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Polyurethane Foam as Matrix for One-Step Laccase Immobilization

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

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RESUMO

Lacases são biocatalisadores importantes devido à sua capacidade de oxidar moléculas fenólicas complexas utilizando oxigênio molecular e sem a necessidade de peróxido de hidrogênio. Sua aplicação abrange a clarificação de sucos e vinhos, o tratamento de resíduos com a degradação de corantes, o tratamento da água com a remoção de disruptores endócrinos e micropoluentes, assim como várias outras. Para melhorar suas propriedades e facilitar sua utilização, a imobilização da lacase em suportes vem sendo feita. Neste trabalho, a lacase de Trametes versicolor foi imobilizada com sucesso in situ em espumas rígidas de poliuretano comercial e oriunda de óleo vegetal (Lac-PUF e Lac-bioPUF). A concentração enzimática no processo de imobilização foi investigada. Os estudos para a seleção da concentração de lacase nos suportes mostraram que a melhor atividade foi obtida com 0.2% em peso para as duas espumas. Ensaios de degradação usando o ácido 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic) (ABTS) como substrato foram realizados em diferentes temperaturas. Através do plot de Arrhenius, a energia de ativação foi calculada. Após a imobilização, a energia de ativação foi reduzida de 15.68 kJ•mol-1 da enzima livre para 9.97 kJ•mol-1 e 10.94 kJ•mol-1 para Lac-PUF e Lac-bioPUF, respectivamente. Comparada à enzima livre, a lacase imobilizada apresentou maior valor de K_m e maior valor de V_{max}, o que indica que após o procedimento de imobilização, a lacase diminuiu a afinidade para com o substrato, porém apresentou aumento da atividade catalítica. Esse aumento pode estar relacionado ao caráter hidrofóbico das espumas. Os estudos de armazenamento mostraram que a Lac-PUF e Lac-bioPUF podem ser utilizadas durante 30 dias, quando armazenadas à temperatura ambiente, com atividade retida de 35% e 25% e, quando avaliada a estabilidade dos derivados enzimáticos acondicionados sob refrigeração (4 °C), as atividades aferidas foram de 53% e 25% em relação a sua atividade inicial, respectivamente. A lacase imobilizada reteve cerca de 60% da atividade inicial após dois ciclos de oxidação do ABTS. O procedimento de imobilização da lacase permitiu a fácil separação da enzima da mistura de reação, bem como a sua reutilização. Os resultados obtidos possibilitam a imobilização e empacotamento in situ do biocatalisador em um reator tubular, proporcionando seu uso em diversas aplicações de interesse industrial.

Palavras-chave: Lacase. Imobilização. Espuma de poliuretano. Materiais de base biológica. Biotransformação.

RESUMO EXPANDIDO

Introdução

Não é atual o fato de que enzimas vêm sendo utilizadas para substituir catalisadores químicos convencionais. As enzimas apresentam um alto grau de especificidade, seletividade e são capazes de biocatalisar reações em condições mais brandas de pH e temperatura do que os catalisadores químicos. A lacase (EC 1.10.3.2) é uma enzima extracelular da classe das oxirredutases que contém quatro cobres em seu sítio catalítico. Essas enzimas são capazes de catalisar a oxidação de compostos fenólicos, como mono-, di- e polifenóis, aminofenóis, metoxifenóis e aminas aromáticas, com a redução de quatro elétrons do oxigênio formando água, sem o uso de peróxido de hidrogênio. Além disso, na presença de um mediador que aumenta seu potencial redox, possui também a capacidade de catalisar a oxidação de compostos não fenólicos.

Esta enzima tem sido investigada em muitas pesquisas devido a sua versátil aplicação. São usadas para síntese de corantes, na síntese de compostos farmacêuticos, na clarificação de vinhos e sucos assim como no tratamento de efluentes, para degradar compostos recalcitrantes que o tratamento convencional não é capaz. No entanto, o uso de enzimas como as lacases em seu estado livre se torna limitado pois são sujeitas à inativação por diversos fatores químicos, físicos e biológicos durante a aplicação catalítica ou durante seu armazenamento. Como uma alternativa para melhorar a estabilidade operacional das enzimas, as mesmas vêm sendo imobilizadas em suportes sólidos, que, além de melhorar as propriedades da enzima também possibilitam o reuso das mesmas em sucessivos ciclos catalíticos.

Diversos tipos de suporte vêm sendo desenvolvidos com este propósito. Estes suportes podem ser sintetizados a partir de vários materiais: orgânicos (naturais ou sintéticos), inorgânicos, assim como híbridos e compósitos. Dentre eles estão os polímeros sintéticos, versáteis materiais que podem ser sintetizados de inúmeras formas e que possuem grupos funcionais em suas superfícies, o que facilita a ligação da enzima com o suporte. Um polímero que tem sido utilizado com sucesso para processos de imobilização são as espumas de poliuretano (PUF). Estas espumas são resistentes a solventes e também são biocompatíveis. São obtidas reagindo um diol com um diisocianato.

Considerando isso, o presente trabalho teve como objetivo imobilizar a enzima lacase em espumas rígidas de poliuretano, uma comercial e uma obtida a partir de óleo vegetal, usando um biopoliol obtido a partir da glicerólise enzimática do óleo de mamona. Além disso, as condições ótimas de pH e temperatura, estabilidade de armazenamento e reutilização foram investigadas usando 2,2-azinobis-3-etilbenzotiazolina-6-sulfonato (ABTS) como substrato. Também foi realizado um estudo cinético para ambos os derivados enzimáticos obtidos.

Objetivos

Objetivo geral

Realizar a imobilização *in situ* da enzima lacase em espumas rígidas de poliuretano comercial e obtida a partir da glicerólise enzimática de óleo vegetal.

Objetivos específicos

- Avaliar a atividade enzimática da lacase livre e imobilizada, diante de diferentes

parâmetros físicos e químicos;

- Caracterizar os suportes desenvolvidos por morfologia (SEM) e superfície (FTIR);
- Determinar os parâmetros cinéticos (K_m e V_{max}), a energia de ativação (Ea) e a entalpia de ativação (ΔH *) em relação ao substrato;
- Avaliar a reutilização do biocatalisador após imobilização em espumas de poliuretano.

Metodologia

Para a obtenção do biopoliol usado na síntese da espuma de poliuretano obtida a partir de óleo vegetal, foi realizada a glicerólise enzimática do óleo de mamona. A reação foi conduzida em reator de vidro encamisado, com agitação mecânica a 2500 rpm, 70 °C por 3 h, utilizado lipase (Novozym 435) como biocatalisador. Depois do término da reação, a lipase foi separada por filtração a vácuo. Posteriormente, o produto obtido (biopoliol) foi separado em um funil de separação.

Lacase de *Trametes versicolor* foi imobilizada por aprisionamento através da polimerização em massa em reação de uma etapa durante a síntese das espumas de poliuretano. A concentração de enzima foi variada de 0.1 a 0.3 wt% em relação à massa total de monômeros. Em ambas as imobilizações, a lacase liofilizada foi adicionada ao poliol e posteriormente foi adicionado o diisocianato. A imobilização da lacase em espuma comercial (Lac-PUF) foi realizada utilizando diisocianato e poliol comercial, em uma proporção 77 g de NCO por 100 g de OH. Os reagentes foram agitados mecanicamente (2500 rpm) por 60 segundos. A imobilização da lacase em poliuretano feito a partir de óleo vegetal (Lac-bioPUF) ocorreu utilizando o biopoliol sintetizado, 1 wt% de água como agente expansor e diisocianato, em uma proporção 1:1 NCO:OH. Os reagentes foram agitados mecanicamente (2500 rpm) por aproximadamente 90 segundos.

Os suportes com e sem enzima foram caracterizados por espectroscopia no infravermelho com transformada de Fourier (FTIR) e por microscopia eletrônica de varredura (MEV). Para avaliar a eficiência do processo de imobilização, foram realizadas análises da atividade enzimática da lacase frente à diferentes condições químicas e físicas. A medida de atividade foi realizada avaliando a oxidação do 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS). A influência da temperatura na atividade da enzima livre e imobilizada foi investigada em 20, 30, 40 e 50 °C, utilizando tampão pH 4 citrato-fosfato 0.1 M. A energia de ativação (Ea) e a entalpia de ativação (ΔH*) foram calculadas. O efeito do pH foi avaliado a 40 °C para tampão citrato-fosfato (0.1M) em pH 3, 4, 5 e 6.

Os parâmetros cinéticos de Michaelis-Menten, a constante de afinidade enzima-substrato (K_m) e a velocidade máxima catalítica (V_{max}) foram calculados a partir dos resultados de atividade enzimática para diferentes concentrações de substrato (1.25 até 10 mM de ABTS em solução aquosa). A estabilidade operacional foi avaliada através de sucessivos ciclos de oxidação do ABTS. A estabilidade ao armazenamento da enzima imobilizada foi investigada medindo a atividade da lacase imobilizada armazenada em temperatura ambiente e em geladeira (4 °C) ao longo de 30 dias.

Resultados e Discussão

A partir dos dados obtidos para o processo de imobilização, a utilização de lacase a uma concentração de 0.2 wt% apresentou resultados promissores. Quando a concentração de lacase foi 0.3 wt% uma diminuição na atividade enzimática foi observada. Com os resultados

para a caracterização dos suportes pôde-se observar que a enzima foi imobilizada em ambas as espumas de poliuretano e sem modificar a estrutura das células poliméricas.

Depois de imobilizada, a enzima apresentou diferentes atividades frente às temperaturas analisadas. A lacase livre e imobilizada em espuma de poliuretano comercial (Lac-PUF) apresentaram maior atividade a 50 °C enquanto a lacase imobilizada em espuma de poliuretano obtida a partir de óleo vegetal (Lac-bioPUF) apresentou maior atividade a 40 °C. Os valores calculados para *Ea* e ΔH^* foram menores para a enzima imobilizada (Lac-PUF e Lac-bioPUF) que para a lacase livre, indicando que o processo de imobilização diminuiu a energia necessária para ativar o complexo enzima-substrato. Frente aos diferentes pHs testados, os resultados obtidos indicaram que a imobilização em ambos os suportes deslocou o pH ótimo da enzima para pH 4, sendo que ela livre apresentou maior atividade em pH 3.

Os parâmetros cinéticos de Michaelis-Menten foram calculados a partir da linearização de Lineweaver-Burk. Os valores de K_m da Lac-PUF (5 mM) aumentaram 1.8 vezes e 11.2 vezes para a Lac-bioPUF (30 mM) comparado com a enzima livre (2.67 mM), indicando uma diminuição na afinidade da lacase com o substrato usado após o processo de imobilização. No entanto, os valores obtidos para V_{max} da Lac-PUF e Lac-bioPUF aumentaram depois da imobilização. Estes resultados indicam que o procedimento de imobilização causou modificações opostas na afinidade (K_m aumentou, afinidade diminuiu) e na máxima capacidade catalítica (aumentou), o que influencia o tempo da biocatálise.

No final de 30 dias de armazenamento em temperatura ambiente, a lacase imobilizada, Lac-PUF e Lac-bioPUF, reteve 35 e 25% da atividade inicial, respectivamente. Já quando armazenada a 4 °C, 53 e 25% de atividade residual foi observado para Lac-PUF e Lac-bioPUF, respectivamente. A determinação da estabilidade operacional do biocatalisador é importante para determinar a força de ligação entre a enzima e o suporte. Quando a capacidade de reuso da enzima imobilizada foi avaliado, observou-se que depois de dois ciclos catalíticos, ambas as espumas utilizadas para imobilização permitiram que a lacase mantivesse aproximadamente 60% da atividade apresentada no primeiro ciclo. Não obstante, os biocatalisadores puderam ser facilmente separados do meio reacional.

Considerações Finais

- A lacase de *Trametes versicolor* foi imobilizada *in* situ com sucesso utilizando espumas rígidas de poliuretano comercial e obtida a partir de óleo vegetal como suportes.
- A partir dos dados apresentados, pode-se concluir que o melhor suporte para imobilização de lacase foi a espuma de poliuretano comercial.
- Em geral, ambas as espumas de poliuretano demonstraram melhorar as propriedades enzimáticas e possibilitaram sua reutilização.
- Este estudo sugeriu que esses biocatalisadores têm grande potencial para serem projetados para um reator de leito empacotado e, em seguida, serem usados em sucessivas reações catalíticas de biodegradação.

Palavras-chave: Lacase. Imobilização. Espuma de poliuretano. Materiais de base biológica. Biotransformação.

ABSTRACT

Laccases are important biocatalysts due to their ability to oxidize complex phenolic molecules using molecular oxygen and without the need for hydrogen peroxide. Its application includes the clarification of juices and wines, the treatment of residues with the degradation of dyes, the treatment of water with the removal of endocrine disruptors, and micropollutants, as well as several others. To improve its properties and facilitate its use, the laccase immobilization on supports has been done. In this work, the *Trametes versicolor* laccase was successfully immobilized *in situ* in rigid foams of commercial polyurethane and derived from vegetable oil (Lac-PUF and Lac-bioPUF). The enzymatic concentration in the immobilization process was investigated. Studies to select the laccase concentration in the supports showed that the best activity was obtained with 0.2% by weight for both foams. Degradation tests using 2,2'-azino-bis- (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate were carried out at different temperatures. Through the Arrhenius plot, the activation energy was calculated. After immobilization, the activation energy was reduced from 15.68 $kJ \cdot mol^{-1}$ of the free enzyme to 9.97 $kJ \cdot mol^{-1}$ and 10.94 $kJ \cdot mol^{-1}$ for Lac-PUF and Lac-bioPUF, respectively. Compared to the free enzyme, the immobilized laccase showed a higher K_m value and a higher V_{max} value, which indicates that after the immobilization procedure, the laccase decreased the affinity for the substrate, but showed an increase in catalytic activity. This increase may be related to the hydrophobic character of the foams. The storage studies showed that Lac-PUF and Lac-bioPUF could be used for 30 days, when stored at room temperature, with the retained activity of 35% and 25%, and, when evaluated the stability of enzymatic derivatives packaged under refrigeration (4 °C), the measured activities were 53% and 25% concerning their initial activity, respectively. The immobilized laccase retained approximately 60% of the initial activity after two cycles of ABTS oxidation. The laccase immobilization procedure allowed for easy separation of the enzyme from the reaction mixture, as well as its reuse. The results obtained allow the immobilization and packaging in situ of the biocatalyst in a tubular reactor, providing its use in several applications of industrial interest.

Keywords: Laccase. Immobilization. Polyurethane foam. Bio-based materials.

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LIST OF ABBREVIATIONS AND SYMBOLS

Δabs	Absorbance variation
ε	Molar extinction coeficiente
ΔH^*	Enthalpy of activation
AA	Amino acids
ABTS	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
APTES	3-aminopropyl(triethoxy)silane
ACE	Acetaminophen
bioPUF	Bio-based polyurethane foam
BPA	Bisphenol A
BPF	Bisphenol F
BPS	Bisphenol S
BSA	Bovine serum albumin
CLEA	Cross-linked enzyme aggregate
CLEC	Cross-linked enzyme Crystal
CPC	Controlled porosity carrier
CS	Chitosan
DFC	Diclofenac
DMHBA	3,4-dimethoxy-5-hydroxybenzoic acid
ED	Endocrine disruptores
Ea	Activation energy
FTIR	Fourier transform infrared spectroscopy
GA	Glutaraldehyde
HBT	1-hydroxybenzotriazole
HMD	Hexamethylenediamine
HNTs	Halloysite nanotubes
IPDI	Isophorone diisocyanate
Lac-PUF	Laccase immobilized in commercial polyurethane foam
Lac-bioPUF	Laccase immobilized in bio-based polyurethane foam
MANAE	Monoaminoethyl-N-aminoethyl
NP	Nonylphenol

OP	Octylphenol
PAN	Polyacrylonitrile
PHA	polyhydroxyalkanoates
PU	Polyurea
PUF	Polyurethane foam
PUs	Polyurethanes
R	Gas constant
SA	3,5-dimethoxy-4-hydroxybenzaldehyde
SEM	Scanning electron microscopy'
SMZ	Sulfamethoxazole
STZ	Sulfathiazole
E^0	Redox potential
2,4-DNP	2,4-dinitrophenol

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CONCEPTUAL DIAGRAM

"POLYURETHANE FOAM AS MATRIX FOR ONE-STEP LACCASE IMMOBILIZATION"

What?
Laccase *in situ* immobilization into rigid commercial and bio-based polyurethane foams by entrapment method.
Why?
• There is a growing interest in the use of the enzyme laccase for degradation of complexes phenolic molecules, aiming to replace chemical catalysts; • Laccase immobilization has been done for further improvement of enzyme stability, reuse and to facilitate the biocatalyst separation after application; • Supports for laccase immobilization have been investigated, concerning materials properties and costs; • Polyurethane foams have been used for successfully immobilize some enzymes and had enhanced its properties since it is a rigid porous polymeric matrix with C=O groups on the surface, that enables strong enzyme-support binding.
Hypotheses
• Does the laccase immobilization into polyurethane foams by *in situ* synthesis occur successfully? • Is the support capable of attaching a large amount of laccase? • Does the immobilization procedure improve laccase stability by enabling the maintenance of the catalytic activity under not mild reaction conditions? • Can the final heterogeneous biocatalyst be easily separated from the reaction medium and be applied in several consecutive catalytic cycles?

