; JOHANNES, C., MAJCHERCZYK, A., HÜTTERMANN, 1996; MAJCHERCZYK; JOHANNES; HÜTTERMANN, 1998).

Johannes *et al.* (1996) observed anthracene degradation to anthraquinone after 72 hours of incubation at room temperature and pH 4.5, using free laccase and ABTS. The results showed anthracene degradation yields of 35% for assays without a mediator. Deradation reached 47, 75 and 90% for experiments mediated by ABTS in the respective concentrations of 0.005, 1 and 2 mM. This capacity of ABTS to improve the anthracene biodegradation yield in anthraquinone was confirmed by Collins *et al.* (1996), including the oxidation of benzo(a)pyrene, in 24-hour experiments at 27 ^{o}C and pH 5.

Table 3: Overview of some relevant biodegradation studies by laccase of 10 listed PAHs by USEPA as priority pollutants.

		PAHs[1] maximal removal										
Operating Conditions[2]	Scale	Nap	Flu	Phe	Ant	Fla	Pyr	BaA	Chr	BbF	BaP	Reference
Free, ABTS, pH 5, 37 ^{o}C, 24 h.	mg L^{-1}	-	-	-	> 85%	-	-	-	-	-	100%	Collins *et al.* (1996)
Free, HBT, pH 4,5; 37 ^{o}C, 24 h.	mg L^{-1}	-	> 90%	> 5%	100%	> 3%	48%	53%	0%	10%	>90%	Majchercyk *et al.*(1998)
Free, ABTS/HBT, pH 4, 24 ^{o}C, 1 h.	mg L^{-1}	-	-	> 90%	> 95%	>91%	> 40%	-	-	-	>97%	Cho *et al.* (2002)
Free, ABTS, pH 4, 30 ^{o}C, 24 h.	mg L^{-1}	-	-	-	90%	-	-	-	-	-	>88%	Dodor *et al.* (2004)
Immobilized, ABTS, pH 4.5, 30 ^{o}C,24h	mg L^{-1}	-	-	-	80%	-	-	-	-	-	>81%	
Free, ABTS, pH 5, 25 ^{o}C, 72 h.	mg L^{-1}	0%	> 80%	0%	> 80%	-	-	-	-	-	-	Koschorreck *et al.* (2008)
Free, ABTS, 37 ^{o}C, 24 h.	µg L^{-1}	0%	> 10%	> 6%	100%	> 6%	> 10%	> 32%	> 2%	>2%	100%	Wu *et al.* (2008)
Immobilized, ABTS, pH 7, 37 ^{o}C, 48h.	mg L^{-1}	-	-	-	42%	-	-	-	-	-	-	Hu *et al.* (2009)
Free, HBT, pH 5,1; 30 ^{o}C, 72 h.	mg L^{-1}	-	-	-	100%	-	-	-	-	-	-	Arca-Ramos *et al.* (2012)
Free, ABTS, pH 7, 25 ^{o}C, 24 h.	µg L^{-1}	> 35%	-	> 40%	> 45%	-	-	> 35%	> 30%	>25%	>25%	Niu *et al.* (2013)
Immobilized, ABTS, pH 7, 25 ^{o}C, 24h.	µg L^{-1}	> 85%	-	> 85%	> 80%	-	-	> 95%	> 95%	>95%	>95%	
Free, ABTS, pH 5, 25 ^{o}C, 48 h.	µg L^{-1}	2%	30%	2%	100%	1%	2%	> 22%	1%	<1%	>97%	Li *et al.* (2014)
Free, pH 5, 25 ^{o}C, 48 h.	mg L^{-1}	> 93%	-	> 88%	> 63%	-	-	-	-	-	-	Bautista *et al.* (2015)
Immobilized, pH 5,0, 25 ^{o}C, 48 h.	mg L^{-1}	> 81%	-	> 77%	> 56%	-	-	-	-		-	
Free, pH 4,2; 30 ^{o}C, 24 h.	-	-	-	> 50%	> 68%	-	-	> 51%	-	>68%	-	Xu *et al.* (2020)
Free, pH 7, 25 ^{o}C, 120 h.	mg L^{-1}	> 72%	-	-	> 94%	-	-	-	-	-	>30%	Perini *et al.* (2020)

[1]PAHs: Naphthalene (Nap), Fluorene (Flu), Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Fla), Pyrene (Pyr), Benzo(a)anthracene (BaA), Chriseno (Chr), Benzo(b)Fluoranthrene (BbF) and Benzo(a)pyrene (BaP). [2]Conditions: free or immobilized laccase, with mediator ABTS/HBT or without, pH value, temperature and incubation time.[3]Considering residential use.

Similar to ABTS (laccase-mediator), another compound is HBT, which was effective in degrading anthracene and benzo(a)pyrene, in addition to other PAHs (acenaphthylene, acenaphthene, fluorene, and perylene) with removal close to 100% in 72 hours, at room temperature and pH 4.5 (MAJCHERCZYK *et al.*, 1998). This behavior of the mediators, of raising the rate of enzymatic degradation of anthracene and benzo(a)pyrene PAHs was observed in experiments conducted by another laccase of fungal origin, of *C. hirsutus*, benzo(a)pyrene being more rapidly oxidized when mediated by ABTS and the anthracene when mediated by HBT (CHO *et al.*, 2002).

Subsequent studies sought to evaluate new technologies associated with enzymatic oxidation of anthracene by *T. versicolor* laccase in different conditions of the reaction medium. In an attempt to maintain enzyme stability under these conditions and reproduce the high yields of known biodegradation for large-scale applications.

Dodor *et al.* (2004) used the *T. versicolor* laccase immobilized in inorganic kaolinite support. They compared the degradation yield of anthracene and benzo(a)pyrene in tests carried out with the free and immobilized enzyme. In comparison with the free enzyme, immobilized laccase improves the stability at temperature, pH, inhibitors, and storage. The reuse and recovery of the enzyme at the end of the process was also evaluated. After 24 h of incubation in a shaker, pH 4.5, and 30 ^{o}C, in medium containing acetone to increase the solubility of PAHs and ABTS, the degradation efficiencies of the assessed PAHs, using enzyme immobilized were as efficient as with the free enzyme. These results indicated a new perspective for the application of immobilized laccase in the processes of bioremediation.

In this context, the oxidation potential of several PAHs by *T. versicolor* laccase in the remediation of contaminated soil samples was evaluated by Wu *et al.* (2008). Results indicated 100% efficiency of anthracene and benzo(a)pyrene removal, in the presence of 1 mM of the ABTS mediator, in a 24-hour incubation.

The capacity of anthracene degradation by fungi has drawn attention to researchers to test the potential of nanostructured silica SBA-15 as support for laccase immobilization and to evaluate cytotoxicity and genotoxicity of the resulting solution (HU *et al.*, 2009). After 48 hours of oxidation, there was a partial conversion of anthracene to anthraquinone, in the order of 42% in the presence of HBT and 12% in the presence of ABTS. Thus, the resulting solutions exhibited significant cytotoxicity due to the residual presence of anthracene.

The use of a reactor with two immiscible phases was evaluated degradation of anthracene by the *T. versicolor* laccase (ARCA-RAMOS *et al.*, 2012; ARCA-RAMOS

et al., 2014; ARCA-RAMOS *et al.*, 2015), with the use of silicone oils, sunflower oil and olive pomace, as a phase organic was tested. The higher yield observed in anthracene oxidation, obtained with the use of olive-pomace oil in the presence of the surfactant Triton-X (ARCA-RAMOS *et al.*, 2014), led the group to develop a new system for the application of the technique for removing anthracene from contaminated soils in a two-phase bioreactor immiscible, with the use of olive-pomace oil (ARCA-RAMOS *et al.*, 2015). The results of the 48-hour incubation tests led to a complete removal of the anthracene, being considered viable the reuse of the aqueous and organic phases in successive batches of anthracene degradation.

The immobilization of the laccase on nanostructured silica support SBA-15 to increase enzymatic stability in anthracene oxidation processes, proposed by Hu *et al.* (2009) was improved by Bautista *et al.* (2015). The latter authors proceeded with the modification of the support surface, with the introduction of a amine functional group, promoting the fixation of the biocatalyst, by the methods covalent bonding in the aldehyde functional group and adsorption in the surface pores of silica. The conversion values obtained after 48 hours of incubation, when compared with the results obtained with the use of the free enzyme, were 82%, 73%, and 55% for the respective naphthalene, phenanthrene, and anthracene PAHs.

Increasing the stability of the enzyme under non-ideal conditions of pH and temperature, without restrictions on the degradation performance of PAHs, added to the possibility of recovery and reuse of the enzyme, are attractive to consider in the choice of the laccase immobilization technique for degradation of PAHs, for application in the remediation of contaminated areas.

2.6 CONSIDERATIONS ABOUT THE STATE OF ART

There is a variety of work in the literature reporting the enzymatic catalysis of oxidation reactions of PAHs by *T. versicolor* laccase, however, the available data are, mostly, related to controlled systems (pH, temperature, presence of expensive mediators, absence of inhibitors, and others). These controlled systems are considered ideal to achieve high degradation yields, but they are not yet capable of solving practice cleanup PAH polluted sites, as expected from a current trend. On this way, the present study investigated the potential application of two integrated bioremediation strategies, based on emulsification and enzymatic oxidation, to carry out the degradation of PAHs by free and immobilized laccase on polyurethane foam.

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3 SURFACTANT-ENHANCED *IN-SITU* ENZYMATIC OXIDATION: A BIOREMEDIATION STRATEGY FOR OXIDATION OF POLYCYCLIC HYDROCARBONS IN CONTAMINATED SOILS AND AQUIFERS

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) degradation by laccases has been often evaluated on optimal conditions, in presence of expensive mediators. However, the application of this process on remediation of contaminated areas is not clearly understood. The aim of this work is to provide an alternative strategy closer to a real application. Batch experiments were performed on contrasted PAH contaminated model and a real (groundwater) solution at room temperature for 2 and 5 days, respectively. Analyses of anthracene, benzo(a)pyrene, naphthalene and laccase activity were performed. In model solution at pH 7 without ABTS, 50.59% of anthracene and 42.15% of benzo(a)pirene were removed. The laccase-mediated oxidation was then applied to four groundwater contaminated samples from abandoned gas station areas. The harsh chemical and biological conditions of groundwater may have influenced enzyme stability, and consequently PAH oxidation rates. After 5 days of incubation, groundwater sample 2 resulted in 94.37% of anthracene oxidation, producing anthraquinone. The final anthracene concentration was lower than the intervention value of international groundwater protection and cleanup policies, demonstrating the capability of laccases to mediate PAH oxidation until safe concentration, when applied directly into groundwater and, thus, making the proposed strategy an interesting alternative to remedy polluted sites

Keywords: Bioremediation strategies; Enzymatic degradation; PAH biodegradation.

3.1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are contaminants occurring in industrial and urban areas, which represent a threat for human health because of their recognized carcinogenic and mutagenic effects (ABDEL-SHAFY; MANSOUR, 2016). A range of contaminated soils and groundwater sites have been reported and the sources include steel making or coking plants of petroleum drilling, fuel stations, and other activities with heavy use of hydrocarbon fuels and oils (KUPPUSAMY *et al.,* 2017).

These pollutants occur in the subsurface environment sorbed to soil surfaces, dissolved in groundwater and in non-aqueous phase liquids (NAPLs) (BESHA *et al.,* 2018*)*. Due to their tendency to be sorbed to soil materials and lower water miscibility, isolated NAPLs remediation technologies based on *in-situ* plume treatment are not suitable to remediate PAH site. On the other hand, integrated remediation technologies based on surfactant-enhanced remediation (SER) have been developed to improve PAHs transfer efficiency from NAPLs/soils into the aqueous phase, increasing the availability of PAHs in groundwater (LIANG *et al.,* 2017. These SER' integration allows to apply *in-situ* treatment technologies without excavation, to NAPLs remediation in soil and groundwater, such as pump-and-treat system, *in-situ* bioremediation and *in-situ* chemical oxidation (ISCO) (LAMICHHANE; KRISHNA; SARUKKALIGE, 2017).

