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# Production strategies and environmental applications of fungal enzymes

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TESE DE DOUTORAMENTO

# PRODUCTION STRATEGIES AND ENVIRONMENTAL APPLICATIONS OF FUNGAL ENZYMES

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# Production strategies and environmental applications of fungal enzymes

Dna. Sandra González Rodríguez

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# Production strategies and environmental applications of fungal enzymes

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*De parvis grandis acervus erit*

## Abstract

The need to apply greener technologies to reduce environmental impact has led to the search for biotechnological processes that allow the valorisation of waste and the minimisation of the use of toxic compounds.

In this sense, the use of fungal enzymes as biocatalysts is an attractive alternative due to the avoidance of extreme conditions, the reduction of waste generated and the possibility of reuse. However, these catalysts may have some drawbacks, such as the high cost of their production due to the formulation of culture media and the final purification process. For this reason, this Thesis evaluates different residues to produce fungal ligninolytic enzymes. Furthermore, the efficiency of the cocktails obtained was analysed and compared with commercial enzymes.

Chapters 2, 3 and 4 are focused on the evaluation of different agro-industrial residues as substrates for enzyme production using different fungal strains. Fermentations under submerged and solid-state conditions (SmF and SSF, respectively) were tested at flask scale and those that provided the highest activity values were scaled up to a 4-30 L bioreactor, and the cocktails obtained were concentrated by ultrafiltration for subsequent application as biocatalysts. In addition, to determine the potential of the enzyme cocktails obtained, proteomic analysis was used as a tool to identify all the enzymes present in the medium. On the other hand, the environmental impacts associated with enzyme production were estimated using the Life Cycle Assessment (LCA) methodology.

Laccase, manganese peroxidase (MnP) and unspecific peroxygenase (UPO) enzyme cocktails were successfully produced with a lignocellulosic stream from the organosolv process, wheat straw and bioethanol by-products, respectively. Factors related to the dilution of the wastes, inoculum percentage or the need for supplementation were essential to optimise enzyme production. In addition, SmF showed the highest production values for all enzyme cocktails produced, so the scale of the production process was performed in liquid medium. After evaluating the feasibility of the valorisation of agro-industrial residues for enzyme production, the enzyme cocktails and several commercial enzymes were applied in different environmental applications.

Chapter 5 describes the use of different laccases to activate organosolv lignin aiming to increase its reactivity for application as bioadhesive precursor. The modification was focused on the demethylation of lignin through the optimisation of different factors affecting laccase activity, availability of the substrate and improvement of enzyme-substrate interaction. The demethylation degree of the modified lignin was evaluated analysing the methanol released to the media and different analytical techniques such as FT-IR, MALDI and SEC were employed to determine the structural changes. After optimisation, the demethylation of lignin improved by 239 %, showing a decrease in methyl and hydroxyl groups and an increase in molecular weight due to an oligomerisation process. All these structural modifications may increase the reactivity of lignin and thus facilitate its use as a bioadhesive precursor.

MnP and UPO enzyme cocktails were considered for the degradation of different emerging contaminants (ECs) in Chapter 6. Five pharmaceutical compounds and personal care products (PPCPs) and one polycyclic aromatic hydrocarbon (PAH) were studied. Complete elimination of all ECs was possible, with the exception of carbamazepine, for which only 30 % elimination was achieved after 24 h of MnP treatment, which represents the highest elimination achieved to date with enzymatic treatment using peroxidases. BPA and hormones were degraded in less than 1 h at optimal conditions, whereas pyrene required 3.5 h. Moreover, the identification of metabolites obtained after enzymatic treatment of BPA, EE2 and pyrene was addressed with the use of $^{14}$C-labelled compounds. Although no metabolites were identified for BPA and EE2 degradation, it was detected a percentage of mineralisation of these compounds between 0.5 and 4 % with MnP cocktail. In the case of pyrene, using thin-layer chromatography (TLC) and liquid chromatography coupled to mass spectrometry (LC-MS), it was possible to confirm that the final transformation products were a result of a polymerisation reaction.

**Keywords:** residues valorisation, enzymes, biocatalysis, fermentation, fungi, lignin, life cycle assessment, emerging contaminants, proteomic analysis, bioadhesive precursor, biodegradation

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# ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **AaP** | *Agrocybe aegerita* peroxygenase |
| **ABTS** | 2,2′- azino-bis-3-ethylbenzothiazoline-6-sulfonic-acid |
| **APO** | Aromatic peroxygenase |
| **ANOVA** | Analysis of variance |
| **AO** | Alcohol oxidase |
| **ATP** | Adenosine triphosphate |
| **ATR** | Attenuated total reflectance |
| **BLASTP** | Basic local alignment search tool for proteins |
| **BPA** | Bisphenol A |
| **BRF** | Brown rot fungi |
| **CBZ** | Carbamazepine |
| **CDL** | Chemiluminescence detector |
| **CID** | Charge-injection device |
| **CPO** | Chloroperoxidase |
| **CrP** | *Coprinellus radians* peroxygenase |
| **DBH** | 2,5-dihydroxy benzoic acid |
| **DDGS** | Distillers's dry grain solubles |
| **dd$H_2O_2$** | Doubled distilled water |
| **DMP** | 2,6-dimethoxyphenol |
| **DyP** | Dye-decolorizing peroxidase |
| **EC** | Emerging contaminant |

| | |
|---|---|
| **EDC** | Endocrine disrupting chemical |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ESI** | Electrospray ionization |
| **E1** | Estrone |
| **E2** | 17β-estradiol |
| **EE2** | 17α-ethinylestradiol |
| **FET** | Freshwater ecotoxicity |
| **FRS** | Fossil resource scarcity |
| **FT-IR** | Fourier transform infrared spectroscopy |
| **FU** | Functional unit |
| **GAE** | Gallic acid equivalent |
| **GP-SSF** | SSF supplemented with glucose and peptone |
| **GRAS** | Generally regarded as safe |
| **GW** | Global warming |
| **HBT** | 1-hydroxybenzotriazole |
| **HN-CT** | Human non-carcinogenic toxicity |
| **HPLC** | High performance liquid chromatography |
| **LC-MS** | Liquid chromatography coupled to mass spectrometry |
| **HRP** | Horseradish peroxidase |
| **IR** | Ionizing radiation |
| **$K_La$** | Oxygen mass transfer coefficient |
| **LCA** | Life cycle assessment |

| | |
|---|---|
| **LDA** | Lignin-degrading auxiliary |
| **LE** | Ligninolytic enzymes |
| **LiP** | Lignin peroxidase |
| **LMS** | Laccase mediator system |
| **MALDI** | Matrix-assisted laser desorption/ionization |
| **MCO** | Multicopper oxidases |
| **$M_n$** | Number average molar mass |
| **MET** | Marine ecotoxicity |
| **MiP** | Manganese independent peroxidase |
| **MnP** | Manganese peroxidase |
| **MS** | Mass spectrometry |
| **MS/MS or MSMS** | Tandem mass spectrometry |
| **MW** | Molecular weight |
| **$M_w$** | Average molar mass |
| **NADP** | Nicotinamide adenine dinucleotide phosphate |
| **NCBI** | National center for biotechnology information |
| **nHPLC** | Nano high-performance liquid chromatography |
| **NPT** | Number of total peptides |
| **NPU** | Number of unique peptides |
| **NS-SSF** | SSF without supplementation |
| **PAH** | Polycyclic aromatic hydrocarbon |
| **PASEF** | Parallel accumulation-serial fragmentation |

| | |
|---|---|
| **PCP** | Personal care product |
| **PPCP** | Pharmaceutical and personal care products |
| **PSM** | Peptide spectrum matches |
| **QTOF** | Quadropole time-of-flight |
| **ROS** | Reactive oxygen species |
| **SDS-PAGE** | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| **SEC** | Size exclusion chromatography |
| **SmF** | Submerged fermentation |
| **SRF** | Soft rot fungi |
| **SSF** | Solid state fermentation |
| **TE** | Terrestrial ecotoxicity |
| **TIMS** | Trapped ion mobility spectrometry |
| **TLC** | Thin-layer chromatography |
| **ToF** | Time of flight |
| **UHPLC** | Ultra-high performance liquid chromatography |
| **UPO** | Unspecific peroxygenase |
| **VP** | Versatile peroxidase |
| **WRF** | White rot fungi |
| **WSE** | Wheat straw extract |
| **$Đ_M$** | Molar mass dispersity |

# RESUMO

O emprego de encimas como biocatalizadores en diversos procesos de mellora ambiental leva estudándose dende hai varios anos. Non obstante, a pesar de que o seu uso require de condicións menos agresivas en comparación con catalizadores químicos, a súa presenza en procesos biotecnolóxicos segue a estar limitada a campos específicos. Esta tendencia é debida principalmente a que o proceso de produción de encimas e a súa posterior purificación poden supoñer altos custos, sendo isto unha clara desvantaxe á hora de escollelas como catalizadores de diversos procesos biotecnolóxicos fronte a tecnoloxías mais rendíbeis. Por ende, é necesario unha procura de estratexias que permitan a redución dos custos de produción. Unha posibilidade sería o emprego de substratos de menor valor engadido e a análise da viabilidade do uso de cócteis encimáticos sen purificar.

Partindo desta situación, a presente tese de doutoramento céntrase na mellora dos procesos de produción de encimas fúnxicas a través do emprego de distintos tipos de residuos como substrato, así como na aplicación dos cócteles encimáticos en distintos procesos de mellora ambiental, coa fin de avaliar se o seu potencial é comparábel ao das encimas comerciais. Para a consecución deste obxectivo, a investigación levada a cabo nesta tese estruturouse nos capítulos indicados a continuación:

**Capítulo 1. Introdución e contexto.**

Este capítulo pretende servir de marco teórico para a investigación recollida nesta tese. Pon unha banda, realízase unha revisión bibliográfica da produción de encimas relacionadas co metabolismo da lignina, que inclúen en maior medida lacasas e peroxidasas. Abórdanse cales son os principais microorganismos produtores destas encimas, os métodos de produción comunmente empregados e os factores a ter en conta para estimular a produción encimática. Dentro da produción de encimas, faise especial énfase na análise do emprego de residuos agroindustriais como substratos principais para a produción de encimas ligninolíticas.

Asemade, tamén se analizan diversas aplicacións desta clase de encimas no ámbito biotecnolóxico, facendo especial énfase nas aplicacións avaliadas nesta tese, que son a obtención de precursores derivados da lignina para a síntese de bioadhesivos e a biotransformación de contaminantes emerxentes. En relación ó punto anterior, tamén se leva a cabo unha revisión dos principais parámetros que permiten unha mellora da acción catalítica das encimas, cos cales se busca unha mellora da súa estabilidade e/ou da actividade encimática.

Unha vez definido o contexto actual global da tese, xorden dúas cuestións fundamentais:

- Cal é o modo de fermentación máis axeitado para a produción de encimas ligninolíticas mediante a valorización de residuos agro-industriais?
- Presentan os cócteles encimáticos producidos a través da valorización de residuos un potencial catalítico suficiente para ser empregados no campo da biotecnoloxía ambiental en comparación coas encimas comerciais?

**Capítulo 2. Valorización dos licores negros procedentes do proceso *organosolv* de madeira para a produción de encimas ligninolíticas.**

Tal e como se indica con anterioridade, a necesidade de reducir os custes de produción das encimas é crucial para que estas sexan competitivas fronte a outros tipos de catalizadores. Ademais, débese ter presente o contexto actual, no que se fomenta unha economía circular que promova a boa xestión dos residuos, xa sexa mediante a súa redución ou mediante estratexias de valorización. Tendo en conta estas dúas premisas, neste capítulo abórdase o uso de correntes residuais xeradas na industria madeireira como substrato principal para a produción de lacasa co fungo *Ganoderma lucidum*. Concretamente, empregouse un residuo denominado licor negro, cun elevado contido en hemicelulosa e compostos fenólicos, sendo estes fundamentais para inducir a produción de encimas ligninolíticas.

Empregándose este residuo como fonte de carbono para o crecemento de fungo, evítase o emprego de compostos de alto valor engadido como pode ser a glucosa, polo que se reduce o custo global do proceso de produción. Así mesmo, o alto contido en compostos fenólicos pode inhibir o crecemento doutros microorganismos competidores, atallando deste xeito un dos maiores retos presentes nas fermentacións con fungos, que é a posible proliferación de bacterias ou levaduras cun crecemento máis rápido cos fungos.

A estratexia abordada neste capítulo baseouse na produción dun cóctel encimático cunha alta presenza de actividade lacasa mediante o emprego de fermentacións en estado somerxido e estado sólido. Para o primeiro tipo de fermentación empregouse unha dilución do licor negro suplementada con peptona como fonte de nitróxeno e trazas de cobre (Cu) e manganeso (Mn) para inducir a expresión das encimas ligninolíticas, mentres que para a

fermentación en estado sólido empregáronse como soporte espumas de poliuretano embebidas na dilución de licor negro empregado para as fermentación en estado somerxido. Dada a maior produción de lacasa na fermentación en estado somerxido, esta escalouse a un reactor de 4 L para a obtención de maiores cantidades de encima para unha posterior aplicación do cóctel encimático como biocatalizador.

**Capítulo 3. Produción de encimas ligninolíticas a través da valorización de palla de trigo.**

Continuando coa estratexia de uso de residuos como substrato principal para a produción de encimas, este capítulo enfócase no emprego de palla de trigo como principal fonte de carbono para o crecemento do fungo *Irpex lacteus* e a produción dun cóctel encimático con actividade peroxidasa dependente e independente da presenza de manganeso (MnP e MiP, respectivamente). Este substrato foi seleccionado por ser un dos materiais lignocelulósicos máis estendidos a escala global.

A palla de trigo foi empregada como soporte para o crecemento do fungo en estado sólido, estudando se por si soa é suficiente para o correcto crecemento microbiano e produción de encimas, ou se, pola contra, é preciso un aporte externo de carbono e nitróxeno. Por outra banda, estudouse a produción encimática de *I. lacteus* en estado somerxido empregando un extracto da palla de trigo obtido mediante tratamento térmico. Avaliáronse distintas dilucións do extracto e tamén o efecto do tipo de fonte de nitróxeno suplementaria empregada (orgánica e inorgánica).

Os mellores resultados acadados nas fermentacións en estado somerxido levaron a un escalado do proceso nun reactor de 30 L nas condicións óptimas (extracto diluído e suplementado con peptona e glicosa). Este escalado permitiu acadar valores de actividade encimática similares aos obtidos a escala matraz, pero cunha redución do tempo de produción. O cóctel encimático producido foi concentrado e analizado mediante análise proteómica e electroforese en xel, co fin de obter máis información sobre a súa composición e, polo tanto, do seu potencial catalítico. Esta análise permitiu detectar a presenza doutras encimas presentes en menor proporción como proteasas, celobiohidrolasas e dehidroxenasas. Ademais, o contraste dos resultados obtidos na electroforese coa bibliografía existente sobre as encimas producidas con fungo *I. lacteus* permitiu relacionar a actividade MnP cunha peroxidasa dependente do manganeso de cadea corta e a actividade

independente de Mn cunha peroxidasa descolorante (DyP, siglas procedentes do termo en inglés *dye-decolorizing peroxidase*).

Tamén, co obxectivo de estudar a viabilidade medioambiental do proceso de produción de encimas co fungo *I. lacteus* a escala laboratorio, empregouse a metodoloxía de Análise de Ciclo de Vida (ACV), a cal puxo de manifesto que os impactos derivados da produción están ligados principalmente ao consumo enerxético.

**Capítulo 4. Valorización de produtos secundarios da síntese de bioetanol para a produción de peroxixenasa inespecífica.**

Este capítulo ten como obxectivo a produción dunha encima recentemente descuberta: a peroxixenasa inespecífica (UPO, siglas procedentes do termo en inglés *unspecific peroxygenase*). Para a súa obtención empregouse o fungo *Agrocybe aegerita* cultivado nun medio baseado en residuos xerados na industria de síntese de bioetanol. Estes residuos teñen un alto contido proteico, que con anterioridade se relacionou ca indución da expresión da peroxixenasa inespecífica con *A. aegerita*.

Continuouse coa estratexia de produción seguida nos Capítulos 3 e 4, empregando como substrato para a fermentación en estado somerxido a corrente líquida obtida na fermentación de xeración de bioetanol, denominada vinaza. Por outra banda, para a produción encimática en estado sólido empregouse o residuo derivado do secado da vinaza, que son os grans solubles de destilería (DDGS, siglas procedentes do termo en inglés *distillers's dry grain soluble*). Na fermentación en estado sólido empregouse o DDGS como soporte e única fonte de nutrientes para o crecemento de *A. aegerita*, obténdose produción de UPO e lacasa, sendo a de UPO a maior produción en estado sólido publicada ata a data.

En estado somerxido, os nutrientes do medio de fermentación foron aportados exclusivamente pola vinaza, da cal se avaliaron distintas dilucións para estudar a súa repercusión sobre a indución da produción de UPO. Asemade, avaliáronse inóculos de distintas idades, xa que diversos estudos constatan que a idade do inóculo pode ter un impacto substancial na indución da expresión encimática e no tempo requirido para a obtención dos valores máximos de actividade.

A optimización en estado somerxido levou á escolla das condicións óptimas de produción empregando unha dilución de vinaza do 50 % (v:v) e un inóculo

de 14 días. Baixo estas condición escalouse a produción a un reactor de 4 L, no cal se reduciu o tempo de produción e se acadaron valores similares de actividade encimática.

Dado que segue sen estar claro que compostos ou condicións inducen a expresión da UPO, recorreuse á análise proteómica para estudar a expresión encimática antes e despois da detección da actividade UPO no medio de fermentación, con fin de relacionar a activación de certas rutas metabólicas coa expresión da UPO. Esta análise permitiu identificar encimas como a UPO, lacasa e DyP, e diversas proteínas relacionadas con funcións de almacenamento, defensa frente a toxinas e produción e control de radicais libres trala detección de actividade UPO no medio. Asemade, tamén se detectaron cambios no metabolismo de azúcares complexos e fontes nitroxenadas durante a fermentación en estado somerxido.

Ao igual que no capítulo 3, realizouse unha avaliación do impacto ambiental xerado polo proceso de produción do cóctel encimático producido por *A. aegerita*, poñéndose novamente de manifesto que o requirimento enerxético está detrás da maioría de impactos xerados.

**Capítulo 5. Funcionalización da lignina *organosolv* mediante desmetilación encimática para a formulación de bioadhesivos.**

Debido á necesidade de encontrar opcións máis sostibles para a síntese de bioadhesivos, nos últimos anos está en auxe a busca de novos precursores destes compostos. Neste sentido, as materias primas renovables como a lignina sitúanse no punto de mira. Non obstante, este polímero natural presenta unha baixa reactividade, polo que é necesario unha modificación previa de parte dos seus grupos funcionais para aumentar a súa viabilidade como precursor de bioadhesivos.

Neste capítulo téntase demostrar que o uso de encimas ligninolíticas como a lacasa pode lograr un aumento da reactividade da lignina. Concretamente, abordouse a modificación de lignina *organosolv* con lacasas comerciais e co cóctel encimático obtido no Capítulo 2 a través dun proceso de desmetilación, que foi avaliado medindo a liberación de metanol. Tras unha análise inicial na que a lacasa comercial do fungo *Trametes versicolor* resultou lograr un maior grado de desmetilación, continuouse a optimización de distintos factores cruciais para a efectividade da desmetilación encimática da lignina. Deste xeito, avaliáronse parámetros fundamentais para a estabilidade encimática,

como son a temperatura e o pH, estudando rangos destes factores nos que as lacasas reportan as actividades máis elevadas. Posteriormente, dado que a lignina é un substrato cunha baixa solubilidade en auga, tentouse mellorar a súa solubilidade no medio de reacción a través da adición de distintos axentes tensioactivos coma o PEG-600 e o Tween-80, obténdose unha mellora considerable da solubilidade co emprego de Tween-80. Trala optimización destes factores, o seguinte paso centrouse en intentar mellorar a eficiencia da reacción mediante o emprego dun mediador que mellorase o contacto encima-substrato. Consideráronse dous dos mediadores máis habituais nas reaccións con lacasa, un mediador natural (vainillina) e un artificial (1-hidroxibenzotriazol, HBT), acadando os mellores valores de desmetilación co uso de HBT.

Finalmente, o proceso de modificación encimática trasladouse a un biorreactor de 150 mL para poder estudar o efecto da aireación e avaliar a transformación encimática da lignina co conxunto de variables optimizadas. Nestes experimentos corroborouse que o suministro de aire favorecía lixeiramente a desmetilación da lignina, pero que os parámetros cun efecto máis significativo sobre o grado de desmetilación acadado foron a mellora da solubilidade da lignina ca adición de Tween-80 e o emprego do HBT como mediador. Tendo en conta toda a batería de factores optimizados, logrouse mellorar o grado de desmetilación inicial nun 239 %.

A modificación da lignina *organosolv* tratada encimaticamente verificouse a través da análise con distintas técnicas analíticas como a espectroscopía infraroxa por transformada de Fourier, (FT-IR), a desorción/ionización láser asistida por matriz (MALDI) e a cromatografía de exclusión de tamaño (SEC). Estas análises permitiron constatar o aumento do peso molecular na lignina modificada e o descenso da sinal ligada os grupos metilo/metoxilo, o cal é indicativo dunha maior reactividade e de que se acadou certo de grado de oligomerización que axuda a reforzar á estrutura polimérica.

**Capítulo 6. Degradación encimática de contaminantes emerxentes.**

A presenza de concentracións significativas de certos contaminantes emerxentes (ECs) nas augas residuais e masas de auga naturais levou á busca de métodos de degradación destes compostos co obxectivo de reducir a súa toxicidade.

Este capítulo aborda a biotransformación de produtos farmacéuticos e de coidado persoal (bisfenol A, hormonas e carbamazepina) e tamén dun composto modelo de hidrocarburo policíclico aromático (pireno), para avaliar a capacidade dos proceso encimáticos na eliminación de contaminantes emerxentes.

A eliminación dos contaminantes estudouse co cóctel encimático con actividade MnP e MiP obtido no Capítulo 3, co cóctel enzimático con actividade UPO obtido no Capítulo 4 e cunha UPO comercial. Con ambas encimas se avaliaron distintos parámetros condicionantes da actividade encimática como a dose de encima, o pH ou a concentración de $H_2O_2$ (cofactor necesario para o ciclo catalítico de ambas encimas). En todas as condicións estudadas conseguiuse a completa degradación dos ECs, agás no caso da carbamazepina, da cal só se conseguiu unha eliminación do 30 % tras 24 h de reacción coa MnP debido ao seu carácter recalcitrante. Non obstante, esta eliminación é a máis alta reportada ata a data co emprego dun método encimático. No caso do bisfenol A e hormonas, logrouse a degradación total en menos dunha hora nas mellores condicións, namentres que para o pireno foron necesarias 3,5 h e observouse que a acción da UPO foi máis eficiente que a da MnP para a súa degradación. Para as hormonas e bisfenol A as condicións establecidas como óptimas para a UPO comercial empregáronse tamén co cóctel encimático obtido no Capítulo 4, obtendo resultados de degradación similares aos acadados coa encima comercial.

En xeral, os maiores valores de adición de $H_2O_2$ ensaiados resultaron nunha redución do tempo necesario para a completa eliminación dos contaminantes, aínda que as doses deste composto empregadas para a reacción encimática coa MnP e UPO varían considerablemente.

Para complementar o estudo da degradación de contaminantes, tentouse identificar os metabolitos xerados na degradación dalgúns dos compostos estudados. Para isto empregáronse bisfenol A, 17$\alpha$-ethynylestradiol (EE2) e pireno marcados co isótopo $^{14}C$, o cal permite o rastrexo da radioactividade xerada por estes átomos e a súa relación con posibles metabolitos. No caso do pireno, logrouse identificar que os produtos de degradación son resultado dun proceso de polimerización, namentres que para o bisfenol A e EE2, constatouse que as encimas empregadas son capaces de mineralizar ditos compostos, chegando a mineralizar case un 4 % de bisfenol A tras 6,5 h de reacción co emprego do cóctel enzimático con actividade MnP.

**Capítulo 7. Discusión xeral e conclusións.**

Neste capítulo esbózanse as principais conclusións derivadas da investigación levada a cabo nos distintos capítulos desta tese, tentando dar resposta ás distintas cuestións expostas no capítulo de introdución. Asemade, estas conclusións tamén dan pé a que xurdan novas propostas que pretenden ampliar o coñecemento xerado nesta tese.

Respecto a se é posible a produción de encimas a partir da valorización de residuos e cal é o método óptimo de produción, nos Capítulos 2, 3 e 4 expóñense distintas estratexias de emprego de residuos agroindustriais para a produción exitosa de distintas encimas (lacasa, MnP e UPO). A escolla do residuo a empregar dependerá maiormente do tipo de microorganismo produtor e da encima que se pretende producir. Deste xeito, para a produción de lacasa e manganeso peroxidasa, a selección dun residuo cunha pequena proporción de lignina é fundamental para a indución da expresión destas encimas, que ademais están estimuladas pola presenza doutros elementos traza no medio, como poden ser o Mn ou o Cu. No caso da produción da peroxixenasa inespecífica, conclúese que é necesario que o medio estea composto por residuos cun alto contido proteico, xa que a análise proteómica constatou que a síntese desta encima dáse paralelamente á activación de rutas metabólicas de degradación de fontes complexas de nitróxeno. Non obstante, aínda que a vinaza resultou ser un substrato adecuado para a produción de UPO, implicou o emprego de condicións de esterilización do medio máis agresivas, polo que nesta liña sería interesante a busca de residuos de composición similar derivados de procesos que non empreguen microorganismos.

Da mesma maneira que a escolla do tipo de residuo a empregar como substrato inflúe na produción das encimas, tamén o fai o tipo de fermentación que se empregue para o crecemento do fungo. Para todas as encimas producidas acadáronse valores máis altos de produción e nun período máis curto no estado somerxido, aínda que o estado sólido segue a presentar certas vantaxes, como son a menor probabilidade de contaminación causada por microorganismos competidores e un menor gasto enerxético.

Aínda que o uso de residuos agroindustriais resultou óptimo para a produción das distintas encimas, atendendo aos resultados obtidos no análise de ciclo de vida dos procesos de produción de MnP e UPO, queda patente que de cara a reducir o impacto medioambiental derivado destes procesos deben

de adoptarse medidas enfocadas á redución do consumo enerxético. Neste sentido, a avaliación de distintas configuracións dos reactores ou a avaliación de factores que non se tiveron en conta nesta tese podería de ser de gran relevancia. Asemade, o estudo dos impactos medioambientais asociados aos procesos de produción a maior escala, resultaría na obtención de datos máis realistas de cara a implantación a nivel industrial. Na mesma liña, a realización dun estudo económico tamén sería de interese para poder avaliar a viabilidade do emprego de cócteles encimáticos como biocatalizadores.

En relación á segunda cuestión abordada nesta tese, enfocada á aplicación das encimas a distintos procesos encimáticos, nos Capítulos 5 e 6 demóstrase que estes biocatalizadores teñen o potencial necesario para a modificación de substratos naturais en prol dunha mellora da súa reactividade, e que tamén son capaces de degradar distintos contaminantes emerxentes.

O emprego de lacasas para a mellora da reactividade da lignina demostrou que estas encimas son capaces de oxidar os grupos metilo/metoxilo e de reducir o contido en grupos hidroxilo, características que se atribúen a unha mellora da reactividade. Tamén se constatou que a acción destas encimas depende en gran medida de factores que inflúen notoriamente na súa estabilidade ou actividade, ou de compostos que facilitan a dispoñibilidade do substrato no medio de reacción. Aínda que o seguimento da desmetilación da lignina se fixo analizando a cantidade de metanol liberado, as técnicas de FT-IR, MALDI e SEC resultaron ser de gran utilidade para a caracterización da lignina modificada e a verificación dos cambios estruturais ligados ó aumento da reactividade. Neste sentido, o produto obtido na transformación encimática da lignina *organosolv* semella ser un candidato óptimo para a síntese de bioadhesivos, aínda que un futuro traballo a continuar sería a avaliación experimental da aplicación deste polímero modificado nunha formulación real.

Por último, a aplicación dos cócteles encimáticos con actividade MnP e UPO acadou a completa biotransformación de todos os contaminantes emerxentes estudados, coa excepción da carbamazepina, que só se conseguiu degradar parcialmente coa MnP. Aínda que o aumento do tempo de reacción ou a dose de encima empregada xunto co uso de mediadores podería levar a un incremento da porcentaxe de degradación.

Demostrouse que os parámetros estudados (dose de encima, concentración de cofactores e pH) inflúen en gran medida no rendemento da eliminación dos

contaminantes, o cal parece estar máis ligado á actividade encimática que á natureza do contaminante diana. As encimas estudadas acadaron resultados de degradación semellantes no caso dos PPCPs, namentres que a UPO resultou ser a mellor candidata para a degradación de pireno ao requirir un menor tempo de reacción. A análise mediante cromatografía líquida e espectrometría de masas dos metabolitos xerados na transformación encimática do pireno reflicte que o mecanismo de reacción catalizado polas encimas foi a polimerización da molécula. Ademais, demóstrase que o emprego de cócteles encimáticos pode ser tan eficiente coma o uso de encimas purificadas que implican un maior custo de produción.

Aínda que os ensaios de degradación de contaminantes emerxentes se realizaron considerando as concentracións habituais destes compostos en augas residuais e masas de auga naturais, sería interesante o estudo da degradación en concentracións maiores, para avaliar como afecta este incremento á actividade encimática, e se pode existir unha inhibición da reacción encimática por interacción cos metabolitos xerados. Asemade, resulta de interese un estudo máis exhaustivo da mineralización de BPA e EE2 coa MnP, xa que incrementando o tempo de reacción se poderían acadar maiores porcentaxes de mineralización. En relación a degradación de pireno, o uso de neutralizadores de radicais libres como o ácido ascórbico durante a reacción encimática podería dar lugar a un mecanismo de hidroxilación evitando os procesos de polimerización.

# CHAPTER 1

## INTRODUCTION AND CONTEXT

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Detailed information available at "Manuscripts considered in this thesis: rights, contributions and quality indicators", page 227.

## 1.1. Ligninolytic fungi

Ligninolytic fungi encompass all fungal strains that have the ability to depolymerise and mineralise lignin. This group of fungi consists of basidio and ascomycetes present in wood and soil (Pozdnyakova et al., 2022). Lignin degradation is achieved through their extracellular, nonspecific, and oxidative enzyme system, which includes different extracellular oxidoreductases produced during the secondary metabolism of fungi (Knežević et al., 2013). The production of these enzymes has not been reported for lower fungi such as Zygomycota and Chytridiomycota, although some studies suggest that they may also be involved in wood degradation in nature (Fukasawa et al., 2011).

### 1.1.1. Traditional strains

Most ligninolytic fungi are referred to as wood-rot fungi. Although this concept does not refer to any species or taxonomy, it is a generic term for a group of fungi that grow on various forms of wood substrates on which a parallel wood-rotting process develops (Wendiro et al., 2019). These saprophytic fungi are classified according to their morphology and type of wood decay into three main categories:

- White rot fungi (WRF): This subgroup is represented by basidiomycetes, and approximately 90 % of the species included in the wood-rotting fungi group are WRF (Janusz et al., 2017). The enzyme system of these fungi allows them to degrade lignin, cellulose and/or hemicellulose (Goodell et al., 2008). For this reason, most studies focusing on the production and application of ligninolytic enzymes (LEs) use this class of fungi.
- Brown rot fungi (BRF): These fungi are able to metabolise polysaccharides such as cellulose and hemicelluloses, but do not metabolise lignin, although they depolymerise and modify it extensively (Umezawa et al., 2020). As in the case of WRF, this group is also composed of basidiomycetes, such as species like *Schizophyllum commune*, *Fomes fomentarius* and *Serpula lacrymans* (Kumar, 2014). Their main mechanism of lignin transformation is an oxidative process followed by an enzymatic saccharification system, but they are not able to attack the aromatic ring (Illman and Highley, 1989; Umezawa et al., 2020).

- Soft rot fungi (SRF): Several asco- and deuteromycota fungi that cause soft rot decay. The ability of these fungi to degrade lignin is limited because deep penetration of soft rot only takes place under high humidity conditions, where their fungal hyphae penetrate the wall cells and create cylindrical cavities (Kumar, 2014). However, some species of the Xylariales order currently grouped with soft rot fungi have previously been included in the WRF cluster (Janusz et al., 2017).

Although all fungi included in these groups are able to degrade lignin, only WRF carry out lignin mineralisation into $CO_2$ and $H_2O$ (del Cerro et al., 2021). In earlier stages of wood decomposition the main fungi involved are basidiomycota (WRF and BRF) , while ascomycetes (SRF) are dominant in the late stages (Fukasawa et al., 2011).

The most experimentally studied WRF is *Phanerochaete chrysosporium*, due to its rapid growth and fully sequenced genome (Khalil et al., 2021; Martinez et al., 2004). This fungus has been used in numerous investigations focused on the production of LEs and their application to the degradation of various pollutants (Cameron et al., 2000; Singh and Chen, 2008). Nevertheless, other fungi such as *Trametes*, *Irpex* and *Ganoderma* sp have also been studied for the same purposes (Marco-Urrea et al., 2009). All these species present different aspects that make them great candidates for LEs production, such as rapid growth, development at room temperature or a wide variety of globally distributed strains.

The set of enzymes commonly expressed by WRF includes lignin peroxidases (LiPs), manganese peroxidases (MnPs), $H_2O_2$-generating enzymes and laccases. These oxidoreductases have a high potential that makes them especially attractive for different biotechnological applications such as soil remediation and degradation of polycyclic aromatic hydrocarbons (PAHs) (Ellouze and Sayadi, 2016; Günther et al., 1998).

### 1.1.2. Novel strains

In recent years, interest in the search for strains capable of producing LEs has grown exponentially. The evolution of genomic and proteomic techniques has also contributed to a better understanding of the complex enzymatic systems of fungi (Brenelli et al., 2019; Riyadi et al., 2020), leading to the discovery of new species for LEs production.

Different novel strains have received attention in the last two decades, including fungi from the basidiomycota and ascomycota divisions. These fungi, although not necessarily growing on wood, possess an enzymatic system that allows lignin degradation. In this sense, efforts are focused on increasing the knowledge of fungal strains capable of producing novel LEs, such as heme-thiolate haloperoxidases or lignocellulosic-active enzymes (Lankiewicz et al., 2023; René Ullrich et al., 2004), which are included in the group of lignin-degrading auxiliary (LDA) enzymes, as they are involved in lignin degradation but no evidence is provided that lignin is degraded by their action alone (Janusz et al., 2017).

Specifically, within the aforementioned heme-thiolate haloperoxidases, the production of unspecific peroxygenase (UPO) has received special attention in the last two decades due to their broad versatility and similarities to cytochrome P450 (Hofrichter et al., 2015). Several studies have evaluated the production of this enzyme using different strains such as *Agrocybe aegerita*, *Marasmius rotula* or *Chaetomium globosum* (Kinner et al., 2021). Although some of these fungi require longer fermentation times for enzyme production than WRF, the enzyme potential of the cocktail may be higher than that excreted by WRF. For example, *A. aegerita* (Figure 1.1), known to be a major producer of UPO, can also express laccases and peroxidases (Isikhuemhen et al., 2009). For this reason, studying the optimisation of the enzyme production is essential to obtain more competitive biocatalysts from an economic point of view.

**Figure 1.1.** *A. aegerita* grown in Fernbach flask.

Moreover, several studies have evaluated the performance of heme-thiolate haloperoxidases in the oxidation of several compounds, including organopollutants classified as extremely dangerous for the environment (Karich et al., 2017; Torres and Aburto, 2005).

## 1.2. Ligninolytic enzymes

Ligninolytic enzymes is the official term to define lignin-modifying enzymes (LMEs), primarily produced by WRF and composed mainly by hemeperoxidases (LiP, MnP and versatile peroxidase (VP)) and phenol oxidase (laccase). However, in the last decades some types of enzymes such as aromatic peroxygenases and dye-decolorizing peroxidases (DyP) have gained relevance to be included within this group due to their performance in lignin degradation (Catucci et al., 2020; Kinne et al., 2011). These enzymes have been also classified as LDA enzymes, which are enzymes that are not able to degrade lignin by themselves and are involved in the final stages of lignin degradation (Janusz et al., 2017).

All these enzymes are in the spotlight as biocatalysts due to this ability to degrade lignocellulosic materials and phenolic and non-phenolic lignin-model compounds such as veratryl alcohol or dyarilpropane structures (da Silva Vilar et al., 2022; Reddy et al., 2003; Ten Have et al., 2001).

### 1.2.1. Laccase

Laccase (EC 1.10.3.2) is an extracellular copper-containing enzyme belonging to the superfamily of multi-copper oxidases (MCOs), a group of enzymes with different substrate affinities and a wide range of biological functions (Janusz et al., 2020). This enzyme represents the oldest enzyme ever described and was discovered in 1883 in the exudates of the Japanese tree *Rhus vernicifera* by Yoshida (1883), although it was necessary to wait more than a century for the characterisation of this enzyme as a metal-containing oxidase by Gabriel Bertrand (Alcalde, 2007). Different organisms have the ability to produce laccases, including plants, as well as insects and bacteria, but the main producing microorganisms are fungi. Although the most studied functions of fungal laccases are related to lignin modification, many other roles have been suggested for these enzymes, such as involvement in fruiting body development, cell wall reconstitution or pathogenic processes (Alcalde, 2007).

According to their redox potential, laccases can be classified as low, medium and high redox potential enzymes. Low redox potential enzymes are found in bacteria, plants and insects, whereas high redox potential laccases are widely distributed in fungi (Brugnari et al., 2021). Fungal laccases have a redox potential close to 800 mV, which allows them electron abstraction from substrates, being able to act as redox mediators in the attack on lignin by laccase (Janusz et al., 2020). For example, laccase from *Trametes versicolor*, which is one of the most studied laccases, has a redox potential of 790 mV (Alcalde et al., 2002). This high redox potential of fungal laccases allows their application in different fields such as the food industry, biobleaching, wastewater treatment, biosensor enhancement, soil bioremediation, organic synthesis and cosmetic industry (Rodríguez Couto and Toca Herrera, 2006).

