Universidade do Minho
Escola de Engenharia

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Laccase: a green catalyst for biosynthesis of poly-phenols

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Tese de Doutoramento em Engenharia Química e Biológica

Trabalho efetuado sob a orientação do
Professor Doutor Artur Cavaco-Paulo
e da
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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Abstract

Laccase: a green catalyst for biosynthesis of poly-phenols

Laccase is one of the multi-copper oxidases which can catalyze the oxidation of phenols, aromatic amines and other compounds using oxygen as the terminal electron acceptor. As an environment protecting biocatalyst, laccase shows great potential applications in various fields and industries, including textile industry, pulp and paper industry, environmental pollutant conversion and others In the field of synthesis, laccase catalyzed reactions have been widely investigated. However, the mechanism of laccase catalyzed aromatic compounds still needs to be further explored. The research and development of laccase on the polymer making study meet with requirement of the green chemistry, which will promote the application of the synthetic polymer materials. This PhD thesis intends to explore new strategies and mechanisms for the synthesis of aromatic polymers using different laccase forms and different reactors. Aromatic polymers were prepared using laccase from ascomycete *Myceliophthora Thermophila*. Different modifications of laccase were designed to improve its performance, including PEGylation and/or immobilization. Molecular modeling simulations were used to predict the geometry and structural changes of laccase. During the polymerization process, three reactors with different energy environment were applied, and the function of high-energy environment was investigated with the assistance of homology modelling and molecular simulation. The polymers obtained were characterized and applied onto different fabrics to achieve multi-functional fabrics. This study will provide fundamental knowledge for the polymer synthesis by laccase, and offer the theoretical and technical support for the establishment of high efficiency laccase-catalyzed synthesis system.

Firstly, the polymerization of catechol was conducted using laccase as catalyst. Polyethylene glycol (PEG) was used in both non-covalent and covalent modification of laccase, then the performance of modified laccase was evaluated. The catalytic performance of different laccases was compared in both aqueous and gel phases. The results show that both non-covalent and covalent modification of laccase using PEG (PEGylated laccase) could promote the polymerization and improve the polymerization yield, as well as the degree of polymerization. However, these events were only detected in aqueous state. Molecular simulation shows that the presence of PEG slows down the inactivation of laccase. Later, the immobilization of laccase and PEGylated laccase was performed with epoxy resins as the carrier, using PEG also as linker compound to connect laccase and epoxy resin. The performance of immobilized laccase in aqueous solution for polymerization was explored and the structure of the polymer formed was proposed. After immobilization, the half-life time of laccase was improved, as well as the stability. The MALDI-TOF MS analysis showed that, when epoxy-native laccase, epoxy-PEGylated laccase and epoxy-PEG-laccase were used, the degree of polymerization was enhanced with the presence of immobilized laccase.

Secondly, different reactors namely water bath, ultrasonic bath and high-pressure homogenizer were applied to perform the polymerization of catechol. The activity and stability of laccase in those reactors were discussed. The pathway and mechanism of laccase synthesis in high-energy environment were investigated. The polymers were characterized to speculate their structures. The results showed that high-energy environment promote the interaction between enzyme and substrate, incrementing the polymerization yield. The conversion yield when using the ultrasonic bath and high-pressure homogenizer was higher than when water bath was applied. Laccase under ultrasound and high-pressure homogenization showed less stability compared with under normal water bath, however, the polymerization proceeds earlier than this inactivation, thus no obvious negative effect on the synthesis was detected. The performance of native, PEGylated and Epoxy-PEGylated laccases was studied under the high-pressure homogenizer. Their activity and stability were compared and the corresponding polymers produced were evaluated. Both PEGylated laccase and Epoxy-PEGylated laccase showed the greatest catalytic properties and stability.

Afterwards, catechol and *p*-phenylenediamine were polymerized using polyester (PET), cotton and wool as enzyme container in a high-pressure homogenizer using laccase as catalyst. The functionalization of fabrics was achieved via *in-situ* polymerization of aromatic substrate onto fabric containers. Both polymers, poly(catechol) and poly(*p*-phenylenediamine), present good thermal stability and resistance to thermal degradation, as well as free radical scavenging ability. Colored polymers were generated which conferred color to the fabrics. The scanning electron microscopy (SEM) observation shows uniform distribution of the polymer on the surface of cotton, wool and PET. All the fabrics reveal color fastness to washing, sunlight and rubbing. The conductivity of fabrics was determined after treatment with poly(*p*-phenylenediamine) and all the fabrics showed good conductivity. Both polymers are able to confer antimicrobial activity to all the coated fabrics against Gram positive (*S. aureus*) and Gram-negative (*E.coli).* The cytotoxicity tests performed on functionalized fabrics revealed that both polymer diffusion and porous fabric structure may affect cell viability, which could be avoided by the adjustment of the polymer concentration.

This project aims to study the impact of the enzyme modification and processing conditions on the structure of the multi-functional reaction products, and aims to set up a high efficiency reaction system for laccase-catalyzed synthesis of a wide range of phenolic compounds. The oligomers/polymers obtained are supposed to show different functions to be applied on textile, medical and other areas.

Keywords

Laccase, polymerization, protein modification, high-energy environment, fabric functionalization

Resumo

Lacase: um catalisador verde para a biossíntese de polifenois

A lacase é uma das oxidases multicobre que é capaz de oxidar compostos fenólicos, aminas aromáticas e outros compostos usando o oxigénio como aceitador de electrões. Sendo um biocatalisador ambientalmente aceite, a lacase mostra elevado potencial em diversas áreas de investigação assim como ao nível industrial, incluindo a Industria Têxtil, a Industria do papel, e na área de conversão de agentes poluentes, entre outros. No campo da síntese, as reações catalisadas pela lacase têm sido intensamente investigadas, no entanto o mecanismo de catálise de compostos aromáticos necessita de ser estudado em maior profundidade. A pesquisa e desenvolvimento da lacase na produção de polímeros atendem aos requisitos da química verde, o que promoverá uma vasta aplicação dos materiais poliméricos sintéticos. Esta tese de doutoramento pretende explorar novas estratégias e mecanismos para a síntese de polímeros usando lacase em diferentes formas e diferentes tipos de reatores. Os polímeros aromáticos foram produzidos usando a lacase do fungo ascomicete *Myceliophthora Thermophila*. Foram desenhadas diferentes modificações para a lacase de modo a incrementar a sua performance, que incluíram a PEGilação e/ou imobilização. Ao mesmo tempo foram realizadas simulações de modelação molecular de forma a prever a geometria e as modificações estruturais da enzima. Durante o processo de polimerização, foram estudados três tipos de reatores com distintos níveis de energia associados. Os polímeros obtidos foram caracterizados e aplicados em diferentes substratos de forma a obter tecidos multi-funcionais. Este estudo visa fornecer conhecimento fundamental sobre a síntese de polímeros pela lacase promovendo ao mesmo tempo suporte teórico e técnico para o estabelecimento de sistemas catalíticos eficientes.

Primeiramente, a polimerização do catecol foi feita usando a lacase como catalisador. O polietilenoglicol (PEG) foi usado na modificação não covalente e covalente da lacase sendo a sua performance posteriormente avaliada. A performance catalítica das diferentes lacases PEGiladas foi comparada em fase aquosa e em gel. Os resultados demonstram que a lacase modificada, quer de forma não-covalente ou covalente (lacase PEGilada), é capaz de promover a polimerização do catecol, aumentando quer o grau de conversão quer o grau de polimerização. No entanto, estes eventos foram unicamente detetados em fase aquosa. Os estudos de modelação molecular demonstram que a presença do PEG desacelera a desativação da enzima em fase aquosa. Posteriormente, foi efetuada a imobilização da lacase nativa e da lacase PEGilada em resina epoxy, usando polietilenoglicol como agente de ligação entre a enzima e o suporte. A performance de polimerização em fase aquosa das enzimas imobilizadas foi explorada e a estrutura dos polímeros obtidos foi proposta. Depois de imobilizada, o tempo de meia-vida da lacase foi incrementado assim como a sua estabilidade. A análise de MALDI-TOF MS revelou um aumento do grau de polimerização quando as enzimas imobilizadas, epoxy-lacase nativa, epoxy-PEGilada e epoxy-PEG-lacase, foram usadas como catalisador.

Posteriormente, foram estudados diferentes reatores para a polimerização do catecol, nomeadamente banho termostatizado com agitação orbital, banho de ultrassons e homogeneizador de alta pressão. A atividade e

estabilidade da lacase nesses reatores foram primeiramente avaliadas assim como o mecanismo de síntese do catecol em presença de ambientes de elevada energia. Os polímeros obtidos foram caracterizados sendo proposta a sua estrutura final. Os resultados demonstraram que ambientes de maior energia fornecem uma maior interação entre a enzima e o substrato, incrementando assim o rendimento de polimerização. O grau de conversão aquando da utilização quer do banho de ultrassons quer do homogeneizador de alta-pressão foi superior ao obtido quando a polimerização foi efetuada no banho termostatizado com agitação orbital. A lacase sob efeito de ultrassons ou do homogeneizador de alta pressão revelou menor estabilidade que quando sob o efeito do banho termostatizado. No entanto, dado que a polimerização ocorre antes dessa inativação, os ambientes de maior energia não provocam efeito negativo sobre a síntese do polímero. A performance catalítica das lacases, nativa, PEGilada e epóxi-PEGilada, foi avaliada quando sob o efeito do homogeneizador de alta pressão. Foi comparada a atividade e estabilidade assim como os correspondentes polímeros produzidos. As formas da lacase, PEGilada e Epoxi-PEGilada, revelaram a maior atividade catalítica assim como maior estabilidade.

Em seguida, o catecol e a *p*-fenilenodiamina foram polimerizados no homogeneizador de alta-pressão pela lacase usando sacos feitos de poliéster (PET), algodão e lã que serviram de recipientes da enzima. A funcionalização dos tecidos foi conseguida através da polimerização *in situ* dos substratos aromáticos sobre os tecidos contendo a enzima. Ambos os polímeros, poli(catecol) e poli(*p*-fenilenodiamina), apresentam aceitável estabilidade dimensional e resistência à degradação térmica, assim como capacidade de eliminação de radicais livres. Foram obtidos polímeros com cor com capacidade de coloração dos tecidos usados. As observações efetuadas por microscópio eletrónico de varredura mostram uma distribuição uniforme do polímero na superfície dos tecidos de poliéster, algodão e lã, tendo os mesmos revelado alguma resistência à lavagem, à luz solar e à fricção. Os tecidos após funcionalização com poli(*p*-fenilenodiamina) revelaram condutividade elétrica.

Ambos os polímeros produzidos foram capazes de conferir capacidade antimicrobiana aos tecidos com eles funcionalizados contra bactérias Gram positiva (*S. aureus*) e Gram negativa (*E.coli).* Ensaios de toxicidade efetuados nos tecidos funcionalizados revelaram que a difusão dos polímeros, depende da porosidade dos tecidos, e afeta diretamente a viabilidade celular, que pode ser aumentada ajustando a concentração do polímero.

Este projeto visa o estudo do impacto da modificação da enzima e das condições de processamentos na estrutura dos produtos de reação com funções múltiplas, tendo ao mesmo tempo o objetivo de estabelecer um sistema reacional de catálise de uma panóplia de compostos fenólicos pela lacase. Os oligomeros e/ou polímeros produzidos poderão ter diferente aplicações em áreas como têxtil, médica, entre outras.

Palavras-chave

Lacase, polimerização, modificação de proteínas, ambiente de elevada energia, funcionalização de tecido

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List of Abbreviations

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

APS: ammonium persulfate

BPA: bisphenol-A

DHB: 2,5-dihydroxybenzoinc acid

DMSO: dimethyl sulfoxide

DP: degree of polymerization

EDC: dichloroethane

ET: electron transfer

FF: force field

HBT: 1-hydroxybenzotriazole

HPH: high-pressure homogenizer/homogenization

LAC: laccase

LMS: laccase-mediator system

MALDI-TOF MS: Matrix Assisted Laser Desorption/Ionization-Time of flight Mass spectrometry

MCD: magnetic circular dichroism

MD: molecular dynamic

MPEG: methoxy poly (ethylene glycol)

Mn: number average molecular weight

Mw: weight average molecular weight

MTX: methotrexate

NMR: nuclear magnetic resonance

NPT: isothermal-isobaric ensemble

NVT: canonical ensemble

OD: optical density

PBS: phosphate-buffered Saline

PEG: poly(ethylene glycol)

PEGMDE: poly(ethylene glycol) monododecyl ether

PET: polyester

PZ: promazine

SA: Simulated Annealing

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM: scanning electron microscope

SPC: simple point charge

Sulfo NHS: N-hydroxysulfosuccinimide

$T_{5\%}$: temperature where polymers reach 5% of weight loss

$T_{50\%}$: temperature where polymers reach 50% of weight loss

TCFOH: the total content of free OH

TEMED: tetramethyl ethylenediamine

TGA: thermogravimetric analysis

TNC: tri-nuclear cluster

US: ultrasonic bath

UV-vis: ultraviolet and visible spectrophotometer

WB: water bath

XAS: x-ray spectroscopy

1,4-PDA: 1,4-bis(acryloyl)piperazine

4-ABSA: 4-aminoazobenzene-4-sulfonic acid

4-ABN: 4-aminobenzonitrile

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Motivation and Thesis Outline

Scope and objectives

The main goal of this project was to produce functional phenolic polymers using laccase as green catalyst. We foresee that a modified laccase and a reactor with high energy environment would lead to high polymerization yields and high degree of polymerization.

To achieve these goals, laccase was modified with polyethylene glycol (PEG), and immobilized onto epoxy resins. Polyethylene glycol was used as a spacer between laccase and the epoxy resins. These modified laccases are expected to have a better thermostability and a better accessibility towards substrate compared with native laccase. In this thesis, three different reactors were used to perform the synthesis of phenolics catalyzed by laccase, namely a water bath, an ultrasonic bath and an high-pressure homogenization. Reactors like ultrasound and high-pressure homogenizer with high energy environment are expected to promote the synthesis of phenolic compounds using laccase enzyme or modified laccase enzyme. The polymers obtained are expected to retain a better thermostability and less toxicity which could be applied in textile, medical, biosensor and other areas.

The main focus points of this work are:

- Laccase modification by Polyethylene glycol
- Immobilization of laccase onto epoxy resins
- Molecular simulation of geometry conformation of laccase and modified laccase
- Evaluation of the effect of reactors with different energy on the catalyzes reaction
- Set up the synthesis system for the phenolic polymerization
- Characterization of the oligomers/polymers

Thesis layout

This thesis is divided into six chapters, being chapters 2, 3, 4 and 5 dedicated to reporting of the experimental work. The contents of each chapter are summarized below:

Chapter I. Laccase: A green catalyst for biosynthesis of poly-phenols (State-of-art about laccase-catalyzed polymerization)

The purpose of this chapter is to present a comprehensive overview of the laccase-catalyzed polymerization of phenolic compounds using the oxygen in the air. The applications reviewed herein present laccase as a green alternative to the current physicochemical methods which are environmentally unfriendly, costly and less specific. This class of enzymes are of great relevance both as a model for structure/function relationship studies as well as green tools for biotechnology industries. This chapter contextualizes the field of application of the subject of this thesis.

The review of these concepts is presented on the following publication:

Jing Su, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. Laccase: A green catalyst for biosynthesis of poly-phenols. Critical reviews in biotechnology, 2017, 38(2), 294-307.

Chapter II. PEGylation greatly enhances laccase polymerase activity

The chemical modification of laccase to increase its stability will be conducted. The chemical PEGylation was performed by following the methodology described by Daly et al. PEGylation is the covalent attachment of one or more molecules of PEG to a protein. PEGylation can improve thermal stability and reduce deactivation of the enzyme, and also increase the biocatalyst lifetime. Molecular dynamic simulations were conducted to understand the molecular behavior of laccase upon laccase modification and laccase catalyzed polymerization.

Chapter 2 is based on the following publication:

Jing Su, Jennifer Noro, Ana Loureiro, Madalena Martins, Nuno. G. Azoia, Jiajia Fu, Qiang Wang. Carla Silva, Artur Cavaco-Paulo. PEGylation greatly enhances laccase polymerase activity. ChemCatChem, 2017, 9, 3888-3894.

Chapter III. Exploring PEGylated and immobilized laccases for catechol polymerization

The native and PEGylated laccase forms were immobilized onto epoxy resin according to the method previously described by Berrio et al. The epoxy resins were chosen as supports due to their well-known chemistry described in literature. Reported data show considerable enhancement of laccases stability when silanized and glutaraldeyde-activated silica nanoparticles are used as

supports. The immobilization of laccase onto epoxy resins-Eupergit C and a substantial stabilization effect against pH and temperature was observed upon immobilization. The effect of different laccase modifications like PEGylation and immobilization on the activity and stability of laccase were evaluated, and the effect of different laccase forms on the polymerization was studied.

Chapter 3 is based on the following publications:

Jing Su, Jennifer Noro, Ana Loureiro, Madalena Martins, Nuno. G. Azoia, Jiajia Fu, Qiang Wang. Carla Silva, Artur Cavaco-Paulo. PEGylation greatly enhances laccase polymerase activity. ChemCatChem, 2017, 9, 3888-3894.

Jing Su, Jennifer Noro, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. Exploring pegylated and immobilized laccases for catechol polymerization, AMB Express, 2018, 8, 134.

Chapter IV. The effect of high-energy environments on the structure of laccase-polymerized poly(catechol)

The role of different high-energy environments on the laccase-assisted polymerization of catechol was evaluated. For this, three different reactors were used namely a water bath (WB), an ultrasonic bath (US) and a high-pressure homogenizer (HPH). The polymerization was followed during time by UV-Vis spectra analysis of the color change. The produced polymers were characterized by Matrix Assisted Laser Desorption/Ionization-Time of flight Mass spectrometry (MALDI-TOF) and 1H NMR. The activity and stability of laccase during processing was evaluated to study the effect of high-energy environments on the activity and stability of laccase. Molecular dynamic simulations were conducted to understand the molecular behaviour of laccase upon different energy environments.

Chapter 4 is based on the following publication:

Jing Su, Tarsila G. Tallian, Jennifer Noro, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. The effect of high-energy environments on the structure of laccase-polymerized poly(catechol). Ultrasonics Sonochemistry, 2018,48,275-280.

Chapter V. Enzymatic polymerization of catechol under high-pressure homogenization for the green coloration of textiles

Laccase was used in this chapter to catalyze the polymerization of catechol under high-pressure homogenization for the green coloration of textile substrates. The polymerization of catechol was conducted using laccase in different forms. All enzyme forms were deposited inside a polyester fabric bag during the experiments. The oxidation of catechol conducted under high-pressure homogenization can be an efficient methodology for the *in situ* coloration of textiles. This experimental is set-up as a promising greener coloration/coating methodology involving milder conditions than the normally used in textile processes.

Chapter 5 is based on the following publication:

Jing Su, Jennifer Noro, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. Enzymatic polymerization of catechol under high-pressure homogenization for the green coloration of textiles. Journal of cleaner production, 2018, 202, 792-798.

Chapter V. Can laccase-assisted processing conditions influence the structure of reaction products? (Discussion and Conclusions)

Chapter 6 contains a summarized discussion focused on the contributions of this work towards a better understanding of laccase-catalyzed polymerization of phenolics. The conclusions and discussion presented at this chapter point out laccase enzyme as the main trigger point for a cascade of oxidation reactions which brings to the formation of complex polymeric structures. The particular case-studies about poly(catechol) oxidation demonstrated that both enzyme modifications and reactors might lead to the formation of different polymers.

Chapter 6 is based on the following publication:

Jing Su, Jiajia Fu, Carla Silva, Artur Cavaco-Paulo**.** Can laccase-assisted processing conditions influence the reaction products? Trends in biotechnology, 2019, DOI: 10.1016/j.tibtech.2019.03.006.

Appendix sections

The thesis ends with the appendix section that includes a list of the author's publications in the related fields.

Chapter I

Laccase: A green catalyst for biosynthesis of poly-phenols

This chapter is based on the following scientific paper:

Jing Su, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. A green catalyst for biosynthesis of poly-phenols, Critical Reviews in Biotechnology, 2017, 38(2), 294-307.

Abstract

Laccases (benzene diol: oxidoreductases, EC 1.10.3.2) are able to catalyse the oxidation of various compounds containing phenolic and aniline structures using dissolved oxygen in water. Laccase structural features and catalytic mechanisms focused on the polymerization of aromatics compounds are reported. A description about the most recent research on the biosynthesis of chemicals and polymers is made. Selected applications of this technology are covered as well as the advantages, shortcomings and future needs related with the use of laccases.

Keywords: laccase, oxidoreductase, oxidation, polymerization, phenolic

1.1 Historical overview of laccase and its application

Laccases (EC1.10.3.2) are a family of multi-copper containing oxidoreductases enzymes which are able to catalyze the oxidation of various aromatic compounds with the reduction of molecular oxygen to water[1]. During the process it produces an only byproduct water using air oxygen in a catalytic cycle during which four substrate molecules are oxidized. The fact that laccase can use dissolved oxygen as an oxidative source lead to intensive research. Laccases are probably one of the most promiscuous enzymes since they can catalyze a wide range of substrates, and they have gathered much attention for polymer synthesis due to their efficiency in mild reaction conditions[2]. First discovery of laccases can date back to 1883 based on the observation of rapid hardening of latex from Japanese lacquer trees in the presence of air[3-5], and it was named after isolation and purification[6,7]. Since then, laccase activity has been found in other plants species (e.g. mango, mung bean, peach), certain prokaryotes (e.g. *Azospirillumlipoferum*) and various insects, with the most biotechnologically useful laccases being predominantly of fungal origin (e.g. *Ascomycetes, Deuteromycetes, Basidiomycetes*)[8-11]. White-rot fungi from *Basidiomycetes* are the highest producers of laccases. Laccases in fungi carry out a variety of physiological roles including morphogenesis, fungal plant-pathogen interaction, stress defense, and lignin degradation[12,13]. Since laccases have been found in higher plants, prokaryotes, insects, fungi and lichens[14], such widespread detection indicates that the laccase redox process is ubiquitous in nature.

Over the past decades, oxidoreductases have attracted the efforts of many researchers in the environmental and biotechnological fields because of their great potential to be alternative catalysts to the conventional chemical synthetic processes with no hazardous side effects[15]. Normal chemical-catalyzed reactions can lead to irreversible destruction of the desired sub-structures and formation of unwanted by-products. In this regard, laccases have emerged as important enzymes as they are not only eco-friendly but they also work under mild conditions.

Laccases have received attention in biotechnological processes due to their catalytic and electro-catalytic properties, including food, textile, cosmetics, medicine and nanobiotechnology industries. Application areas of laccases are increasing mainly because of their wide substrates range. Laccases catalyze direct oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, and aryl diamines as well as some inorganic ions[12,13, 16]. Some substrates cannot be

oxidized solely because of their steric hindrance or the high redox potential, but they can be oxidized by small laccase-radicalized mediators.

Polymer synthesis by laccases has been reviewed by different researchers highlighting the ability of laccase to synthesize polymers[10, 17-22]. Laccase-catalyzed polymerization of phenols and their various derivatives can give rise to complicated polymeric structures. Moreover, during the organic synthesis, laccase-catalyzed polymerization have a number of selective and efficient reactions based on various substrates, leading to different polymers. Laccases show excellent biochemical properties and provide a unique alternative to organic synthesis. This article reviews the current knowledge on laccase structure, associated catalytic mechanisms and applications as green catalyst. It focus on some emerging trends of laccase applications, highlighting the polymer synthesis.

1.2 Laccase catalytic mechanism and properties

Laccases are glycoproteins which often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular weight of the monomer ranges from about 50 to 130 kDa. The carbohydrate moiety of laccases consisting of mannose, acetylglucosamine, and galactose which ranges from 10 to 45% of the protein mass. This carbohydrate moiety is believed to be responsible for the stability of the enzyme.

Laccases catalyze four-electron substrate oxidations, resulting in reductive cleavage of a dioxygen bond; Cu metal atoms within the enzymes play key roles in the reduction of O_2 to H_2O. The Cu atoms of laccases include one copper of type 1 (Cu_1), one of type 2 (Cu_2) and two of type 3 (Cu_3) (Figure 1.1).

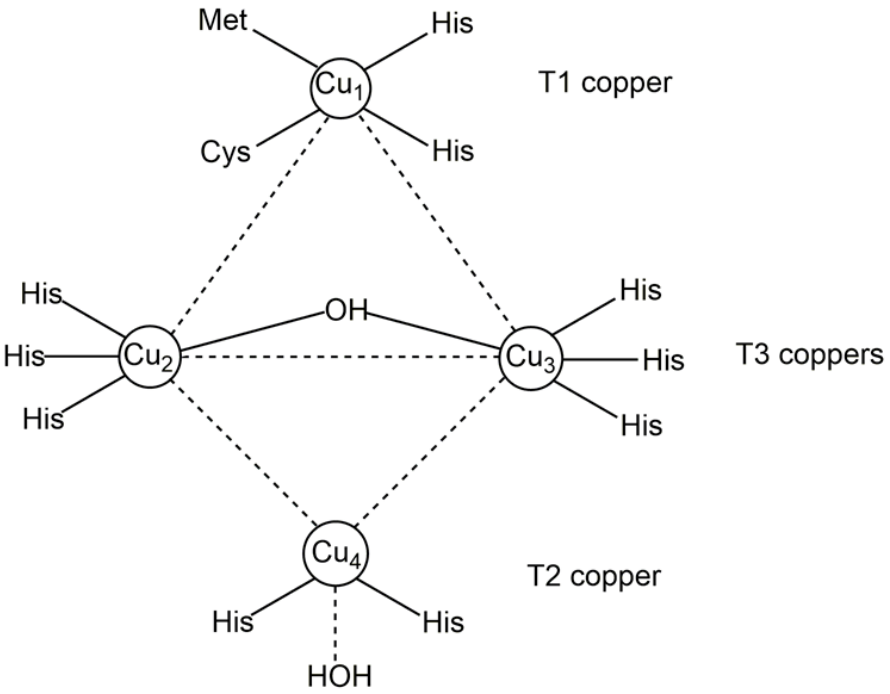

Figure 1.1. Active site of laccase CotA from *Bacillus subtilis* (adapted from Enguita et al, 2003).