Due to their operating costs, the pump-and-treat systems have been replaced by *in-situ* chemical (ISCO) or biological (bioremediation) oxidation technologies to destroy chemical contaminants in place, but feasibility study is generally required on a case-by-case.

While ISCO involves the injection of reactive chemical oxidants (e.g. permanganate, Fenton's reagent, ozone, persulfate) into groundwater or soil to destroy pollutants (HULING; PIVETZ, 2006; LEMAIRE *et al.,* 2019), the integrated technology surfactant enhanced *in-situ* chemical oxidation (S-ISCO) involves the reactive transport of surfactant and co-solvent (BESHA *et al.,* 2018*)*. Furthermore, ISCO can be integrated with other technologies such as biological treatment to achieve the clean-up objectives (HULING; PIVETZ, 2006; LEMAIRE *et al.,* 2019; VALDERRAMA *et al.,* 2009). Moreover, the addition of high doses of the oxidant affects the viability of later biodegradation (PELUFFO *et al.,* 2018).

As an environmentally friendly process and current trends, different bioremediation strategies have been used to the treatment of contaminated water. For the purpose of removing metals in wastewaters, phytoremediation has been studied (SALEH; AGLAN; MAHMOUD, 2019; SALEH; AGLAN; MAHMOUD, 2020). In case of integrated technology surfactant enhanced bioremediation (SEBR), to remedy polluted soils and groundwater, microorganisms including bacteria, fungi and algae have been applied to decompose pollutants dissolved in surfactant solutions (MOHAN *et al.,* 2006; LIANG *et al.,* 2017). These bioremediation processes have been considered as a promising cleanup technology due to its cost-effective and environmentally friendly traits, when compared to S-ISCO (LIANG *et al.,* 2017). Nevertheless, few days to

several weeks are required and the pollutants biotransformation occurs due to the activity of some enzymes, that must be produced by these microorganisms (AYDIN *et al.,* 2017; HADIBARATA *et al.,* 2012; UHNÁKOVÁ *et al.,* 2009; XIE *et al.,* 2015). Oxygenase, dehydrogenase and ligninolytic enzymes have been reported to PAHs degradation (BALDANTONI *et al.,* 2017; HARITASH; KAUSHIK, 2009; PANG; LI; ZHANG, 2015).

Recently, many studies have been reported to evaluate PAHs enzymatic degradation aiming to remediation purposes (KUPPUSAMY *et al.,* 2017). However, these works did not consider some factors related to practical aspects for application of the remediation processes and technologies. For ex-ample, enzymatic treatments with PAHs contaminated soils samples in batch reactors have been studied as remediation purposes (ARCA-RAMOS *et al.,* 2015; PELUFFO *et al.,* 2018; WU *et al.,* 2008), but the practical feasibility depends on soil excavation and reactor assembly, added to treatments with chemical reagents.

The latest techniques are based on the use of surfactant solution for soil washing, integrated with oxidation technologies to destroy chemical contaminants in place, such as S-ISCO (BESHA *et al.,* 2018; WANG *et al.,* 2013) or SEBR (LIANG *et al.,* 2017; MOHAN *et al.,* 2006), whose applications choice depend on the limiting factors as treatment time and operation cost, respectively. The proposed strategy based on surfactant-enhanced *in-situ* enzymatic oxidation is similar, but the direct use of enzyme as oxidizing agent replaces the use of chemical oxidants or microorganisms. Therefore, advantages are obtained due to lower operation cost than S-ISCO and less treatment time in comparison to SEBR. Enzymes are less harmful to environment, act as biocatalyst and are not consumed throughout reaction (DEMARCHE *et al.,* 2012; RAO *et al.,* 2014), being able to apply less injections campaigns in situ than chemical oxidants or micro-organisms, thus making possible to reduce the remediation's cost and time.

Taking into account the purposes listed above, and aiming to make the surfactant-enhanced *in-situ* enzymatic oxidation process a viable alternative closer to a real application, the feasibility of anthracene and benzo(a)pyrene oxidation by fungal laccase was investigated for the first time in the literature in the present work, in a model and a real (groundwater) solution. Furthermore, some understanding of the influences of enzyme stability on pH and presence of mediator were bringed, as well as some groundwater properties on oxidation efficiency. Four groundwater samples were treated in the laboratory by batch experiments with laccase to determine the efficiency

of PAH oxidation and the product of laccase biotransformation of PAHs, by using anthracene as a model aromatic compound.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals and enzyme

Anthracene (Ant), benzo(a)pyrene (BaP), naphthalene (Nap), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Tween 80 and *T. versicolor* laccase (activity of 0.5 U mg^{-1}) were obtained from Sigma-Aldrich Corporation, São Paulo, Brazil. Acetone, acetonitrile, dichloromethane and the other chemicals were purchased from Merck (Brazil).

3.2.2 Enzyme characterization

Enzyme stability to pH was evaluated by preparing 10 mL of enzyme solutions (0.67 mg mL^{-1}) with citrate buffer (0.1 M, pH 5). The solutions were then incubated in a water bath with controlled pH of 3, 4, 5, 6, 7 and 8. Periodically, 1 mL of each solution was withdrawn and the activity was then assayed at 25 °C. This experiment was repeated at least three times.

3.2.3 Effect of pH and mediator on PAHs biodegradation by laccase on model spiked solution

The ability of laccase from *T. versicolor* to oxidase PAHs was investigated at pH values of 5 and 7 in either the presence or the absence of mediator ABTS. The PAHs biodegradation treatments were performed in 20 mL reactants in 125 mL Erlenmeyer flasks containing 0.15 U mL^{-1} of laccase dissolved in citrate-phosphate buffer (pH 5 and 7) and 1% (v/v) Tween 80 to assess the impacts of reaction conditions (pH and presence of mediator ABTS) on the laccase catalysis. Ant and BaP dissolved in acetone was added to all treatments to make the final concentrations 12.48 mg L^{-1} of Ant and 5.00 mg L^{-1} of BaP. The concentration of the mediator ABTS reached 1 mM in the reaction mixture. Reaction flasks were incubated in a horizontal shaker at the speed of 100 rpm at 25 °C for 48 h. PAHs were extracted with 1.5 mL dichloromethane and analyzed by HPLC. Samples for HPLC analysis were evaporated and redissolved in acetone. All samples were performed in duplicate. Control samples for each PAHs concentration without enzyme were also prepared using the same procedure.

3.2.4 Groundwater sampling and analysis

The groundwater samples used in the experiments were selected from two abandoned fuel station areas, Joinville-SC, Brazil, due to the availability of groundwater monitoring structure. Four non polluted groundwater samples were collected according to USEPA method (BARCELONA, *et al.*, 1985).

Their geographical coordinates were indicated and their physico-chemical properties (temperature, pH, oxidation reduction potential–ORP, conductivity and turbidity) were analyzed on field by HI 9828 HANNA multi-parameter probe (Hanna Instruments, Woonsocket, USA), and they kept refrigerated (4 °C) until the use. Before using, the samples were membrane filtered (25 μm) to remove the suspended solids and then contaminated with the PAHs of interest.

3.2.5 Enzymatic PAHs biodegradation behavior on real municipal groundwater samples

The experiments were carried out in conditions closer to reality, in search of strategy validation, by surfactant-enhanced *in-situ* enzymatic oxidation of PAHs in contaminated soils and aquifers. Laboratory treatability (e. g., for S-ISCO treatment) testing using contaminated site soil and groundwater are crucial before implementing full-scale others remediation systems (ETHICAL CHEM, 2019)

The four groundwater samples were used on experiments with the addition of surfactant Tween 80 and were contaminated with 12.48 mg L^{-1} of Ant, 5.00 mg L^{-1} of BaP and 32.00 mg L^{-1} of Nap, just as would be groundwater after soil washing, to simulate field application of surfactant-enhanced *in-situ* enzymatic oxidation. The PAHs concentrations were improved by the surfactant addiction, as highest PAHs concentrations that should be found on groundwater, after PAH contaminated soil was washed by surfactant solution. The physico-chemical and microbial properties of groundwater samples were kept on experiments, except for the parameter suspended solids. It was used low agitation (100 rpm) on experiments, only to favor the minimum mass transfer phenomena of natural occurrence in groundwater.

In these experiments, almost the same conditions were used as those experiments that evaluated the effect of pH and mediator on PAHs biodegradation by laccase, except for groundwater samples, naphthalene addition and incubation time. Batch biodegradation experiments were performed in duplicate at 25 ± 2 °C in 125 mL Erlenmeyer flasks and stirred at 100 rpm for 5 days. Laccase dissolved in citrate-phosphate buffer (pH 7) to final concentration 0.15 U mL^{-1} was mixed with

groundwater sample, surfactant Tween 80 (1% v/v) and PAH's dissolved in acetone to make 20 mL reactants.

After 5 days of incubation, the PAHs (Ant, BaP and Nap) removal efficiency was determinated in four groundwater samples and resulting solution of the better one was chosen to identify oxidation product by GC–MS analysis. The scheme of methodological sequence procedure is illustrated in Figure 5, highlighting and detailing the groundwater experiments, which seek to simulate strategy application.

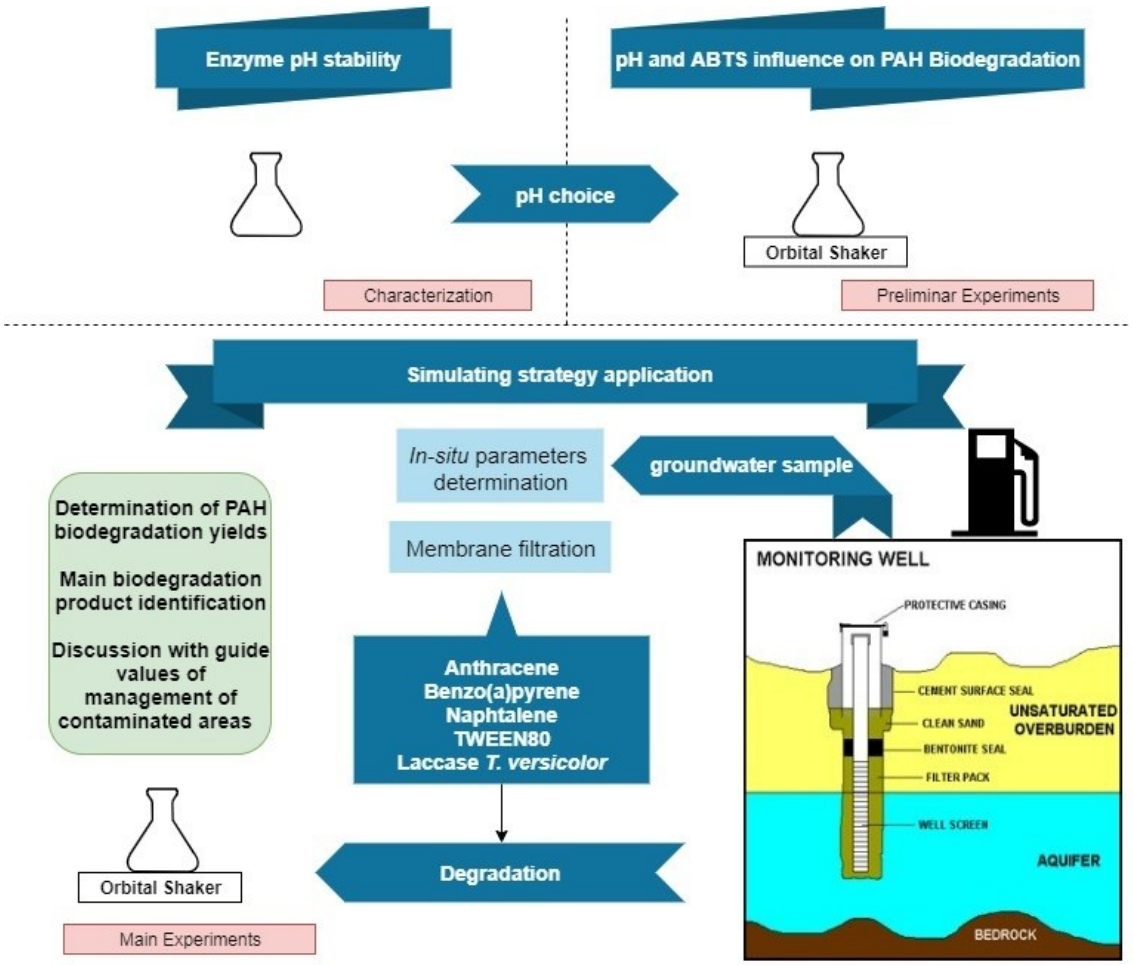
3.2.6 Enzyme activity assay and PAHs analysis

Laccase activity was determined by measuring the oxidation of ABTS at 30 °C. Briefly, a 1 mL reaction mixture was prepared including 0.8 mL ABTS (0.18 mM), 0.1 mL phosphate-citrate buffer (0.05 mol L^{-1} phosphate-citrate, pH 5.0 at 25 °C), and 0.1 mL enzyme solution. The increase in absorbance at 420 nm was monitored with a spectrophotometer (UV-1601PC UV visible, Shimadzu) to determine laccase activity (ε_{420} = 36,000 M cm^{-1}) by the equation: $\Delta A \times 20 \times 10^6/36{,}000$, where ΔA is the increment of absorbance per min when it is stable. One unit of laccase activity is defined as the amount of enzyme able to oxidize 1μmoL ABTS per minute.