CHAPTER 1

In this chapter, we have a brief introduction to the research developed and its general and specific objectives.

1 INTRODUCTION

The commercial use of a large number of enzymes in multiple industrial areas has increased in the past decades due to advancements in modern biotechnology. Notwithstanding, its industrial application can be limited, since enzymes are sensitive and unstable, present low operational stability, difficult separation from the reaction medium and the infeasibility of its reuse, features that are not ideal for a catalyst and undesirable in most synthesis (HANEFELD; GARDOSSI; MAGNER, 2009). An efficient strategy to minimize these problems is the enzyme immobilization, a technique that solves the protein solubility problem, improves the control of the reaction, avoids product contamination by the enzyme and permits the use of different reactor configurations (GARCIA-GALAN *et al.*, 2011). Enzyme immobilization consists of the cross-linking of the enzymes forming aggregates, or the attachment of enzyme molecules into or onto a solid matrix by physical adsorption, covalent binding, or entrapment methods (SHELDON, 2011; SHELDON; VAN PELT, 2013).

To successfully immobilize an enzyme, the supports must offer high stability, availability, be a low-cost material, and have high affinity to the bound enzyme. Materials of different origins can be used with this purpose and can be divided into organic, inorganic, and hybrids or composites. The affinity between the functional groups of the support and the binding enzyme may allow the formation of an effective attachment of the enzyme in the matrix (ZDARTA *et al.*, 2018a). Polymeric materials present many functional groups in their structure involving linkages of any reactive component on the enzyme and provide very strong bindings in immobilized derivative (DATTA; CHRISTENA, 2013).

An important class of polymers is the polyurethanes (PUs). This polymer consists of a chain of organic units connected by urethane links. These materials are synthesized by reacting a monomer containing at least two alcohol groups (polyol) and a monomer containing at least two isocyanate functional groups (NCO) in a polyaddition reaction (BERNARDINI *et al.*, 2015; ROZMAN *et al.*, 2003). Polyurethanes can be synthesized by using a wide range of vegetable oils as renewable sources to replace the petrochemical polyols partially or totally, such as sunflower, palm, rapeseed, soybean and castor oils (BERNARDINI *et al.*, 2015; NARINE *et al.*, 2007).

Enzymes such as lipase, pectinase, invertase, and inulinase have been immobilized in polyurethanes made with petrochemical and renewable sources, and the final biocatalyst,

proved to be stable against more extreme conditions of pH and temperatures, as well as during storage and, also, made it possible to reuse the biocatalyst in successive catalytic cycles (BRESOLIN *et al.*, 2019; BUSTAMANTE-VARGAS *et al.*, 2015; CADENA *et al.*, 2010, 2011; NYARI *et al.*, 2016; SILVA *et al.*, 2013).

An enzyme that deserves attention is laccase, mainly due to its low substrate specificity they carry-out the one-electron oxidation of monophenols, diphenols, and polyphenols as well as di-amines, aromatic amines, and many others, with simultaneous reduction of oxygen to water without the need for hydrogen peroxide (ZDARTA; MEYER; PINELO, 2018). Laccases have been applied for oxidizing phenolic compounds, in organic synthesis, to environmental pollutant treatment as decolorization and emerging pollutants removal (BAYRAMOGLU; KARAGOZ; ARICA, 2018; BRUGNARI *et al.*, 2018; GARCÍA-MORALES *et al.*, 2018; LONAPPAN *et al.*, 2018; NAGHDI *et al.*, 2018; PEREIRA *et al.*, 2020; REDA; HASSAN; EL-MOGHAZY, 2018; SALAMI *et al.*, 2018; SU *et al.*, 2018; TAHERAN *et al.*, 2017; WELLINGTON; GOVINDJEE; STEENKAMP, 2018; ZHANG *et al.*, 2017a). Also, this versatile enzyme is used in clarification in food industry, for anti-proliferative activity on cancer cells and in the manufacture of biosensors for phenolic compounds detection (KIM *et al.*, 2018; MEI *et al.*, 2015; MILENA *et al.*, 2015; MOHTAR *et al.*, 2019; NAZARI; KASHANIAN; RAFIPOUR, 2015).

Laccase has been successfully immobilized in polymeric matrices: polyacrylonitrile (PAN) beads (CATAPANE *et al.*, 2013); poly(glycidyl methacrylate) brushes in microspheres of polyestyrene-divinyl benzene (BAYRAMOGLU; KARAGOZ; ARICA, 2018); polyurea microspheres (JIANG *et al.*, 2017); nylon 6 film and nylon fibers 6 (FATARELLA *et al.*, 2014). However, there are considerable polymeric matrixes for being tested to produce a quality immobilized derivative of laccase for industrial processes.

Considering the exposed above, the goal of this work was to study the one-step immobilization of a commercial *Trametes versicolor* laccase using rigid foams of commercial and bio-based polyurethanes as matrices.

1.1 OBJECTIVES

1.1.1 General Objective

Evaluate laccase immobilization by one-step reaction into commercial and bio-based polyurethane foams by entrapment method.

1.1.2 Specific Objectives

- Evaluate the enzymatic activity of free and immobilized laccase, in front of different physical and chemical parameters;
- Characterize the developed matrix by morphology (SEM) and surface (FTIR);
- Determine the kinetics parameters (K_m and V_{max}), the activation energy (Ea) and enthalpy of activation (ΔH^*) relative to the substrate;
- Evaluate the reuse of the biocatalyst after immobilization on PU foams.

CHAPTER 2

In this chapter, a literature review about the oxidoreductase enzyme laccase and the supports that have been recently used for its immobilization is presented. This review chapter was submitted to the Chemical Engineering Journal in December 2019, and it is under review.

2 ELUCIDATING THE CHOICE FOR A PRECISE MATRIX FOR LACCASE IMMOBILIZATION: A REVIEW

ABSTRACT

The use of enzymes as biocatalysts has grown widely. Laccase, a versatile oxidoreductase enzyme, has been used as a catalyst in a multidisciplinary field. This enzyme is used for clarification in the food industry, in the chemical industry for organic synthesis of synthetic pharmaceuticals and dyes, in the treatment of pollutants in the textile industry for dyes discoloration and endocrine disruptor removal. It is also used in the manufacture of biosensors for phenol detection among other applications. In general, laccases are capable of oxidizing phenolic compounds and, using low molecular weight compounds as a mediator to increase their redox potential, they are capable of oxidizing non-phenolic compounds as well. To improve the enzyme properties and to enable its reuse, the immobilization of the laccase onto support has been done. Various types of supports can be used: inorganic, organic (natural or synthetic), and also hybrids. Unlike lipase, which is effectively immobilized and already commercialized, laccase has at its catalytic site copper atoms that make it difficult to stabilize under the operating conditions of the final biocatalyst. There is a need to find a suitable way to immobilize this enzyme and then enable the commercialization of a stable biocatalyst, resistant to process conditions, and with a long shelf life. This review summarizes the most recently used support materials in academic studies. Moreover, the properties of the final biocatalyst and its application are discussed. This work intends to provide the foundation to facilitate the choice of support for laccase immobilization in future works.

Keywords: Laccase, enzyme, immobilization, support, matrix, carrier

2.1 INTRODUCTION

Biocatalysts gained special attention in recent years due to their specific properties along with the concern towards sustainable environmental technology. Enzymes are versatile biocatalysts and great substitutes for traditional chemicals used as catalysts due to they exhibit great chemo-, stereo-, and regio-selectivity under mild conditions (BILAL; IQBAL, 2019). However, several problems related to its instability under operational conditions as rapid loss

of activity and difficulties in enzyme and product recovery have limited its industrial applications (DESKA; KONCZAK, 2019). To improve their catalytic properties under process conditions increasing its stability in larger pHs and temperature ranges and allowing the enzyme recovery for further reuse, enzyme immobilization onto support has been done. The use of pre-existing solids (matrix) to immobilize proteins is perhaps the most widespread strategy of enzyme immobilization (GARCIA-GALAN *et al.*, 2011). Laccases, copper-containing oxidoreductases, are biocatalysts used in food, textile, the paper industry as well as for bioremediation processes (DESKA; KONCZAK, 2019; LEGERSKÁ; CHMELOVÁ; ONDREJOVI, 2018; MILENA *et al.*, 2015; ZHU *et al.*, 2018). Lipases are successfully immobilized and there are commercial enzymes, however, the same does not occur with laccases due to the presence of copper in their catalytic sites, a fact that hinders the improvement of stability. The development of solid support and an appropriate method to immobilize laccase that would improve laccase stability as well as facilitate its reuse in the application of interest is of utmost importance. This review intends to describe the last trends in laccase immobilization using solid supports and has the purpose to facilitate the choice of the material used for laccase immobilization, based on what has been recently done.

2.2 LACCASE

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are extracellular multicopper enzymes (BALDRIAN, 2006) that belong to a small group of enzymes named blue copper proteins or the blue copper oxidases, which are characterized by containing 4 catalytic copper atoms in their site (COPETE *et al.*, 2015). Laccases can carry out one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water, without the use of hydrogen peroxide. Moreover, they can oxidize non-phenolic compounds in the presence of electron transfer mediator molecules that enable them to overcome their lower redox potential (450–800 mV) towards these compounds (BA *et al.*, 2013; JULIO POLAINA AND ANDREW P. MACCABE, 2007; KUNAMNENI *et al.*, 2007). These multicopper enzymes were first described by Yoshida, in 1883, when studying the exudate of *Rhus vernicifera*, a Japanese lacquer tree (YOSHIDA, 1883) and for this reason, it was named “laccase”. Subsequently, it was demonstrated to be a fungal enzyme (THURSTON, 1994). In contrast with the past, nowadays many sources of laccase are known in nature: various bacteria (YUAN *et al.*, 2018), insects (WANG *et al.*, 2018), some vascular plants (WAN *et*

al., 2010) and an ample amount of fungi (NIHEI *et al.*, 2018). Laccases usually present a low substrate specificity and their catalytic competence varies widely depending on its source (DURÁN *et al.*, 2002). The optimal temperature and pH range from 20 to 60 ºC and 3 to 9 respectively, and depend on the substrate (MOGHARABI; ALI, 2014).

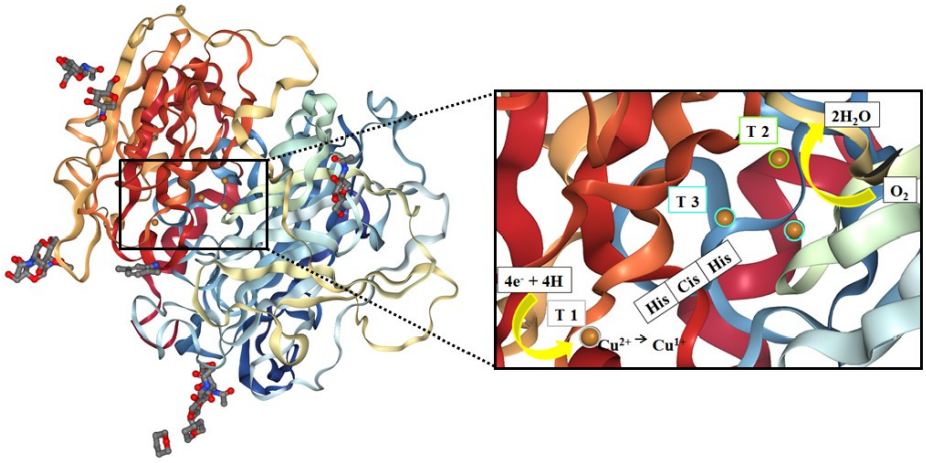
They catalyze the four-electron reduction of molecular oxygen to water with one-electron oxidation of reducing substrate, without the presence of hydrogen peroxide. Laccases can oxidize *o*- and *p*-diphenols, aminophenols, methoxy-substituted phenols, benzenethiols, polyphenols, polyamines, hydroxyindols, some aryl diamines and a range of other compounds (JULIO POLAINA AND ANDREW P. MACCABE, 2007). Laccase participates in the cross-linking of monomers (IKEDA *et al.*, 2001), degradation of polymers (FUJISAWA; HIRAI; NISHIDA, 2014), and ring cleavage of aromatic compounds (KAWAI; NAKAGAWA; OHASHI, 1999). In organic synthesis, laccase is also broadly used and the reaction products are dimers and oligomers derived from the coupling of reactive radical intermediates (KUNAMNENI *et al.*, 2008; RANIMOL *et al.*, 2018).

2.2.1 Molecular properties of laccase

As previously mentioned, the laccases are generally enzymes containing at least one copper atom with, frequently, three additional coppers bound to specific sites of its structure and no other co-factor. These enzymes are found to be made of a sequence of a polypeptide containing about 500 amino acids (AA) residues and linked to saccharide molecules. The AA residues are arranged in three domains of similar β-barrel type architecture, which are related to the small blue copper proteins such as azurin or plastocyanin (GIARDINA *et al.*, 2010; PIONTEK; ANTORINI; CHOINOWSKI, 2002). These coppers can be classified according to their spectroscopic and paramagnetic properties in three redox groups: type-1 (T1), type-2 (T2) e type-3 (T3), as illustrated in Figure 1 (BENTO *et al.*, 2010; JONES; SOLOMON, 2015; JULIO POLAINA AND ANDREW P. MACCABE, 2007; KUNAMNENI *et al.*, 2007). The crystal structure of laccase was downloaded from Protein Data Bank (PDB ID: 1KYA). For the T1 and T3 Cu, some laccases have a “low” redox potential (E^0) of ≈0.4–0.5 V (*versus* the normal hydrogen electrode), while others have a “high” E^0 of ≈0.7–0.8 V. However, the E^0 of the T2 Cu appears to be ≈0.4 V for both low and high E^0 laccase groups (SHLEEV *et al.*, 2005). The reduction of molecular oxygen is accompanied by one-electron oxidation of reducing substrates. Blue copper oxidases contain one T1 copper, a mononuclear copper, sited in domain 3 where it is bound to histidine, cysteine, and often to methionine residues, which

is presumably the primary oxidation site. The blue multicopper oxidases typically employ at least three additional coppers: one T2 and two T3 coppers arranged in a trinuclear copper cluster in which center is embedded at the interface of domains 1 and 3 where they are coordinated to histidine, the latter being the site at which the reduction of molecular oxygen takes place. A histidine-cysteine-histidine tripeptide connects T1 and T2/T3, disulfide of cysteine-cysteine binds domains 1 and 2 and domains 1 and 3, and an extensive loop bridges the domains 2 and 3 to provide a stable structure (ANDBERG *et al.*, 2009; BA; VINOTH KUMAR, 2017; JONES; SOLOMON, 2015; KALLIO *et al.*, 2011). The knowledge of characteristics of proteins is essential once it can directly affect the immobilization procedure or the performance of the final biocatalyst. For laccases, the copper can interact with some supports forming chelates with the active site, destabilizing the catalytic property.

Figure 1- Activity site and catalytic oxidation cycle system of laccase.