In the laccase-catalyzed oxidation process Cu_1 is the primary electron acceptor. The electrons are next transferred via a highly conserved His-Cys-His tripeptide to a tri-nuclear cluster (TNC) which includes Cu_2 and Cu_3 atoms. Then electrons reduce O_2 to H_2O[17, 23]. Since Cu_1 has a wide cavity on the enzyme surface, a large range of substrates can be accommodated. Cu_1 plays the role of a door that offers entry for substrate electron to the catalytic site. Meanwhile Cu_1 controls the catalytic rate, during the whole catalytic process reduction of Cu_1 is rate-limiting[12, 24]. The redox potential of Cu_1 is relatively low allowing electron abstraction[25-27]. With the four "electrons" transferred from Cu_1, the two Cu_2/Cu_3 ions arranged in a triangular manner, facilitate dioxygen binding, leading to reduction of molecular oxygen[12, 17].

Generally, laccases catalyze phenolic substrates, which loose one electron and one proton and form phenoxy radicals that are stablished by resonance into the respective quinone structures or covalently coupled to oligo- or polymeric products. It has been described that laccases can promote homo- or hetero-molecular coupling reactions. During reactions between phenols or quinonoid systems and primary amines, new C-O, C-N or N-N products are formed. Aromatic amines have been mainly used as nucleophiles in phenol reactions catalyzed by laccases[28]. Recently, aromatic amines used as substrates of laccase were described in the oligomerization of aniline to produce

conducting polyaniline or in the polymerization of aniline derivatives[29].

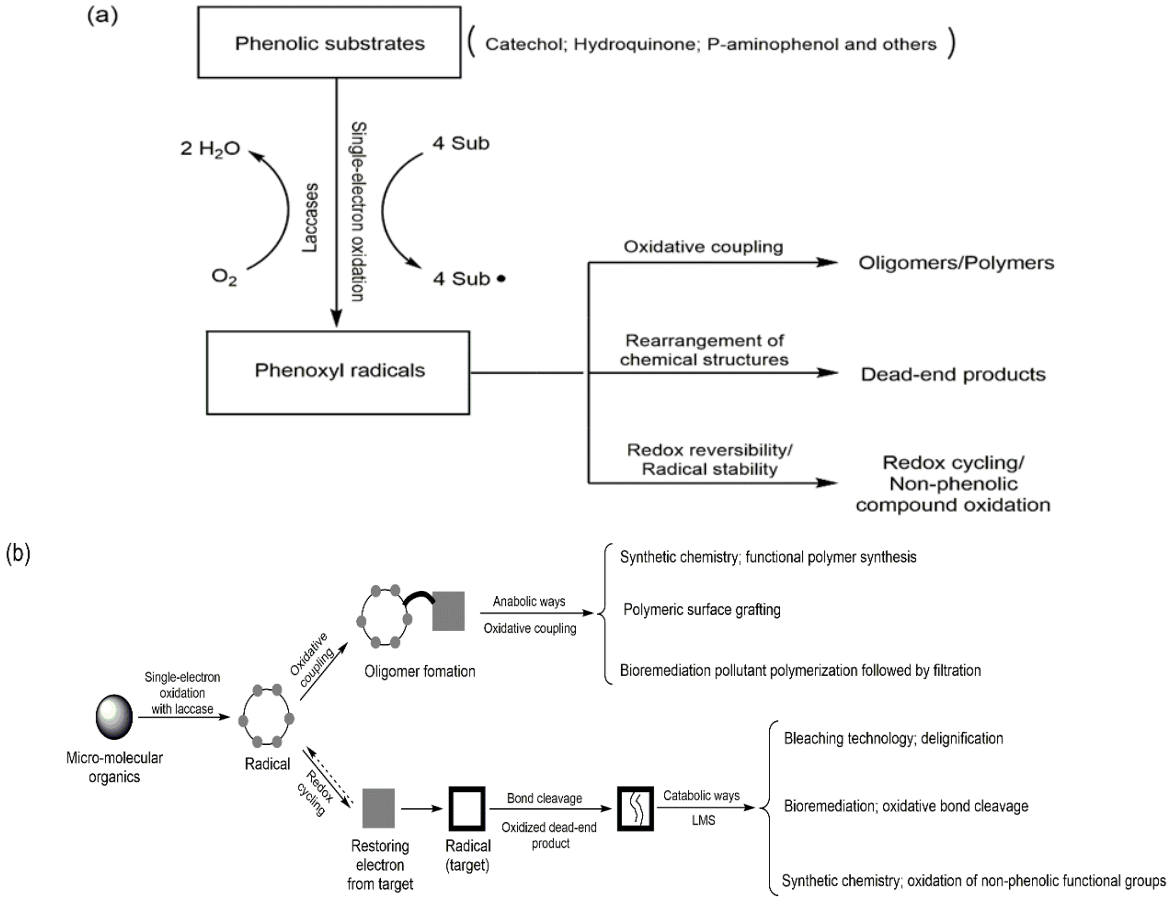

Figure 1.2. (a) Representative oxidation reactions of phenolic substrates catalyzed by laccase; (b) Bifunctional actions of small organic reactions with laccase (LMS: laccase-mediator system) (adapted from Jeon et al.,2013).

Oxidation of phenolic substrates with laccase is summarized in Figure 1.2 (a) and (b). Enzymatic reactions will be much greener if substrates are natural since laccase catalysts are also natural. In recent researches, laccase applications in synthetic chemistry based on phenols have a trend to mimic natural anabolism, predicting the role that laccases and phenols play in *in vivo* anabolic processes[17, 30]. Naturally occurring phenols used in the synthetic reactions are considered the key substrates of laccase, and the synthetic path seems to be limited to oxidative coupling of natural phenols *per se* or their cross-coupling into preformed biomacromolecules (Figure 1.3).

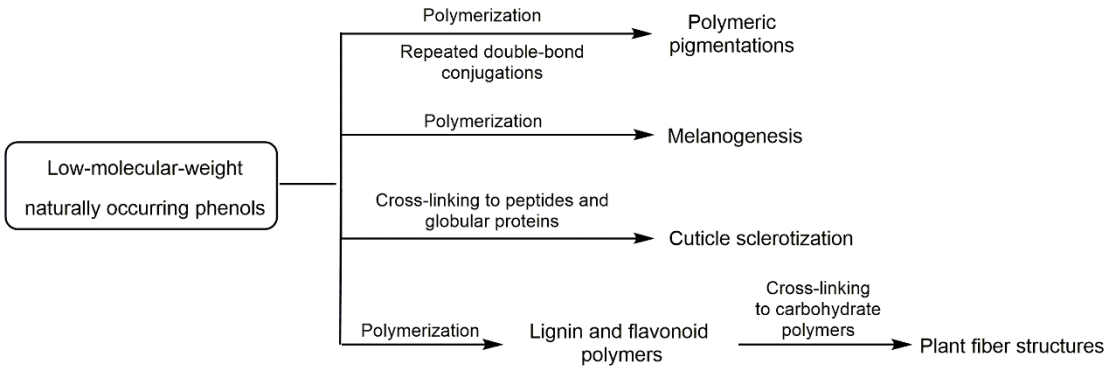

Figure 1.3. Laccase-catalyzed *in vivo* anabolic pathways showing the low molecular weight phenol roles played as laccase substrates (adapted from Jeon et al.,2013).

Normally laccase catalytic properties can be attributed to the following three major steps: (i) T1 copper is reduced by accepting electrons from the reducing substrate; (ii) Electrons are transferred from T1 copper to the tri-nuclear T2/T3 cluster; (iii) Molecular oxygen is activated and reduced to water at the tri-nuclear T2/T3 cluster. As can be seen in Figure 1.4(a), the catalytic mechanism of laccase involves a four-electron reduction of the dioxygen molecule to water at the enzyme copper sites[21, 28]. Oxygen molecule interacts with the completely reduced trinuclear cluster (T2/T3) via a 2e- process (k ≈ 2 × 106 M-1s-1) to produce the peroxide intermediate which contains the dioxygen anion[31]. One oxygen atom of the dioxygen anion bound to the T2 and T3 copper ions and the other oxygen atom bound to T3 copper ion. Then, the peroxide intermediate undergoes a second 2e- process (k > 305 s-1)[32], and the peroxide O-O bond is spited to produce a native intermediate which is a fully oxidized form with the three copper centers in the trinuclear site mutually bridged by the product of full O_2 reduction with at least one Cu-Cu distance of 3.3 Å. This native intermediate form of laccase was confirmed by x-ray spectroscopy (XAS) and magnetic circular dichroism (MCD) by Solomon *et al.*[33]. Furthermore it has been proved via model studies and calculations that the three copper centers in the trinuclear cluster are all bridged by a μ3-oxo ligand[34]. This structure has a single μ3-oxo bridged trinuclear CU(II) complex at the center of the cluster, with the second oxygen atom from O_2 either remaining bound or dissociated from the trinuclear site as shown in the native intermediate structure in Figure 1.4(b). This μ3-oxo bridged structure of the native intermediate provides a relatively stable structure that serves as the thermodynamic driving force for the 4e- process of O_2 reduction, and also provides efficient electron transfer (ET) pathways from

T1 site to all of the copper centers in the trinuclear cluster[34]. This efficient ET pathways lead to the fast reduction of the fully oxidized trinuclear cluster in the native intermediate to generate the fully reduced site in the reduced form for further turnover with O_2. The native intermediate can slowly convert to a completely oxidized form called "resting" laccase which has the T2 copper isolated from the couple-binuclear T3 centers.

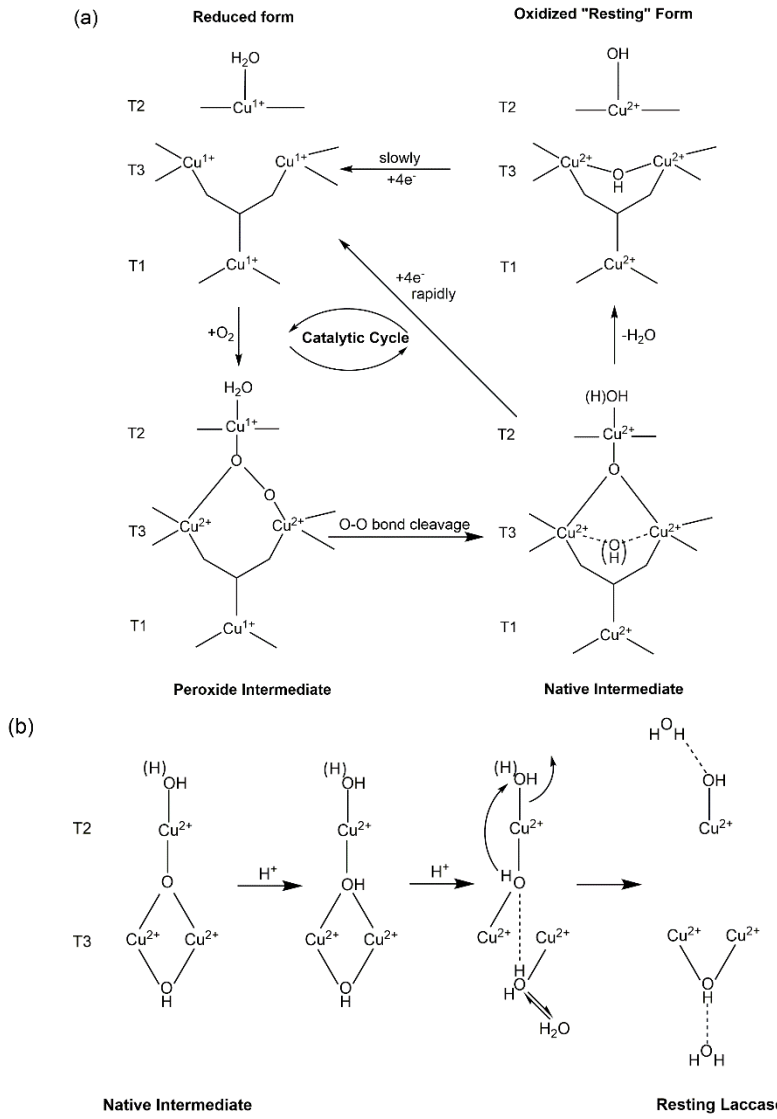

Figure 1.4. (a) Catalytic cycle of laccase showing the mechanism of four-electron reduction of a dioxygen molecule to water at the enzyme copper sites; (b) Proposed decay mechanism of the native intermediate to the resting laccase (adapted from Witayakran et al., 2008).

The decay of the native intermediate to the resting enzyme proceeds via successive proton-assisted

steps as illustrated in Figure 1.4 (b)[35]. The first proton binds at μ3-oxo center and the other proton binds at T3 OH- bridge. Finally, the three copper centers in the trinuclear cluster are uncoupled to form the resting form of laccase. The slow decay of the native intermediate is due to the rearrangement of the μ3-oxo-bridge, the rate limiting step, from inside to outside of the cluster. The T1 site of this resting laccase can be reduced by a substrate. However, the electron-transfer rate onto the trinuclear cluster (T2/T3) is too low to be significant for catalysis[33, 36].

1.3 Laccase substrates

Laccases can catalyze the oxidation of a variety of compounds, including polyphenols, amino-phenols, polyamines, lignin, aryl diamines, and a number of inorganic ions (Figure 1.5)[37,38]. To accomplish it, laccase abstracts an electron from a substrate to produce a free radical, and reduces oxygen to water, as seen in Figure 1.6 (a). The simplified scheme of laccase-catalyzed oxidation is illustrated in Figure 1.6 (b).

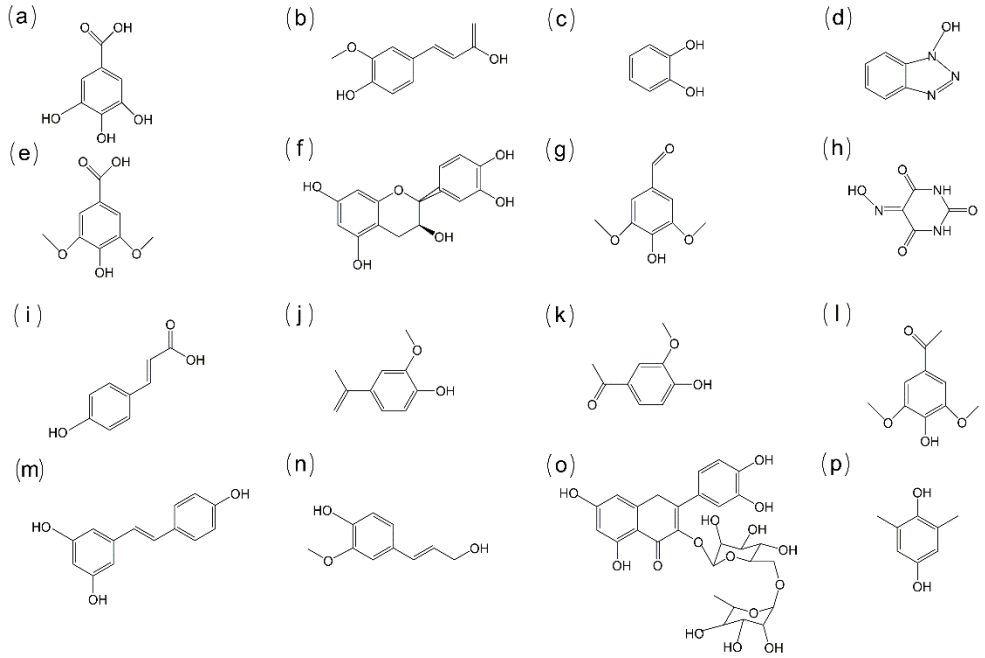

Figure 1.5. Chemical structures of small organics used for laccase bifunctionality: (a) gallic acid, (b) ferulic acid, (c) catechol, (d) 1-hydroxybenzotriazole, (e) syringic acid, (f) catechin, (g) syringaldehyde, (h) violuric acid, (i) p-coumaric acid, (j) vanillin, (k) acetovanillone, (l) acetosyringone, (m) resveratrol, (n) coniferyl alcohol, (o) rutin, (p) 2,6-dimethoxy-1,4-benzohydroquinone (adapted from *Jeon et al.,2013*).

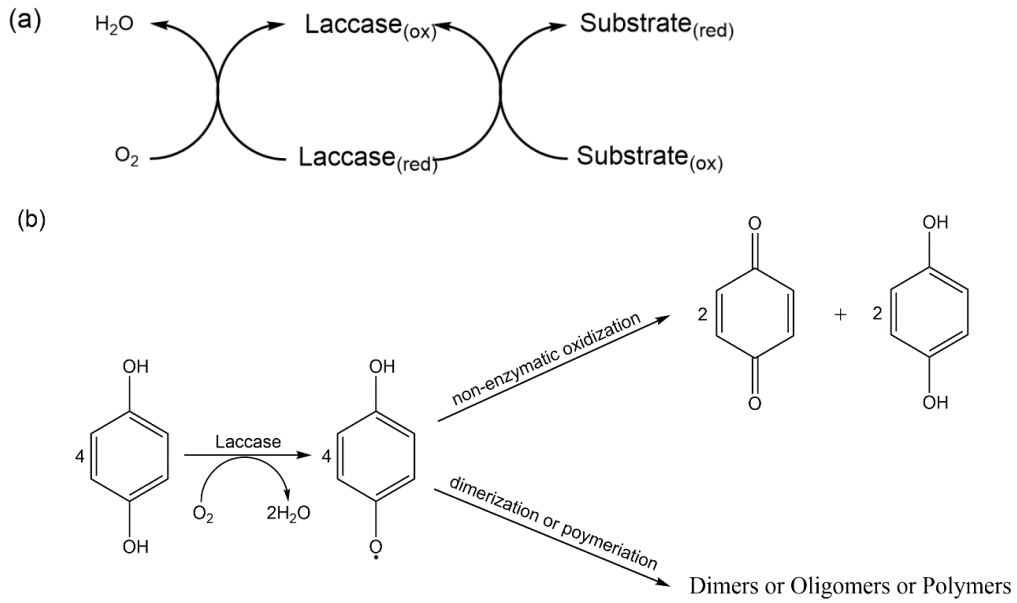

Figure 1.6. (a) Scheme of laccase-catalyzed redox cycles for substrate oxidation; (b) oxidation of hydroquinone by laccase.

Phenol groups are considered typical laccase substrates due to their low redox potentials (see Table 1 for redox potential of different laccase substrates). Phenols are oxidized to phenoxyl free radicals by coupling-based polymerization or radical rearrangement *per se*, yielding dead end products. However, depending on phenoxyl radical stability, reversibility of the oxidation may be observed; such reactions use the phenolic substrates as laccase mediators[39]. Radical-based coupling or redox recycling of phenolic substrates improves the versatility of laccase catalytic action because non-laccase substrates can serve as oxidation targets.

Table 1.1 Redox potential (mV) of different laccase substrates

Laccase substrate	Redox potential (mV)
2,2-azino-bis(3ethylbenzthiazoline-6-sulfonic acid) - ABTS	680 (vs. NHE)[71]
1-hydroxybenzotriazole - HBT	1080 (vs. SHE [72]
Syringaldazine	270 (vs. SCE)[73]
Promazine - PZ	540 (vs. SCE)[73]
Hydroquinone	170 (vs. SCE)[73]

Phenoxyl radicals are coupled with non-laccase substrates, thus allowing for formation of new heteromolecular dimers. Otherwise, the phenoxyl radicals feature restored PhOH bonds, resulting from cleavage of the benzylic C–H bonds of oxidation targets. Such hydrogen atom transfer oxidation, involving phenolic compounds, gives non-laccase substrates the properties of reactive radicals, finally leading to various types of enzymatic biotransformations. The non-specific nature of oxidations performed by laccases, combined with the use of enzyme immobilization technology, has encouraged the use of the enzymes in green organic synthesis[2, 10, 30, 40].

During phenol polymerization reactions with laccases, aromatic amines have been mainly used as nucleophiles (Figure 1.7). The coupling of a typical substrate of laccase, for example, a substituted hydroquinone with primary amines usually occurs as nucleophilic amination on the aromatic ring by the substitution of hydrogen, halides or alkyl groups. Elongation of the reaction time, the increase of reaction temperatures or the reactions with excess of amine, will promote the formation of diaminated products[24, 28].

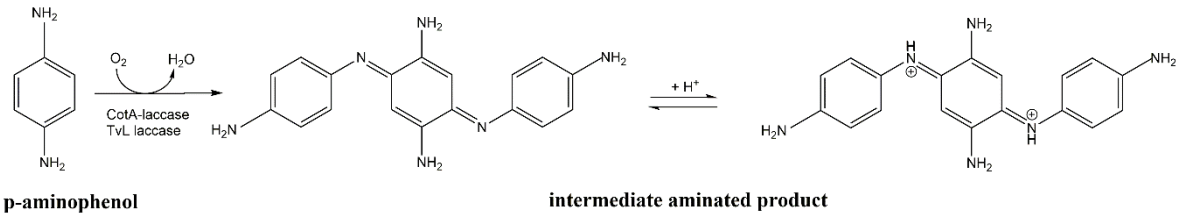

Figure 1.7. Scheme of p-aminophenol polymerization reaction with laccase using amines as nucleophiles.

Oxidation of phenol compounds in the crude extract of the residual compost of *Agaricus bisporus* using laccase was studied by Trejo-Hernandez and other researchers. Phenol, guaiacol, 2,6-dimethoxyphenol were oxidized to insoluble products and ventril alcohol was transformed to a soluble aldehyde[41,42]. The relative activity of the compost extract, measured in terms of the time required to oxidize the substrates, was 2,6-dimethoxyphenol> guaiacol>phenol>ventril alcohol>aniline.

Monolignols are phytochemicals acting as source materials for the biosynthesis of both lignans and lignin. The laccase-catalyzed oxidation reactions of main monolignols including coniferyl alcohol, isoeugenol and ferulic acid were studied by Chen and his co-workers. Coniferyl alcohol and isoeugenol were oxidized with laccase from Rhus vernicifera (tree) and Pycnoporus coccineus (fungus) in acetonewater (1:1, v/v)[2, 42, 43]. These oxidations followed a first order rate law. The catalytic rate of the oxidation of isoeugenol and coniferyl alcohol was compared between laccases, and it was found that Pycnoporus laccase-catalyzed oxidation is three to seven times faster than Rhus laccase-catalyzed oxidation. As shown in Figure 1.8(a) both the monolignol and the laccase influence the rate of the oxidation.

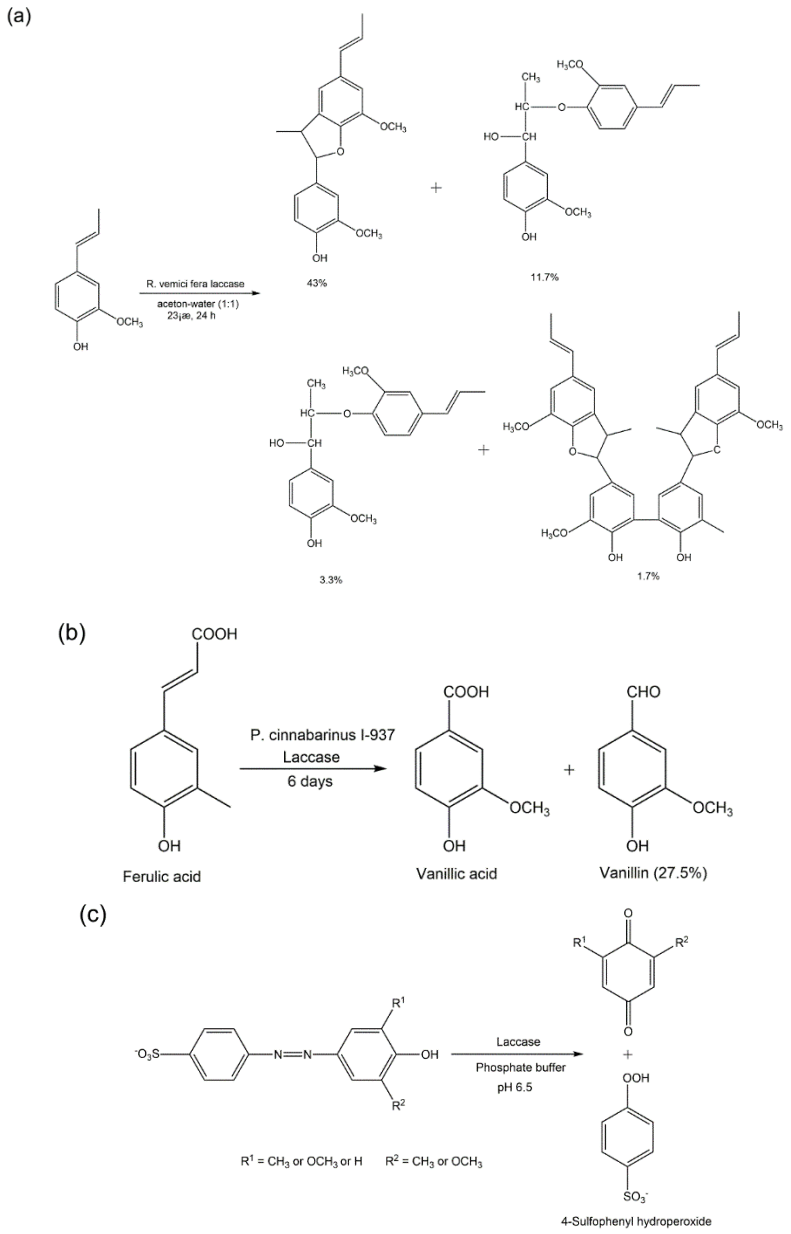

Figure 1.8. (a) Dimer and tetramer products resulting from the oxidation of isoeugenol alcohol by laccase (adapted from Witayakran); (b) Biotransformation of ferulic acid by laccase; (c) The oxidation of phenolic azo dyes by laccase.

The transformation of ferulic acid was examined by Nishida and Fukuzumi[44]. The white rot fungus, *Trametes versicolor*, was cultivated in a medium including ferulic acid, glucose and ethanol under aerobic conditions in submerged culture. The ferulic acid was transformed into coniferyl alcohol, coniferylaldehyde, dihydroconiferyl alcohol, vanillic acid, vanillyl alcohol, 2-methoxyhydroquinone

and 2-methoxyquinone. The biotransformation of ferulic acid in cultures of the white-rot fungus *Pycnoporus cinnabarinus* I-937 was also studied by Falconnier and co-workers[45]. When produced, the enzyme countered the vanillin formation by promoting the polymerization of ferulic acid into lignin-like polymers (Figure 1.8(b)).