PAHs concentrations were analyzed by a Thermo Fisher Scientific Finnigan Surveyor high performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, USA) with a diode array detector. A Agilent Pursuit 3 PAH column (4.6 mmx 100 mm, particle size 3μm), using a mobile phase with acetonitrile-water mixture (0 min, 60:40; 7 min, 90:10; 13.5 min, 100:0; 16 min, 60:40; 21 min, 60:40; gradient elution 21 min) at a constant flow rate 0.5 mL min^{-1}, was used to separate PAHs.

The products of PAHs oxidation by laccase from *T. versicolor* were analyzed by a gas chromatography-mass spectrometer (Agilent 5975C, Agilent Inc., Palo Alto, USA) equipped with a GC column (HP-5MS, 30 mm £ 0.25 mm). The oven temperature was programmed from 100 °C with a 2-min hold and a 10 °C min^{-1} increment to 300 °C with a 10 min hold.

Figure 5: Schematic representation of experiments



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3.3 RESULTS AND DISCUSSION

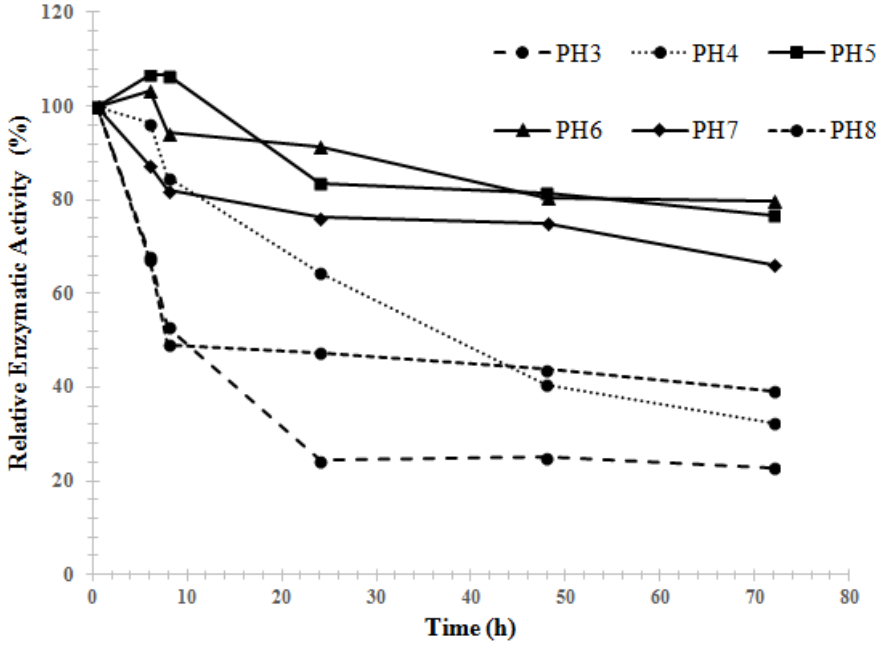
3.3.1 Effect of pH on enzyme stability

Results revealed that values of pH 3, 4 and 8 could lead to a corresponding decrease in the enzyme stability (Figure 6). The activity of the enzyme before incubation in water was defined as the control and attributed a relative activity of 100%.

At values of pH 5 and 6, highest values of relative enzymatic activity (80%) after 72 h of incubation were observed. A decrease on enzyme stability was also observed at the pH 7 condition, in comparison with pH 5 and 6 conditions. At pH 7 the relative enzyme stability was 70% after 72 h. Others studies related that the pH 5 was the optimal for this enzyme (ARCA-RAMOS *et al.,* 2014; COLLINS *et al.,* 1996; LI *et al.,* 2014), which was the same pH condition observed in this study, but considering

enzyme stability conditions. These studies have evaluated degradation efficiency of PAHs removal by *T. versicolor* laccase at pH 5 in the presence of mediator compounds.

Figure 6: Effect of pH on *Trametes versicolor* laccase stability



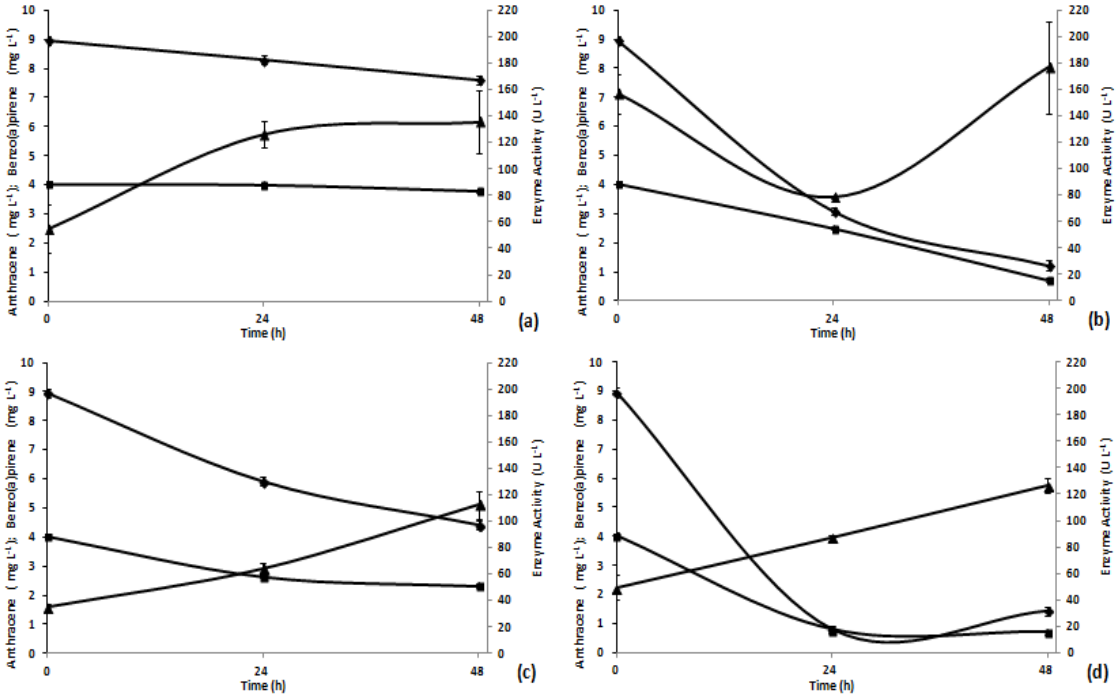
Source: from author.

When it comes to apply the enzyme laccase for *in-situ* treatment of contaminated groundwater, the maintenance of pH 5 would be limited to the remediation applications, for techniques where pH conditions can be controlled as pump and treat technique, using external pH-controlled reactor. For the proposal strategy, where enzyme would be applied directly into groundwater, a more neutral pH range is required, such as pH 7, considering pH range of natural groundwater conditions similar to groundwater conditions reported (LI *et al.,* 2017). These facts led to the choice of pH values of 5 and 7 to evaluate the PAHs oxidation by laccase on neutral pH range and its behavior in comparison with the optimal/stable pH range, usually reported in other studies.

3.3.2 Effect of pH and ABTS on PAHs biodegradation by laccase

It was used a mixture of Ant and BaP at the total concentration of 12.48 mg L^{-1} and 5.00 mg L^{-1} with equal concentrations of each PAH, at pH 5 and at pH 7, and in the presence or absence of ABTS as mediator, to evaluate PAHs kinetics and oxidation yield over time (Figure 3).

Figure 7: Batch degradation experiments by free laccase at T= 25 °C, with Tween 80 (1% v/v) and different pH and presence/abstention of mediator ABTS conditions: a) pH = 5; b) pH = 5 and ABTS (1 mM); c) pH = 7; d) pH = 7 and ABTS (1 mM). ♦, anthracene; ■, benzo(a)pyrene; ▲, laccase volumetric activity.



Source: from author.

On these pH conditions, laccase from *T. versicolor* had higher stability, however pH 5 was the optimal and pH 7 was neutral range condition, as usually found in groundwater.

The profiles of the enzymatic treatment in model solution were determined and for both pH conditions laccase from *T. versicolor* was demonstrated stable. Regarding to the degradation efficiency of Ant and BaP, an inverse relationship with the enzymatic activity was ob-served, when considering only the pH conditions of the experiments.

Thus, the experimental conditions of Figure 7 (c) and (d) (pH 7) presented the highest degradation values of Ant and BaP and the lowest values of enzymatic activity when compared to experiments at conditions presented in Figure 7 (a) and (b) (pH 5).

When analyzing the presence of the compound ABTS which acts as a mediator, (b) and (d) conditions, it can be observed an increase of PAHs degradation compared to the experiments of the same conditions in the absence of mediator, (a) and (c) conditions. The oxidation of PAH was reported to be considerably enhanced by the presence of mediator ABTS (ARCA-RAMOS *et al.,* 2014; COLLINS *et al.,* 1996; JOHANNES; MAJCHERCZYK; HUNTEERMANN, 1996; WU *et al.,* 2008), and the

same increase on Ant and BaP oxidation was obtained on treatments by the addition of ABTS.

It was possible to observe a highest degradation efficiency of PAHs (91.03% of Ant and 80.28% of BaP) after 48 h in the experimental conditions presented in Figure 7 (d) at pH 7 with ABTS, which was close results from previous report (DODOR *et al.,* 2004; HU; WANG; HWANG, 2009; LI *et al.,* 2014) under very similar treatment conditions using laccase from *T. versicolor*. Even though the highest and fastest PAHs biodegradation condition were in the presence of ABTS conditions, this would not be the best condition to apply *in-situ* enzymatic oxidation, when considering the technique operating cost. The main influencing factor is related to ABTS cost, that is an expensive reagent to use for this purpose and could be a financial constraint.

Ant was removed to 50.59% from the reaction mixture during 48 h incubation, followed by BaP which were oxidized to 42.15%, in experimental condition presented in Figure 3 (c) at pH 7 without ABTS. This condition showed a tendency of PAHs decrease to 48 h, which suggests that could be even more degradation above this time. At this moment, another influencing factor should be considered, as the usual pH groundwater condition, similar to the groundwater samples (Table 4) collected from two gas station areas.