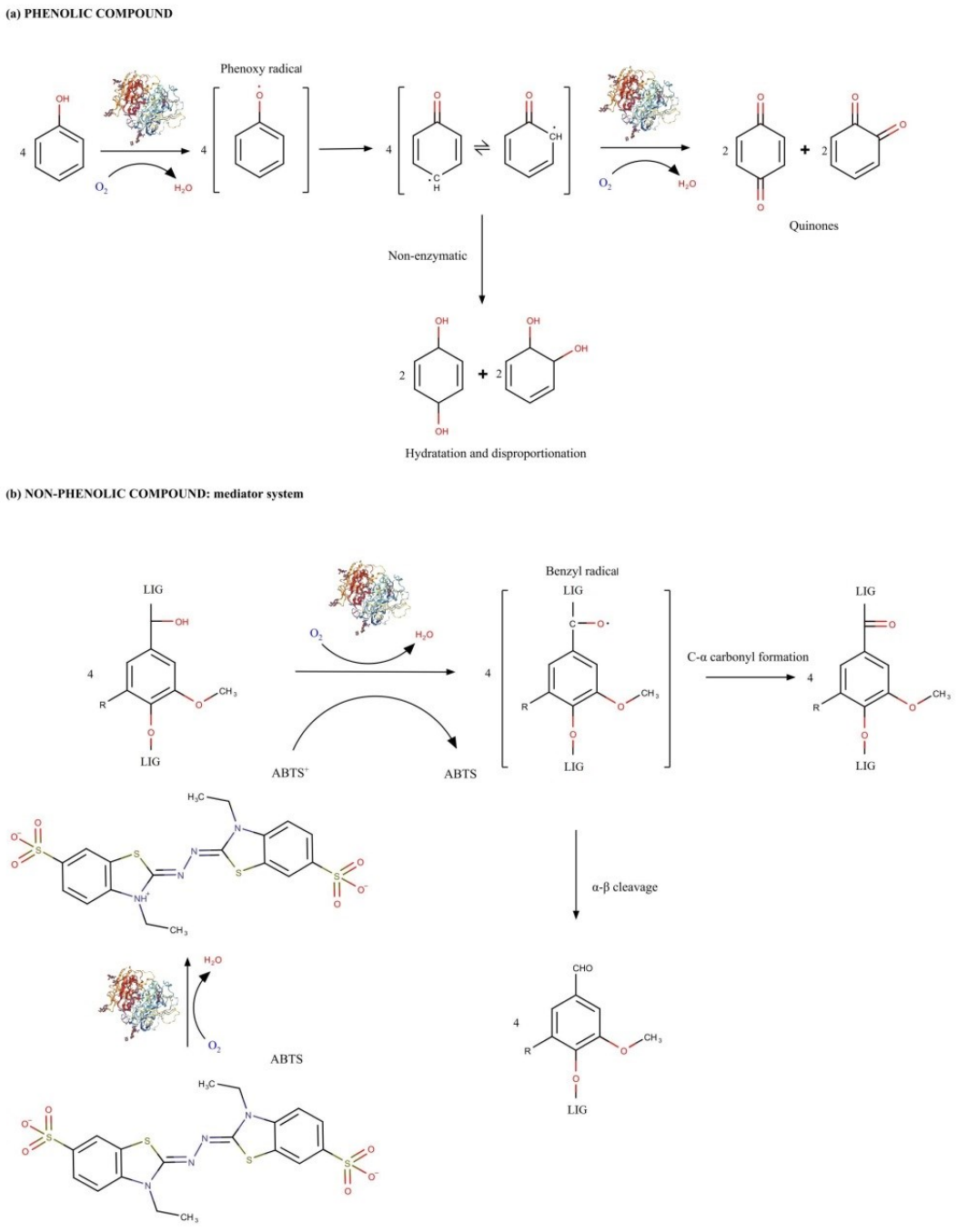
Source: Modified from Protein Data Bank (PDB ID: 1KYA).

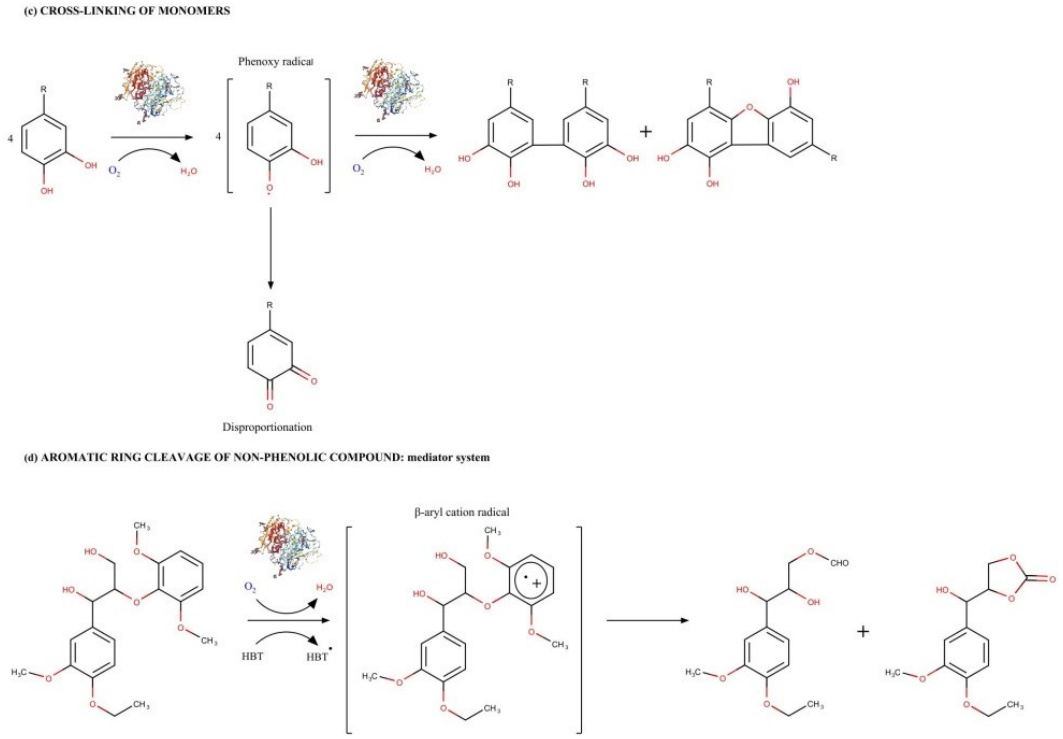
2.2.2 Mechanism of action

Substrate oxidation appears to be via one-electron oxidation coupled to a four-electron reduction, where four molecules of the substrate are oxidized to reduce dioxygen to water (BARRIOS-ESTRADA *et al.*, 2018; STRONG; CLAUS, 2011). The formation of quinoid derivatives or homo-molecular dimers coming from an intermolecular nucleophilic attack from the radicals formed by reaction with laccases can be observed. The initial free radical is typically unstable and can convert phenol into a quinone in a second enzyme-catalyzed step, or may undergo non-enzymatic reactions such as hydration by the reaction with hydroxyl group from the water or even disproportionation and/or may partake in a

polymerization reaction giving an amorphous insoluble melanin-like product by spontaneous disproportionation (see Fig. 2.a) (STRONG; CLAUS, 2011; THURSTON, 1994). There are reports of the reaction of the radical formed after laccase oxidation with the solvent used in the reaction (WANG *et al.*, 2019). The units composing these dimers are linked by C-C or C-O bonds by oxidative condensation, oxidative phenolic coupling, or oxidative coupling (HAUTPHENNE; PENNINCKX; DEBASTE, 2016). After a certain reaction time, this coupling can lead to the formation of oligomers or polymers, which may increase the reaction viscosity, increasing diffusional limitations (MOGHARABI; ALI, 2014). If the reaction of interest involves polymerization, the support choice has to be made up with this concern. Avoiding porous materials may be a good option. Cross-linking of monomers occurs as shown in Fig. 2.c. After laccase-catalyzed oxidation, the unstable formed radicals begin coupling with other radicals forming dimers that react with each other leading to polymerization, as in the case of artificial *urushi* synthesis (IKEDA *et al.*, 2001). In the case of non-phenolic compounds, such as lignin, a laccase mediated system can be used. This system works with the use of auxiliary molecules that are capable of increasing laccase redox potential to over 1.1 V, which allows the oxidation of substrates that without the mediator laccase would have no or minimal activity. The mediator acts as an electron diffuser, enabling the oxidation of large molecular weight compounds (KUNAMNENI *et al.*, 2008). In Figure 2, in items (b) and (d) there is the oxidation scheme of non-phenolic compounds using the laccase mediated system. The laccase mediator system enables other reactions ways and the formation of other reaction products. As shown in Fig. 2 (b), the use of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a mediator led to the breaking of the lignin polymer chain because the ABTS couples in the phenolic groups in lignin to form complexes with hydrophilic character and promote the cleavage of Cα-Cβ of non-phenolic sites, depolymerizing lignin (ARCHIBALD *et al.*, 1997). In Fig. 2 (d) the ring cleavage of an aromatic lignin compound mediated by 1-hydroxybenzotriazole (HBT) is schematized and shows that with the formation of an aryl cation radical from methoxylated aromatic substrate occurs the aromatic ring cleavage of the lignin exemplified (KAWAI; NAKAGAWA; OHASHI, 1999).

Figure 2 - Scheme for laccase action mechanism: (a) direct oxidation and coupling reaction of phenolic compounds by laccase, (b) laccase mediator system for carbonyl formation and cleavage of non-phenolic compounds, (c) cross-linking of monomers and (d) laccase mediator system for aromatic ring cleavage.





Source: Modified from Archibald *et al.* (1997).

2.2.3 Applications

The laccases capacity to catalyze a wide range of aromatic compounds made them attractive, from detoxification and decolorization of various phenolic pollutants until applications in the food industry. There are some emerging trends on laccase applications, showing how laccase is a good substitute for chemical catalysts. Moreover, its usefulness is proven by its use in oxidizing phenolic compounds (BRUGNARI *et al.*, 2018; NAGHDI *et al.*, 2018; ZHANG *et al.*, 2017b), as well as its suitability for organic synthesis (SU *et al.*, 2018; WELLINGTON; GOVINDJEE; STEENKAMP, 2018), environmental pollutant treatment such as decolorization (GARCÍA-MORALES *et al.*, 2018; REDA; HASSAN; EL-MOGHAZY, 2018; SALAMI *et al.*, 2018) and endocrine disruptors (ED) removal (BAYRAMOGLU; KARAGOZ; ARICA, 2018; LONAPPAN *et al.*, 2018; TAHERAN *et al.*, 2017). Manufacture of biosensors for phenolic compounds detection (MEI *et al.*, 2015;

MOHTAR *et al.*, 2019; NAZARI; KASHANIAN; RAFIPOUR, 2015) and even the anti-proliferative activity on cancer cells (KIM *et al.*, 2018). A more detailed approach to the application is beyond the scope of this review and can be found in some specific references (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013; PEZZELLA; GUARINO; PISCITELLI, 2015; PRAJAPATI; MINOCHEHERHOMJI, 2018).

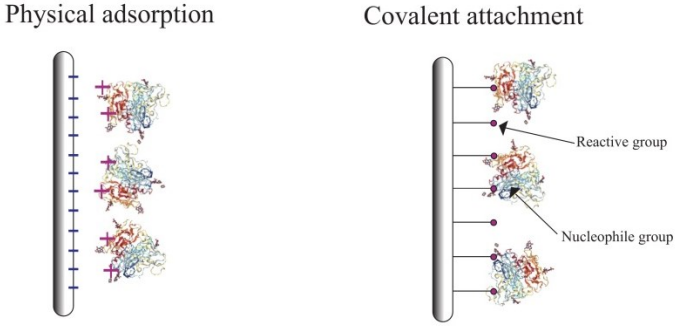
2.3 IMMOBILIZATION

During the past few years, the biotechnological industries have been searching for technologies to satisfy current market demands to increase the stability and reusability of biocatalysts. Once the use of enzymes in the free form is related to low operational stability, high prices, and failure in enzyme reuse and/or product recovery, among other factors (CAROLINA *et al.*, 2018; DATTA; CHRISTENA, 2013), alternatives should be evaluated to make the use of these catalysts possible at industrial levels. The immobilization allows the easy recovery of enzyme once its attachment to an insoluble support, rapid termination of enzyme assay, and high reusability in a few catalytic cycles, which will reduce the operational cost (LIU; CHEN; SHI, 2018). Furthermore, after immobilization, the storage stability, chemical, and thermal resistance are usually improved (BERNAL; RODRÍGUEZ; MARTÍNEZ, 2018; DROZD *et al.*, 2018). Those features are gained due to the interactions between the enzyme and a matrix (immobilization), which stabilizes the peptide structure of the biocatalyst and results in an improvement of enzyme stability (ZDARTA; MEYER; PINELO, 2018). The immobilization of enzymes on different supports may improve their activities, due to the limitation of enzymatic movement and better substrate diffusion at higher temperatures (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). However, the immobilization processes may result in conformational alterations of the enzyme, heterogeneity of the enzyme on the support, and a slight loss of activity (FERNÁNDEZ-FERNÁNDEZ; SANROMÁN; MOLDES, 2013). Attention should be paid to the decrease in catalytic efficiency, which can be related to several factors, including enzyme leakage, partial inactivation caused by its utilization in repeated cycles and formation of high-molecular-weight products through oxidation and/or polymerization (that might partially inhibit the enzyme's active sites, which significantly limits the transfer of substrates) (JI *et al.*, 2017; TARR, 2003).

Methods for enzyme immobilization can be divided into three categories (SHELDON; VAN PELT, 2013):

The binding to support can be physical or chemical (Fig.3): physical methods involve the creation of non-specific interactions rather via hydrogen bonds and ionic and hydrophobic interactions. The chemical changes in the enzyme are limited (keeps their free features) and the retention of high catalytic activities is observed (DATTA; CHRISTENA, 2013; DURÁN *et al.*, 2002; ZDARTA; MEYER; PINELO, 2018). Chemical immobilization methods mainly include *(i)* enzyme attachment to the matrix by covalent bonds, *(ii)* cross-linking between enzyme and matrix, and *(iii)* enzyme cross-linking by multifunctional reagents. The creation of strong chemical bonds, between the biocatalyst and matrix, significantly reduces leakage of the enzyme and enhances its reusability (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011; ZDARTA; MEYER; PINELO, 2018).

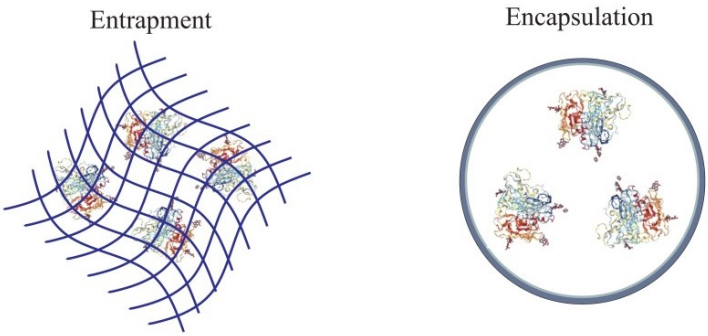
Figure 3 - Scheme for laccase immobilization by binding to support. Adsorption by ionic forces (left). Covalent binding between enzyme and support surface (right).



Source: from the author.

The entrapment immobilization is the inclusion of an enzyme in a polymer network, which is typically an organic or an inorganic polymeric matrix (see Fig. 4) (SHELDON; VAN PELT, 2013). The enzyme barely suffers alteration, but the method has some disadvantages, such as continuous leakage of the enzyme due to variable pore-size, uneven distribution in the gel, and reduced substrate accessibility to the enzyme by diffusional limitation (RODRÍGUEZ-DELGADO *et al.*, 2015).

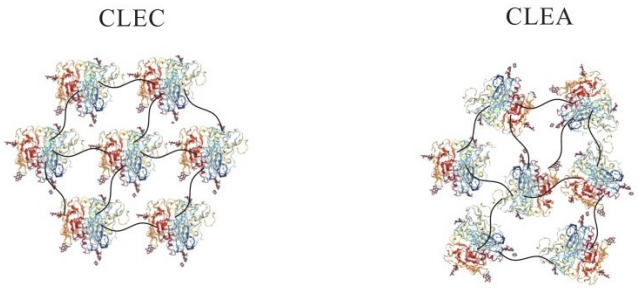
Figure 4 - Scheme for laccase immobilization by entrapment by physical interactions. Entrapment immobilization in a porous matrix (left). Encapsulation of laccase (right).