The oxidation of ferulic acid by laccase to synthesize phenolic colorants was also investigated[46]. Mustafa and co-workers found that this kind of oxidation in a biphasic hydro-organic system (perfectly mixed) composed of ethyl acetate and sodium-phosphate buffer could result in intermediate stable yellow products. These products could be solved in organic solvent which decreased the activity of laccase and the rate of non-enzymatic reactions, thus preventing the further polymerization of the intermediate. It was suggested that this yellow-colored product could be applied as food colorants.

As the largest group of colorants, azo dyes have excellent coloring properties and can be oxidized by laccase[47]. Laccase from *Pyricularia oryzae* was used for the oxidation of phenolic azo dyes. Renganathan and Chivukula found that the azo dyes can be oxidized to 4-sulfonylhydroperoxide, a quinone compound, and other products. During the laccase oxidation phenoxy radicals were formed which were further oxidized into a quinone and 4-sulfonylhydroperoxide. It suggested that laccase oxidation can result in the detoxification of azo dyes.

Aromatic amines used as laccase substrates are described in the oligomerization of aniline to produce conducting polyaniline or polymers of aniline derivatives. 1,4-phenylenediamine and 4-aminophenol are widely used as dye precursors or oxidation-based compounds for the dyeing of hair. These substrates produce colored compounds as hair dyes, which are of great interest because of healthy demands for direct contact with human skin. Laccases are thought to be excellent candidates for the production of biological colorants under mild conditions[48].

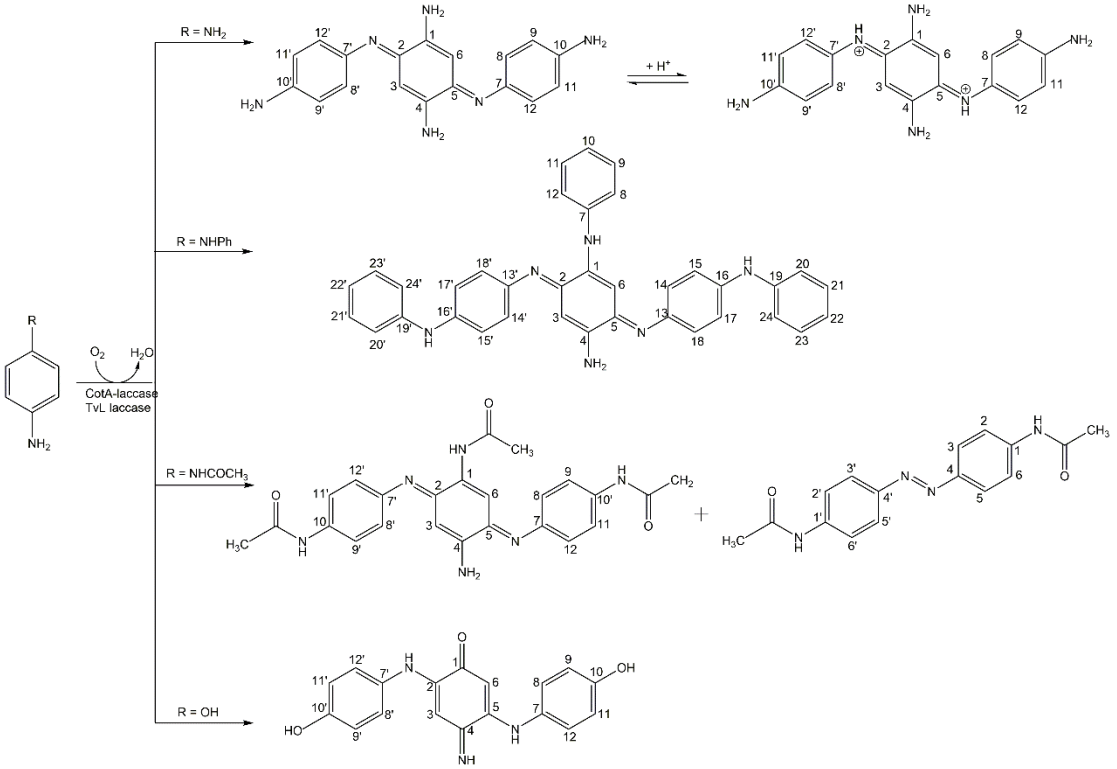

Figure 1.9. Structures of the main products formed in laccase reactions with *p*-substituted aromatic amines. 1,2,3): the aromatic nature of benzoquinonediiminic structures; 4): enzymatic oxidized 4-APA intermediate; 5): 2,5-diaminated 1,4-quinonemonoiminic structure.

The oxidation of 1,4-PDA, 4-ADA and 4- APA results in the formation of trimers with a 1,4-substituted-2,5-benzoquinonediimine skeleton (1–3, Figure 1.9). The oxidation of 4-AP, with a hydroxyl group results in the formation of the trimeric structure 5, with a 1,4-benzoquinonemonoimine skeleton substituted at positions 2 and 5. The aromatic nature of the benzoquinonediiminic structures (1, 2 and 3) was confirmed by the presence of multiple signals in the range 5.5–7.8 ppm and 90-160 ppm in both ^{1}H and ^{13}C NMR spectra, respectively[28].

The oxidation product of 1,4-PDA, the trimer 1,4-diamino-2,5-benzoquinonediimine (1) was isolated in two forms 1a and 1b (Figure 1.9), showing distinct colors (orange and dark purple, respectively) and distinct water solubility. The oxidation of 4-AP leads to formation of a brown solid (5) with a 2,5-diaminated 1,4-quinonemonoiminic structure. The presence of the hydroxy group on C-1, from which a quinonic structure could be easily obtained, should be the responsible for the formation of the 1,4-benzoquinonemonoimine skeleton. This trimeric structure is different from the one reported

before for 4-AP oxidation, leading to the formation of the 1,4-substituted-2,5-benzoquinonediimine trimer[49].

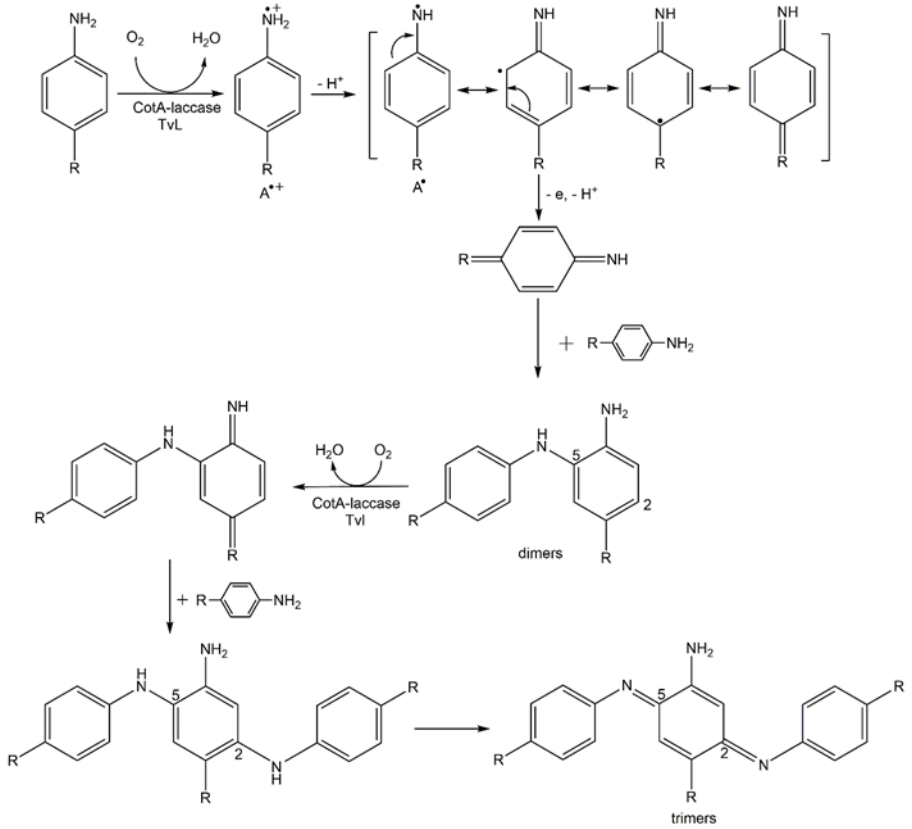

Figure 1.10. A proposed oxidative pathway for the formation of the disubstituted benzoquinonediimine trimers from laccase and aromatic amines (adapted from Sousa et al., 2013).

Figure 1.10 shows the proposed pathway that leads to the formation of the disubstituted benzoquinonediimine trimers. The initial step of the laccase oxidation process should be the electron abstraction followed by deprotonation of the substrate. The end products are expected to be two short-lived intermediates: an aminium cation radical ($A^{+\cdot}$) or a neutral radical species ($A^{\cdot}$), the aminyl radical, in keeping with species previously detected during laccase oxidation of 1,4-PDA by ferricyanide[50] and as intermediates of the amines oxidation by cytochrome P450 enzyme[51]. Thus, electron-donor substituents in the *p*-position, by stabilizing the radical cation, are expected to lower the transition state energy and speed up the enzymatic oxidation, whereas electron-withdrawing substituents do retard it.

Therefore, starting from the radical intermediates, which are also susceptible to sequential self-conjugation, the reaction proceeds through the formation of the benzoquinonediimine intermediate

and NC coupling in the activated ortho position (C-5) to the amino group to form a homo molecular dimeric structure. After the first coupling, the second addition on the aromatic ring will be performed in the para position (C-2) relative to the first covalent CN bond site and the central ring is stabilized by resonance[28].

According to this pathway, the stability of the radicals seems to be of great importance for the efficiency and the presence of electron-donating substituents on the aromatic ring are considered to be a key factor for this stability.

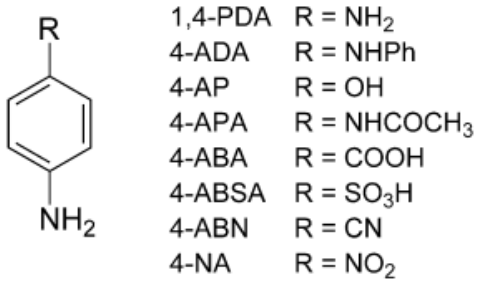

Figure 1.11. Representative structures of p-substituted aromatic amines.

Considering the electrochemical studies of substrates, difference in the redox potential between oxidoreductases and substrates plays an important role in enzymatic reactions, which influence if the reaction can occur and how fast the reaction come to proceed. The aromatic amines can be divided into *p*-electron-donor groups (1,4-PDA, 4-ADA, 4-AP, 4-APA) and *p*-electron-withdrawing groups (4-ABA, 4-ABSA, 4-ABN, 4-NA)[28], of which compounds with only *p*-electron-donor groups can be oxidized by laccase enzymes because *p*-electron-donor group converts recalcitrant amine molecules into laccase substrates by increasing the electronic density on the amine group (Figure 1.11).

There are some reports showing that templates were used successfully for controlling the chemical structure of polymers during the polymerization process[52]. These templates promote the formation of the poly(catechol) and poly(aniline), as shown by UV absorption spectroscopy. Works related with the use of PEG as template proposed that interactions of phenol with templates (possibly via H-bonds) led to the observed regioselectivity in the polymerization reaction[52-55]. It is not exactly clear how the templates enhance the polymerization reaction, whether they interact with substrate

and/or only keep polymers soluble[52,53,55,56]. PEG is also thought to protect the laccases of being entrapped inside the formed polymer[57,58] (Figure 1.12).

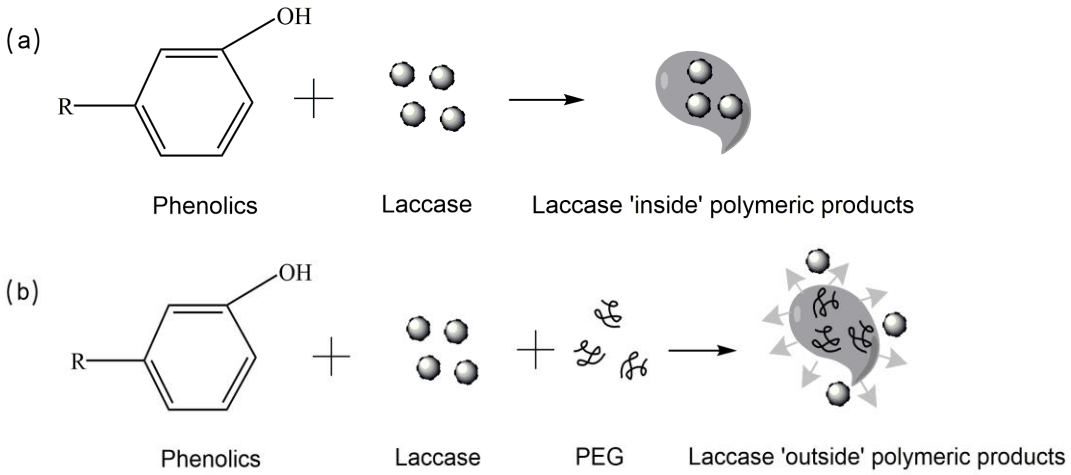

Figure 1.12. (a) Laccase-based polymerization of phenolic polymers; (b) proposed mechanism for the polymerization using PEG as template.

The use of chemical structure-controlling templates for enzymatic polymerization and the preparation of polymers with a defined chemical structure is still a challenge. Understanding the way template exerts its effect on a molecular level would help on the development and design of new and sustainable polymerization systems[52].

1.4 Laccase polymerization of phenolics

Laccase has been reported to oxidize a variety of phenolic compounds, which would acquire specific functional properties in terms of molecular weight, dispersity, degree of crosslinking, crystallinity, and inter- and intramolecular bonding[59]. Laccases are able to catalyze the transformation of phenol derivatives through an oxidative coupling reaction, resulting in the formation of less soluble and high molecular weight polymer compounds[11, 27, 60]. The products of laccase-catalyzed oxidation of natural phenols are diverse, ranging from dimers to macromolecules. The properties of such synthesized materials may be further engineered, depending on the type of phenolic monomers used for oligomer and polymer synthesis.

Recent developments about laccase-based synthetic applications have shown that the mimicking

of laccase-dependent *in vivo* phenolic anabolism can offer new opportunities for the development of eco-friendly applications. Novel functional properties imparted by special physical or chemical features of the synthesized materials have been of great interest for biotechnological applications. Several studies have shown that laccase-catalyzed polymerization of natural phenols, including rutin[21], epigallocatechin gallate and catechin[61], yields antioxidant materials more powerful than the natural monomeric phenols. The fact that such *in vitro* synthetic processes enhance antioxidant capacity is consistent with the fact that high-molecular-weight natural polyphenols are much stronger antioxidants than low-molecular-weight polyphenols of plants. Natural phenols have the ability to be coupled to well-known antibiotic compounds, to synthesize new drugs, or may be cross-coupled with macro-matrices to functionalize surfaces. Methyl catechol was used in reactions with antibiotics such as ampicillin and cefadroxil, that gave rise to heterodimer coupled products via nuclear amination, but the antibiotic potencies of the resulting derivatives were similar to those of the original antibiotics[40].

1.5 Laccase polymerization reactions: practical applications

Textile dyes occupy an important fraction of chemical industry market. Although consumption of dyes by the textile industry already accounts for two-thirds of the total dyestuff market, recent world dyes and organic pigments industry have estimated a further 3.5 % annual growth for the period 2013–2018[24]. Due to their toxicity and the harsh operative conditions required for their synthesis, this growth has been accompanied by strict legislation regarding removal of dyes from industrial effluents and has encouraged the development of ecofriendly processes for their disposal. In this scenario, laccases have shown to play a pivotal and double role: being efficient biocatalysts for biodegradation of synthetic dyes and attractive enzymes for coupling reactions leading to the production of new colored products.

Small colorless aromatic compounds such as phenols, aminophenols, diamines are oxidized by laccase to aryloxy radicals may undergo further non-enzymatic reactions leading to formation of colored products. Experimental evidences have shown the ability of laccase to catalyze the formation of colored products (from yellow/brown to red and blue) by oxidation of benzene derivatives containing at least two substituents (comprising amino, hydroxyl, and methoxy groups)[62].

Colorful materials are synthesized by coupling reactions resulting in oligomer or polymer synthesis. Chromophore formation is achieved via repetitive double-bond conjugation[1, 63, 64].

Derivatization of various phenolic and non-phenolic substrates may represent a strategy to expand their range of application such as bio-coloration. Textile industry is the main area in which bio-coloration has been valuable. Such polymerized flavonoids are generally dark brown, and the extent of coupling to fabric surfaces can be manipulated by varying pH, temperature and the extent of mechanical agitation[1, 63].

Apart from its application in textile dyeing, enzymatically controlled coloration is relevant in the fields of cosmetic production, owing to the eco-friendly features of the synthesized dyes[62, 64]. The use of pprovidesenols from plant fibers in laccase-catalyzed polymerization, yields diverse colors in the visible spectrum, thus allowing a pallet of colors to be precisely formulated.

Traditional chemical based hair coloring products are often irritant, difficult to handle and not safe. Hydrogen peroxide (H_2O_2) and phenylenediamines are the most used chemicals in hair dying. They are allergenic and carcinogenic[65] and can, respectively, cause severe hair damage[66,67]. Laccase can act as an alternative oxidizing agent, substituting H_2O_2. Laccase-based hair dying is an emerging field of research. Deep black colored polymers were achieved after laccase polymerization of catechin and catechol for application in cosmetics for hair coloration[64, 66].The authors indicated that the laccase-catalyzed polymerization of natural phenols to produce polymeric hair dyes would be a promising and applicable 'green' technology in the cosmetic industry.

In the cosmetic field, an increasing interest has also been focused on laccase application in the formulation of some personal hygiene products, including deodorant, toothpaste, mouthwash, detergent and soap. The importance of these oxidoreductases as efficient catalysts in these fields is underlined by the increasing number of patents registered in the last years[68]. Samuelson (Patent US 6569651) claim an enzymatic polymerization of anilines or phenols around a template[69], and Barfoed present the enzymatic method for textile dyeing, which the polymer dyes obtained can be applied on cotton, fur, leather, silk and wool[70].

Food industry benefits from laccase application in different sectors and for multiple purposes: from modification of food sensory parameters and texture to improvement of products shelf-life and determination of certain compounds in beverages. In fact, many laccase substrates, mostly

phenols, thiol-containing proteins and unsaturated fatty acids are fundamental components of various foods and beverages, thus their modification may lead to new functionalities, quality improvement and cost reduction.

1.6 Final Remarks

Laccases are ancient enzymes with a promising future. Since the first laccase-based commercial product, launched in 1996 by Novozyme (Novo Nordisk, Denmark), widespread companies have been engaged in producing this enzyme in several formulations and for different purposes, mainly for the textile and food industries.

As presented in this review, laccase-assisted reactions have potential for a vast range of applications. Research about the modification of natural polymers like lignin and cellulose and their application in wood and paper industries represent an important portion of the applicative research done so far using laccases. Other emerging sectors on which laccase has been object of exploitation are the bio-sensing and fuel cells, cosmetic, biopolymer synthesis, food and textile industries. The use of laccases as green catalysts for the synthesis of high-added value organic compounds is emerging as a new sector of applicative research, although still poorly exploited.

Despite this plethora of applications, laccase potentialities are not fully exploited, due to several issues related with the costs and enzyme efficiency. Enzyme-producing companies have been directing their efforts to the improvement of enzyme activity and/or stability through immobilization and protein engineering techniques. Other stabilization approaches have been also considered, namely enzyme functionalization, medium engineering, addition of polymers and surfactants, among others.

The applications reviewed herein present laccase as a green alternative to the current physicochemical methods which are environmentally unfriendly, costly and less specific. This class of enzymes are of great relevance both as a model for structure/function relationships studies as well as green tools for biotechnology industries.

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Chapter II

Laccase: PEGylation greatly enhances laccase polymerase activity

This chapter is based on the following scientific paper:

Jing Su, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo.PEGylation greatly enhances the laccase polymerase activity, ChemCatChem, 2017, 38(2),294-307.

Abstract

Laccase catalyzes the oxidation and polymerization of phenolic compounds in the presence of oxygen. Herein, we report for the first time that a previous pegylation of laccase enhances the polymerase activity by 3-fold comparing with the native enzyme, as confirmed by UV-Vis spectroscopy. The polymerization of catechol increased only 1.5-fold when polyethyleneglycol (PEG) was added to the medium reaction. Molecular dynamic simulations suggest the formation of a mixable complex of polycatechol and PEG, which is responsible to push the reaction forward. In a negative control experiment set, all catalysts were entrapped inside acrylamide gels and here the native laccase showed a relatively higher activity. These results suggest that the mobility of PEG is a key feature for the enhancement of the reaction.

Keywords: catechol; laccase; polymerization; template; polyethylene glycol (PEG)

2.1 Introduction

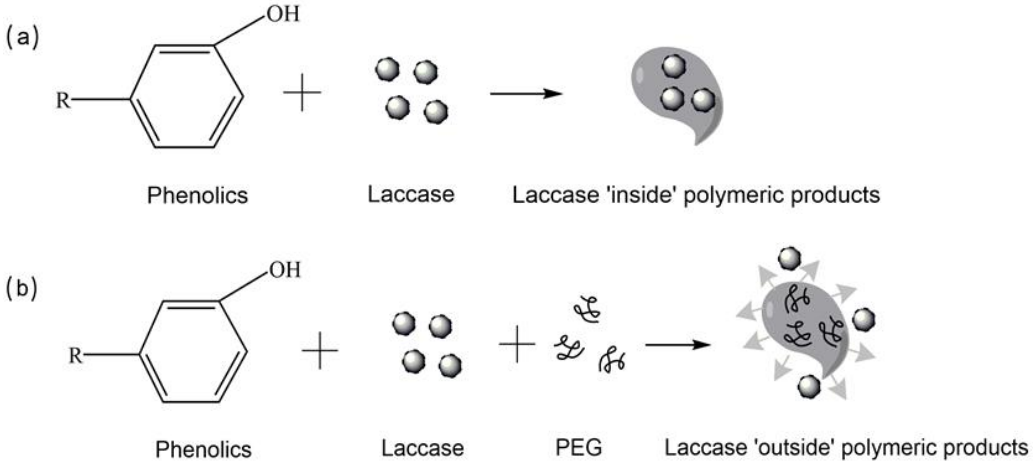
Laccases have been investigated for their ability to catalyze the oxidation of various substrates such as phenols, aminol compounds and their derivatives. These enzymes are mostly considered as extracellular proteins that are fairly stable when kept at neutral pH, under room temperature, with a broad substrate specificity, catalytic activity, effective pH and working temperature ranges. They have received much attention for their potential use in a wide variety of applications such as biosensors, bioremediation, green synthesis, green biodegradation of xenobiotics and other areas[1]. Recently, phenolic polymers have attracted an increasing attention as novel materials based on their excellent properties and applications in several areas. The enzymatic synthesis using laccase under mild reaction conditions has been considered as an alternative process for the polymerization of phenolic polymers replacing the use of toxic chemicals[2-5].

The control of the chemical structure of polymers obtained by enzyme-catalyzed template polymerization has also been studied. Generally, template polymerization is defined as a process in which the monomer units are organized by interactions with a template macromolecule[6]. Peter

Walde[7] and his co-workers considered template as a type of additive which help controlling the outcome of a polymerization reaction. The chemical structure of the polymers obtained through enzyme-catalyzed reactions can be controlled by the addition of chemical structure-controlling templates. Two general strategies concerning the mode of action of templates during polymerization were proposed: a) the direct interaction between the template and the reacting monomers and/or the growing polymer chains and b) the structuring of the reaction medium in such a way that the polymerization reaction takes place spatially confined. In the first case, the reaction is called "template-assisted" polymerization. The monomers and the growing polymer chains are assumed to interact with the template, which can influence the chemical structure of the polymers obtained, e.g. increasing reaction regioselectivity. In the second case, the reaction system is divided in two regions by the template: in one region the monomers are soluble and undergo polymerization, while in the other region the monomers and the growing polymer chains are not soluble and less accessible to the enzyme. The templates mainly affect the morphology of the polymers obtained, namely the particle geometry and size, porosity, and chemical structure. Kim[2, 8] and co-workers suggested that the presence of the template can influence the polymerization rate of the monomer compared with the blank polymerization and also that the properties of the final products, such as the molecular weight and the stereo regularity. The enzymatic polymerization of phenolic substrates by horseradish peroxidase was studied by using PEG as template in an aqueous medium. The addition of the additive produced a mixable complex of polyphenol and PEG as precipitates in high yields. The formation of a phenol-PEG complex was verified by hydrogen bonding interaction. It was confirmed that the amount of PEG strongly affected the polyphenol yield[2]. These authors also studied the enzymatic oxidative polymerization of phenol by the addition of poly(ethylene glycol) monododecyl ether (PEGMDE) template to the medium. They observed that the addition of this additive pushed forward the polymerization in water[1, 9]. The presence of PEGMDE template in the aqueous medium greatly improved the regioselectivity, yielding polyphenol with a phenylene unit content near 90%. Aron Steevensz[1] showed that PEG extended the lifetime conversion of the phenolics into polymers. When a 'PEG effect' was observed, there was a linear relationship between the amount of additive used and the amount of precipitate, suggesting a strong interaction between products and PEG, confirmed by NMR spectroscopy. PEG only enhances the laccase-catalysed

oxidative coupling of certain substrates, depending on the substituent positioning of the substrate and the functionality of the polymers formed[10].

Katy and co-workers[11] examined the feasibility of using laccase to treat synthetic wastewater containing bisphenol-A (BPA) where PEG was thought to reduce enzyme inactivation. Its addition to the medium had a significant protective effect on the activity of laccase. It is inferred that an interaction between PEG and the polymeric products resulted in the protection of the enzyme (Scheme 1). However, the mechanism of enzyme protection by PEG need to be deepen studied.



Scheme 2.1. Proposed mechanism for laccase polymerization in the absence (a) and in the presence of PEG (b)[12].

Polymerization using PEG with different molecular weight was investigated[13,14]. Studies with laccase demonstrated that the effectiveness of PEG as a protective additive was dependent on its molecular weight. By using low-molecular weight PEG, an effective complex with polymeric products could not be accomplished due to PEG's biodegradability trend. At the same time, high-molecular weight PEG gave rise to an increase of the solution viscosity hindering polymer production[2]. PEG with molecular weight between 3000-4000 Da was revealed as the most effective and environmental friendly alternative[15], protecting laccases of being entrapped inside the formed polymers. It has been hypothesized that PEG attaches to phenolic polymers forming a mixable complex, whereas the enzyme remains in the solution to proceed polymerization, as proposed in Scheme 2.1.