The active site of fungal laccases contains four catalytic copper (Cu) atoms, which are responsible for their catalytic action due to their mediation in the oxidation-reduction process. These Cu atoms are arranged in three sites designed as T1, T2 and T3, although the T2 and T3 sites are assembled. The catalytic cycle begins with the T1 site containing a Cu atom, which changes its oxidation state from $Cu^{2+}$ to $Cu^{+}$ by acting as an electron acceptor. Subtraction of electrons from the substrate leads to the formation of an unstable cation radical, which can undergo an oxidation reaction mediated by a second enzymatic reaction or follow a non-enzymatic reaction such as hydration or polymerisation. The electrons accepted by T1-Cu are transferred via the cystidine-histidine dipeptide pathway to the T2/T3 center composed of three Cu atoms, where the reduction of $O_2$ into $H_2O$ takes place. Given that the reduction of $O_2$ requires four cationic forms of substrate, the stoichiometry of the enzymatic reaction involves four molecules of reducing substrate for each $O_2$ molecule (Alcalde, 2007; Wong, 2009). Figure 1.2 shows the catalytic cycle of laccases.

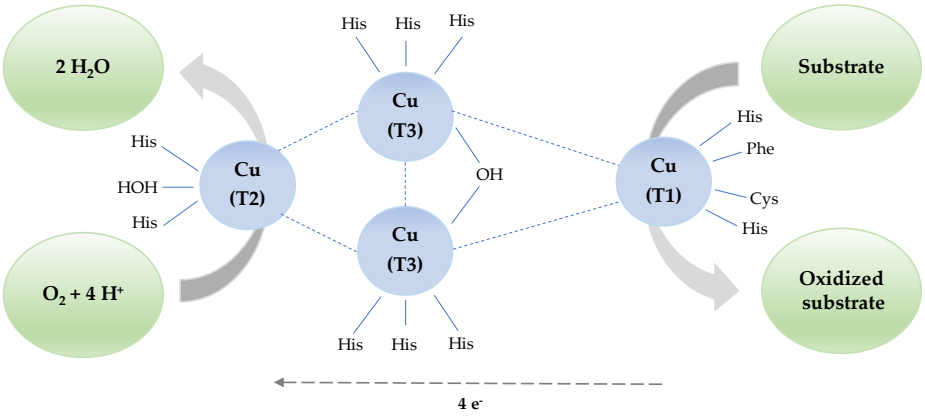



**Figure 1.2.** General catalytic cycle of laccases (adapted with permission from Rodríguez-Delgado et al (2017). Copyright 2017, Springer Nature).

### 1.2.2. Manganese peroxidase

MnP (EC 1.11.1.13) is a $H_2O_2$-dependent heme enzyme belonging to the oxidoreductases family (Kumar and Arora, 2022). It was discovered in the fungus *P. chrysosporium* (Glenn and Gold, 1985) and is found in many microorganisms such as bacteria and fungi. In WRFs, which are the main producers of this enzyme, it is considered to be expressed as a primary enzyme during lignin catabolism, sharing 43 % identity with LiP sequences, which is another major enzyme involved in the fungal enzyme system developed for lignin degradation (Hofrichter, 2002). Due to its versatile catalytic performance, MnP has been used in different processes such as biobleaching, pollutant removal or dye decolorisation (Eibes et al., 2006; Moreira et al., 2003; Qin et al., 2014).

The molecular structure of this enzyme, with a molecular weight between 25 and 68 kDa, contains two $Ca^{2+}$ ions and five disulfide-bridging elements, which have the function of maintaining the structure of the MnP active site. This active site consists of several amino acids (AA) such as the proximal histidine ligand, H-bonded to an aspartic acid residue and a distal peroxidase-binding side pocket containing catalytic histidine and arginine residues (Kumar and Chandra, 2020).

In relation to their C-terminal tail, MnPs of WRF are classified into short, long or extra-long types. Short MnPs are 20–30 AA shorter in comparison to long and extra-long MnPs, and the fifth cysteine disulphide bond near the $Mn^{2+}$

binding site present in long and extra-long MnPs is not present in these enzymes. Furthermore, short MnPs have a higher genetic similarity to LiPs and VPs than long or extra-long MnPs (Hofrichter et al., 2010) and some of them (e.g. *Pleurotus ostreatus* MnP, and *Pleurotus eryngii* MnP) are capable of oxidising aromatic substrates in the absence of $Mn^{2+}$ (Heinfling et al., 1998; Sarkar et al., 1997). Nevertheless, the mechanism responsible for the $Mn^{2+}$-independent oxidation of aromatic substrates by short MnPs is still unknown (Li et al., 2019).

Fortunately, the mechanism of long and extra-long MnPs is well known. Their catalytic cycle starts with the transfer of two electrons from the heme group to $H_2O_2$ in the resting state, leading to the production of Compound I and water. Compound I then undergoes oxidation of the substrate with the corresponding formation of Compound II and free radicals. Finally, Compound II is able to catalyse the oxidation of $Mn^{2+}$ to $Mn^{3+}$, and this cation performs the oxidation of compounds containing aromatic rings. It should be noted that Compound I can oxidize $Mn^{2+}$ or a substrate molecule, but Compound II requires the presence of $Mn^{2+}$ for its reaction. When $Mn^{3+}$ is formed, then it reacts non-specifically with phenolic compounds by removing an electron and a proton from the substrate molecule. $Mn^{2+}$ promotes the reversion of MnP to its original form (Hofrichter, 2002; Wong, 2009). Figure 1.3 summarises a general catalytic cycle of long and extralong MnPs.

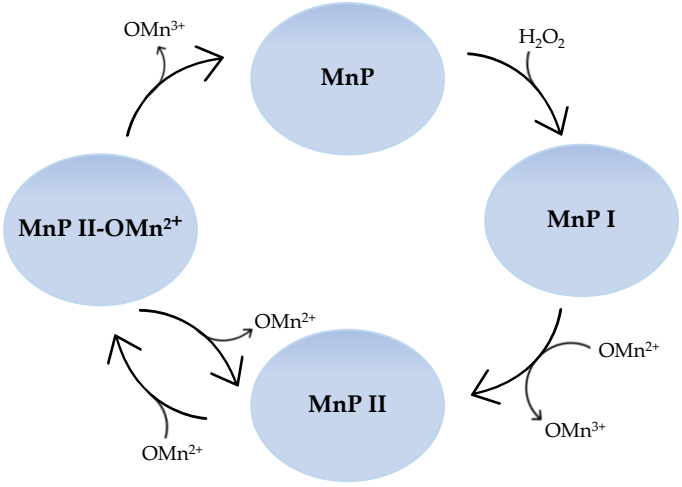



**Figure 1.3.** General catalytic cycle of long and extralong MnPs (adapted with permission from Chowdhary et al. (2019). Copyright 2019, Springer Nature).

### 1.2.3. Unspecific peroxygenase

UPOs (EC. 1.11.2.1) are peroxygenases belonging to the heme-thiolate family with peroxygenase (insertion of one O atom) and peroxidase activity (subtraction of one electron) (Kinner et al., 2021). It was discovered almost 20 years ago by Ullrich et al. (2004) in the fungus *Agrocybe aegerita*. However, in the first years after its discovery, this enzyme was designated as haloperoxidase or aromatic peroxygenase due to the AA sequence similarities and hydroxylation activity on halides and aromatic compounds characteristic of these enzymes, and it was not until 2011 that it was renamed as UPO.

What is most remarkable about UPOs is that in addition to the common catalytic cycle of hemoperoxidases, these enzymes are also capable of performing different non-specific reactions attributed to cytochrome P450 monooxygenases, but with the advantages of being more stable and requiring only $H_2O_2$ as a co-substrate, which acts as an electron acceptor and oxygen donor (Kinner et al., 2021; Ullrich and Hofrichter, 2005).

This enzyme has been secreted by several fungi, such as *Agrocybe* (*Cyclocybe*) *aegerita*, *Coprinellus* (*Coprinus*) *radians*, *Marasmius rotula*, *Chaetomiun globosum*, *Coprinus verticillatus*, *Leptoxyphium fumago*, *Marasmius wettsteinii* and *Psathyrella aberdarensis* (Kinner et al., 2021). The function of these enzymes in nature is still unclear, but it has been suggested that they are probably related to different physiological roles in metabolite synthesis, detoxification processes and interaction with antimicrobial peptides released by host plants (Hofrichter et al., 2015). Despite this unknown role in the fungal enzyme system, their ability to oxidise a wide range of compounds such as alkenes, chlorinated benzenes, halogenated biphenyl ethers, nitroaromatics, polycyclic aromatic hydrocarbons (PAHs), phthalates, steroids, etc. has been explored by different authors (Karich et al., 2017; Peter et al., 2013; Ullrich et al., 2018).

UPOs can be classified according to their structural characteristics and affinity for substrates into two main families: Family I of short UPOs and Family II of long UPOs. Long UPOs have a molecular weight around 44 kDa, whereas short UPOs are between 32 and 29 kDa in size and usually do not have a signal peptide (Hofrichter et al., 2020). Furthermore, long UPOs have been detected only in basidiomycetes and ascomycetes, whereas short UPOs are more widespread within fungal phyla (Hofrichter et al., 2015; Pecyna et al., 2009).

The structure of both enzymes is different. Long UPOs are monomeric proteins, whereas short UPOs are mostly dimeric. In addition, the former has internal disulphide bridges and arginine as a charge stabiliser and in the case of short UPOs, these bridges are external and stabilised by histidine. Although both families have similar conserved structural motifs, there are relevant differences in the organisation of the heme channel (in topology and dimension), leading to different affinity for the substrates. For example, short UPOs accept large substrates, whereas long UPOs are more active with smaller compounds (Monterrey et al., 2023). Only a few crystal structures of UPOs are available in Protein Data Bank (PDB): one from *A. aegerita* (PDB: 2YOR for wild-type and 5OXU for the PaDa-I secretion variant) (Piontek et al., 2010; Ramirez-Escudero et al., 2018) and another from *Marasmius rotula* (PDB: 5FUK; unpublished, available at PDB). From the cited structures, it is elucidated that the core is mostly composed of $\alpha$-helices that organise the active site around the heme group. The active site is mainly covered by aromatic residues, implying a preference for slightly hydrophobic substrates (Rotilio et al., 2021).

The catalytic mechanism of UPOs is based on a combination of the catalytic cycle of hemo-peroxidases and cytochrome P450 monooxygenases (Dunford 1999, Montellano y De Voss, 2005). They are able to oxygenate C-H bonds in a similar way to P450 through their peroxide "shunt" pathway and also have the ability to oxidise phenols like heme peroxidases. UPOs can transfer an oxygen atom contained in the peroxide (H-O-O-R) to various organic substrates. The target substrates can undergo hydroxylation, epoxidation and heteroatom oxygenation reactions and, in turn, spontaneous dealkylation, deacylation or (re)aromatization (Hofrichter and Ullrich, 2014). In addition, UPO also catalyses single-electron oxidations, such as the abstraction of single electrons from phenolic hydroxyl groups, acting similarly to conventional peroxidases (Figure 1.4).

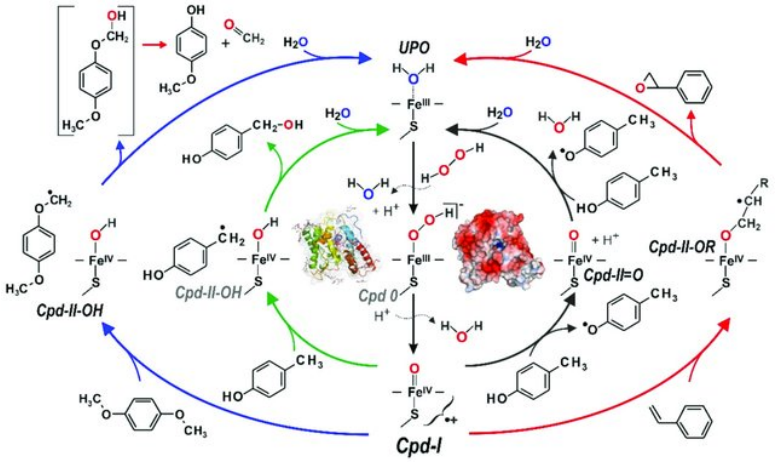



**Figure 1.4.** Catalytic cycles of unspecific peroxygenase using three different substrates. Left: benzylic hydroxylation of p-cresol to 4-hydroxybenzyl alcohol (inner green arrows), O-dealkylation of 1,4-dimethoxybenzene to p-hydroxyanisole (outer blue arrows) via an unstable hemiacetal intermediate (in square brackets); right: epoxidation of styrene to styrene oxide (outer red arrows) and oxidation of p-cresole to two corresponding phenoxy radicals (inner black arrows) (reproduced with permission from Hofrichter et al. (2022). Copyright 2022, The authors, published by MDPI).

## 1.3. Production of ligninolytic enzymes

Interest in LEs production has increased in recent decades due to the need to find new catalysts that are high-performing, biocompatible and biodegradable. The main consideration to carry out the production of an enzyme is the evaluation of different microorganisms that are able to produce the enzyme of interest. These microorganisms are wild strains that are isolated and studied to understand the set of enzymes that make up their enzyme system. The use of wild strains is related to traditional enzyme production, where fermentations with these microorganisms are carried out by studying and optimizing various factors affecting cell growth and enzyme expression. However, in recent years, the use of genetically modified microorganisms is becoming more relevant, as the use of heterologous hosts allows the improvement of enzyme titers and the reduction of production costs, as well as the synthesis of enzymes "*à la carte*".

### 1.3.1. Production of wild-type enzymes

Species that can produce LEs without any genetic modification are designed as natural or homologous hosts and are responsible for the production of the wild-type version of these enzymes. The most commonly used microorganisms for the production of LEs are WRF, due to its capability to express one or various LEs. The use of natural or synthetic media similar in composition to the medium in which these fungi grow in nature is required, and various factors involved in cell growth and enzyme induction (e.g., pH, aeration, temperature) must be optimised to achieve high titers of activity.

There are two typical strategies for enzyme production with native strains based on the conditions under which fermentations are carried out: solid state fermentation (SSF) and submerged state fermentation (SmF). The former uses solid substrates and fungal growth occurs under static conditions, replicating the natural conditions in which the fungus grows. This fermentation strategy is simple and only requires ensuring optimal humidity and temperature for the development of the selected species.

Different authors have evaluated SSF with WRF such as *P. chrysosporium*, *Pleurotus ostreatus*, *T. versicolor*, *Irpex lacteus* or *Ganoderma lucidum* for LEs production (Gupte et al., 2007; Rodrigues et al., 2019). For other hand, SmF require a liquid medium in which the fungus grows under controlled temperature and agitation (Elisashvili et al., 2010; Olajuyigbe et al., 2018). Although the use of SmF is more widespread at the industrial level due to the easier management of the fermenter, better control over aseptic conditions and shorter times to obtain the enzymes, SSF is still being investigated due to higher process yields and cost-effective production compared to SmF (Musoni et al., 2015).

Commonly, the production of wild-type enzymes requires a preliminary flask-scale optimisation step and then, the process is developed to reactor scale. In the case of fungal LEs, most of them are extracellular enzymes, so the downstream process for obtaining the enzymes is simpler than for intracellular enzymes. The downstream process requires the removal of biosolids from the enzymatic crude. If isolation of the enzyme is required, a purification process is necessary and is usually carried out by chromatographic techniques. However, the level of purification depends on

the final application of the product and is in many cases unnecessary due to the possibility to apply the crude enzyme.

### 1.3.2. Production of recombinant-type enzymes

In recent years, the use of recombinant microorganisms with WRF genes has gained special attention in the field of LEs production due to the advantage of increased productivity, reduced time and lower costs (Debnath and Saha, 2020). Recombinant protein expression, also called heterologous expression, involves the use of a host microorganism into which a gene from a native enzyme producer is inserted, encoding the necessary instructions for enzyme expression. In general, the procedure followed involves the following steps: cloning of the gene of interest into an expression vector, transformation of the host microorganism (expression system), selection process of the transformants, growth of the microorganism and expression in bioreactors, and finally recovery and purification of the protein (Ongley et al., 2013).

Host microorganisms commonly used to produce recombinant-type LEs are different types of fast-growing yeasts, bacteria or filamentous fungi (e.g. *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei*), due to their faster growth compared to fungi expressing the enzymes of interest. Specifically, *Escherichia coli* is probably the most studied candidate due to its faster growth and the extended knowledge about it (Zelena et al., 2014). However, in the case of LEs expression its use is less common due to the inability of bacteria to perform post-translational modifications typical in eukaryotes, such as glycosylation on peroxidases secreted by basidiomycota that confer them a functional structure (Demain and Vaishnav, 2009). Moreover, recombinant proteins expressed by this system accumulate in the cytoplasm or in the periplasmic space with the corresponding formation of aggregates that can inactivate the enzyme and decrease its solubility (Fischer et al., 1992). For this reason, although there are other bacterial hosts that can reduce these obstacles (Terpe, 2006), yeasts are the best candidates for expressing LE, in addition to their status as GRAS (generally regarded as safe) microorganisms (Baghban et al., 2019). The most commonly used yeast strains for heterologous expression are *Pichia pastoris* and *Saccharomyces cerevisae* (Demain and Vaishnav, 2009).

For example, yeasts such as *P. pastoris* have been widely used for the expression of different LEs, reaching production levels that can also be higher

than those obtained with natural hosts (Table 1.1). In addition, genetic engineering also allows the introduction of several modifications in the genetic code that are able to visibly change the enzyme structure to make it more suitable for specific substrates. Another advantage is the option to introduce genes that confer resistance to a specific antibiotic compound, which can be used during fermentation to avoid contamination by external microorganisms and thus reduced production yield (Nordén et al., 2011).

**Table 1.1.** Production of ligninolytic enzymes by heterologous expression using *P. pastoris* as host.

| Enzyme | Origin microorganism | Scale (L) | Activity (U/L) | Reference |
|---|---|---|---|---|
| Lignin peroxidase | *Phanerochaete chrysosporium* | 10 | 4480 | (Majeke et al., 2020) |
| Manganese peroxidase | *Phanerochaete chrysosporium* | 2 | 2500 | (Jiang et al., 2008) |
| Laccase | *Trametes versicolor* | 5 | 18,123 | (Li et al., 2014) |
| Unspecific peroxygenase | *Agrocybe aegerita* | 2500 | 30,000 | (Tonin et al., 2021) |

However, there are also several bottlenecks in the use of this technology. One of them is that heterologous expression focuses mainly on the production of a specific enzyme, without taking into account that other enzymes must be present in the medium for the proper functioning of the enzyme. For example, $H_2O_2$-dependent enzymes requires the presence of enzymes that produce $H_2O_2$. In fermentations with wild-type strains, the set of enzymes produced by the microorganism ensures the optimal functioning of the enzyme system, so that the enzymes can act as catalysts while being excreted. In heterologous expression, it is possible to overcome this shortcoming by including more genes to ensure the expression of different enzymes or by using tandem systems such as *S. cerevisae* and *P. pastoris*, but this leads to an increase in the complexity of expression.

## 1.4. Factors involved in the expression of ligninolytic enzymes

Environmental conditions have a direct impact on the regulation of fungal metabolism in nature, so the control of different parameters during fungal fermentation is critical to induce LEs expression. In the production of LEs on

a laboratory or industrial scale, these parameters can be divided into those related to the composition of the medium and those concerning the operating conditions of the fermenters.

### 1.4.1. Medium composition

#### *1.4.1.1. Carbon source*

In general, filamentous fungi are able to use carbon (C) provided by monosaccharides, disaccharides and oligosaccharides. The preference for one source or another depends on the type of C compounds present in their natural environment. Although there are several studies assessing the influence of the type of C source on LEs production (Raghuwanshi et al., 2022; Wang et al., 2016), it is unclear how fungi detect complex carbohydrates in biomass and how this detection can be translated into an intracellular metabolic response that induces the expression of one enzyme or another (Wu et al., 2020).

The preferred source of C for growth and enzyme production differs for each type of fungus. For example, Bettin et al. (2009) found that laccase activity in *Pleurotus sajor-caju* was higher when the main source of C was glucose compared to lactose. However, lactose proved to be the best C source for laccase production by *Pseudotrametes gibbose* (Elisashvili and Kachlishvili, 2009).

On the other hand, the expression of LEs seems to be related to low concentrations of C in the medium (Galhaup et al., 2002; Lú-Chau et al., 2018; Saparrat et al., 2002). Lu-Chau et al. (2018) suggested that the boosting of LEs production with *G. lucidum* and *I. lacteus* at low glucose concentration could be related to physiological changes involved in the change from primary to secondary metabolism.

#### *1.4.1.2. Nitrogen source*

Nitrogen (N) limitation can be determinant in the induction of the secondary metabolism and therefore, in the enzyme expression. The most studied WRF, *P. chrysosporium*, only produces LiP and MnP under nitrogen-limiting conditions (Reddy and D'Souza, 1994). Although some WRFs such as *Bjerkandera sp. BOS55* do not show this correlation between nitrogen limitation and LEs expression, and rich-N medium are required for optimal LEs expression (Kaal et al., 1993; Moreira et al., 2000). This difference may be

the result of LEs production in some species being carried out by metabolic pathways different from those traditionally studied for WRFs.

Moreover, the type of N source also has a relevant impact on LEs expression. Several authors have evaluated the impact of different N sources, including organic and inorganic compounds (Kanwal and Reddy, 2011; Levin et al., 2010; Reddy and Kanwal, 2022). The use of organic compounds such as yeast extract or casein as nitrogen source reported the highest laccase production for *Morchella spongiola* (Reddy and Kanwal, 2022). In addition, Levin et al. (2010) also reported higher levels of MnP production by *Trametes* sp using organic nitrogen compounds such as glutamic acid and supplementation with vitamins such as thiamine.

#### *1.4.1.3. Inducers*

The inducer is a particular molecule that stimulates the synthesis of the relevant enzyme and is typically a substrate for the enzyme (Vrsanska et al., 2015). Commonly, inductors are classified into natural and synthetic inducers. In the case of WRF lignocellulosic substrates can act as inducers, as they have a small concentration of lignin that can stimulate LEs production. In this regard, different lignocellulosic residues have gained special attention, as several studies have demonstrated the performance of these residues as optimal substrates for fungal growth and expression of enzymes such as laccases and/or peroxidases (Kanwal and Reddy, 2011; Wan and Li, 2012).

Other inducers of LEs expression are metals, which are required in trace amounts by fungi, although they can be toxic in excess (Baldrian, 2003). Metals essential for fungal growth include Cu, iron, zinc, nickel, manganese and molybdenum (Gadd, 1993). Once metals enter the fungal cell, they have the ability to affect metabolic reactions and thus the production of extracellular enzymes (Baldrian, 2003). Specifically, for WRF there are two essential metals: Mn and Cu (Vrsanska et al., 2015), which can also act as co-factors in the catalytic cycle of some LEs. Several studies have reported the effect of supplementing the fermentation medium with Cu on laccase production, which can regulate enzyme expression at the transcriptional level (Collins and Dobson, 1997; Soden and Dobson, 2001). In addition, MnP production has also been increased by adding traces of Mn to the fermentation medium (Lueangjaroenkit et al., 2019).

Finally, other organic compounds that can act as inducers of LEs production in WRF are phenolic compounds, ethanol and surfactants. In the case of ethanol, its capacity to increase laccase production in different species of WRF has been demonstrated (Hernández et al., 2015; Lee, IY., Jung, KH., Lee, 1999), although in *Pycnoporus sanguineus* its inductor effect has been also related to the nitrogen conditions of the medium (Hernández et al., 2015). On the other hand, several authors have used surfactants such as Tween-80 to stimulate extracellular enzyme secretion in both SmF and SSF (Gassara et al., 2010; Teodoro et al., 2018). It has been suggested that the mode of action of this surfactant is related to increased cell permeability caused by changes in the composition and content of unsaturated acids in the cell membrane, which may facilitate nutrients transfer (Okumura et al., 2021). In addition, the presence of surfactants in the medium may also stabilise extracellular proteins (Webster et al., 2021).

### 1.4.2. Operational parameters

Different abiotic factors such as pH, temperature, aeration or agitation are crucial for fungal growth and can influence the activation of metabolic pathways responsible for enzyme expression. Bioreactors allow setting fixed values for most of these parameters, thus ensuring the most suitable conditions for fungal growth and enzyme production.

#### *1.4.2.1. Temperature*

This factor can affect fungal enzyme expression by its effect on chemical reactions and also, at extreme levels, on cellular components. Temperature must be optimal for the set of reactions that make up fungal metabolism (Keller, 2019). In addition, high temperatures can affect the lipid composition of cell membranes, which may jeopardize cell viability, and the structure of enzymes, affecting their functionality (Aaronson et al., 1982; Agustí-Brisach and Armengol, 2012).

Most fungi are mesophilic and usually grow at temperatures between 5 and 35 °C, with optimal growth at around 25-30 °C (Daou et al., 2021). However, some fungal species can tolerate low or high temperatures and are classified as psychrophilic and thermophilic, respectively (Godswill Awuchi et al., 2022).

#### *1.4.2.2. pH*

The pH requirements for fungal growth differ according to species. Most ligninolytic fungi grow in environments with pH between 3 and 7, with optimal growth around pH 5 if nutrient requirements are met (Ali et al., 2017; Pardo et al., 2006). In general, pH is adjusted by adding alkaline solutions such as NaOH or by buffering the medium. Moreira et al. (2000) tested the production of MnP using *Bjerkandera sp. BOS55* with both strategies and concluded that no remarkable difference in LEs production was observed under standard conditions. Only a negative impact on MnP production was observed at high agitation and absence of buffer, which resulted in a 30 % decrease in MnP activity.

#### *1.4.2.3. Agitation*

Ensuring homogeneous conditions of the culture medium is the main objective of agitation, so as to guarantee the distribution of spores, an adequate distribution of nutrients and oxygen, avoiding mass transfer limitations (Suryanarayan, 2003). In SmF, it has been suggested that agitation could improve the removal of volatile metabolic products, which prevents the formation of agglomerates. In addition, it favors heat exchange, which allows optimal conditions for microbial growth (Gassara et al., 2013a). However, fungal morphology can be affected by agitation. Once fungal granules are formed, strong agitation could have negative effects such as pellet breakage (Veiter et al., 2018). This mechanical stress can also contribute to induce sporulation in WRF, as these microorganisms use spore formation as a survival mechanism, which can negatively impact enzyme production (Su et al., 2012).

#### *1.4.2.4. Oxygen*

Aeration is primarily aimed at supplying the oxygen necessary for fungal growth and $CO_2$ removal. In addition, aeration is also responsible for heat and moisture transfer between the solid/liquid and gas phase. However, excessive aeration can lead to stress conditions that can affect the morphology of filamentous fungi (Shojaosadati and Babaeipour, 2002). For this reason, the optimisation of aeration is key to enhance the growth of the microorganism and the production of primary and secondary metabolites (Gassara et al., 2013a).

#### *1.4.2.5. Inoculum*

Another relevant factor affecting enzyme production is the inoculum used. Morphology, age and ratio to the fermentation medium are crucial for fungal growth and thus enzyme production. Lueangjaroenkit et al. (2018) studied two different morphological inocula for LEs production with *Trametes polyzona*, observing that the use of a pellet-grown inoculum increased MnP and laccase activities compared to fermentations where an inoculum was used as free-form mycelial clumps (4.8 and 2 times higher, respectively). In addition, inoculum age was evaluated for laccase production with *Cerrena unicolor* by Rola et al. (2013), finding that fermentations performed with inocula of different ages could result in a notable difference in the maximum LEs activities achieved.

## 1.5. Valorisation of agro-industrial wastes for production of ligninolytic enzymes

The global expansion of industry, together with concerns about environmental impacts, led to the search for alternative processes that allow the valorisation of waste streams. In recent years, several studies have focused on the evaluation of different strategies to integrate waste valorisation as a sustainable alternative for processes focused on obtaining high value-added products under a circular economy perspective (Arias et al., 2022; Santiago et al., 2020).

In this field, the use of different wastes for the cultivation and production of LEs by WRF has received special attention, as these wastes can be optimal sources of C for fungal growth (Maller et al., 2011). The use of natural media instead of synthetic media is more beneficial for reducing the cost of enzyme production. However, the composition of the waste stream will be decisive for the induction of the expression of one type of enzyme or another.

By 2050, it is estimated that the increase in demand for agricultural products will be around 50 %, which will lead to an increase in the waste and secondary flows generated (Arias et al., 2022; Ramírez-Pulido et al., 2021). Most of this waste is released into the environment without any treatment, most commonly solid waste is landfilled or incinerated. Therefore, considering the large quantities generated on a global scale and the need for a process that

allows their valorisation, the use of agro-industrial wastes seems to be a suitable option for LEs production.

Agro-industrial waste can be divided into two categories: agricultural waste and industrial waste. The first group can be divided into those wastes generated after harvesting crops (e.g., leaves, stalks, stems) or those obtained after processing crops into alternative valuable compounds (e.g. molasses, bagasse, husks), while the second includes wastes produced by food processing industries such as, juice, meat or fruit industries (Sadh et al., 2018).

### 1.5.1. Agriculture residues

Although agricultural residues can be used for production of biofuels, biomaterials or energy, their composition is also suitable for LEs production due to their abundance of nutrients such as hemicellulose, cellulose and lignin (Capanoglu et al., 2022). Specifically, the lignin content of, for example, nut shells, can reach 30-40 % (Howard et al., 2003). In addition, these wastes are relative inexpensive, which implies a reduction in the cost of enzyme production (Rodríguez Couto and Sanromán, 2005).

Although these residues have been used in SmF and SSF, their use as solid substrates in SSF is more popular, as they can be used as a support for fungal growth while they provide the nutrients required by the microorganisms. The bioconversion of agricultural residues through SSF technology has been established as an eco-friendly and economical option (Marzo et al., 2019). In this regard, several authors have evaluated the use of legume and cereal peels or empty fruit bunches in the production of laccases and peroxidases (Risdianto et al., 2012; Zhao et al., 2015). Enzyme production titers obtained with the valorisation of these wastes (Table 1.2) are in the range of those obtained with synthetic substrates. Thus, the use of agricultural wastes as substrates for LEs production under SSF conditions can be a suitable alternative to manage these waste streams (Kachlishvili et al., 2012). Furthermore, solid waste can be used in its raw state or only requiring particle size reduction by mechanical pretreatment, which increases the surface area that facilitates the access of the microorganism to the substrate.

**Table 1.2.** Studies on production of ligninolytic enzymes in solid state fermentations employing agriculture residues.

| Microorganism | Substrate | Enzyme | Activity (U/L) | Reference |
|---|---|---|---|---|
| *Marasmius* sp | Rice straw | Laccase | 1116 | (Risdianto et al., 2012) |
| *Trametes hirsuta* | Empty fruit bunches | Laccase | 220 | (Risdianto et al., 2012) |
| *Trametes versicolor* | Corn cob | Laccase | 400 | (Risdianto et al., 2012) |
| *Irpex lacteus F17* | Pine sawdust, rice straw, soybean powder | MnP | 950 | (Zhao et al., 2015) |
| *Lenzites betulina* | Wheat bran | MnP | 740 | (Kachlishvili et al., 2012) |
| *Trametes ochracea* | Wheat bran | Laccase | 29,800 | (Kachlishvili et al., 2012) |
| *Pleurotus eryngii* | Banana peel | VP | 10,800 | (Palma et al., 2016) |

Because of the predominantly solid nature of agriculture residues, their use as substrate for SmF usually requires a previous pretreatment to integrate them into the liquid medium. In this sense, thermal pretreatments are the most employed for this purpose, as they can break the structure of solids to increase the solubility of organic matter at the time that can serve as sterilisation step of the medium. Nevertheless, it is important the control of temperatures and time applied in this pretreatment in order to avoid the release of compounds that could have inhibitory effect on fungal growth as for example furfural and its derivates (Yee et al., 2018). Table 1.3 summarises LEs production in SmF with different agriculture wastes employing different WRF.

**Table 1.3.** Studies on production of ligninolytic enzymes in submerged state fermentations employing agriculture residues.

| Microorganism | Substrate | Enzyme | Activity (U/L) | Reference |
|---|---|---|---|---|
| *Lenzites betulina* | Mandarin peel | Laccase | 43600 | (Kachlishvili et al., 2012) |
| *Lenzites betulina* | Walnut pericarp | Laccase | 76700 | (Kachlishvili et al., 2012) |
| *Lenzites betulina* | Wheat branr | MnP | 1550 | (Kachlishvili et al., 2012) |
| *Pleurotus ostreatus 98* | Mandarin peel | Laccase | 2375 | (Mikiashvili et al., 2006) |
| *Pleurotus ostreatus 98* | Grapevine cutting sawdust | Laccase | 82 | (Mikiashvili et al., 2006) |

The production values of LEs using SmF based on the valorisation of agricultural residues differ depending on the type of residue used and the fungal strain. Kachlishvili et al. (2012) observed that laccase production in walnut pericarp based medium by *Lenzites betulina* was almost 2-times higher than the production with the same strain in mandarin peel (MP) based medium. Moreover, Mikiashvili et al. (2006) evaluated laccase production of *P. ostreatus* in SmF using MP and grapevine cutting sawdust (GCS), observing that enzyme production was considerably higher in SmF with MP (2375 U/L in MP vs 82 U/L in GCS), probably due to the presence of water-soluble aromatic compounds (flavones and flavonols) able to stimulate LEs expression.

### 1.5.2. Industrial wastes

Industrial wastes include residues from agricultural industrial processing, including coffee grounds, bagasse, degummed fruits and legumes, milk serum, cellulose, etc. However, other wastes such as effluents from soap factories, distilleries, paper mills and mineral processing are also considered industrial wastes that come from the processing of agricultural raw materials (Yusuf, 2017).

Several studies have reported the growth of LE-producing fungal strains on different agro-industrial wastes, confirming their suitability as substrates for the production of this type of enzymes (Pant and Adholeya, 2007; Zaier et al.,

2021). Moreover, once LEs production takes place, the enzyme system is able to reduce the toxicity of these wastes through the transformation of phenolic compounds, decolorisation, and reduction of N content (Bohacz and Korniłłowicz-Kowalska, 2020; Pant and Adholeya, 2007; Raghukumar et al., 2004).

Different residues from agricultural industrial processes have been evaluated for LEs production (Table 1.4). For example, vinasse, a liquid waste stream from bioethanol synthesis industry, has been used as a sole source of nutrients and supplemented with different C sources to produce laccase with *Trametes* sp in SSF (Ahmed et al., 2022). In this study, Ahmed et al. (2022) use an SSF technology in which the fungus immobilised on polyurethane foam cubes was grown with different dilutions of vinasse. It was found that after the end of fermentation, replacing the liquid stillage fraction with a fresh stillage fraction could lead to even higher enzyme yields, reaching a laccase production of 361 U/L. In addition, this study also reports a decolorisation of the waste stream and the decrease of phenolic, chemical oxygen demand and biological oxygen demand concentrations, confirming that LEs production is also linked to the detoxification of the waste stream.

Gassara et al. (2010) evaluated fish and brewery wastes, pulp and paper sludge and apple waste (pomace) for LEs production with *P. chrysosporium*, achieving MnP production with all tested wastes. For laccase production, it was observed that the use of copper sulfate as an inducer can trigger enzyme production in all waste-based media, whereas LiP was only found in fermentations with pomace and pulp industry sludge as substrates and with the addition of veratryl alcohol as an inducer of enzyme expression. A maximum MnP activity of 631 U/g was reached with pomace and veratryl alcohol as inducer and a maximum laccase activity of 739 U/g with brewery waste and copper sulfate as inducer, after 8 and 14 d, respectively.

**Table 1.4.** Production of ligninolytic enzymes using agrowastes.

| Microorganism | Substrate | Enzyme | Activity | Reference |
|---|---|---|---|---|
| *Trametes* sp | Sugarcane vinasse | Laccase | 361 U/L | (Ahmed et al., 2022) |
| *Phanerochaete chrysosporium* | Apple pomace | MnP | 631 U/g | (Gassara et al., 2010) |
| *Phanerochaete chrysosporium* | Brewery waste | Laccase | 739 U/g | (Gassara et al., 2010) |
| *Phanerochaete chrysosporium* | Apple pomace | Laccase | 720 U/g | (Gassara et al., 2010) |
| *Phanerochaete chrysosporium* | Pulp and paper sludge | MnP | 488 U/g | (Gassara et al., 2010) |
| *Pleurotus eryngii* | Cherry waste | MnP | 2263 U/L | (Akpinar and Ozturk Urek, 2020) |
| *Pleurotus eryngii* | Cherry waste | Laccase | 4677 U/L | (Akpinar and Ozturk Urek, 2020) |
| *Pleurotus eryngii* | Cherry waste | LiP | 70 U/L | (Akpinar and Ozturk Urek, 2020) |

## 1.6. Biotechnological applications of ligninolytic enzymes

The need to apply biocatalysts as LEs in industry and biotechnology is growing rapidly due to their potential use in a wide range of processes (Maciel et al., 2010). Potential applications of LEs include numerous fields such as chemical, pulp and paper, food, paper, textile and cosmetic industries and bioremediation processes (Figure 1.5). The LEs complex is involved in the degradation of various xenobiotic compounds and dyes. This ability to transform xenobiotic substances into polymeric products makes these enzymes an effective biocatalyst for bioremediation purposes (Maciel et al., 2010; Yadav and Yadav, 2015).