Although the addition of mediator ABTS resulted in high degradation efficiency of PAHs, two influencing factors were considered in the next experiments on groundwater samples, aiming to apply *in-situ* enzymatic oxidation strategy.

3.3.3 PAH enzymatic degradation efficiency on real groundwater samples

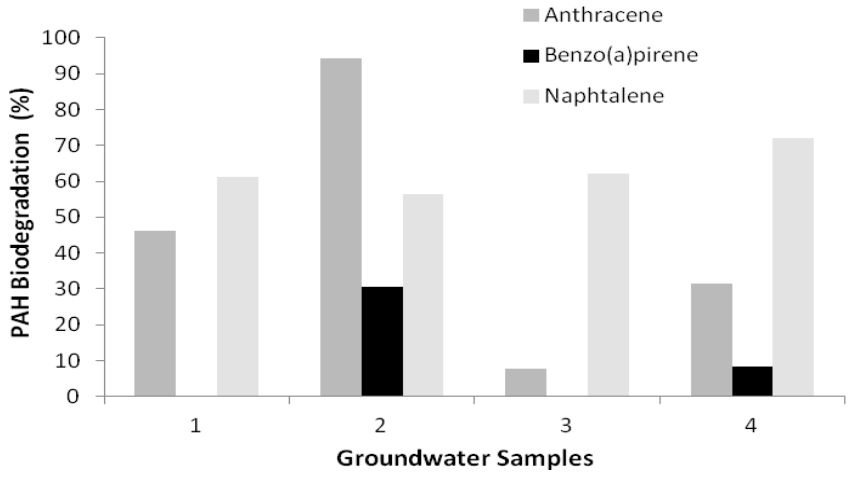
The *in-situ* groundwater parameters were determined during sampling (Table 4). While high values of conductivity and turbidity were observed in groundwater sample 4, low value of oxidation reduction potential (ORP) was detected in groundwater sample 2. Groundwater samples exhibited similar physicochemical properties among them in terms of temperature and pH, very close to previous batch conditions in model solution (25 °C and pH 7), which is positive in order to validate the directly use of laccase in groundwater bioremediation.

Table 4: *In-situ* parameters: physicochemical properties contents of the four groundwater samples

Ground-water Sample	Geographical Coordinates	T (^{o} C)	pH	Oxidation Reduction potential (ORP)	Conductivity ($\mu S\ m^{-1}$)	Turbidity (NTU)
1	26°16'14"S 48°49'15"O	22.5	7.22	62.9	173.5	22
2	26°17'01"S 48°50'26"O	22.0	7.62	32.6	105.9	210
3	26°17'01"S 48°50'27"O	21.9	7.48	45	176.3	19.65
4	26°16'60"S 48°50'26"O	22.0	7.22	55	846.3	905

The four samples were contaminated to final concentrations of 12.48 mg L^{-1} of Ant, 5.00 mg L^{-1} of BaP and 32.00 mg L^{-1} of Nap, with addition of surfactant Tween 80 (1% v/v) to increase the PAH availability. The efficiency of PAH oxidation by laccase in batch experiments were determined (Figure 8) and the product of anthracene laccase-transformation was identified. While the original pH of the groundwater samples was kept constant, the temperature of batch experiments was 25 °C, slightly above of the original groundwater samples temperatures (Table 4). The samples were then membrane filtered to remove the suspended solids.

Figure 8: Biodegradation yields of anthracene, benzo(a)pyrene and naphthalene in four groundwater samples at 25 °C, with Tween 80 (1% v/v).



Source: from author.

The results obtained showed that laccase catalytic efficiency on PAHs biodegradation may be affected by different groundwater samples in different ways.

Naphthalene (two fused rings), which has lower molecular weight, was degraded to a similar yield on four samples, reaching 65% of average degradation yield on batch experiments, unlike than observed to anthracene and benzo(a)pyrene. The physico-chemical properties and organic matter of groundwater samples did not affect the degradation capacity of Nap, but limited the yield rate, that could have reached values above 90% as reported using with free laccase at pH 4.5 in model solution (BAUTISTA; MORALEZ; SANZ, 2015).

The other two PAHs degraded to different rates on four samples, and the low degradation rate was observed for BaP in comparison with Ant. One inverse proportion between degradation rate and molecular weight was described for anthracene (three fused rings) and benzo(a)pyrene (five fused rings) enzymatic degradation in real soil samples. A faster anthracene degradation rate was observed (95%) after five months, whereas 50% of BaP after nine months (BALDANTONI *et al.,* 2017).

The highest PAHs degradation yields were observed in sample 2 (94.37% of Ant and 30.43% of BaP) and in sample 4 (72.08% of Nap), after 120 h of incubation. In sample 2, under close conditions (pH and without ABTS) compared to experiment (c) (Figure 7) more degradation of Ant was observed, possibly by the longer incubation time. The yield in sample 2 was considerably higher than those 42% of anthracene oxidation by immobilized laccase, obtained in pH 7, after 48 h in presence of mediator (HU; WANG; HWANG, 2009).

Degradation of 94.37% of Ant in sample 2 after 5 days, obtained in laboratory treatability of surfactant-enhanced *in-situ* enzymatic oxidation strategy, was a successful result in comparison than 96% reduction of PAHs in 14 days using S-ISCO applications with persulfate radical (WANG *et al.,* 2013).

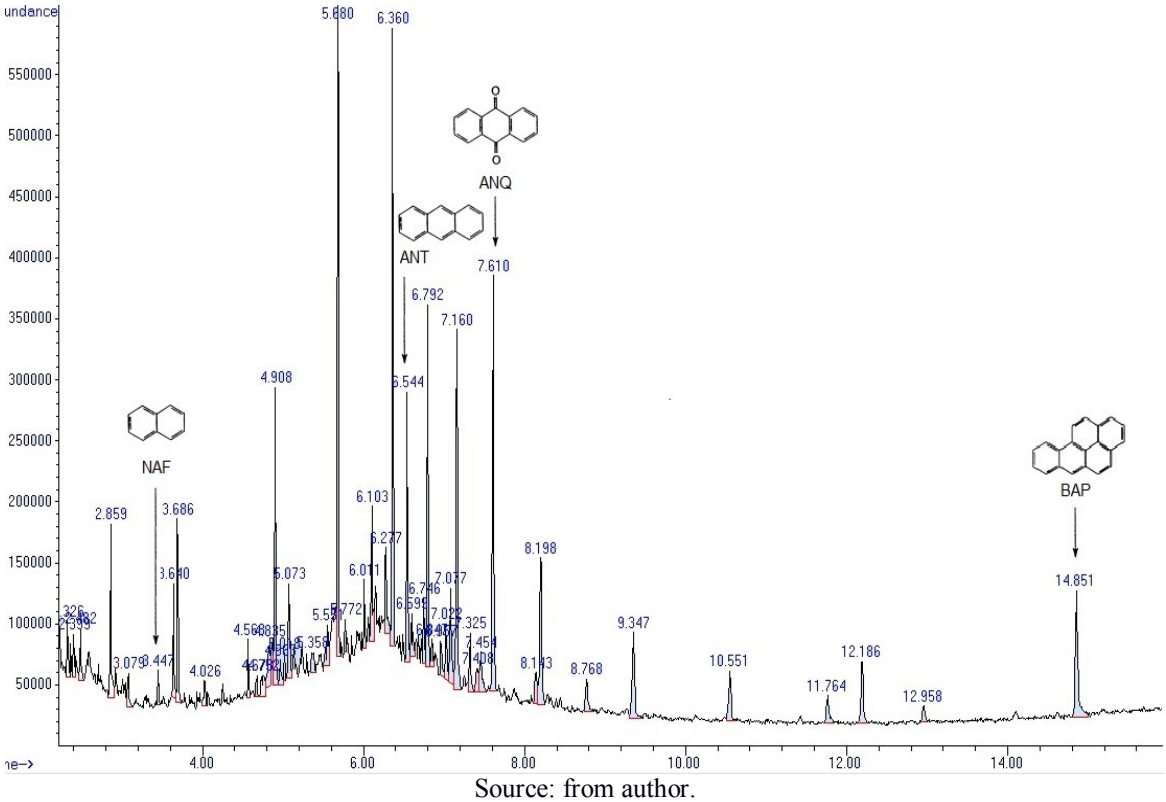
3.3.4 Product identification of anthracene biodegradation

Although the main objective of this work was to evaluate PAHs degradation by laccase, aiming at application by a surfactant-enhanced *in-situ* enzymatic oxidation bioremediation strategy, it is also important to identify degradation products after the incubation. From the literature, after 48 h of process, immobilized laccase in presence of mediator resulted in 42% of anthracene oxidation, which produced anthraquinone (ANQ). The cytotoxicity and genotoxicity of Ant and ANQ were evaluated and the results illustrated that ANQ is less toxic than Ant as well (HU; WANG; HWANG, 2009). The gas chromatography-mass spectrometry (GC–MS) analysis, after anthracene

oxidation by laccase from *T. versicolor*, indicated that 9,10-anthraquinone was the main product of anthracene degradation. Microtox test suggested that laccase treatment of anthracene would not increase the acute toxicity of contaminated site (LI *et al.,* 2014).

The batch experiment with laccase from *T. versicolor* in groundwater sample 2 presented the highest Ant degradation (94.37% of Ant). The product of this anthracene oxidation by laccase was determined by GC-MS analysis, according to the gas chromatogram presented in Figure 9. The results indicated the appearance of a lot of peaks at different retention times, which is probably from contaminated amount of groundwater, collected from abandoned gas station area. Despite this, the gas chromatogram resulted in appearance of a peak at retention time of approximate 7.61 min, which can be further identified as ANQ based on the mass spectrum, as in other studies (HU; WANG; HWANG, 2009; LI *et al.,* 2014).

Figure 9: Gas chromatogram of oxidation of Polycyclic Aromatic Hydrocarbons by laccase in groundwater sample (2) after 120 h incubation. Detection of naphthalene, anthracene; 9,10-anthraquinone and benzo(a)pyrene was by gas chromatography-mass spectrometry analysis.



Source: from author.

3.3.5 Groundwater protection and cleanup policies

This study brought, for the first time, batch experiments for PAHs biodegradation by laccase directly in groundwater samples, validate as an application of a bioremediation strategy. The final PAHs concentrations detected in samples were an important result on the management of contaminated sites and must be evaluated with guideline values of groundwater quality. International groundwater policies from some countries (e.g. Brazil, Ireland and New Zealand) regulate investigations and cleanup actions as necessary to protect human health and the environment. Each policy (CETESB, 2014; EPA, 2003; NZME, 1997), presents a guideline values for groundwater based on substance concentration values which above are potential risks to human health, considering the most restrictive exposure scenario, where include drinking contaminated groundwater as one of direct route of exposure. The regulations of other countries do not list groundwater guideline values, so they must be calculated case by case, considering all applied direct routes of exposure.

While the latest law in Brazil, which guides values for soils and groundwater (CETESB, 2014), lays down the concentration of these substances (intervention values), on experiment 2 after 120 h incubation it was detected 0.70 mg L^{-1} of Ant, 3.47 mg L^{-1} of BaP and 13.91 mg L^{-1} of Nap. The final 0.70 mg L^{-1} of Ant detected in sample 2 was lower than the intervention value (0.90 mg L^{-1}). Similarly, the residual sample of Ant detected was lower than 10.00 mg L^{-1}, interim value of Irish policy (EPA, 2003) and 1.00 mg L^{-1}, summary of generic water acceptance criteria (Potable) of New Zealand law (NZME, 1997). In this case, if it was in a real process of contaminated area management, it would have achieved the cleanup goal and the remediation process could have ceased. Considering the exposure scenario of Brazilian, Irish and New Zealand policies, it would be possible to conclude that are no more risks to human health, for anthracene concentration in groundwater that reached safe levels for groundwater protection and resource conservation.