Source: from the author.

The cross-linking of the enzyme particles can be by creating a cross-linked enzyme aggregate (CLEA) or creating a cross-linked enzyme crystal (CLEC) (see Fig. 5) (ZDARTA; MEYER; PINELO, 2018). CLEAs are easily made by mixing an aqueous solution of the enzyme with an aqueous solution of glutaraldehyde (GA), nevertheless, CLEAs exhibited low activity retention, poor reproducibility, and low mechanical stability and, owing to their gelatinous nature, were difficult to handle (SHELDON, 2011; XU; YANG, 2013). The CLECs have better stability but it requires the crystallization of the enzyme, a laborious and costly procedure (SHELDON, 2011).

Figure 5 - Self-immobilization method. Cross-linked enzyme crystal (CLEC) (right) and cross-linked enzyme aggregate (CLEA) (left).



Source: from the author.

Depending on the functional group present on the support surface the enzyme attachment will be given differently. If in the surface of the support there are -OH, C=O and -NH_2 groups only entrapment methods can be applied, but when there are -OH, COOH, C=O, -

SH, -NH_2 in the surface, adsorption of the enzyme can occur (ZDARTA; MEYER; PINELO, 2018). A surface with -OH, COOH, C=O, -SH, and -NH_2 enables covalent bond formation (ZDARTA *et al.*, 2018a).

For immobilizing an enzyme three things should be known: *(i)* the selection of support material, with suitable surface chemistry, which defines the binding chemistry for enzyme attachment; *(ii)* the evaluation of experimental conditions during the process, to optimize operational performance (i.e. high immobilization yield and expressed activity) and *(iii)* the characterization of the catalytic behavior of the resulting biocatalyst under operational conditions (SHELDON; VAN PELT, 2013).

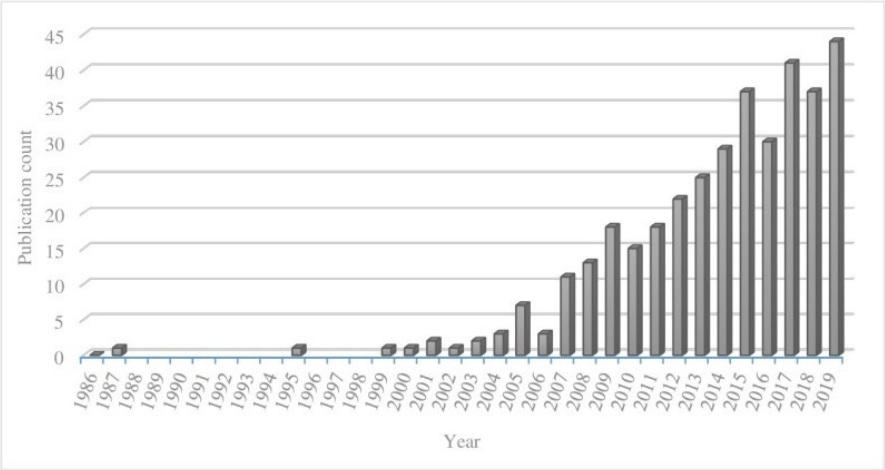
Since the beginning, one should have the application in mind. Once its application is defined it will be possible to find the best support, the best way of enzyme's attachment to the support and thus the catalytic behavior of the biocatalyst will be better.

2.4 MATERIALS USED AS SUPPORT FOR LACCASE IMMOBILIZATION

The characteristics of the support are of fundamental importance to determine the success of the final biocatalyst. Ideal support should: *(i)* be of low cost and eco-friendly (avoiding to increase the cost of the process and still generate an environmental problem), *(ii)* be inert after immobilization (for do not interact with the reaction medium), *(iii)* have thermal and mechanical resistance (to withstand the process conditions), *(iv)* enhance the enzyme specificity (improve interactions between the molecule of the substrate and the active site of the enzyme, targeting the product of interest), *(v)* be able to pack a large amount of enzyme (large pore size will cause a notable fall in the surface area, while small pore size will barely exclude the protein), *(vi)* prevent undesired protein adsorption and denaturation and *(vii)* be able to shift in the pH and temperature optimum for the enzyme action to the desired value for the process (RODRIGUES *et al.*, 2013; SIRISHA; JAIN; JAIN, 2016).

Scientific researches about laccase immobilization have been reported in the literature since 1986, as shown in Figure 6. The visual ascendancy in the number of articles related to "laccase" and "immobilization" shows us the increasing interest in the development of new supports for these biocatalysts. Aiming better stability and commercial performance, a large diversity of matrices is developed. The support matrices can be classified according to their chemical composition as (a) inorganic materials, (b) organic materials, and (c) hybrids and composite materials.

Figure 6 - Publications data for “laccase” and “immobilization” in articles by Scopus, 26th July.



Source: from the author.

The most diverse types of materials used for laccase immobilization and a general overview of these materials as matrices are presented in Table 1. It shows laccase immobilized on different supports as well as its source and applications. The support type, immobilization method, support affinity, support size, the surface area of the support, enzymatic loading on support, application of the biocatalyst obtained, its activity under application, the stability of the immobilized laccase and its reuse are specified below, taking into account the material used as support.

2.4.1 Inorganic materials

Inorganic supports are widely used to laccase immobilization due to their high thermal, mechanical, and microbial resistance (HARTMANN; KOSTROV, 2013). They include several silica-based and other oxide-based materials. Furthermore, two main features distinguishing inorganic supports are rigidity and porosity (HARTMANN; KOSTROV, 2013; ZUCCA; SANJUST, 2014). The typical rigidity of the inorganic supports ensures the invariance of pore diameter/pore volume (ZUCCA; SANJUST, 2014). Thus, inorganic materials are commonly used for the immobilization of laccases. Some examples of the various inorganic materials, their use for immobilization of laccase, and their final application are presented in Table 1.

2.4.1.1 Silica

Silica and its derivatives are the most used materials as supports for enzyme immobilization. The hydrophilic character of the silica surface, as well as the presence of many hydroxyl groups, leads to immobilization of biomolecules not only via adsorption but also by the creation of covalent bonds and even by encapsulation (ZDARTA; MEYER; PINELO, 2018). Salami and coworkers immobilized laccase on epoxy-functionalized silica (gel) using cyclohexyl-isocyanide as a crosslinker agent and observed that isocyanide functional group as an active reagent in immobilization process caused a significant increase in immobilization yield (from 20 to 50 $mg \cdot g^{-1}$). They obtained a degradation of 43, 49, 34, 36, and 57% without a mediator and 98, 99, 78, 97, and 100% with 3,4-dimethoxy-5-hydroxybenzoic acid (DMHBA) as a mediator (See Table 1) (SALAMI *et al.*, 2018). Laccase has a carbohydrate moiety, which causes a spatial configuration problem and makes some of its free amino groups inaccessible for immobilization and, as a result, immobilization yield decrease (BERKA *et al.*, 1997).

In another study, Mohammadi and co-workers used similar epoxy-functionalized silica to remove phenol, *p*-chlorophenol, and catechol. In the absence of the active reagent isocyanide, they obtained an immobilization yield of 30 mg of protein in 1 g. With a distribution particle size of 0.063-0.2 mm, free laccase from *Myceliophthora thermophile* showed stability at pH 3-5, and low stability at 40 and 45 ºC. The immobilized enzyme maintained pH stability and improved temperature resistance. About 24 and 40% of phenol and p-chlorophenol degradation was obtained after 24 h treatment, respectively (MOHAMMADI *et al.*, 2018).

An interesting example of silica support use is presented by Dehghanifard *et al.* In their study, they immobilized laccase from *Trametes versicolor* on nano-porous CPC (controlled porosity carrier) silica beads with a surface area of 40 ± 5 $m^2 \cdot g^{-1}$ and particle size distribution of 355–600 mm, to degrade 2,4-dinitrophenol (2,4-DNP). The immobilized laccase was more resistant to pH variations and the maximum degradation of 2,4-DNP was raised to 91% on 12 h contact time at 40 °C. The immobilization led to an increase in K_m value. On the other hand, the immobilized laccase maintained its activity for more than 85% after 30 cycles of use (DEHGHANIFARD *et al.*, 2013).

Rahmani and co-workers immobilized laccase from *Trametes versicolor* on CPC silica beads for the removal of sulfathiazole (STZ) and sulfamethoxazole (SMZ) (RAHMANI

et al., 2015). The immobilized laccase showed higher relative activity (85.4%) after incubation at 70 °C for 2 h when compared to free laccase, with a significantly sharp decrease in its relative activity (25.6%) as well. Also, the K_m value for immobilized laccase was lower than that free laccase for both sulfonamides tested, which can mean that the structure of the matrix reduced diffusional limitation in the transport of the substrates. Another silica-based support is the fumed silica nanoparticles. Hommes and co-workers used this matrix with 390 ± 40 $m^2 \cdot g^{-1}$ of superficial area and aggregates of particle size of 7 nm to immobilize laccase from three different sources and then to remove ^{14}C-labeled Bisphenol A with the environmentally relevant concentration. They obtained, after optimization of the immobilization process, enzymatic loading of 0.77 ± 0.12 $U \cdot mg^{-1}$, 4.77 ± 0.24 $U \cdot mg^{-1}$ and 2.70 ± 0.05 $U \cdot mg^{-1}$, for crude laccase from *Phoma* sp.*, Coriolopsis polyzona,* and *Thielavia genus*, respectively. The biocatalyst with *Thielavia genus* laccase, which showed better results, retained 95.8 ± 4.8% of its initial activity after 7 days (HOMMES *et al.*, 2012). As reported by Wehaydi *et al.*, the immobilization of laccase from *Polyporus durus* ATCC 26726 on nanoporous zeolite-X (ZX) reduced 5.42 $kJ \cdot mol^{-1}$ the activation energy (*Ea*) of the catalytic reaction and also improved the thermal stability: after treatment at 60 °C immobilized laccase retained 93.5% of initial activity and the free laccase retained only 5%. When investigated against two dyes, Remazol Brilliant Blue R and Acid blue 225, the biocatalyst ZX-laccase were capable of 100% removal, so this final biocatalyst can be used in textile industries for wastewater treatment (WEHAIDY *et al.*, 2019).In summary, laccases can be immobilized on several types of silica-based materials, usually with high efficiencies. However, it is also noticed that the limitation of this material is the change in the affinity of the enzyme to its substrate, probably caused by conformational changes in the enzyme caused by the immobilization procedure.

2.4.1.2 Metal oxides

Inorganic metal oxides (e.g titania, alumina, and magnetic iron oxides II and III) have been used widely for laccase immobilization (HOU *et al.*, 2014; JI *et al.*, 2017; SINGH *et al.*, 2015). These materials present a high surface area, which allows a higher enzymatic loading and lower mass transfer resistance to the substrates (ASURI *et al.*, 2006). The structures have hydroxyl groups (OH) at the surface, which makes the functionalization of these materials to be favored (NGUYEN *et al.*, 2014) and, consequently, the covalent bonding of the laccase to the matrix is possible, increasing its resistance to pH and temperatures

(ASGHER *et al.*, 2014). Laccase from *Pycnoporus sanguineus* CS43 was immobilized on titania nanoparticles by García-Morales and his team in order to degrade acetaminophen (ACE) and diclofenac (DFC), pharmaceutical active compounds, in the groundwater. They functionalized TiO_2 with 3-aminopropyl(triethoxy)silane (APTES) to provide amino groups as anchor points and glutaraldehyde as a cross-linker. It was observed an increase in relative activity with more than 90% in contrast to 65% for free laccase. Although the immobilization process did not generate any significant change over the K_m values, it is important to take into account that there was good preservation of the affinity between the immobilized enzyme and substrate. In this study, when applied, 68% of DCF was removed after 8 h and 90% of ACE was removed after 2 h (GARCÍA-MORALES *et al.*, 2018).

The physical adsorption of laccase on titania nanoparticles provides the maintenance of its conformational structure and makes possible the support reuse. Hou *et al.* functionalized titania nanoparticles with APTES, dispersed into a GA solution, washed, and then suspended it into laccase solution. The resulting biocatalyst presented 60.1 ± 0.3 $m^2 \cdot g^{-1}$ of surface area and enzymatic loading of 0.085 ± 0.011 $U \cdot mg^{-1}$ of support and, also, the K_m showed to be only 2.5 times lower than the free enzyme (HOU *et al.*, 2014). Another inorganic metal oxide used for enzyme immobilization is alumina (COSTA *et al.*, 2001; ZILLE *et al.*, 2003). Singh and his group used alumina pellets functionalized with APTES (to increase the hydrophobicity of the final biocatalyst) for melanoidin degradation. They observed an increase in the enzymatic activity upon immobilization (886 $U \cdot L^{-1}$ compared to 176 $U \cdot L^{-1}$ for soluble laccase) and, on the other hand, the enzymatic stability did not increase beyond pH 4.5 but increased at higher temperatures. The thermal stability is possibly due to strong linkages between the oxide groups of APTES on the support and amino groups of the enzyme, which creates a stable three-dimensional enzyme structure (SINGH *et al.*, 2015). As can be seen, the inorganic oxides used as support for laccase exhibited good results and easy immobilization methods. These materials also present thermal, chemical, and mechanical resistance, behavior which is of great significance.

2.4.1.3 Clays

Inorganic sources such as mineral clays are used as immobilization support because of its cost-efficient, abundance on nature, facility of reusability, low mass transfer resistance, and low microbial corrosion (BASYARUDDIN *et al.*, 2005). Some clays, as the halloysite, clay, kaolin *et cetera*, have been used in the last few years as a low-cost matrix for laccase

immobilization (HU; ZHAO; HWANG, 2007; OLSHANSKY *et al.*, 2018; SONGURTEKIN *et al.*, 2013). Wen *et al.* used kaolinite as support for laccase attachment for degradation of malachite green effluent with the coexistence of Cd (II), a heavy metal that is released into the environment along with the dye. In the presence of 3,5-dimethoxy-4-hydroxybenzaldehyde (SA, a redox mediator), Kaolin-Laccase could degrade over 98% of malachite green, but it lost nearly 50% of its activity after two cycles (WEN *et al.*, 2019). This behavior can be ascribed to the enzyme leaching during the washing process, once the physical adsorption immobilization had weak bonds between enzyme and support (LIU *et al.*, 2012).

A study about the adsorption of laccase on sepiolite was made by Olshansky *et al.* and they realized that the immobilization process increased its activity by 250 ± 40% compared to the non-adsorbed enzyme (OLSHANSKY *et al.*, 2018). Montmorillonite can be used as well, as done by Songurtekin *et al.* These authors immobilized laccase on montmorillonite functionalized with histidine (His-Mt/Lac) for applying on flow injection analysis of phenols. When compared, the thermal stability of the histidine modified clay minerals was found lower than that of unmodified ones and pH 5.5 is the optimum for His-Mt/Lac biosensor (SONGURTEKIN *et al.*, 2013). In summary, this kind of support can be economic and has an easy immobilization process, but on the other hand, it is important to have in mind the possibility of enzyme leaching.

Table 1 - Materials of inorganic, organic, and hybrid origin used for laccase immobilization and its use for phenols and similar removal.