In the present work, we study the role of PEG as template on the laccase-assisted polymerization of catechol. To achieve this, the enzymatic polymerization of the phenolic monomer was conducted

using different conditions, namely a) in the absence of PEG; b) in the presence of PEG and c) with a previously PEGylated laccase. The produced polymers were characterized by Matrix Assisted Laser Desorption/Ionization-Time of flight Mass spectrometry (Maldi-TOF). UV spectra analysis was conducted for the analysis of the colour change during polymerization.The immobilization of all catalysts on acrylamide gels was performed to test the reaction under reduced mobility of PEG. Molecular dynamic simulations were conducted to understand the role of PEG during laccase-assisted polymerization of catechol.

2.2 Experimental Section

2.2.1 The PEGylation of laccase

Laccase from *Myceliophthora Thermophila* was PEGylated using the procedure reported by Daly et al[16]. Briefly, 14.0mL of 12mg/mL laccase were reacted with 20kDa, O-[2-(6-Oxocaproylamino)ethyl]-O′-methylpolyethylene glycol at pH=5, 10mM sodium phosphate buffer with 20mM sodium cyanoborohydride. A control reaction without mPEG was also conducted in every experiments. The reactions were stirred rapidly for 4 or 17 h at 4 °C. After 10min. of mixing, the reagents were completely dissolved, and an aliquot (namely time 0 h) was taken, as well as at each time point of reaction, 4 and 17h. These samples were ultrafiltrated using a 30kDa cellulose membrane mounted in a ultrafiltration apparatus to separate the unbounded PEG. The PEGylated enzyme was then freeze-dried and analysed by SDS-PAGE electrophoresis. The samples for the SDS-PAGE analysis were assembled as follows: 0.001mL of a 4X loading buffer were added to 0.005 mL of the sample, mixed, and heated for 1 min at 98 °C in a digital heatblock. 10% acrylamide gels, containing 1% SDS were run at 40mA for 60 min and silver-stained to visualize PEGylation.

2.2.2 Enzymatic-assisted polymerization of catechol in solution

Catechol polymerization was processed by incubating 10 mg/mL of monomer in different solutions: a) 98 mg/mL native laccase; b) 98 mg/mL native laccase + with 0.5mg/mL PEG (3-4kDa); and c) 98 mg/mL PEGylated laccase, in acetate buffer (pH=5). The reactions were performed in a water bath at 40 °C for 8 hours. Afterwards the powder was washed with water by centrifugation and dried under vacuum for posterior Maldi-TOF analysis.

2.2.3 Enzymatic-assisted polymerization of catechol in acrylamide gels

A different approach for the polymerization of catechol was assessed by using laccase previously immobilized onto acylamide gels. The acrylamide gels gels were produced by mixing in different well spots of a 6-well microplate 0.7mL of acrylamide (30%), 1.3 mL of Tris-HCl (0.5 M, pH 6.8) and 2.97mL of distilled water. Then 10 µL of TEMED (tetramethylethylenediamine) and 37.5 µL of APS (ammonium persulfate) (10%) were added and mixed. Afterwards, the enzyme (98 mg/mL) was added to each spot: native laccase; native laccase + PEG and PEGylated laccase, and 30 minutes later the gels were formed. A control gel without catalyst was also considered. After gels production, the polymerase activity of entrapped catalyst was evaluated by adding 5mg/mL of catechol into the gels and let the reaction proceed for 8h at 40 °C.

2.2.4 Determination of Total Content of Free OH groups

The total content of free OH groups before and after polymerization was performed using the Folin-Ciocalteu spectrophotometric method. The monomer and polymer solutions dissolved in DMSO (100 µL) were added to the mixture of Folin-Ciocalteu reagent (500 µL) and distilled water (6 mL), and the mixture was shaken for 1 minute. Then Na_2CO_3 solution (15%, 2mL) was added to the mixture and shaken for 1 minute. Later the solution was brought up to 10 mL by adding distilled water. After 2 h, the absorbance at 750 nm (25°C) was measured. The Total content of free OH was assessed by plotting a gallic acid calibration curve (from 1 to 1500 µg/ml). The equation of the gallic acid calibration curve was $A=0.2977c+0.0368$, and the correlation coefficient was $r^2=0.9988$.

2.2.5 Polymers characterization

The polymerization reactions were followed by UV-vis spectroscopy using a 96-quartz microplate reader. The polymer products obtained were characterized by Matrix assisted laser desorption/ionization-Time of flight mass spectrometry (MALDI-TOF).

MALDI-TOF mass spectra were acquired on a Bruker Autoflex Speed instrument (Bruker Daltonics GmbH) equipped with a 337 nm nitrogen laser. The matrix solution for MALDI-TOF measurements was prepared by dissolving a saturated solution of 2,5-dihydroxybenzoinc acid (DHB) in 100 %

ethanol. Samples were spotted onto a ground steel target plate (Bruker part n° 209519) and analysed in the linear negative mode using factory-configured instrument parameters suitable for a 1-10 kDa *m/z* range (ion source 1: 19,5kV; ion source 2: 18,3kV). Time delay between laser pulse and ion extraction was set to 130 ns, and the laser frequency was 25 Hz.

2.2.6 Calculation of average polymerization degree

The Mn (number average molecular weight) and Mw (weight average molecular weight) of polycatechol obtained after oxidation was obtained by Maldi-TOF direct analysis and according to the equations:

1) $M_n = \frac{\sum n_i M_i}{\sum n_i}$

2) $M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i}$

3) $Pd = \frac{Mw}{Mn}$

Where n_i is the relative abundance of each peak; M_i is the *m/z* correspondent to each peak; **Pd** is the polydispersity.

Molecular dynamic simulations were performed with GROMACS 4.6, using GROMOS 54a7 force field. The molecules were parametrized using the Automated Topology Builder (ATB). The system size was chosen according to the minimum image convention, taking into account a cutoff of 1.4 nm. Unbonded interactions were calculated using a twin-range method, with short and long range cutoffs of 0.8 and 1.4 nm, respectively. Neighbor searching was carried out up to 1.4 nm and updated every five steps. A time step of integration of 2 fs was used. A reaction field correction for the electrostatic interactions was applied using a dielectric constant of 54. The single point charge model was used for water molecules. The initial systems were energy minimized for 2000 steps using the steepest descent method, with all heavy atoms harmonically restrained using a force constant of 1000 kJ/mol nm2. The systems were initialized in the canonical ensemble (NVT) for 50 ns, with all heavy atoms harmonically restrained using a force constant of 1000 kJ/mol nm2. The simulation was then continued for 50 ns in the isothermal-isobaric ensemble (NPT), with the heavy atoms harmonically restrained with the same force constant. Pressure control was

implemented using the Berendsen barostat, with a reference pressure of 1 bar, 0.5 ps of relaxation time, and isothermal compressibility of 4.5 × 105 bar. Temperature control was set using the V-rescale thermostat at 300 K. The solutes and the solvent molecules were coupled in separated heat baths, with temperatures coupling constants of 0.025 ps in the first two initialization steps and with 0.1 ps for the rest of the simulations. The simulations were carried out for 10 ns.

2.3 Results and Discussion

2.3.1 PEGylation of laccase

PEGylation is the covalent attachment of one or more molecules of PEG to a protein. PEGylation can improve thermal stability and reduce deactivation of the enzyme, and also increase the biocatalyst lifetime. In this work, the PEGylation of laccase was performed and confirmed by SDS-PAGE electrophoresis (Figure 2.1).

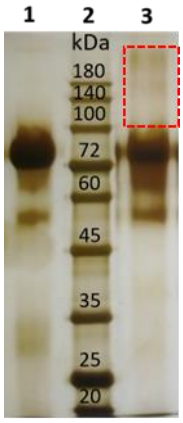


Figure 2.1. SDS-PAGE analysis of laccase after PEGylation with 20kDa PEG, using silver staining for protein detection; native laccase 5 μg (lane 1), GRS protein Marker Blue standards 2.5 μg (lane 2), PEGylated laccase 5 μg (lane 3).

In order to confirm laccase PEGylation, the samples were analyzed by SDS-PAGE. The gel was stained with silver for protein detection (Figure 2.1). The silver staining shows a smear between 100-180kDa for PEGylated samples (area in a red rectangle), confirming the increase of the molecular weight corresponding to the presence of the PEG.

2.3.2 Laccase-assisted polymerization of catechol in the presence of PEG

From previous studies, it is known that in normal cases native laccase produced low amount of polymer and gave rise to low polymerization degrees. This is attributed to the interactions between laccase and the reaction products leading to enzyme inactivation. PEG (polyethyleneglycol) is considered as a particularly effective additive for laccase protecting it against inactivation. In fact, some reports showing the successful use of PEG to enhance the formation of polymers during the polymerization process have been described[7]. Herein, a series of experiments were conducted to analyze the role of PEG during catechol polymerization. The laccase-assisted polymerization of catechol was carried out in acetate buffer (pH=5): a) in the absence of PEG, b) in the presence of PEG (3-4kDa) and c) with PEGylated laccase (20kDa), using air oxygen as oxidizing agent. During reaction, oligomers and polymers are formed until their solubility limit is reached and powdery precipitates collected by centrifugation.

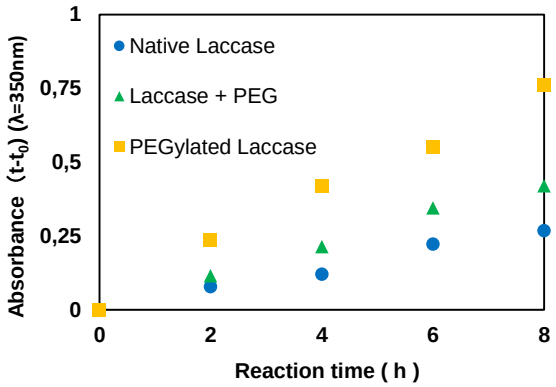


Figure 2.2. UV absorption at 350nm during polymerization of catechol under different conditions.

Figure 2.2 shows the UV absorbance of poly(catechol) in absence and in the presence of PEG in acetate buffer (pH 5). By addition of PEG to the system, higher values of absorbance at 350nm are detected (2-fold increase), especially when PEGylated laccase is used (3-fold of color increase). The differences of intensity are related with different polymerization degrees resulting from the differentiated hydrogen bonding interactions between the hydroxyl groups of catechol and PEG which are suggested to occur via "zip mechanism"[2,7]. After laccase addition, the reaction mixtures rapidly changed from colorless to brown or dark brown due to the quinones formed during oxidation. The color differences can be attributed to the amount of soluble oligomers in the reaction

supernatant[16] resulting from the presence of PEG in the medium. High polymerization degree is obtained when PEG is present, even in the free form of linked to laccase (average DP 8) (Table 1). This is a step forward comparing with the reported results where authors described only higher DPs after addition of an additive to the medium[2]. PEGylation of laccase is responsible, not only for a higher degree of polymerization, but also for the production of a higher amount of poly(catechol) evidenced by the amount of powder obtained after freeze-drying (data not shown).

It has been established that PEG effect is less pronounced when crude enzymes are used[2]. Herein, we used a commercial native laccase obtained from Novozymes containing on its composition specific additives and stabilizers, which can also contribute for the difference observed between samples containing PEG. After PEGylation, the PEGylated protein undergoes an ultrafiltration step that eliminates a large amount of these stabilizers and hence the PEG effect is more pronounced. Our results also suggest that the interaction of the additive with the enzyme may contribute for the PEG effect. A robust and less inactivated enzyme is obtained after PEGylation, explaining the high amount of polymer after reaction.

2.3.3 Enzymatic polymerization of catechol in the presence of immobilized laccase onto acrylamide gels

The use of laccases in practical industrial applications is still limited due to their high cost and low stability[17]. To overcome these limitations, the immobilization of laccase has been studied and many immobilization methods reviewed[18]. Several techniques are applied during the immobilization procedure based on chemical and physical mechanisms such as cross-linking, adsorption, entrapment and encapsulation. One of the most widely used systems of laccase immobilization is the entrapment in a polymer lattice, poly-acrylamide gel, obtained by polymerization/cross-linking of acrylamide. This type of immobilization has proved to be a particularly easy and effective way to immobilize enzymes or other proteins[19].

Herein, acrylamide gels were prepared to serve as supports for laccase and PEGylated laccase immobilization. As previously described for the polymerization in solution, three different experiments were performed. Native laccase, native laccase + PEG and PEGylated laccase were immobilized during acrylamide gels production and used afterwards for the polymerization of

catechol (Figure2.3). UV absorbance data acquired after polymerization revealed that, contrarily to the experiments in solution, PEG did not display the same template role. When the catalyst is immobilized into acrylamide gels, the addition of PEG did not favor enzyme mobility hindering reaction progression and resulting in lower polymerization degrees (Figure 2.3).

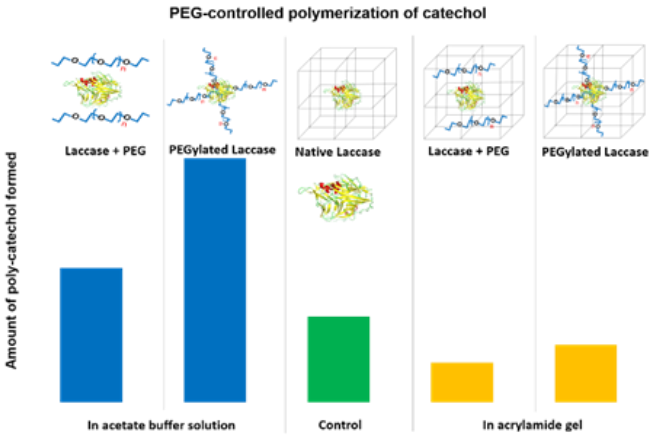


Figure 2.3. Comparison of UV absorbance (%) of poly(catechol) polymerized by laccase in solution and by laccase immobilized onto acrylamide gels for 8h (λ=350 nm; control: native laccase in solution and in gel corresponds to 100%).

The restrictions imposed by this three-dimensional network structure might constrain enzyme mobility and block reaction progression. Being entrapped inside gel, PEG is not able to work as template and form the typical complexes with the polymer (Scheme 2.2). These results indicate that mobility is an important feature to consider on systems using this additive as a template for phenolics polymerization.

The mechanism of catechol polymerization by laccase has been already proposed by others[20] and defines repeating units of oxyphenylene after reaction. To confirm PEG role as template, free in solution or chemically bond to laccase, we estimate the amount of oxyphenylene units by measuring the total content of free OH in all the mixtures after reaction (Table 1). Folin-Ciocalteau method measured the total OH after catechol polymerization and the values are normalized considering the total content of free OH of catechol as 1 (100%). From Table 1, it can be depicted a decrease of the total free OH after catechol polymerization in the presence of PEG, being more pronounced for samples polymerized by PEGylated laccase. As the content of OH groups decrease as more chains

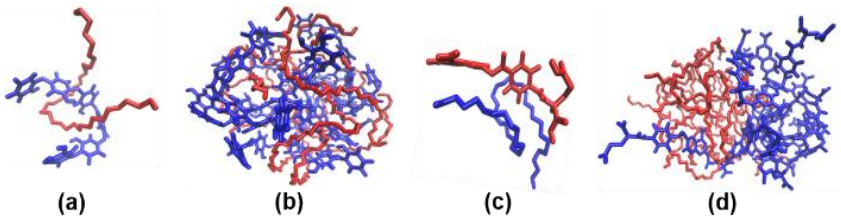
of oxyphenylene are present, confirming higher degrees of polymerization. From Table 1, one can highlight the high stability of PEGylated laccase during polymerization. A lower specific activity gave rise to higher polymerization yields compared with non-PEGylated laccase applied. PEGylation played here a crucial role on protecting enzyme for deactivation allowing PEG to work as a reaction template. The data also revealed that laccase PEGylation would be a strategic tool to improve reaction yields as confirmed by the absorbance and total content of free OH data (Table 1). Moreover, the processing drawbacks related with the enzyme cost can be undertaken by a 3-fold reduction of enzyme needed to proceed the reaction and reach similar or higher conversion levels.

Table 2.1: Polymerization of catechol in solution (specific activity of laccase, protein concentration, absorbance at 350nm, total content of free OH, Mn, Mw, Pd and average degree of polymerization*) (*calculated by Maldi-TOF analysis –see details in experimental section)

	Protein conc.	Specfic activity ($U/mg_{protein}$)	OD after 8h (350 nm)	Free OH (catechol as 1.00)	M_n, M_w, pd *	Average DP
Native Laccase	98 mg/mL	6.50	0.268	0.498	748, 776, 1.03	7
Laccase + PEG		6.50	0.420	0.414	831, 850, 1.02	8
PEGylated Laccase		2.11	0.760	0.329	833, 849, 1.02	8

Molecular modelling simulations were performed to predict the interaction at a molecular level between PEG/poly(catechol) and PEG/methotrexate (MTX). Poly(catechol) model with 6 repeating units (in blue) and a PEG model (in red) with 12 units were used for simulations (Scheme 2a). Methotrexate model with 1 unit (in blue) and 12 units of PEG (in red) were also used for simulations (Scheme 2c). The images obtained resulted from a short molecular dynamic simulation performed with GROMACS, using gromos54a7 force filed in water. Poly(catechol) demonstrates the ability to interact with PEG, forming mixable complexes, as depicted in scheme 2b). These results corroborate the experimental data, where a 2-fold improvement of DP was observed when PEG was present on the system, preserving the enzyme from being inactivated. The enzyme is therefore able to proceed with the reaction in solution. When PEGylated enzyme is used one can expect that the

complex formed between PEG and the products of polymerization would remain close to the catalyst endorsing a protective effect without compromising its catalytic behavior. At the same time, the products of polymerization in the presence of PEG are inert to enzyme and less accessible in solution, restraining its polymerization for higher DPs. This is evidenced by the formation of higher amount of products after polymerization in the presence of PEG. The use of this additive as template does not correspond necessarily to higher DPs but is the key factor to obtain higher yields of polymerization and increased amount of poly(catechol).



Scheme 2.2. Molecular dynamic simulations of catechol and methotrexate (MTX) polymerization showing: (a) 6 repeating units of poly(catechol) (in blue) and 12 repeating units of PEG (in red); (b) final mixable complex between poly(catechol) and PEG; (c) 1 unit of MTX (in red) and 12 repeating units of PEG (in blue); (d) final non mixable complex between poly-MTX and PEG.

In order to predict if PEG would serve as template for other enzymatic-assisted polymerization reactions we investigated the potential of α-chymotrypsin (from bovine pancreas) for the polymerization of methotrexate (MTX) in the presence of this additive. This serine protease has been described to catalyze the oligomerization of dipeptides in aqueous media[21].

The results achieved when MTX was polymerized using native α-chymotrypsin revealed a DP=5 with polymerization yield: ŋ= 80%. The role of PEG as a template of MTX polymerization was also evaluated. For this α-chymotrypsin was PEGylated with mPEG (5kDa) and further used for MTX polymerization[23]. From the results obtained, no differences on the DP and amount of polymer were observed after polymerization with PEGylated α-chymotrypsin (Table 2; 1a) and 1b)). The molecular modelling simulations also corroborate these findings, showing that poly-MTX does not complexate with PEG (Scheme 2d).

Table 2.2 The role of PEG on the polymerization of MTX and catechol with different substrates using α-chymotrypsin and laccase, respectively (DP-degree of polymerization was calculated by Maldi-TOF analysis; relative polymerization yield was calculated as OD increase relatively to control for samples polymerized by laccase; for samples polymerized by chymotrypsin the yield was obtained by the quotient between the number of moles of the isolated polymer and the number of moles of the monomer)

		Enzyme (w/v)	PEG (kDa)	DP	Relative conversion yield
1a)	**α-chymotrypsin + MTX**	10 %	-	5	100%
1b)	**PEGylated α-chymotrypsin + MTX**	10 %	5	4	57%
2a)	**Laccase + catechol**	20%	-	7	100%
2b)	**Laccase + PEG + catechol**	20%	3-4	8	150%
2c)	**PEGylated laccase + catechol**	20%	5	8	270%

These results indicate that different enzymes present variations in their affinities for the polymeric precipitate as well as on the sensitivity to additives like PEG. Moreover, the different hydrophobicity of the polymer precipitates are modulated by the nature of the initial monomers. A product-additive relationship may be related with the functionality and position of the substituents of the substrate used[1]. Also, the optimal PEG Mw would vary between enzymes, due to inactivation phenomena or to the nature of the products that interact with the enzymes during reaction, which are different among experiments and may alter the role of PEG as a template of the polymerization reaction[1].

2.3.4 Characterization of the polymers

The enzymatic-assisted polymerization was confirmed by MALDI-TOF analysis (Figure 2.4). The data achieved reveal peaks of poly(catechol) corresponding to average polymerization degrees of 7, 8 and 8, when laccase, "laccase + PEG" and "PEGylated laccase" were applied, respectively. From this data one can establish that the reaction improvement is more reliable using PEGylated laccase, since despite the similar DP, the enzyme is highly protected and higher amount of poly(catechol), as supported by powder quantification (data not shown). The PEG retained by the products of polymerization was detected in sample b) corresponding to the polymerization assisted

by free PEG. If PEG remains even after several washing, it indicates that it is strongly complexated with the isolated products and there is no need of its elimination by an additional step.

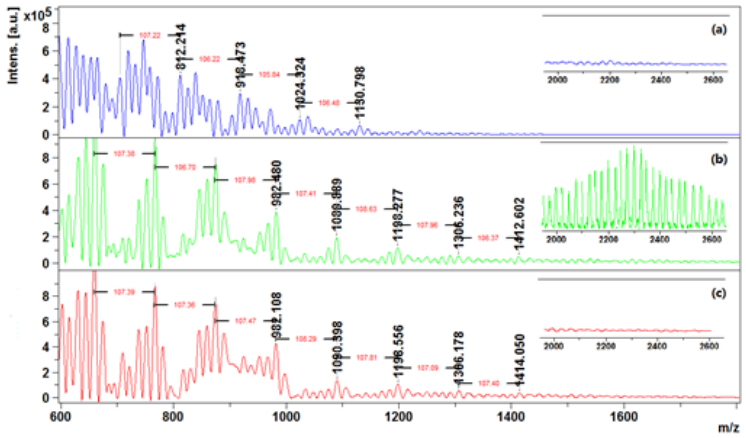


Figure 2.4. MALDI-TOF analysis of poly(catechol) polymerized using: (a) laccase; (b) laccase + PEG; (c) PEGylated laccase; the zoom area between 2000-3000 *m/z* highlights the presence of PEG after polymerization.

2.4 Conclusions

Laccase from *Myceliophthora Thermophila* was effective on the polymerization of catechol demonstrating to be an attractive alternative to conventional chemical processes. The role of PEG as additive on the enzymatic polymerization of catechol was evaluated. Higher polymerization degrees and increased amount of polymer were achieved in the presence of PEG in the medium.

A previous PEGylation of laccase showed a great potential for future applications since it allowed to obtain the same yields of polymerization as when free PEG is used, despite the lower specific activity resulted from PEGylation process. The novelty of this study relies on the effect of laccase PEGylation on the polymerization of poly(catechol). A higher degree of polymerization as well as higher amount poly(catechol) is obtained. The mobility was found to be a crucial property for the success of "template-assisted" polymerization. "PEG effect" is not perceptible after laccase entrapment due to enzyme constrains when entrapped in acrylamide supports. The effect of PEG as template is also highly dependent, as reported previously[1], on the type and structure of monomer. Additive's polymeric structure and interaction with the enzyme may be contributing factors to the template effect. Since we have focused our study only on the polymerization of

catechol, the effect the PEG on the laccase-based polymerization of other compounds is hard to predict. Our data on laccase after ultrafiltration suggest that PEG effect on crude enzymes might be reduced.

The mechanism of "PEG effect" on laccase-based polymerization is not clearly understood, molecular dynamics data suggest that PEG needs to interact (mix well) with the polymer formed. Here, a new environmentally friendly system was designed for the enzymatic polymerization of catechol opening space for further studies and optimization steps.

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Chapter III

Exploring PEGylated and immobilized laccases for catechol polymerization

This chapter is based on the following scientific publication:

Jing Su, Jennifer Noro, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. Exploring PEGylated and immobilized laccases for catechol polymerization, AMB Express, 2018, 8, 134.

Abstract

Laccases have been reported for their ability to eliminate hazardous phenolic compounds by oxidative polymerization. The exploitation of the oxidative behavior of different laccase forms, namely free/native, free/PEGylated, immobilized/native and immobilized/PEGylated, was assessed in this study. We found that PEGylated and immobilized laccase forms have differentiated catalytic behavior revealing distinct conversion rates and differentiated poly(catechol) chains, as confirmed by UV-Visible spectroscopy, by the total content of OH groups and by MALDI-TOF spectroscopy. The synergy underlying on the immobilized/PEGylated enzyme forms reveal to be responsible for the highest conversion rates and for the longer polymers produced.

Keywords: Laccase; polyethylene glycol; immobilization; PEGylation; polymerization

3.1 Introduction

Laccases are multicopper proteins considered as one of the most promiscuous enzymes since they can catalyze a broad spectrum of aromatic compounds and their derivatives[1]. This makes laccases promising biocatalysts for applications in biotechnological processes, including the detoxification of industrial effluents, textile and petrochemical industries, polymer synthesis, bioremediation of contaminated soils, wine and beverage stabilization, medicine and cosmetic ingredients[2]. One of the potential applications is on the removal of environmental and industrial pollutants, like phenolic compounds[3, 4]. Several studies have been reporting the polymerization of catechol highlighting different aspects related with the enzymatic catalysis like enzyme performance, degree of polymerization, conversion rate, among others. A study conducted by Gavrillas *et al.* reported the catechol biotransformation into high molecular species with purified laccase in contrast with the low molecular weight species obtained with a commercial enzyme[5]. Aktaş and co-workers studied the kinetics of laccase polymerization reaction and confirmed that a general enzyme kinetics saturation response was observed for catechol substrate during their

oxidation, which is probably due to the reduced laccase stability or to a drop in the dissolved oxygen concentration[6]. Although there is a great commercial potential for laccase applications, its instability in harsh conditions is considered a huge issue and strategies need to be explored to stabilize laccase either by chemical modification, immobilization or other techniques[7, 8]. Moreover, the elimination of the harsh solvents generally applied is also a concern related with the enzymatic oxidation reactions involving these types of compounds. Several studies have highlighted the identification of reaction products generated by the degradation of phenolic contaminants in water by laccases, however lacking information about the NMR of the final structures obtained[8-11].