3.4 CONCLUSIONS

A large number of studies that evaluate PAHs enzymatic oxidation working on optimal pH and temperature conditions with expensive mediator compounds to achieve high degradation yields are already presented in the literature. But they are not yet capable of solving practice cleanup PAH polluted sites, as expected from a current trend. On this way, the present study investigated the potential application of an integrated *in-situ* bioremediation strategy, based on emulsification and enzymatic

oxidation, to carry out the degradation of PAHs by laccase from *T. versicolor*. The influence of participation of an oxidative mediator (ABTS) and enzyme stability on pH results were demonstrated to remove polycyclic aromatic hydrocarbons model samples. In spite of the known positive impact on PAH degradation yield promoted by ABTS, at pH 7 without mediator, significant amounts of anthracene and benzo(a)pirene were oxidized after 48 h incubation, with a decay tendency. 120 h of laboratory treatability testing using four contaminated groundwater samples demonstrated the potential of laccase of *T. versicolor* oxidize PAHs. Even though some enzymatic fraction could be inactivated due to the harsh chemical and biological conditions of the real groundwater, the *T. versicolor* laccase was able to trigger efficient oxidative reaction. Furthermore, GC–MS analysis after incubation indicated presence of anthraquinone, known to be product of anthracene oxidation by laccases, which is less toxic than the substrate as well. In this case, residual anthracene amount was lower than the intervention values of international groundwater protection and cleanup policies. In other words, it was the condition in the exposure scenario that there is no more risks to human health.

For the proposal integrated *in-situ* bioremediation strategy, this study provides a novel perspective, since they are for the first time the targets of real's groundwater samples. Based on the results, surfactant-enhanced *in-situ* enzymatic oxidation can be considered an interesting alternative to remedy polluted soils and groundwater against chemical oxidation (S-ISCO), mainly considering anthracene oxidation by laccase. Further investigations are necessary to improve the enzyme stability against the groundwater conditions, in order to avoid inactivation. The next step in the study would be collect groundwater and soil samples from an anthracene contaminated site, to perform batch tests followed by column study, before applying field scale trails.

4 PERSPECTIVE FOR PRACTICAL APPLICATION OF LACCASE IMMOBILIZED ON COMMERCIAL POLYURETHANE FOR PAHs REMEDIATION PURPOSES

ABSTRACT

There is growing concern about developing treatment technologies for the hazardous Polycyclic Aromatic Hydrocarbons (PAHs), because the rising levels of these compounds in the environment by human activities. The application of laccase (enzyme) has been evaluated as one of the most promising treatments. Thus, laccase immobilization on polyurethane foam (PUF) a low-cost material was evaluated for bioremediation batch mode of simulated groundwater using a combination of 16 polycyclic aromatic hydrocarbons as model pollutants. Conditions closer to a real contaminated site(non-optimal) were considered on our experimental design, leading to the formation of new degradation intermediaries, even more degraded than the usual ones The bioremediation of PAH in mg L^{-1} using immobilized laccase on PUF reached 92.35% of removal yield for anthracene (Ant) and 97% for benzo(a)pyrene (BaP). After degradation, the biodegradation products were identified diisooctyl phthalate and tetradecane. The biodegradation mechanism were proposed, where PAHs oxidation processes and aromatic ring fission led to quinone and diethyl phthalate formation. Then through the latter processes besides, polymerization and methylation, lead to the identified biodegradation product formation. The immobilized enzyme improvement in the removal yield of 8 of 14 PAHs tested in μg L^{-1}, compared to the free counterpart. Laccase immobilized on PUF achieved final anthracene concentration of 0.95 mg L^{-1}, up to 38 μg L^{-1} of chrysene (77% removal)and 98 μg L^{-1} of pyrene (32% removal), under the intervention limits of environmental protection policies. Thus, hence laccase immobilized on PUF in bioreactors profile as a potential approach for PAHs bioremediation for an *ex-situ* treatment.

Keywords: Bioremediation; immobilized laccase; polyurethane foam; enzymatic biodegradation; PAHs.

4.1 INTRODUCTION

The contamination of groundwater, surface water, and drinking water by Polycyclic Aromatic Hydrocarbons (PAHs) is one of significant concern worldwide because of high ecological risks and their potential as carcinogens to humans

(ABDELSHAFY; MANSOUR, 2016; BALL; TRUSKEWYCZ, 2013; EOM *et al.*, 2013). Due to these effects, the U.S. Environmental Protection Agency (USEPA) has listed 16 PAHs as priority pollutants (LLOBET *et al.*, 2006). The primary anthropogenic sources of PAHs in the environment are heavy oil, as crude oil, many refined and residual oils, and their oily sludge (XU *et al.*, 2020). In urban areas, processes that use fossil oil and fuels are the potentially polluting activities, such as gas stations, steel making, or coking plants of petroleum drilling and other activities (KUPPUSAMY *et al.*, 2017).

It is common to detect in PAHs contaminated sites the occurrence of leaks, spills, floor, and soil infiltration (primary mechanisms), and release of contaminants, through infiltration and dispersion in the soil, down to lower layers of soil and groundwater (secondary mechanisms) (KUPPUSAMY *et al.*, 2017; LIANG, *et al* 2017; SUN, *et al.*, 2019). PAHs also can be found in surface water and sediment of rivers due to contamination sources activities (ADENIJI; OKOH; OKOH, 2019; LI; LI; LIU, 2017). Each country has its our environmental protection and clean-up policy, which provides PAHs concentration limits in soil and groundwater, guiding the remediation management of contaminated area (Table 3, presented in Chapter 2).

PAHs site remediation needs combined and integrated techniques to achieve the clean-up goals in soil and groundwater. The integration of techniques is commonly used, for example, the use chemical oxidation with surfactants (OSSAI et al., 2020). Treatment with PAHs contaminated soils samples in batch reactors has been studied for remediation purposes, which in practical terms would need soil excavation (GRAY; BANERJEE; FEDORAK, 1994; PELUFFO *et al.*, 2018; XIE *et al.*, 2015). Nevertheless, soil excavation has been carried out at a majority of PAH contaminated sites until the 1990s, and due to high cost has been replaced from others techniques. For instance of the integrated technologies (KUPPUSAMY *et al.*, 2017), PAHs are transferred from soils into the aqueous phase in groundwater by surfactant flushing and soil washing, known as surfactant-enhanced remediation (SER) (LIANG *et al.*, 2017). Once released into groundwater, PAHs can be degraded by some *in-situ* treatment technologies such as pump-and-treat system, *in-situ* bioremediation, and *in-situ* chemical oxidation (ISCO) (LAMICHHANE; KRISHNA; SARUKKALIGE, 2017).

The use of microorganisms and enzymes for PAHs oxidation (AYDIN *et al.*, 2017; KUPPUSAMY *et al.*, 2017; MOHAN *et al.*, 2006) is an current environmental trend.I In field application, would be associated to *in-situ* treatment techniques of pump-

and-treat systems or *in-situ* bioremediation. Laccases are well-known for their ability to degrade anticancer drugs (PEREIRA *et al.*, 2019) and other organic compounds (BAYRAMOGLU; KARAGOZ; ARICA, 2018; GARCÍA-MORALES *et al.*, 2018; UHNAKOVÁ *et al.*, 2009). Laccases have also been reported to degrade PAHs (BALDANTONI *et al.*, 2017; HARITASH, KAUSHIK, 2009; PANG, LI, ZHANG, 2015) in the presence or absence of mediators, such as 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diam-moniumsalt (ABTS) and 1-hidroxybenzothyazole (HBT) (COLLINS *et al.*, 1996; JOHANNES, MAJCHERCZYK; HÜTTERMANN, 1996; LI *et al.*, 2014; WU *et al.*, 2008). There is still no consensus on the use of mediators, or in the best mediators should be used. However, there is an empty space on literature that must be explored, evaluating PAHs degradation by laccases without high-cost mediator and in non-optimal pH experiment conditions.

The optimal pH range of laccases is between 4 and 5, which also is the condition where laccase has high stability (COLLINS *et al.*, 1996; MAJCHERCZYK *et al.*, 1998). However, the observed pH range in groundwater detected in other studies usually is a more neutral range (pH 6 to 7) (ADENIJI; OKOH; OKOH, 2019; LI; LI; LIU, 2017). Thus, in order to increase the enzyme stability, the immobilization on solid materials is known as a promising technology (BAUTISTA; MORALEZ; SANZ, 2010). According to Kim *et al.* (2006), enzyme immobilization could improve enzyme stability, increase the reuse potential, prolong the lifetime, and reduce the enzyme amount of required. Laccases immobilized on different supports have been developed for degradation of many organic compounds. Immobilization in kaolinite, improvements were observed the stability at temperature, pH, inhibitors, and storage (DODOR *et al.*, 2004). Immobilization on silica caused better pH and temperature stability, however degradation yields were slightly lower than those for free laccase (BAUTISTA; MORALEZ; SANZ, 2015; HU *et al.*, 2009).

Polyacrylonitrile beads were used as support to immobilized laccase, showed complete degradation of nonylphenol and octylphenol (CAPATANE *et al.*, 2013). One novel synthetic polymer, poly(glycidyl methacrylate), was developed as a laccase support, and 90% of bisphenol A and 100% of Congo Red dye was removed in batch experiments (BAYRAMOGLU; KARAGOZ; ARICA, 2018). These synthetic organic polymers used as support to immobilize laccase do have hydrophobic character, and high standard activities have been shown onto biodegradation of organic compounds.

Based on synthetic organic polymers' properties, commercial polyurethane foam (PUF) has been shown as efficient support to other enzymes. In the case of

lipases, properties as stability in pH and temperature conditions were enhanced by PUF immobilization (BRESOLIN *et al.*, 2019; NYARI *et al.*, 2016). Results showed high removal capability of nonylphenol polyethoxylates by laccase from *T. versicolor* immobilized on PUF, due to biodegradation and adsorption on the support (STENHOLM *et al.*, 2020). In this sense, the use of PUF a low-cost material as support for laccase has potential to make efficient PAHs degradation under non-optimal conditions without expensive mediators.

It is also expected to make a current trend possible to field application, considering practical aspects and environmental protection policies Despite the variation in each protection policy with guiding values for soil and groundwater, some details caught our attention, as concentration scale. Usually is in mg kg^{-1} in soil and μg L^{-1} in groundwater (CETESB, 2014; DTIV, 2000; NZME, 1997). Besides, most of the state of art studies of Table 3 (in Chapter 2) have been worked in an aqueous medium, presence of mediator ABTS and optimal pH conditions (range between 4 and 5); they were also evaluated in mg L^{-1} concentration scale. On real contaminated site remediation, high PAHs removal efficiency obtained in mg L^{-1} scale could not be sufficient to achieve safe concentration to protect human health and environment protection, since guide values are set to μg L^{-1} instead of mg L^{-1}. In these cases, the contaminated site probably would need another complementary treatment to achieve clean-up remediation goals.

Thus, the aim of the present study was to evaluate biodegradation of 16 PAHs by *T. versicolor* laccase immobilized on commercial polyurethane in a model solution and a groundwater sample. Different PAHs concentration scales were used in the laboratory in descontinuos assays, without any oxidation mediator compound, in pH and temperature values close to the reality of a contaminated site. Anthracene and benzo(a)pyrene were used as models PAH for products of laccase-transformation identification.

4.2 MATERIAL AND METHODS

4.2.1 Chemicals

Anthracene (Ant), benzo(a)pyrene (BaP), naphtalene (Nap), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Tween 80 and *T. versicolor* laccase (activity of 0.5 U mg^{-1}) (Sigma-Aldrich Brazil). Acetone, acetonitrile, dichloromethane and the other chemicals were purchased from (Merck Brazil). Polymeric methylene

diphenyl diisocyanate (pMDI - specflex NE 134) and commercial polyol were kindly donated by Dow Chemistry.