	Support and immobilization method	Support size (a) and surface area (b)	Enzymatic loading	Substrate	Degradation	Process conditions	Reuse	Reference
Inorganic materials	Epoxy-functionalized silica (gel) + cyclohexyl isocyanide as crosslinker [(c)]		With isocyanide: 50 mg·g Whithout: 20 $mg \cdot g^{-1}$	Acid orange 156, acid red 52, coomassie brilliant blue, methyl violet and malachite green	No mediator: 43%, 49%, 34%, 36%, 57% With DMHBA: 98%, 99%, 78%, 97%, 100%	pH: 4-5 T:35-45°C	61% of initial activity at 8th cycle	(SALAMI *et al.*, 2018)
	Epoxy-functionalized silica (gel) [(c)]	(a) 0.063-0.2 mm	30 $mg \cdot g^{-1}$	Phenol, p-chlorophenol and catechol	24% and 40% of phenol and p-chlorophenol, 24h 95% catechol, 2h	pH:3-5 T:35-45°C	61% of initial activity at 5th cycle	(MOHAMMADI *et al.*, 2018)
	CPC nano-porous silica beads + GA [(a)]	(a) 355–600 mm pore size <100 nm (b) 40 ± 5 $m^2 \cdot g^{-1}$		2,4-dinitrophenol	91%, 12h	pH: 5 T:40°C	85% of initial activity at 30th cycles	(DEHGHANIFARD *et al.*, 2013)
	CPC silica beads + GA [(a)]			Sulfamethoxazole and sulfathiazole	76%, 85%	pH: 5 T: 50°C	63% and 82.6% of initial activity at 10th cycles	(RAHMANI *et al.*, 2015)
	Fumed silica nanoparticles + GA [(a)]	(a) 7 nm (b) 390 ± 40 $m^2.g^{-1}$	*i)*0.77 ± 0.12 $U \cdot mg^{-1}$ *ii)*4.77 ± 0.24 $U \cdot mg^{-1}$ *iii)*2.70 ± 0.05 $U \cdot mg^{-1}$	^{14}C-labeled bisphenol A	*ii)* 1.38 ± 0.03 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ *iii)* 1.69 ± 0.02 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$	pH: 3-9 T:25°C	95% of initial activity at 7th cycle	(HOMMES *et al.*, 2012)
	Titania nanoparticles + APTES + GA [(a)]	(a) 21 nm		Acetaminophen and diclofenac	90%, 2h 68%, 8h	pH: 2-4 T:40°C		(GARCÍA-MORALES *et al.*, 2018)
	Titania nanoparticles [(b)]	(b) 60.1±0.3 $m^2 \cdot g^{-1}$	0.085 ± 0.011$U \cdot mg^{-1}$	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)	Remaining activity of 0.12 $U \cdot mg^{-1}$	pH: 7 T:25°C		(HOU *et al.*, 2014)
	Alumina pellets + APTES + GA [(a)]	(a) 3 mm (b) 160 $m^2 \cdot g^{-1}$	5-6 $mg \cdot g^{-1}$	Melanoidin	50%, 8h	pH: 4.5 T:35°C	90% of initial activity at 10th cycle	(SINGH *et al.*, 2015)

Organic Materials	Kaolinite [(b)]		12.55 mg·g^{-1}	Malachite green	98%, 5h	pH: 3-6 T:40°C	50% of initial activity at 2nd cycle	(WEN *et al.*, 2019)
	Sepiolite [(b)]	Pore volume 0.44 cm^3·g^{-1} (b) 340 m^2·g^{-1}						(OLSHANSKY *et al.*, 2018)
	Montmorillonite + Histidine [(b)]	Basal spacing 1.32 nm		Detection of phenols		pH: 5.5 T:15°C		(SONGURTEKIN *et al.*, 2013)
	Chitosan [(c)]		98% efficiency 2.254 µg	Indigo carmine	100%, 8h	pH: 9-13 T: 80°C	44% of initial activity at 2nd cycle	(JAISWAL; PANDEY; DWIVEDI, 2016)
	Alginate-gelatin mixed gel [(c)]		86.7% efficiency 0.7 mg·g^{-1}	Azo dyes anthraquinone and triphenylmethane	80%	pH: 5-7 T: 40°C	65% of initial activity at 7th cycle	(REDA; HASSAN; EL-MOGHAZY, 2018)
	MANAE-agarose [(b)]		18 mg·g^{-1} 120±6 U·g^{-1}	Bisphenol A	50%, 20 min	pH: 5-8 T:50-55°C	90% of initial activity at 15th cycle	(BRUGNARI *et al.*, 2018)
	Hippospongia communis skeleton [(b)]	(a) 20 µm		Bisphenol A, bisphenol F and bisphenol S	96%, 95%, 53%, 24h	pH: 4-5 T:30-40°C	30, 40 e 20% removed respectively at 5th cycle	(ZDARTA *et al.*, 2018b)
	Polyacrylonitrile beads + GA [(a)]		0.28 mg·g^{-1}	Nonylphenol and octylphenol	100%, 6h	pH: 5-6 T: 45-50°C		(CATAPANE *et al.*, 2013)
	Poly(glycidyl methacrylate) brushs in microspheres of polyestyrene-divinyl benzene [PS-co-DVB-g-P(CCMA)] [(a)]	(a) 210–422 µm (b) 32.4 m^2·g^{-1}	47.8 mg·g^{-1}	Bisphenol A and congo red dye	89%, 100%, 2h	pH: 5-6 T:30-45°C	53-67% of initial activity at 10th cycle	(BAYRAMOGLU; KARAGOZ; ARICA, 2018)
	Poliurea microspheres + GA [(a)]	(a) 11 µm	20.63 mg·g^{-1}	Remazol brilliant blue R	64%, 5h	pH: 3 T:50°C	92% of initial activity at 3rd cycle	(JIANG *et al.*, 2017)

	Nylon 6 film *(i)* and nylonfibers 6 *(ii)* + GA [(a)]	Pore diameter 200 nm (b) *(i)* 0.45 mm^2 *(b)(ii)*1050 mm^2	*(i)* 0.04 $mg \cdot g^{-1}$ *(ii)* 0.17 $mg \cdot g^{-1}$	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)		pH: 4.5 T*(i)*:55°C T*(ii)*:50°C	*(i)*21% and *(ii)*29% of initial activity at 7th cycle	(FATARELLA *et al.*, 2014)
Hybrids and Composites	Fe_3O_4@SiO_2@Kit-6-NH_2 + GA [(a)]	Pore diameter 5.8 nm (b) 238.2 $m^2 \cdot g^{-1}$	84.4% efficiency	Delignification of olive pomace	74%	pH: 4.5 T:35°C	71% of initial activity at 11th cycle	(AMIN *et al.*, 2018)
	Polyamide 6/chitosan nanofiber + *(i)* GA-BSA-GA and *(ii)* GA-HMD-GA [(a)]	Fiber diameter 185±19 nm	*(i)* 64.1 ± 7.9 $mg \cdot g^{-1}$ *(ii)* 72.9 ± 14.6 $mg \cdot g^{-1}$	Bisphenol A and 17-α-ethinylestradiol	92%, 100%, 6h	pH: 5 T:37°C	50% of initial activity at 3rd cycle	(MARYŠKOVÁ *et al.*, 2016)
	Fe_3O_4-halloysite nanotubes-Chitosan + GA [(a)]		90 $mg.g^{-1}$	Anti-proliferative applications on liver (HepG2), lung (H460), cervix (Hela) and stomach (AGS) cancer cells	Immobilization enhanced antiproliferative activity on Hela cells			(KIM *et al.*, 2018)

* Immobilization methods: covalent attachment [(a)]; physical adsorption [(b)] and entrapment [(c)].

2.4.2 Organic materials

Several materials of organic origin have been employed as support for the immobilization of laccases, both natural and synthetic polymers, utilizing many techniques of immobilization (See Table 1). Organic materials can also be obtained with controlled porosity, but they are usually very sensitive to pressure and/or pH (ZUCCA; SANJUST, 2014).

2.4.2.1 Natural Polymers (Biopolymers)

A wide variety of natural polymers such as agar, alginate, gelatin, carrageenan, chitosan/chitin, agarose, collagen, and cellulose have been used as support for laccase immobilization due to their easily activated chemical structure (BANSAL *et al.*, 2018; JUS *et al.*, 2011; MAKAS *et al.*, 2010). They can bind to proteins and enzymes reversibly and irreversibly, are available in large quantities, most are inexpensive, and show high thermal and mechanical resistance by cross-linking with bifunctional reagents (DATTA; CHRISTENA, 2013). Some of the most used organic materials for laccase immobilization are chitosan and chitin (JIA *et al.*, 2006). This interest is due to the excellent biological properties of these compounds, such as low cost, easy availability, biodegradability, biocompatibility and non-toxicity properties and high mechanical strength (JIA *et al.*, 2006).

In this sense, Jaiswal and co-workers used chitosan to immobilize laccase from *Carica papaya* by covalent bonds and physical adsorption for dye discoloration. They utilized entrapment technique without any cross-linker, obtaining an immobilization yield of 98% with no leaching either during the bead formation or washing. This laccase-chitosan material removed 100% of indigo carmine after 8 h of treatment and, the pH range to immobilized laccase increased from 8 to an alkaline pH range of 9-13 (JAISWAL; PANDEY; DWIVEDI, 2016). Besides chitosan, alginate and gelatin are also economic sources for the biocatalyst design. They are commonly used not only for laccase but also for the immobilization of other groups of enzymes. Alginate with gelatin was used as support for laccase through the entrapment immobilization process, with an immobilization yield of 86.7%. Despite the increase in K_m (assigned to diffusion limitations and steric hindrances caused by the entrapment), the removal percentage of dyes color was about 80% (REDA; HASSAN; EL-MOGHAZY, 2018).

Agarose is also used due to being a neutral and linear polysaccharide, which is generally extracted from red algae including *Gracilaria* and *Gelidiella* (ZHANG *et al.*, 2018).

Monoaminoethyl-N-aminoethyl agarose gel (MANAE-agarose) was used by Brugnari and co-workers to immobilize the laccase from *Pleurotus ostreatus*. The procedure resulted in an immobilization yield of 100% with an enzymatic loading of 120 ± 6 $U \cdot g^{-1}$, which was able to degrade 50% of bisphenol A after 20 min and maintained 90% of initial activity after 15 cycles (BRUGNARI *et al.*, 2018).

A novel material, *Hippospongia communis* skeletons, was used by Zdarta and co-workers to immobilize laccase from *Trametes versicolor* for bisphenols removal. *H. communis* skeletons with fibers of about 20 μm of diameter and, based on FTIR and SEM results, effective deposition of the laccase was confirmed. When the derivative was tested for degradation of Bisphenols A, F, and S over five catalytic cycles it was observed that even in the fifth removal cycle, about 30% of BPA, 40% of BPF and 20% of BPS was degraded after 24 h (ZDARTA *et al.*, 2018b). It has been demonstrated that natural organic materials are a good choice because of their easy immobilization procedure, low cost, and eco-friendly appeal.

2.4.2.2 Synthetic Polymers

Synthetic polymers are versatile materials that might have a porous surface, several sizes, and shapes that can be chosen for the application of interest. Ion exchange resins, polystyrene, polyamides, polyacrylonitrile belongs to this class (ZDARTA; MEYER; PINELO, 2018). These polymers, due to the presence of many functional moieties in the structure, allow the creation of relatively strong interactions between the enzymes and the support (DATTA; CHRISTENA, 2013).

Catapane and co-workers immobilized laccase on polyacrylonitrile (PAN) beads, with glutaraldehyde used both as a spacer arm and as a bifunctional reagent to bind covalently the aminoaril derivatives useful for enzyme immobilization. The biocatalyst was used in a fluidized bed reactor to degrade nonylphenol (NP) and octylphenol (OP), showing a complete degradation after 6 and 5 h, respectively. Moreover, the K_m has slightly increased for both substrates (CATAPANE *et al.*, 2013).

Bayramoğlu *et al.* (2018) developed a novel synthetic polymer, which contained a cyclic carbonate group to facilitate the enzyme immobilization. The support was synthesized from [poly(styrene-co-divinylbenzene)] microsphere under a carbon dioxide atmosphere with grafting of poly(glycidyl methacrylate). With a specific surface area of 32.4 $m^2 \cdot g^{-1}$, 210-422 μm of microspheres diameter, they obtained an enzymatic loading of 47.8 $mg \cdot g^{-1}$ of

support. After 2 h of treatment, 89 and 100% of bisphenol A and Congo Red dye was degraded, respectively. The initial activity remained about 53-67% after 10 cycles. The space between enzyme and support surface was provided by the poly(glycidyl methacrylate) brushes and the covalent attachment of the enzymes on the cyclic carbonates groups helped to improve the operational stability of the final biocatalyst (BAYRAMOGLU; KARAGOZ; ARICA, 2018).

In another study, uniform polyurea (PU) microspheres were prepared through precipitation polymerization using isophorone diisocyanate (IPDI) as the only monomer in a mixed solvent of water-acetonitrile and then it was activated using GA to attach aldehyde groups on their surface, followed by immobilization of laccase via direct covalent bonding. These microspheres, with a diameter between 8 and 12 μm, led to an enzymatic loading of 20.63 $mg \cdot g^{-1}$ of support and were capable to remove 64% of Remazol Brilliant Blue R, after 5 h of treatment (JIANG *et al.*, 2017).

Nanostructured polymers such as polyamides represent excellent support due to their high surface area (KIM; GRATE; WANG, 2006). Nylon 6 film and nanofiber carriers (prepared by electrospinning method) were investigated as matrixes for laccase immobilization. Laccase from *Trametes versicolor* was covalently immobilized onto spacer-arm attached carriers after acidic hydrolysis. The amount of immobilized enzyme on the nylon film (0.45 mm^2 of surface area) and nanofibers (1050 mm^2 of surface area) was 59.4 and 71.0%, respectively. The use of the hydrolysis activation step has shown better immobilization performance than the non-activated ones because it enables the glutaraldehyde binding to the support and, posteriorly, the formation of the imine bond (Shiff's base) between aldehyde groups of glutaraldehyde and the amino groups of lysine protein residue (FATARELLA *et al.*, 2014).

In synthesis, many synthetic organic polymers are used as support to immobilize laccase, varying according to its final application. The final properties of the biocatalyst can widely vary depending on the polymer characteristics. This kind of material usually does not have hydrophilic character, behavior which facilitates its easy separation at the end of the process and, as can be observed, high standard activities have been shown onto the application.

2.4.3 Hybrids and composites

The idea of combine features the characteristics of inorganic and organic materials has attached a lot of attention in recent years, with the formation of so-called hybrid materials (ZDARTA; MEYER; PINELO, 2018). Combining properties of the precursor materials, hybrid materials maximize their qualities also expanding their practical application (MEI; HAN; WU, 2018). Since there are inorganic and organic materials, three types of hybrids do exist: inorganic-inorganic, organic-organic, and inorganic-organic (ZDARTA *et al.*, 2018a; ZDARTA; MEYER; PINELO, 2018). Materials that have been extensively used in a variety of applications are the magnetic nanoparticles with a functionalized surface (AMSTAD; TEXTOR; REIMHULT, 2011). The magnetic iron oxide nanoparticles can be covered with a thin layer of organic molecules, polymers, and also by inorganic materials such as silica and metal oxide (TENG *et al.*, 2012). These materials have attracted attention due to its high surface area, controllable pore diameter, uniform pore size distribution and strong magnetic response (LOPEZ *et al.*, 2019). Through magnetic induction, the material can be easily separated from the process (GAO; CHEN, 2013).

Amin *et al.* (2018) covered magnetite nanoparticles with amorphous silica for the attachment of Ordered Mesoporous SiO_2 (KIT-6) and after that, the core-shell Fe_3O_4@SiO_2@Kit-6 nanoparticles had the NH_2 functionalization with APTES and then laccase was attached to Fe_3O_4@SiO_2@Kit-6-NH_2 with glutaraldehyde. This inorganic-inorganic hybrid biocatalyst was applied to determine the delignification potential of the immobilized enzyme with olive pomace. With a high superficial area of $238.2 m^2 \cdot g^{-1}$ and an immobilization yield of 84.4%, the immobilized laccase exhibited improvement on thermal, pH, and storage stabilities with a great performance for reusability, since it was able to keep 71% of its initial activity after 11 cycles.

Marysková and co-workers reported the use of laccase from *Trametes versicolor* immobilized onto organic-organic hybrid polyamide 6/chitosan (PA6/CHIT) nanofibers modified using two different spacers: bovine serum albumin (BSA) and hexamethylenediamine (HMD). The final biocatalysts (PA6/CHIT-GA-BSA-GA and PA6/CHIT-GA-HMD-GA) presented very similar results when applied for biodegradation of endocrine-disrupting chemicals bisphenol A and 17-α-ethinylestradiol and removed 92 and 100%, respectively (MARYŠKOVÁ *et al.*, 2016). It should also be mentioned that hybrid supports built from two synthetic polymers may also be used for laccase immobilization. The

number of combinations that can be made of materials to change the matrix characteristics (as hydro-affinity) is uncountable.