In previous works we, and others, have assessed the effect of PEG on the polymerization of catechol by laccase from *Myceliophthora Thermophila*[12-14]. We have found that the previous PEGylation of the enzyme lead to a 3-fold polymerization activity increase while the single addition of PEG (0.5 mg/ml) to the medium increased only 1.5-fold, when compared with native enzyme. Experiments in a porous acrylamide gel[15], where all the catalysts were "frozen", show no effect of PEG on laccase polymerase activity[14]. The presence of PEG[16, 17] increased the average DP by one unit (from 7 to 8) and it is believed that a good mixing between PEG and poly(catechol) enhanced the reaction, as suggested by Molecular Dynamic Studies.

Herein, we aim to study the role of both chemical PEGylation and immobilization on the polymerase activity of laccase. The chemical PEGylation was performed by following the methodology described by Daly *et al.*[18]. The native and PEGylated laccase forms were immobilized onto epoxy resin according to the method previously described by Berrio *et al.*[19] (see reactions in Figure 3.1). The epoxy resins were chosen as supports due to their well-known chemistry described in literature[20-23]. Reported data show considerable enhancement of laccases stability when silanized and glutaraldeyde-activated silica nanoparticles are used as supports[24]. Jimmy and co-workers studied the immobilization of laccase onto epoxy resins-Eupergit C[19] and a substantial stabilization effect against pH and temperature was observed upon immobilization.

The polymerization of catechol was conducted using: a) native laccase immobilized onto epoxy resin and b) PEGylated laccase immobilized onto epoxy resin and c) native laccase immobilized onto PEG-activated resin. Control assays comprising the oxidation of catechol namely with free/native laccase and free/PEGylated laccase, were also performed. UV-Visible spectroscopy was

conducted to follow the color change during polymerization. The precipitated polymers were washed with water and methanol to separate the enzyme and the unreacted monomer from the oligomers. The different powder fractions obtained after each washing step were characterized by the Total content of free OH groups (TCFOH), Nuclear Magnetic Resonance spectroscopy (^{1}H NMR) and Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) spectrometry.

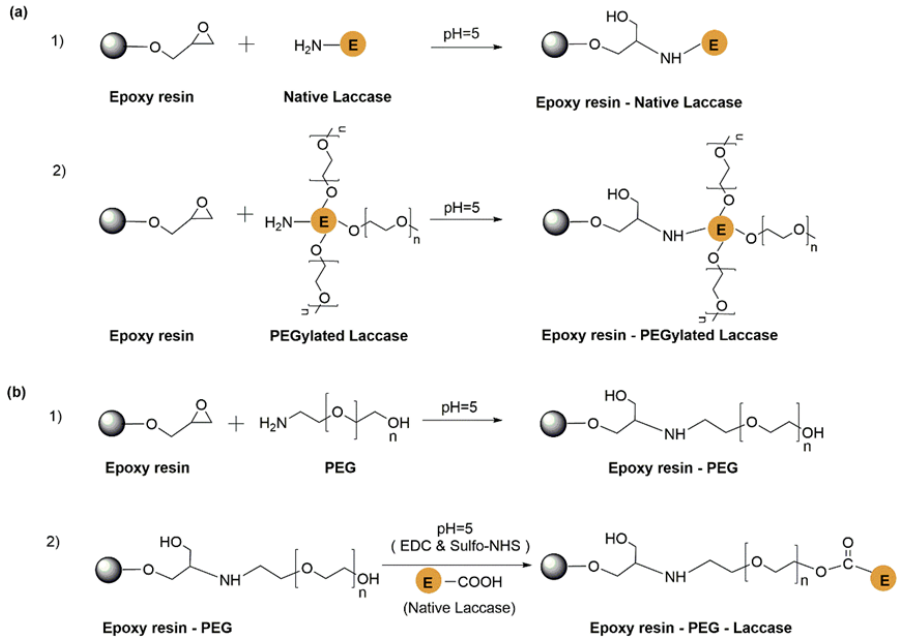

Figure 3.1. Reactional schemes proposed for the immobilization of laccase onto epoxy resins: a) covalent immobilization of native laccase (a1) and laccase PEGylated (a2); b1) activation of epoxy resin with (2-aminoethyl) polyethylene glycol (3kDa); b2) covalent immobilization of native laccase onto PEG-activated resin via EDC/sulfo NHS method.

3.2 Materials and methods

3.2.1 PEGylation of laccase

Laccase from *Myceliophthora Thermophila* (supplied by Novozymes, Denmark) was PEGylated as previously reported[14] using the procedure reported by Daly et al[18]. Briefly, 14.0mL of 12mg/mL laccase were reacted with 20kDa, O-[2-(6-Oxocaproylamino) ethyl]-Omethylpolyethylene glycol at pH=5, 10mM sodium phosphate buffer with 20mM sodium cyanoborohydride. A control reaction without mPEG was also conducted in every experiment. The reactions were stirred rapidly for 17 h

at 4 °C. After 10min of mixing, the reagents were completely dissolved, and an aliquot (namely time 0 h) was taken. These samples were ultra-filtrated using a 30kDa cellulose membrane mounted in an ultrafiltration apparatus. The PEGylated enzyme was then freeze-dried and analyzed by SDS-PAGE electrophoresis.

3.2.2 Immobilization of native and laccase PEGylated onto epoxy resin supports

The immobilization of native and laccase PEGylated onto epoxy methacrylate resins (Purelite Lifetech ECR enzyme immobilization resins: 300-600Å) was conducted as follows: 2 mg/mL laccase (native or PEGylated) in 0.5 M acetate buffer (pH 5.0) were mixed with epoxy methacrylate (50 mg/mL) and then stirred for 48 hours at 4 °C. The powder was then washed several times with water by centrifugation and dried under vacuum[19].

3.2.3 Immobilization of native laccase onto PEG-activated epoxy resin supports

Firstly, PEG (*O*-(2-Aminoethyl) polyethylene glycol): 3 kDa was used to activate epoxy by nucleophilic attack, as follows: 50 mg/mL PEG in 0.5 M acetate buffer (pH 5.0) was add to epoxy (50 mg/mL) and stirred for 48 hours at 4 °C. The powder was washed with water by centrifugation and dried under vacuum. Afterwards, to 5 mg/mL laccase prepared in 0.1 M MES solution (100ml), were add 40 mg of EDC and 110mg sulfo-NHS (N-hydroxysuccinimide) and mixed for 15 minutes using orbital agitation at room temperature. The reaction was stopped with 140 µL 2-mercaptoethanol, and the pH adjusted to 7 with PBS (Phosphate-buffered Saline) solution[25]. The final step consisted to add the previously PEG-activated epoxy (10 mg/mL) into the mixed solution and let react for 2 hours at room temperature with agitation. Finally, the samples were centrifuged with amicon tubes (100 kDa) to separate the unbound PEG and the free laccase.

3.2.4 Enzyme stability and half-life time of all laccase forms

The effect of temperature on the enzyme activity and stability of laccase was studied. For this the different forms of laccase were incubated with acetate buffer (pH=5) at 40, 50 and 60 °C, for 150h. The activity of laccase was measured against ABTS according to the methodology described

by Childs and Bardsley[26]. The half-life time of the different forms of laccase was calculated according to the Equation[27]:

Half-life time $\left(t_{\frac{1}{2}}\right) = \frac{\ln 2}{K}$, where $K = (\ln U_0 - \ln U_t) / t$

U_0: enzyme activity at time zero;

U_t: enzyme activity at time t;

t: time of incubation.

U: one U is defined as the amount of enzyme that catalyzes the conversion of 1µmol of substrate (ABTS) per minute.

3.2.5 Enzymatic-assisted polymerization of catechol by free laccase forms

Catechol polymerization was processed by incubating 5 mg/mL of monomer in different solutions: a) 100 U/mL native laccase, b) 100 U/mL PEGylated laccase, in acetate buffer (pH=5) (normalized concentration). The reactions were performed in a water bath at 40 °C for 8 hours. Afterwards the powder was washed with different solvents, namely 5 water washings and 2 methanol washings, under centrifugation, to separate the maximum amount of protein and the non-reacted catechol from the small and big oligomers. Further all the powder fractions recovered from water and methanol extraction step were freeze-dried for posterior analysis. The polymerization reactions were followed during time by UV-Visible spectrometry using the same dilutions for all the solutions obtained.

3.2.6 Enzymatic-assisted polymerization of catechol by immobilized laccase forms

5 mg/mL of catechol were incubated with: Epoxy-native laccase, Epoxy-laccase PEGylated and Epoxy-PEG-laccase, separately. As controls, free/native laccase and immobilized/native were used to polymerize catechol using the same conditions. The reactions were performed in a water bath at 40 °C for 8 hours using 100 U/mL enzyme. Afterwards the polymers were washed with water to remove the protein and separate the oligomers formed from the epoxy by centrifugation with amicon 10 kDa followed by freeze-drying. Then the freeze-dried powder was washed with different solvents, namely 5 water washings and 2 methanol washings, under centrifugation, to separate the maximum amount of protein and the non-reacted catechol from the small and big oligomers.

Further all the powder fractions recovered from water and methanol extraction step were freeze-dried for posterior analysis. The polymerization reactions were followed during time by UV-Visible spectrometry using the same dilutions for all the solutions obtained.

3.2.7 Mass spectra analysis

The new polymers were analyzed by Matrix-Assisted Laser Desorption/Ionization with time-of-flight (MALDI-TOF) using 2,5-dihydroxy benzoic acid (DHB) as the matrix (≥99.5%). The mass spectra were acquired on an Ultra-flex MALDI-TOF mass spectrophotometer (Bruker Daltonics GmbH) equipped with a 337nm nitrogen laser. For this, the samples were dissolved in a TA30 (30% acetonitrile/70% Trifluoroacetic acid) solution and mixed with a 20 mg/mL solution of DHB (1:1). Then a volume of 2 μL was placed in the ground steel plate (Bruker part n° 209519) until dry. The mass spectra were acquired in analyzed using in linear positive mode.

3.2.8 ^{1}H NMR spectra

The precipitates obtained after washing with water and methanol under centrifugation were dissolved in a deuterated solvent, DMSO-d_6, for ^{1}H NMR evaluation. The amount of OH groups on the samples was evaluated by ^{1}H NMR spectra after addition of two water drops to the previous samples in DMSO-d_6. The spectra were acquired in a Bruker Advance III 400 (400 MHz) using the peak solvent as internal reference.

3.2.9 Determination of the Total Content of Free OH groups

The total content of free OH groups before and after polymerization was performed using the Folin-Ciocalteu spectrophotometric method. The monomer and polymer solutions dissolved in DMSO (100 μL) were added to the mixture of Folin-Ciocalteu reagent (500 μL) and distilled water (6 mL), and the mixture was shaken for 1 minute. Then Na_2CO_3 solution (15 *wt*%, 2mL) was added to the mixture and shaken for 1 minute. Later the solution was brought up to 10 mL by adding distilled water. After 2 h, the absorbance at 750 nm (25°C) was measured. The Total content of free OH was assessed by plotting a gallic acid calibration curve (from 1 to 1500 μg/ml). The equation of

the gallic acid calibration curve was A=0.2977c+0.0368, and the correlation coefficient was r^2=0.9988.

3.3 Results

3.3.1 Reaction turnover and amount of poly(catechol) formed

The amount of poly(catechol) produced after catalysis with the different forms of laccase, namely free/native LAC, free/PEGylated LAC, Epoxy/native LAC and Epoxy/PEGylated LAC, was evaluated by means of UV/visible spectroscopy. The results reveal that the presence of PEG greatly enhanced the amount of polymer produced [28, 29], either in solution (data from our previous work) or when linked to the enzyme (1.5-fold of increase) (Figure 3.2). The catalysis of poly(catechol) with immobilized PEGylated laccase was also enhanced relatively to the immobilized native form. Comparing the catalysis with both, free/native and immobilized/native laccase forms, one can observe a slight decrease of the polymer amount. As expected, the catalytic activity decreases after immobilization due to lower mobility of the catalysts which has some molecular space occupied by the epoxy support.

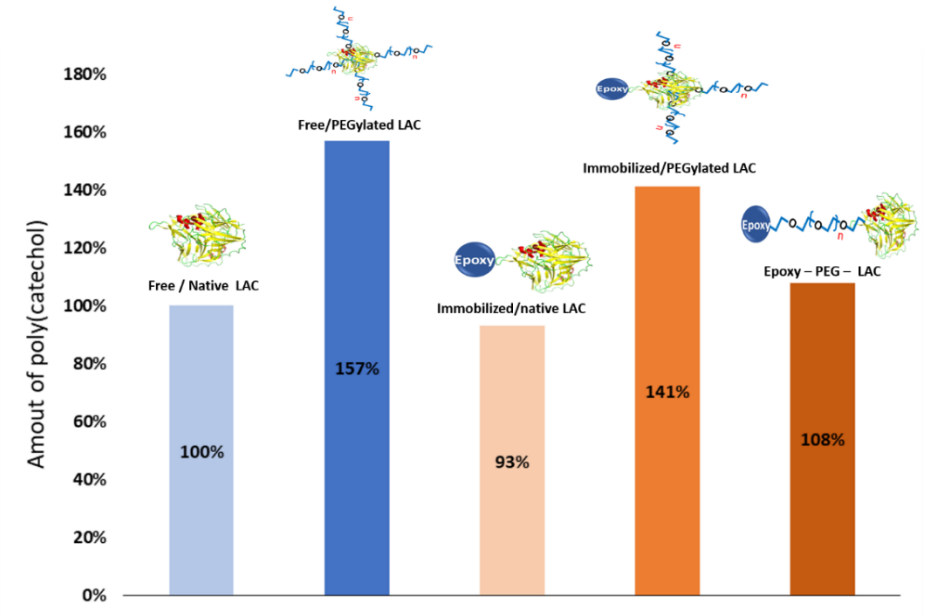

Figure 3.2. Amount of poly(catechol) after catalysis with: free/native LAC, free/PEGylated LAC, immobilized/native LAC, immobilized/PEGylated LAC and immobilized onto epoxy activated/native; the polymerization was conducted for 8h at 40 °C using 100U/mL_{enzyme} (λ=400 nm; free/native laccase corresponds to 100%) (% of polymer produced was calculated by UV measurements).

It is noteworthy that the stability of native and chemically modified laccases was evaluated, and the results confirm the loss of activity of all the enzyme forms particularly pronounced at 60 °C. Free/PEGylated LAC and immobilized/PEGylated LAC present a higher stability than free/native enzyme confirming the stabilization role of both PEG and of the immobilization step (see Table S1).

3.3.2 The role of laccase PEGylation and immobilization on catechol polymerization

After polymerization our goal was to perceive if we were in the presence of size-differentiated polymers and in which extent the PEGylation of the catalysts would influence the conversion rate of reaction and the final polymerization degrees.

From the data obtained one can infer that the amount of polymer produced is not the solely alteration observed when the chemically modified and immobilized enzymes were applied (Table 1). The nature of the polymer formed is quite different in size when PEGylated LAC is applied but is more evident when the PEGylated catalyst is in the immobilized form. The average DP increases from 7 to 8 when native/PEGylated is used but increases more considerably when the immobilized laccase form is used (DP=7 versus DP=10, comparing free and immobilized native laccase; DP=8 versus DP=14, comparing free and immobilized PEGylated laccase).

The MALDI-TOF data (Figure 3.3 and Table 3.1) also reveal that the biocatalysis using the solid support lead to the formation of poly(catechol) with a higher polymerization degree (DP=10 versus DP=7) comparing both free/native and immobilized/native enzyme. The immobilized/PEGylated form present higher DP than free/PEGylated one (DP=14).

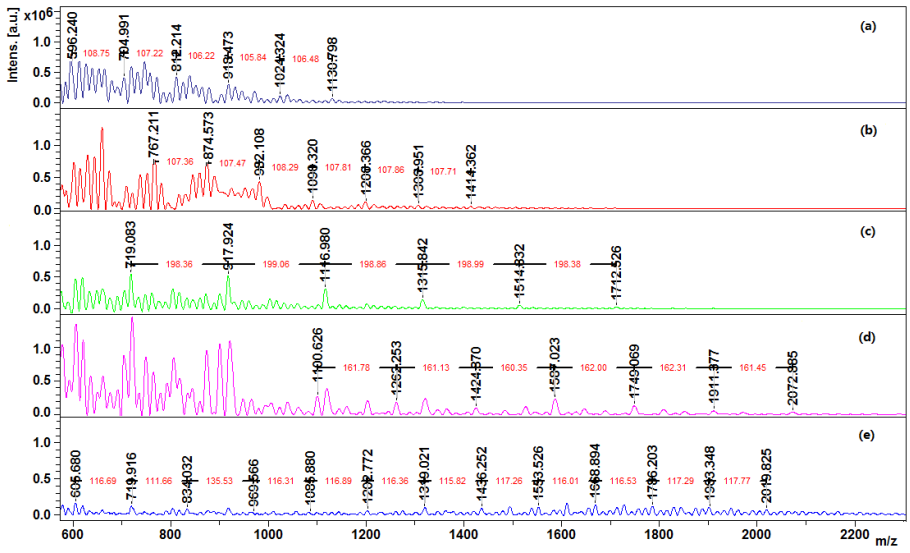

Figure 3.3. MALDI-TOF mass spectra of poly(catechol) catalyzed by: (a) free/native LAC; (b) free/PEGylated LAC; (c) immobilized/native LAC; (d) immobilized/PEGylated and (e) native immobilized onto PEG-activated resin.

The determination of the total content of free OH groups by different methodologies, namely Folin-Ciocalteu[30, 31] and ^{1}H NMR methods allowed us to distinguish different catalytic behaviour between all the laccase forms used for catechol polymerization. Comparing both native and PEGylated laccase forms, the data obtained show a significant decrease of the total content of OH groups for the modified form, even on free or in the immoblized form (see Figure 3.S1).

By ^{1}H NMR spectra analysis one can perceive broad signals observed between δ_H 6.0 and 8.5 ppm, corresponding to the aromatic peaks of poly(catechol). This indicates that we are in the presence of complex mixtures after polymerization with all enzyme forms (Figure 3.S1). Due to spectra complexity no polymer structure can be elucidated. Still, another broad signal can be detected between δ_H 8.5 and 10.5 ppm, which corresponds to OH signal group. This assumption was confirmed by D_2O addition to the NMR tube (data not shown). The OH content contained in all the reaction samples was calculated by ^{1}H NMR integration and are presented in table 1.

Table 3.1. Polymerization of catechol under different experimental conditions (laccase amount; OD at 400nm; total content of free OH, calculated by Folin-Ciocalteu method and by ^{1}H NMR; average degree of polymerization and dispersity) (*average degree of polymerization calculated by MALDI-TOF analysis; one U is defined as the amount of enzyme that catalyzes the conversion of 1µmol of substrate (ABTS) per minute per mg of protein used)

LAC	Activity (U/mL)	OD – 8h (400 nm)	Amount of OH groups		(Mn; Mw)*	Average DP*	D_M (dispersity)
			By Folin-Ciocalteu	By ^{1}H NMR			
Free / native	100	0.926	0.515	0.500	748; 776	7	1.03
Free / PEGylated		1.452	0.293	0.310	833; 849	8	1.02
Immobilized/native		0.857	0.192	-	1088; 1118	10	1.03
Immobilized/PEGylated		1.301	0.202	0.110	1460; 1498	14	1.02
Epoxy-PEG-LAC		1.003	0.075	0.320	1570;1600	15	1.02

The immobilization of the catalysts, in the native or PEGylated form, influenced greatly the laccase performance as demonstrated in Figure 3.4. Free/native LAC in solution conditions yields polymers with the typical dark brown color presenting a typical UV/Vis spectrum (Figure 3.4, in purple). When immobilized, the enzymes produce green-brown polymers with a different spectra behavior (Figure 3.4, in green).

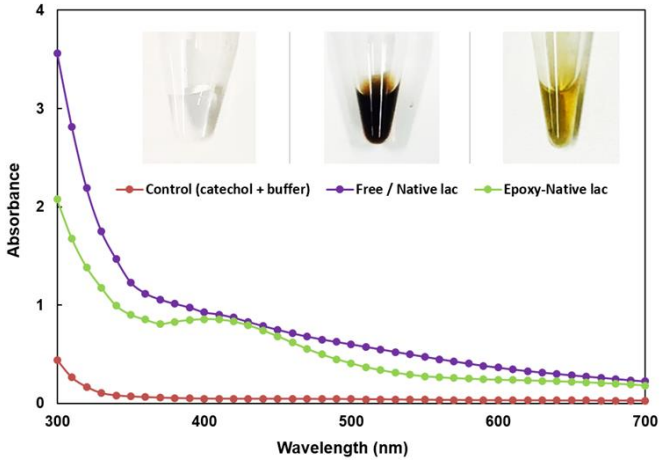

Figure 3.4. UV-Visible spectra of poly(catechol) after polymerization using: control (buffer + catechol) (red line), free/native laccase (purple line) and immobilized/native laccase (green line); the polymerization was performed using 100U/mL$_{enzyme}$ for 8h at 40 °C.

3.4 Discussion

The catalytic ability of different laccase forms (free/native LAC, free/PEGylated LAC, immobilized/PEGylated LAC and Epoxy-PEG-LAC) to polymerize catechol was evaluated. Overall the data from Table 1 and Figure 3.2, considering the amount of poly(catechol) formed and the polymerization degrees obtained, indicates that PEGylated laccase, free or immobilized onto the epoxy resin, seems to be the ideal catalyst for catechol polymerization. Previously Modaressi and co-workers described the protective role of PEG on laccase activity[28]. Herein, we confirm also that the presence of PEG linked to the enzyme greatly enhanced the amount of polymer produced, as set by UV-Visible spectroscopy. The color change of the catechol solution is associated with a bathochromic shift and an increase in the UV-Vis absorption intensity indicates a greater degree of π-conjugation thus confirming polymerization[32]. The results demonstrate other potentiality of PEG, which can not only be used as stabilizer, in solution or linked to the enzyme, but also as a spacer/linker for the immobilization of laccase onto solid supports[16].

The free hydroxyl content and MALDI-TOF data (Table 3.1, Figure 3.3), the UV/visible spectra (Figure 3.4) indicate that, independently on the amount of poly(catechol) produced, the different laccase forms used yield size-different polymers. The PEGylation and immobilization of the catalyst influence greatly the catalytic behavior of laccase. The role of PEG as template for polymerization reactions is not new, some works have already reported the role of this additive as template to assist polymerization reactions[14, 28, 33]. Previously we also confirmed that the oxidation of phenolics like catechol is favored when conducted in the presence of PEG, even in solution or linked to the enzyme. The PEGylation of laccase enhanced the polymerase activity by 3-fold compared with the native enzyme, while PEG in solution only increased polymerization by 1.5-fold. Our results were corroborated by molecular dynamics simulations which suggested the formation of a mixable complex between PEG and the poly(catechol) formed, pushing the reaction forward. Herein, our data also confirms these previous findings, showing higher conversion rates and longer polymers when PEGylated enzyme is applied. Moreover, the only report about the effect of PEGylated MtL on other substrates[16] revealed that Kcat and Km are not significantly affected by PEGylation, confirming our previous assumptions.

Several reports highlight the conformational changes suffered by enzymes when immobilized which

may alter their catalytic properties[20, 23, 34]. However, it is also established that when immobilized, the enzymes became stable and less susceptible to the medium interference like obstruction of the catalytic active site by the new polymers formed. Our data indicates that immobilized laccase gave rise to a higher decrease of the free hydroxyls, indicating the formation of a longer polymer. From UV/Visible spectra data one can predict a synergistic effect between PEGylation and immobilization, since immobilized/native enzyme did not reveal higher performance when compared with free/native catalyst. On the other hand, when PEGylated laccase form, or even native enzyme is immobilized onto PEG activated resin, the conversion increases and longer polymers are obtained. The immobilization of the catalyst together with the presence of the PEG are expected to stabilize the enzyme allowing the reactions to proceed longer with the formation of polymers with higher polymerization degrees. Comparing with several works reported in literature, the modified laccase forms herein presented are promising catalysts to efficiently produce longer polymers[35, 36].

The literature related with the enzymatic polymerization of catechol report a typical polymer structure where the catechol units are connected by ether linkages. The reaction occurs by oxygen-carbon bonding at the *para*-position of the other monomeric unit. This position is more prone to be involved in the reaction binding rather than the *orto*-position, which involves more stereochemical impediments[35-39] . Both formation of quinoid derivatives or homomolecular dimers coming from an intermolecular nucleophilic attack from the radicals formed by reaction with laccases have been described. The units composing these dimers are linked by C-C or C-O bonds by oxidative condensation, oxidative phenolic coupling or oxidative coupling. After a certain reaction time, this coupling can lead to the formation of oligomers or polymers.

The complexity to identify these oligomers as well as to establish the corresponding polymerization reaction pathways, especially when different laccase forms are applied, is still a major drawback to overcome. The structure identification of some polymeric products is not completely clear and need deeper investigations. Moreover, despite the high number of works reporting the enzymatic polymerization of catechol, no elucidation about the polymer NMR is presented, neither about the fractionation of the final polymers obtained. The authors generally base their structural proposals on the previous findings reported previously. Contrarily to what is reported, after enzyme and unreacted catechol removal, we were not able to predict a possible polymer structure. The ^{1}H NMR

and C-13 NMR (not shown) reveal a very broad spectra which does not allow the correct prediction of the possible polymer structures. Even though, the structure generally assigned in literature as being the final poly(catechol) is also not identified in all the spectra acquired after catalysis with all enzyme forms. From all the spectra obtained one can infer that we are in the presence of a panoply of different polymer structures with differentiated lengths, however impossible to identify.

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Chapter IV

The effect of high-energy environments on the structure of laccase-polymerized poly(catechol)

This chapter is based on the following scientific publication:

Jing Su, Tarsila G. Tallian, Jennifer Noro, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. The effect of high-energy environments on the structure of laccase-polymerized poly(catechol). Ultrasonics Sonochemistry, 2018,48, 275-280.

Abstract

The laccase polymerization of catechol was performed using different reactors namely a water bath (WB), an ultrasonic bath (US) and a high-pressure homogenizer (HPH). The total content of free OH and the MALDI-TOF spectra of polymers obtained demonstrated that reactions are favored in the presence of high-energy environments. Higher conversion yields and polymerization degrees (DP) were obtained after polymerization using US or HPH. Molecular dynamic simulation studies supported these findings by revealing a more open enzyme active site upon environments with high molecular agitation. The higher mass transport generated by US and HPH is the main feature responsible for a higher substrate accessibility to the enzyme which contributed to produce longer polymers.