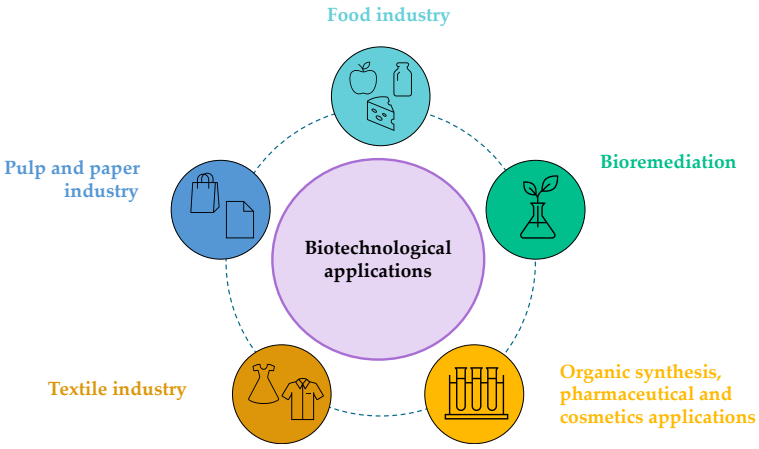



**Figure 1.5.** Biotechnological applications of ligninolytic enzymes.

In addition, the use of enzymes as biocatalysts can be more effective than other conventional treatments, although it is important the evaluation of the environmental impacts associated to enzymatic treatment to confirm their suitability for environmental purposes.

### 1.6.1. Biomaterial precursors

In recent years, the need to find alternatives to the current synthesis of different products involving the use of non-renewable resources or the generation of negative environmental impact is in the spotlight (Delgado-Sánchez et al., 2022). LEs are considered as promising candidates to replace conventional chemical processes in several industries (Maciel et al., 2010). Some studies have reported the transformation of natural polymers by LEs to improve their structure and make them more suitable for different industrial applications (Chen et al., 2019; Huber et al., 2016; Zhu et al., 2021). For example, related to the synthesis of bioadhesives, enzymatic modification of materials such as starch or lignin has been evaluated to obtain transformed polymers with characteristics that increase their reactivity or obtain a more homogeneous network. Enzymatic treatment of these polymers can promote their oxidative activation through a polymerisation initiated via radical mechanism (Pizzi, 2014).

Huber et al. (2016) evaluated the polymerisation of kraft lignin and lignosulfonate by a laccase from *Myceliophthora thermophila* immobilised on polypropylene beads. The results of this study show that the molecular

weight (MW) of lignosulfonate increased 12-fold after 24 h of enzymatic treatment, whereas in the case of kraft lignin the increase in MW was considerably lower (1.7-fold). This increase in MW could improve the dispersion properties of these materials, which is key for their use as plasticizers.

Another field where enzymatic modification of polymers is becoming more relevant is the food industry, where this technique is used to facilitate the formation of network structures towards improved properties (Chen et al., 2019; Zhu et al., 2021). Zhu et al. (2021) studied the transformation of potato flour by laccase and peroxidase, achieving a more ordered protein structure and greater stability after enzymatic treatment, which favor the formation of protein-protein and/or protein-starch cross-linking networks, leading to gelation. On the other hand, Chen et al. (2019) studied the combination of heat treatment with laccase-catalysis for the synthesis of double network gels using soy protein isolate and sugar beet pectin as raw materials. This study reported that increasing laccase concentrations led to a reduction of the time required for gel formation, linked to an improvement of its viscosity.

In recent years, the use of laccase mediator systems (LMS) has also gained special attention due to their ability to directly generate polymers that could not be produced by conventional chemical synthesis (Aktaş and Tanyolaç, 2003). The use of mediators has shown an enhancement of the catalytic action of laccase, achieving strong oxidation of the treated polymers. The evaluation of the treatment of an industrial kraft pine lignin with a partially purified laccase from *Fusarium proliferatum* in the absence and presence of 2,2′- azino-bis-3-ethylbenzothiazoline-6-sulfonic-acid (ABTS) as mediator was carried out by González Arzola et al. (2006). The modification with LMS led to a higher degree of oxidation and depolymerisation than transformation with laccase alone, demonstrating that the use of mediators can improve enzymatic treatment to facilitate the biotechnological use of industrial lignin.

Moreover, Qiu et al. (2023) studied the regioselective C6-OH oxidation of corn starch using the laccase-TEMPO system with the aim of improving the functional properties of this natural polymer, such as water absorption capacity. Specifically, using TEMPO as a mediator in the enzymatic oxidation, an increase in the carboxyl content was obtained, which resulted in an improvement of 34.1-110.1 % in the water absorption properties of oxidised corn starch relative to the unmodified raw material. This improvement in

water absorption properties increases the potential of this material for use in the adhesive, paper and textile industries.

### 1.6.2. Degradation of emerging pollutants

Emerging contaminants (ECs) are defined by the US EPA (United States – Environmental Protection Agency) as new chemicals without regulatory status and whose impact on environment and human health is unclear. Due to the industrialisation and other human anthropogenic activities, the presence of these compounds in the environment is rising significantly (Deblonde et al., 2011). Therefore, the evaluation of environmentally friendly technologies is essential to remove these contaminants. The degradation of ECs present in the environment can be addressed by enzymes, which through their catalytic cycle have the capability of transform these compounds into non-toxic forms. In addition, the enzymatic treatment allows a more specific degradation in comparison with other processes because the removal mechanism completely depends on the enzyme-substrate interaction (Saravanan et al., 2021).

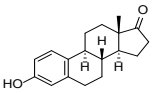
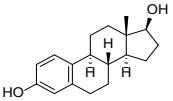
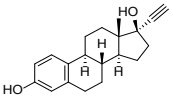
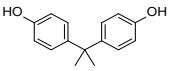
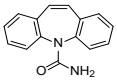
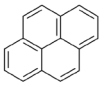
#### *1.6.2.1. Pharmaceutical and personal care products*

Pharmaceuticals and personal care products (PPCPs) include a wide range of organic compounds, including pharmaceutical drugs and compounds used in different personal care products (PCPs) such as lotions, fragrances, sunscreens, etc. Since the use of these compounds is expanded globally, these contaminants and their metabolites can enter the aquatic environment because they are not removed in conventional wastewater treatment (Daughton and Ternes, 1999; Liu and Wong, 2013).

Some of these compounds are classified as endocrine disruptors (EDCs) due its similitude with natural steroid hormones and consequently to produce negative health effects in the organisms (Grelska and Noszczyńska, 2020; Vandenberg et al., 2009). These EDCs include compounds such as estrogens, which are sex hormones that can be classified as natural or synthetic hormones and which in recent years have been detected in significant concentrations in the environment due to their use in contraceptive drugs. The hormones most typically found in wastewater includes estrone (E1) and 17β-estradiol (E2) (natural hormones) and 17$\alpha$-ethinylestradiol (EE2) (synthetic hormone) (Ying et al., 2002). Moreover, other compounds such as bisphenol A (BPA), that is typically used to make polycarbonate plastics and epoxy

resins for packaging of personal care product or foods, can act as EDC (Vandenberg et al., 2009). The molecular structure and characteristics of these compounds are shown in Table 1.5.

**Table 1.5.** Characteristics of different emerging contaminants studied in this Thesis.

| Compound | Molecular weight (Da) | Water solubility (mg/L) | Structure |
|---|---|---|---|
| Estrone | 270.4 | 30 |  |
| 17β-estradiol | 272.4 | 3.6 |  |
| 17α-ethinylestradiol | 296.4 | 11.3 |  |
| Bisphenol A | 228.3 | 12 |  |
| Carbamazepine | 236.3 | 17.7 |  |
| Pyrene | 202.25 | 0.135* |  |

*Extracted from Mackay and Shiu (1977).

Several studies have been focused on the enzymatic treatment of EDCs, mainly using LEs such as laccase, MnP or LiP. The efficacy of enzymatic treatment lies in the phenolic structure of EDCs and the low substrate specificity of LEs (Cajthaml et al., 2009). Several studies have reported elimination rates higher than 80 % for different EDCs using commercial laccases from *T. versicolor* and *M. thermophila*. (Becker et al., 2017; Onaizi and Alshabib, 2021).

However, most of these studies were performed with commercial enzymes. In this regard, although a few authors have evaluated the use of unpurified

enzymes or crude enzyme extracts, their use as biocatalysts is less extended (de Freitas et al., 2017; Eibes et al., 2011; Taboada-Puig et al., 2016). Further research with these types of biocatalysts is needed to make enzymatic treatment a suitable option for EDC removal.

Another type of PPCPs that have received special attention are those with a recalcitrant nature, such as carbamazepine (CBZ, Table 1.5) and trimethoprim. In order to consider enzymatic treatment as a suitable option for the removal of these compounds it is important to elucidate which type of enzymes are most involved in the degradation of these contaminants, since the removal depends on the binding efficiency of the contaminants in the catalytic region (Bilal et al., 2022). Although the use of laccase have reported significant degradations of CBZ (Alharbi et al., 2019), other types of LE, such as VP or LiP, have degradation rates of less than 10 % (Eibes et al., 2011; Zhang and Geißen, 2010). In addition, the combined use of LEs to tertiary wastewater treatment technologies, such as UV radiation, can also increase the removal performance of these compounds (Tufail et al., 2021). However, such combined systems require high energy consumption and can also result in the generation of toxic by-products (Mohapatra et al., 2014).

#### *1.6.2.2. Polycyclic aromatic hydrocarbons*

PAHs are compounds that can be generated in the combustion processes associated with the oil, coal and gas industries. These chemicals are highly toxic and are considered persistent pollutants due to the negative impact of their complex mixtures on the environment and the long periods required for their natural degradation (Anyanwu et al., 2020; Patel et al., 2020). Its structure is composed by two or more fused benzene rings, without presence of heteroatoms and substituents on the polycyclic rings. Attending to the number of rings that integrate its structure, PAHs can be classified into two main categories: light PAHs (containing up to four rings) and heavy PAHs (containing more than 4 rings). In general, a higher molecular weight and angularity of PAHs is associated with an increase in the hydrophobicity and electrochemical stability (Sahoo et al., 2020).

Several technologies have been evaluated for their removal from the environment, including strong oxidising agents such as Fenton chemicals or ozone (Jonsson et al., 2006). Due to the ability of LEs to destabilise the bonds that make up the PAH structure (Chang et al., 2002), enzymatic treatment is a

suitable method to remove these compounds in an environmentally friendly way, although its effectiveness is highly dependent on the PAH structure, among other factors (Nzila, 2018).

Most PAHs degradation studies focus on their removal from soils or solid substrates. In this regard, several studies have employed *in vivo* assays with WRF to degrade these contaminants (Jove et al., 2016; Novotný et al., 2004). PAHs degradation in aqueous media has received less attention, but some studies have reported the degradation of PAHs with LEs in aqueous media containing organic solvents. Under these conditions, avoiding enzyme deactivation becomes crucial (Eibes et al., 2005). Eibes et al. (2006) studied the degradation of anthracene, dibenzothiophene and pyrene (Table 1.5) by MnP in presence of acetone in order to increase the solubility of PAHs. Different enzyme doses and reaction times were tested, reaching degradation rates close to 100 % with MnP doses between 550 and 1340 U/L in 7 h for anthracene and 24 h for pyrene and dibenzothiophene. In addition, the transformation products were analysed, verifying that the enzymatic transformation of anthracene and pyrene was achieved by an oxidation process involving OH·.

Other studies evaluated the enzymatic degradation of PAHs using surfactants such as Tween-80 to improve the solubility of these compounds and immobilising the enzyme on magnetic particles. This allows for a reusable biocatalyst that can be used in several cycles, although the removal efficiency of PAHs decreased after several cycles (Deng et al., 2022).

## 1.7. Factors controlling the enzyme activity

Considering that LEs are involved in different metabolic processes, their activity can be modulated by different factors including the concentration of the compounds involved in the enzymatic reactions, temperature, pH, the nature of the reaction medium and the presence of compounds that can act as activators or inhibitors. Therefore, the study of these factors is essential to ensure optimal functionality and reaction yields.

### 1.7.1. Substrate, enzyme and product concentrations

The kinetics of enzyme reactions is highly influenced by the concentrations of substrate, enzyme and products. Modifications in their concentrations can lead to a change in the enzymatic reaction, which is schematically described according to a multi-step mechanism (Equation 1.1) (Olkiewicz et al., 2022):

$$E + S \underset{kr}{\overset{k_f}{\rightleftarrows}} ES \underset{k_{catr}}{\overset{k_{cat}}{\rightleftarrows}} EP \underset{k_{asoc}}{\overset{k_{dis}}{\rightleftarrows}} E + P$$

**Eq. 1.1**

In this mechanism, first the enzyme (E) and substrate (S) bind to each other forming the enzyme-substrate complex (ES). Then, the substrate is transformed into the product(s), giving an enzyme-product complex (EP). Finally, the product(s) detach from the enzyme, releasing the enzyme to react again. $K_f$, $k_r$, $k_{cat}$, $k_{catr}$, $k_{dis}$, $k_{asoc}$ are forward rate constant, reverse rate constant, catalytic rate constant, catalytic reverse rate constant, dissociation constant and association constant (Olkiewicz et al., 2022).

Nevertheless, this equation can be simplified to Equation 1.2 (known as Michaelis-Menten equation), that describes reversible binding of E and S into the ES complex, and irreversible conversion of the complex into the product (P) and the free E.

$$E + S \underset{k_r}{\overset{k_f}{\rightleftarrows}} ES \overset{k_{cat}}{\rightarrow} E + P$$

**Eq. 1.2**

Michaelis-Menten equation is explained by the mathematical model indicated in Equation 1.3, where $K_M = k_r + k_{cat}/k_f$ is the Michaelis constant, roughly the dissociation constant of the complex and $V_{max} = k_{cat}\,[E]_0$ is the maximum reaction velocity and $[E]_0$ is the initial enzyme concentration.

$$\upsilon = \frac{d[P]}{dt} = \frac{V_{max}[S]}{K_M + [S]}$$

**Eq. 1.3**

In this kinetic model, which can only be considered when the substrate concentration greatly exceeds the enzyme concentration or when the energy released during the reaction is high, the influence of substrate and enzyme concentration on the efficiency of the enzymatic reaction is noteworthy. However, if the assumptions required for the use of the Michaelis-Menten equation are not met, the use of more complex models is necessary (Tummler et al., 2014; Wong et al., 2015), and also, in the case of the presence of inhibitors it is required the inclusion of inhibitor effects in the mathematic model.

A common enzymatic inhibition may be that produced by the products generated during the enzymatic reaction, which commonly have similarities with the original substrate and may interact with the active site reducing the amount of free active sites for binding with substrate molecules, with a

consequent decrease in the efficiency of the enzymatic reaction. In addition, the reaction products obtained in the oxidation of typical substrates of multicopper oxidases, such as ABTS, can react with organic sulfhydryl compounds and form complexes with the Cu atoms of the enzyme, leading to enzymatic inhibition (Valles et al., 2020).

Pamidipati and Ahmed (2020) also observed a decrease in laccase activity using different concentrations of common intermediates generated during lignin degradation. Therefore, intermediates may also cause competitive inhibition that decreases the yield of the enzymatic reaction.

### 1.7.2. Temperature

The study of the optimum temperature of an enzyme is crucial for its application as a biocatalyst. In general, chemical reaction rate and enzyme activity increase with increasing temperature. At moderate temperatures, the rate of enzyme deactivation is negligible and can be ignored, but at higher temperatures the deactivation is noticeable due to the compromise of the enzyme structure. If the enzyme reaction is carried out at the optimum temperature, a balance between reaction yield and enzyme activation/deactivation is established (Wojcik and Miłek, 2016).

The effect of temperature in reaction rate is explained by the Arrhenius equation (Equation 1.4) that was proposed in the 19th century based on empirical observations. In this equation the rate constant (k) is expressed in function of a pre-exponential factor (A), the activation energy ($E_a$), the universal gas constant (R) and the temperature in kelvin (T). $E_a$ is defined as the energy barrier which has to be overcome by the relative translational motion of the reactants in order for the reaction to occur (Arcus et al., 2016; Menzinger and Wolfgang, 1969). In the practice, the increase in temperature allows a higher initial level of energy, which will be translated in a reduction of $E_a$, facilitating the reaction performance (Grahame et al., 2015).

$$k = A \cdot e^{-\left(\frac{E_a}{RT}\right)} \qquad \textbf{Eq. 1.4}$$

On the other hand, the effect of high temperatures in the structure of the enzyme is responsible of the decrease in the enzyme activity. In general, fungal LEs can show higher activities at high temperatures, but this activity is not maintained during a long period of time, so optimal temperatures for their use as biocatalysts are in the range of 20 and 40 °C (Preethi et al., 2013; Thoa

et al., 2022; Zhou et al., 2023). Lueangjaroenkit et al. (2019) studied the stability of different laccases and peroxidases from *Trametes polyzona* between 20 and 90 °C and observed that although some of them showed maximum activity at 90 °C, it was not maintained for a long period of time, and temperatures up to 50 °C showed a fast activity loss (Lueangjaroenkit et al., 2019).

However, there is a group designated as thermostable enzymes that encompasses different enzymes whose primary structure makes them more resistant to proteolysis and chemical denaturation. These enzymes show greater stability than mesophilic enzymes because their structure has higher levels of non-polar AA that increase the hydrophobicity of the proteins affecting the catalytic site and the rigidity of the proteins (Che Hussian and Leong, 2023; Das and Gerstein, 2000). Thermostable LEs are naturally produced by a limited number of fungal hosts, as for example the laccase produced by *M. thermophila* (Yao et al., 2020). Interestingly, recombinant technology allows the expression of LEs with some structural modifications focused on the improvement of its thermal stability (Mateljak et al., 2019). Undoubtedly, these enzymes are promising options for biotechnological processes that require the use of high temperatures.

### 1.7.3. pH

The pH plays a crucial role in enzyme activity. As the pH of the medium changes, the charge state of the ionizable groups in the enzyme structure changes, which can lead to protonation or deprotonation of AA residues, resulting in denaturation of the enzyme, non-denaturing changes in structure, dissociation of oligomers and/or changes in activity rate (Grahame et al., 2015).

In general, the catalytic cycle begins with the transfer of a proton from a protein residue (proton donor) to the substrate, and for this the proton donor must be protonated. In addition, a nucleophilic attack on a substrate atom or the stabilisation of a positively charged intermediate is common during the enzymatic reaction mechanism, which is accomplished by the action of an AA residue with a free lone pair or a negative charge (Nielsen and McCammon, 2003). Considering these general characteristics of the catalytic cycle, pH has an important effect on the functionality of enzymes, and some studies have been performed in order to elucidate how pH can alter the interaction of the active site of LEs with substrates (Mauk et al., 1998; Mehra et al., 2018).

The optimal pH to maximize the performance of LEs varies from one type of enzyme to another. For most common LEs secreted by WRF, the optimal pH is situated in a range between acid and neutral values. Several studies have demonstrated that pH in which MnP present its maximum and constant activity is between 3.5 and 4.5 (Couto et al., 2006; Kong et al., 2016). For LiP, the pH optimum also lies in the range observed for MnP (Couto et al., 2006).

Most fungal laccases show their optimum activity for the oxidation of phenolic substrates in a pH range of 3-5.5 and become inactive as the pH approaches neutral and alkaline values. (Baldrian, 2006; Xu, 1997). Finally, UPOs present a different trend in relation to their optimal pH range, being this value more near to neutral pH. Several studies have observed a better performance for aryl alcohols oxidation in pH values between 5 and 7, depending on the strain productor of the enzyme (Gröbe et al., 2011; René Ullrich et al., 2004).

### 1.7.4. Solvents

Enzymes need a certain level of water in their structures to preserve their original conformation, which allows them to maintain their activity and functionality. Moreover, one of the reagents in hydrolytic reactions is water, so its presence in the reaction medium is essential for the reaction kinetics (Rezaei et al., 2007).

In nature, enzymes usually act as catalysts under aqueous conditions. For this reason, the application of enzymes as biocatalysts is usually performed in aqueous buffers. However, to meet the demands of some industrial applications focused on the synthesis of poorly water-soluble compounds, it is necessary to explore other alternatives to aqueous reaction systems (van Schie et al., 2021).

Among the non-aqueous media used for biocatalysis, organic solvents are the most employed (Wang et al., 2016). In these solvents, enzyme activity depends on partition coefficient (log P), functional groups and molecular structure of organic solvent (Liu et al., 2010). Enzyme activity is higher in organic solvents with a log P higher than 4, which is indicative of their hydrophobic nature (Wang et al., 2016). In addition, the functional groups of the organic solvent may be located near the active site or hydrophobic regions of the enzyme, which may cause a reorientation of the side chains of some AA, affecting the

affinity and specificity of the substrate, as well as the hydration of the enzyme (Lousa et al., 2013; Wang et al., 2016).

In the last years, ionic liquids (ILs) have also gained attention as solvents for enzymatic reactions. ILs, also called molten salts, are organic salts melting below 100 °C. These solvents have high thermal stability, insignificant vapor pressure, and moderate polarity. In addition, their physicochemical properties (e.g., viscosity, melting point, polarity, and hydrogen bond basicity) can be modified by simply changing anions or cations (Mora-Pale et al., 2011). It was also observed a correlation between the hydrophobicity of IL and a higher stability and activity of the enzyme, as for organic solvents (Lozano et al., 2001).

The last type of solvents that have been evaluated for biocatalytic reactions are supercritical fluids, which are materials above their critical temperature and critical pressure. Their properties can be modulated by changes in pressure and temperature, so they may be a suitable option for enzymatic reactions with specific requirements (e.g. enantioselectivity) (Wang et al., 2016). Although only a few studies evaluate the application of LEs in supercritical fluids, it has been demonstrated their suitability for the degradation of several pollutants such as textile dyes (Cristo et al., 2010).

### 1.7.5. Mediators

Enzyme activity can be improved by the use of mediators, which are compounds able to act as electron shuttle between the substrate molecule and the enzyme to reduce steric limitations and kinetic restrictions (Bhardwaj et al., 2022). However, mediators are non-essential molecules, because enzyme reaction can take place also in the absence of these compounds. Enzymes are able to oxidise mediators and generate radical species that can be more oxidative than the LE by itself (Bhardwaj et al., 2022). The efficiency of mediators can be evaluated through the analysis of different factors such as the affinity of the enzyme for the mediator, the stability of the oxidised intermediates generated and the oxidation potential of the intermediate compounds (Lloret et al., 2010).

Laccase is the most studied LE together with the use of mediators, in a system designed as laccase mediator system (LMS) that have been evaluated in the degradation of several compounds such as pesticides, phthalates and

pharmaceuticals (Jin et al., 2016; Parra Guardado et al., 2019; Wang et al., 2023).

Among the most commonly used mediators are natural compounds such as lignin-related phenolic compounds (syringaldehyde, p-coumaric acid, etc.) vanillin and synthetic mediators (i.e. ABTS or TEMPO) (Bhardwaj et al., 2022; Cañas and Camarero, 2010). The improve of enzyme activity by mediators strongly depends on the type of mediators used and the concentration. Lloret et al. (2010) studied the effect of different natural and synthetic mediators in concentrations ranging from 0.1 to 2 mM with laccase from *M. thermophila* on the degradation of pharmaceuticals compounds and concluded that, in general, the degradation increased together with an increased concentration of mediator.

### 1.7.6. Co-factors

Co-factors are essential molecules required to observe enzymatic activity (Di Cera, 2006; Gohara and Di Cera, 2016). For example, enzymes as MnP and UPO need the presence of $H_2O_2$ to start their catalytic cycle. This molecule act as electron receptor and allows the formation of an intermediate capable of attacking the substrate molecules. Moreover, MnP also requires the presence of $Mn^{2+}$ and a dicarboxylic acid, which are also involved as co-factors in the catalytic cycle.

Although these compounds are crucial and in their absence the enzyme reaction does not take place, high concentrations can cause inhibitory effects. In this sense, high $H_2O_2$ concentrations can lead to the production of excessive reactive species during enzymatic oxidative process, which can modify the enzyme active site in a mechanism designed as "suicide inactivation" (Gribas et al., 2016).

### 1.7.7. Inhibitors

Inhibitors are compounds that can interfere with the active site of the enzyme and affect the enzyme-substrate interaction (Lopina, 2017). In relation to enzyme inhibitors, depending on their mode of action, they can be classified into reversible and irreversible. The first one, leads to the deactivation of the enzyme through non-covalent interactions, which are easily reversible, and the second one inactivates an enzyme through a covalent bonding to a particular group of the active site. Therefore, a reversible inhibitor can

dissociate from the enzyme, whereas in the case of an irreversible inhibitor the bond is so strong that the inhibition cannot be reversed (Lopina, 2017).

Reversible inhibitors can be mainly divided into competitive and non-competitive. A competitive inhibitor has a similar structure to the molecule that may be the substrate of the enzyme, thus it competes with the substrate for binding to the active site of the enzyme. Although the competitive inhibitor does not act, it prevents the interaction between the active site and the substrate (Hollenberg, 2008). In the case of non-competitive inhibitor, it has the ability to interact with the free enzyme or with the enzyme-substrate complex, because its binding site is not the active site of the enzyme (Buker et al., 2019). Nevertheless, the presence of a non-competitive inhibitor can modify the three-dimensional conformation of the enzyme, leading to changes in the conformation of the active site.

The main molecules than can act as enzyme inhibitors are metal ions, organic compounds and large bioorganic molecules (Lopina, 2017). For example, many metal ions result in a negative impact of laccase activity, specially $Hg^{2+}$ (Couto and Herrera, 2006; Juárez-Gómez et al., 2018). Moreover, organic compounds such as hydrazide-hydrazones and several sulfhydryl compounds have been tested as inhibitors of *T. versicolor* laccase (Johannes and Majcherczyk, 2000; Maniak et al., 2020). In addition, chelating agents such as potassium cyanide, sodium azide or ethylenediaminetetraacetic acid (EDTA) can also be inhibitors of LEs activity because of their capacity of chelating Cu atoms (Couto and Herrera, 2006).

## 1.8. Objectives and structure of this Thesis

This section presents the main objective of this Doctoral Thesis, as well as the scientific questions posed and the strategies adopted to try to answer them, which are defined as research objectives. In addition, the structure of the Thesis is described in the different chapters.

### 1.8.1. Main objective

The goal of this Thesis is evaluating the suitability of different agro-industrial wastes for the production of fungal LEs, trying to search an alternative to conventional fermentation media that requires the use of high-value compounds. In addition, it is also intended to demonstrate the catalytic

potential of the enzyme cocktails obtained in the improvement of a natural polymer reactivity and in the oxidation of emerging pollutants.

### 1.8.2. Research questions

Taking into account the main objective of the Thesis and the existing knowledge about LEs production and their biotechnological applications, two research questions were proposed:

- Which type of fermentation is most suitable for the production of LEs through the valorisation of agro-industrial wastes?

- Do enzyme cocktails produced from the valorisation of waste streams have sufficient catalytic potential to be applied in the field of environmental biotechnology compared to commercial enzymes?

### 1.8.3. Research objectives

Trying to give an answer to the research questions contemplated, and after a state-of-art analysis of the main fields covered, the following research objectives were approached:

- Valorisation of different waste streams using SmF and SSF, including agricultural residues and agro-industrial by-products as carbon and nitrogen sources for fungal growth and induction of LEs expression.

- Application of enzyme cocktails to the modification of a natural polymer to improve its structure and make it suitable as raw material for the synthesis of bioadhesives, and application of enzyme cocktails in the degradation of different emerging pollutants.

### 1.8.4. Thesis structure

This Thesis is structured following the roadmap shown in Figure 1.6. An initial state-of-art was addressed in Chapter 1 to define the framework of the thesis, followed by the definition of the main objective and the research questions developed. Subsequently, different chapters aim to answer the research questions posed.

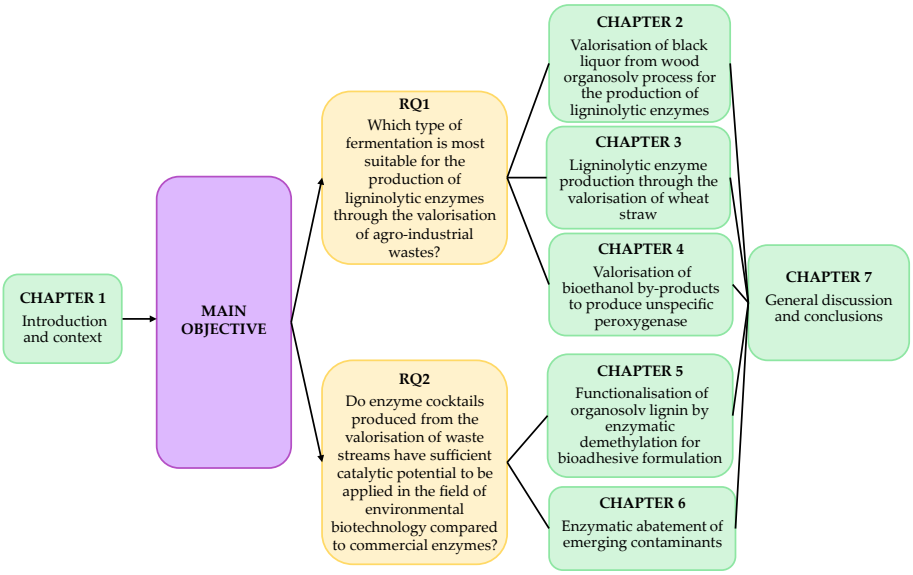



**Figure 1.6.** Structure of the PhD Thesis.

Chapters 2, 3 and 4 focused on the production of different LEs cocktails through the valorisation of wastes streams from different origin. Chapter 2 aims the production of laccase with *G. lucidum* using the residual liquid fraction (black liquor) of the organosolv process, Chapter 3 focuses on the production of MnP by *I. lacteus* from fermentation media based on raw wheat straw and extracts obtained from this material and Chapter 4 pursues the production of UPO by *A. aegerita* using by-products generated in the bioethanol industry, including vinasse and dried distillers's grains with solubles (DDGS).

In Chapters 5 and 6, an evaluation of the enzyme cocktails produced in the previous chapters as catalysts in different biotechnological processes was carried out. Chapter 5 deals with the modification of organosolv lignin with laccases to increase its reactivity and transform it into a viable precursor for the synthesis of bioadhesives, while Chapter 6 studies the removal of different emerging contaminants with the enzyme cocktails with MnP and UPO activity. Finally, Chapter 7 summarises the main conclusions derived from the Thesis and discusses new issues that may arise from the results obtained.

# CHAPTER 2

## VALORISATION OF BLACK LIQUOR FROM WOOD ORGANOSOLV PROCESS FOR THE PRODUCTION OF LIGNINOLYTIC ENZYMES

**SUMMARY**

White rot fungi (WRF) are characterised by the production of extracellular ligninolytic enzymes (LEs), which are capable of degrading a polymer as complex as lignin due to their high oxidation potential and non-specific character. Although ligninolytic fungi have been the topic of numerous studies in recent years, the complexity of the production of these enzymes, typically linked to secondary metabolism and their application at large-scale requires further research and development. Therefore, the search for new substrates to formulate the culture media that allow the production of enzymes in an efficient way at low cost is especially relevant. The valorisation of industrial waste streams rich in lignin is here presented as a promising alternative to foster LEs production. In this sense, the work presented in this chapter focuses on the production of LEs (laccase and manganese peroxidase) by *Ganoderma lucidum* in a fermentation medium based on black liquor, a by-product of the organosolv process of beechwood. Fermentations were carried out in solid (SSF) and submerged (SmF) state. High titers of laccase activity were achieved in the SmF while manganese peroxidase (MnP) activity was less relevant. The enzyme production was scaled-up to a 4 L reactor, achieving a laccase production of 2417 U/L, which is an 18.7 % decrease in enzyme production with respect to the flask scale, but it allowed a reduction in the time required to achieve peak activity. Finally, a proteomic analysis of the enzyme cocktail was performed in order to evaluate the main enzymes present at the end of the fermentation.

## 2.1. Introduction

### 2.1.1. Ligninolytic enzymes production and wood industry streams valorisation

The performance in the production of LEs is highly dependent on the microorganism, substrate and cultivation method (Elisashvili et al., 2008). Although several microorganisms are capable of producing LE, the most efficient producers of these proteins are fungi belonging to the WRF class (Plácido and Capareda, 2015). Among this class of fungi one that has gained special relevance in recent years has been *G. lucidum*. This fungus, which has been extensively studied for its medicinal and nutritional properties (El Sheikha, 2022), is also known to be a LE producer. The main lignin-modifying enzymes expressed by *G. lucidum* are laccases and heme peroxidases (Zhou et al., 2018). Although several studies have evaluated the use of *G. lucidum* to produce LEs, some of them are focused on the utilisation of high-value substrates rather than the use of waste streams (Arora and Gill, 2001; Kuhar and Papinutti, 2014). The use of industrial wastes can lead to a dual purpose: enzyme production and waste valorisation.

In this regard, lignin-rich waste streams can stimulate LEs production in *G. lucidum*. Furthermore, it is relevant to search for residues that are produced in large quantities on a global scale, such as waste derived from wood industry (Adhikari and Ozarska, 2018). Specifically, the black liquor associated with chemical pulping of cellulose is a promising candidate to boost LEs production. In particular, black liquor from the organosolv process, although much less abundant than Kraft liquor, has chemical oxygen demand (COD) values above those allowed for discharge and with low biodegradability levels, due to the presence of lignin and their derivatives.

In addition the high hemicellulose content in the stream ensures the presence of a C source to ensure fungal growth, and the high phenolic content can prevent the proliferation of other competing microorganisms, such as several bacteria species (Pacheco-Ordaz et al., 2018). Moreover, the fungal treatment of this liquid fraction from the organosolv process can detoxify this stream increasing its biodegradability (García-Torreiro et al., 2018).

### 2.1.2. Objectives of this chapter

Despite the suitable composition of black liquor to enhance enzyme production, the potential applications of this hemicellulose-rich waste stream were only explored in a few studies (García-Torreiro et al., 2018; Lú-Chau et al., 2018; Narra et al., 2020). The integral valorisation of organic waste streams through different biotechnological processes constitutes a challenge that pursues a double benefit: efficient waste management and the production/recovery of high value-added compounds. In addition, this allows adding an economic value to waste streams that are initially of no interest. Among these high value-added compounds, LEs are of particular interest for their potential application in different biocatalytic processes. This is one of the reasons why the formulation of fermentation media incorporating waste streams such as black liquor offers a possibility that needs to be evaluated for possible large-scale development.

A complete source of nutrients can be provided by these wastes, in which it may be necessary to add additional nutrients but in much lower concentration than in synthetic media. In this sense, the cost of enzyme production can be reduced, making enzymatic processes more competitive in comparison with other conventional technologies.

As a summary, it should be noted that the research carried out in this chapter has as its main objective the production of LEs, focusing mainly on the production of laccase. In addition, as a parallel objective, the valorisation of a residual stream such as black liquor is sought.

## 2.2. Materials and methods

### 2.2.1. Chemicals, raw material and microorganism

$MnSO_4$, $H_2O_2$, 2,6-dimethoxyphenol (DMP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), $CuSO_4$ and Antifoam 204 (A6426) were acquired from Sigma-Aldrich. Glucose, $KH_2PO_4$ and $NaC_2H_3O_2$ were purchased from Panreac, meal peptone from Cultimed, yeast extract from iNtRON Biotechnology and $MgSO_4 \cdot 7H_2O$ from Fluka.

Black liquor from the organosolv process of beechwood was used to prepare the fermentation medium. This stream was provided by the Fraunhofer Center for Chemical-Biotechnological Processes (CBP) (Germany). The characterisation of this waste fraction is shown in Table 2.1.

**Table 2.1.** Characteristics of the concentrated stream from the hemicellulosic fraction resulting from the organosolv treatment of beech wood.

| | |
|---|---|
| **pH** | 5.5 |
| **Total reducing sugars (g/L)** | 225 |
| **Total nitrogen (mg/L)** | 748 |
| **Glucose (g/L)** | 10.8 |
| **Xylose (g/L)** | 144.3 |
| **Rhamnose (g/L)** | 44.1 |
| **Xylose oligomer (g/L)** | 75.7 |
| **Acetic acid (g/L)** | 17.1 |

The fungus *G. lucidum* was isolated from mushroom spent substrate, kindly provided by Hifas da Terra S.L. (Pontevedra, Spain). The strain was stored in Petri dishes with a Kimura medium below -4 °C.

### 2.2.2. Fermentations

#### *2.2.2.1. Inoculum preparation*

For the fungal culture under static conditions, three plugs of active mycelia were transferred from Petri dishes to Fernbach flasks with 100 mL of Kimura medium (glucose 20 g/L, peptone 5 g/L, yeast extract 2 g/L, $KH_2PO_4$ 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 mg/L) and maintained at 30 °C for 7 d. After this step, the contents of the Fernbach flasks were crushed in a blender and used as inoculum for the Erlenmeyer flasks.

Regarding the operation in the reactor, the content of the crushed Fernbach (10 mL) was transferred to Erlenmeyer flasks with Kimura medium (90 mL) previously autoclaved at 110 °C for 30 min. Erlenmeyer flasks were incubated at 30 °C for 7 d, and after that the culture broth from the flasks was used as inoculum for reactor operation (10 % v:v).

#### *2.2.2.2. Solid state fermentation at flask scale*

Polyurethane foam cubes were used as support for *G. lucidum* growth in SSF. Each flask was filled with 0.5 g of polyurethane foam cubes with a volume of approximately of 1 $cm^3$ (1 cm × 1 cm × 1cm). To ensure the required supply of nutrients for fungal growth in each flask, a volume of 18 ml of a black liquor dilution (1:37) supplemented with meat peptone (2 g/L), $MnSO_4$ (0.5 mM) and

$CuSO_4$ (0.15 mM) at pH 4.5 was added, ensuring that the foam cubes were thoroughly soaked.

Erlenmeyer flasks with the soaked cubes were sterilised in an autoclave (Raypa AES-7) for 30 min at 110 °C and inoculated with 2 mL of inoculum (10 % v:v). The Erlenmeyer flasks were incubated for 21 d at 30 °C in a humidity-saturated environment under static conditions. All experiments were conducted in duplicate. For enzyme harvesting, the content of each Erlenmeyer flask was transferred to a 100 mL syringe and the fermentation medium absorbed into the polyurethane foam cubes was recovered. After that, the recovered medium was centrifuged at 4500 rpm for 5 min and the supernatant was withdrawn to analyse the enzyme activity and pH.