Among these hybrid materials, there are the inorganic-organic hybrids, which have combinations of inorganic materials with natural polymers, for example, chitosan (ALVER; METIN, 2017) or alginates (THANH *et al.*, 2016), as well as synthetic polymers like poly(4-vinyl pyridine) (BAYRAMOGLU; YILMAZ; ARICA, 2010).

Kim *et al.* (2018) synthesized a super-magnetized core-shell nanoparticle using magnetic Fe_3O_4 and chitosan (CS) functionalized with halloysite nanotubes (HNTs). The hybrid material (termed as Fe_3O_4-HNTs-CS) was used as a carrier to laccase immobilization and then applied the final biocatalyst to test the anti-proliferative activity against cancer cell lines by the liver (HepG2), lung (H460), cervix (Hela) and stomach (AGS). With an enzymatic loading of 90 $mg \cdot g^{-1}$, Fe_3O_4-HNTs-CS-Lac showed significant cytotoxicity against all studied cancer cell lines (KIM *et al.*, 2018).

Such hybrids systems have the ability to precisely design the properties of the final biocatalyst and, as well, the process in which they will be applied (DESKA; KONCZAK, 2019). Due to the high variety of possibilities, these supports are a choice that presents amazing results at the final catalytic behavior.

2.5 GENERAL OVERVIEW

The ideal support for usage on industrial applications should be inert, rigid, inexpensive, eco-friendly, and also should present thermal and mechanical resistance. The most different types of materials can be successfully used as support for laccase immobilization preserving and/or improving their catalytic efficiency. In the first moment, both application and operational costs have to be crucial factors to get the right choice of support. Furthermore, for the development of efficient immobilization protocols must be considered the use of materials with an appropriate surface area, in order to promote a good enzyme-substrate interaction, as well as increase the stability and the reuse of the catalyst. Table 2 presents a summary considering the advantages and disadvantages of the different material sources used for laccases immobilization.

Properly choosing the support to immobilize the laccase can be a challenge. With porous supports a large surface area is obtained, so a large amount of enzyme can be immobilized, and then more catalytic sites would be obtained in a smaller volume. However,

depending on the size of the substrate and in case of polymerization of the catalysis products this may not be a good option due to diffusional limitations. When inorganic supports were used it was possible to observe good catalytic efficiency, usually, they obtained a removal greater than 60% (See Table 1) in different applications. More attention should be paid when the inorganic gel was used, without mediator removals of about 24-50% were obtained. This decrease is caused by diffusional limitations.

When natural organic supports, such as alginate, chitosan, and agarose were used it was observed that the catalytic behavior showed a good performance. These supports are inexpensive, eco-friendly, and easy to handle. However, once they are hydrophilic, the separation procedure can be difficult. In contrast with that, synthetic polymers may have a high surface area, which makes them capable of loading a large amount of enzyme and its water affinity can be changed at the synthesis moment. Although they are usually synthesized with petroleum-based monomers, they are not renewable.

Table 2 - A summary of the properties of materials used as support for laccase immobilization.

	Advantages	*Disadvantages*
Inorganic materials	Reusability of the support, easy surface functionalization, high thermal and pH stability	Possibility of unspecific interactions, without surface modification, have weak laccase-support interactions, may present diffusional barriers
Biopolymers	Biocompatibility, eco-friendly, easy immobilization procedures	Usually needs functionalization, may present diffusional barriers
Synthetic polymers	High surface areas, capable of retaining a large amount of enzyme, strong binding, innumerous shapes and sizes	Difficult preparation procedures, usually not eco-friendly
Hybrids and composites	Strong and multipoint binding, limited enzyme leakage, possible customization	Expensive

Source: Modified from Zdarta; Meyer; Pinelo, 2018.

Thereafter, hybrid supports can be tailored targeting the behavior wanted under application, can be easily separated, presents high mechanical resistance and high removal efficiencies. Although several supports can be used for practical applications, should be weighed the costs, the immobilization procedure, and the operational stability, always with the application in mind. At the same time that inorganic carriers and organic carriers are low-costs materials, when applied it requires a filtration procedure. In another way, hybrids and composites materials can be easily separated, presents high mechanical resistance and high

removal efficiencies. Nevertheless, the cost of the immobilization procedure and consequently the cost of the final biocatalyst will be increased but, instead of that, the reuse possible after immobilization already lowers the price of the catalytic process.

2.6 TRENDS ON LACCASE IMMOBILIZATION: BIO-BASED MATERIALS

The worldwide market for plastics has been growing rapidly, and plastics polymers have been producing typically from petroleum-based chemicals. Strategies for a sustainable circular economy set the requirement for the wide use of bio-based materials, which are also biodegradable in various environments (BRIASSOULIS; MISTRIOTIS, 2018). Some synthetic polymers are made with specific modifications and used as matrices for enzymes immobilization, which can be an alternative for some not eco-friendly materials. Bresolin and co-workers synthesized green polyurethane foam (PUF) from a bio-polyol obtained by enzymatic glycerolysis and used it as a carrier for lipase NS-40116 immobilization. With an apparent density of 0.19 ± 0.03 g·cm^{-3} and an immobilization yield of $94 \pm 4\%$, the attachment of the enzyme to the support had increased its activity and the K_m remained almost the same. Lastly, the final support maintained their properties (compared to the common PU), and also it is considered an environment-friendly material (BRESOLIN *et al.*, 2019). A biocatalyst made with bio PU and laccase could be used for wastewater treatment, once it is robust support, easy separation and even can be applied to a continuous reactor.

Biomimetic or bioinspired silica is another technology that has been employed to reduce the impact of materials derivatives by fossil sources on nature. *In vitro* silica formation occur through reactions derived or similar to those occurring *in vivo* (CAZABAN; WILSON; BETANCOR, 2017). Production of nanostructured particles is carried out at close to neutral pH and room temperature under aqueous conditions (BEGUM *et al.*, 2015). Cazaban *et al.* made bio-inspired silica to use as lipase support for the synthesis of fatty acid methyl esters. A hyperactivation effect of the enzyme was observed when immobilized on the biomimetic inspired silica functionalized supports with a 420% increase in activity when offering 2.5 $mg_{protein} \cdot g_{support}^{-1}$ (CAZABAN *et al.*, 2018). The developed support was used for laccase immobilization and, after that, applied for phenol oxidation processes.

Nowadays, as a consequence of the global concern about reducing waste and contribute towards resource and environmental sustainability, the consumers demand green and sustainable products with minimal environmental impact (AHMED; LEFERINK;

SCRUTTON, 2019). The synthesis of bio-based polymers can be made by monomers from renewable sources and/or using enzymatic and microbial catalysis. Lignocellulosic materials are an abundant, inedible, and renewable resource which were recently used for the synthesis of several monomers by fermentation procedures, such as dicarboxylic acids, diamines, diols, phenylpropanoids and benzoic acids, which can be used to synthesize bio-based polymers like polyester, polyamides, polyurethanes, polyimides, and polybenzoxazoles (KAWAGUCHI; OGINO; KONDO, 2017; MENON; RAO, 2012). The microorganism metabolic engineering is also progressing and making possible the synthesis of monomers and polymers with clean routes (CHUNG *et al.*, 2015). Chemo-enzymatic routes have broadly been used to obtain bio-based polymers, such polyester and polyhydroxyalkanoates (PHA) (from bio-based monomers such as diacid monomers, chiral hydroxy acids and chiral lactones), terpene based biopolymers (from monoterpenes limonene, β-myrcene and α-, β-pinene) and polyurethanes (from diamines and polyols) (AHMED; LEFERINK; SCRUTTON, 2019). All these bio-based materials are a less aggressive alternative for some already based matrices that are generally used for laccase attachment. With a cautious study of the difference caused by the presence of a bio-sourced material or by its production method, these materials can become excellent supports for laccase immobilization.

2.7 CONCLUSIONS

Laccase has been used for the most diverse oxidative reactions. To improve its characteristics in the application and for being able to reuse it, the immobilization of this biocatalyst has been applied. The immobilization of the laccase leads to an increase in pH and temperature optimum range, mechanical resistance and facilitates the completion of the reaction due to the easy separation of the biocatalyst. The source of the material is of great importance at the time of choice. For each application, there is a better catalyst to be developed. The variety between them is immense, so before starting the whole process, one has to weigh all the factors involved, and then the intended objectives will be achieved.

CHAPTER 3

In this chapter, the methodologies and results concerning the laccase immobilization in rigid commercial and bio-based polyurethane foams were carefully described. Also, the final biocatalysts were physically and chemically characterized, and the operational behavior was investigated.

3 LACCASE IMMOBILIZATION USING COMMERCIAL AND BIO-BASED POLYURETHANE FOAMS AS SUPPORT

3.1 INTRODUCTION

In the latest decades has grown the concern for cleaner technologies. Biocatalytic systems are well known for their high catalytic activity, selectivity, and specificity under benign experimental and environmental conditions, a remarkably safer alternative than chemical and physical procedures (MAHMOODI; SAFFAR-DASTGERDI; HAYATI, 2020; MATEO *et al.*, 2007). Laccases (benzenediol: oxygen oxidoreductase E.C.1.10.3.2) are extracellular multicopper enzymes which are characterized by containing 4 catalytic copper atoms in their site (COPETE *et al.*, 2015). These enzymes are capable of catalyzing the one-electron oxidation of phenolic compounds, such as mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines, with the four-electron reduction of oxygen to water, without the use of hydrogen peroxide (BA *et al.*, 2013; FATARELLA *et al.*, 2014; KUNAMNENI *et al.*, 2008). Laccases have been investigated in many researchers due to their versatile catalytic applications. They can be used in organic synthesis of pharmaceuticals and dyes, beverage clarification, and also in the treatment of pollutants as dyes discoloration and endocrine disruptor chemicals degradation (DESKA; KONCZAK, 2019; LEGERSKÁ; CHMELOVÁ; ONDREJOVI, 2018; MILENA *et al.*, 2015; ZHU *et al.*, 2018). However, enzymes in their free state have some disadvantages such as instability under operational conditions, the rapid loss of activity, and difficult enzyme separation from the reaction products, limiting their industrial applications (DESKA; KONCZAK, 2019).

Enzyme immobilization has been used to intensify biocatalytic processes, since it enables the catalyst separation from the reaction mixture, improves the pH and temperature conditions of the enzyme under application and allows the reuse of the biocatalyst in successive catalytic cycles (FATARELLA *et al.*, 2014; HOU *et al.*, 2014). Immobilization can be done in different supports materials: inorganic, organic (natural or synthetic), and hybrids. Among this, synthetic polymers are versatile materials presenting many functional groups in the structure that allows the creation of relatively strong linkages between enzymes and support (DATTA; CHRISTENA, 2013).

In recent years, polyurethane foams (PUF) have been investigated as support for enzymes immobilization due to mechanical and solvent resistance and are also biocompatible

(BUSTAMANTE-VARGAS *et al.*, 2015; CADENA *et al.*, 2011). PUF, a porous polymeric matrix that has a repeating unit of urethane bonds in the structure, enables the *in situ* immobilization of enzymes using the entrapment method in the impermeable polymeric matrices during the foam expansion. (NYARI *et al.*, 2016; SILVA *et al.*, 2013). Obtained by reacting a diol (OH) and a diisocyanate (NCO) group in a polycondensation reaction, the PUF characteristics depend on the nature of the monomers used, changing the structure parameters as crosslink, density, urea content and pore cell structure (BERNARDINI *et al.*, 2015; CADENA *et al.*, 2010). PUF monomers are typically produced from petroleum-based chemicals that raising economic and environmental concerns (KAWAGUCHI; OGINO; KONDO, 2017).

Based on the need to find support that better operationally stabilizes the laccase and, given the utilization of a low-cost material for the immobilization of the enzyme, this work was done. This study intended to evaluate the one-step laccase immobilization into commercial polyurethane foam (PUF) and bio-based polyurethane foam (bioPUF), using a biopolyol obtained by enzymatic glycolysis of castor oil in a solvent-free system. Also, the optimal conditions of pH and temperature, storage stability, and reusability were investigated using 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) as a substrate. Furthermore, a kinetic study for both enzymatic derivatives was also carried out.

3.2 MATERIAL AND METHODS

3.2.1 Material

Laccase from *Trametes versicolor* (EC 1.10.3.2) and 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS, 99%) were purchased from Sigma-Aldrich (USA). Castor oil (Campestre Industry), commercial glycerol (Vetec, 99.5%), Tween 80 (Vetec, 65-80 mg (OH/g), and Novozym 435 (*Candida antarctica* B, kindly donated by Novozymes, Brazil, A/S) were used to obtain the biopolyol. Polymeric methylene diphenyl diisocyanate (pMDI - specflex NE 134) and commercial polyol were kindly donated by Dow Chemistry. Dibasic phosphate P.A. and citric acid were purchased from Neon (Brazil).

3.2.2 Biopolyol synthesis

Biopolyol synthesis was performed according to the glycerolysis method previously described by Valério *et al.* (2010). Castor oil and glycerol (molar ratio 1:6), 16 wt% Tween 80 as a surfactant, and 9 wt% of immobilized Novozym 435 as catalyst were added into a jacketed glass reactor. The reaction mixture was mixed and kept at 70 °C, 600 rpm for 3 h. The products obtained were vacuum filtered to remove the lipase. The solution containing two phases was separated using a separating funnel. Biopolyol was stored in the dark for later use.

3.2.3 Enzyme immobilization

Laccase from *Trametes versicolor* was immobilized by entrapment method through bulk polymerization in one step reaction during polyurethane foams synthesis. The enzyme concentration used ranged from 0.1 to 0.3 wt%. The laccase immobilization into commercial polyurethane foam (Lac-PUF) was carried out at room temperature. Lyophilized laccase was added to the commercial polyol and mixed with the help of a glass stick. After that, the diisocyanate was added and the expansion occurred after 60 seconds of mechanical stirring (2500 rpm), using 77 g of NCO per 100 g OH, according to manufactory instructions. The immobilization of laccase into bio-based polyurethane (Lac-bioPUF) was carried out as described previously by Bresolin *et al.* (2019). Briefly, the molar ratio diisocyanate to biopolyol (NCO:OH) used was 1:1 and distilled water was applied as a blowing agent (1 wt% concerning the mass of the monomers). The water and the lyophilized laccase were added to the biopolyol and mixed with the help of a glass stick. Thereafter, the diisocyanate was added. The monomers were mechanically stirred (2500 rpm) for about 90 seconds at room temperature. Both enzymatic derivatives, Lac-PUF and Lac-bioPUF, were stored at 4 °C for further use.

3.2.4 Determination of laccase activity

The oxidation rate of ABTS to $ABTS^{+}$ by laccase was monitored to determine the activity of the free and immobilized laccase (GARCÍA-MORALES *et al.*, 2018; RAHMANI *et al.*, 2015). For the free laccase activity, 0.3 mL of a laccase solution (1 $mg \cdot mL^{-1}$) 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. 0.3 mL of Milli-Q water was used instead of the free laccase as blank. For 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) in a HACH test tube. 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 was calculated using the molar extinction coefficient of ABTS (ε_{420} = 36,000 M^{-1} cm^{-1}) as shown in Equation 1 (CHILDS; BARDSLEY, 1975). One activity unit (expressed in $U \cdot L^{-1}$) is defined as the amount of enzyme necessary to oxidize 1 µmol of ABTS per min. All analysis were carried out in triplicate.

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

Where:

$\frac{U}{L}$: enzymatic activity

Δabs: absorbance variation

V: total reaction volume

ε: molar extinction coefficient

d: step length

v: sample volume

t: reaction time

3.2.5 Characterization of the developed materials

The characterization of the polyurethane foams with or without laccase was performed by Fourier transform infrared spectroscopy (FTIR) in Agilent Technologies (Cary 660 FTIR). Samples were analyzed in a zinc selenide tip and evaluated by transmittance in the region of 4000 - 660 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans. Structural morphologies of synthesized materials were analyzed by scanning electron microscopy (SEM) analysis with field emission (SEM-JEOL JSM-6390LV). The polyurethanes foams were cut and fixed with carbon tape on a stub and coated with gold.