Keywords: catechol; polymerization; laccase; ultrasonic bath; high-pressure homogenizer

4.1 Introduction

Wastewater discharged from processing industries, like oil refineries and petrochemical, contain dissolved organic pollutants such as phenols and substituted phenolic compounds, which are toxic and hazardous to the environment, unless they are treated properly[1]. A number of technologies have been proposed for the removal of phenolic compounds, like catechol, from wastewater aqueous solutions. These include destructive processes such as chemical oxidation, coagulation, solvent extraction, liquid membrane permeation and adsorption, adsorptive micellar flocculation, ultrafiltration, and biological methods[2]. Although chemical methods are mostly applied, milder reaction conditions (like mild pH and low temperature) are often preferred. Due to their versatile biochemical properties, high protein stability, and breadth of substrate spectrum, laccases are the key promiscuous and environmentally friendly biocatalysts, able to catalyze various aromatic compounds[3]. Previous studies indicate that flavonoids like catechol represent an important and versatile substrate which are polymerized via laccase-catalyzed oxidation, and the products of

reaction are regarded as valuable redox polymers with excellent matrix functionalities applied in several fields[4, 5]. In our previous studies we chemically modified laccase from *Myceliophthora Thermophila* by PEGylation with 20 kDa poly(ethylene glycol) methyl ether, and use it to polymerize catechol without solvents addition, using a water bath as reactor device under mild pH conditions (pH 5). We have found that it is possible to control the structure of the poly(catechol) produced by PEGylated laccase polymerization[6]. However, as other studies reported, low polymerization yields were obtained after laccase catalysis in the absence of external stimulus.

Ultrasound has been extensively used in enzyme catalyzed biotransformations aiming to intensify the reaction processes and obtain higher yield of products in short periods of time[7-10]. Several works have been reporting laccase catalysis assisted by ultrasound however lacking information about the polymer structure obtained using differentiated devices[9, 11, 12].

High pressure homogenization as well as ultrasounds are known to produce cavitation. Comparison studies have been performed between both methods which reveal that hydrodynamic cavitation offers a better control over operating parameters, being more energy-efficient and less sensitive to reactor geometry[13]. However, until now few reports have been presenting the use of hydrodynamic cavitation to assist polymerization being the acoustic cavitation the only tool used for this purpose.

Our aim in this study is to evaluate the role of different high-energy environments on the laccase-assisted polymerization of catechol. For this, three different reactors were used namely a water bath (WB), an ultrasonic bath (US) and a high-pressure homogenizer (HPH). The polymerization was followed during time by UV-Vis spectra analysis of the color change. The produced polymers were characterized by Matrix Assisted Laser Desorption/Ionization-Time of flight Mass spectrometry (MALDI-TOF) and ^{1}H NMR. The activity and stability of laccase during processing were evaluated. Molecular dynamic simulations were also conducted to understand the molecular behavior of laccase under high-energy environments.

4.2 Experimental part

4.2.1 Materials

Laccase from *Myceliophthora Thermophila* was supplied by Novozymes, Denmark. Catechol, sodium carbonate, Folin-Ciocalteu reagent and MALDI-TOF matrices were purchased from Sigma Aldrich, Spain. Deuterated chloroform and dimethyl sulfoxide were obtained from Cortecnet, France.

4.2.2 Evaluation of enzyme stability and half-life time quantification

The effect of high-energy environments on the activity and stability of laccase was evaluated. For this, laccase was incubated under the same conditions used for catechol polymerization: the enzyme (100 U/ml) was incubated in acetate buffer (pH=5) at 40 °C for 2 hours using different reactors namely a water bath, an ultrasonic bath and a high-pressure homogenizer. Aliquots of enzyme solution were taken at different periods of incubation and the activity of laccase was measured against ABTS according to the methodology described by Childs and Bardsley [14]. The half-life time of native laccase was calculated according to the Equation [15]:

Half-life time $\left(t_{\frac{1}{2}}\right) = \frac{\ln 2}{K}$, where $K = (\ln U_0 - \ln U_t) / t$

U_0: enzyme activity at time zero;

U_t: enzyme activity at time t;

t: time of incubation;

U: one U is defined as the amount of enzyme that catalyzes the conversion of 1µmol of substrate (ABTS) per minute.

4.2.3 Laccase-assisted polymerization of catechol using different reactors

Catechol polymerization was processed by incubating 5 mg/mL of monomer with 100 U/mL laccase in acetate buffer (pH=5). The reactions were performed in three different reactors namely a water bath (Grant, United Kingdom), an ultrasonic bath (USC600TH, VWR International Ltd., USA; frequency 45 kHz and power of 120 W) and a high-pressure homogenizer (Emulsifex-C3, Avestin, Canada; 500-2000 bar, 50 Hz), at 40 °C for 2 hours. During reactions, the temperature of the homogenizer container was monitored using a thermometer. Afterwards the powder was

washed with water by centrifugation to remove the maximum amount of protein and dried under vacuum for further ^{1}H NMR and MALDI-TOF characterization.

4.2.4 UV-Visible spectra analysis

The polymerization was followed by UV-Vis spectroscopy using a 96-quartz microplate reader (Biotek Synergy Mx, Shimadzu, Japan).

4.2.5 1H NMR spectra

The precipitates obtained after washing and centrifugation were dissolved in deuterated solvents, DMSO-d_6 and $CDCl_3$, for ^{1}H NMR evaluation. The spectra were acquired in a Bruker Avance III 400 (400 MHz) using the peak solvent as internal reference.

4.2.6 MASS SPECTRA analysis

The polymers were analyzed by Matrix-Assisted Laser Desorption/Ionization with time-of-flight (MALDI-TOF) using 2,5-dihydroxy benzoic acid (DHB) as the matrix (≥99.5 %). The mass spectra were acquired on an Ultra-flex MALDI-TOF mass spectrophotometer (Bruker Daltonics GmbH) equipped with a 337nm nitrogen laser. For this, the samples were dissolved in a TA30 (30 % acetonitrile/70 % TFA) solution and mixed with a 20 mg/mL solution of DHB (1:1). Then a volume of 2 µL was placed in the ground steel plate (Bruker part n° 209519) until dry. The mass spectra were acquired in linear positive mode.

4.2.7 Determination of Total Content of Free OH

The total content of free OH groups was determined before and after polymerization using the Folin-Ciocalteu spectrophotometric method. The monomer and polymer solutions dissolved in DMSO (100 µL) were added to the mixture of Folin-Ciocalteu reagent (500 µL) and distilled water (6 mL), and the mixture was shaken for 1 minute. Then Na_2CO_3 solution (15 wt %, 2 mL) was added to the mixture and shaken for 1 minute. Later the solution was brought up to 10 mL by adding distilled water. After 2 h, the absorbance was measured at 750 nm (25 °C). The total content of free OH was assessed by plotting a gallic acid calibration curve (from 1 to 1500 µg/ml). The equation of

the gallic acid calibration curve was A=0.2977c+0.0368, and the correlation coefficient was r^2=0.999.

4.2.8 Molecular modeling and molecular dynamic simulations

Molecular Dynamics (MD) simulations were performed with the native laccase structure originated by homology modelling: the *Myceliophthora thermophila* amino acid sequence was obtained from the Gi number identifier (Gi 10058140) published in 2003 among several laccases structures[16] and accomplished by searching this number on UniProt server[17]. After that, we use the Swiss-model server[18, 19] to determine the laccase 3D structure under study, building a model from the most similar laccase template, *Melanocarpus albomyces,*[20] with 75 % of similarity.

Laccase was modelled with the simple point charge (SPC) water model in an octahedral box with a hydration layer of at least 1.5 nm between the peptide and the walls. Na^+ ions were added to neutralize the simulation boxes. Three stages of energy minimization were performed using a maximum of 50000 steps with steepest descent algorithm, due to the size of the system. Position restraints were applied in all heavy atom at the first step, followed by position restraints in the main chain atoms for the second step, and no restraints were used for the last step of energy minimization. The systems were initialized in a NVT ensemble, using Berendsen[21] algorithm, with the coupling constant τ_T = 0.10 ps, to control temperature at 310 K (40 °C) and simulate the experimental conditions used. After that, a NPT initialization step was performed, with V-rescale[22] and Parrinello-Rahman barostat[23] algorithms to couple temperature and pressure at 313 K and 1 atm, respectively. The following coupling constants were considered: τ_T = 0.10 ps and τ_p = 2.0 ps. Position restraints (with force constant of 1000 kJ·mol^{-1}·nm^{-2}) were applied to all protein heavy atoms in NVT initialization, and in the main chain in NPT step. The positions of copper atoms in the active site were frozen to maintain the coordination with histidine. Simulated Annealing[24, 25] method was performed during 10 ns to gently carry the system from 36 to 70 °C, simulating the experimental temperature conditions. After this procedure, one of the frames at 36 °C and other at 70 °C were submitted to classical molecular dynamics during 30 ns, without position restraints, and with the same NPT ensemble described above. All simulations were performed using the GROMACS 4.5.4 version[26-28], within the GROMOS 54a7 force field (FF)[29]. The Lennard-Jones

interactions were truncated at 1.4 nm and using particle-mesh Ewald (PME)[30] method for electrostatic interactions, also with a cut-off of 1.4 nm. The algorithm LINCS[31, 32] was used to constrain the chemical bonds of the enzyme and the algorithm SETTLE[33] in the case of water. From MD simulations, we computed the middle structure at 36 °C and 70 °C, through a single linkage analysis, implemented on GROMACS[26]. This technique adds structures that are below a RMSD (Root Mean Square Deviation) cut-off, generating more or less populated clusters and, within the largest cluster, it finds a middle structure that is the most representative of the whole simulation. We look at these central structures to analyze the differences in the active site, in both cases.

Molecular dynamics/docking data is shown in Supporting Information (Appendix) to provide additional information about the *Myceliophthora thermophila* active site and its interactions with catechol at target temperatures.

4.3 Results and discussion

4.3.1 Characterization of the poly(catechol)

We have previously set-up the optimal conditions for the laccase-assisted polymerization of catechol using a water bath. The optimal conditions were considered herein for the polymerization using the high-pressure homogenizer. During catechol polymerization, the changes in the UV-Visible region were recorded to follow the reaction along time. Figure 3.1 presents the spectra, after saturation, of poly(catechol) after 2 h of polymerization using different reactors. All spectra present a typical peak at 300 nm which intensity increases after polymerization probably due to a new molecular arrangement and polymer formation. The intensity increment is more evident for the solutions polymerized under high-energy environments (US and HPH). Respecting to control (conducted in the same conditions without enzyme), a new peak can be depicted at around 430 nm for all the polymer solutions. The increase of absorption is directly related with the amounts of polymers in solution which is remarkable higher for samples polymerized in the presence of high-energy environments.

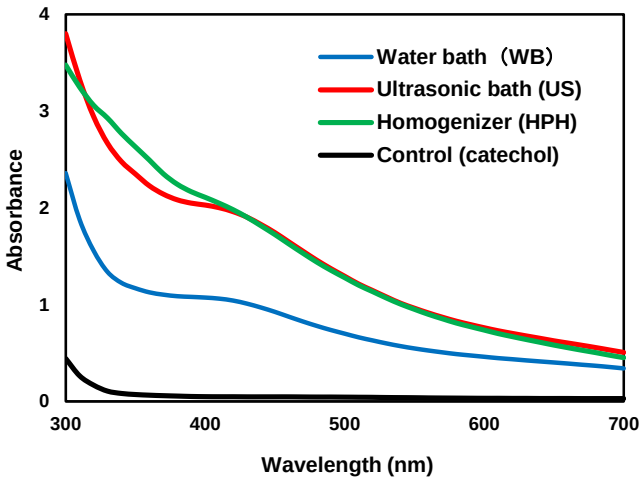

Figure 4.1. UV-Visible spectra of poly(catechol) after polymerization with 100 $U/mL_{laccase}$ for 2 h at 40 °C using: water bath (blue line); ultrasonic bath (red line); high-pressure homogenizer (green line); control (buffer + catechol, without laccase) (black line) (the controls performed on the 3 reactors present similar spectral behavior).

In order to predict the influence of high-energy environments on the polymer structure of the newly formed poly(catechol), we measured the total content of free OH in the polymer mixtures. As shown in Table 1, when ultrasound and high-pressure homogenization are applied, a lower content of free OH is observed, confirming a higher amount of polymer produced. The positive role of cavitation was not only perceived for the improvement of the conversion yields but was also effective on producing polymers with high molecular weight, as demonstrated by MALDI-TOF results (Figure 4.2). Despite the decrease of the total free OH observed, the spectra of products obtained using the WB show the formation of polymers with low average molecular weight. It is expectable that the reduced time of incubation (2h) will give rise, in this case, to low amount of polymer which is hard to ionize during MALDI-TOF evaluation. The spectra that resulted from catalysis using both US and HPH, show average polymerization degrees of 8 and 6, respectively for US and HPH, while for the water bath is 5. The effects resulting from acoustic and hydrodynamic cavitation are mainly high levels of transport. In both cases, sonication is most likely affected by physical rather than chemical activation, by increasing the mass transfer and, by sweeping the polymer surface, creating more propagation sites[34]. In the case of hydrodynamic cavitation, considering the data obtained, one can predict a more homogeneous chain growth, hence a narrower distribution of the molecular weight (Figure 4.2). A comparison between both high energy devices, ultrasonic bath and homogenizer,

must be considered for a further practical application. According to reported in literature the homogenizer presents some advantages in large-scale operation relatively to its counterpart. It is proved to be more energy-efficient, easier to generate and less sensitive to the geometry of the vessel[13]. The energy input required by the hydrodynamic cavitation reactor is lower since it converts its pressure energy into kinetic energy and its distribution is more homogeneous allowing a continuous operation[35].

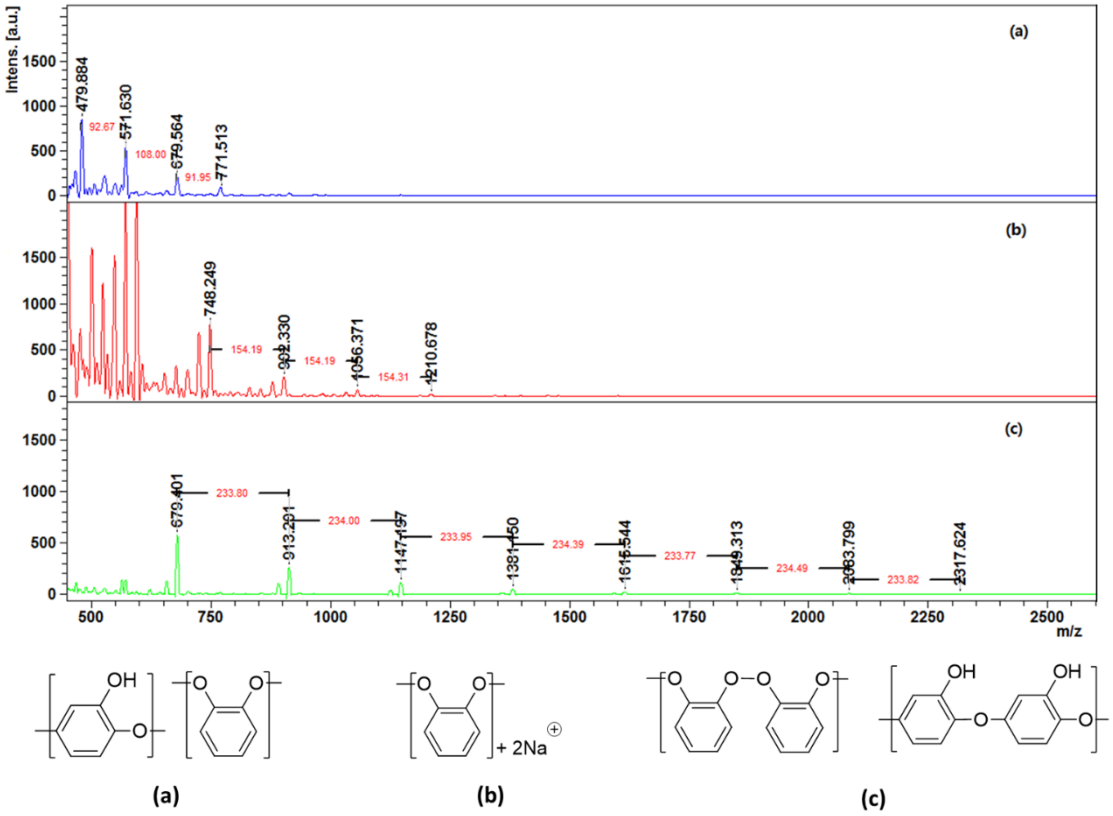

Figure 4.2. MALDI-TOF spectra of poly(catechol) after polymerization with 100 U/mL$_{laccase}$ for 2 h at 40 °C using: **(a)** water bath; **(b)** ultrasonic bath and **(c)** high-pressure homogenizer; at the top-down image are represented the possible polymer structures:(**a**: [M/(M-O)]; **b**: [M+2Na^{+}]; **c**: [2M+Na^{+}-5H^{+}], M represents the catechol monomeric unit).

Table 4.1. Laccase-assisted polymerization of catechol using different reactors: water bath (WB), ultrasonic bath (US) and high-pressure homogenizer (HPH) (conversion yield, total content of free OH, and Mn, Mw)

	Reactors		
	Water bath (WB)	Ultrasonic bath (US)	High-pressure homogenizer (HPH)
Conversion yield(%)Δ	73.75	83.60	86.20
TCFOH·	0.55	0.29	0.39
Mn, Mw*	547, 560	818, 836	609,689

*Calculated by MALDI-TOF spectra analysis

ΔCalculated by polymer weighting

·The values are normalized considering the total content of free OH of catechol as 1.00 (100%)

The differentiated polymerization observed when high-energy reactors were used was also confirmed by ^{1}H NMR analysis (Figure 4.S1). ^{1}H NMR spectra recorded in DMSO-d_6 showed no significant differences between the structure of the polymers obtained using water bath, ultrasonic bath or high-pressure homogenizer. However, when the spectra were recorded in $CDCl_3$ (Figure 4.S1), we observed significant polymer solubility differences depending on the reactor used. The starting material, catechol, was highly soluble in this solvent, but the polymers produced using ultrasound and homogenizer were extremely insoluble. Considering that only unreacted catechol is solubilized in $CDCl_3$, it was possible to compare its amount in all spectra. The amount of unreacted catechol is higher for samples incubated using a water bath followed by ultrasonic bath and high-pressure homogenizer. Taking this into consideration we might conclude that when the polymerization is conducted under high-energy environments, either acoustic or hydrodynamic cavitation, a high conversion of catechol into poly(catechol) occurred.

4.3.2 Laccase activity and stability during catalysis

Proteins are sensitive to high temperatures and it is expected that either ultrasound or high-pressure homogenization might lead to activity loss[9, 36, 37]. To predict the effect of the different high-energy environments used, the activity of laccase was evaluated during processing. Figure 4.3 shows the residual activity of laccase when processed using different reactors. The results obtained

show that after incubation in the water bath the enzyme retains almost 80 % of its initial activity. When high-energy environments (US or HPH) are applied, the half-life of the enzyme is greatly reduced (Table 4.2). However, since polymerization occurs in the first 0,5-1h of incubation, the activity loss will not restrict the final amount of poly(catechol) produced.

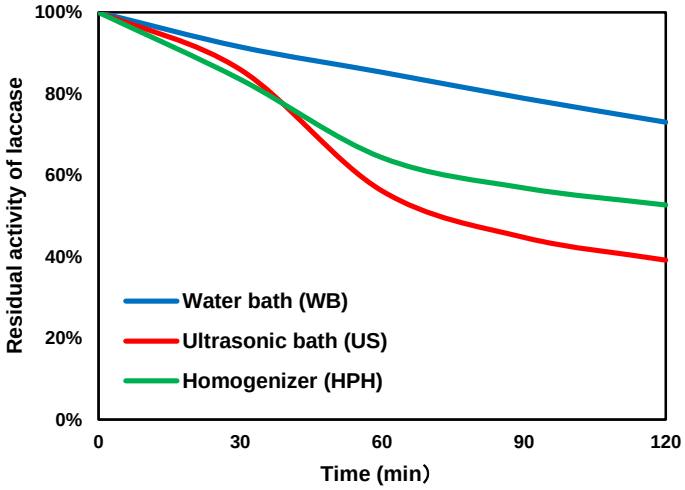

Figure 4.3. Residual activity of laccase (40 °C, pH 5) using: water bath (WB) (blue line); ultrasonic bath (US) (red line) and high-pressure homogenizer (HPH) (green line); the measurements were performed until enzyme loosed 50 % of the initial activity; for all conditions, the initial laccase activity was considered as 100 %.

Table 4.2. Half-life time of laccase incubated in different reactors

	Water bath (WB)	Ultrasonic bath (US)	High-pressure homogenizer (HPH)
Half-life time (h)	41.30	1.35	2.50

4.3.3 Simulation of laccase behavior under heating conditions

Extreme local temperature and pressure are properties generally related with the cavitation phenomena which might affect enzymatic activity. The cavitation reactors used herein are not exception and an increase of the local temperature was observed during time by thermometer monitoring. For this reason, it was imperative to simulate the behavior of the enzyme in conditions of heating increase. Simulated Annealing (SA)[24, 25] method was performed to study the behavior of

laccase under heating. This was performed by increasing the temperature during Molecular Dynamic (MD) simulations. The temperature was increased from 310 to 344K (36 °C to 70 °C), in 10 ns. Afterwards the simulation was conducted 30 ns with the initial structure at 36 °C and the last frame at 70 °C, from SA method. Figure 4.4 highlights the active site of the middle structure of laccase under different temperatures.

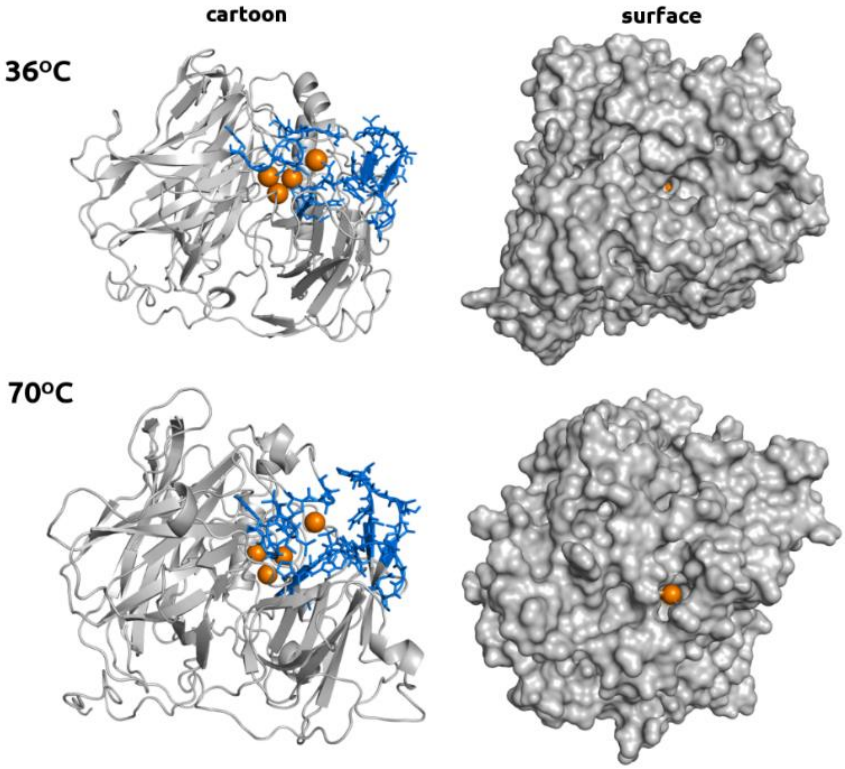

Figure 4.4. Middle structures of laccase at 36 °C and 70 °C in cartoon (left) and surface (right) representations; active site and cavities, for catechol access to the T1 copper site, are highlighted using amino acid side chains in blue or the surface shape; laccase is represented in grey and copper atoms in orange.

After simulation one can infer that at 36 °C, the enzyme is very stable, with the typical front access to the T1 copper in the active site. At 70 °C, the active pocket is more opened in several directions, which is easily observed analyzing both representations. The accesses from back, top and front to the T1 copper atom, facilitate the catechol entrance and the polymerization process. At higher temperatures, well-defined cavities can be observed leading to a greater facility of the substrate to enter the cavity and reach the copper. These data allowed us to speculate that enlarged catalytic pocked might favor the formation of larger polymer and/or the formation of different types of

poly(catechol) (as shown in Figure 4.2).

Our experimental data shows that under high-energy environments the enzyme is active during time enough to process the polymerization of the substrate, but the tendencies of enlargement of the catalytic pocket might also lead later to a progressive loss of enzyme activity.

Further molecular dynamics/docking data (Fig. 4.S2 and Fig. 4.S3) suggest that apparently at 40 °C catechol stays closer to T1 copper and at 70 °C it stays close to T2/T3 site, which would be consistent with the loss of enzyme activity[38], but the polymers are formed any way. Molecular dynamic tools used herein seems do not explain completely our system. We do think that the increased high mass transport effects provided by ultrasound and high-pressure homogenization will contribute to a faster kinetics and increased molecular weight of the poly(catechol) formed.

4.4 Conclusions

The role of high-energy environments on the polymerization of catechol by laccase was evaluated. The data obtained revealed that a higher mass transport occurring in US and HPH improves greatly the polymerization conversion yields. Longer polymers are obtained especially when the reaction is conducted in a high-pressure homogenizer (HPH). The decrease of enzyme activity observed during processing did not hampered the polymerization since it occurs mainly in the first half-hour where the catalyst maintained 80 % of its residual activity. One can also highlight that heating generated by high-energy environments favored the polymerization process as we confirmed by molecular dynamic simulations.

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Chapter V

Enzymatic polymerization of catechol under high-pressure homogenization for the green coloration of textiles

This chapter is based on the following scientific publication:

Jing Su, Jennifer Noro, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. Enzymatic polymerization of catechol under high-pressure homogenization for the green coloration of textiles. Journal of cleaner production, 2018, 202, 792-798.