#### *2.2.2.3. Submerged fermentation at flask scale*

SmF was carried out in 250 mL-Erlenmeyer flasks, with a working volume of 100 mL. The supplemented black liquor dilution used in SSF was used as fermentation medium. Erlenmeyer flasks containing 90 mL of the final medium were autoclaved at 110 °C for 30 min and inoculated with 10 mL of inoculum. The Erlenmeyer flasks were incubated on an orbital shaker (COMECTA 100D Incubation Shaker) at 30 °C and 150 rpm for 16 d. Fermentations were carried out in triplicate with an initial pH of 4.5. Enzyme activity, pH and total nitrogen were determined in the supernatant of the samples taken periodically during the fermentation. For this purpose, 3 mL samples were taken and centrifuged at 4500 rpm for 5 min to remove the biomass.

#### *2.2.2.4. Submerged fermentation at reactor scale*

The enzyme production was performed in a 4 L stirred tank bioreactor (Biostat Bplus, Sartorius Stedim Biotech S.A.). The fungus grown in Erlenmeyer flasks (see section 2.2.2.3) was used as inoculum at 10 % (v:v). The fermentation medium was prepared as described for SSF at flask scale (see section 2.2.2.2). The conditions for medium sterilisation were 100 °C and 60 min. The bioreactor was operated for 2 d at controlled temperature (30 °C), under mechanical agitation (100-150 rpm), air supply (1-2 L/min) and pH monitoring. Foaming was automatically controlled by the addition of antifoam through a peristaltic pump.

### 2.2.3. Recovery and concentration of the enzymatic cocktail

The culture broth collected at maximum laccase activity in the 4 L reactor was filtered through filter paper (Whatman No.1, Maidstone). Afterwards, it was passed through a microfiltration membrane (Filtron Minisette System, Pall Corporation; 0.2 μm cut-off) and finally concentrated by ultrafiltration with a 10 kDa membrane (Filtron Minisette System, Pall Corporation). The cell-free concentrated crude, designated as enzymatic cocktail, was stored at -20 °C.

### 2.2.4. Shotgun proteomic analysis by mass spectrometry

The concentrated enzyme cocktail was processed in solution by trypsin digestion, reduction-alkylation and finally desalted using ZipTip-μC18 material (Merck Millipore). The obtained sample was analysed using the nanoUHPLC-TIMS-QTOF technique. Peptide samples (0.3 μg of protein) were injected onto a timsTOF Pro (Bruker) equipped with a nano-electrospray source (CaptiveSpray) and a TIMS-QTOF analyser. The chromatographic analysis was performed using a nanoELUTE chromatograph (Bruker) with a ReproSil C18 column (50 × 0.075 mm, 1.9 μm, 120 Å, Bruker). The nHPLC was set up with binary mobile phases that included solvent A (0.1 % formic acid in dd$H_2O$), and solvent B (0.1 % formic acid in acetonitrile). The analysis time was 20 min, in which B/A solvent ratio was gradually increased.

For MS acquisition, a CID fragmentation and nanoESI positive ionization mode was applied. The PASEF-MSMS scan mode was set for an acquisition range of 100-1700 m/z. MS/MS spectra was processed with PEAKS Studio (Bioinformatics Solutions) software for protein identifications using a database with all protein sequences available in NCBI protein database for *G. lucidum* taxonomy. The label-free module from PEAKS Studio was used for protein semi-quantification. The parameters analyzed from this analysis were the accession number (Accession number of the protein from NCBI database), area (the area under the curve of the peptide feature found at the same m/z and retention time as the MS/MS scan), n° peptides (peptides identified for each protein), n° unique peptides (number of peptide sequences unique to a protein group), avg. mass (promedium molecular weight) and coverage (calculated by dividing the number of amino acids in all peptides found by the total number of amino acids in the whole protein sequence). The Spec value is based on peptide spectrum matches (PSM) and was used as indicator for the relative abundance of the proteins in each sample (Ma et al., 2003).

### 2.2.5. Analytical protocols

Laccase activity was measured by spectrophotometric monitoring of the oxidation of 5 mM ABTS to the cation radical $ABTS^{+}$ at 436 nm ($\varepsilon_{436}$ = 29,300/(M·cm)) in sodium-acetate buffer (pH 5) at room temperature. MnP activity was measured by monitoring the oxidation DMP in the presence of $Mn^{2+}$ (1 mM) at 30 °C and pH 4.5. Changes in absorbance over time were monitored at 468 nm. In both cases, one unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of product per minute.

For all spectrophotometric measurements a UV-visible spectrophotometer (Shimadzu UV-1800) was used.

## 2.3. Results and discussion

### 2.3.1. Solid state fermentation at flask scale

SSF was performed using polyurethane foam cubes as support for fungal growth and a solution of black liquor supplemented with peptone (2 g/L) and a low concentration of Mn and Cu salts was added as main nutrients source to induce laccase and MnP production. Figure 2.1 shows the activity enzyme profile during the fermentation. MnP presented the maximum activity in the first stage of the fermentation, with its maximum value at 226 U/L on day 4, while laccase production presented the highest titers on day 15 of fermentation (338 U/L). Nevertheless, it is noteworthy that laccase activity showed several peaks of production in the initial stage, which is in agreement with previous studies of laccase production in SSF by *G. lucidum*, presenting the maximum production of laccase in the first week of fermentation (Rodrigues et al., 2019). Although enzyme production generally follows an upward trend until the maximum production is achieved, the occurrence of several peaks of activity before the maximum production value is reached has also been observed by other authors (Chevreuil et al., 2022).

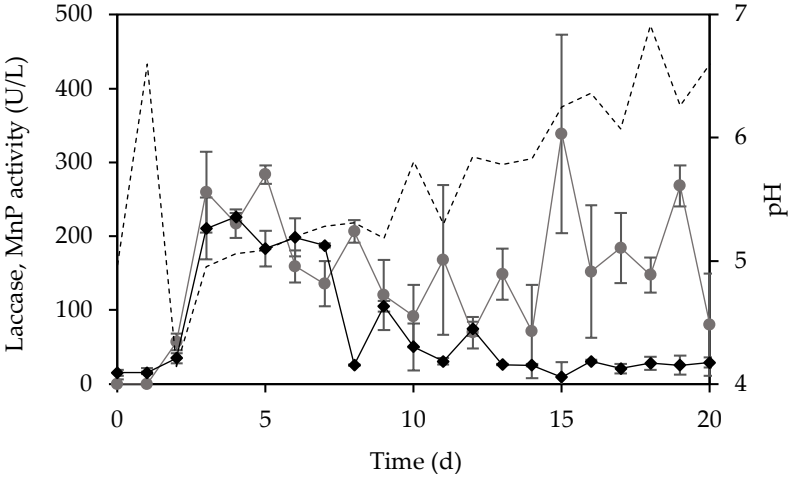



**Figure 2.1.** Evolution of laccase activity (•), MnP activity (♦) and pH (--) in SSF using polyurethane foams cubes soaked with black liquor-based medium.

SSF is particularly advantageous in the cultivation of basidiomycete fungi due to the similarity of the culture conditions to those characteristic of the natural environment in which these fungi grow (Rani et al., 2009). The use of an inert support as polyurethane foam entails a series of benefits in comparison with the use of natural substrates. In SSF using natural substrates as supports, the solid medium is disintegrated, leading to geometrical and physical changes of the fungal support. In addition, the enzyme recovery from an inert support is simpler (Ooijkaas et al., 2000).

A comparison of the maximum activities with previous works is not possible due to the different methods used to measure enzyme activity. However, if production times are compared with previous studies of SSF by *Ganoderma* strains, the time required to achieve the highest laccase titer in this study is higher compared to several studies considering agricultural residues as substrate (Nisa et al., 2014; Rodrigues et al., 2019). However, the initial levels of laccase production in this study appear at a fermentation time similar to that reported by Nisa et al. (2014) and Rodrigues et al. (2019).

Regarding MnP production, the use of agro-industrial residues as support and nutrient source allows the production of MnP in fermentation times similar (4-6 d) to those reported in this research (Nisa et al., 2014).

### 2.3.2. Submerged fermentation at flask scale

A dilution of black liquor was used as fermentation medium for *G. lucidum* growth and LEs expression in SmF. This medium was enriched with peptone as nitrogen source and with $CuSO_4$ and $MnSO_4$ to favor the production of laccase and MnP, as $Cu^{2+}$ and $Mn^{2+}$ have been shown to be relevant modulators of LEs biosynthesis in WRF, especially in the production of laccases (Baldrian and Gabriel, 2002; Wariishi et al., 1988).

Figure 2.2 shows laccase and MnP activities measured throughout the fermentation. Laccase production started after 48 h of fermentation, reaching its maximum value on day 6 (2971 U/L). However, similar levels of laccase activity were maintained on successive days of fermentation, following the trend of laccase production with different WRF (including *G. lucidum*) in black liquor-based medium (García-Torreiro et al., 2018). This high level of laccase achieved may be favoured by the phenolic content of black liquor, as high levels of oil-soluble aromatic/phenolic compounds have been shown to increase LEs production (Kapich et al., 2004). In addition, there has been no limitation on the amount of nitrogen available in the fermentation medium, with total nitrogen concentration remaining above 100 mg/L throughout fermentation (data not shown).

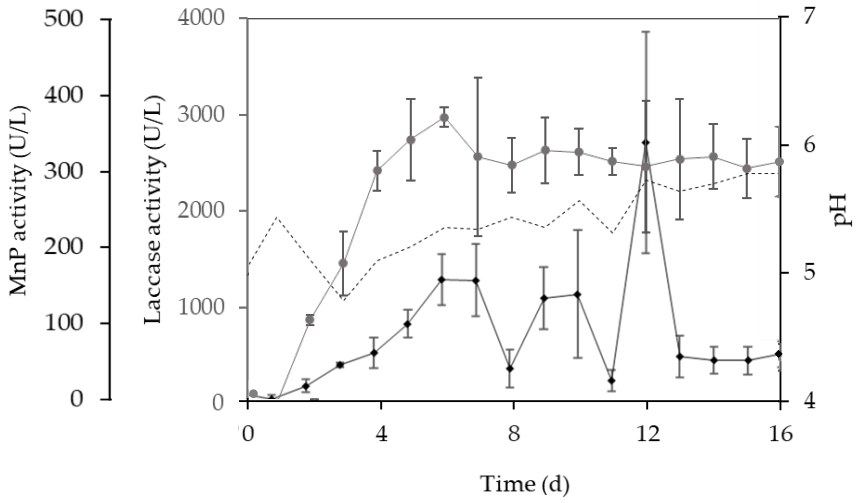



**Figure 2.2.** Evolution of laccase activity (•) on grey axis, MnP activity (♦) on black axis and pH (--) in SmF with black liquor-based medium.

In relation to MnP production, enzyme production started in parallel to laccase production, however, the increase of MnP activity was slower than laccase. The maximum MnP activity was reached on day 12 with 341 U/L. This result indicates that black liquor is suitable for MnP expression by *Ganoderma lucidum*, in comparison with other lignocellulosic wastes in which laccase but not MnP production was reported (Maganhotto De Souza Silva et al., 2005; Songulashvili et al., 2006).

Comparing these results with those obtained by García-Torreiro et al. (2018) who considered the same strain for SmF in hemicellulose-rich liquor, it is remarkable that a higher yield of laccase production is achieved in the SmF present in this chapter, obtaining a two-fold increase in laccase production (2971 U/L vs 1497 U/L) for similar fermentation periods. Although the same dilution of the waste stream and same supplements were used, the peptone concentration was twice that reported by García-Torreiro et al. (2018), so this may be the reason for the difference between the maximum laccase activities achieved.

Comparison of LEs production reported in this chapter with that achieved by *Ganoderma* sp on other types of substrates is difficult due to the different methods used to measure enzymatic activity. However, the times required for maximum enzymatic activity were lower than those described by other authors who reported 14 d to achieve maximum production (Mäkelä et al., 2013; Papinutti and Forchiassin, 2003).

Regarding pH, a peak (5.45) was observed when both enzymes started to be expressed (day 1), followed by a progressive decrease until day 3. After that, pH tended to increase until the end of fermentation, with a maximum of 5.8. This evolution in pH is common in SmF by basidiomycetes, in which pH tends to increase proportionally to enzyme activity, but with values between 4.5 and 7, which are optimal for LEs activity (Cen et al., 2022; Ullrich et al., 2005).

### 2.3.3. Scale-up of submerged fermentation

Scale-up of SmF with supplemented diluted black liquor achieved a maximum laccase and MnP production of 2417 and 55 U/L, respectively (Figure 2.3). For both enzymes the titers obtained were lower than those obtained in the flask scale, which may be due to the addition of antifoam.

Antifoaming compounds can have a negative impact on the growth of microorganisms due to an alteration of the permeability of cell membranes (Routledge, 2012). Mechanical agitation and aeration can also produce stress conditions that can lead to fragmentation of fungal granules, which would influence enzyme expression (Tinoco-valencia et al., 2014). However, at bioreactor scale these conditions are necessary to guarantee homogeneity of the medium that ensures a correct diffusion of nutrients.

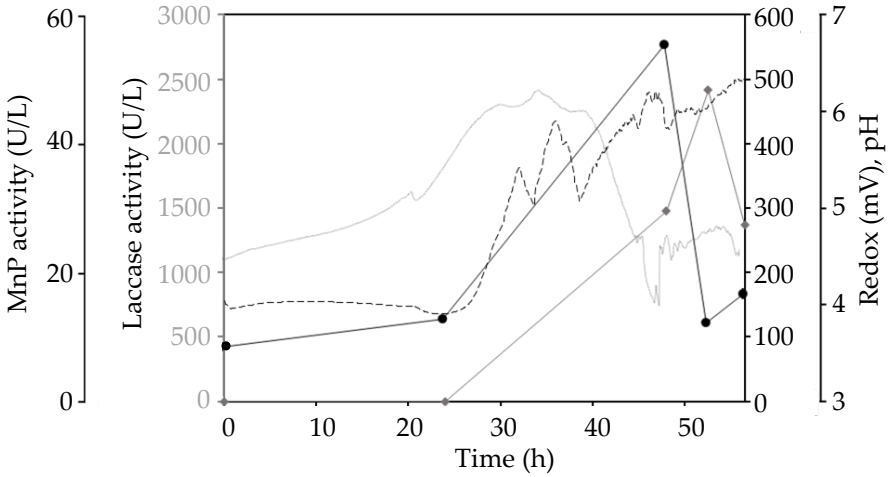



**Figure 2.3.** Evolution of pH (-), redox potential (··), laccase activity (♦) on grey axis and MnP activity (●) on black axis during the fermentation in a 4 L bioreactor.

However, the time required to obtain the maximum laccase production was reduced from 6 d at flask scale to 2 d in bioreactor. Moreover, comparing the laccase production obtained in the 4 L reactor with a previous work that also studied SmF by *G. lucidum* in black liquor based-medium, the laccase titers were 4-fold higher (Lú-Chau et al., 2018).

As for pH, the trend was different from that observed at flask scale. In the bioreactor, the pH value increased faster than in the flask scale, with a maximum value of 6.2. At the end of fermentation, it decreased to the initial value, whereas at the flask scale, no decrease in pH was observed at the final stage. These higher pH values in the reactor may be the result of the addition of antifoam. Nevertheless, this trend agrees with that reported by Lú-Chau et al. (2018) in SmF with *G. lucidum* in black liquor-based medium, which also

presented the highest pH (around 6.2) at the stage where laccase was expressed.

The redox value started to increase at the stage where laccase production began. It maintained a faster upward trend during the first hours and then grew more slowly until the maximum laccase production was reached. This redox evolution agrees with that reported by Lú-Chau et al. (2018), but the maximum redox value was higher in the present study (500 mV vs 400 mV), which makes sense considering that laccase production was higher.

Finally, the use of black liquor in the formulation of the fermentation medium may also allow a decrease in the toxicity of this waste stream, derived mainly from its high content of recalcitrant compounds such as lignin, tannins, etc. It has been reported that treatment of this residue with *G. lucidum* implies a 12-fold reduction in the initial toxicity of the diluted black liquor (García-Torreiro et al., 2018), related to toxicity to inhibition of *Vibrio fischeri* growth (Bulich and Isenberg, 1981). Although the research presented in this chapter did not address toxicity tests, a decrease in the colour intensity of the medium after fungal treatment was observed (Figure A2.1), which is likely related to the decrease in phenolic compounds reported by García-Torreiro et al. (2018).

### 2.3.4. Identification of enzyme cocktail proteins

In order to identify the proteins present in the enzyme cocktail produced by *G. lucidum*, a shotgun proteomic analysis was performed. By shotgun proteomic analysis, 11 enzymes were identified as shown in Table 2.2. The main proteins present in the enzyme cocktail were laccases, with more than 50 % of total coverage. Other enzymes present in smaller proportions, with less than 2 unique peptides, were beta-actins, beta-glucosidases and polyubiquitin.

Most of described fungal laccases have a molecular weight (MW) between 60-70 kDa. Nevertheless, the MW of the laccase detected by proteomic analysis (56.3 kDa) is in the range observed for laccases isolated from other *Ganoderma* species (Kumar et al., 2021; Umar and Ahmed, 2022). This lower MW could be a competitive advantage over other laccases, even those with higher redox potential, since a better access to the substrate could be achieved. In general,

laccases from *Ganoderma* sp belong to middle redox potential laccases, as its redox potential is between 430 and 710 mV (Sharma et al., 2013).

Beta-actins, the proteins detected in higher proportion after laccases, belong to a cytoskeletal filament protein class, which are involved in different cellular functions, including cell motility. In relation to beta-glucosidases, their functions and expression have been widely studied in fungi (Korotkova et al., 2009). The main function of these enzymes in WRF is the hydrolysis of different polysaccharides present in lignocellulosic materials, leading to the release of glucose (Sørensen et al., 2013). Therefore, the expression of this enzyme is expected in fermentation by *G. lucidum* in black liquor-based medium.

Finally, polyubiquitin, an enzyme also present in the enzyme cocktail, was shown to play an important role in fungal development, stress tolerance, an virulence (Wang et al., 2019). In fact, several studies suggests that it can be involved in proteasome degradation (Gilkerson et al., 2015; Gutierrez et al., 2018).

**Table 2.2.** List of proteins identified in the shotgun proteomics analysis of the enzyme cocktail from *G. lucidum*. Proteins are ranked by their Spec number, calculated with Peaks software and which can be correlated with their abundance in the sample.

| NCBI Accession | Description | Area | Nº peptides | Nº unique peptides | Avg. Mass (kDa) | Coverage (%) |
|---|---|---|---|---|---|---|
| gi\|117959694 | laccase | $5.00 \cdot 10^3$ | 8 | 3 | 56.29 | 19 |
| gi\|117959692 | laccase | $5.00 \cdot 10^3$ | 8 | 3 | 56.26 | 19 |
| gi\|558633451 | laccase partial | $2.86 \cdot 10^4$ | 7 | 2 | 56.61 | 18 |
| gi\|564733409 | beta-actin | 0 | 3 | 3 | 41.52 | 12 |
| gi\|558633473 | laccase partial | 0 | 2 | 1 | 54.94 | 4 |
| gi\|1701710131 | beta-glucosidase 3 | 0 | 1 | 1 | 85.40 | 1 |
| gi\|2103683182 | transcription factor bHLH7 | 0 | 1 | 1 | 105.75 | 1 |
| gi\|545693750 | polyubiquitin | 0 | 1 | 1 | 18.20 | 6 |
| gi\|564733421 | polyubiquitin | 0 | 1 | 1 | 18.20 | 6 |
| gi\|564733417 | polyubiquitin | 0 | 1 | 1 | 35.66 | 3 |
| gi\|564733419 | polyubiquitin | 0 | 1 | 1 | 42.74 | 2 |

## 2.4. Conclusions

Black liquor from the organosolv process of wood industry, has been shown to be a suitable nutrient source for *G. lucidum* growth and LEs expression. The type of fermentation has a significant impact on enzyme production, especially laccase activity. SmF resulted in higher laccase production than SSF, while in relation to MnP production, both fermentation types achieve similar enzyme production results. Scaling up the fermentation to a 4 L reactor resulted in a reduction of the maximum enzyme activity for both enzymes, probably due to the addition of antifoam, and/or the mechanical agitation which can negatively affect fungal growth. However, the time required to reach the maximum enzyme activities was significantly reduced.

Finally, the presence of laccase in the concentrated enzyme cocktail obtained in the reactor could be verified by proteomic analysis and could match with *G. lucidum* laccases previously described in the literature.

## 2.5. Annexes Chapter 2

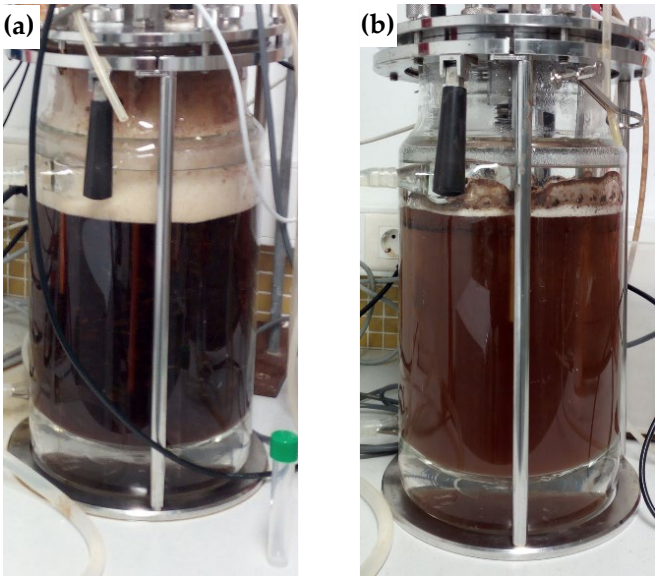



**Figure A2.1.** Aspect of fermentation medium of SmF by *G. lucidum* in 4 L reactor with diluted black liquor at **(a)** day 0 and **(b)** day 2.

# CHAPTER 3

## LIGNINOLYTIC ENZYMES PRODUCTION THROUGH THE VALORISATION OF WHEAT STRAW

Part of the results of this chapter have already been published as "González-Rodríguez S, Lu-Chau TA, Trueba-Santiso A, Eibes G, Moreira MT. Bundling the removal of emerging contaminants with the production of ligninolytic enzymes from residual streams. Appl Microbiol Biotechnol. 2022; 106(3):1299-1311. https://doi.org/10.1007/s00253-022-11776-7".

Detailed information available at "Manuscripts considered in this thesis: rights, contributions and quality indicators", page 227.

**SUMMARY**

Enzymes offer interesting features as biological catalysts for industry: high specificity, activity under mild conditions, accessibility and environmental friendliness. Being able to produce enzymes in large quantities and having them available in a stable and reusable form reduces the production costs of any enzyme-based process. Agricultural residues have recently demonstrated their potential as substrates to produce LEs by different white rot fungi (WRF). In this chapter, the biotechnological production of manganese peroxidase (MnP) by *Irpex lacteus* was conducted through solid state fermentation (SSF) with wheat straw as substrate and submerged fermentation (SmF) using wheat straw extract (WSE). The enzyme cocktail produced also showed manganese-independent activity (MiP), related to the presence of a short MnP and a dye-decolorizing peroxidase (DyP) which was confirmed by shotgun proteomic analyses. In view of the enhanced production of LEs in SmF, different parameters such as WSE concentration and nitrogen source were optimized. The highest enzyme titers were obtained with a medium formulated with glucose and peptone (339 U/L MnP and 15 U/L MiP). The scale-up to a 30 L reactor achieved similar activities, demonstrating the feasibility of enzyme production from the residual substrate at different production scales. Moreover, the Life Cycle Assessment (LCA) methodology was applied in order to study the environmental impact of the enzyme production process at 30 L reactor scale.

## 3.1. Introduction

### 3.1.1. Peroxidases production by *Irpex lacteus* and valorisation of waste streams

WRF include a broad spectrum of fungal species that produce LEs such as laccases, lignin peroxidase (LiP), MnP and other peroxidases, whose main function in nature is lignin mineralisation (Yadav and Yadav, 2015). Among WRF, *I. lacteus* has shown a significant role in different biotechnological fields due to the production of extracellular oxidative and hydrolytic enzymes (Novotný et al., 2009). Genome analysis reveals that the main ligninolytic enzyme of *I. lacteus* is MnP, with a major role in lignin degradation (Yao et al., 2017). However, depending on the substrates used and fermentation conditions, other ligninolytic enzymes, such as versatile peroxidase (VP) and laccase are also produced (Rothschild et al., 2002). For example, Salvachúa et al. (2013b) described the joint production of peroxidases with Mn(II)-dependent and Mn(II)-independent activities in fermentations with *I. lacteus*, which increases the range of use of its enzyme cocktail in different biodegradation processes.

Among the different fermentation media that allow the correct growth of the fungus and enzyme expression, several studies have focused on the valorisation of waste streams for the production of fungal LEs in recent years (Gupte et al., 2007; Pena et al., 2012; Xu et al., 2009). From the point of view of the fungal culture, two options can be considered for enzyme production: SmF or SSF. The former has been applied to a wider range of processes since the design, operation and control of the fermenter is relatively simple (Musoni et al., 2015). However, SSFs also offer some advantages, such as less complexity in downstream processing, low costs and energy, lower sterility demands and culture conditions similar to natural fungal habitat; but, with the negative aspects of heterogeneity, limited mixing and oxygen transfer (Couto and Sanromán, 2006; Hölker et al., 2004).

Although the technical aspects of fungal fermentations have been extensively studied, only a few studies focus on the environmental impact associated with the enzyme production process. Therefore, further research on the environmental impact implications of enzyme production processes is required.

### 3.1.2. Objectives of this chapter

Although different wastes have been considered for fermentations with *I. lacteus*, valorisation of solid wastes is usually performed only in SSF. However, this type of culture offers fewer advantages than SmF, since the latter allows greater homogeneity in terms of cell concentration, nutrients and products. Therefore, the search for new formulations for fermentation media based on waste streams that can be used in SmF is relevant. In addition, the biotechnological process of enzyme production with *I. lacteus* has focused mainly on MnP, leaving aside the study of other enzymes that can also be produced by this fungus. In this sense, the correct characterisation of the enzyme cocktail produced can provide a better knowledge of the enzymes expressed depending on the culture medium used, providing a more efficient application of the catalytic potential of this cocktail.

With this in mind, the work described in this chapter is focused on the valorisation of an agro-industrial waste (wheat straw) for the production of an enzyme cocktail with a high catalytic potential, in which most of the enzymatic activity is attributed to the MnP enzyme. In addition, proteomic analysis and LCA methodology have been applied to further characterise the enzyme cocktail and assess the environmental sustainability of its production.

## 3.2. Materials and methods

### 3.2.1. Chemicals, raw material and microorganism

$MnSO_4$, $C_2H_3NaO_2$, $H_2O_2$, 2,6-dimethoxyphenol (DMP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and Tween-80 were provided by Sigma-Aldrich. Glucose, casein hydrolysate, $KH_2PO_4$, $NaC_2H_3O_2$ and $CaCl_2$ were purchased from Panreac, meat peptone from Cultimed, yeast extract from iNtRON Biotechnology and $MgSO_4 \cdot 7H_2O$ from Fluka. Reagents for protein gen electrophoresis were purchased from ThermoFisher Scientific and GLUCOSE-TR kit for glucose quantification was purchased from Spinreact.

Wheat straw was obtained from a local supplier (Carral, Spain) and stored at room temperature until use. The contents of cellulose and hemicellulose, as well as lignin in the wheat straw, are presented in Table A3.1. These parameters are decisive in identifying the concentration of the C source present in the wheat straw for the formulation of the fermentation medium.

The fungus *I. lacteus*, strain Fr. 238 617/93 was obtained from the Culture Collection of Basidiomycetes (CCBAS) of the Academy of Sciences of the Czech Republic, Prague.

### 3.2.2. Fermentations

#### *3.2.2.1. Inoculum preparation*

For the fungal culture under static conditions, three plugs of active mycelia were transferred from Petri dishes to Fernbach flasks with 100 mL of Kimura medium (Chapter 2, section 2.2.2.1) and maintained at 30 °C for 7 d. After this step, the contents of the Fernbach flasks were crushed in a blender and used as inoculum for the Erlenmeyer flasks.

#### *3.2.2.2. Solid state fermentations at flask scale*

Wheat straw was cut into 2 cm particles before being used as substrate and support for fungal growth. To evaluate whether wheat straw was sufficient for fungal growth, SSF was performed with two different media, one with wheat straw moistened with distilled water and the other with wheat straw moistened with a solution of C and N sources (4 g/L glucose and 1 g/L peptone). Each 250 mL Erlenmeyer flask containing 3 g of wheat straw and 9 mL of culture medium was sterilized for 45 min at 110 °C (RAYPA AES-75) and inoculated with 1 mL of inoculum. The Erlenmeyer flasks were incubated for 21 d at 30 °C in a humidity-saturated environment under static conditions. The initial pH of the medium in all experiments was 4.5. All experiments were conducted in duplicate.

For enzyme harvesting, the contents of each Erlenmeyer flask were extracted with distilled water at a liquid/solid ratio of 10:1. The Erlenmeyer flasks were then shaken in an orbital shaker (C24 Incubator Shaker, New Brunswick Scientific) for 1 h at 175 rpm to facilitate the separation of the enzyme adsorbed on the wheat straw. The harvested liquid was then filtered through a cellulose filter and centrifuged for 10 min at 10,000 rpm prior to the determination of enzyme activity in the supernatant.

#### *3.2.2.3. Submerged fermentations at flask scale*

WSE supplemented with different types of nitrogen sources and also two conventional media based on typical composition were tested to stimulate fungal growth. WSE was obtained by adding 75 g of wheat straw to 1 L of

distilled water, autoclaving the mixture at 121 °C for 20 minutes, and finally removing the solids by filtration. The WSE composition is shown in Table A3.2. To assess whether the concentration of WSE had any impact on enzyme production, experiments were carried out with the raw extract and with a diluted solution of the extract (25 % in distilled water). Both media were supplemented with glucose and peptone (or casein hydrolysate) as shown in Table 3.1. The addition of Tween-80 (0.5 mL/L) as a surfactant was intended to enhance enzyme production.

**Table 3.1.** Supplementation of the different media elaborated with diluted and raw WSE.

| | Glucose (g/L) | Peptone (g/L) | Casein hydrolysate (g/L) | Tween-80 (mL/L) |
|---|---|---|---|---|
| **Raw WSE** | 4 | 1 | - | 0.5 |
| | 4 | - | 1 | 0.5 |
| **Diluted WSE (25 %)** | 4 | 1 | - | 0.5 |
| | 4 | - | 1 | 0.5 |

Erlenmeyer flasks containing 90 mL of the final medium were autoclaved at 110 °C for 45 min and inoculated with 10 mL of inoculum. The Erlenmeyer flasks were incubated on an orbital shaker (Innova 4000 Incubation Shaker, New Brunswick Scientific) at 30 °C and 150 rpm for 7 d. Fermentations were carried out in triplicate with an initial pH of 4.5. The sampling strategy considered the periodic withdrawal of 3 mL of culture medium under sterile conditions, which were centrifuged for 10 min at 10,000 rpm to remove fungal biomass. The supernatant was used to monitor the culture pH and to analyze MnP and MiP activities.

To compare the enzyme production using wheat straw based media with other conventional media, two of the most typical culture media for the growth of white rot fungus were tested: Kimura medium (see 3.2.2.1) and modified Kirk medium (glucose 10 g/L, ammonium tartrate 0.5-10 g/L, sodium acetate 2.72 g/L, $KH_2PO_4$ 2 g/L, $CaCl_2$ 100 mg/L, mineral salts 10 mL/L, thiamine 2 mg/L, $MnSO_4$ 84.51 mg/L) at pH 5.5.

### *3.2.2.4. Submerged fermentation at reactor scale*

The scale-up of enzyme production by *I. lacteus* under SmF was carried out in a 30 L stirred tank bioreactor (Biostat Cplus, Sartorius Stedim Biotech S.A.). The fungus grown in Erlenmeyer flasks was used as inoculum at 10 % v:v.

The medium was prepared with diluted WSE (25 % in distilled water) and supplemented with 4 g/L of glucose and 1 g/L of peptone at pH 4.5. The reactor was operated for 3 d with temperature (30 °C), agitation (150 rpm) and air flow (10 L/min) control. Other parameters measured on-line were redox potential, dissolved oxygen and pH. The morphology of the fungus along the fermentation was evaluated using an optical microscope (Axioskop 2 plus, Zeiss) connected to a digital camera (Cool Snap, Roper Scientific) with a resolution of 1392 × 1040.

### 3.2.3. Recovery and concentration of the enzymatic cocktail

The culture broth collected at maximal MnP and MiP activities in 30 L reactor was concentrated following the same protocol as indicated in Chapter 2 (section 2.2.3). The cell-free concentrated crude, designated as enzymatic cocktail, was stored at -20 °C.

### 3.2.4. Protein identification

#### *3.2.4.1. Protein gel electrophoresis*

The presence of different enzymes in the cocktail was confirmed separating them by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in denaturing conditions (SDS-PAGE). For this, the protein concentration of the enzyme cocktail was estimated using a Pierce™ BCA Protein Assay Kit. Electrophoresis was run for triplicate aliquots with 17 ng protein/well. As molecular weight marker the PageRuler™ Plus Prestained Protein Ladder from 10 to 250 kDa was included. Bis-Tris NuPAGE 4-12 % gel was used and electrophoresis was run at 200 V.

#### *3.2.4.2. Shotgun proteomic analysis by mass spectrometry*

Shotgun proteomic analysis of the concentrated enzyme cocktail was performed as described in Chapter 2 (section 2.2.4). MS/MS spectra was processed with PEAKS Studio (Bioinformatics Solutions) software for protein identifications using a homemade database with all protein sequences available in NCBI protein database for *I. lacteus* taxonomy. The label-free module from PEAKS Studio was used for protein semiquantification. The parameters analysed from this technique are shown in Table A3.3.

### 3.2.5. Analytical protocols

Different parameters were analysed throughout the fermentations, including pH, glucose concentration and enzyme activity. Glucose concentration was determined by using an enzymatic quantitative kit. MnP activity was measured by monitoring the oxidation of DMP in the presence of $Mn^{2+}$ (1 mM) at 30 °C and pH 4.5. MiP activity was determined as above but lacking $Mn^{2+}$. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of product per minute. Changes in absorbance over time were monitored at 468 nm. Laccase activity was measured by monitoring the oxidation rate of 0.267 mM ABTS to its cation radical ($ABTS^{\cdot+}$) at 420 nm ($\varepsilon_{420}$ = 36,000/(M·cm)) in McIlvaine buffer (pH 3.0) at room temperature., following the protocol described by Muñiz-Mouro et al. (2017). One unit (U) of activity was defined as the amount of enzyme forming 1 μmol of $ABTS^{\cdot+}$ per min. All spectrophotometric measurements were carried out on a Shimadzu UV-1800 spectrophotometer.

For the determination of wheat straw composition, it was employed an adapted protocol from NREL (National Renewable Energy Laboratory, Golden, USA). After an acid hydrolysis, it is possible a separation of sugar fractions. The concentration of hemicellulose and cellulose were calculated from the concentration of the corresponding monomeric sugars using an anhydro correction of 0.88 and 0.90 for C5 and C6 sugars, respectively.

### 3.2.6. Life Cycle Assessment

The methodology of LCA was applied in order to evaluate the environmental impacts associated to MnP production with *I. lacteus* in a 30 L-reactor. In accordance with the ISO 14040:2006 standard, the goal and scope of the evaluation were defined as the analysis of the environmental feasibility of MnP production in SmF with *I. lacteus* from a cradle-to-gate approach, which allows analyzing all the stages required to produce the enzyme cocktail with MnP and MiP activity. Maintenance and transport activities were not included in the scope of the assessment.

The general scheme of the process is depicted in Figure 3.1, with the identification of the different stages. Stage I corresponds to inoculum preparation (section 3.2.2.1), Stage II to fermentation (section 3.2.2.4) and Stage III to downstream process (section 3.2.3).

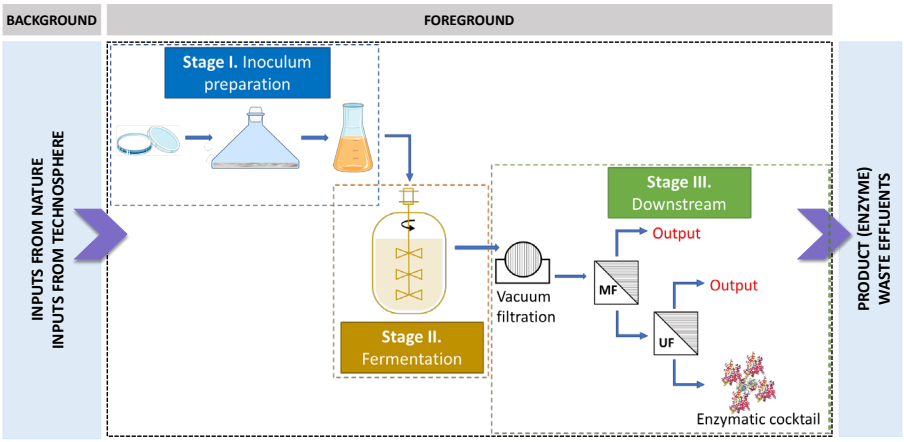



**Figure 3.1**. General scheme of the MnP production process according to a Life Cycle Assessment perspective.

The functional unit (FU) selected for the environmental assessment was one unit of MnP. In order to perform the analysis, life cycle inventory was compiled according to experimental data (Table A3.4). For the inventories, Ecoinvent was selected as the database, since it provides all the information on the main inputs necessary for the production of MnP. In the case of the energy needs of the process, an allocation of the systems was made to consider only the energy required for heating, agitation or sterilization of the units used (Petri dishes, Fernbach flasks, etc.). This consideration was also made with the energy generated by the compressor consumed for reactor aeration, taking into account the flow used by the bioreactor.

The methodology selected to perform the environmental assessment was the ReCiPe 2016 hierarchist MidPoint method V1.03World (2010), which includes several midpoint impact indicators, among which the following were considered: global warming (GW), ionizing radiation (IR), terrestrial ecotoxicity (TE), freshwater ecotoxicity (FET), marine ecotoxicity (MET), human non-carcinogenic toxicity (HN-CT) and fossil resource scarcity (FRS).