3.2.6 Properties of free and immobilized laccase

3.2.6.1 Optimum temperature

The influence caused by temperature for free and immobilized laccase was investigated by measuring the ABTS oxidation reaction at different temperatures (20, 30, 40,

and 50°C). For both free and immobilized enzyme, the activation energy (*Ea*) was calculated by the Arrhenius plot as in the Equation 2 and then the enthalpy of activation (ΔH^*) were calculated at 40 °C (ABDEL-NABY *et al.*, 2017).

$$Slope = \frac{-E_a}{2.303\ RT} \quad (2)$$

Where the gas constant (R) = 8.314 J·K.mol and (T) absolute temperature in Kelvin.

3.2.6.2 Optimum pH

To evaluate the optimum pH activity for free and immobilized laccase, enzyme activity was measured at different pH and 40 ºC. Phosphate-citrate buffers (0.1M) on pH 3, 4, 5 and 6, and 0.1 $mg \cdot mL^{-1}$ of free enzyme and immobilized laccase.

3.2.6.3 Effect of substrate concentration

A range of ABTS concentration was tested (1.25 to 10 mM aqueous solutions). For the assays, 0.1 $mg \cdot mL^{-1}$ of the enzyme was used, with activities of 1038 $U \cdot L^{-1}$, 13219 $U \cdot g^{-1}$ (270 $U \cdot L^{-1}$) and 13577 $U \cdot g^{-1}$ (242 $U \cdot L^{-1}$) for free laccase, Lac-PUF, and Lac-bioPUF, respectively. Free and immobilized enzymes were assayed with 0.1 M citrate-phosphate buffer pH 4 and 40 °C for these substrate concentrations. The Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) were calculated from Lineweaver-Burk plot, from the intercepts at x- and y-axis of the plot, respectively, as shown the Equation 3:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} \quad (3)$$

Where:

V: reaction rate

V_{max}: maximum reaction rate

K_m: Michaelis-Menten constant

$[S]$: substrate concentration

3.2.6.4 Operational stability of immobilized laccase

The operational stability of immobilized laccase (Lac-PUF and Lac-bioPUF) was assessed through consecutive ABTS oxidation cycles. The residual activity assay was carried out at 40 ºC, 250 rpm and pH 4, and was assayed as described earlier. The activity of cycle 1

was taken as 100%. After each cycle, the immobilized laccase was washed three times with 0.1 M phosphate-citrate buffer pH 4. The residual activity was calculated as per Equation 4.

$$\textit{Residual activity (\%)} = [\textit{Activity in cycle } t_i \textit{ (U/g)} \times 100] / \textit{activity in cycle } t_1 \textit{ (U/g)} \quad (4)$$

Where:

Activity in cycle t_1*:* enzymatic activity in the first catalytic cycle

Activity in cycle t_i*:* enzymatic activity in the succeeding cycles ($i \neq 1$)

3.2.6.5 Storage stability

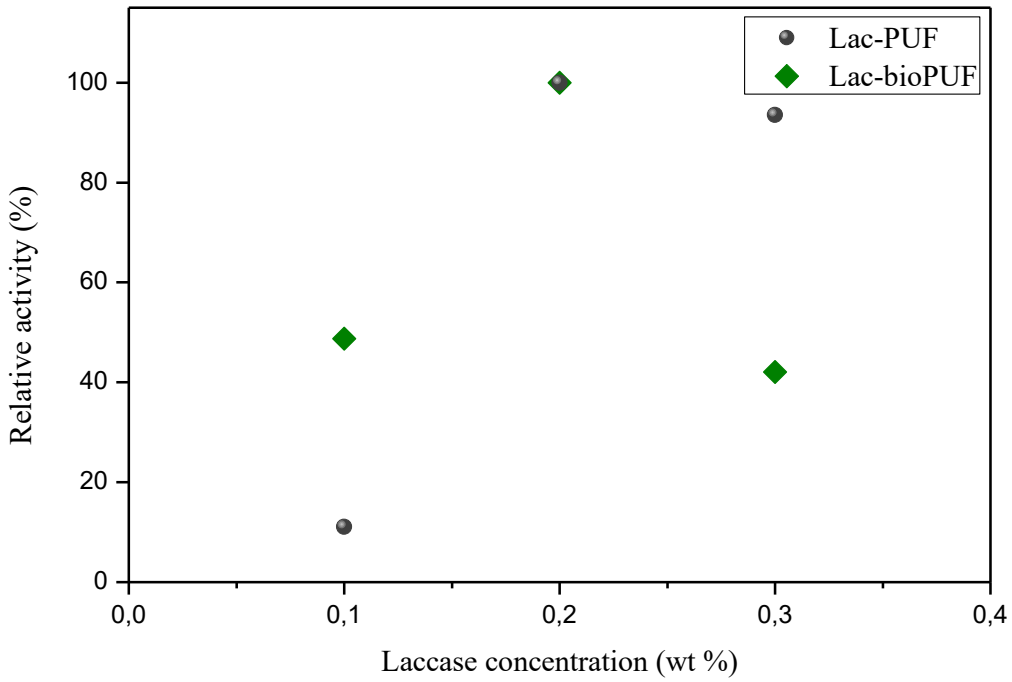
Storage stability was measured over 30 days. Samples of free and immobilized laccase were stored dry, without any buffer, at 4 °C and at room temperature, and the enzyme activity was monitored with ABTS oxidation. The storage stability was plot as residual activity considering the activity on the first day as 100%.

3.3 RESULTS AND DISCUSSION

3.3.1 Enzyme immobilization

Evaluation of the enzyme concentration used for immobilization was done observing the relative activities of the biocatalysts. Figure 7 shows the effect of the amount of enzyme used in the immobilization procedure. According to the results obtained, with 0.2 wt% of the enzyme the highest activity was obtained. Whilst the laccase concentration was 0.3 wt% a decrease in the relative activity was observed for both Lac-PUF and Lac-bioPUF. Similar results were also obtained for some supports previously used (KADAM; JANG; LEE, 2017; WEN *et al.*, 2019). This decrease occurred due to agglomeration or crowding of laccase molecules onto the surface of the support when a high enzyme concentration was used (LIU *et al.*, 2012). The agglomeration could limit the dispersion of laccase, change the conformation, and lead to a decrease in laccase activity (WEN *et al.*, 2019).

Figure 7 - Evaluation of the laccase concentration in immobilization procedure.



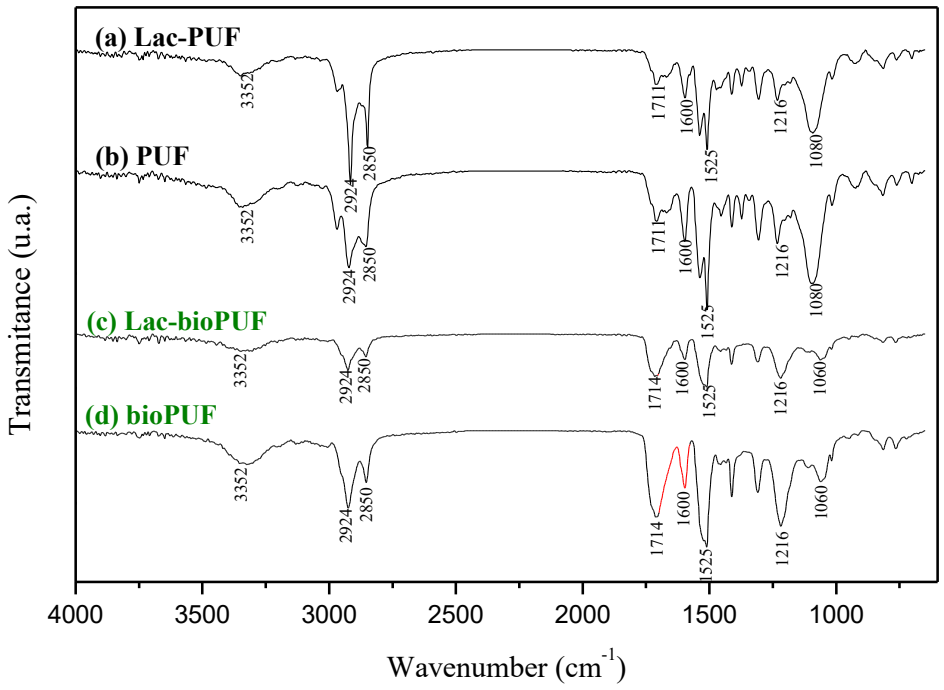
Source: from the author.

3.3.2 Characterization of the developed materials

To identify the functionals groups present in the structure of the polyurethane foams and to confirm the effective laccase immobilization, FTIR spectroscopy was used. Figure 8 shows the chemical transformations of the foams after the immobilization assay. The region at 1750 - 1700 cm^{-1} can be attributed to the carbonyl bonds from urethane. The absence of isocyanate peak (-NCO) was observed in the region of 2270 cm^{-1}, indicating that there is no presence of residual diisocyanate. It was possible to identify the groups -C-O-C, $-CH_2-O-CH_2$ and -C=C at 1216 cm^{-1}, 1060 $cm^{-1,}$ and 1525 cm^{-1}, respectively. Also, the presence of symmetric vibration of methyl linkage at 2924 cm^{-1} and 2850cm^{-1} was observed. The characteristic peak of O-H bond (between 3400 and 3300 cm^{-1}) after immobilization with laccase ((a) and (c)), is decreased in intensity. This tendency is shown due to the enzyme-PUF linkage region and the interaction between enzyme and support (BADRI *et al.*, 2010; BRESOLIN *et al.*, 2019; STUART, 2012). In the biocatalytic systems, as shown in the FTIR spectrum, changes in the intensity of the signals attributed to amide I band (1600 cm^{-1}) can be

noted. According to the analysis, the formation of both polyurethanes foam and the attachment to the matrix were successfully performed.

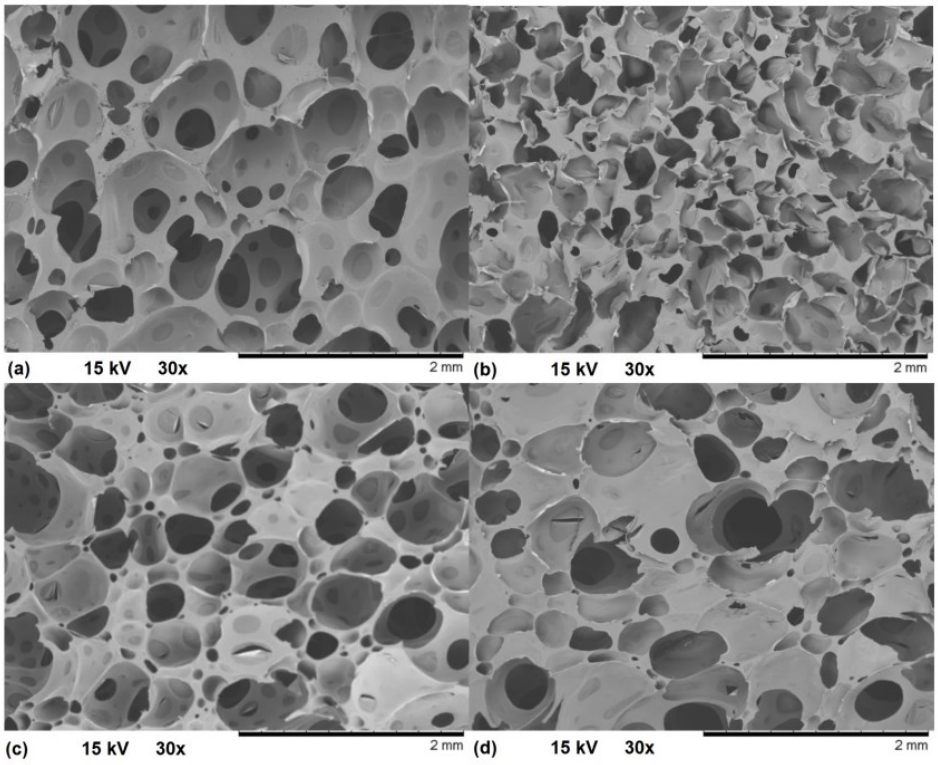
Figure 8 - Fourier transformed infrared spectra (FTIR) of (a) immobilized laccase in commercial polyurethane foam, (b) commercial polyurethane foam, (c) immobilized laccase in bio-based polyurethane foam and (d) bio-based polyurethane foam.



Source: from the author.

The morphology of commercial and bio-based foam before (Fig 9a, 9c) and after immobilization (Fig 9b, 9d) was determined based on SEM analysis. As shown in Figure 9, the immobilization procedure caused a change in cell size. However, the closed pore structure remained the same. Both materials (PUF, Lac-PUF, bioPUF, and Lac-bioPUF) presented homogeneous cells. During the expansion occurs the formation of CO_2, which is released, forming the bubbles. This characteristic of PU is responsible for the conformation of the foam and affects directly the mechanical resistance and can lead to operational problems when applied (BRESOLIN *et al.*, 2019).

Figure 9 - SEM images for commercial and bio-based polyurethane foams: (a) bio-based polyurethane foam, (b) laccase immobilized in bio-based polyurethane foam, (c) commercial polyurethane foam, and (d) laccase immobilized in commercial polyurethane foam.



Source: from the author.

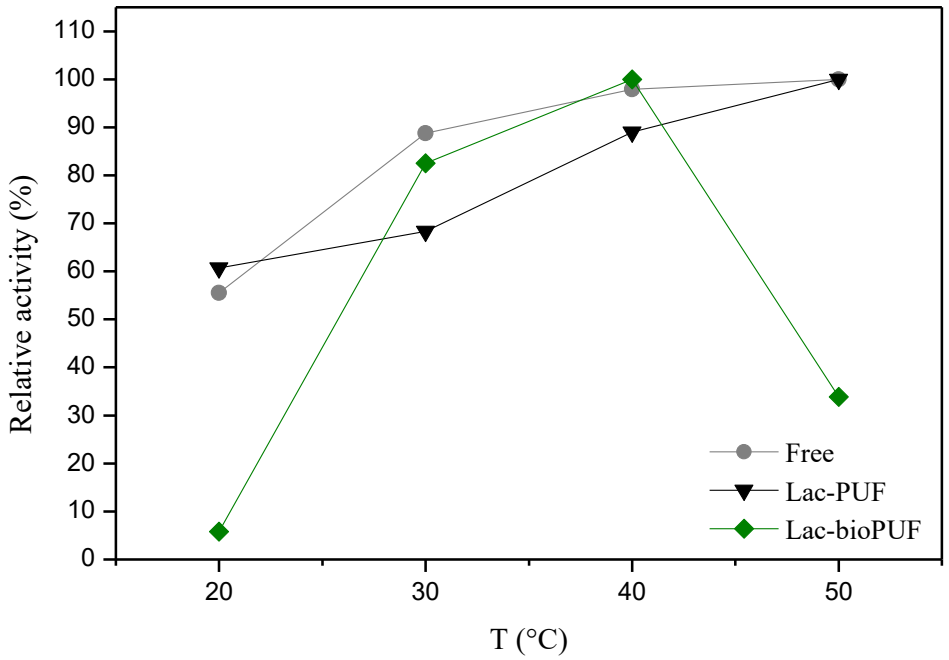
3.3.3 Properties of free and immobilized laccase

3.3.3.1 Optimum temperature

The effect of temperature on the enzymatic activity was tested over a temperature range from 20 to 50 °C, at pH 4. Relative activities of both the free and immobilized laccase as a function of temperature were compared in Figure 10. Free laccase shows high activities in temperatures between 30 and 50 ºC. For Lac-bioPUF the optimal temperature is ranging from 30 to 40 ºC. In the lower and higher temperature tested, (was 20 and 50 ºC) the activity decrease drastically, while, oxidation activities were higher than 68%, for Lac-PUF, in temperatures between 30 and 50 °C. Similar results were obtained by Zdarta *et al.* (2018b) when laccase from *Trametes versicolor* was immobilized into *Hippospongia communis* sponging scaffolds and applied for the oxidation of bisphenol A (ZDARTA *et al.*, 2018b). As observed, at 30 °C the Lac-PUF was only 1.3 folds lower than that of the free enzyme and

Lac-bioPUF. However, the optimum temperature of free laccase and Lac-PUF was slightly higher than that of the bio-based support material with a shift from 40 to 50 °C.

Figure 10 - Optimum temperature of the free and immobilized laccase.