4.2.2 Laccase Immobilization on commercial polyurethane foam

Laccase from *T. versicolor* was immobilized *in-situ* with commercial reagents of polyurethane foam (PUF) based on Bresolin *et al.* (2019) and Daronch (2020). A 0.2 wt% laccase in Milli-Q water was added to commercial polyol and mixed with the help of a glass stick. After that, methylene diphenyl diisocyanate was added and the expansion occurred after 60 seconds of mechanical stirring (2,500 rpm), using 77 g of NCO per 100 g OH, according to supplier instructions. The immobilized laccase was stored at 4 °C until further use.

4.2.3 Enzyme activity and PAHs analysis

The activity of the free and immobilized laccase were determined by the oxidation rate of ABTS to $ABTS^{+}$. For determination of the free laccase activity 0.3 mL of a laccase solution in Milli-Q water (1 mg mL^{-1}) and 0.3 mL of aqueous ABTS (5 mM) were added to 2.4 mL of phosphate-citrate buffer (0.1 M, pH 3) in a quartz cuvette. A sample of 0.3 mL of Milli-Q water was used instead as a blank.

For determination of immobilized laccase activity, a piece of foam (about 5x5x5 mm in size and 15 mg in weight) was added into 0.4 mL of 5 mM ABTS solution and 3.6 mL phosphate-citrate buffer solution (pH 3). The reaction occurred incubated at 30 °C and 250 rpm for 5 min. A change in absorbance at 420 nm was monitored using a UV/Vis spectrophotometer (HACH, DR5000) and the laccase activity (U L^{-1}) was calculated using the molar extinction coefficient of ABTS (ε_{420} = 36,000 M^{-1} cm^{-1}) using the equation:

$$\frac{U}{L} = \frac{\Delta Abs.V}{\varepsilon.d.v.t} \quad (1)$$

Where ΔA is the increment of absorbance per min when it is stable, V *i*s the total reaction volume, d is the step length, v is the sample volume and t: reaction time. One unit of laccase activity (U/L) was defined as the amount of enzyme able to oxidize 1 μmoL of substrate per minute. All analyses were carried out in triplicate.

The PAHs were analyzed with a standardized gas chromatography/mass spectrometry procedure, using the recommended method by the USEPA for detection and measurements of organic pollutants in aquatic environments (USEPA method 8270). The analysis was carried out in Inova Laboratório e Engenharia (Laboratory and

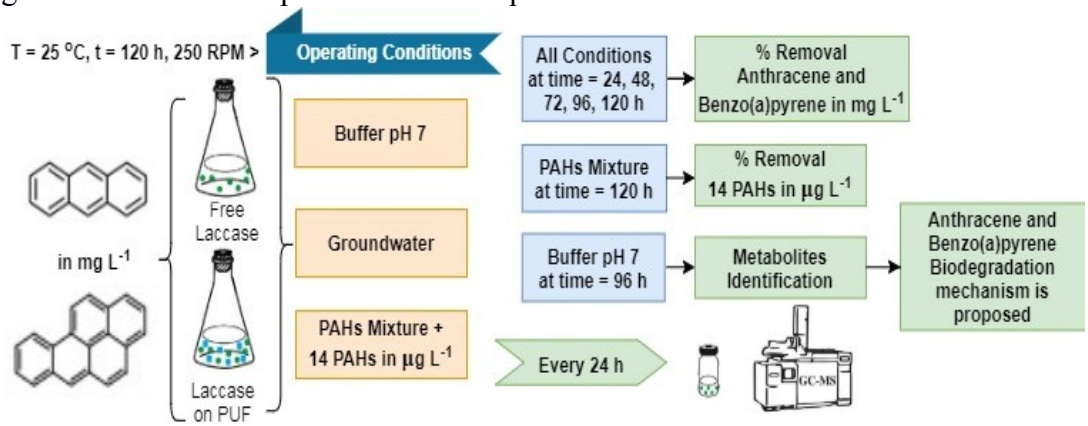
Engineering), whose quality assurance and analytical competence were evaluated by Supelco® quick turn proficiency test. The standard sample PE1173 from Sigma-Aldrich for PAHs was used on test, which is intended for water pollution/wastewater. A gas chromatograph-mass spectrometer (Agilent 5975C, Agilent Inc., Palo Alto, USA) equipped with a HP-5MS column (30 mm x 0.25 mm) was used. The oven temperature was programmed from 100 °C with a 2-min hold and a 10 °C min^{-1} increment to 300 °C with a 10-min hold (PERINI *et al.*, 2020). Sample containing PAHs (1.5 mL) was extracted three times using 1.5 mL of dichloromethane into a test-tube under agitation in a vortex mixer. After this step, the extract was evaporated at room temperature and redissolved in 1.5 mL acetone, adapted from USEPA method 8270.

The obtained ions in FULL (SCAN) mode of MS scan analysis were compared according to the similarity (*m/z*) of the data available in the National Institute of Standards and Technology library (NIST, 2009). The match factor values were used for compound identification based on qualities of the mass spectra determined by the NIST Match and Reverse Match (R. Match) factor methods (KOO; KIM; ZHANG, 2013).

4.2.4 Tests for PAHs removal

The ability of immobilized laccase to remove anthracene, and benzo(a)pyrene in mg L^{-1} concentration range was investigated at three conditions; buffer solution (pH 7), real groundwater sample and in the presence of a mixture of 14 PAH in µg L^{-1} concentration (PAHs mixture condition). The same conditions were evaluated using free laccase, as shown in Figure 10. Both PAHs in mg L^{-1} range were used as model PAH for biodegradation products of laccase-transformation identification and mechanism assessment. The removal of anthracene, and benzo(a)pyrene in mg L^{-1} and a mixture of 14 PAHs in µg L^{-1} were measured in PAH mixture condition.

Figure 10: Schematic representation of experiments.



Source: from author.

All conditions of batch removal experiments were performed in triplicate at 25 ± 2 °C in 125 mL Erlenmeyer flasks, with 20 mL reactant during five days in an orbital shaker. Surfactant Tween 80 (1% v/v) and PAHs dissolved in acetone were added to an erlenmeyer flask in amount to reach the final concentrations 12.48 mg L^{-1} of Ant and 5.00 mg L^{-1} of BaP (PERINI *et al*., 2020). While in free laccase tests 150 U L^{-1} were used , in immobilized laccase tests ten pieces (about 5x5x5 mm in size and 20 mg in weight each), making 7 U L^{-1}, were used. Initial laccase activity in tests using immobilized enzymes was lower than free laccase due to limitations on maximum supportable amounts and lower specific activity. Thus, most of the previous study conditions of the group (PERINI *et al.* 2020) were maintained, including 150 U L^{-1} in free laccase tests, however, agitation was increased to 250 rpm in all conditions due to make easy immobilized enzymes treatability.

Buffered condition was carried out in citrate-phosphate buffer (pH 7) and PAHs mixture condition with a standard solution containing other 14 PAHs dissolved in acetone, to make the final concentrations (μg L^{-1}): naphthalene (Nap) 1000, acenaphthene (Ace) 426, acenaphthylene (Acy) 72, fluorene (Flu) 84, phenanthrene (Phe) 1000, fluoranthrene (Fla) 162, pyrene (Pyr) 144, benzo(a)anthracene (BaA) 150, chrysene (Chr) 165, benzo(b)fluoranthene (BbF) 131, benzo(k)fluoranthene (BkF) 60, dibenzo(a;h)anthracene (DaA) 88, benzo(g;h;i)perylene (BgP) 72 and indeno(1,2,3-cd)pyrene (IcP) 202.

The other test condition contained an unpolluted groundwater sample collected from an abandoned gas station area and stored at 4 °C until the use. The samples were initially passed through a vacuum filtration system (25 μm) to remove the sample's suspended solids. The following physico-chemical properties were read *in-situ* by HI 9828HANNA multiparameter probe (Hanna Instruments, Woonsocket, USA): 50 ORP (oxidation-reduction potential), 197.7 μSm^{-1} (conductivity), 25.3 NTU (turbidity) 20.5 ^{o}C (temperature) and pH 6.82. The observed properties in groundwater were similar to those detected in other studies (ADENIJI; OKOH; OKOH, 2019; LI; LI; LIU, 2017).

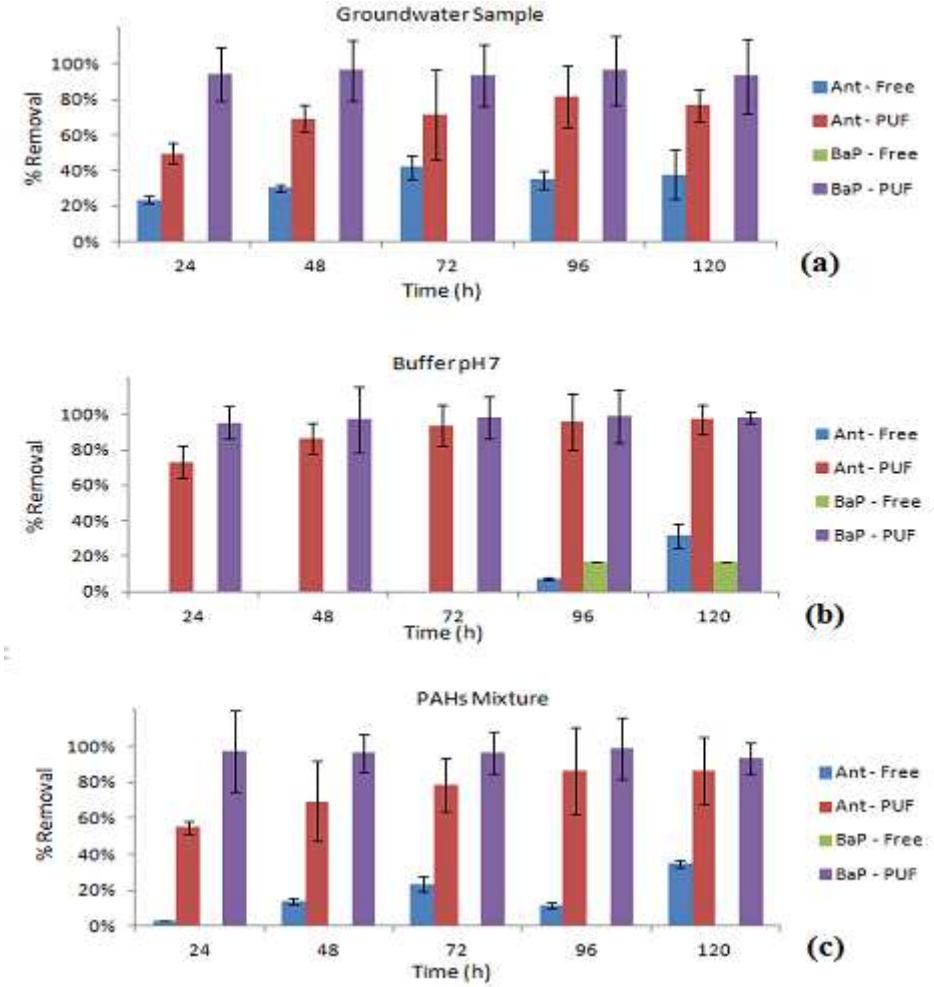
Tests for PAHs removal by free and immobilized laccase on PUF in 3 conditions (buffer pH 7, groundwater and PAHs mixture) were performed in triplicates for 120 hours, with 1.5 mL of medium samples collected every 24 h for the GC-MS analysis.

4.3 RESULTS AND DISCUSSION

4.3.1 Removal capability of Ant and BaP, in mg L^{-1} concentration range, by free and immobilized laccase on PUF

Although many studies suggest the importance of redox mediators in the oxidation of PAHs by laccases, this work pursues closer to field reality of groundwater conditions and avoid operating at high-cost conditions. Experiments were carried out in non-optimal conditions, without any mediator compound. Because of their higher capacity to be degraded by laccase, anthracene, and benzo(a)pyrene have been chosen as models PAHs for carrying out the mg L^{-1} concentration range. The removal efficiencies of PAHs reacting by free and immobilized laccase on PUF for 120 h, were determined at 24 h intervals in 3 experimental conditions: Buffer pH 7, PAHs mixture, and Groundwater (Figure 11).