The environmental impacts of each stage were evaluated, as well as the impacts associated with the global production process of the concentrated enzyme cocktail. The environmental loads represented in the figures are expressed as percentages in the characterisation stage.

## 3.3. Results and discussion

### 3.3.1. Solid state fermentation at flask scale

Wheat straw was used as substrate and support for the SSF of *I. lacteus*. The additional supply of glucose (4 g/L) and peptone (1 g/L) as carbon and nitrogen sources was evaluated in terms of MnP production (Figure 3.2). MnP activity was higher in the SSF without supplementation (NS-SSF, 176.0 ± 8.6 U/L), compared with those of the SSF supplemented with glucose and peptone (GP-SSF, 53.6 ± 11.8 U/L). Several studies have demonstrated that low N and C concentrations in SSF can stimulate secondary metabolism in WRF, which is responsible of LEs production (Elisashvili et al., 2008; Iqbal et al., 2011). More specifically, Elisashvili et al. (2008) have established that nitrogen supplementation in SSF with wheat straw had a negative impact on enzyme production by *Pleurotus ostreatus*. It is likely that the fungus first consumes easily metabolizable nutrients, and then initiates the secretion of LEs as a strategy to access the C source present in wheat straw (Elisashvili et al., 2008). This fungus presents a high capacity to degrade acid-insoluble lignin (Zuo et al., 2018), which is the most abundant type of lignin present in the wheat straw used in this study (Table A3.1). This ability to degrade lignocellulosic material present in the deeper layers of wheat straw is directly related to a higher production of LEs.

It is also noteworthy that the time required to reach the maximum MnP production was similar in both SSF, being 9 d in the NS-SSF and 7 d in the GP-SSF. Once the maximum activity was reached, a decrease in MnP activity was observed. SSF studies using non-pretreated wheat straw showed different fermentation periods to reach maximum MnP activity, varying from 14 to 23 d (Dias et al., 2010; Salvachúa et al., 2013b). Nevertheless, SSF studies using other agro-industrial wastes, such as olive biomass, have shown longer fermentation times (30 d) to reach maximum enzyme production (Martínez-Patiño et al., 2018).

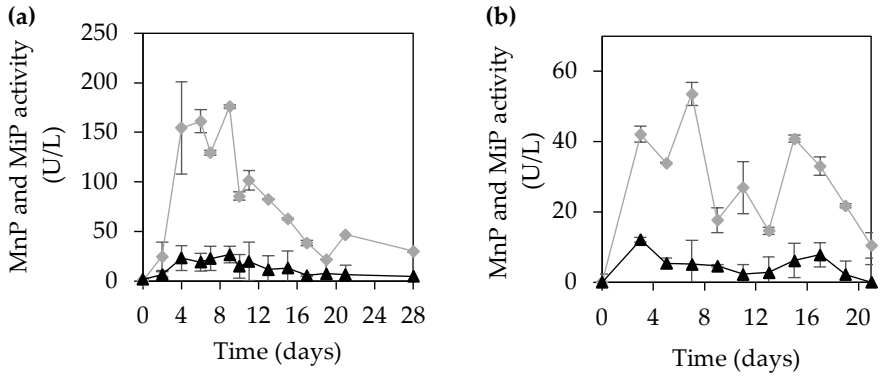



**Figure 3.2.** Enzyme activities during SSFs: **(a)** MnP (♦) and MiP (▲) activities in the NS-SSF with wheat straw as only C and N source; **(b)** MnP (♦) and MiP (▲) activities in the GP-SSF with wheat straw, glucose and peptone.

Regarding MiP activity, its profile in NS-SSF shows production peaks similar to the maximum in earlier stages of the fermentation, whereas in GP-SSF the highest activity is reached in the initial phase of the fermentation, followed by a decrease of the activity. The maximum MiP activities (26.6 ± 2.8 and 12.1 ± 2.3 U/L) were reached on days 3 and 9 for the SSF without and with extra nutrient addition, respectively.

The pretreatment of wheat straw used in SSF can influence enzyme production. Despite the potential of enzyme production in SSF, the period of time required to obtain maximum production is too long, which was one of the reasons why the production of LEs in SmF was addressed.

### 3.3.2. Submerged fermentation at flask scale

Submerged fermentation was conducted using WSE supplemented with glucose and peptone, to ensure a nutrient-balanced medium for an optimal fungal growth. In addition, to evaluate whether any of the substances present in the extract could affect the growth of the microorganism, SmF was performed using the crude WSE and the diluted extract (25 % WSE in distilled water). Enzyme production was higher for fermentations with diluted extract (Figure 3.3-b), which could be related to the lower glucose consumption in the medium with the raw extract. The presence of possible inhibitory compounds in WSE may affect fungal growth and thus glucose consumption.

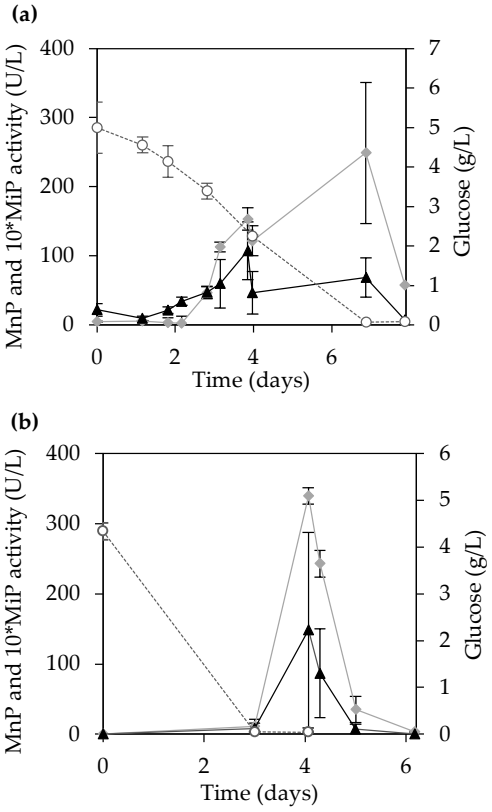



**Figure 3.3.** Evolution of MnP activity (♦), MiP activity (▲) and glucose concentration (○) in the SmF with **(a)** raw WSE and **(b)** diluted WSE both supplemented with glucose and peptone.

Maximum activities were reached on day 4 in the fermentation with diluted extract (339.4 ± 11.6 U/L of MnP; 14.8 ± 13.9 U/L of MiP). In the fermentation with raw extract (Figure 3.3-a) the maximum MiP activity was 10.7 ± 6.1 U/L, and the maximum MnP activity 249.0 ± 51.0 U/L. Interestingly, the production of enzymes when using 100 % WSE was faster (activity started on day 2 compared to day 4 in diluted extract), which could be due to dissolved lignin derived compounds acting as inducers of LEs production. The effect of aromatic compounds acting as inducers has been previously described, and it was suggested that LEs synthesis may function as a defense mechanism against chemical stress (Elisashvili et al., 2010). Although the production of

enzymes was faster in the raw extract and maintained for a longer period, the maximum production was achieved in the diluted extract after 4 d.

It was also evaluated whether a change in the type of N source could affect enzyme production. For this, casein hydrolysate was used as N source instead of peptone, in addition to the nutrients provided by WSE. As in the previous fermentations with peptone, both raw and diluted WSE were used. Figure 3.4 shows that the highest MnP activity was achieved with the medium with the raw WSE (227.9 ± 21.9 U/L at day 6), and the activity in the diluted medium was 96.7 ± 6.3 U/L, also at day 6. For MiP activity, the diluted WSE performed similarly to raw extract, reaching maximum activities of 18.0 ± 9.5 and 12.2 ± 3.5 U/L after 6 and 3 d, respectively.

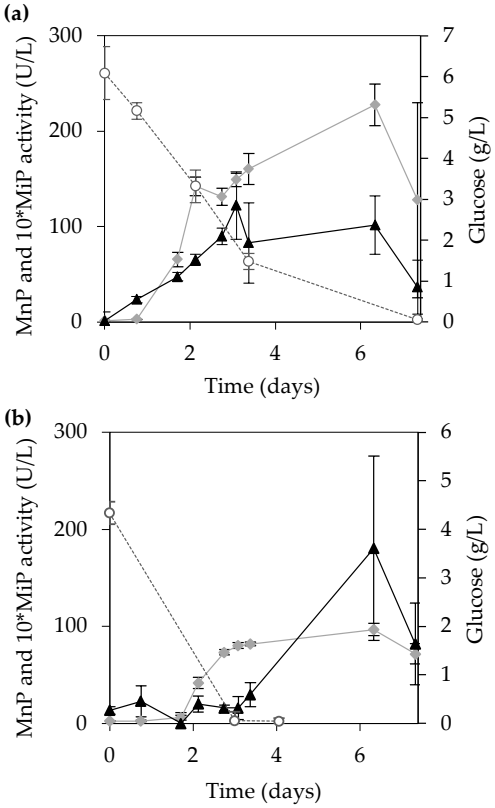



**Figure 3.4.** Evolution of MnP activity (♦), MiP activity (▲) and glucose concentration (○) in the SmF with **(a)** raw and **(b)** diluted WSE, both supplemented with glucose and casein hydrolysate.

The choice of the type of nitrogen sources can have an important impact in fungus development. Previous studies reported that organic compounds perform better than inorganic compounds in fungal SmF, due to their ability to stimulate fungal growth (Juwon and Emmanuel, 2012). The use of peptone was reported as booster of enzyme production in several studies (Lú-Chau et al., 2018; Pinheiro et al., 2020). Moreover, it is noteworthy that for all tested conditions MiP was produced in lower values than MnP, in agreement with other reports on *I. lacteus* (Salvachúa et al., 2013b). Specifically, the maximum levels of MnP activity were approximately 10 times higher than the maximum levels of MiP activities.

In addition, to have a reference of the enzyme production of *I. lacteus* and to evaluate the impact of the use of WSE, SmF with *I. lacteus* was carried out using two of the most common conventional media for fungal culture: Kirk and Kimura (Kimura et al., 1990; Tien and Kirk, 1988). In these experiments only the Mn(II)-dependent activity was studied. Table 3.2 shows the MnP production throughout the 14-day fermentation. The highest MnP activity (100.3 ± 26.5 U/L) was reached in Kimura medium on day 8, while in the case of Kirk medium the maximum production peak (60.4 ± 6.7 U/L) was reached on day 14.

**Table 3.2.** MnP activity (U/L) during SmF on conventional media.

| | Day 2 | Day 4 | Day 6 | Day 8 | Day 10 | Day 12 | Day 14 |
|---|---|---|---|---|---|---|---|
| **Kirk** | 18.5 ± 0.5 | 22.6 ± 2.0 | 46.0 ± 11.8 | 45.4 ± 14.4 | 51.7 ± 15.2 | 51.7 ± 2.5 | 60.4 ± 6.7 |
| **Kimura** | 17.3 ± 2.2 | 38.2 ± 2.2 | 57.2 ± 8.0 | 100.3 ± 26.5 | 98.4 ± 18.4 | 31.0 ± 8.7 | 22.3 ± 1.9 |

It should be noted that all SmFs with WSE-based medium showed similar or higher MnP production than SmFs in conventional media, which makes WSE a good alternative for the production of LE, with the advantage that it is possible to valorise an agricultural waste in the context of a circular economy approach. The fact that wheat straw contains significant levels of manganese (50 mg/L) (Hofrichter et al., 1999) leads to more suitable conditions that favor MnP production. Laccase activity was also measured in all fermentations with wheat straw-based media, but no laccase activity was detected in any of the trials.

### 3.3.3. Scale-up of submerged fermentation

The SmF with maximum enzyme production was scaled up to a total volume of 30 L in a stirred tank reactor, obtaining a maximum MnP production of 345 U/L. During the operation of the reactor, different parameters were measured online (Figure 3.5). The analysis of the redox potential and pH profile allows to determine an indicator of increased enzyme production. Regarding the pH, before the onset of enzyme production, the pH value decreased to around 4.4 and after the peak of MnP production, pH increased again till 4.7. On the other hand, the redox potential underwent an increase as more enzyme was produced, reaching its maximum value (500 mV) in parallel to the peak of enzyme production (Lú-Chau et al., 2018).

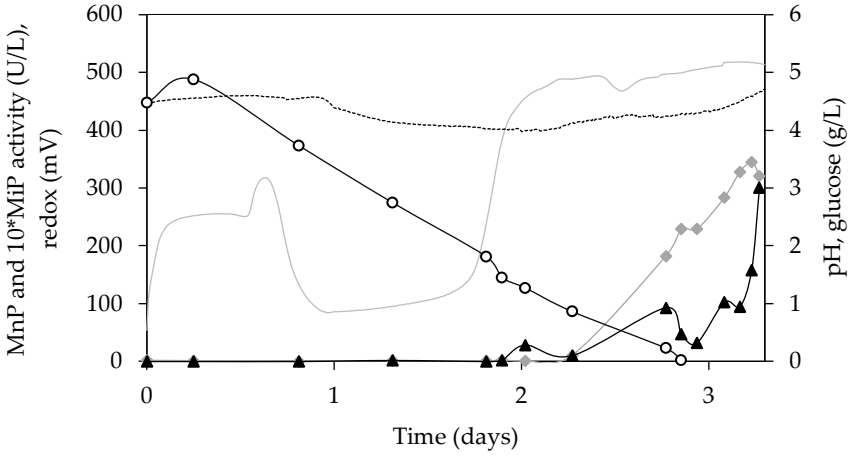



**Figure 3.5.** Evolution of pH (- -), redox potential (-), MnP activity (♦), MiP activity (▲) and glucose concentration (○) during the fermentation in a 30 L fermenter.

Another remarkable fact is that enzyme production started once the glucose concentration was below 0.8 g/L, similar to the trend observed in Erlenmeyer flask experiments. This is mainly because low glucose levels induce a phase shift from primary to secondary metabolism, a phase in which the fungus begins to synthesize LEs (Iqbal et al., 2011).

At the morphological level, it was observed that the morphology of the fungus varied during the fermentation. Initially, the fungal pellets had a well-defined spherical shape (Figure A3.1-a) because the addition of Tween-80 favours their agglomeration (Q. Li et al., 2018). As fermentation progresses, due to the

shear stress that the fungus undergoes by continuous agitation and aeration, the mycelium fragmented into smaller amorphous pieces (Figure A3.1-b) reaching a point where it started to form spores (Figure A3.1-c,d,e), trying to conserve its genetic material. WRF sporulation only occurs under certain conditions of metabolic and mechanical stress associated with agitation, conditions in which the fungus uses spore formation as a survival mechanism (Su et al., 2012).

### 3.3.4. Identification of enzyme cocktail proteins

Due to the detection of Mn(II)-dependent and Mn(II)-independent activities, the different proteins present in the concentrated enzyme cocktail were separated using SDS-PAGE, as a tool to differentiate the enzymes responsible for these activities. Figure 3.6 shows the migration pattern of triplicate cocktail aliquots. The band with the highest intensity corresponds to a molecular weight of 53 kDa, while also other bands with molecular weights of 89, 59 and 44 kDa are visible.

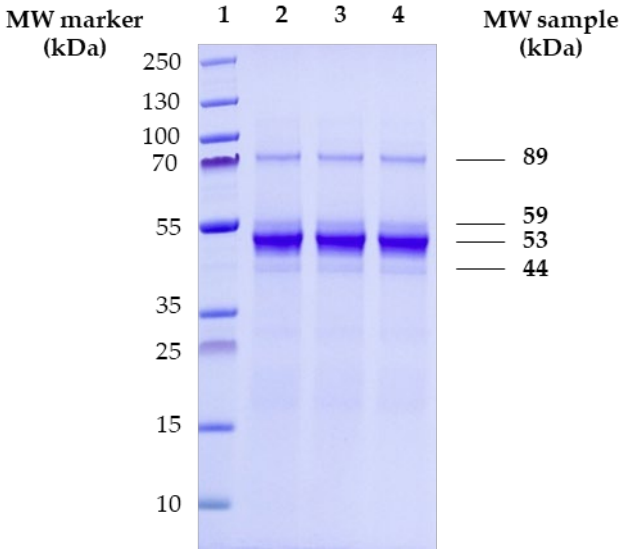



**Figure 3.6.** SDS-PAGE of triplicate aliquots from the concentrated enzyme cocktail obtained in the SmF with *I. lacteus* (lanes 2-4). Molecular weight marker is shown in lane 1.

By shotgun proteomic analyses, 18 enzymes were identified in the enzyme cocktail as shown in Table A3.3 (considering only the proteins with >2 unique

peptides). Most of them correspond to peroxidases, and among them, interestingly, long-chain MnPs, one short-chain MnP and one DyP. Other enzymes present in the enzyme cocktail included proteinases, cellobiohydrolases and dehydrogenases.

The production of enzyme cocktails with MnP and MiP activities has been documented for different WRF species (Chen et al., 2015; Duan et al., 2018). However, very few articles analyze whether this activity is due to a single enzyme or to a set of enzymes. The expression of a short MnP with both MnP and MiP activity has already been described for *I. lacteus* (Li et al., 2019). Therefore, it is essential to determine its presence in the obtained enzyme cocktail, as it could offer some advantages due to its shorter C-terminal length that can increase the catalytic properties by a better interaction with the substrate molecule (Li et al., 2019).

Considering the SDS-PAGE electrophoresis results, it is clear that the proteins present in highest abundance correspond to a molecular weight (MW) of 53 kDa. However, in shotgun proteomic analyses the most abundant proteins were MnPs with MWs ranging from 37.25 – 40.25 kDa. This is in agreement to Shin et al. (2005), who reported a purified MnP from *I. lacteus* with a MW of 38.3 according to their MALDI-TOF analyses, while in SDS-PAGE its band was located in 53 kDa. Also, in Wang et al. (2002), an isolated MnP from *Bjerkandera adusta* showed an apparent MW in SDS-PAGE of 43 kDa in SDS-PAGE and 36.6 kDa by MALDI-TOF. These differences can be explained by the fact that MnPs are glycosylated proteins, and high carbohydrate contents can affect migration in SDS-PAGE. Another band observed in the gel at 44 kDa might correspond to a short MnP. Its MW is in agreement with other values reported in the literature for short MnPs of *I. lacteus* in SDS-PAGE (43-45 kDa) (Chen et al., 2015; Duan et al., 2018). By mass spectrometry one enzyme identified with 5 unique peptides corresponds with a short MnP of *I. lacteus* previously characterised by Chen et al. (2015) (GenBank: AGO86670.2).

On the other hand, the presence of a band at the MW of 59 kDa, could correspond to a DyP. Salvachúa et al. (2013a) have already described a DyP produced with *I. lacteus* on wheat straw with a molecular weight of 57 kDa in SDS-PAGE. The presence of this enzyme together with short MnP would justify the MiP activity measured in the enzyme cocktail. Accordingly, proteomic analysis detected a DyP with 17 unique peptides.

Finally, a weak band was observed in SDS-PAGE at a corresponding MW of 89 kDa which could not be explained by any of the proteins identified by shotgun proteomics. One possibility is that this band is composed by the choline dehydrogenases detected in shotgun with MW of 65.87 kDa. Nonetheless it cannot be discarded that this might be result of the artificial dimerization of an enzyme during the sample processing for SDS-PAGE, which is rare but have already been described, especially for highly hydrophobic proteins (Rath et al., 2009).

Regarding the abovementioned, the proteomic analysis confirmed the presence of different peroxidases in the enzyme cocktail and is a more precise tool for the identification of enzymes and their corresponding MW in mixed cocktails.

### 3.3.5. Environmental profile associated to enzyme production

In order to evaluate the components of the life-cycle inventory that contributes the most to the environmental impacts, the LCA methodology was used to the complete process of MnP production. Figure 3.7 shows the environmental profile of the different stages of the process, in which it is possible see that stage II (fermentation) is the largest contributor to the impacts estimated for all the categories selected. Nevertheless, stage I (inoculum preparation) also had a significant contribution to environmental burdens of the complete process of MnP production. Table A3.5 shows the average values by impact category and process stage.

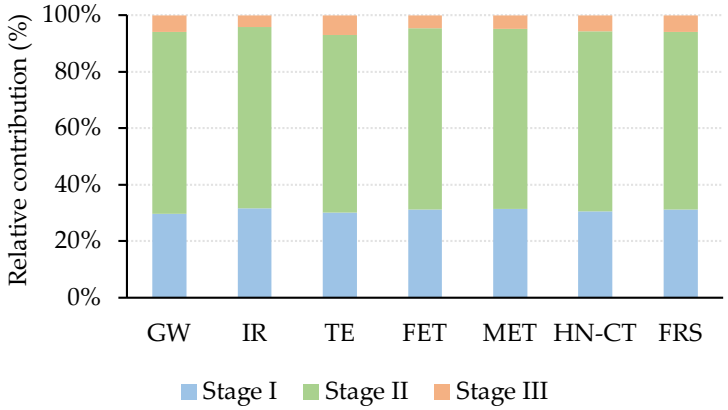



**Figure 3.7.** Environmental impact contributions per process stage in MnP production with *I. lacteus* in 30 L reactor.

To try to determine why these stages contribute the most to the overall environmental impact of the process, the environmental profile of each stage was analyzed separately. Figure 3.8 presents the contributions to the environmental impact at the different stages of MnP production. It shows the breakdown distribution of environmental impacts for each category in terms of energy required, water consumption, chemicals and treatment of the output streams. Energy consumption was the main issue in all categories evaluated. For the correct growth of the fungus and the expression of enzymes, a series of environmental conditions such as suitable temperature, agitation and aeration are necessary, which imply the use of equipment with a considerable energy demand for several days. Nevertheless, for stage III, in which it is only required energy for the operation of the peristaltic pumps that allows the flow of the enzyme cocktail to the membranes of micro and ultrafiltration, electricity had less weight in the environmental impacts than in previous stages. The second main contributor to the impacts in stage III was the use of chemicals (except for terrestrial ecotoxicity category) due to the addition of NaOH for the cleaning of the filtration membranes.

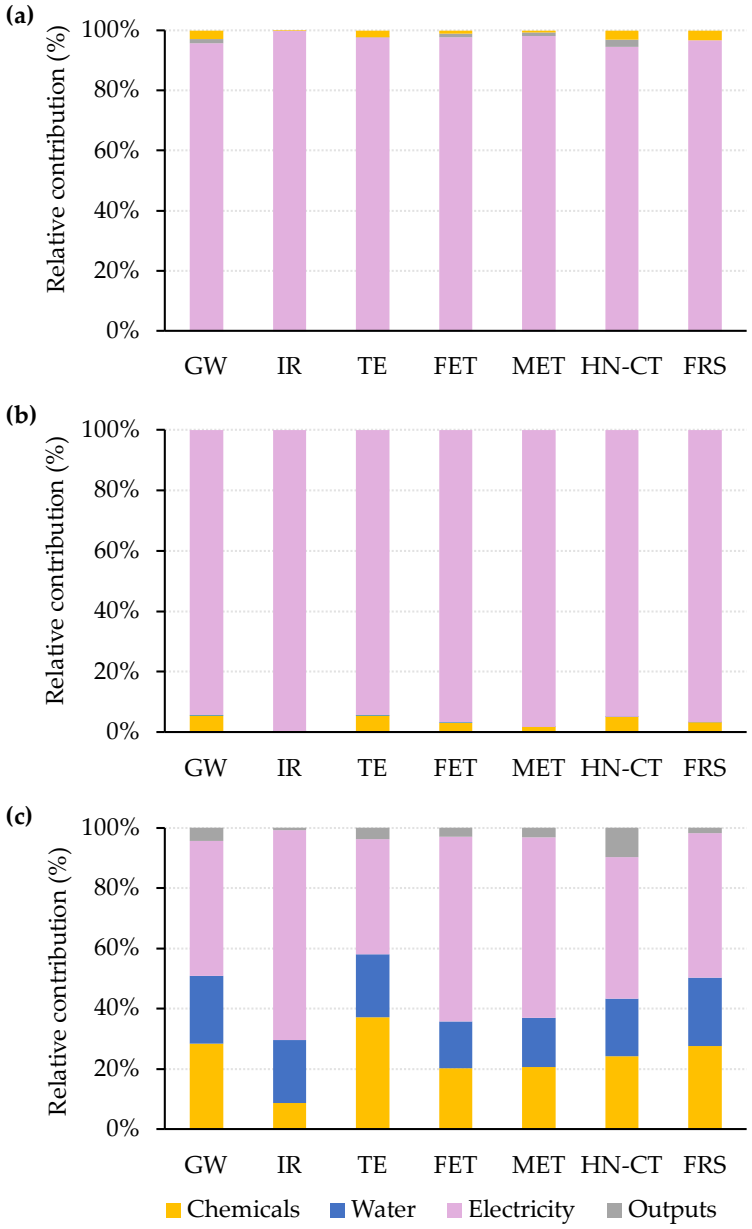



**Figure 3.8.** Environmental impact contributions in the different stages of MnP production with *I. lacteus* in 30 L reactor: **(a)** Stage I- Inoculum preparation, **(b)** Stage II- Fermentation and **(c)** Stage III- Downstream.

Finally, if the electricity consumed in stages I and II is disaggregated, it is possible to see the contribution of each piece of equipment to the environmental impacts of each category studied (Figure 3.9). For stage I, the equipment that caused the largest environmental impacts were the shaker and autoclave, due to the wide period required for the growth of the inoculum and the conditions necessary to prepare the wheat straw extract and sterilize the fermentation medium. In the case of stage II, the autoclave and compressor were the main contributors to environmental profile associated to energy consumption, even taking into account that only the energy necessary to produce the air flow supplied to the reactor was considered.

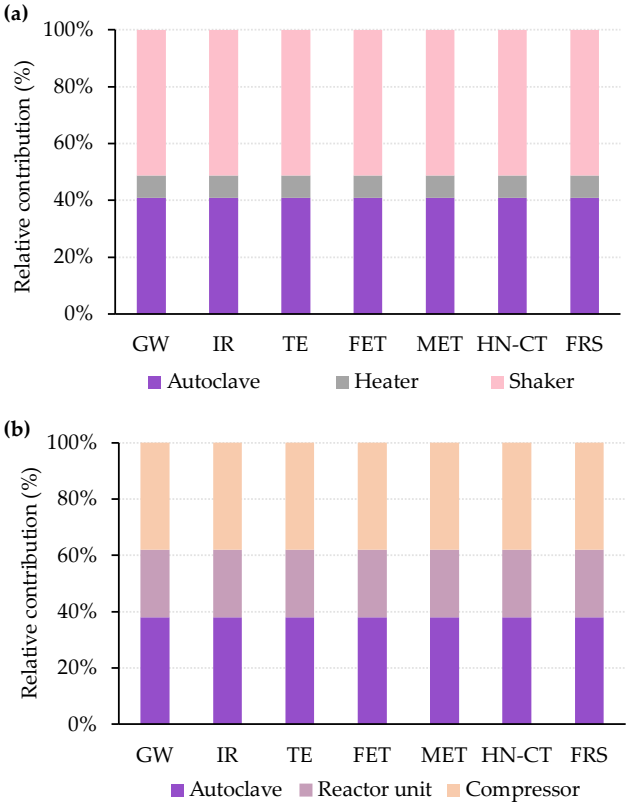



**Figure 3.9.** Environmental impact contributions of the energy consumption of each equipment in: **(a)** Stage I- Inoculum preparation and **(b)** Stage II- Fermentation.

Considering that the energy requirement was the main critical point identified in the global process, different alternatives must be explored to reduce this contribution. In this sense, improving process efficiency or reducing fermentation time could be crucial. A possible alternative to achieve this goal could be to optimise the reactor configuration, which could enhance the environmental conditions for fungal growth and lead to a reduction in enzyme production time or an increase in enzyme activity per batch. For example, Yang et al. (2012) evaluated the effects of different impeller configurations on fungal physiology and the subsequent improvement of the production of high-valued compounds. Improved impeller configuration led to a 25 % reduction in energy requirement due to improved mixing efficiency, fluid homogeneity, and mass transfer.

## 3.4. Conclusions

Wheat straw is a great alternative substrate to support the growth of *I. lacteus* and to induce the production of LEs in both SSF and SmF. It was demonstrated that higher titers of enzyme production can be achieved in WSE based medium in comparison with conventional media. It was also demonstrated that the supply of different types of supplementary nitrogen sources had a significant effect on the enzymatic activities obtained in the submerged state.

Most studies evaluating lignocellulosic residues for fungal fermentation have achieved the production of enzyme cocktails with Mn(II)-dependent activity, but there are a few studies reporting the production of an enzyme cocktail with both Mn(II)-dependent and Mn(II)-independent activity. Proteomic analysis has allowed the identification of other enzymes present in the enzyme cocktail such as DyP and dehydrogenase, which can increase the biotechnological applications of the cocktail. Moreover, the environmental impact associated with the production of the enzyme cocktail was also evaluated, where the fermentation step contributed to a considerable part of the environmental impacts, especially due to energy consumption.

## 3.5. Annexes Chapter 3

**Table A3.1.** Wheat straw composition.

| | |
|---|---|
| **Total lignin (%)** | 49.94 ± 0.52 |
| **Acid insoluble lignin (%)** | 44.90 ± 1.24 |
| **Acid-soluble lignin (%)** | 5.59 ± 0.36 |
| **Cellulose (%)** | 8.46 ± 0.18 |
| **Hemicellulose (%)** | 8.37 ± 0.18 |
| **Ash (%)** | 0.547 ± 0.365 |

**Table A3.2.** Characteristics of raw wheat straw extract.

| | |
|---|---|
| **pH** | 6.66 |
| **Total nitrogen (mg/L)** | 69.4 ± 0.3 |
| **Total organic carbon (mg/L)** | 924.2 ± 4.1 |
| **Total reducing sugars (mg/L)** | 224 ± 8 |
| **Glucose (mg/L)** | 92 ± 16 |

**Table A3.3.** List of proteins identified in the shotgun proteomics analysis of the enzyme cocktail from *I. lacteus*. Proteins are ranked by their Spec number, calculated with Peaks software and which can be correlated with their abundance in the sample.

| NCBI Accession | Description | Area | Nº Peptides | Nº Unique Peptides | Avg. Mass (kDa) | Coverage (%) |
|---|---|---|---|---|---|---|
| AQT03613.1 | manganese peroxidase 2 | $4.45 \cdot 10^4$ | 28 | 5 | 37.8 | 45 |
| AGO86670.2 | short manganese peroxidase | $4.45 \cdot 10^4$ | 28 | 5 | 37.83 | 45 |
| AZJ17947.1 | manganese peroxidase | $1.37 \cdot 10^5$ | 22 | 20 | 37.94 | 50 |
| AZJ17945.1 | manganese peroxidase | $1.37 \cdot 10^5$ | 22 | 20 | 38.24 | 49 |
| ANA52681.1 | dye-decolorizing peroxidase partial | $1.01 \cdot 10^5$ | 17 | 17 | 48.42 | 29 |
| AZJ17942.1 | manganese peroxidase | $3.26 \cdot 10^4$ | 5 | 3 | 30.32 | 21 |
| AZJ17939.1 | manganese peroxidase | $1.99 \cdot 10^4$ | 5 | 4 | 37.25 | 15 |
| sp\|P17576.1\|CARP_IRPLA | RecName: Full=Polyporopepsin | $3.60 \cdot 10^4$ | 7 | 7 | 35.05 | 14 |
| prf\|\|1512141A | Asp protease | $3.60 \cdot 10^4$ | 7 | 7 | 35.05 | 14 |
| BAA00467.1 | aspartic proteinase precursor partial | $3.60 \cdot 10^4$ | 7 | 7 | 35.05 | 14 |
| pdb\|1WKR\|A | Chain A Polyporopepsin | $3.60 \cdot 10^4$ | 7 | 7 | 35.04 | 14 |
| AQT03612.1 | manganese peroxidase 1 | $1.26 \cdot 10^4$ | 6 | 6 | 37.92 | 16 |
| AZJ17938.1 | manganese peroxidase | $1.26 \cdot 10^4$ | 6 | 6 | 40.25 | 15 |
| AZJ17940.1 | manganese peroxidase | $2.11 \cdot 10^2$ | 5 | 3 | 37.96 | 14 |
| BAD16575.1 | cellobiohydrolase | $5.86 \cdot 10^2$ | 6 | 6 | 54.84 | 11 |
| ALJ82906.1 | mitochondrial choline dehydrogenase | $9.06 \cdot 10^1$ | 4 | 4 | 65.87 | 9 |
| ALJ82895.1 | choline dehydrogenase | $8.92 \cdot 10^2$ | 3 | 3 | 65.87 | 6 |
| BAG48183.1 | cellobiohydrolase II | $4.55 \cdot 10^2$ | 2 | 2 | 47.28 | 5 |

**Table A3.4.** Global process inventory for the MnP production at 30 L bioreactor (considering as FU one unit of MnP).

| | Inputs from Technosphere | | | | Outputs | | | |
|---|---|---|---|---|---|---|---|---|
| | *Material/Electricity* | *Value* | *Unit* | *Comments and assumptions* | *Material* | *Value* | *Unit* | *Comments* |
| **Stage I** | Agar | $6.24 \cdot 10^{-5}$ | g | Considered as generic organic chemical | Biowaste | $1.22 \cdot 10^{-2}$ | g | Petri dish and wheat straw |
| | Glucose | $7.12 \cdot 10^{-2}$ | g | | Polystyrene | $2.76 \cdot 10^{-3}$ | g | Petri dish |
| | Meat peptone | $1.78 \cdot 10^{-2}$ | g | Considered as protein feed | | | | |
| | $KH_2PO_4$ | $1.91 \cdot 10^{-4}$ | g | Considered as generic inorganic chemical | | | | |
| | $MgSO_4$ | $4.68 \cdot 10^{-5}$ | g | | | | | |
| | Yeast extract | $3.83 \cdot 10^{-4}$ | g | Considered as protein feed | | | | |
| | Sawdust (*Pinus radiata*) | $8.32 \cdot 10^{-6}$ | g | | | | | |
| | Wheat straw | $3.60 \cdot 10^{-1}$ | g | | | | | |
| | HCl | $3.05 \cdot 10^{-3}$ | mL | | | | | |
| | Polystyrene | $2.76 \cdot 10^{-3}$ | g | Petri dish | | | | |
| | Distilled water | $6.48 \cdot 10^{-4}$ | $dm^3$ | Considered as deionized water | | | | |
| | Electricity | $1.75 \cdot 10^{-3}$ | kWh | | | | | |
| **Stage II** | Meat peptone | $5.62 \cdot 10^{-3}$ | g | Considered as protein feed | Biowaste | $1.20 \cdot 10^{-1}$ | g | Wheat straw |
| | Glucose | $2.25 \cdot 10^{-2}$ | g | | | | | |
| | Wheat straw | $1.20 \cdot 10^{-1}$ | g | | | | | |
| | HCl | $1.02 \cdot 10^{-3}$ | mL | | | | | |
| | Distilled water | $5.81 \cdot 10^{-3}$ | $dm^3$ | Considered as deionized water | | | | |
| | Electricity | $3.54 \cdot 10^{-3}$ | kWh | | | | | |
| **Stage III** | NaOH | $3.33 \cdot 10^{-2}$ | g | | Biowaste | $4.18 \cdot 10^{-3}$ | g | Fungi biomass |
| | Distilled water | $1.66 \cdot 10^{-3}$ | $dm^3$ | Considered as deionized water | Wastewater | $5.94 \cdot 10^{-3}$ | $dm^3$ | |
| | Tap water | $9.36 \cdot 10^{-2}$ | $dm^3$ | | | | | |
| | Electricity | $1.61 \cdot 10^{-4}$ | kWh | | | | | |

**Table A3.5.** Midpoint values per impact category and process stage.

| Impact category | Acronym | Unit | Total | Stage I | Stage II | Stage III |
|---|---|---|---|---|---|---|
| Global warming | GW | kg $CO_2$ eq | $2.41 \cdot 10^{-3}$ | $7.14 \cdot 10^{-4}$ | $1.55 \cdot 10^{-3}$ | $1.41 \cdot 10^{-4}$ |
| Stratospheric ozone depletion | SOD | kg CFC11 eq | $2.38 \cdot 10^{-9}$ | $4.99 \cdot 10^{-10}$ | $1.78 \cdot 10^{-9}$ | $9.94 \cdot 10^{-11}$ |
| Ionizing radiation | IR | kBq Co-60 eq | $1.16 \cdot 10^{-3}$ | $3.68 \cdot 10^{-4}$ | $7.45 \cdot 10^{-4}$ | $4.86 \cdot 10^{-5}$ |
| Ozone formation, Human health | OF | kg $NO_x$ eq | $4.44 \cdot 10^{-6}$ | $1.34 \cdot 10^{-6}$ | $2.79 \cdot 10^{-6}$ | $3.12 \cdot 10^{-7}$ |
| Fine particulate matter formation | FPM | kg PM2.5 eq | $3.73 \cdot 10^{-6}$ | $1.13 \cdot 10^{-6}$ | $2.34 \cdot 10^{-6}$ | $2.54 \cdot 10^{-7}$ |
| Ozone formation, Terrestrial ecosystems | OF | kg $NO_x$ eq | $4.49 \cdot 10^{-6}$ | $1.36 \cdot 10^{-6}$ | $2.82 \cdot 10^{-6}$ | $3.16 \cdot 10^{-7}$ |
| Terrestrial acidification | TA | kg $SO_2$ eq | $9.60 \cdot 10^{-6}$ | $2.92 \cdot 10^{-6}$ | $6.11 \cdot 10^{-6}$ | $5.69 \cdot 10^{-7}$ |
| Freshwater eutrophication | FE | kg P eq | $2.92 \cdot 10^{-6}$ | $8.49 \cdot 10^{-7}$ | $1.93 \cdot 10^{-6}$ | $1.43 \cdot 10^{-7}$ |
| Marine eutrophication | ME | kg N eq | $6.85 \cdot 10^{-7}$ | $1.08 \cdot 10^{-7}$ | $5.27 \cdot 10^{-7}$ | $4.87 \cdot 10^{-8}$ |
| Terrestrial ecotoxicity | TE | kg 1,4-DCB | $7.23 \cdot 10^{-3}$ | $2.17 \cdot 10^{-3}$ | $4.55 \cdot 10^{-3}$ | $5.10 \cdot 10^{-4}$ |
| Freshwater ecotoxicity | FET | kg 1,4-DCB | $2.50 \cdot 10^{-4}$ | $7.77 \cdot 10^{-5}$ | $1.61 \cdot 10^{-4}$ | $1.14 \cdot 10^{-5}$ |
| Marine ecotoxicity | MET | kg 1,4-DCB | $3.13 \cdot 10^{-4}$ | $9.79 \cdot 10^{-5}$ | $2.00 \cdot 10^{-4}$ | $1.48 \cdot 10^{-5}$ |
| Human carcinogenic toxicity | HCT | kg 1,4-DCB | $2.03 \cdot 10^{-4}$ | $5.53 \cdot 10^{-5}$ | $1.14 \cdot 10^{-4}$ | $3.39 \cdot 10^{-5}$ |
| Human non-carcinogenic toxicity | HN-CT | kg 1,4-DCB | $3.80 \cdot 10^{-3}$ | $1.16 \cdot 10^{-3}$ | $2.42 \cdot 10^{-3}$ | $2.15 \cdot 10^{-4}$ |
| Land use | LU | $m^2$a crop eq | $3.46 \cdot 10^{-4}$ | $5.53 \cdot 10^{-5}$ | $2.86 \cdot 10^{-4}$ | $4.71 \cdot 10^{-6}$ |
| Mineral resource scarcity | MRS | kg Cu eq | $6.27 \cdot 10^{-6}$ | $1.79 \cdot 10^{-6}$ | $3.76 \cdot 10^{-6}$ | $7.20 \cdot 10^{-7}$ |
| Fossil resource scarcity | FRS | kg oil eq | $6.21 \cdot 10^{-4}$ | $1.93 \cdot 10^{-4}$ | $3.91 \cdot 10^{-4}$ | $3.59 \cdot 10^{-5}$ |
| Water consumption | WC | $m^3$ | $1.41 \cdot 10^{-4}$ | $1.50 \cdot 10^{-5}$ | $3.33 \cdot 10^{-5}$ | $9.24 \cdot 10^{-5}$ |

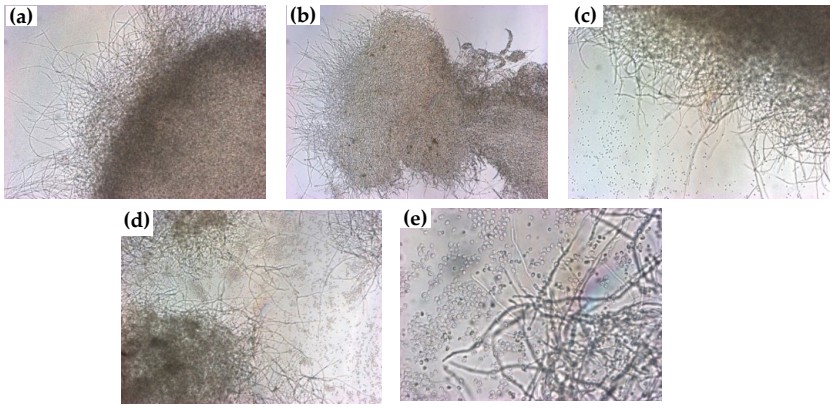



**Figure A3.1.** Microscopy images of fermentation samples taken from the 30 L reactor at: **(a)** day 0 (10x), **(b)** day 1 (10x), **(c)** day 2 (10x), **(d)** day 3 (10x), and **(e)** day 3 (40x).