Abstract

Laccase from *Myceliophthora thermophila* was used in this work to catalyze the polymerization of catechol under high-pressure homogenization for the green coloration of textile substrates. The polymerization of catechol was conducted using laccase in different forms, namely native laccase, PEGylated laccase and PEGylated laccase immobilized onto an epoxy resin. All three enzyme forms were deposited inside a polyester fabric bag during the experiments. The amount of polymer obtained was similar when using the three enzyme forms and its dispersion in water/DMSO mixture lead to powder particles of about 30-60 nm. The immobilized and PEGylated enzymes lead to poly(catechol) with 13 and 10 units, respectively, while the native form gave rise to shorter polymers (DP=8). We have shown that the oxidation of catechol conducted under high-pressure homogenization can be an efficient methodology for the *in-situ* coloration of textiles. The polymers produced by this methodology stained strongly the textile container, revealing this experimental set-up as a promising greener coloration/coating methodology involving milder conditions than the normally used in textile processes.

Keywords: catechol; laccase; high-pressure homogenization; oxidation; coloration

5.1 Introduction

Phenolic compounds, such as catechol, are released to the environment from a variety of industrial sources since they are often used as industrial reagents in the production of rubber, dyes, pesticides, colors, plastics, pharmaceuticals, and cosmetics[1]. When organic compounds like catechol are released into the environment, they can accumulate in the soil, groundwater and surface water, and therefore become an issue of great environmental concern. The presence of these potentially toxic chemicals may be able to transform into teratogenic or carcinogenic agents to life[2-6]. The increased demand of the industry to develop environmentally friendly methodologies lead to the development of enzymatic processes for its pollutant removal from wastewater [1, 7].

Laccase-catalyzed polymerization has received much attention from researchers in the last decades due to its ability to oxidize both phenolic and non-phenolic compounds as well as highly recalcitrant environmental pollutants, making it useful for applications on several biotechnological processes[8, 9]. Laccases, a family of multi-copper containing oxidoreductases, are probably one of the most promiscuous enzymes considering their excellent catalytic properties. They are capable of oxidizing a wide range of different substrates with or without mediators under mild conditions, and for these reasons several researchers have been applying them on the polymer synthesis[10-12]. As typical laccase substrates, phenols, namely catechol, and its derivatives can generate various functional polymers based on diverse monomers, which may be applied in several fields like medicine, cosmetics, food and textiles. During synthesis, laccases are known to provide a unique alternative to organic synthesis compared with conventional chemical-catalyzed synthetic processes. The oxidation of catechol using laccase as catalyst has been studied. Saha et al. investigated a two-step treatment method for the removal of phenol, benzenediols, and an equimolar mixture of phenol and benzenediols from water and demonstrated that the proposed enzymatic method is a viable alternative means to remove phenol and benzenediols from industrial wastewaters [13]. Tušek *et al.* tested two different methods to immobilize laccase from *Trametes versicolor* and compared them for the catechol polymerization using different reactors[1]. They found the successful catechol oxidation using immobilized laccase in different reactor systems, batch and continuous, micro and macro size.

Different reactors have been explored to proceed the enzyme-catalyzed reactions[14-16]. Generally, water bath is the most common device used to promote mechanical agitation and improve the mass transport effects. However the absence of external stimulus reveal low polymerization yields mainly due to inefficient agitation and thus low mass transport is achieved[17]. These results lead the researchers to investigate the use of different devices able to promote higher levels of conversion. They found that when ultrasound devices were applied, cavitation effects enhanced the transport of substrate molecules to the enzyme improving catalysis and accelerating the reactions[18-21]. Several works have been reporting enzyme catalysis assisted by high-pressure homogenization (HPH)[22, 23], and their results reveal HPH as a promising method to improve the enzyme application under cavitation effects. High-pressure homogenization, as well as ultrasound, are known to

produce cavitation, which may accelerate reactions and increment the mass transport phenomena[24, 25]. Until now and from our knowledge, the use of HPH on the polymer synthesis is still poorly explored.

In previous studies, we conducted the catechol polymerization with laccase from *Myceliophthora thermophila* using different apparatus, namely a water bath, an ultrasonic bath and a high-pressure homogenizer[26]. The results obtained showed higher conversion yields and polymerization degrees when both high-energy reactors were used compared to water bath reactor. Molecular dynamic simulations performed also demonstrated that under these conditions the enzyme presented a more open active site which we consider the main factor for the higher substrate accessibility to the enzyme therefore favoring the production of longer polymers. High-pressure homogenization was thus considered as a promising technique for catechol polymerization.

In this study, our goal was to evaluate the catalytic ability of different forms of laccase to polymerize catechol under high-energy environments (high-pressure environments). The catalysis was thus conducted using native laccase, PEGylated laccase and laccase immobilized onto an epoxy resin (Epoxy-PEGylated laccase) under high-pressure homogenizer (HPH) (Figure 5.1). In this study, the non-soluble enzyme form was contained within a polyethylene terephthalate (PET) bag, as well as the other forms for control purposes. The objective was to constrain the immobilized enzyme movement along the machine and allow only the liquid solution to recirculate. The polymerization was followed during time by UV-Vis spectra analysis to monitor color change. The produced polymers were characterized by weight measurement, ^{1}H NMR, and TGA. The particle size of the polymer powders was analyzed by dynamic light scattering analysis. We aim with this work to evaluate the potentialities of the high-pressure homogenization as energy source for the enzymatic-catalysis reactions with different laccase forms and explore the possibilities to obtain polymers with coloring performance via a green methodology. Generally, fabric dyeing processes imply the use of extreme conditions like high temperatures and/or highly acidic or alkaline pHs. Polyphenolic components from laccase reactions are produced at milder conditions and could be used for green coloration.

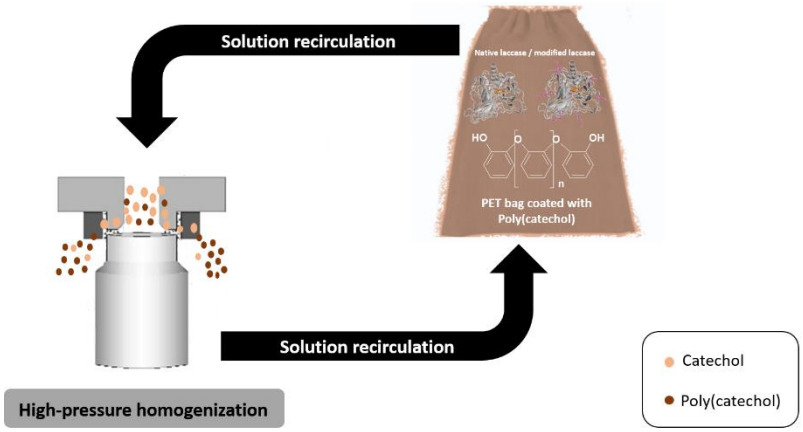

Figure 5.1. Representation of polymerization of catechol with native and modified laccases under high-pressure homogenization using a PET bag as enzyme container.

5.2 Materials and methods

5.2.1 Enzyme and reagent sources

Laccase from *Myceliophthora thermophila* (Novozymes A/S) was supplied by Novozymes, Denmark. Catechol, poly(ethylene glycol) methyl ether and sodium carbonate were purchased from Sigma Aldrich, Spain. Deuterated dimethyl sulfoxide was obtained by Cortecnet, France.

5.2.2 PEGylation of laccase

Laccase from *Myceliophthora thermophila* was PEGylated as previously reported[27] using the procedure of Daly *et al.*[28]. Briefly, 14.0 mL of 12 mg/mL laccase were reacted with 20 kDa, poly(ethylene glycol) methyl ether at pH 5.0 phosphate solution 100 mM with 20 mM sodium cyanoborohydride. A control reaction without $_{m}$PEG was also conducted in every experiment. The reactions were stirred rapidly for 17 h at 4 °C. After 10 min of mixing, the reactants were completely dissolved, and an aliquot (namely time 0 h) was taken, as well as at each time point of reaction. These samples were ultrafiltrated, and washed several times with water, using a 30 kDa cellulose membrane mounted in an ultrafiltration apparatus, to separate the free PEG. Afterwards the final solution was freeze-dried.

5.2.3 Immobilization of PEGylated laccase onto epoxy resin supports

The immobilization of PEGylated laccase onto epoxy methacrylate resins (Purelite Lifetech ECR enzyme immobilization resins: 300-600Å) was conducted as follows: 2 mg/mL PEGylated laccase in 0.5 M acetate buffer (pH 5.0) were mixed with epoxy methacrylate (50 mg/mL) and then stirred for 48 hours at 4 °C. The powder was then washed several times with water by centrifugation and dried under vacuum.

5.2.4 Evaluation of enzyme activity and stability

The effect of high-pressure on the activity and stability of native laccase, PEGylated laccase and Epoxy-PEGylated laccase, was evaluated. For this, the three forms of laccase were incubated under the same conditions used for catechol polymerization: 100 U/mL enzyme were incubated in acetate buffer (pH=5) at 40 °C for 3 hours, using a high-pressure homogenizer. The enzymes were exposed for longer time in order to ensure the accurate stability during time. Aliquots of enzyme solution were taken at different periods of incubation and the activity of laccase was measured against ABTS according to the methodology described by Childs and Bardsley[29].

5.2.5 Enzyme-assisted polymerization of catechol

Catechol polymerization was processed by incubating 50 mM of monomer in different solutions: a) 100 U/mL native laccase and b) 100 U/mL PEGylated laccase, c) 100 U/mL Epoxy-PEGylated laccase, in acetate buffer (pH=5). The immobilized enzyme was confined in a polyethylene terephthalate (PET) bag and placed in the sample receptor of the high-pressure homogenizer. For control reasons, the other enzyme forms were also placed inside the PET bag. Afterwards the catechol solution was added and the homogenization proceed for 2 hours (corresponding to 360 homogenization cycles). During the reaction, the top of the feed port was covered with a parafilm with tiny holes to allow the air oxygen entrance without extra oxygen supply. The starting temperature was set to 40 °C and the temperature was monitored during processing to follow the inherent increase during high-energy device processing. Next the polymer powder was collected from the HPH device by dissolution with dimethyl sulfoxide to solubilize the insoluble polymers and posteriorly dried under vacuum for ^{1}H NMR analysis.

5.2.5 ^{1}H NMR

^{1}H NMR spectra were acquired in a Bruker Avance III 400 (400 MHz). DMSO-d_6 was used as deuterated solvent, using the peak solvent as internal reference.

5.2.6 Determination of Total Content of Free OH groups

The total content of free OH groups before and after polymerization was performed using the Folin-Ciocalteu spectrophotometric method. The monomer and polymer solutions dissolved in DMSO (100 µL) were added to the mixture of Folin-Ciocalteu reagent (500 µL) and distilled water (6 mL), and the mixture was shaken for 1 minute. Then Na_2CO_3 solution (15 %, 2 mL) was added to the mixture and shaken for 1 minute. Later the solution was brought up to 10 mL by adding distilled water. After 2 h, the absorbance at 750 nm (25 °C) was measured. The total content of free OH was assessed by plotting a gallic acid calibration curve (from 1 to 1500 µg/ml). The equation of the gallic acid calibration curve was A=0.2977c+0.0368, and the correlation coefficient was r^2=0.9988.

5.2.7 Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) was carried out in a Perkin Elmer TGA 4000 equipment. Calibration was performed with metals, such as Nickel, Alumel and Perkalloy, based on their Curie Point Reference. All the analyses were performed with a 6 mg sample in aluminum pans under a dynamic nitrogen atmosphere between 100 and 700 °C. The experiments were run at a scanning rate of 20 °C/min.

5.3 Results and discussion

5.3.1 Effect of chemical modifications on enzyme's catalytic activity

PEGylation and immobilization processes are known to conduct to more stabilized catalysts however reducing their specific activity[30, 31]. Herein, one could observe that after PEGylation and immobilization process a ratio of activity among native laccase, PEGylated laccase and Epoxy-

PEGylated laccase of (1: 0.7: 0.55) was observed, revealing an activity loss after the chemical modification process, as also confirmed previously by others[30].

5.3.2 UV/Visible spectra monitoring during catechol polymerization

During laccase-assisted polymerization of catechol under high-pressure homogenization, all the reaction mixtures changed colour from colourless to dark brown, and the UV-Vis spectra was recorded to follow the reaction (Figure 5.2). From data obtained, it can be seen all spectra present a typical peak around 300 nm which increases in intensity after polymerization due to new molecular arrangement and polymer formation. The spectra of catechol incubated with enzymes present a new peak at around 430 nm confirming polymerization.

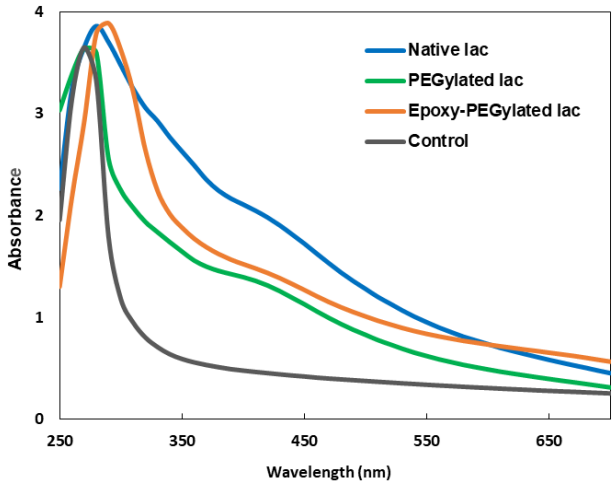

Figure 5.2. UV/Visible spectra of poly(catechol) polymerized by different enzyme forms using HPH; the spectra was acquired after 2 h of incubation.

Generally an increase in the UV-Vis absorption intensity indicates a greater degree of π-conjugation correlated with the occurrence of polymerization[32], and this increase is directly related with the amount of soluble poly- or oligomers, which is remarkable higher for samples polymerized in the presence of native laccase in this study. However, differences in UV-Vis spectra are related with the amount of soluble poly- or oligomers and are not attributed to the precipitate[17]. Precipitates with darker colour in the reaction solution correspond to insoluble polymers formed and cannot be quantified by UV-Vis spectra, being therefore quantified by weight measurement. Besides this, the

polymers coated onto PET fabric were also quantified by weighting and considered for the total amount of polymer produced and yield of polymerization (Table 5.1). The data obtained reveal higher reaction yield when PEGylated laccase is used as catalyst. These findings are in accordance with our previous findings which reveal that PEGylation of laccase greatly enhances catechol polymerization, and the molecular dynamic simulations suggested that PEG serves as template forming a complex with the newly formed polymer, pushing the reaction forward[27].

As confirmed by others, UV/Visible data reveal that laccase catalysis of catechol assisted by high-pressure homogenization provide a mild route to produce polymers and may offer a green alternative to the environmentally unfriendly, costly and less specific physico-chemical methods[33-36].

Table 5.1 Polymerization yield of poly(catechol) using different enzyme forms

	Weight of polymer coated onto fabric (mg)	Weight of polymer powder recovered from solution (mg)	Total weight of polymer (mg)	Yield of reaction* (%)
Native laccase	7.6	22.8	30.4	60.8
PEGylated laccase	9.1	27.1	36.2	72.8
Epoxy-PEGylated laccase	7.4	24.1	31.5	63.0

* Yield of reaction = (weight of polymer recovered from solution + weight of polymer coated onto fabric) / total weight of monomers) *100%

5.3.3 Enzyme stability during enzymatic oxidation process

All enzyme forms used herein were subjected during processing to the high-pressure homogenization heating conditions which might affect their catalytic performance. To predict the effect of high-pressure homogenization on enzyme activity and stability, an experiment was conducted using the same conditions as applied for catechol polymerization without addition of monomer. The activity of the enzyme was evaluated during time by evaluation at different periods of incubation. According to Figure 5.3, as the processing time increased, a decrease of activity is observed for all the enzymes tested, however, displaying a different decay. For native laccase it is evident a deep decrease of the activity on the first periods of incubation. The residual activity was maintained only in around 55% after 2 hours of incubation. The PEGylated and immobilized/PEGylated laccase forms, showed a different behavior, a slow drop of the activity was

verified during time and the residual activity after 2 hours of incubation remained at around 75% and 73%, respectively. We might speculate that the different deactivation behavior observed is probably due to the stabilization effect of the PEG and of the immobilization which, in the case of the modified enzymes, delayed the enzyme deactivation[37, 38].

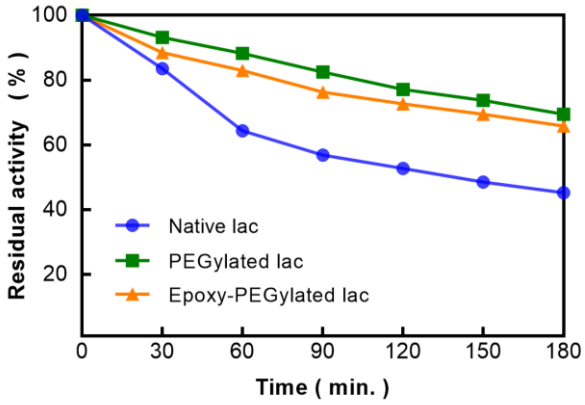

Figure 5.3. Residual activity (%) of all enzyme forms when incubated in the same conditions as the used for catechol polymerization under high-pressure homogenizer.

5.3.4 ^{1}H NMR characterization of poly(catechol)

Figure 5.4 shows the spectrum of catechol and poly(catechol) after polymerization. As it can be seen by ^{1}H NMR data, the aromatic peaks of the polymers suffer a chemical shift from δ_H 6.58 and 6.72 to δ_H 6.88 and 6.96 ppm. No other peaks are observed in the spectra, and it is noteworthy that all the powder is completely soluble in the DMSO-d_6 for analysis. The OH peak of catechol is observed at δ_H 8.8 ppm as a singlet (proton a). In the polymers spectra, this peak disappears, while a small singlet is observed at δ_H 8.48 ppm, which is assigned as the terminal OH of the polymers (proton d). The patterns turn to be more complex in the polymers, but the calculation of the coupling constants is still possible. Catechol has coupling constants of J= 6.0 and 3.6 Hz, corresponding to the *orto* and *meta* coupling constants, respectively. The same coupling constants were obtained for the polymers obtained using the three different laccase forms, suggesting that polymerization reaction occurred via hydroxyl groups in all cases.

COSY bidimensional spectra shows the correlation between the aromatic protons of poly(catechol) (Figure 4C). The pattern of the peaks (two doublet of doublets), and the COSY analysis corroborates

the previous proposed structure, which suggests that the polymerization of catechol occurs by the oxygen atoms.

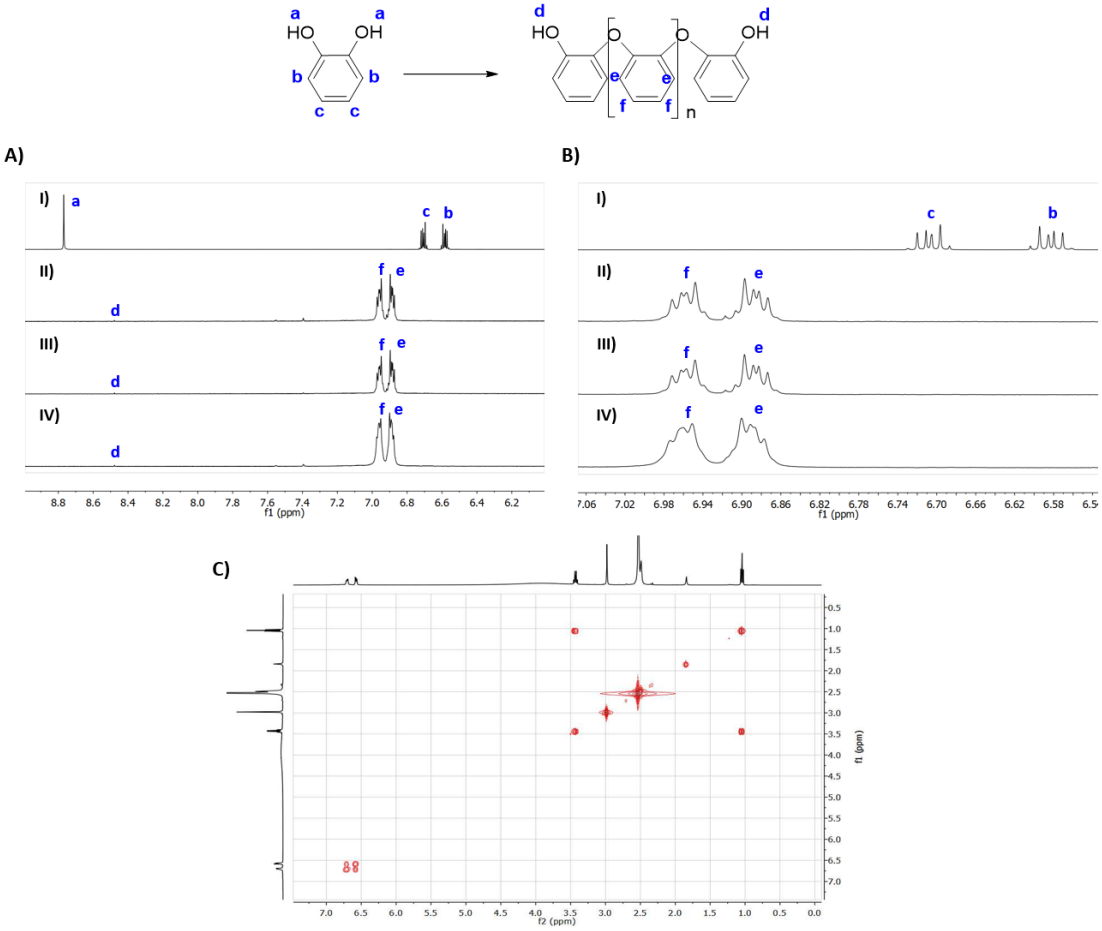

Figure 5.4. A) ^{1}H NMR spectra of: **I)** catechol; **II)** Poly(catechol) using Native laccase; **III)** Poly(catechol) using PEGylated laccase; **IV)** Poly(catechol) using epoxy-PEGylated laccase; **B)** expansion of the ^{1}H NMR spectra in A); **C)** COSY spectra of poly(catechol) using epoxy-PEGylated laccase as catalyst (recorded in DMSO-d_6).

To confirm the role of different laccase forms on the polymerization, the amount of oxyphenylene units was estimated by measuring the total content of free OH in all the mixtures after reaction (Table 5.2). Folin-Ciocalteau method measured the total OH after catechol polymerization and the values are normalized considering the total content of free OH of catechol as 1 (100 %). From Table 5.2, it can be depicted a decrease of the total free OH after catechol polymerization in the presence of PEGylated laccase being more pronounced for samples polymerized by Epoxy-PEGylated laccase. As the content of OH groups decrease as more chains of oxyphenylene are present, confirming higher degrees of polymerization, which is accordance with the ^{1}H NMR results. The average

polymerization degrees are 8, 10 and 13, when native laccase, PEGylated laccase and Epoxy-PEGylated laccase were applied, respectively. From this data it was possible to infer the role of enzyme form on the polymerization degree. One can establish that using the later PEGylated laccase and Epoxy-PEGylated laccase higher DP and higher amount of poly(catechol) is obtained, confirming our previous findings[26] (Table 5.1). The green, high-energy efficient and low cost methodology applied[39], high-pressure homogenization, played however a crucial role on improving the polymerization of catechol. Comparing with previous findings using native laccase in a high-pressure homogenizer, we found herein an increase of the DP from 6 to 8. Moreover, the poly(catechol) polymerized by PEGylated and epoxy-PEGylated laccases suffered an increase of the final DP when homogenization is applied, in comparison with processing in a water bath[27]. Hydrodynamic cavitation, the type of cavitation inherent to the high-pressure homogenizer, is generated by the flow of liquid through a simple geometry such as venture tubes or orifice plates under controlled conditions. When the pressure at the throat falls below the vapor pressure of the liquid, the liquid flashes, generating a number of cavities, which subsequently collapse when the pressure recovers downstream of the mechanical constriction[40]. These liquid jets activate the solid catalyst and increase the mass transfer to the surface by disruption of the interfacial boundary layers as well as dislodging the material occupying the active sites[41]. In comparison with acoustic cavitation, this type of technology is more energy efficient and low processing costs are associated. Moreover, the use of a biological catalyst replacing the chemicals generally used, provide a green approach for the catechol detoxification through polymerization[42].

The use of a PET fabric bag as enzyme container might also be significant to improve the polymerization of catechol. It was not expectable a high affinity of the newly polymers within the surface of PET fabric due to a lack of bond formation between the polymers and the PET fiber. However, we can speculate that the pairing of the aromatic part of the newly formed polymers with the aromatic chains of PET (stacking interaction) might favor the polymerization and increase the amount of polymer at the surface of the support.

The effect of high-pressure homogenization on the particle size of the polymers obtained was also evaluated. Our previous findings reveal particle size of poly(catechol) catalysed by native laccase, PEGylated laccase and Epoxy-PEGylated laccase using a water bath of 135.70 ± 0.68 nm, 155.90

± 2.35 nm and 162.70 ± 1.56 nm, respectively[27]. The use of hydrodynamic cavitation for catechol polymerization promoted a significant decrease of poly(catechol) particle's size, as presented in Table 2. Small, narrow and monodisperse polymer populations, in the range of 30-60 nm, are obtained for all the enzyme forms used. As confirmed by others[25], the high-pressure homogenizer operated at high dynamic pressure is effective in reducing the particle size of the polymers. The combination of shear, grinding and cavitation, promote the collapse on the surface, particularly of powders, producing enough energy to cause fragmentation and thus reducing the final achievable particle size of the polymers[25].

Table 5.2 Degree of polymerization (DP) calculated by ^{1}H NMR; particle size of polymer powders (polydispersity; count rate) and total content of free OH groups

Poly-catechol	Degree of polymerization (DP) by ^{1}H NMR	Size (nm) (50%H2O / 50%DMSO)	Polydispersity (PDI)	Count rate	Total content of free OH (catechol as 1.0)
Native laccase	8	39.26 ± 0.42	0.11	474.1	0.4849
PEGylated laccase	10	55.77 ± 1.60	0.11	170.8	0.3634
Epoxy-PEGylated laccase	13	61.86 ± 2.11	0.17	263.3	0.3215

5.3.5 Thermal properties of poly(catechol)

The thermal properties of the synthesized poly(catechol) were investigated by thermogravimetric analysis (TGA). Depending on the conditions of polymerization, differences in poly(catechol) TGA thermal behavior can be found in the literature[17]. In our work, the substrate catechol only shows one-step decomposition, losing all the weight from 100 to 230 °C (shown in Fig. 5.S.1). Herein, poly(catechol) synthesized by different laccase forms exhibited two distinct stages of weight loss, at around 120 °C, corresponding to 5 % of weight loss, and at around 400 °C, corresponding to 50 % of weight loss (Table 5.3). Like others previously reported, the new polymers formed presented a very slow degradation rate above 200 °C[17]. We also find a residue of around 40 %, namely when modified enzymes were used, up to 700 °C, which is also in accordance with previous data reported[43, 44]. Compared with catechol, which presented a vestigial amount of residue up to 700 °C, the new polymers formed demonstrated high stability to thermal degradation, especially when

polymerized by the modified forms of laccase, PEGylated or epoxy-PEGylated laccase under high-pressure homogenization.