Figure 11: Removal of anthracene (Ant) and benzo(a)pyrene (BaP) by free laccase and laccase immobilized on PUF in batch experiments, at reaction times 24, 48, 72, 96, 120 h, in conditions Groundwater Sample (a), Buffer pH 7 (b), PAHs Mixture (c) at T = 25 °C with 1% Tween 80 (v/v).



Source: from author.

The mediator compound's dependency was evidenced on the effects of BaP degradation by the free enzyme in buffer pH 7 condition, which may have negatively influenced. A positive percentage was detected only after 96 h, where 16.5% of BaP removal was reached; lower than obtained in our previous work, which was above 30% (PERINI *et al.*, 2020). No significant elimination of BaP without ABTS has been related to other studies (COLLINS *et al.*, 1996). In these conditions, after 120 h, the highest remaining relative laccase activity amoung other conditions were observed, almost 77% was enough to promote more BaP removal. On the other hand, an increase of Ant was observed from 7.1% removal in 96 h to almost 35% after 120 h, time that Ant removal rate by free laccase showed up close in all three conditions.

For Ant removal by free enzyme, the results showed higher removal than BaP, but it was quite lower than by immobilized laccase on PUF. Buffer pH 7 was the worst condition for Ant removal by free laccase, while 37.9% and 34.5% were obtained in groundwater and Standard PAHs conditions, respectively. Theses Ant removal percentages were lower than those reported in other studies without mediators (BAUTISTA; MORALEZ; SANZ, 2015; XU *et al.*, 2020). However, they above mentioned works operate adjusted at optimal pH conditions (range between 4 and 5) where laccase have higher activity. Under neutral pH range between 6 and 7, as in this work, anthracene degradation by free laccase present a different behavior, as the presence of several compounds in medium (groundwater sample and Standard PAHs condition) enhanced the removal, the presence of citrate and phosphate ions of buffer solution affected negatively Ant removal until 96 h.

Figure 11 shows a significant removal of both PAHs by immobilized laccase on PUF compared with free laccase for all tested conditions. The highest removal rate reached 97.1% for Ant and 99.2% for BaP by laccase on PUF in condition Buffer pH7. Close removal yields of BaP were obtained in the other two conditions, approximately 3% lower. Maximal Ant removal using laccase on PUF was 81.6% for real groundwater condition and 92.4% for simulated PAHs condition (PAHs mixture). The final 0.952 mg L^{-1} achieved Ant concentration was lower than New Zealand guide values showed in Table 2 (1.000 mg L^{-1}), and close to the Brazilian limit (0.900 mg L^{-1}).

After 120 h, almost 46% of remaining relative laccase activity was detected for laccase on PUF in buffer pH 7 condition. The presence of other compounds in both other conditions, standard PAHs and groundwater, seems to decrease both free and immobilized laccase activity. While 47% of free laccase relative activity was observed in groundwater condition, only 26% of remaining relative laccase activity were detected

for laccase on PUF in groundwater and PAHs mixture; which was the same amount apply for free laccase in PAHs mixture. However, compared with Figure 11, PAHs removal yields by free and immobilized laccase had little relation to laccase activity, although significant, as observed by Wu *et al.* (2008).

Furthermore, in the first 24 h, almost all benzo(a)pyrene was removed by immobilized laccase at all conditions. In contrast, anthracene removal was 73.1% in buffer pH 7 condition and approximately 50% in the other two, which could be associated with the ring number of the PAH. Some authors (NIU *et al.*, 2013) also observed higher removal of 5 and 6 rings PAHs, more than 95%, in comparison to 3 rings PAHs, more than 80% for Ant using immobilized laccase (Table 3), attributed to the support adsorption. The removal associated to adsorption will be investigated after biodegradation product identification as product identification of anthracene and benzo(a)pyrene biodegradation.

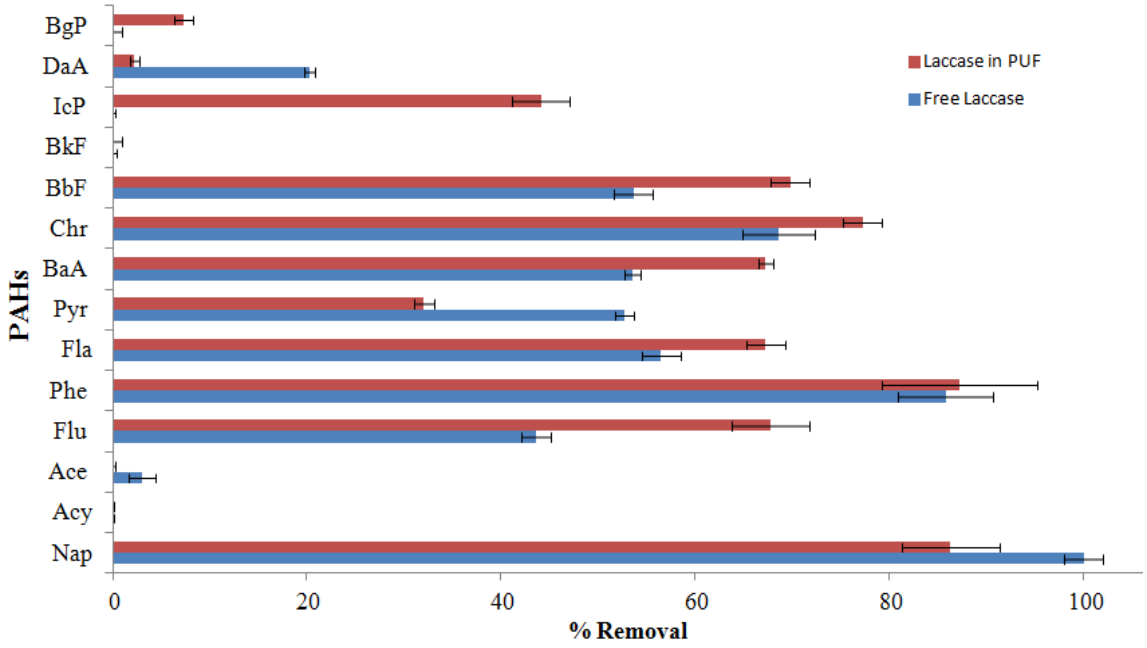
4.3.2 Removal capability of the 14 PAHs mixture, in μg L^{-1} concentration range, by free and immobilized laccase

The degradation of simulated PAHs which are listed as priority pollutants were also evaluated at condition PAHs mixture but in μg L^{-1} range. The purpose to observe their influence on the transformation of benzo(a)pyrene and anthracene, which likely occurs due to competitiveness and inhibition of the enzyme metabolism (HARITASH; KAUSHIK, 2009; RAO *et al.*, 2014). According to some authors listed in state of the art, laccase also has the capability to degrade other PAHs, besides Ant and BaP, and were included in the medium in μg L^{-1} rangeto evaluate their removal.

Guidelines for assessing and managing the contaminated site of PAHs fixed limits in μg L^{-1} for groundwater contaminants. Usually, these limits are different according to each country and policy decisions regarding tolerable levels of risk for the derivation of groundwater acceptance criteria, as shown in Table 2. In this direction, it is applicable to work in μg L^{-1} range when the objective is to envision and associate the under developing process to applications in the remediation of contaminated PAHs sites.

As shown in Figure 12, the results were positive, since 8 of 14 compounds were removed by free laccase and had the removal percentage enhanced when immobilized laccase was used. Removal of 53.6% of BaA by free laccase and 68.8% of BbF by laccase on PUF were consistent with other previous results, also operating without mediators using free laccase as well, but at pH 4.2 (XU *et al.*, 2020).

Figure 12: Percentage removal of 14 Polycyclic Aromatic Hydrocarbons in μg L^{-1} concentration range by free laccase (blue) and laccase immobilized on polyurethane foam (red) in batch experiments, after 120 h, in condition PAHs mixture, at T = 25 °C with 1% Tween 80 (v/v).



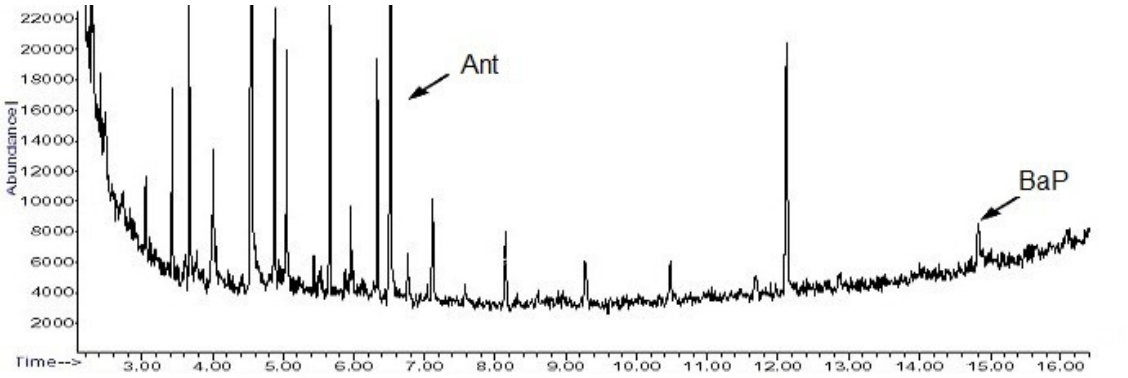
Source: from author.

In the present study, 7 of 14 EPA PAHs tested were degraded, achieving more than 50% removal, by free laccase. The two notable were naphthalene (100%) and phenanthrene (85.8%). Besides, removal of 8 of 14 PAHs evaluated in μg L^{-1} range was improved by using of laccase immobilized on PUF when compared to free laccase. Immobilized laccase removed 77.3% of chrysene up to 34 μg L^{-1}, and 32.1% of pyrene up to 98 μg L^{-1}, whose final concentration values are lower than acceptance criteria of Brazilian and New Zealand law, respectively.

4.3.3 Identification of anthracene and benzo(a)pyrene biodegradation products

The oxidation of PAHs by laccase has been reported, and the oxidation products were detected by gas chromatography-mass spectrometry (GC-MS) analysis. Our results showed that ANT and BAP removal increased in experiments with laccase immobilized in PU foam compared to free laccase, in all three evaluated conditions. The condition of free laccase in pH 7 buffer solution in time 96 h was chosen to detect what compounds were formed by degradation of both PAHs. Chromatogram was indicated in Figure 13, containing peaks of of anthracene and benzo(a)pyrene biodegradation by free enzyme.

Figure 13**:** Gas chromatogram of sample after 96 h of oxidation of anthracene and benzo(a)pyrene in condition pH 7 buffer solution (control sample), by free laccase.



Source: from author.

The biodegradation products observed after removing of anthracene and benzo(a)pyrene by free and immobilized laccase were evaluated by match quality test after GC-MS scan analysis. The mass spectra (*m/z*) of the formed products in the chromatograms were compared with the National Institute of Standards and Technology library data (NIST, 2009). After this procedure, based on Koo; Kim; Zhang, (2013) method, identified compounds with fair and poor match factors values were eliminated. Table 5 shows some parameters for 1,2 benzenedicarboxylic acid diisooctyl ester (diisooctyl phthalate) and tetradecane, which were identified as Ant and BaP biodegradation products, obtained after GC-MS scan analysis and match quality test.

Table 5: Biodegradation products retention times (tR), fragmentation using GC-MS (SCAN mode), qualities of the *m/z* determined by the Match (NIST) and Reverse Match (R. Match) and respective abundance value peaks by free and immobilized laccase on PUF.