# CHAPTER 4

## VALORISATION OF BIOETHANOL BY-PRODUCTS TO PRODUCE UNSPECIFIC PEROXYGENASE

Part of the results of this chapter have already been published as "González-Rodríguez S, Trueba-Santiso A, Lu-Chau TA, Moreira MT, Eibes G. Valorization of bioethanol by-products to produce unspecific peroxygenase with *Agrocybe aegerita*: Technological and proteomic perspectives. N Biotechnol. 2023; 76:63-71. https://doi.org/10.1016/j.nbt.2023.05.001".

Detailed information available at "Manuscripts considered in this thesis: rights, contributions and quality indicators", page 227.

**SUMMARY**

Unspecific peroxygenases (UPOs) (EC 1.11.2.1) represent an interesting subclass of oxidoreductases with peroxygenase and peroxidase activity. These characteristics allow their use as industrial biocatalysts on a wide range of applications. In this chapter, the biotechnological production of UPO by *Agrocybe aegerita* using bioethanol by-products as substrate is addressed. Solid-state and submerged fermentations (SSF and SmF) were evaluated, achieving the highest titers of UPO and laccase activities in SmF using vinasse as nutrient source. Optimal UPO production of 331 U/L was achieved in 50 % (v:v) vinasse medium with an inoculum grown for 14 d. These conditions were applied for the operation of a 4 L reactor, reaching an UPO activity of 265 U/L. Proteome expression before and after UPO production was analyzed by shotgun proteomic analysis to identify potential inducers of UPO expression. UPO and laccase, as well as proteins related to the production of reactive oxygen species (ROS) and the defense against excess of ROS were detected. Interestingly, metabolism of complex nitrogen sources was activated once UPO was expressed, supporting that media with high nitrogen content could act as inducers of UPO.

## 4.1. Introduction

### 4.1.1. Unspecific peroxygenase potential and the importance of medium composition on its expression

The discovery of UPO family is relatively recent, being described for the first time in the basidiomycetous fungus *Agrocybe aegerita* in 2004 (René Ullrich et al., 2004). Although it was initially cataloged as haloperoxidase or aromatic peroxygenase (APO) due to the partial similitude of its amino acid sequence with the heme chloroperoxidase (CPO) from the ascomycete *Caldariomyces fumago* and due to the hydroxylation activity found on halides and aromatic compounds, some years later this class of enzymes was renamed as unspecific peroxygenases (UPOs, E.C. 1.11.2) (Hofrichter et al., 2015; Hofrichter and Ullrich, 2006; René Ullrich et al., 2004). These hemo-thiolate enzymes are particularly interesting for their combination of the catalytic cycle of heme peroxidases with the ability to perform some nonspecific reactions commonly attributed to cytochromes P450 (Kinner et al., 2021). Nevertheless, in contrast to cytochromes P450, UPOs only need $H_2O_2$ as co-substrate, which act as both electron acceptor and oxygen donor (Linde et al., 2020; Ullrich and Hofrichter, 2005).

Since this peroxygenase is produced during secondary metabolism, its production requires a long time, from 2 to 4 weeks of cultivation (Kinner et al., 2021). The composition of the culture medium has a direct impact on the accomplishment of UPO production. Previous reports suggest that a high nitrogen content, which can be provided by plant-derived compounds such as soybean meal or soybean peptone, may trigger UPO expression (Gröbe et al., 2011; René Ullrich et al., 2004), although this relationship has not yet been elucidated. Therefore, it seems essential to investigate the effects of new substrates with high nitrogen content. Accordingly, the liquid side-stream (vinasse) from the bioethanol production process and the solid fraction, which mainly correspond to distillers's dried grains with solubles (DDGS), are interesting candidates as nitrogen source in the fermentation medium.

In addition, it is important to analyse the factors involved in UPO expression in order to identify the most suitable culture medium conditions to induce the expression of this enzyme. The physiological role of UPO in nature is still uncertain, different activities having been proposed, including metabolite synthesis, detoxification processes of lignin degradation and interaction with

antimicrobial peptides released by host plants (Hofrichter et al., 2015). Bormann et al. (2022) suggested that, in addition to external culture conditions, a type of fungal autoregulation may influence UPO synthesis and secretion.

### 4.1.2. Objectives of this chapter

The most studied fermentation media to produce UPO are based on high-value compounds such as soybean meal or glucose, so it is important to give more preference to fermentations that are developed from residues or low value compounds rich in C and/or N that will be used as substrates in the formulation of the culture medium.

This work proposes an alternative use of by-products from the bioethanol industry in the formulation of the fermentation media suitable for UPO production by *A. aegerita*, demonstrating that different types of fermentation (SSF and SmF) are optimal for fungal growth and UPO expression. In addition, due to the lack of information about the factors that induce the production of this enzyme, it is especially important to use techniques that allow to better understand the metabolic mechanisms that lead to the expression of the target enzyme. In this sense, proteomic analysis provides valuable information on the changes in proteome expression at different times of the fungal culture with the aim of relating other cellular activities to the induction of these enzymes.

## 4.2. Materials and methods

### 4.2.1. Materials

#### *4.2.1.1. Chemicals, raw material and microorganism*

$H_2O_2$, veratryl alcohol, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and $MgCl_2$ were purchased from Sigma-Aldrich, malt extract from Biolife italiana, $KH_2PO_4$ and ethylenediaminetetraacetic acid (EDTA) from Panreac, yeast extract from iNtRON Biotechnology, $MgSO_4{\cdot}7H_2O$ from Fluka and sodium dodecyl sulfate (SDS) from Invitrogen. Vinasse and DDGS were provided by Bioetanol Galicia S.A. (Teixeiro-Curtis, Spain). The vinasse was stored at -18 °C, whereas DDGS was stored at room temperature until use. The characterisation of DDGS kindly provided by Bioetanol Galicia S.A. is shown in Table 4.1.

**Table 4.1.** Composition of DDGS provided by Bioetanol Galicia S.A.*.

| | |
|---|---|
| **Starch and solubles (% owb**)** | 3.9 |
| **Ash (% owb**)** | 4.3 |
| **Crude fibre (% owb**)** | 7.4 |
| **Fat (% owb**)** | 12.3 |
| **Humidity (%)** | 9.9 |
| **Protein (% owb**)** | 27.7 |

*Personal communication.
**owb: on wet basis.

The fungus *A. aegerit*a, strain DSM 22459, was obtained from the German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig.

### 4.2.2. Fermentations

#### *4.2.2.1. Inoculum preparation*

For mycelium production in static cultures, three plugs of active mycelia were transferred from Petri dishes to Fernbach flasks with 100 mL of malt extract-based medium (malt extract 7.5 g/L, yeast extract 2 g/L, $KH_2PO_4$ 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 mg/L) and maintained at 28 °C for 14-28 d. After this step, the content of the Fernbach flasks was crushed and used as inoculum for the Erlenmeyer flasks.

#### *4.2.2.2. Solid state fermentation at flask scale*

DDGS was used as support and substrate for SSF of *A. aegerita*. The DDGS pellets were crashed using a pestle to increase the surface area of the substrate. Each 100 mL Erlenmeyer flask containing 3 g of DDGS and 7.5 mL of distilled water was sterilized for 20 min at 121 °C (RAYPA AES-75) and inoculated with 750 μL of inoculum. The Erlenmeyer flasks were incubated for 42 d at 28 °C in a humidity-saturated environment under static conditions. The moisture content was replenished weekly to ensure optimal humidity conditions for fungal growth. All experiments were conducted in duplicate.

For enzyme extraction, 30 mL of distilled water was added to each Erlenmeyer flask and incubated for one hour at 175 rpm on an orbital shaker (C24 Incubator Shaker, New Brunswick Scientific) to promote separation of the

adsorbed enzyme on the DDGS. The extracted liquid was then filtered through a cellulose filter and centrifuged for 10 min at 10,000 rpm before measuring enzyme activity and pH in the supernatant.

#### *4.2.2.3. Submerged fermentation at flask scale*

Different dilutions of vinasse were evaluated as fermentation media: 25, 50 and 75 % (v:v) of vinasse. Next, the inoculum age was evaluated using fungal biomass grown in Fernbach flasks for 14, 21 and 28 d, respectively. Erlenmeyer flasks containing 112.5 mL of the final medium were autoclaved at 110 °C for 40 min and inoculated with 12.5 mL of inoculum. The Erlenmeyer flasks were incubated on an orbital shaker (Innova 4000 Incubation Shaker, New Brunswick Scientific) at 28 °C and 120 rpm for 40 d. Fermentations were carried out in triplicate at an initial pH of 5.5, that was adjusted by addition of 1 M NaOH. Periodically, 750 µL samples were withdrawn and centrifuged for 10 min at 13,500 rpm to remove fungal biomass. The supernatant was used to monitor culture pH, $H_2O_2$ concentration and to analyse UPO and laccase activities.

#### *4.2.2.4. Submerged fermentation at reactor scale*

The enzyme production was conducted in a 4 L stirred tank bioreactor (Biostat MD, B. Braun Biotech). The fungus grown in Erlenmeyer flasks for 3 weeks was used as inoculum at 20 % (v:v). The medium was prepared with a 50 % dilution of vinasse in distilled water (v:v) and adjusted to a pH of 5.5 with 1M NaOH. The conditions of medium sterilisation and inoculum percentage were selected after testing different sterilisation times (40-70 min) and temperatures (110-121 °C), selecting as optimal conditions 70 min of sterilisation under 121 °C. Shorter times and lower temperatures led to the contamination of the culture by yeasts, which probably came from the vinasse used for the formulation of the culture medium. In addition, a percentage of 20 % of inoculum was used in order to promote faster fungal growth and avoid contamination. The bioreactor was operated for 17 d at controlled temperature (25 ± 3 °C), under mechanical agitation (100-120 rpm), air sparging (2 L/min) and pH monitoring.

### 4.2.3. Protein identification and shotgun proteomic analysis

Biomass sampled from Erlenmeyer flasks of SmF before and after UPO activity was detected (7 and 16 d of fermentation, respectively) was

lyophilised. Each extraction was performed in triplicate using aliquots of 100 mg dry biomass, that were finally pooled for MS analyses. To obtain the total proteome fractions, 1.5 mL of extraction buffer containing 1.5 % SDS, 10 mM EDTA, 20 mM $MgCl_2$ in 50 mM Tris buffer was added to the dry biomass that was further digested at 90 °C for 20 minutes, cooling the samples each 5 minutes. Next, zirconium glass beads were added to the sample and cell disruption was performed in a bead beater for a total of 15 minutes (alternating 3 min beating and 1 min in ice). Samples were then centrifuged at 5000 g, 4 °C for 20 min. Supernatants were collected, proteins were precipitated with two consecutive steps of cold acetone (approximately 4 volumes per sample volume) at -20 °C and resuspended in 1 mL resuspension buffer (molecular grade water with 1 M urea and 10 mM EDTA).

Aliquots of 0.3 μg of protein were processed in solution by trypsin digestion, reduction-alkylation and finally desalted using ZipTip-μC18 material (Merck Millipore). The peptide samples were injected onto a timsTOF Pro (Bruker) equipped with a nano-electrospray source (CaptiveSpray) and a tims-QTOF analyser. The chromatographic analyses were performed using a nanoELUTE chromatograph (Bruker) with a ReproSil C18 column (50 x 0,075 mm, 1.9 μm, 120 Å, Bruker). The nHPLC was configured with binary mobile phases that included solvent A (0.1 % formic acid in dd$H_2O$), and solvent B (0.1 % formic acid in acetonitrile). The analysis time was 20 minutes, in which B/A solvent ratio was gradually increased.

For MS acquisition, a CID fragmentation and a nanoESI positive ionization mode was used. PASEF-MSMS scan mode was applied for an acquisition range of 100-1700 m/z. MS/MS spectra was processed with by PEAKS Studio (Bioinformatics Solutions) software for protein identifications using a homemade database with all protein sequences available in NCBI for *A. aegerita* and *Cyclocybe aegerita* taxonomies. Amino acid sequences from those accessions corresponding to 'Unnamed protein' were manually blasted in NCBI BLASTP against all fungi kingdom and the protein description with the highest identity. For this analysis, the amino acid (AA) sequences of those peptides detected in the samples from day 7 and 16 of fermentation were analysed with Unipept desktop tool v.4.3. The label-free module from PEAKS Studio was used for protein semiquantification. The Spec value is based on peptide spectrum matches (PSM) and was used as indicator for the relative abundance of the proteins in each sample (Ma et al., 2003).

### 4.2.4. Analytical protocols

UPO activity was measured by monitoring the oxidation of 20 mM veratryl alcohol into veratraldehyde at 310 nm ($\varepsilon_{310}$ = 9.3/(M·cm)) in presence of 10 mM $H_2O_2$. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of product per minute. Laccase activity was determined following the protocol described in Chapter 3 (section 3.2.5). Changes in absorbance over time were monitored using a UV-visible spectrophotometer (Shimadzu UV-1800, Shimadzu). $H_2O_2$ was measured with semi-quantitative peroxide test strips (colorimetric, 0.5-25 mg $H_2O_2$/L, MQuant®, Merck).

AA profile was determined for a lyophilised dilution 50 % (v:v) of raw vinasse, a lyophilised sample at the beginning of the SmF and a lyophilized sample obtained at the end of the SmF. For this analysis, an acid hydrolysis of the proteins was carried out for 24 h at 110 °C using HCl 6 N. After protein hydrolysis was completed, the hydrolysate was diluted with distilled water and filtered through a 0.45 μm filter (Filter Lab). The derivatisation of standards and samples was carried out according to Gálvez et al. (2019). The identification of AA was done through HPLC (Alliance 2695 model, Waters, Milford, MA, USA), using a scanning fluorescence detector (model 2475, Waters) according to Munekata et al (2020). The quantification was done using the external standard technique with AA standard (Amino Acid Standard H, Thermo). The results are expressed as mg per 100 g of sample.

### 4.2.5. Life Cycle Assessment

The Life Cycle Assessment (LCA) methodology was used to evaluate the environmental impacts associated with the production of UPO by *A. aegerita*. The operation data of the 4 L reactor were extrapolated to the operation of a 30 L reactor, considering an identical type of fermenter as the one used to scale-up MnP production in Chapter 3. The reason behind the selection of a larger reactor volume is that the data used for process modeling should come as much as possible from semi-pilot or demonstration scale bioreactors as a first choice. The data associated with the energy and resource consumptions of a process at small scale may be distorted by the large influence of energy on the final environmental impact results. The rationale for this methodology is based on ISO 14040:2006.

Accordingly, the goal and scope of the assessment were defined as the analysis of the environmental feasibility of UPO production in SmF by *A.*

*aegerita* from a "cradle-to-gate" approach. The same considerations assumed for the LCA of MnP production explained in Chapter 3 (section 3.2.6) were taken, including the allocation of systems. The functional unit (FU) selected for the environmental assessment was one unit of UPO. The life cycle inventory to perform the analysis is compiled in Table A4.1, assuming that the bioreactor is operated for 16 d (time required to achieve maximum UPO production). The methodology selected to perform the environmental assessment was the ReCiPe 2016 hierarchist MidPoint method V1.06 World (2010), which includes several mid-point impact indicators, among which the same ones evaluated for the production of MnP in Chapter 3 were considered.

The environmental impacts of each stage were evaluated, as well as the impacts associated with the production process of the concentrated enzyme cocktail. The environmental loads represented in the figures are expressed in terms of characterisation percentages.

For the downstream stage, recovery percentages after Amicon filtration of enzyme cocktail produced at flask scale by *A. aegerita* were employed, as no concentration of the enzyme cocktail for fermentation by *A. aegerita* was performed for the 4 L reactor. All other data were estimated from downstream process of MnP production (Chapter 3).

### 4.2.6. Statistical analysis

In order to determine whether there were significant differences between the maximum UPO production values in SmF, a one-way analysis of variance (ANOVA) was performed using the software R software (version 4.0.5, R Core Team, 2021). A significance level ($\alpha$) of 0.05 was considered for all the statistical analyses. The results from the statistical analysis are shown in Figure A4.1.

## 4.3. Results and discussion

### 4.3.1. Solid state fermentation at flask scale

DDGS was used as the sole substrate and support for the growth of *A. aegerita*, achieving complete colonisation of the substrate in the Erlenmeyer flask after 14 d (Figure 4.1), indicating that DDGS is a suitable substrate for *A. aegerita* growth without the need for external nutrient supplementation. Other substrates used for *A. aegerita* culture under solid-state conditions included agricultural lignocellulosic wastes such as wheat straw and cotton waste

(Philippoussis et al., 2001), kiwi fruit peels and peanut shells (Vipotnik et al., 2021) or orange peels and distillery grape stalks (Nicolini et al., 1993).

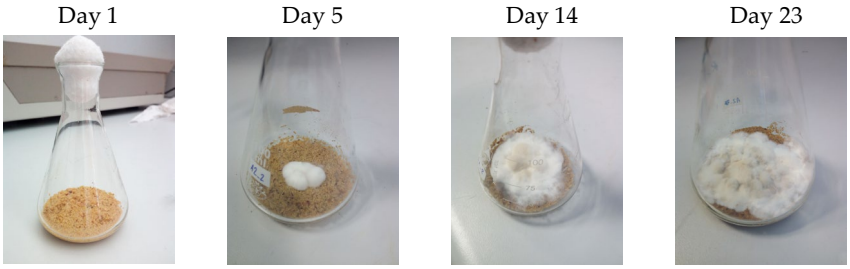



**Figure 4.1.** Growth of *A. aegerita* in SSF with DDGS as substrate.

However, it is important to note that fungal growth is not always indicative of peroxygenase production by *A. aegerita*, as previously reported by Pecyna et al. (2009). In DDGS medium, laccase and UPO were produced in SSF (Figure 4.2). Laccase was expressed earlier than UPO, reaching its maximum activity on day 21 (10.2 ± 1.6 U/g DDGS). It has been previously reported that nitrogen rich materials, with high protein content, can stimulate laccase production (Mishra and Kumar, 2007). These values agree with those reported by Vipotnik et al. (2022) who reached a maximum laccase activity of 11.31 U/g in the third week of *A. aegerita* cultures. In contrast, in the present study UPO activity was first detected 14 d after the onset of laccase production, once laccase activity began to decline. Maximum UPO activity titers (2.4 ± 0.2 U/g DDGS) were reached after 33 d of fermentation. The pH showed a gradual increase to reach a value of 7.6 at 20 d of fermentation, which was maintained thereafter and finally decreased to pH 7.2 at the end of the culture. To our knowledge, only two previous studies evaluated UPO production under SSF (Liers et al., 2011; Reina et al., 2017). In these works, a maximum value of 1.2 U/g was obtained by *A. aegerita* growing on barley medium supplemented with dried olive-mill residue (Reina et al., 2017), while UPO activities below 0.4 U/g were reached using different media and/or with different strains. Therefore, DDGS proved to be an efficient substrate for laccase and UPO production, with UPO production being the highest reported to date in SSF.

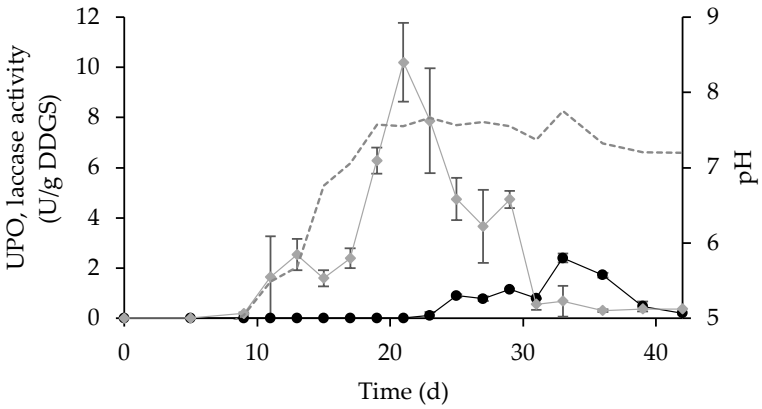



**Figure 4.2.** Parameters measured during SSF with DDGS: UPO activity (•), laccase activity (♦) and pH (--).

### 4.3.2. Submerged fermentation at flask scale

Different dilutions of vinasse were evaluated in order to establish their optimal concentration to maximize UPO production in SmF. In addition, the influence of inoculum age was evaluated, as it may affect the maximum enzyme activity and the time of peak production (Rola et al., 2013).

Concentrations between 25 % and 75 % v:v (vinasse:$H_2O$) were evaluated as fermentation medium. The objective was to study whether any of the compounds present in the substrate could have an inhibitory effect on fungal growth and/or enzyme production. Figure 4.3 shows the evolution of enzyme production during 38 d of fermentation. UPO activity (Figure 4.3-a) seems to be affected by the vinasse concentration of the medium, with the highest production titres in 50 % (v:v) assay (331 ± 229 U/L) on day 23. The medium with 25 % (v:v) of vinasse showed the lowest UPO production (47 ± 4 U/L), which can be related to a protein deficit, since previous studies reported that high concentrations of organic nitrogen were required for UPO production by *A. aegerita* (Gröbe et al., 2011; René Ullrich et al., 2004). In this sense, Ullrich et al. (2004) observed that a 2-fold increase in N source concentration (soybean meal) led to an increase of about 40 % in UPO production. Nevertheless, they also showed that UPO production highly depends on the strain of *A. aegerita* employed, since by evaluating eight different strains reached activities ranging between 5 and 1336 U/L, employing the same fermentation medium (René Ullrich et al., 2004).

It is noteworthy that the time required to obtain maximum production (19 days) in 25 % (v:v) was shorter than in the other dilutions tested. In the case of the 75 % (v:v) experiment, the maximum UPO production of 167 ± 191 U/L was reached on day 32. The large difference between the replicate values can be expected given the long fermentation periods. Previous works where fermentation times exceed 30 d have also shown significant differences between replicates (Reina et al., 2017).

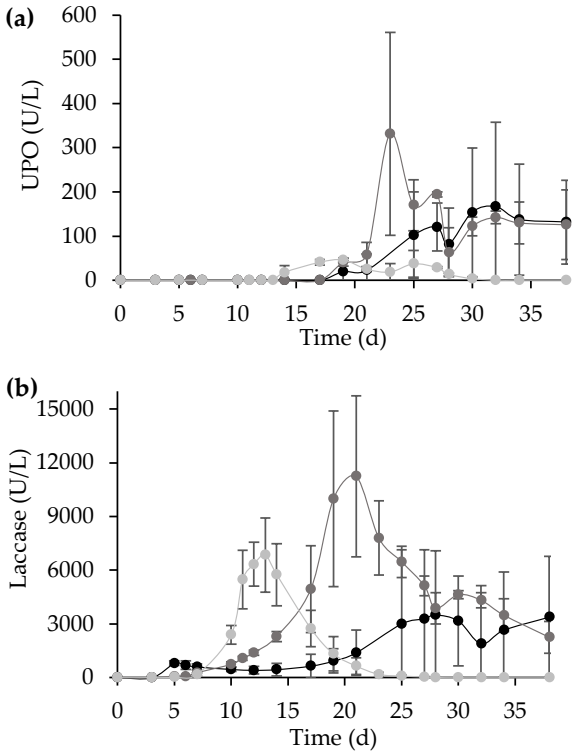



**Figure 4.3.** Evolution of **(a)** UPO and **(b)** laccase activity in the SmF with 25 % (•), 50 % (•) and 75 % (•) vinasse medium.

With reference to laccase production (Figure 4.3-b), a similar trend was observed. The time required to reach maximum activity increased with vinasse concentration. The highest production was obtained in 50 % (v:v) medium, reaching 11,252 ± 4501 U/L on day 21. This activity was much higher than those previously achieved in our laboratory also using vinasse but with another basidiomycetous fungus (Pena et al., 2012) and with different

substrates such as a residual stream from the organosolv process (García-Torreiro et al., 2018). Production reached in 25 and 75 % (v:v) media were also significant (6848 ± 2062 and 3499 ± 3574 U/L on days 13 and 28, respectively), but notably lower than that reached in the 50 % (v:v) medium. The high laccase activity can also be consequence of the residual ethanol remaining in the vinasse, which has been suggested to act as inducer of laccase production (Manavalan et al., 2013). In this regard, Hernández et al. (2015) reported that ethanol only functions as an inducer of laccase when simple nitrogen sources are not available in the culture medium, but in the presence of complex nitrogen sources, such as those present in vinasse, ethanol can have a positive synergistic effect on laccase production. Other studies focusing on UPO production with different strains of *A. aegerita* in conventional medium have reported higher UPO expression (2021 U/L) but lower laccase production, supporting the hypothesis of laccase induction by ethanol from vinasse (René Ullrich et al., 2004).

As for the pH trend (Figure A4.2), UPO production started at pH values close to 8, with a maximum activity around pH 8.5. This pH profile is similar to that reported by Ullrich et al. (2004) in fermentations with *A. aegerita* in soybean meal-based medium.

After optimising the percentage of stillage used to maximise UPO production, the effect of inoculum age on UPO production was studied. Inoculum age can play a crucial role in fungal metabolic activity and growth (Shah et al., 2014). In order to evaluate its potential effect on UPO expression, inocula from 2 to 4 weeks were used to cultivate *A. aegerita* under submerged conditions. Additionally, $H_2O_2$ concentration was monitored since its production may be related to UPO secretion.

As depicted in Figure 4.4 and Figure 4.5, inoculum age seems not to have a very strong effect on the onset of UPO and laccase production in the vinasse based medium.

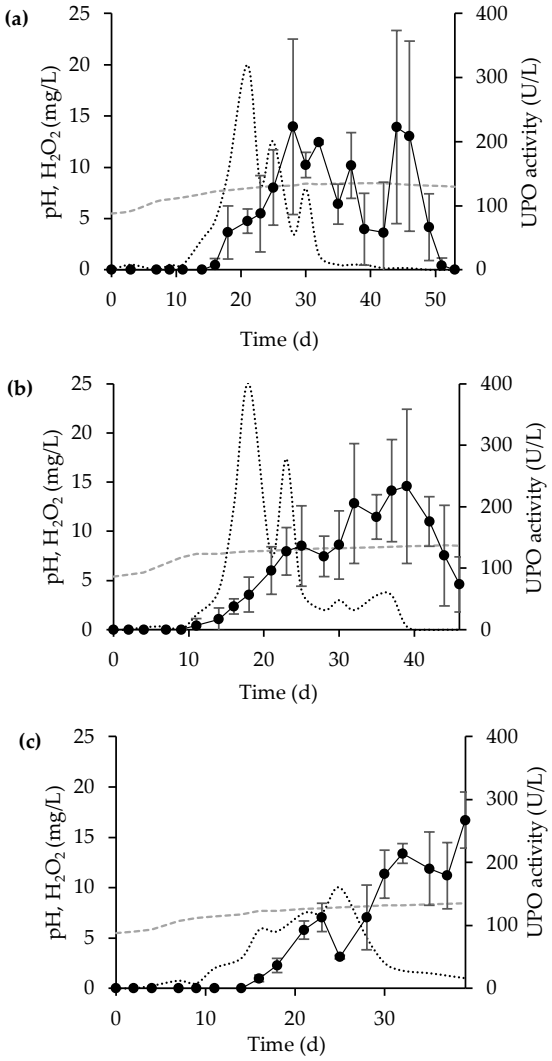



**Figure 4.4.** Evolution of UPO activity (•), pH (--) and $H_2O_2$ (··) in SmF in vinasse-based medium 50 % v:v with inocula of different age: **(a)** 14 days, **(b)** 21 days and **(c)** 28 days.

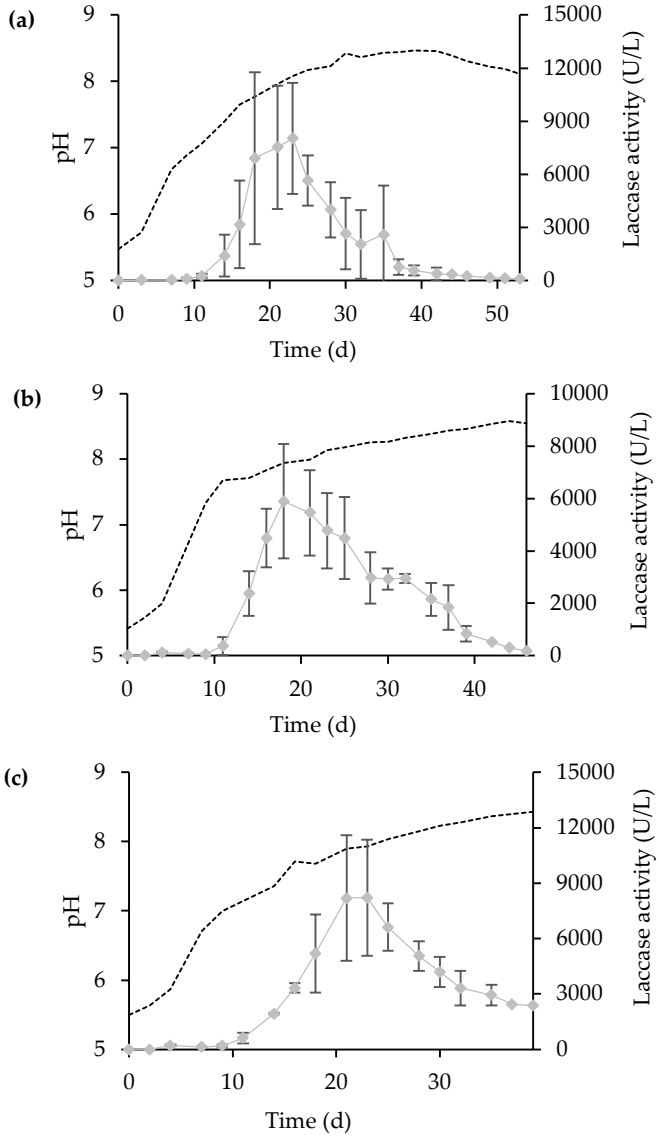



**Figure 4.5.** Evolution of laccase activity (♦) and pH (--) in SmF in vinasse-based medium 50 % v:v employing inocula of different age: **(a)** 14 days, **(b)** 21 days and **(c)** 28 days.

As observed in SSF, laccase production in SmF (Figure 4.5) started earlier in the culture (day 3), whereas UPO production (Figure 4.4) started between days 11 and 16. Although production values using inocula of different ages do not show significant differences (statistical analysis in Figure A4.1, p-value 0.88) the time required to reach maximum UPO production was shorter in the fermentation with 14-day-old inoculum, where a production of 223 ± 137 U/L was obtained on day 28. In terms of laccase activity, younger and older inocula led to similar titers (8030 ± 3124 and 8219 ± 3148 U/L on day 23, respectively). On the other hand, the intermediate-aged inoculum required a shorter period to obtain the highest laccase activity (18 d), but only an activity of 5899 ± 2176 U/L was achieved.

The presence of $H_2O_2$ was detected before the onset of UPO production (Figure 4.4), increasing to a maximum before the peak of UPO production. This trend in the concentration is probably related to the consumption of this compound by UPO and/or other enzymes (e.g. catalases) in different fungal metabolic pathways, leading to a decrease in the concentration of available $H_2O_2$ as enzymatic production increases. In fact, the maximum levels of UPO coincided with the minimum concentration of $H_2O_2$, suggesting that enzyme production could be affected by the lack of $H_2O_2$ required for its catalytic cycle.

Inactivation of UPO due to high $H_2O_2$ concentration could be ruled out because a maximum concentration of 0.74 mM (25 mg/L) was detected, which is lower than the concentration reported by Karich et al. (2016) to completely inactivate UPO from *A. aegerita* at pH 7. On the other hand, $H_2O_2$ concentration could affect laccase activity, as 1 mM hydrogen peroxide was reported to inactivate 76 % of laccase from *Pleurotus pulmonarius* (Marques De Souza and Peralta, 2003).

### 4.3.3. Scale-up of submerged fermentation

The SmF with the medium based on a dilution of 50 % v:v of vinasse was successfully scaled-up to a total volume of 4 L. After several episodes of yeast contamination, probably from the stillage, medium sterilisation was intensified, and inoculum concentration doubled to reduce the possibility of contamination. Fermentation was monitored for 17 d. Both enzymes were expressed, obtaining maximum values of 265 U/L for UPO on day 16 of fermentation, and 9639 U/L for laccase on day 11 (Figure 4.6). As observed in

the Erlenmeyer flask cultures, pH increased throughout the fermentation, with the greatest increase coinciding with the maximum UPO activities. The maximum UPO activity was obtained at pH 8.6, which was similar to the pH reached in the Erlenmeyer scale SmF (8.4), so pH could be used as an optimal indicator of the enzymatic production stage.

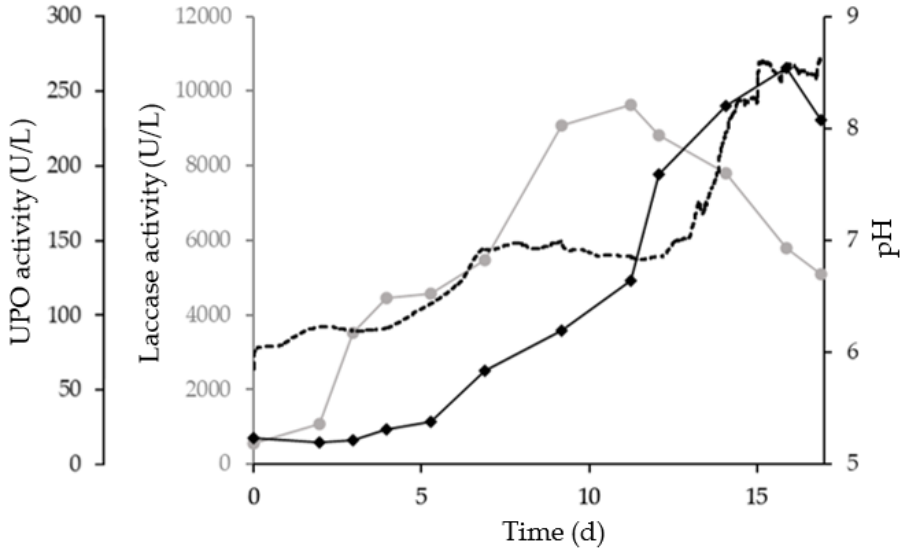



**Figure 4.6.** Evolution of pH (- -), UPO activity (♦) and laccase activity (•) during the fermentation in a 4 L bioreactor.

The UPO and laccase activity values achieved in the reactor were similar to those obtained with Erlenmeyer flasks. Therefore, mechanical stress due to agitation, which affects fungal morphology by causing pellet breakage, does not seem to affect enzyme production (Jafari et al., 2007; Lopez-Ramirez et al., 2018). Interestingly, these maximum titers were achieved in shorter time than at Erlenmeyer flask scale, which could be related to the higher inoculum concentration or to the more efficient aeration system enhancing mass transfer in the bioreactors (Lú-Chau et al., 2018).