Table 5.3 Thermal performance of catechol and poly(catechol) ($T_{5\%}$: temperature where polymers reach 5% of weight loss; $T_{50\%}$: temperature where polymers reach 50% of weight loss; poly(catechol) 1: obtained by catalysis with native laccase; poly(catechol) 2: obtained by catalysis with PEGylated laccase; poly(catechol) 3: obtained by catalysis with epoxy-PEGylated laccase)

	$T_{5\%}$ (°C)	$T_{50\%}$ (°C)	Residual weight (700 °C , wt %)
Catechol	147.89	201.56	0.55
Poly(catechol) 1	118.02	345.06	37.55
Poly(catechol) 2	122.85	414.28	42.45
Poly(catechol) 3	120.51	437.80	40.14

5.3.6 Fabric coloration with poly(catechol)

The use of laccase to synthesize colorants *"in situ"*, from aromatic compounds such as phenols, has been presenting as an efficient way for textiles dyeing at green and mild reaction conditions of pH, temperature and chemical reduction or elimination. Environmental issues have been led to an extensive research on the enzymatic dyeing of protein and cotton fibers[45-47]. However, due to their composition and hydrophobicity, polyester fibers are still poorly explored on this context. Herein, when using a PET bag as enzyme container for the catechol polymerization we found that the newly polymers produced were covering the fiber surface and this coating was stable after washing (Figure 5.4). Comparing with control sample (catechol incubated with buffer using the same high-pressure conditions, without laccase), the PET fabric samples containing poly(catechol) polymerized by native, PEGylated and epoxy-PEG-laccase presented higher coloration. Spectral data reveal different coloration for the different enzymes used. Higher coloration was obtained when using native laccase, followed by PEGylated and epoxy-PEG-laccase. As expected, the modified enzymes gave rise to lower coloration levels since part of the polymers produced by these forms are insoluble and would hardly be attached to the PET surface. Low polymer particles obtained by using native laccase were more prone to colorize the PET fabric surface. It is noteworthy that the

particle size was determined by using a mixture of water/DMSO after polymer recover at the end of the process. We may speculate higher polymer particle size during polymerization with modified enzymes in buffer (data not shown), which hinder a homogeneous covering of the fabric surface.

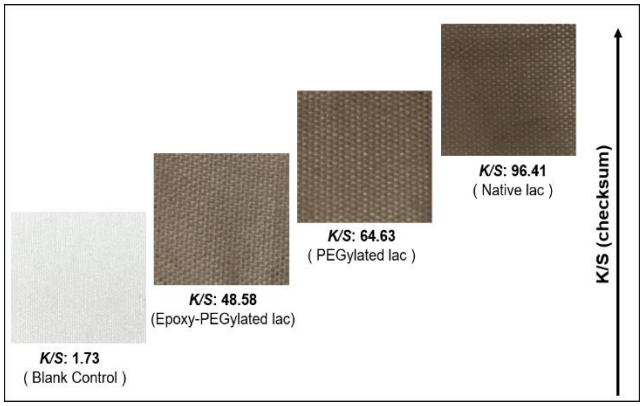

Figure 5.5. PET samples after coloration with the poly(catechol) polymerized by enzymatic catalysis under high-pressure homogenization.

5.4 Conclusions

We found that the enzymatic catalysis using PEGylated and epoxy-PEG laccase, under high-pressure homogenization gave rise to longer polymers with particle sizes between 30-60 nm. The small particle size obtained, especially when native laccase form was used, favored the coloration of a fiber which, in theory, would not be easily colored by these compounds. High-pressure homogenization revealed to be an effective technique for the enzyme-assisted polymerization of catechol. Comparing with other processing methods, hydrodynamic cavitation promoted by the high-pressure homogenizer provides a greener approach for catechol oxidation since it is energy efficient, chemical saving with low costs associated.

The green technology for catechol oxidation is reinforced by the use of laccase as biological catalyst working under mild reactions conditions of pH and temperature without addition of chemicals, using only atmospheric oxygen as electron source for oxidation.

The work presented herein is a step forward the green coloration of synthetic substrates by *in situ* enzymatic oxidation of phenolic compounds. Our results present polymers obtained enzymatically as valuable tools for the green coloration of fibers at lower temperatures. Further exploitation of the

synergism between laccase and high-pressure homogenization technique will allow to obtain a vast palette of colors by oxidation of different concentrations of phenolic monomers.

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Chapter VI

Can laccase-assisted processing conditions influence the structure of reaction products? *Discussion and Conclusions*

This chapter is based on the following scientific publication:

Jing Su, Jiajia Fu, Carla Silva, Artur Cavaco-Paulo. Can laccase-assisted processing conditions influence the structure of the reaction products?, Trends in biotechnology, 2019, 37, 683-686.

Abstract

Laccase is a promiscuous enzyme known to catalyse a wide range of phenolic substrates. Recent data indicate that several poly(catechol) products can be obtained using different processing conditions. The differentiated reaction products may be attributed to changes on the enzyme active site geometry induced by different environmental reaction settings.

Keywords: laccase, processing conditions, protein modification, polymer structure

6.1 Catalytic properties of laccase enzyme

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are biological catalysts belonging to one of the most promising polyphenol oxidases containing copper atoms in the catalytic centre, normally called multicopper oxidases[1-3]. These enzymes show great application in biochemical reactions, giving a major breakthrough in reducing pollution issues through environmentally clean production of materials. Laccases are widely distributed in higher plants, fungi, insects and bacteria[4], where they function in metal oxidation, morphogenesis, stress defence and lignin degradation[5, 6], as well as in detoxification of antimicrobial agents[7]. This enzyme couples the four-electron reduction of oxygen with the oxidation of a broad range of organic substrates, including phenolic and non-phenolic compounds, namely catechin, catechol, gallic acid, ferulic acid, syringaldehyde, vanillin, acetovanillone, coniferyl alcohol, rutin and others[1, 8], and even certain inorganic compounds by one-electron transfer mechanism[9, 10], as represented previously. Laccase uses dissolved oxygen to oxidise its substrates. In nature, laccase participates in lignin biodegradation, breaking down lignin found in woody substrates with phenolic compounds, which are further oxidised into polyphenolics known for their antimicrobial properties. These polyphenolic surfaces are normally found at open wood cuts in nature with the biological role of preventing microbial degradation.

The formation of small oligomers is more prone to occur in laccase-assisted reactions. The enzymatic polymerization via oxidative coupling is constrained by mass transfer and saturation

limitations[6-8]. Despite the awareness of the limitations to obtain high-molecular weight polymers when laccase is used as catalyst, it is also true that the structural features of the polyphenolics obtained and the inherent processing conditions are poorly described in literature[6-8].

6.2 Different approaches for laccase-assisted polymerization of catechol: protein and processing conditions modification

The enhancement of polymerization yield using laccases as catalysts was studied using template compounds like poly(ethylene glycol) (PEG). Free PEG and PEGylated enzymes enhanced the reaction conversation rates leading to higher amount of poly(catechol) formed with differentiated structural units, as shown in Table 6.1. The immobilization of laccase in epoxy resins, with or without PEG as a spacer between the resin and the catalyst, lead to other differentiated structures[11, 12]. Processing conditions were also investigated for the laccase-assisted polymerization of catechol. Three reactors, namely water bath, ultrasonic bath and high-pressure homogenizer, with specific characteristics in terms of agitation, pressure and cavitation, were used during the polymerization processing (Figure 6.1A)[13]. The application of these differentiated processing conditions, especially focusing on low and high-energy environments gave yield several poly(catechol) structural units, which are identified and characterized herein[13].

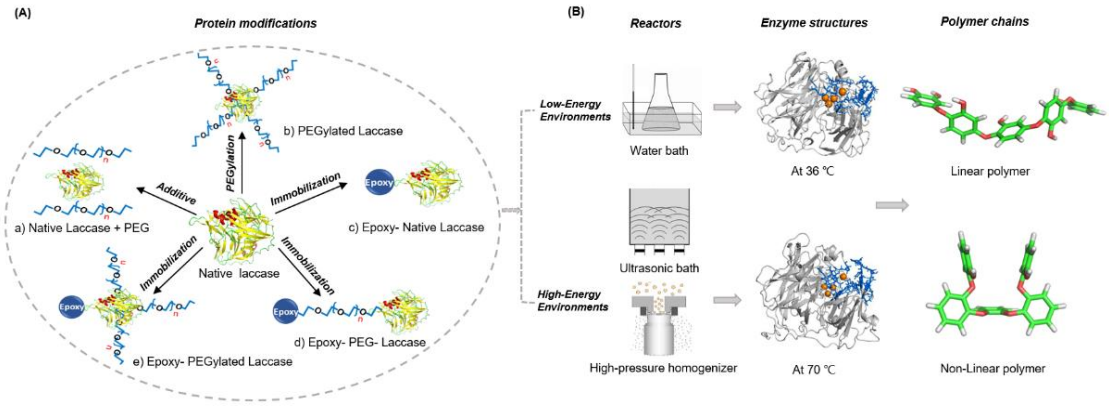


Figure 6.1 Different approaches for laccase modification and processing conditions using high-energy and low-energy reactors for catechol polymerization. (A) Different approaches for laccase modification: a) native laccase + PEG; b) PEGylated laccase; c) Epoxy-native laccase; d) Epoxy-PEG-lac; e) Epoxy-PEGylated lac, using different reactors (water bath, ultrasonic bath and high-pressure homogenizer) to perform the polymerization reactions;

(B) Different processing conditions: at left side are represented the high-energy and low energy reactors used to conduct the laccase-assisted polymerization of catechol; at the centre are represented the middle structures of laccase at 36 °C and 70 °C in cartoon representations; active site and cavities for catechol access to the T1 copper site are highlighted using amino acid side chains in blue; laccase is represented in grey and copper atoms in orange; at right are represented the proposed structures of poly(catechol) after polymerization, obtained by quantum calculation, at B3LYP/6-311++G(d,p) level (down: proposed linear polymer; top: proposed non-linear polymer) (Image adapted with permission from references[12, 13]).

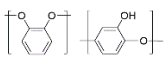
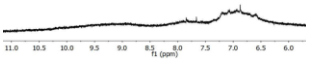
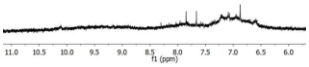
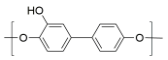
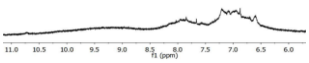
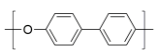
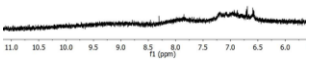
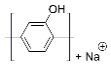
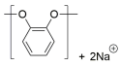
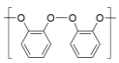
The characterization of the polymers produced using modified laccase forms and different reactors is combined in Table 6.1. The colour variation of the reactional mixtures are displayed macroscopically corresponding to different UV/Vis spectra behaviour, depending of the enzyme and reactor used. In our previous studies the quantification of the total content of free OH groups of poly(catechol) was crucial to evaluate the type of polymer formed. Considering the level of free OH groups in catechol as 1.0, the total content of free OH groups found for the new poly(catechol) is lower (0.5 or less), revealing that the enzymatic reaction might occur either by the formation of an oxidation cascade by formation of ether bonds in the polymeric structure or ablation of OH groups from the phenolic structures. Therefore, the determination of the total content of free OH in the polymer mixtures allowed identification of different polymeric structures when different laccase forms are applied. There is another founding that for a same reactor (WB), the use of a modified enzyme led to a decrease of the total content of free OH[12], when using ultrasonic bath or high-pressure homogenizer the same tendency is displayed[13]. The differentiated repeating units calculated from MALDI-TOF data indicate that, different laccase forms using the same reactor may yield to diverse repeating units as PEGylation and immobilization influence greatly the catalytic behaviour of native laccase[12, 14]. At the same time, when different reactors are used and the reactions are catalysed by the same enzyme different repeating units may also be produced since the acoustic and hydrodynamic cavitation from ultrasound and high-pressure homogenization alter the behaviour of the enzyme's active site cavity which may influence the polymer production.

The most common structure of connected catechol units when polymerized is the ether linkages as described in the literature[1]. The reaction occurs by oxygen-carbon bonding at the *para-* position

of another monomeric unit. The formation of both quinoid derivatives and homomolecular dimers is reported, and the units are linked by C-C or C-O bonds which lead to the formation of oligomers or polymers after a certain reaction time[15].

The ^{1}H NMR spectra (Table 6.1) of the polymers produced by the different enzyme forms revealed a very broad behaviour, because the samples analysed are a mixture of oligomer and polymer species, making complex to predict and identify the accurate polymer structures. The MALDI-TOF analysis enabled the prediction and the proposal of different possible structures depending on the enzyme and reactor used (Table 6.1).

Table 6.1 Characterization of polymers obtained using different enzyme forms and different reactors

Reactor*	Enzyme	Repeating unit[1]	Free –OH content[2]	Possible structures of repeating unit[3]	^{1}H NMR spectra of polymers obtained using different enzyme forms	The colour of mixture
WB	Native lac	92/108	0.51-0.55			
	PEGylated lac	106/107	0.29-0.33			
	Epoxy-native lac	198/199	0.19-0.27			
	Epoxy-PEGylated lac	161/162	0.21-0.25			
	Epoxy-PEG-lac	116/117	0.07-0.16		Not published	
US	Native lac	154	0.24-0.31		Not published	
HPH	Native lac	233/234	0.32-0.39		Not published	

[1] Calculated by MALDI-TOF spectra analysis.

[2] The values are normalized considering the total content of free OH of catechol as 1.00 (100%).

[3] ^{1}H NMR of powder fraction was obtained after washing with water and with methanol (in DMSO-d_6).

* WB: water bath; US: ultrasonic bath; HPH: high-pressure homogenizer. The data were obtained from references [12, 13]

6.3 The polymer prediction: different structures or different oxidation stages?

The prediction of a polymer structure generally requires accurate and clear ^{1}H NMR and C-13 NMR spectra which could match together. The works about the enzymatic polymerization of catechol reported in literature do not clarify the information about the NMR spectra of the polymers formed, neither supply information about the fractionation of the final polymers obtained. From the vast

work performed till now, it was observed that after the removal of enzyme and unreacted substrate, the structures obtained do not match the structures proposed in the related literature. Despite all the efforts, and based only on previous achievements, it is still not possible to confirm an exact possible polymer structure.

However, the information obtained from MALDI-TOF analysis and from Molecular Dynamic simulations showed a new perspective about the data obtained[12, 13]. From MALDI-TOF spectra of the final polymers obtained one can infer that the reaction is favoured in the presence of modified laccases and high-energy environments, revealing high conversion yields of reaction and polymers with high DP, displaying however different repeating units depending on the enzyme and reactor used. The enhancement of laccase reactions seems to show a lower level of Free OH content, which can make hypothesis that the different polymeric structures might be part of the same oxidation stage. Molecular dynamic simulation studies using Simulated Annealing method predicting the behaviour of the enzyme in conditions of heating increase supported these findings by revealing a more open and stable enzyme at higher temperatures [13], associated to high-energy environment reactors, and a higher substrate accessibility to the active site, which may lead to the formation of a mixture of linear and non-linear polymers with higher molecular weight than the products obtained using low energy reactors, as shown in Figure 6.1B. One can deduce that complex polymers with differentiated lengths and structures are produced when the reaction is catalysed by modified laccases and carried out using high-energy environment reactors. The difficulty to identify these oligomers/polymers is still however a major drawback to overcome. Deeper and further studies are needed to explain whether the different structures obtained correspond to products at the final stage or if are still in an intermediate stage of the oxidation process.

6.4 Concluding Remarks

Laccase is a special enzyme due to its broad substrate specificity. The discussions presented at this forum point out this catalyst as the main trigger point for a cascade of oxidation reactions which brings to the formation of complex polymeric structures. The particular case-studies about poly(catechol) oxidation demonstrated that both enzyme modifications and reactors might lead to

the formation of different polymers. Despite the great number of publications related to the production of differentiated poly(catechol) structures, one can hypothesize that all of these structures might be reaction products at different process oxidation stages.

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Appendix

Supporting Information, Scientific publications and Conferences

Supporting Information

Chapter III

Table S1. Half-life time of enzymes *vs* temperature of incubation

Incubation temperature (°C)	Half-life time ($t_{1/2}$) (h) ± SD		
	Native laccase	PEGylated laccase	Epoxy-PEGylated laccase
40	41.3±4.2	48.4±4.3	46.9±4.5
50	21.1±3.5	26.2±3.4	26.7±4.1
60	12.9±2.9	20.4±2.6	19.3±3.9

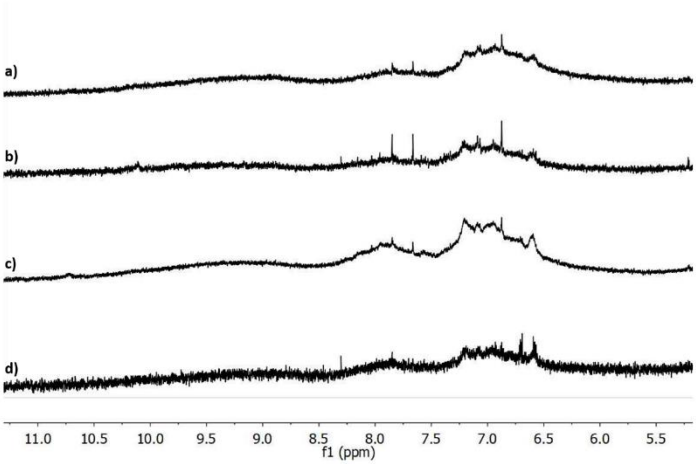

Figure 3.S1: ^{1}H NMR of powder fraction polymerized by **a)** free/native laccase; **b)** free/PEGylated; **c)** immobilized/PEGylated laccase and d) native laccase immobilized onto PEG-activated resin, after washings with water and with methanol (in DMSO-d6).

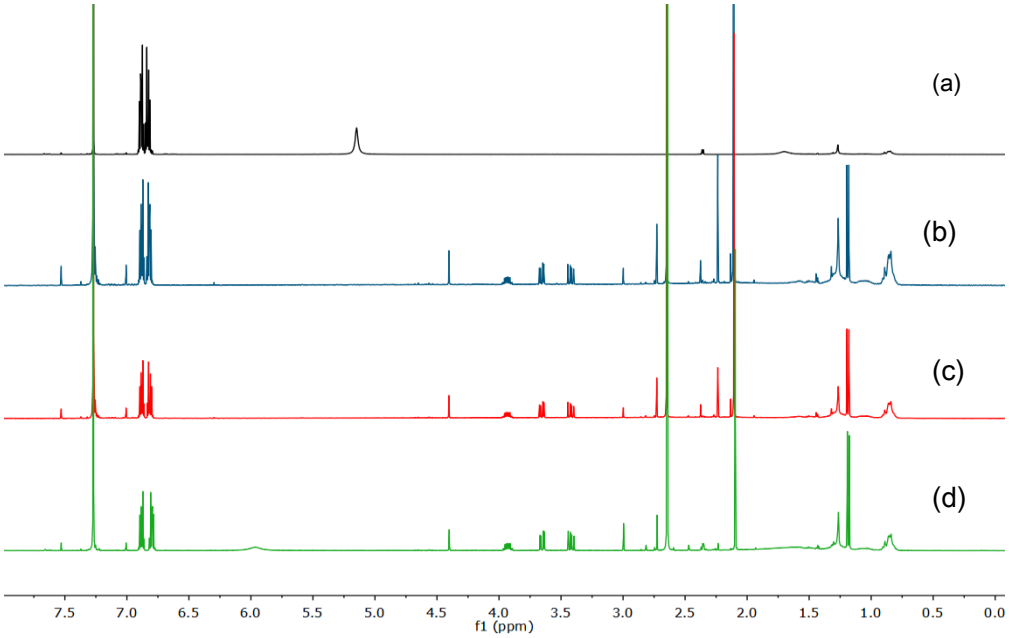

Figure 4.S1. ^{1}H NMR spectra of **(a)** catechol and poly(catechol) polymerized by laccase using **(b)** water bath (WB); **(c)** ultrasonic bath (US) and **(d)** high-pressure homogenizer (HPH) ($CDCl_3$).

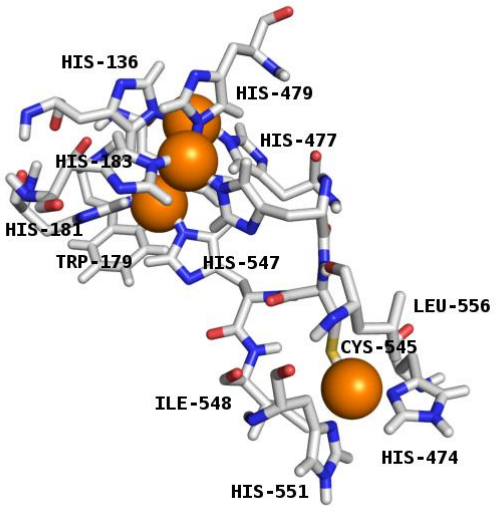

Figure 4.S2. Catalytic site of *Myceliophthora thermophila* (homology model); the three copper centers are represented by orange spheres; Cu2+ at T1 site has a trigonal coordination with HIS-474, HIS-511 and CYS-545. At T2/T3 copper center, Cu2+ atoms are coordinate with several

imidazole groups from histidines; amino acids from active site are represented in sticks (grey for carbon, blue for nitrogen, red for oxygen and yellow for sulfur); the amino acid nomenclature is written in black.

Docking Protocol

Docking experiments were performed with AutoDock 4.0 [1, 2] and prepared with the AutoDock Tools Software [1, 3]. The middle structures obtained from LAC MD simulations at 36 °C and 70 °C, were used as macromolecules and catechol used as ligand. Lamarckian Genetic Algorithm (LGA) was chosen as search algorithm [4]. A grid box was created and centered at T1 Cu ion, in a resolution of 0.375 Å, with the necessary size to involve all copper sites. Grid potential maps were calculated using AutoGrid 4.0. Each docking consisted of 200 independent runs, with a population of 150 individuals, a maximum number of $25x10^6$ energy evaluations (due the torsions of the polymers) and a maximum number of 27,000 generations.

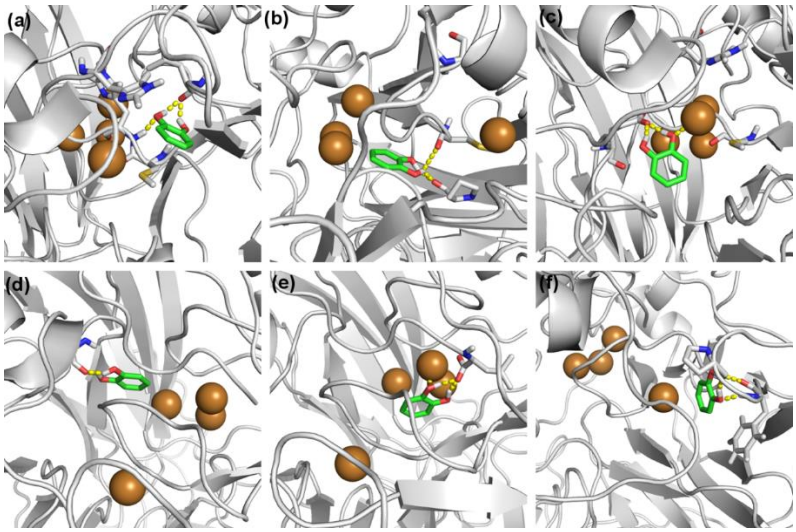

Figure 4.S3. Docking snapshots of catechol complexed with laccase, at 36 °C (a-c) and 70 °C (d-f). Laccase is represented in grey cartoon, interacting amino acids in sticks, catechol in green and copper atoms in orange. The three most populated clusters are shown for each system.

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Chapter V

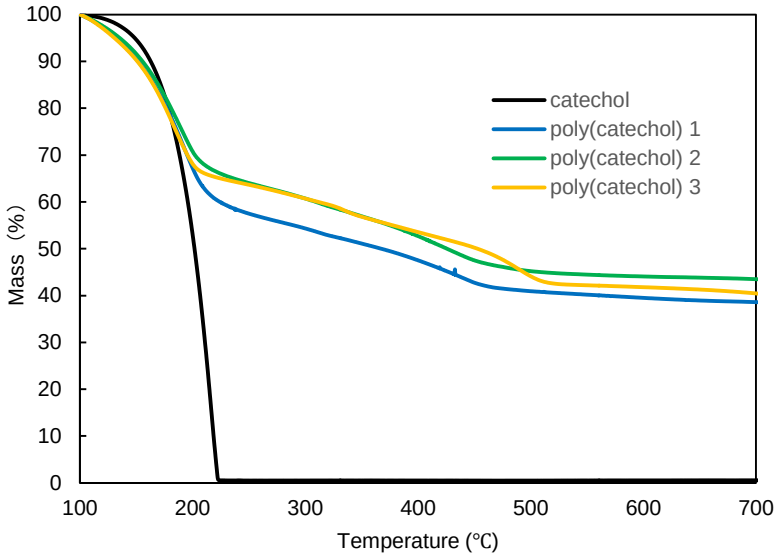

Figure 5.S1: TGA curves of catechol and poly(catechol); poly(catechol) 1: obtained by catalysis with native laccase; poly(catechol) 2: obtained by catalysis with PEGylated laccase; poly(catechol) 3: obtained by catalysis with epoxy-PEGylated laccase).

Scientific articles

1. **Jing Su**, Jiajia Fu, Carla Silva, Artur Cavaco-Paulo**.** Can laccase-assisted processing conditions influence the reaction products. Trends in biotechnology, 2019, 37, 683-686.
2. **Jing Su**, Jiajia Fu, Qiang Wang, Carla Silva, Artur Cavaco-Paulo. Laccase: A green catalyst for biosynthesis of poly-phenols. Critical reviews in biotechnology, 2017, 38(2), 294-307.
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