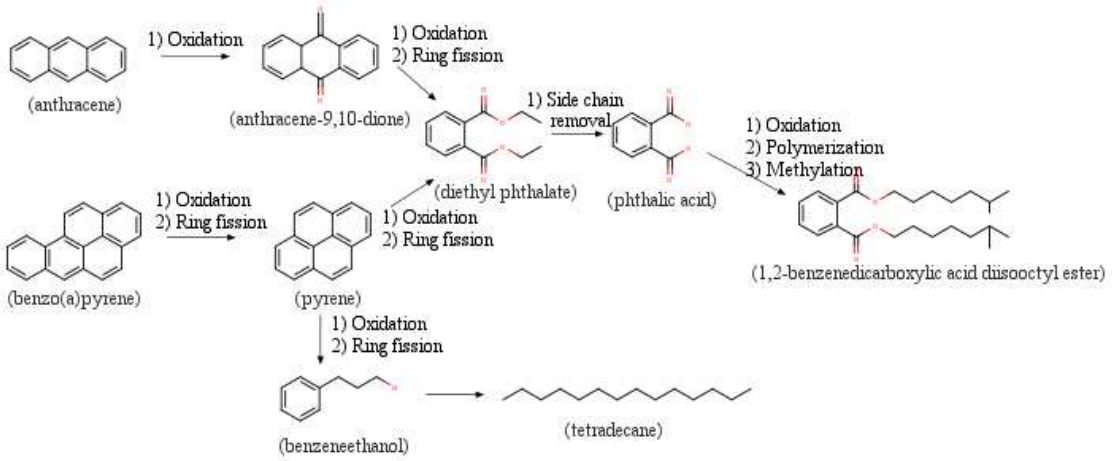
Biodegradation	t_R		Quality of *m/z*		Abundance value peak	
products	min^{-1}	Fragmentation	Match	R.Match	Laccase	Laccase on PUF
Diisooctyl phthalate	12.122	149, 167, 57	738	864	20,472	9,302
Tetradecane	4.541	57, 43, 71	836	921	85,525	719,079

Source: from author.

However, other possible pathways have also been proposed based on biodegradation products identification of PAHs by several microorganisms and enzymes. Although these specific studies did not find quinones, many of the biodegradation products identified here have previously been identified in other studies. Quin *et al.* (2018) have proposed one pathway for biodegradation of BaP, where pyrene and phenanthrene were identified as biodegradation products, whose successive loss of aromatic ring from BaP to Phe occurs similary. On the basis of identified

biodegradation products after GC–MS, the degradation pathway for anthracene and benzo(a)pyrene was proposed. This possible pathway is represented in Figure 14.

Figure 14: Based on the identified biodegradation products through gas chromatography-mass spectrometry analysis, the purpose pathway of anthracene and benzo(a)pyrene degraded by laccase immobilized on polyurethane foam.



Source: from author.

As shown in Figure 14, the laccase oxidation of anthracene occurs in 9th and 10th carbons, resulting in anthraquinone (anthracene-9,10-dione) (ARCA-RAMOS *et al.*, 2015; HU; WANG; HWANG, 2009; LI *et al.*, 2014). After the oxidation and ring fission of this biodegradation product, diethyl phthalate is formed, and subsequently, phthalate acid (SWAATHY *et al.*, 2014; TARAFDAR; SINHA; MASTRO, 2017). The laccase degradation of benzo(a)pyrene results in the oxidation and ring fission, resulting in pyrene (QUIN *et al.*, 2018). After that, the pyrene is oxidized and the ring fission occurs, forming diethyl phthalate, a common biodegradation product of Ant and BaP degraded, probably due to the similarity on molecule structure and ionization potential of anthracene and benzo(a)pyrene (COLLINS *et al.*, 1996; QUIN *et al.*, 2018). The pyrene degradation can also be followed by the formation of benzene ethanol, that is also oxidized by laccase and may lead to ring fission, polymerization, and methylation, forming aliphatic compounds, pathway similar to benzo(a)anthracene's biodegradation (ABO-STATE; SALEH; PARTILA, 2013; PARTILA; MOHAMED, 2019). Once laccase oxidation of compounds starts by forming a radical, a wide variety of compounds may be formed (DARONCH *et al.*, 2020; ZENG; HONG; WAVREK, 2000).

Once the pathway was proposed, the abudance peaks of biodegradation products were considered (Table 5) in condition Buffer pH 7 by free laccase, where 16,55% of BaP was degraded. Thus, for confirm of the occurrence of BaP (98.7%) biodegradation by immobilized laccase on PUF under the same condition. While abundances of approximately 719,079 for tetradecane and 9,302 for diisooctyl phthalate were detected in samples treated with immobilized laccase, for free laccase peaks of 85,525 and 20,472 (Table 5), respectively, were detected for those compounds. This result is a positive indicator for the occurrence of BaP degradation by immobilized laccase, and also Ant, according to the proposed pathway. At this pH range between 6 and 7, free laccase is known for having lower PAH's oxidation yield, specifically for BaP (NIU *et al.* 2013; LI *et al.* 2014). However, these factors were not observed for BaP biodegradation through immobilized laccase, not even in the absence of mediators and presence of other substances (biodegradation products), that could compete or have priority for laccase activities.

Besides biodegradation another effect could also influence the BaP and Ant removal rates by laccase on PUF. Niu *et al.* (2013) noted an improvement in PAHs removal when they used encapsulated laccase in loading spider type reactor fabricated by poly(d,l-lactide-co-glycolide). Their results showed that higher rates were achieved by PAHs degradation combined with the adsorption onto the surface of the fibers. The same effect linked to adsorption influence was observed in the removal of nonylphenol polyethoxylates by immobilized laccase on PUF, in continuous flow circular bioreactor, due to the hydrophobic character of PUF (STENHOLM *et al.*, 2020). In addition to biodegradation, this adsorption phenomenon probably influenced PAHs removal by immobilized laccase in our experiments but in a lighter way. In this sense, higher amount of identified biodegradation product of BaP (tetradecane) was formed by immobilized than free laccase, based on abundance peaks. Considering that in batch experiments with vigorous agitation, PAHs biodegradation was observed, based on our biodegradation products' analysis by free and immobilized laccase in non optimal pH range without expensive mediator ABTS (> \$ 65 g^{-1} – Sigma-Aldrich). Ant, Pyr and Chr were achieved safe levels for groundwater protection in this work, in practical terms of contaminated area management, for those two PAHs specifically on remediation level, could be considered achieved the cleanup goal, the remediation may be stopped and could initiate the monitoring level until rehabilitee. The challenge for feasible this *ex-situ* bioremediation process in the future on-field applications is to evaluate if using commercial laccases (≈ \$ 2 kg^{-1} – Alibaba); the obtained results will be

maintained. The expectation is that process cost can be reduced, eliminating high-cost mediators, chemicals, and enzymes, upon using low-cost support for enzyme immobilization. Indeed, the first step has been taken in this direction.

4.4 CONCLUSIONS

For any PAHs contaminated site cleanup, costs have played an essential role in selecting site remediation alternatives. However, PAHs treatment by laccase evaluated in controlled conditions with high-cost reagents (ABTS and pure enzymes) are in the reverse path. Combination of bioprocess with commercial materials could reduce significantly remediation cost. However, efficiency must be tested and approved, before application in the field. Laccase immobilized on commercial polyurethane foam was evaluated under non optimal conditions without mediator to remove PAHs and its potential for practical bioremediation application. Shortly reviewing state of the art and environmental policies led us to realize that PAHs' scale concentration is an essential factor and must be considered. In comparison with free laccase, was achieved high removal of anthracene and benzo(a)pyrene in mg L^{-1} range by immobilized laccase on PUF and a brief increase of others 8 PAHs removal in μg L^{-1} range. The prospective biodegradation mechanism was proposed based on the match quality analysis of formed diisooctyl phthalate and tetradecane. Comparing of biodegradation products abundance peaks with percentage Ant and Bap removal allowed concluding that more degradation happened. In contrast to protection and cleanup policies, anthracene in mg L^{-1} range, chrysene and pyrene in μg L^{-1} range were reduced in the medium under safe concentration, lower than the intervention values of protection policies. It was positive in the continuous development of a viable technique closer to field application so that a new approach *ex-situ* bioremediation was brought based on pump-and-treat and biotransformation. The cost has already reduced, eliminating high-cost mediators and other chemicals for controlled conditions, using low-cost support for enzyme immobilization. Finally, this process's last challenge is to evaluate if using commercial laccases (E.g. ≈ $ 2 kg^{-1} laccase powder from Alibaba supplier); the obtained results will be maintained being able for field application.

5 CONCLUSIONS

Free and immobilized laccases on PUF were evaluated to biodegrade PAHs under non-optimal without the expensive usual mediator ABTS (> \$ 60 g^{-1} – Sigma-Aldrich). Removal yields of anthracene, benzo(a)pyrene tested in mg L^{-1}, and other 8 PAHs in μg L^{-1} were enhanced by immobilized laccase. The use of free and immobilized laccase allowed to final concentration of anthracene under legal limits of protection policies, which were also achieved for pyrene and chrysene by laccase on PUF. This enzymatic treatment was developed using low-cost support for enzyme immobilization, eliminating high-cost mediators and chemicals for controlled conditions. For field application, the last challenge is to replace with commercial laccases (E.g. ≈ \$ 2 kg^{-1} laccase powder from Alibaba supplier) obtaining similar degradation results, consecrating the proposed cost-reduction bias. Thus, there is potential in two strategies for bioremediation of polycyclic aromatic hydrocarbons by laccases: (I) application of the free laccase, directly, in groundwater to bioremediate polycyclic aromatic hydrocarbons *in-situ*, and (II) using laccase immobilized on PUF in bioreactors by *ex-situ* treatment.

5.1 FEASIBLE PERSPECTIVES ON BIOREMEDIATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY FREE AND IMMOBILIZED LACCASES

The use of laccase for PAHs biodegradation concentration has been studied in the presence of expensive mediators such as ABTS, on controlled pH and temperature conditions, for bioremediation purposes. The significant challenge for make still study bioremediation processes able in the future for on-field application is to consider some issues associated with practical aspects, process cost, and attendance to environmental protection policies. Therefore, these are exactly the considerations care adopted in this study that proposed two potential bioremediation strategies of polycyclic aromatic hydrocarbons by laccases.

It was evaluated in chapter 3 potential of free laccase for PAHs biodegradation in a homogeneous biocatalytic process, called surfactant enhanced *in-situ* enzymatic oxidation for bioremediation. In this strategy, the soil is flushed by a surfactant solution injection, carrying PAHs in micelles to groundwater, where their oxidation occurs by directly laccase solution injection.

The chapter 4 strategy was based on the use of immobilized laccase on commercial polyurethane foams for PAHs biodegradation in a heterogeneous biocatalytic process. Once the PAHs in micelles reached groundwater after soil flushing, they are pumped and treated in an external reactor (*ex-situ*) in this strategy. Thus, this process advantage is reuse of laccase on PUF until it has enzymatic activity for PAHs degradation, considering the medium separation ease.

Considering protection and cleanup policies, for some evaluated PAHs safe levels in groundwater were achieved. The obtained removal yields and final PAHs concentration in both treatments were promising for practical bioremediation application.

5.2 SUGGESTIONS FOR FUTURE WORKS

- Evaluate potential of a low-cost enzyme for polycyclic aromatic hydrocarbons biodegradation by free and immobilized laccases.
- Determine the reuse capacity of immobilized laccase on PUF for polycyclic aromatic hydrocarbons biodegradation.
- Perform batch tests by column study for polycyclic aromatic hydrocarbons biodegradation by free laccase, using groundwater and soil samples from a real contaminated site.
- Determine the adsorbent capability of laccase immobilized on polyurethane foam and of polyurethane foam waste.
- Apply the free laccase and surfactant solution in the oxidation of polycyclic aromatic hydrocarbons in a real contaminated site, based on the surfactant-enhanced enzymatic oxidation technique.
- Apply the laccase immobilized on polyurethane foam in a stirred tank bioreactor for degradation of polycyclic aromatic hydrocarbons from a real contaminated site, through prior pumping.

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