UPO production from *A. aegerita* achieved by Ullrich et al. (2004) was higher and required shorter fermentation periods (maximum UPO activity of 1550 U/L, about 10 mg/L, at day 10 in a 5 L bioreactor), but the laccase production was significantly lower, reaching only 290 U/L compared to 9639 U/L achieved with a vinasse-based medium.

Heterologous expression has allowed higher UPO production values. For example, Tonin et al. (2021) reached a maximum UPO production of 300 mg/L after 7 d (activity of 30,000 U/L by ABTS assay) using *Pichia pastoris* as host microorganism. This suggests that heterologous expression seems the most suitable option to produce UPO. However, it should be noted that obtaining enzyme cocktails with a predominance of several enzyme activities, as laccase and UPO in this work, can result in a synergistic effect when applying these cocktails as biocatalysts in different reactions.

Furthermore, homologous expression may be useful in the study of the natural functions of UPO, which remain unclear. The elucidation of its role in nature is not merely a scientific question but is also important for biotechnological development and ecophysiological considerations of the enzyme (Bormann et al., 2022).

### 4.3.4. Identification of enzyme cocktail proteins

To explore the enzymatic set expressed by the fungus and its cellular activities in different moments of the biotechnological process, shotgun proteomic analysis was performed at two different periods of *A. aegerita* growth in Erlenmeyer flasks: on day 7 of the culture (no UPO activity had yet been detected) and on day 16, when UPO activity was spectrophotometrically confirmed in the supernatant. A total of 328 proteins were identified in this study (after considering only those with a minimum of 2 peptides detected). Table 4.2 summarises the selected identified proteins. The AA sequences from the detected peptides were analysed by Unipept and grouped into Interpro protein categories. Those categories activated, suppressed, or upregulated at day 16 compared to day 7 are shown in Figure A4.3.

**Table 4.2.** List of selected proteins identified in the shotgun proteomic analysis of Erlenmeyer SmF of *A. aegerita* grown with vinasse and their relative abundances on the proteome before and after UPO activity was spectrophotometrically confirmed.

| NCBI Accession | Description | No UPO activity in the medium | | | UPO activity in the medium | | |
|---|---|---|---|---|---|---|---|
| | | Spec | NPT | NPU | Spec | NPT | NPU |
| CAA7262553.1 | Catalase | 43 | 21 | 21 | 25 | 14 | 14 |
| CAA7263457.1 | SOD | 10 | 7 | 6 | 10 | 8 | 0 |
| pdb\|2YP1\|B | UPO | 0 | 0 | 0 | 13 | 8 | 0 |
| CAA7265189.1 | Laccase | 0 | 0 | 0 | 11 | 5 | 0 |
| CAA7270351.1 | Trx-DP | 0 | 0 | 0 | 7 | 5 | 0 |
| CAA7269993.1 | Lectin | 2 | 2 | 2 | 5 | 3 | 0 |
| CAA7266156.1 | SOD | 3 | 3 | 2 | 2 | 2 | 0 |
| CAA7268902.1 | Lectin | 2 | 2 | 2 | 2 | 2 | 0 |
| CAA7262403.1 | UPO | 0 | 0 | 0 | 2 | 2 | 0 |
| CAA7264511.1 | Lectin | 2 | 2 | 2 | 0 | 0 | 0 |
| CAA7264490.1 | DyP | 2 | 2 | 2 | 0 | 0 | 0 |
| CAA7260134.1 | Peptidase | 5 | 4 | 4 | 7 | 4 | 0 |
| CAA7264546.1 | Peptidase | 3 | 1 | 1 | 8 | 2 | 0 |
| CAA7265101.1 | Peptidase | 2 | 2 | 2 | 3 | 2 | 0 |
| CAA7266636.1 | Peptidase | 1 | 1 | 1 | 4 | 2 | 0 |
| CAA7265216.1 | Peptidase | 0 | 0 | 0 | 2 | 2 | 0 |
| CAA7264859.1 | Oxidase | 0 | 0 | 0 | 2 | 0 | 2 |
| CAA7260317.1 | PS | 0 | 0 | 0 | 9 | 5 | 0 |

**NPT:** number of total peptides identified in the sample and corresponding to that protein; **NPU:** number of unique peptides (i.e. high-confidence supporting peptides) that are mapped to only one protein group identified in the sample and corresponding to this protein; **SOD:** superoxidase dismutase; **Trx-DP:** thioredoxin dependent peroxidase; **PS:** phosphate shynthase.

First, shotgun proteomics served to corroborate the production of unspecific peroxygenases (accession numbers: pdb|2YP1|B and CAA7262403.1) and laccase (CAA7265189.1) by *A. aegerita*. According to the specific activities measured on site, the expression of unspecific peroxygenases was detected at day 16 whereas at day 7 no UPO activity was detected. Laccase activity was already detected on day 7 of fermentation, although the values were lower than those obtained on day 16. Interestingly, also a DyP-type peroxidase (CAA7264490.1) was found on day 6, which is known to reduce $H_2O_2$ to water and oxidize phenolic compounds as well as non-phenolic lignin model dimers and veratryl alcohol (Mattila et al., 2022).

A catalase (CAA7262553.1), responsible for the degradation of $H_2O_2$ to $H_2O$ and $O_2$, was detected and its expression was negatively correlated with both UPO expression and the $H_2O_2$ concentration in the medium. It was found to be more abundant before the UPO activity was detected (when the $H_2O_2$ concentration had not yet been detected) than in the second period ($H_2O_2$ concentration detected). This observation might suggest that UPO and catalase follow different induction patterns. Nevertheless, at stage in which catalase was detected, the production of laccase has already started. According to Romanholo Ferreira et al. (2020), catalase can be expressed as response to the presence of recalcitrant coloured compounds derived from vinasse degradation and also to the degraded products produced by ligninolytic enzymes, such as laccase.

NADP-dependent oxidoreductases were overexpressed when UPO was active. These enzymes are involved in providing reducing power for the decomposition of ROS species. Furthermore, two superoxide dismutases (CAA7266156.1 and CAA7263457.1) involved in the intracellular ROS control (Mattila et al., 2022) catalysing the reduction of superoxide ($O^{-2}$) or molecular oxygen ($O_2$) into intracellular hydrogen peroxide ($H_2O_2$), were also detected. However, in this case no significant change in their expression was found among the two conditions studied, suggesting they expression was not related to UPO activity.

Regarding Interpro protein categories: thioredoxin-like family (which contains UPO) was overexpressed at day 16, according to the results abovementioned. Interestingly, other proteins involved in ROS defense were only found at day 16, when UPO activity was detected. Among them, alkyl hydroperoxide reductases, cupredoxins, peroxiredoxins and redoxins were detected. Peroxiredoxins are thiol-dependent redox enzymes responsible for quenching of intracellular ROS and building up the effective cellular defense system in living organisms against oxidative stress. The expression of these enzymes is induced by stressful conditions resulting from the presence of ROS from vinasse degradation, coinciding with the described by Romanholo Ferreira et al. (2020).

Other interesting finding was the presence of lectins at the time in which UPO production starts (sp|Q6WY08.1|ATLE_AGRAE and CAA7264511.1). This type of proteins is proposed as protein reserve in mushrooms and also as a defense mechanism (Sun et al., 2003). Moreover, in the last years these

proteins have been increasingly studied due to its antitumour and antimicrobial activity (Heim et al., 2015; Yang et al., 2009).

Previous literature reports have shown the relevance of nutrient concentrations in the environment to activate UPO production (Gröbe et al., 2011; René Ullrich et al., 2004). Interestingly, those enzymes involved in the transformation of complex sugars, such as beta-galactosidase and glycoside hydrolase were only activated at day 16 (when UPO was active). Pyruvate kinase and ATP synthase activities were, however, suppressed, indicating that glycolysis and growth may be compromised at this stage, which could be attributed to total reducing sugars depletion in the fermentation medium.

In addition, different proteins involved in the metabolism of nitrogen compounds, such as those belonging to the Interpro protein categories arginase (IPR014033) and ureohydrolase (IPR006035; IPR023696; IPR020855) were active only in the second condition (when UPO is active). This suggests that complex nitrogen compounds present in vinasse are decomposed into more simple molecules like urea or ammonia, that can be responsible of the higher pH value of the medium in the stage where UPO is produced. It is remarkable that arginine, the substrate for arginase, is the amino acid with the highest nitrogen content (32 %). Results from AA profiling of fermentation samples from SmF (Figure 4.7) showed a decrease of 71.8 % on the arginine content at the end of the experiment.

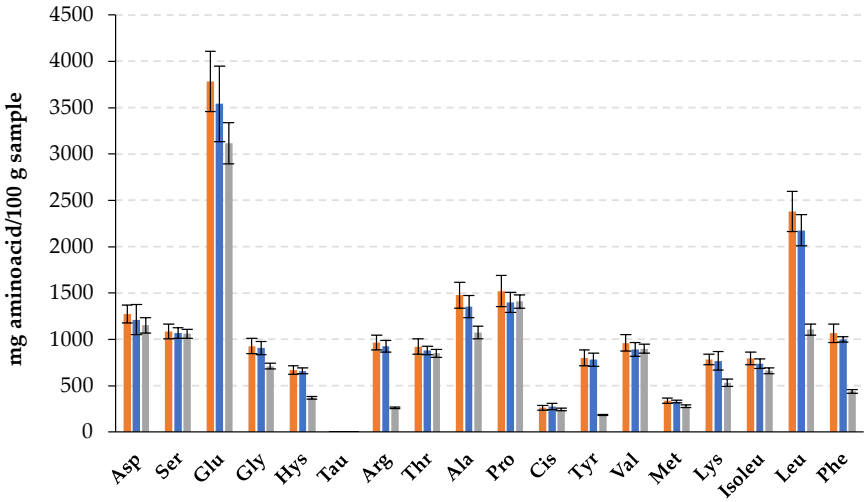



**Figure 4.7.** AA content of initial and final samples of SmF of *A. aegerita* with vinasse based medium (blue and grey, respectively). In orange AA content of raw vinasse. **Asp:** aspartic acid, **Ser:** serine, **Glu:** glutamic acid, **Gly:** glycine; **Hys:** histidine, **Tau:** taurine, **Arg:** arginine, **Thr:** threonine, **Ala:** alanine, **Pro:** proline, **Cis:** cysteine, **Tyr:** tyrosine, **Val:** valine, **Met:** methionine, **Lys:** lysine, **Isoleu:** isoleucine, **Leu:** leucine, **Phe:** phenylalanine.

Other enzymes related to nitrogen metabolism and overexpressed after UPO production are peptidases (CAA7260134.1, CAA7264546.1, CAA7265101.1, CAA7266636.1 and CAA7265216.1) which interact with AA residues for proteolysis (Neto et al., 2018). This could be related to the reduction of amino acids other than arginine. Specifically, peptidases CAA7264546.1 and CAA7265101.1 use proteins with leucine as AA residue as substrate, so they are probably involved in the decline of leucine content (49.1 %) at the end of the fermentation in submerged conditions (Figure 4.7).

Compounds resulting from the proteolysis carried out by peptidases include short-chain peptides, which can act as inhibitors of the fungal growth (Mahindra et al., 2014). Short-chain peptides from the metabolism of complex nitrogen compounds could induce UPO expression. In fact, Pecyna (2015) observed that short-chain peptides from storage proteins of soybean induced UPO production. The author hypothesised that UPO production in ligninolytic fungi might play a role in the chemical warfare among fungi and

plants in real environmental conditions, acting as a response to antimicrobial peptides released by plants preventing from a fungal attack.

Finally, the presence of peroxisomal copper amine oxidase (CAA7264859.1) indicates that the metabolism of primary or secondary amines was also activated during UPO production stage. These enzymes can convert amino acids to keto-acids, with the subsequent release of ammonium and $H_2O_2$. Therefore, these enzymes can be also producers of $H_2O_2$ required for UPO and other enzymes.

The overexpression of the enzyme myo-inositol-1-phosphate synthase (CAA7260317.1) also supports the activation of metabolic pathways focused on the degradation of nitrogen compounds. Apart from their role in fungus growth, these enzymes are also secreted to confer resistance to abiotic stress as high concentration of $H_2O_2$ or NO (Tan et al., 2013), being this last compound produced in the metabolism of complex nitrogen compounds.

Based on the results obtained, the metabolic pathways related to the glycolysis process that employ simpler sugars were suppressed in the second period, when UPO was active, whereas different enzymes involved in the degradation of more complex sugars were overexpressed at this stage. In addition, the metabolism of complex nitrogen sources was activated at this stage, where a variety of enzymes responsible for the metabolism of these compounds (ureohydrolases and peptidases) were detected. Previous literature reports have shown by operational approaches the relevance of nutrient concentrations in the environment to activate UPO production (Gröbe et al., 2011; René Ullrich et al., 2004). Data obtained in this proteomic analysis is in line with previous hypotheses pointing that the metabolism of complex sugars and complex nitrogen compounds could be related to the expression of UPO.

Moreover, it is worth noting the presence of different enzymes related to ROS production and defense. The production of ROS in basidiomyceta fungi is related to different stress agents, such as mechanical damage and starvation (Gessler et al., 2007), whereas the presence of enzymes related to ROS defense is part of the strategy of fungi to maintain its homeostasis in response to the oxidative stress during vinasse degradation (Romanholo Ferreira et al., 2020).

However, these first biochemical evidence deserves further research efforts to reach conclusive theories. Specific time courses allowing the collection of

more proteome samples at different timepoints may help to infer the mechanism for UPO expression.

### 4.3.5. Environmental profile associated to enzyme production

The LCA methodology was used to evaluate the impacts associated with the different components included in the life cycle inventory. The environmental impacts were evaluated for the categories of GW, IR, TE, FET, MET, HN-CT and FRS because they are the most representative categories of the environmental impact of the process.

The impacts associated with the different stages of the UPO production by *A. aegerita* in 30 L reactor are shown in Figure 4.8. Stage II (fermentation) was responsible for the highest proportion of impacts for all categories studied, with 67-69 % of contribution to total impacts. Table A4.2 shows the midpoint values per category and process.

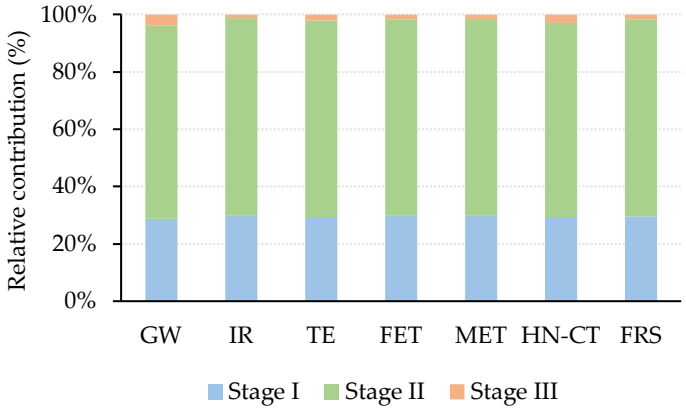



**Figure 4.8.** Environmental impact contributions per process stage in UPO production with *A. aegerita* in 30 L reactor.

Figure 4.9 shows the categorised contributions in the different stages in order to identify the relevant process in the environmental performance. In general, electricity was found to be the main hotspot at all stages of the UPO production process with *A. aegerita*.

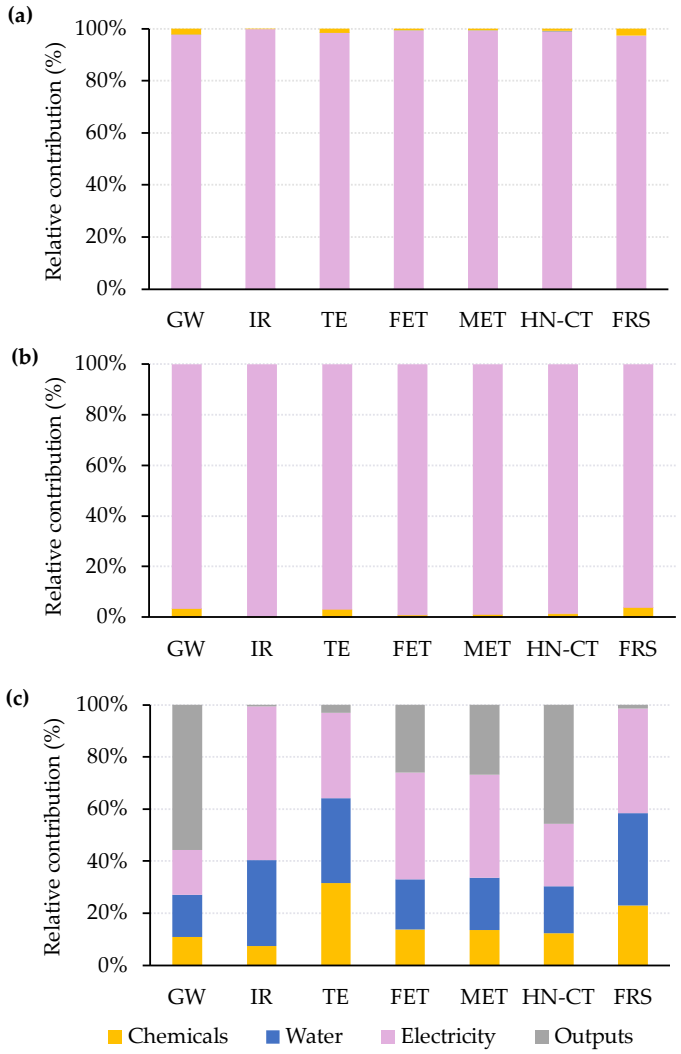



**Figure 4.9.** Environmental impact contributions in the different stages UPO production with *A. aegerita* in 30 L reactor: **(a)** Stage I- Inoculum preparation, **(b)** Stage II- Fermentation and **(c)** Stage III- Downstream.

In stages I and II, the energy consumption caused the highest impacts in all the categories studied. This was expected in view of the need to use different equipment to maintain the optimal conditions for inoculum growth, as well as the different steps of sterilisation of the medium necessary to avoid

unwanted contamination. Meanwhile, in stage III, the main contributors to environmental impacts are electricity and outputs, which include the fungal biomass removed in the filtration step and the wastewater obtained in the membrane cleaning process and discarded fraction in micro and ultrafiltration process. Energy consumption contributed the most to the impacts in IR, FET, MET and FRS categories due to the use of peristaltic pumps to circulate the enzyme cocktail during micro and ultrafiltration steps. In the case of GW and HN-CT categories, the outputs are the major contributors to the impact. It is also noteworthy that for TE category, the energy consumption, outputs generated, and chemical consumed contributed equally to the environmental impact. In the case of chemical use, this term refers to the use of an NaOH solution to clean the micro and ultrafiltration membranes.

Since electricity contributed the most to environmental impacts derived from UPO production with *A. aegerita* in 30 L reactor, this component was disaggregated in order to evaluate the equipment responsible of these impacts in stages I and II (Figure 4.10). The contribution of shaker energy consumption was the most relevant in stage I for all categories evaluated, because the growth of fungus in Erlenmeyer flask was the most time-consuming part (21 d in comparison with 14 d required for the growth in Fernbach and Petri dish) and required a continuous agitation. For stage II, the compressor was the equipment responsible for the majority of environmental impacts, representing 63.6 % of impact contribution for all categories studied, although only the energy required by the compressor to generate the air flow required for reactor operation (15 L/min required vs. 920 L/min of compressor capacity) was considered.

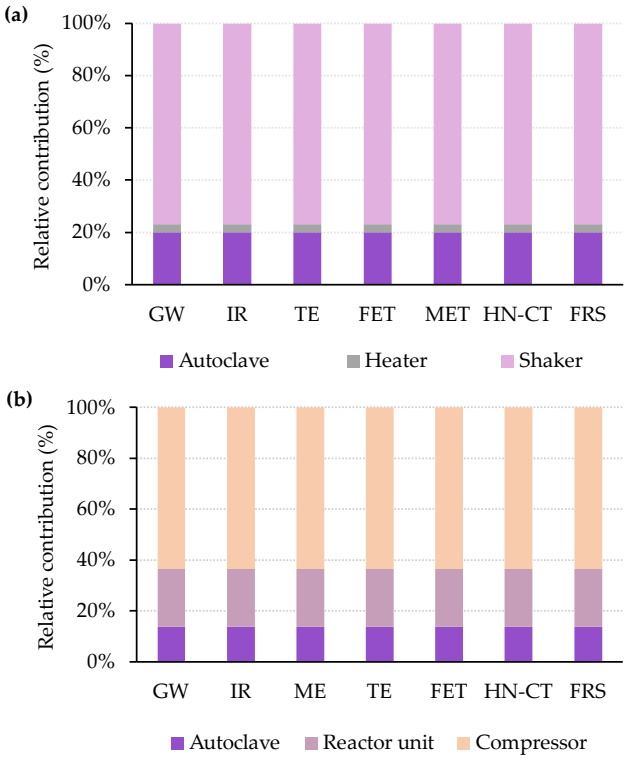



**Figure 4.10.** Environmental impact contributions of the energy consumption of each equipment in: **(a)** Stage I- Inoculum preparation and **(b)** Stage II- Fermentation.

Taking into account the long time required to produce UPO with *A. aegerita*, it is especially relevant to focus the effort in minimising energy consumption with the use of more efficient equipment. In this case, the use of a better agitation configuration should be considered, since the use of 20 % inoculum leads to a high concentration of biomass in the bioreactor, which hinders the correct medium homogenisation. This poor homogenisation of the medium can result in deficient oxygen and nutrients transfer, hence requiring higher air flow or increasing agitation speed. Moreover, the modification of air supply system can also entail an enhance of oxygen transfer. Wong (2013) reported the decrease of the mass transfer coefficient ($K_{La}$) in vinasse in relation with water, so it could be of interest the evaluation of the use of airlift or bubble column reactors. The stress generated by the bubbles on the fluid and the walls on the fluid is very low compared to that caused by the impeller

on the fluid in a stirred tank bioreactor, and this type of reactor consumes less energy than mechanically stirred reactors (Barker and Worgan, 1981).

## 4.4. Conclusions

Bioethanol synthesis residues are adequate substrates for *A. aegerita* growth and the production of an enzymatic cocktail with UPO and laccase activity in solid and submerged conditions due to their high nitrogen content. The highest titer of UPO activity was achieved in submerged fermentation, where the percentage of vinasse in the medium affected enzyme production. Proteomic analysis corroborated the presence of UPO, laccase, DyP, lectins, enzymes related to ROS production and control as well as changes in the metabolism of complex sugars and nitrogen sources during the submerged fermentation.

Finally, the environmental impact associated with the production of the enzyme cocktail at the 30 L reactor scale revealed that stage II (fermentation) contributed the most to the total environmental impact, due to the high energy consumption derived from the long time required to produce the enzyme.

## 4.5. Annexes Chapter 4

**Table A4.1.** Global process inventory for UPO production at 30 L bioreactor (considering as FU one unit of UPO).

| Inputs from Technosphere | | | | Outputs | | | |
|---|---|---|---|---|---|---|---|
| *Material/Electricity* | *Value* | *Unit* | *Comments and assumptions* | *Material* | *Value* | *Unit* | *Comments* |
| Agar | $2.65 \cdot 10^{-5}$ | g | Considered as generic organic chemical | Biowaste | $1.84 \cdot 10^{-2}$ | g | Petri dish |
| Glucose | $7.94 \cdot 10^{-5}$ | g | Considered as generic organic chemical | Polystyrene | $3.51 \cdot 10^{-3}$ | g | Petri dish |
| Malt extract | $1.21 \cdot 10^{-3}$ | g | Considered as generic inorganic chemical | | | | |
| $KH_2PO_4$ | $1.69 \cdot 10^{-4}$ | g | | | | | |
| $MgSO_4$ | $4.01 \cdot 10^{-5}$ | g | | | | | |
| $CaCl_2$ | $5.27 \cdot 10^{-7}$ | g | | | | | |
| **Stage I** Sawdust (*P. radiata*) | $1.06 \cdot 10^{-5}$ | g | | | | | |
| Yeast extract | $3.17 \cdot 10^{-4}$ | g | Considered as fodder yeast | | | | |
| Vinasse | $7.73 \cdot 10^{-4}$ | Kg | Considered as vinasse from sugar beet | | | | |
| NaOH | $9.13 \cdot 10^{-4}$ | g | | | | | |
| Polystyrene | $3.51 \cdot 10^{-3}$ | g | Petri dish | | | | |
| Distilled water | $8.78 \cdot 10^{-4}$ | $dm^3$ | Considered as deionized water | | | | |
| Electricity | $8.48 \cdot 10^{-3}$ | kWh | | | | | |
| Vinasse | $3.43 \cdot 10^{-3}$ | Kg | Considered as vinasse from sugar beet | | | | |
| **Stage II** NaOH | $4.00 \cdot 10^{-3}$ | g | | | | | |
| Distilled water | $3.17 \cdot 10^{-3}$ | $dm^3$ | Considered as deionized water | | | | |
| Electricity | $1.95 \cdot 10^{-2}$ | kWh | | | | | |
| NaOH | $4.23 \cdot 10^{-2}$ | g | | Biowaste | $3.35 \cdot 10^{-1}$ | g | Fungi biomass |
| **Stage III** Distilled water | $2.12 \cdot 10^{-3}$ | $dm^3$ | Considered as deionized water | Wastewater | $7.57 \cdot 10^{-3}$ | $dm^3$ | |
| Tap water | $2.23 \cdot 10^{-1}$ | $dm^3$ | | | | | |
| Electricity | $2.05 \cdot 10^{-4}$ | kWh | | | | | |

**Table A4.2.** Midpoint values per impact category and process stage.

| Impact category | Acronym | Unit | Total | Stage I | Stage II | Stage III |
|---|---|---|---|---|---|---|
| Global warming | GW | kg $CO_2$ eq | $1.17 \cdot 10^{-2}$ | $3.39 \cdot 10^{-3}$ | $7.89 \cdot 10^{-3}$ | $4.64 \cdot 10^{-4}$ |
| Stratospheric ozone depletion | SOD | kg CFC11 eq | $7.22 \cdot 10^{-9}$ | $2.02 \cdot 10^{-9}$ | $5.06 \cdot 10^{-9}$ | $1.43 \cdot 10^{-10}$ |
| Ionizing radiation | IR | kBq Co-60 eq | $5.96 \cdot 10^{-3}$ | $1.78 \cdot 10^{-3}$ | $4.11 \cdot 10^{-3}$ | $7.30 \cdot 10^{-5}$ |
| Ozone formation, Human health | OF | kg $NO_x$ eq | $2.19 \cdot 10^{-5}$ | $6.42 \cdot 10^{-6}$ | $1.50 \cdot 10^{-5}$ | $4.79 \cdot 10^{-7}$ |
| Fine particulate matter formation | FPM | kg PM2.5 eq | $1.85 \cdot 10^{-5}$ | $5.42 \cdot 10^{-6}$ | $1.26 \cdot 10^{-5}$ | $3.88 \cdot 10^{-7}$ |
| Ozone formation, Terrestrial ecosystems | OF | kg $NO_x$ eq | $2.21 \cdot 10^{-5}$ | $6.49 \cdot 10^{-6}$ | $1.51 \cdot 10^{-5}$ | $4.87 \cdot 10^{-7}$ |
| Terrestrial acidification | TA | kg $SO_2$ eq | $4.70 \cdot 10^{-5}$ | $1.38 \cdot 10^{-5}$ | $3.245 \cdot 10^{-5}$ | $8.73 \cdot 10^{-7}$ |
| Freshwater eutrophication | FE | kg P eq | $1.32 \cdot 10^{-5}$ | $3.62 \cdot 10^{-6}$ | $8.13 \cdot 10^{-6}$ | $1.41 \cdot 10^{-6}$ |
| Marine eutrophication | ME | kg N eq | $1.50 \cdot 10^{-6}$ | $3.02 \cdot 10^{-7}$ | $7.60 \cdot 10^{-7}$ | $4.38 \cdot 10^{-7}$ |
| Terrestrial ecotoxicity | TE | kg 1,4-DCB | $3.57 \cdot 10^{-2}$ | $1.05 \cdot 10^{-2}$ | $2.44 \cdot 10^{-2}$ | $7.64 \cdot 10^{-4}$ |
| Freshwater ecotoxicity | FET | kg 1,4-DCB | $1.25 \cdot 10^{-3}$ | $3.71 \cdot 10^{-4}$ | $8.56 \cdot 10^{-4}$ | $2.17 \cdot 10^{-5}$ |
| Marine ecotoxicity | MET | kg 1,4-DCB | $1.58 \cdot 10^{-3}$ | $4.69 \cdot 10^{-4}$ | $1.08 \cdot 10^{-3}$ | $2.86 \cdot 10^{-5}$ |
| Human carcinogenic toxicity | HCT | kg 1,4-DCB | $9.61 \cdot 10^{-4}$ | $2.68 \cdot 10^{-4}$ | $6.22 \cdot 10^{-4}$ | $7.11 \cdot 10^{-5}$ |
| Human non-carcinogenic toxicity | HN-CT | kg 1,4-DCB | $1.83 \cdot 10^{-2}$ | $5.39 \cdot 10^{-3}$ | $1.24 \cdot 10^{-2}$ | $5.38 \cdot 10^{-4}$ |
| Land use | LU | $m^2$a crop eq | $5.10 \cdot 10^{-4}$ | $1.41 \cdot 10^{-4}$ | $3.61 \cdot 10^{-4}$ | $7.95 \cdot 10^{-6}$ |
| Mineral resource scarcity | MRS | kg Cu eq | $3.01 \cdot 10^{-5}$ | $8.63 \cdot 10^{-6}$ | $2.02 \cdot 10^{-5}$ | $1.306 \cdot 10^{-6}$ |
| Fossil resource scarcity | FRS | kg oil eq | $3.15 \cdot 10^{-3}$ | $9.31 \cdot 10^{-4}$ | $2.17 \cdot 10^{-3}$ | $5.46 \cdot 10^{-5}$ |
| Water consumption | WC | $m^3$ | $4.60 \cdot 10^{-4}$ | $7.06 \cdot 10^{-5}$ | $1.67 \cdot 10^{-4}$ | $2.22 \cdot 10^{-4}$ |

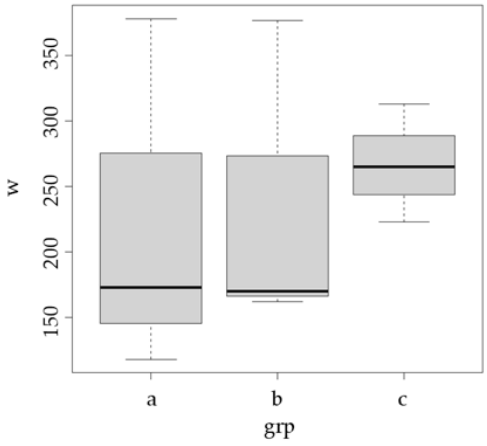



**Figure A4.1.** Statistical analysis (test ANOVA, significance level of 0.05) of maximum UPO production values in SmF with inocula with **(a)** 14 , **(b)** 21 and **(c)** 28 days.

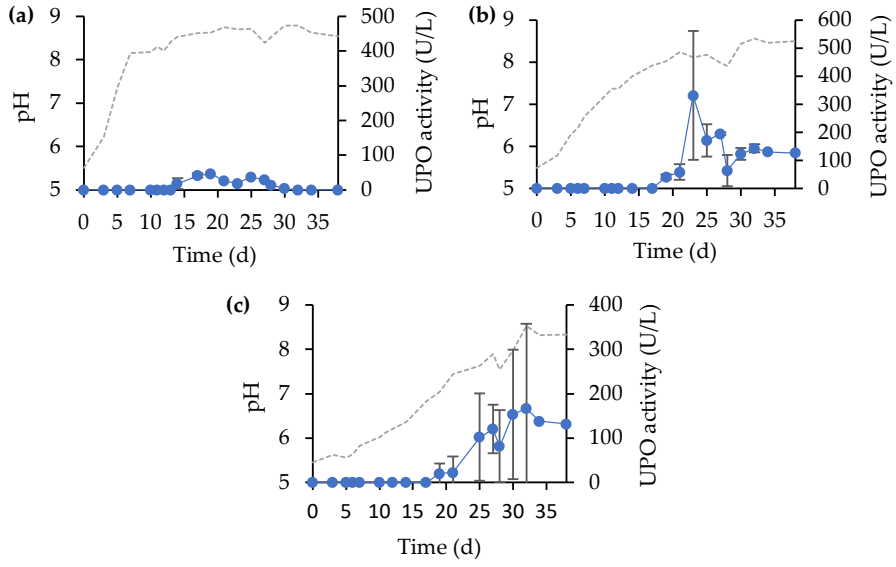



**Figure A4.2**. pH profiles (--) and UPO activity (•) in SmF by *A. aegerita* with **(a)** 25 %, **(b)** 50 % and **(c)** 75 % (v:v) of vinasse.

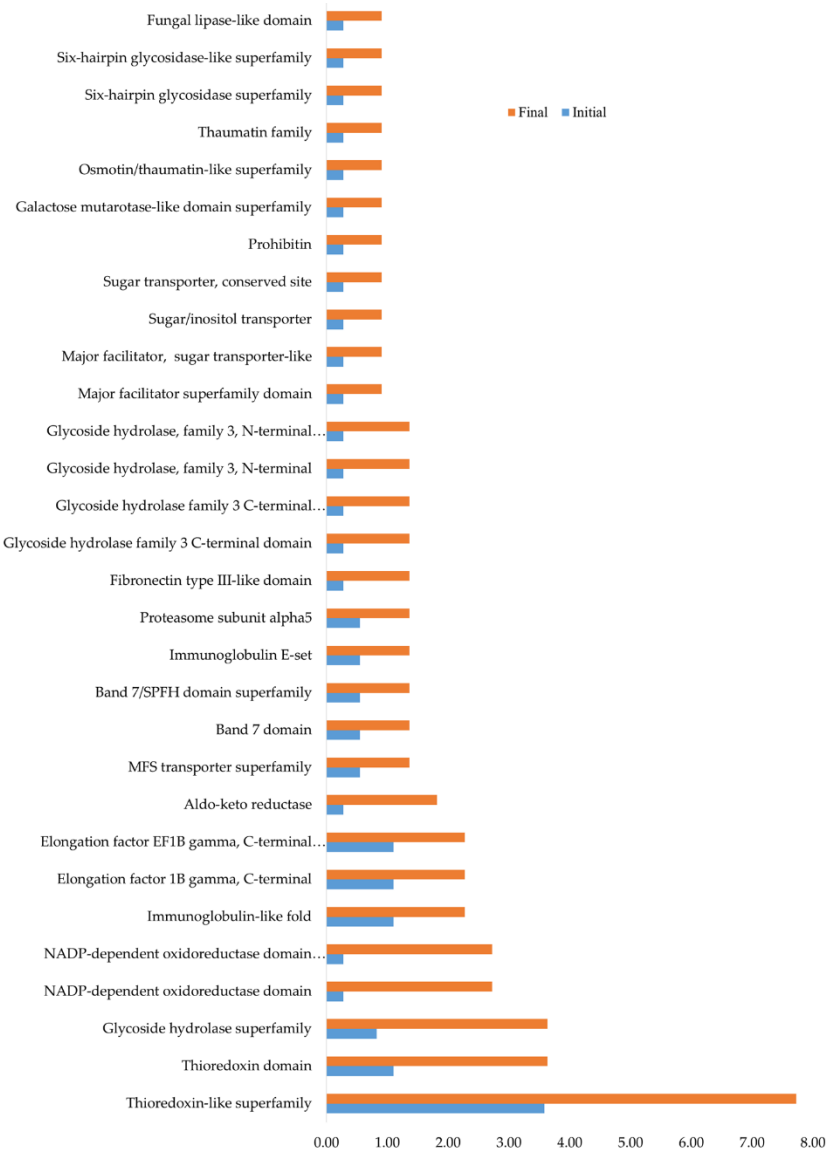



**Figure A4.3.** Interpro protein categories expressed both before (day 7, orange bar) and after (day 16, blue bar) UPO activity was detected and upregulated in the second condition in SmF cultivation of *A. aegerita*. The values are presented in the figure as percentage of peptides corresponding to each category from the total of peptides detected in each sample.

# CHAPTER 5

## FUNCTIONALISATION OF ORGANOSOLV LIGNIN BY ENZYMATIC DEMETHYLATION FOR BIOADHESIVE FORMULATION

The results of this chapter have already been published as "González-Rodríguez S, Lu-Chau TA, Chen. X, Eibes G, Pizzi A, Feijoo G and Moreira MT. Functionalisation of organosolv lignin by enzymatic demethylation for bioadhesive formulation. Ind. Crops Prod. 2022; 186, 115253. https://doi.org/10.1016/j.indcrop.2022.115253".

Detailed information available at "Manuscripts considered in this thesis: rights, contributions and quality indicators", page 227.

**SUMMARY**

Bio-based adhesives offer a sustainable alternative to formaldehyde-based adhesives, which is the most applied product in wood-based panel manufacturing. Despite the undeniable interest of lignin as a bulk chemical for the synthesis of bio-based adhesives, the main problem is its low reactivity. Therefore, it is crucial to study techniques to increase their reactivity, such as demethylation, that can be performed through an enzymatic treatment. One of the parameters to monitor the success of demethylation is the methanol concentration measured in the reaction medium and complementing these results with the use of non-quantitative analyses such as Fourier Transform Infrared Spectroscopy (FT-IR), Matrix-Assisted Laser Desorption/Ionization (MALDI) and Size Exclusion Chromatography (SEC). In order to maximize the action of the enzyme, a preliminary screening was performed with different enzymes, which were added to organosolv lignin in the form of purified enzymes and cocktails. *Trametes versicolor* laccase was selected as the best candidate for the demethylation of organosolv lignin, with a methanol released concentration of 107 mg/L. Based on these results, parameters affecting both enzyme action and enzyme-substrate interaction were evaluated. Optimal working conditions were strongly defined by the use of surfactants that enhanced lignin solubility (Tween-80) and mediators (HBT, vanillin) that favored enzyme catalysis to participate directly in the enzyme catalytic cycle. However, it was also observed that factors such as pH, temperature and aeration had a direct impact on the enzymatic demethylation of lignin. Considering the operational variables that allowed better demethylation values, the amount of methanol released increased to 363 mg/L, which implies an improvement of 239 % compared to previous results. The increase in molecular weight (MW) observed in the SEC analysis and the reduction of the intensity of the FT-IR bands related to the methyl/methoxyl groups also support these results.