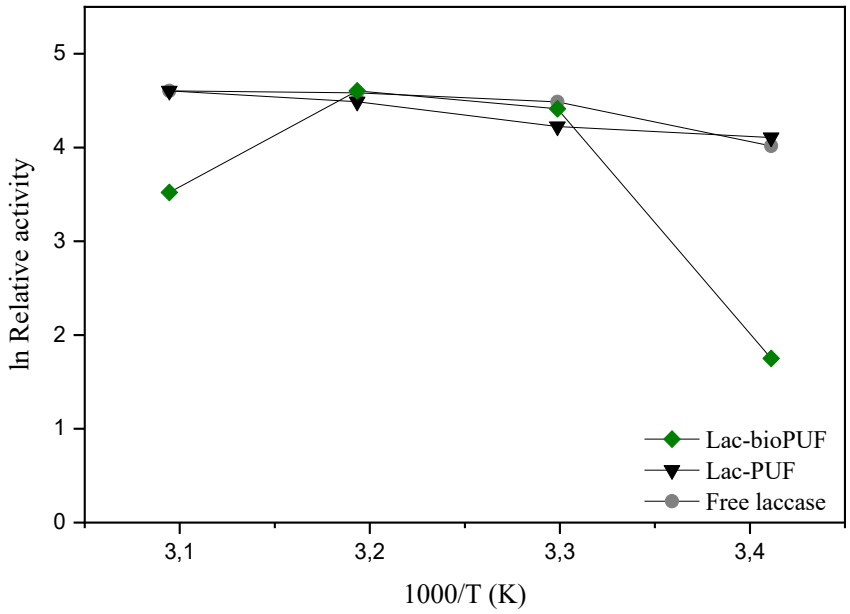


Source: from the author.

The activation energy was calculated by the Arrhenius plot in order to observe the modifications in the enzyme catalytic properties towards the effect of temperature after the immobilization procedure. Figure 11 shows the temperature effect in the activities of free and immobilized laccase. The calculated values of activation energy (*Ea*) of free and immobilized laccase was 15.68 kJ·mol^{-1}, 9.97 kJ·mol$^{-1,}$ and 10.94 kJ·mol^{-1} for free laccase, Lac-PUF, and Lac-bioPUF, respectively. Besides, the enthalpy of activation (ΔH*) was calculated with the obtained values for *Ea*. Enthalpy was lower at 40 °C for Lac-PUF (7.37 kJ.mol^{-1}) and for Lac-bioPUF (8.33 kJ.mol^{-1}) than for the free laccase (13.08 kJ.mol^{-1}). The lower values of activation energy (*Ea*) and the enthalpy of activation (ΔH*) for immobilized laccase indicate the high catalytic efficiency of this biocatalyst, once it requires lower energy for making activated complex enzyme-substrate. Lowering down the *Ea* and ΔH* of laccase after immobilization was previously reported (ABDEL-NABY *et al.*, 2017; WEHAIDY *et al.*,

2019). The tri-phasic nature of Arrhenius plot showed in Figure 11 for Lac-bioPUF explained that the enzyme exhibited two conformations up to optimum temperature (40 °C), and beyond transition point, the immobilized enzyme activity was declined, indicating inactivation at higher temperatures (JAVED *et al.*, 2009).

Figure 11 - Arrhenius plots of temperature data of the free and immobilized laccase.

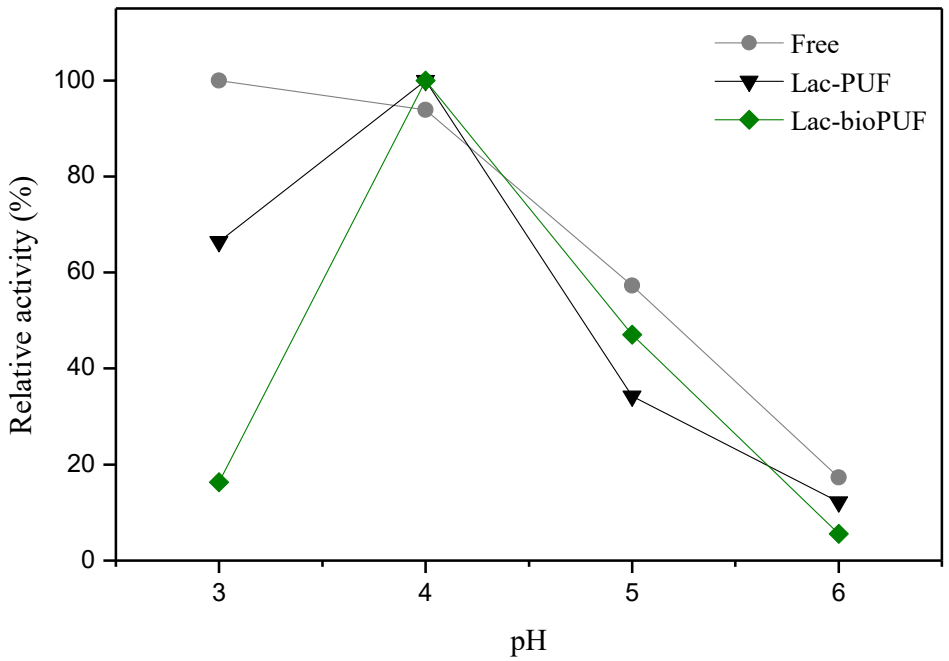


Source: from the author.

3.3.3.2 Optimum pH

In addition to temperature, the pH of the solution significantly affects the laccase catalytic activity. Figure 12 shows the results of the pH relative activity of the free and immobilized enzyme, studied at various pH values (3-6). It is possible to observe in Figure 12 that maximum activity values of the free laccase were at pH 3 and for both immobilized enzymes were obtained at pH 4. Immobilized enzyme activity slightly shifted toward higher pH value as has been reported previously (MOHAMMADI *et al.*, 2018; SALAMI *et al.*, 2018). The pH stability of the free laccase revealed retention of 93% activity at pH 4. For both free and immobilized laccases, activity decreased with the increase of pH (above pH 4). The similarity of the free enzyme and immobilized behavior under higher pH shows that ionization of the amino acid residues at the active site remains unaffected by the immobilization process (WEHAIDY *et al.*, 2019).

Figure 12 - Optimum pH of the free and immobilized laccase.



Source: from the author.

3.3.3.3 Effect of substrate concentration

The kinetic parameters K_m and V_{max} values were determined through the Lineweaver-Burk plot for the ABTS oxidation to evaluate the changes in the substrate-enzyme affinity. Table 3 shows the obtained values for K_m and V_{max} and the specificity constant of free and immobilized laccase. K_m value of Lac-PUF (5 mM) increased 1.8 times compared to free laccase (2.67 mM), and Lac-bioPUF (30 mM) increased 11.2 times. An increase in K_m after immobilization indicates a decrease in the affinity for the substrate comparing with the free enzyme. The increase of K_m can be attributed to internal and external limitations, such as a change in microenvironment of the enzyme, conformational changes of the protein, diffusional resistance to substrate partitioning or as mass transfer resistance and limited accessibility of active sites after immobilization (HOU *et al.*, 2014). Moreover, there are similar studies regarding the increase of K_m value after laccase immobilization which indicates a low affinity of the enzyme towards its substrate in respect do the free form (AMIN *et al.*, 2018; BAYRAMOGLU; KARAGOZ; ARICA, 2018; BRUGNARI *et al.*, 2018; FATARELLA *et al.*, 2014; REDA; HASSAN; EL-MOGHAZY, 2018). This increase in K_m

value shows that the bound formation between the enzyme and support favored the stability of the enzyme but it decreased its flexibility, which is essential for substrate binding (BAYRAMOGLU; KARAGOZ; ARICA, 2018; CELIKBICAK *et al.*, 2014) However, V_{max} value of Lac-PUF and Lac-bioPUF increased after immobilization. These data indicate that the immobilization procedure caused opposite modifications on affinity (K_m increase, affinity decreased) and maximal catalytic capacity (increased), which will influence the catalysis time (BRUGNARI *et al.*, 2018). This behavior was observed using ABTS as a substrate. It is important to note that when another substrate is applied, due to the difference in the characteristics of its structures, the behavior may be different.

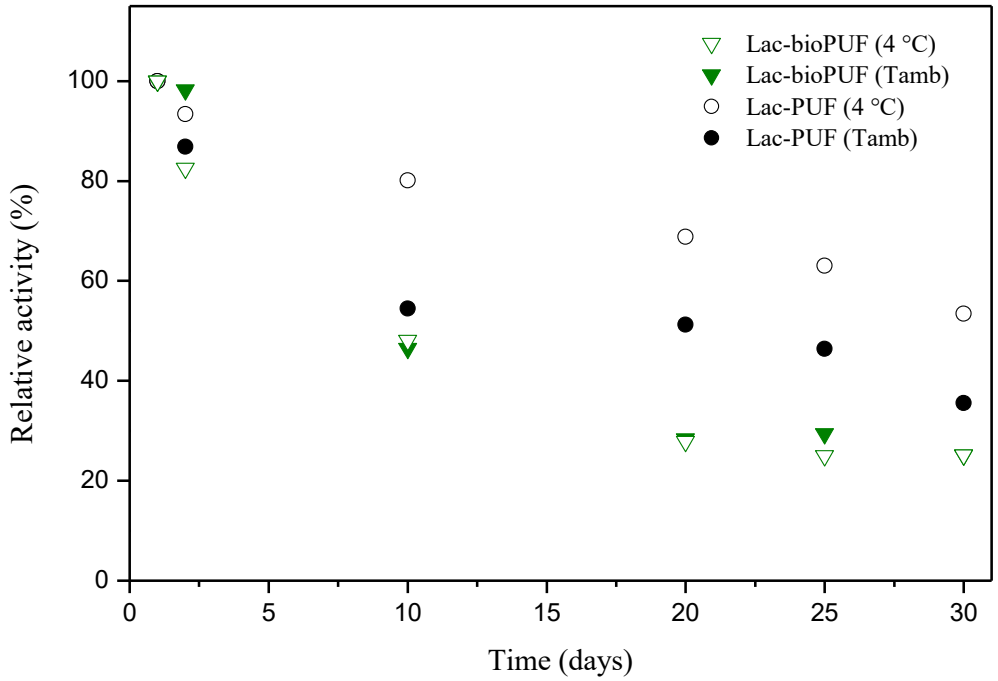
Table 3 - Kinetic parameters of catalysis by free and immobilized *Trametes versicolor.* laccase.

Parameter	**Free**	**Lac-PUF**	**Lac-bioPUF**
K_m **(mM)**	2.67	5.00	30.00
V_{max} **(µmol.L^{-1}.min^{-1})**	1666.67	25000.00	100000.00
$V_{max}.K_m^{-1}$	624.22	5000.00	3333.33
Ea **(kJ.mol^{-1})**	15.68	9.97	10.94
ΔH^* **(kJ.mol^{-1})**	13.08	7.37	8.33

3.3.3.4 Storage stability

At the end of 30 days of storage, at ambient temperature, the entrapped laccases, Lac-PUF and Lac-bioPUF retained 35% and 25% of their original activities, and when stored at 4 °C, 53% and 25% residual activities were observed, respectively. As shown in Figure 13, the laccase immobilized in the commercial polyurethane foam exhibit higher stability for the storage at 4 °C after 30 days. However, the same biocatalyst when stored at ambient temperature had a decrease of 65% in the activity. Laccase immobilized in the bio-based polyurethane did not present to be stable when stored, once in both storage temperatures, a decrease of 75% was observed after the 30 days.

Figure 13 - Effect of storage time on the activities of immobilized *Trametes versicolor* laccase.



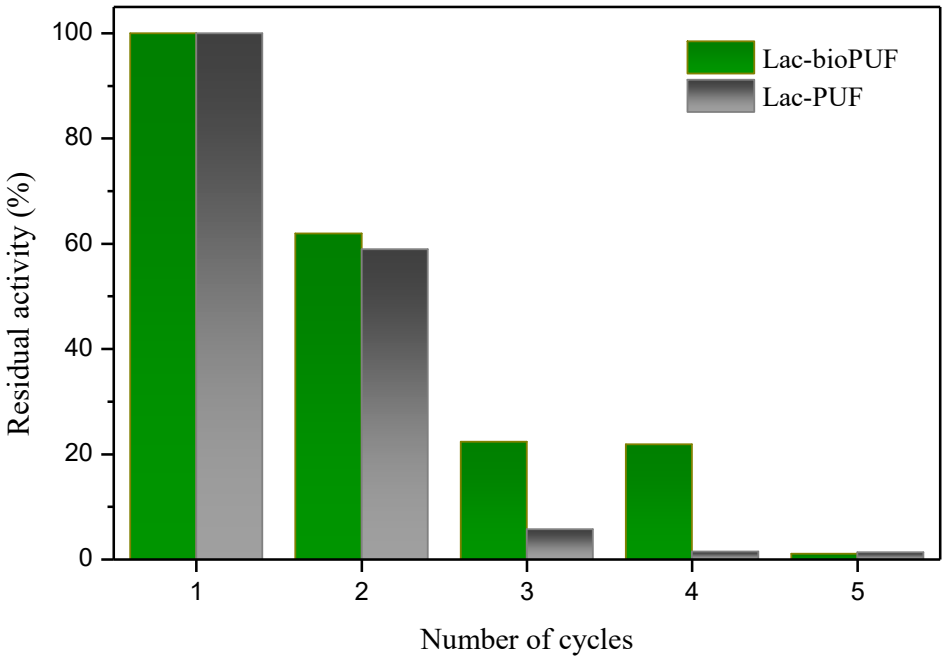
Source: from the author.

3.3.3.5 Operational stability of immobilized laccase

The determination of biocatalyst operational stability is important to evaluate the binding force between the enzyme and the matrix. It has already been proven that free laccase is not capable of retaining its catalytic activity after one degradation cycle and, moreover, the soluble-enzyme separation from the reaction mixture is difficult (ZDARTA *et al.*, 2018b). Reusability of laccase immobilized on the rigid commercial and bio-based polyurethane foam was realized at pH 4 and 40 °C. Figure 14 shows the catalytic behavior of the immobilized enzyme in the degradation of ABTS during 5 cycles. As seen in Figure 14, the immobilized laccase in a catalytic reaction with ABTS as substrate retained about 60% of their initial activity after two catalytic cycles for both foams used. In the third cycle, a loss of more than 80% of the initial activities was observed. The decrease in the catalytic activity after several cycles might point out the weak binding between the enzyme and the support. Partial enzyme leakage from the matrix and the partial inactivation of the immobilized laccase due to its utilization in repeated cycles could cause this decrease in enzyme activity (SADIGHI; FARAMARZI, 2013). Nevertheless, during the handling and analysis of the samples, some

conditions could damage their surfaces, such as multiple washing steps and the agitation used to provide a homogenous reaction mixture (BAYRAMOGLU; KARAGOZ; ARICA, 2018). Laccase immobilized on kaolinite lost 50% of its initial activity after 2 cycles of ABTS transformation (WEN *et al.*, 2019). The same laccase from *Trametes versicolor* immobilized covalently on polyamide 6/chitosan nanofibers lost 50% of its initial activity after 3 catalytic cycles (MARYŠKOVÁ *et al.*, 2016).

Figure 14 - Operational stability of the immobilized laccase in commercial and bio-based polyurethane foams in 0.1 M citrate-phosphate (pH 4) buffer containing 0.5 mM ABTS.



Source: from the author.

3.4 CONCLUSIONS

Laccase from *Trametes versicolor* was successfully immobilized *in situ* using rigid commercial and bio-based polyurethane foams as supports. From the data presented, it can be concluded that the best support for laccase immobilization was the commercial polyurethane foam. In general, both polyurethanes foams showed to improve the enzymatic properties and enabled its reuse. This study suggested that these biocatalysts have great potential to be designed for a packed bad reactor and then be used for several biocatalytic reactions.

4 SUGGESTIONS FOR FUTURE WORKS

- Perform biodegradation and enzymatic degradation tests of polyurethane and bio-based polyurethane foams used as supports, to find out if the material from a renewable source is more easily degraded;
- Functionalize the surface with groups that allow covalent enzyme-support binding and probably improving the laccase features for biocatalysis;
- Apply the biocatalysts in the oxidation of polycyclic aromatic hydrocarbons in contaminated soils and aquifers;
- Apply the biocatalysts obtained in a packed bed reactor for the degradation of anticancer drugs and evaluate the toxicity of the compounds before and after the treatment.

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