## 5.1. Introduction

### 5.1.1. Lignin as promising candidate for bioadhesive formulation

The development of the adhesive market is increasingly widespread (Dinte and Sylvester, 2018). The most widely used alternatives are formaldehyde-based adhesives, such as urea-formaldehyde, phenol formaldehyde and melamine-formaldehyde (Liu et al., 2018; Zhu et al., 2018). However, the evolution of technological processes and legislation in favor of the use of non-volatile adhesives are some of the factors that have driven a trend towards more environmental-friendly processes (Dinte and Sylvester, 2018). In this regard, the use of natural compounds to produce adhesives (bioadhesives) has gained special relevance.

Soy protein is one of the first bio-based adhesives with commercial projection for plywood (Li et al., 2004; Ye et al., 2019). There are also references with promising results for different types of bio-based materials: corn starch, chitosan; but undoubtedly, the most widely used raw material for the formulation of natural-based adhesives is lignin, the second most abundant natural polymer in the world after cellulose (Lange et al., 2013). This is the main reason for the widespread interest in finding potential applications for lignin, including the manufacture of bioadhesives that can replace synthetic resins (Domínguez-Robles et al., 2018; El Mansouri et al., 2011; Ji and Guo, 2018). However, due to its low content of hydroxyl groups, the substitution of the aromatic ring and the steric barrier of its molecular structure, the reactivity of lignin is relatively poor (Kuo et al., 1991; Vázquez et al., 1999). Therefore, it is necessary to modify its main functional groups to increase its reactivity. Many studies have focused on different ways to improve the reactivity of lignin, such as demethylation (Venkatesagowda, 2019), oxidation (Yu et al., 2019) or phenolation (Li et al., 2018). Nevertheless, demethylation seems to be one of the best methods, converting part of the methoxy groups to phenolic hydroxyl groups (Chen et al., 2021).

Four main types of extracellular ligninolytic enzymes are known to be involved in lignin modifications, namely laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP). The most studied enzyme is laccase (EC 1.10.3.2), as it is the most commercially available enzyme and uses molecular oxygen as the final electron acceptor (Venkatesagowda, 2019). More specifically, laccase catalyses the oxidation of

lignin-related phenol-vanillyl glycol, leading to the initial formation of dimers and subsequent polymerization reactions (Lundquist and Kristersson, 1985). Occasionally, it may be necessary to use redox mediators, which act as electron transporters with the purpose of increasing the redox potential of the enzyme (Bourbonnais and Paice, 1990).

### 5.1.2. Objectives of this chapter

Lignin has gained much interest as an alternative material for the formulation of bioadhesives due to its high abundance at world scale. However, the incorporation of unmodified lignin in the formulation of phenolic resins is limited due to poor substitution performance and shows a reduction in bond strength with longer pressing time (Newman and Glasser, 1985) because lignin has lower reactivity compared to phenol. Enzymatic demethylation opens up a biotechnological approach for lignin modification (Venkatesagowda, 2019), provided that the selected enzymes are able to cleave the O-methyl/methoxyl groups of phenolic and some non-phenolic compounds. Accordingly, methanol release can be detected as an indicator of the progress of the reaction.

Based on these assumptions, the present study considers the enzymatic modification of organosolv lignin by different laccases, which was verified by monitoring the degree of demethylation and modification of phenolic groups enabling the cross-linking of lignin monomers. The effects of different conditions and factors were evaluated with the enzyme which showed the highest lignin modification and the best demethylation results. In order to validate the previous results and assess the effect of aeration, the conditions previously established as optimal were evaluated in a 150 mL bioreactor with continuous aeration.

## 5.2. Materials and methods

### 5.2.1. Chemicals, raw material and enzymes

Pullulan standards (ReadyCal-Kit Pullulan, PSS-pulkitr1) used in the SEC analysis were purchased from PSS. Vanillin was purchased from Merck, 1-hydroxybenzotriazole (HBT) was purchased from Fluka, Folin-Ciocalteu reagent was purchased from VWR Chemicals and other analytical chemicals from Sigma-Aldrich. The enzymes used for the methanol measurement protocol: horseradish peroxidase (HRP) (Sigma-Aldrich P6782, 950-2000

units/mg solid) and alcohol oxidase (AO) from *Pichia pastoris* (Sigma A2404, 10-40 units/mg protein), and other chemicals and analytical chemicals were purchased from Sigma-Aldrich.

Organosolv lignin, obtained after an organosolv treatment of beech wood, was kindly provided by the Fraunhofer Center for Chemical-Biotechnological Processes (CBP) (Leuna, Germany). In the fractionation process two phases are obtained: one solid fraction rich in cellulose and a black liquor mainly composed by lignin, C-5 sugars, and degradation products. Lignin was precipitated from this stream by the addition of acidified water and filtered for separation.

Three different types of laccases were used for lignin demethylation: Two commercial laccases from *Trametes versicolor* (Ref. No. 53739; Sigma-Aldrich) (0.9 U/mg) and *Myceliophthora thermophila* (Novozymes 51003®) ($1.44 \cdot 10^3$ U/mL) and an enzyme cocktail with laccase activity produced at lab-scale from *Ganoderma lucidum* (Chapter 2).

**5.2.2. Enzymatic modification of lignin in test tubes**

The experiments of organosolv lignin transformation were conducted in 12 mL polypropylene tubes with 50 mg of lignin added to 5 mL of citrate-phosphate buffer (lignin concentration 1 % w:v). The tubes were sealed with plastic caps and incubated under agitation (1500 rpm, mixing orbit 2 mm) and constant temperature (30 °C) in a thermoshaker incubator (Land Technics MSC-100). The laccase dosage was 50 U/g lignin by adding 15-70 μL of the enzyme. All experiments, as well as the controls with thermally inactivated laccase, were performed in triplicate. Samples were taken at 0, 0.5, 1, 2, 4, 8 and 24 h, which were centrifuged at 10,000 rpm for 10 min to measure methanol content and laccase activity in the supernatant. After that, the solid was washed twice with distilled water and freeze-dried for further analysis.

After testing the different enzymes, an optimisation of different factors at tube scale with *T. versicolor* laccase was conducted. The variables tested on a small scale were: a) pH (3, 4, 5, 6 and 7), b) temperature (20, 30 and 40 ˚C), c) addition of surfactants (150 mM PEG-600, and 1 % v:v Tween-80, which correspond with 398 and 50 μL, respectively) and d) addition of mediators (HBT and vanillin 30 mM, which correspond with 20.3 and 22.8 mg, respectively). The parameters were evaluated following a one factor at a time (OFAT) approach in the given order.

### 5.2.3. Enzymatic modification of lignin in 150-mL reactor scale

Assays were carried out in a 150 mL reactor (Biostat Q, B. Braun Biotech) to study the impact of aeration on laccase activity under more controlled conditions, such as those offered by an automated fermenter with numerous sensors and ports to control lignin modification. The reactor allowed continuous measurement of redox potential, pH, dissolved oxygen and temperature, and had the option of supplying air at a maximum flow rate of 110 mL/min.

In these experiments, the oxygen requirements for lignin oxidation by *T. versicolor* laccase, the addition of a mediator and the use of surfactants to improve lignin solubility were evaluated under optimised pH and temperature conditions at test-tube scale (pH 4 and 30 °C). Controls were performed with thermally inactivated enzyme. Triplicate test samples were withdrawn at 0, 0.5, 1, 2, 4, 8 and 24 h to measure the methanol released and check the enzyme activity described above.

### 5.2.4. Analytical protocols

#### *5.2.4.1. Measurement of laccase activity and methanol content*

Laccase activity was measured by 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) oxidation following the protocol described by Muñiz-Mouro et al. (2017) indicated in Chapter 3 (section 3.2.5).

The degree of lignin demethylation was measured indirectly by the release of methanol during modification by laccase. The methanol concentration was measured using the enzymatic method reported by Mangos & Haas (1996) with some modifications. This protocol uses two enzymes in cascade, an AO with high selectivity towards methanol, and HRP, which uses the hydrogen peroxide generated by AO as a co-factor in the oxidation of ABTS, whose product is monitored spectrophotometrically. The protocol uses three solutions, which were prepared as follows. Solution A was prepared by dissolving ABTS diammonium salt, Sigma A1888) in distilled water (10 mg/mL). Solution B corresponds to 0.65 mg of horseradish peroxidase (HRP) (Sigma-Aldrich P6782) dissolved in distilled water and diluted to 1 mL, 20 μL of that solution diluted to 2 mL with distilled water. Solution C was an AO solution, which was prepared by adding 30 μL of AO solution from *P. pastoris* (Sigma A2404) to 2 mL of distilled water.

Solution A (30 μL, ABTS), solution B (10 μL, HRP) and solution C (10 μL, AO) were pipetted into one well of a microplate. Aliquots (150 μL) of methanol standards (0.1-2.0 mg of methanol/L) or sample diluted in 0.20 M potassium phosphate buffer (pH 7.5) were added. The microplates were placed in a Biotek Powerwave XS2 spectrophotometer, shaken for 2 s, and the kinetic software was started instantly. The absorbance at 420 nm was recorded for 60 s, which was then used to calculate the maximum slope (AU/min) using R statistical software (R-CRAN). A calibration curve was obtained by correlating methanol concentration (0 to 2 mg/L) and the slope of the absorbance at 420 nm vs time. To counteract the sample matrix effect, a volume of the initial sample was added to the methanol standards to achieve the same concentration in the samples.

#### *5.2.4.2. Indirect measurement of lignin solubility*

Two indirect analytical methods were used to determine lignin solubility: ultraviolet absorption at 280 nm and determination of total phenolic content. The phenolic content of the modified lignin and its controls was measured using the Folin-Ciocalteu protocol (Singleton et al., 1965). First, 6 mg of freeze-dried treated lignin was dissolved in 1 mL of 0.2 M NaOH, and then different dilutions were prepared. 75 μL of diluted samples or gallic acid (used as standard) were added to 625 μL of Folin-Ciocalteu reagent (diluted at a ratio of 1:10 and 500 μL of $Na_2CO_3$ (7.5 % w:v)). The mixture was kept in the dark at room temperature for 1 h to complete the reaction, and then the absorbance was measured at 760 nm ($\varepsilon_{760}$ = 1.45 × $10^3$/(M cm)). The sample blank was prepared with 75 μL of methanol instead of the sample. Results were expressed as mg gallic acid equivalent (GAE) per g lignin as an average of two replicates. All spectrophotometric measurements were carried out on a Shimadzu UV-1800.

#### *5.2.4.3. Fourier Transform Infrared Spectroscopy*

FT-IR spectra of the freeze-dried samples of unmodified and demethylated organosolv lignin were recorded on a VARIAN FT-IR 670 spectrometer provided by an attenuated total reflection (ATR) Miracle diamond crystal. The powder samples were placed in the diamond eye (1.8 mm) of the ATR equipment and the sample contact was secured by tightly screwing the clamping device. Each extract was scanned by recording the spectrum with

32 scans with a resolution of 4 $cm^{-1}$ and the spectral width from 400 to 4000 $cm^{-1}$.

#### *5.2.4.4. Size Exclusion Chromatography analysis*

Lignin was analysed by SEC in order to determine the weight-average molar mass ($M_w$) and number-average molar mass ($M_n$). Prior to analysis, 1 g of freeze-dried sample was dissolved in 1 mL of 0.1 M NaOH. An HP-1100 HPLC system was applied to measure the MW of the different lignin samples, using 10 mM NaOH aqueous solution as mobile phase on MCX 100 A 5 μm 8 × 300 mm (PSS) and MCX 1000 A 5μm 8 × 300 mm (PSS) columns coupled in series and with a MCX precolumn 5 μm 8 × 50 mm (PSS). A flow rate of 1 mL/min, a sample injection volume of 20 μL and an IR 1047 A-HP detector were used. Calculations were based on calibration curves obtained from monodisperse pullulan standards (with specific MW of 180-708,000 Da). Quantification of the peak signals was performed with Unichrom software. The determination of $M_w$ and $M_n$ was carried out using an R script programmed based on the algorithm reported by Lopéz-Abelairas et al. (2015). The molar mass dispersity ($Đ_M$) was calculated according to Equation 5.1:

$$Đ_M = M_w/M_n \qquad \textbf{Eq. 5.1}$$

#### *5.2.4.5. Matrix-Assisted Laser Desorption/Ionization analysis*

The analysis were performed following the protocol described by Chen et al. (2021). Samples for matrix assisted laser desorption ionization time-of-flight (MALDI-ToF) analysis were prepared by first dissolving 7.5 mg of the samples in 1 mL of a 50:50 v:v acetone/water solution. Then, 10 mg of this solution was added to 10 μL of a 2,5-dihydroxy benzoic acid (DHB) matrix. The locations dedicated to the samples on the analysis sample holder were first covered with 2 μL of a NaCl solution 0.1 M in 2:1 v:v methanol/water, and pre-dried. Then 1.5 μL of the sample solution was placed on its dedicated location and the plaque was dried again. Red phosphorous was used to standardise the MALDI equipment. MALDI-ToF spectra were obtained using an Axima-Performance mass spectrometer from Shimadzu Biotech (Kratos Analytical, Shimadzu Europe Ltd.) using a linear polarity-positive tuning mode. The measurements were carried out making 1000 profiles. The spectra were precise at +1 Da.

### 5.2.5. Statistical analysis

Statistical analysis of the experimental results was performed using R software (version 4.0.5, R Core Team, 2021). To determine whether there were significant differences between the values of methanol released from laccase, a one-way analysis of variance (ANOVA) was applied. If the ANOVA confirmed the difference, a post hoc analysis (Tukey's HSD) was applied to determine between which values the difference was significant. A significance level ($\alpha$) of 0.05 was considered for all the statistical analysis. The main results of the statistical analysis were included in Annexes (Figure A5.1, Figure A5.2 and Figure A5.3).

## 5.3. Results and discussion

### 5.3.1. Selection of laccase for lignin demethylation

The demethylation of organosolv lignin was studied using laccases of different redox potentials. For this purpose, two commercial laccases from *T. versicolor* and *M. thermophila*, with high ($E_0$= 780 mV) and low ($E_0$= 470 mV) redox potentials, respectively, were used (Singh et al., 2015). Furthermore, crude laccase from *G. lucidum* was evaluated, which has mid-redox potential (Kumar et al., 2021).

As shown in Figure 5.1-a, a higher amount of methanol was released after 24 h in the enzymatic modification of organosolv lignin with *T. versicolor* laccase (107 mg/L), which is related to a higher degree of lignin demethylation (Venkatesagowda, 2019). Previous studies have demonstrated that *T. versicolor* laccase is capable of attacking the methyl/methoxyl groups of lignin, but in most of them this enzyme requires the use of a mediator compound that increases the catalytic potential of the enzyme (Venkatesagowda, 2019). The low-redox potential laccase reached a slightly lower value of methanol released after 24 h (90 mg/L), although it seemed that the demethylation with this enzyme was faster during the first hours of the reaction.

The lower amount of methanol released with *G. lucidum* laccase compared to that obtained with the other enzymes may be a consequence of the presence of inhibitors in the enzyme crude, since a black liquor-based medium from the organosolv treatment of beech wood was used for laccase production. This behaviour was also reported by Ibrahim et al. (2011), who showed that the use

of a laccase crude was less efficient in the demethylation of lignin than the use of the purified enzyme.

In terms of phenolic content (Figure 5.1-b), all three enzymes led to a decrease in phenolic groups. This decrease is similar for the experiments with *T. versicolor* and *M. thermophila* laccases (22 % and 17 %, respectively). Whereas, in the case of the experiments with *G. lucidum* laccase, the reduction in phenolic content was about 3 times lower (6 %). These results show a direct relationship between the methanol released and the reduction of phenolic hydroxyl groups, with a higher reduction of phenolic hydroxyl groups with higher methanol release. In contrast, many studies have reported an increase in phenolic content after chemical oxidation of lignin (Song et al., 2016; F. Wang et al., 2018), which may be attributed to the transformation of oxidized methoxy groups into catechol intermediates. However, Wang et al. (2018) suggested that the absence of catechol intermediates after enzymatic treatment with laccase may be caused by the rapid oxidation of catechol to quinones. In view of the results obtained with *G. lucidum* laccase, we can conclude that the use of a crude laccase produced in a complex medium is not suitable for the enzymatic transformation of lignin.

A statistical analysis (Figure A5.1) of the results of methanol released with *T. versicolor* and *M. thermophila* laccases was performed. It was found that the methanol released in the *T. versicolor* laccase treatment was significantly higher than that of *M. thermophila* (p-value 0.03885). For this reason, optimisation of the enzymatic demethylation of organosolv lignin was continued with *T. versicolor* laccase.

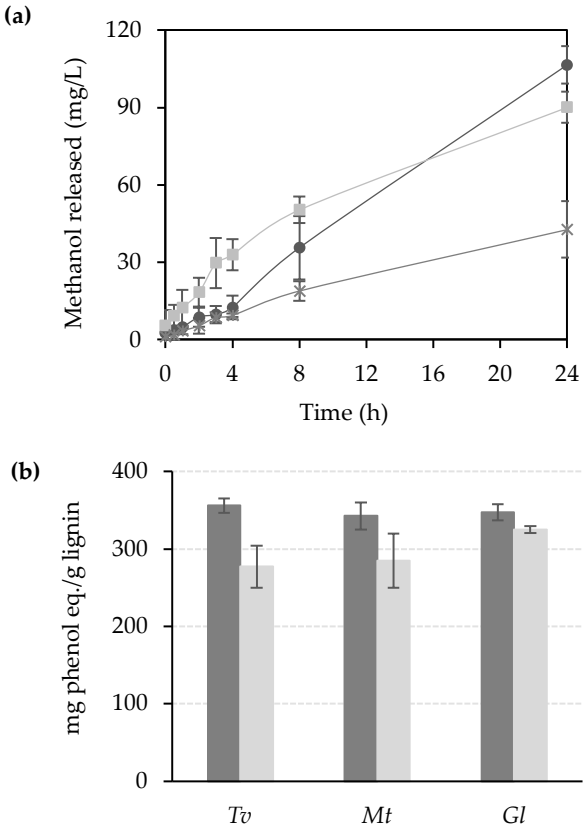



**Figure 5.1.** Parameters measured during the enzymatic treatment of organosolv lignin at pH 6 and 30 °C in 5 mL experiments: **(a)** methanol released with *T. versicolor* (•), *M. thermophila* (■) and *G. lucidum* (×) laccase; **(b)** phenolic content after 24 h of enzymatic transformation with *T. versicolor* (Tv), *M. thermophila* (Mt) and *G. lucidum* (Gl) laccase (■) and their respective controls (■) with inactivated laccases.

### 5.3.2. Influence of pH and temperature on lignin demethylation

The enzymatic modification of organosolv lignin was evaluated in the pH and temperature range most typical of laccase-catalysed reactions. Although lignin is more soluble at basic pH, enzymatic demethylation of lignin by laccase requires the use of neutral or acidic pH (Hämäläinen et al., 2018), as fungal laccase enzymes have an optimal pH range between 2.5 and 7, depending on the type of substrate (Gavrilaş et al., 2012; Kurniawati and

Nicell, 2008; Morozova et al., 2007). Hence, pH values from 3 to 7 were evaluated in this study.

As shown in Figure 5.2-a, the highest lignin demethylation was obtained at acidic pH values. In this regard, values of 120-130 mg/L methanol were detected at pH 3-4 (Figure A5.2). At neutral pH, no methanol release was observed during 24 h. At the same time, the enzyme showed the highest stability at pH 7, with only 6 % loss of activity after 24 h, while at pH 3 laccase lost 75 % of the initial activity (Table A5.1). It is well known that laccase activity generally decreases with increasing pH due to inhibition by hydroxide anion, which binds to the trinuclear T2/T3 site to interfere with electron transfer from the T1 to T2/T3 sites (Xu, 1997). However, enzyme stability is favored by neutral pH.

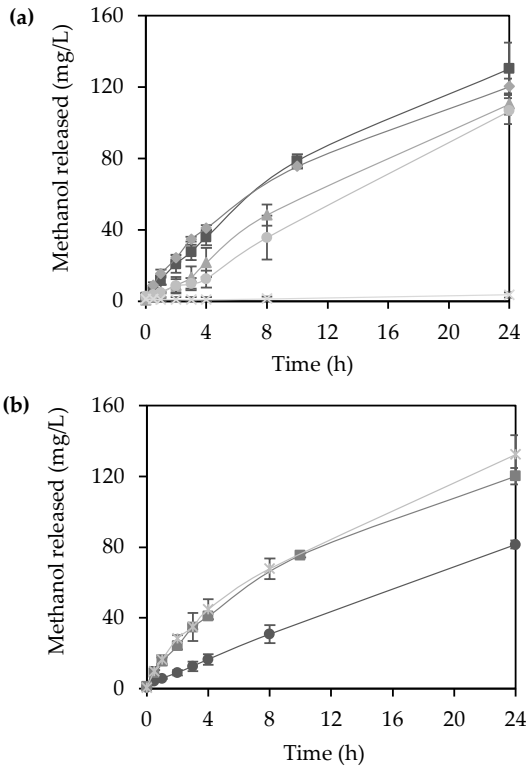



**Figure 5.2.** Methanol released during the enzymatic treatment of organosolv lignin by *T. versicolor* laccase in 5-mL experiments: (a) at pH 3 (■), 4 (♦), 5(▲), 6 (•) and 7 (×); (b) at 20 (•), 30 (■) and 40 °C (×).

In the case of temperature, the optimal activity of fungal laccases is generally between 30 °C and 55 °C (Kumar et al., 2012), although considering the duration of the reaction (24 h), it was decided to use temperatures between 20 °C and 40 °C. As shown in Figure 5.2-b, the highest methanol release was achieved at 40 °C (132 mg/L), while at 20 °C the methanol released was less than 75 % of the value achieved at other temperatures. Statistical analysis (Figure A5.3) shows that there was no significant difference between methanol released at 30 °C and 40 °C (p-value 0.1391). Taking this into account and considering that the loss of enzymatic activity was lower at 30 °C than at 40 °C (Table A5.1), 30 °C was chosen as the optimum temperature to further optimise the enzymatic transformation of lignin.

Lignin treated with *T. versicolor* laccase at optimum pH (pH 4) and temperature (30 °C) was analysed by FT-IR and compared with the original lignin and with a control using inactivated laccase. The observed band assignments and their relative intensity are listed in Table 5.1 and were based on Hansen et al. (2016). The band at 1595 $cm^{-1}$, corresponding to aromatic ring vibrations, was used as the reference to calculate the relative intensities (Rashid et al., 2016). The absorption spectra of the different lignin samples show a similar profile (Figure A5.4), although the relative intensity of certain bands showed differences. The control and untreated lignin (Figure A5.4-a,b) showed similar relative intensities, suggesting that the addition of buffer and inactivated enzyme slightly affected the absorption spectrum. The band at 1216 $cm^{-1}$, that is related with phenolic groups, showed a decrease in the relative intensity, confirming that the phenolic content is affected after the enzymatic transformation at optimum pH and temperature. Methyl and methoxyl groups (with bands at 2935, 2841 and 1456 $cm^{-1}$) showed a decrease in relative intensities. On the other hand, the band associated with unconjugated C=O (1708 $cm^{-1}$) increased the relative intensity.

**Table 5.1.** Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at optimum pH and temperature.

| Wavenumber ($cm^{-1}$) | Assignment | Relative intensity[a] | | | |
|---|---|---|---|---|---|
| | | untreated | control | treated | Δ[b] (%) |
| 3388 | Phenolic OH + aliphatic OH | 0.74 | 0.72 | 0.69 | -3.5 |
| 2935 | $CH_3$ + $CH_2$ | 0.71 | 0.71 | 0.66 | -8.1 |
| 2841 | $CH_3$ + $OCH_3$ | 0.56 | 0.56 | 0.52 | -7.4 |
| 1708 | C=O (Aromatic-Carbonyl) | 0.43 | 0.38 | 0.44 | +15.3 |
| 1456 | $CH_3$ + $CH_2$ | 1.34 | 1.32 | 1.09 | -17.4 |
| 1213 | Phenolic OH | 1.63 | 1.60 | 1.38 | -13.7 |

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 $cm^{-1}$.

[b] Change in relative intensity; a negative value means a decrease, whereas a positive value means an increase.

### 5.3.3. Improvement of lignin solubility

Most of the tests performed on the chemical demethylation of lignin have been carried out in alkaline media to enhance lignin solubility (Evstigneev, 2011). However, when laccase is used to carry out the demethylation, an alkaline pH would negatively affect the activity and stability of the enzyme as it would cause its inactivation within a short period of time. It has been shown that, at basic pH, protein denaturation occurs as a result of the disruption of enzymatic hydrogen bonds by excess $OH^-$ ions (Saoudi et al., 2017). For this reason, enzymatic treatment must be performed at neutral or acidic pH, with the disadvantage of poor lignin solubility.

To mitigate the poor enzyme-substrate contact resulting from this lower lignin solubility, it is important to evaluate the impact of the addition of different surfactants that can increase the amount of solubilised lignin. In this case, Tween-80 and PEG-600 were evaluated as surfactants that can increase the apparent solubility of lignin (Saoudi and Ghaouar, 2019; Xu et al., 2016). First, the impact of the addition of these compounds on the solubility of lignin was evaluated after 17 h. Through two analytical techniques: measurement of the soluble lignin fraction at 280 nm (Figure 5.3-a) and determination of the total phenolic content of the dissolved fraction (Figure 5.3-b) it was possible to identify the effect of the surfactants considered. The first technique is a

direct measurement, while the second is an indirect measurement, in which a higher phenolic content in the soluble fraction can be related to a higher amount of dissolved lignin, since the only compound in the medium that has phenolic groups is lignin.

As shown in Figure 5.3, Tween-80 and PEG-600 improved lignin solubility relative to the citrate-phosphate buffer at pH 4 used previously. Compared to samples at pH 4, the initial solubility increased 1.8 and 5.3-fold using PEG-600 and Tween-80, respectively, when measuring soluble lignin via absorbance at 280 nm. After 17 h, the improvement in solubility compared to the buffered sample was slightly higher, with 2.1 and 7.7-fold increases for PEG-600 and Tween-80, respectively. The same effect was observed when measuring total phenolic content (Figure 5.3-b), with a more pronounced effect for Tween-80 and in particular, after 17 h of incubation (19.8-fold higher, while the initial solubility was 8.0-fold higher than the buffered sample).

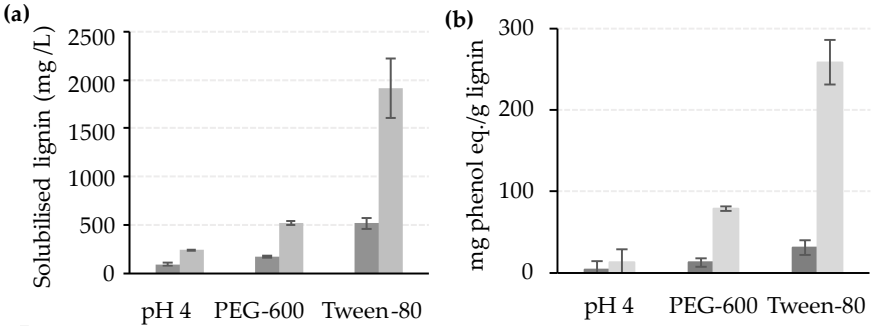



**Figure 5.3.** Measurements of lignin solubility in buffer and different solubilising agents by **(a)** measurement of soluble lignin through absorbance at 280 nm and **(b)** determination of total phenolic content of dissolved fraction, at 0 h (■) and 17 h (■).

After evaluating the positive impact of Tween-80 and PEG-600 on lignin solubility, lignin demethylation was performed in the presence of both compounds. Figure 5.4 shows that the methanol concentrations detected in the experiments with Tween-80 (202 mg/L), and PEG-600 (165 mg/L) were higher than that of the control experiment (120 mg/L). It is known that small length PEG can act as a stabiliser of the laccase structure, while increasing PEG chain length is associated with a decrease in laccase activity (Saoudi and Ghaouar, 2019). The loss of activity in the experiments with Tween-80, PEG-600 and the buffer control was 5.8, 28 and 68.6 %, respectively (Table A5.1).

Both polymers protected the enzyme from inactivation (Saoudi and Ghaouar, 2019; M. Wang et al., 2018), although Tween-80 exerted a more pronounced effect. Therefore, both the increase in apparent solubility of lignin and the stability of laccase were more favorable when Tween-80 was used, so that a higher methanol release was observed.

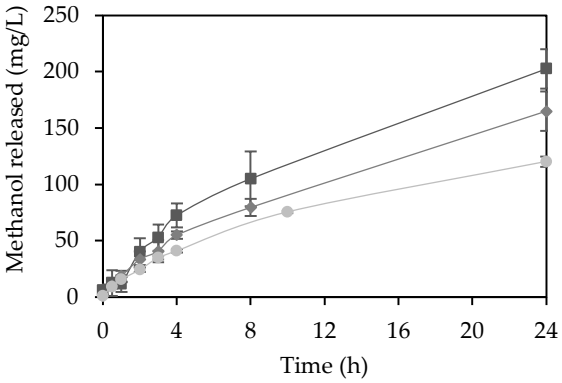



**Figure 5.4.** Methanol released during the enzymatic treatment of organosolv lignin by *T. versicolor* laccase in 5-mL experiments using Tween-80 (■) and PEG-600 (♦) as solubilizing agents, and in the experiment at pH 4 and 30 °C without addition of solubilizing agents (•).

FT-IR analysis of laccase treated samples containing Tween-80 (Table 5.2) showed a decrease in phenolic (1213 $cm^{-1}$) and hydroxyl groups (3338 $cm^{-1}$) and an increase of the carboxyl groups (1708 $cm^{-1}$) as observed previously. However, not all relative intensities corresponding to methyl/methoxyl groups decreased, since the intensities at 2935 and 2841 $cm^{-1}$ corresponding to $CH_3 + CH_2$ and $CH_3 + OCH_3$, respectively, increased after treatment. However, it should be noted that the presence of the surfactant increased the intensity of these bands, as observed when comparing the control (presence of Tween-80) and the original lignin. Therefore, Tween-80 could be masking part of the expected reduction in the content of methyl/methoxyl groups, which may be obscured in the FT-IR spectra of Tween-80 treated lignin and its control (Figure A5.4-c).

**Table 5.2.** Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at optimum pH and temperature and the presence of Tween-80.

| Wavenumber ($cm^{-1}$) | Assignment | Relative intensity[a] | | | |
|---|---|---|---|---|---|
| | | untreated | control | treated | Δ[b] (%) |
| 3388 | Phenolic OH + aliphatic OH | 0.74 | 0.64 | 0.57 | -11.9 |
| 2935 | $CH_3 + CH_2$ | 0.71 | 0.99 | 1.07 | +7.4 |
| 2841 | $CH_3 + OCH_3$ | 0.56 | 0.81 | 0.88 | +8.7 |
| 1708 | C=O (Aromatic-Carbonyl) | 0.43 | 0.56 | 0.64 | +13.0 |
| 1456 | $CH_3 + CH_2$ | 1.34 | 1.47 | 1.31 | -10.6 |
| 1213 | Phenolic OH | 1.63 | 1.65 | 1.34 | -18.9 |

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 $cm^{-1}$.

[b] Change of the relative intensity with respect to the control with inactivated laccase; a negative value means a decrease, whereas a positive value means an increase.

### 5.3.4. Laccase mediator system

The use of compounds acting as mediators is common in polymer oxidation by laccase, due to the ability of these compounds to enhance the redox potential of the enzyme (Nguyen et al., 2016). To increase lignin demethylation, two types of mediators were evaluated: a natural compound (vanillin) and a synthetic compound (HBT). In the presence of these compounds, laccase generates radicals that are capable of oxidising phenolic and some non-phenolic lignin models (Crestini et al., 2003; Kupski et al., 2019).

Figure 5.5 shows the methanol released both with laccase mediator system (LMS) and with the joint use of HBT and Tween-80. Methanol released (86.2 mg/L) in the LMS with vanillin was lower than that achieved in the experiment at the same pH and temperature without mediator (120.1 mg/L). In the HBT experiment, the methanol released increased to 172 mg/L, which is 1.43 times the amount of methanol released without mediator. The combined use of HBT and Tween-80 resulted in 368 mg/L methanol released. This high increase is an indicator of the high yield of both compounds in the demethylation reaction. The role of HBT in the oxidation of laccase together with the increase of lignin solubility by Tween-80 had a high impact on the demethylation process.

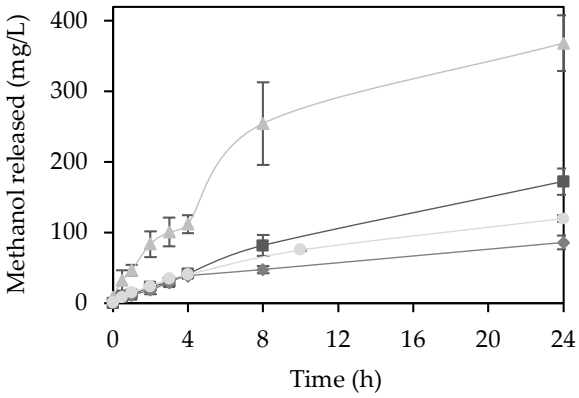



**Figure 5.5.** Methanol released during the enzymatic treatment of organosolv lignin by *T. versicolor* laccase at pH 4 and 30 °C in 5-mL experiments using HBT (■) and vanillin (♦) as mediators, in the experiment with the addition of Tween-80 and HBT (▲) and in the experiment at pH 4 and 30 °C without the addition of surfactant or mediator (●).

It is also notable that the loss of laccase activity due to the addition of HBT was somewhat reduced due to the presence of Tween-80 (Table A5.1). In this regard, the presence of HBT resulted in the loss of 45.8 % of the initial activity; whereas when Tween-80 was also added, this value was reduced to 19.8 %. This loss of activity was lower than that reported in previous studies, even using longer reaction times (Bourbonnais et al., 1997). FT-IR analysis of the LMS-treated lignin showed a similar trend, with a slight increase in carbonyl groups, and a decrease in methyl/methoxyl and hydroxyl groups (Table 5.3). The complete lignin spectra after enzymatic treatment with the addition of HBT and its control are included in Annexes (Figure A5.4-d).

**Table 5.3.** Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at optimum pH and temperature and the presence of HBT.

| Wavenumber ($cm^{-1}$) | Assignment | Relative intensity[a] | | | |
|---|---|---|---|---|---|
| | | untreated | control | treated | Δ[b] (%) |
| 3388 | Phenolic OH + aliphatic OH | 0.74 | 0.71 | 0.67 | -6.1 |
| 2935 | $CH_3$ + $CH_2$ | 0.71 | 0.72 | 0.68 | -6.1 |
| 2841 | $CH_3$ + $OCH_3$ | 0.56 | 0.57 | 0.55 | -3.3 |
| 1708 | C=O (Aromatic-Carbonyl) | 0.43 | 0.38 | 0.41 | +7.7 |
| 1456 | $CH_3$ + $CH_2$ | 1.34 | 1.36 | 1.09 | -19.5 |
| 1213 | Phenolic OH | 1.63 | 1.64 | 1.24 | -24.2 |

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 $cm^{-1}$.

[b] Change of the relative intensity with respect to the control with inactivated laccase; a negative value means a decrease, whereas a positive value means an increase.

### 5.3.5. Aeration

It has been shown that oxygen supply has a remarkable effect on laccase-mediated oxidation of organosolv lignin (Ortner et al., 2015). For this reason, after optimisation of the main parameters related to laccase activity, the impact of adding an oxygen source to the reaction medium was evaluated. For this purpose, the demethylation process at pH 4 and 30 °C was carried out in a 150 mL-reactor to ensure better control of the continuous air supply and thus maintain the oxygen concentration above 80 % of its saturation value throughout the experiment.

The first set of experiments were carried out in the absence of mediator and surfactant in the reaction medium. The methanol released in the 150 mL non-aerated experiment (76.15 mg/L, Figure 5.6) was substantially lower than the previous control (120.1 mg/L). This difference may be associated with an under-quantification of the methanol release, due to its partial vaporization. It should be noted that, unlike the experiments performed in 5 mL tubes, the reactor system is open, which probably contributes to the loss of methanol in the vapor phase. In fact, when comparing the FT-IR results of both experiments under the same operating conditions but at different scales, the spectra in the fingerprint region are very similar, suggesting a similar effect (Figure A5.4-b,e).

However, in comparative tests performed in the presence and absence of oxygen, methanol release increased from 76 mg/L to 107 mg/L when air was supplied (Figure 5.6), although no significant differences were observed at the initial times, which is indicative of the fraction of oxygen that existed in the reactor headspace. In contrast, at longer reaction times, the need for an external oxygen supply to ensure optimal enzyme activity is noticeable (Figure A5.5).

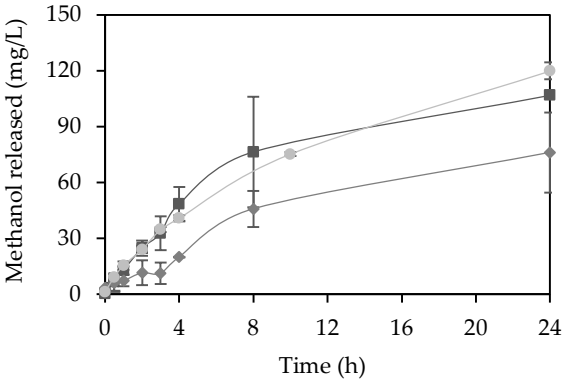



**Figure 5.6.** Methanol released during the enzymatic treatment of organosolv lignin by *T. versicolor* laccase at pH 4 and 30 °C in 150 mL-reactor with aeration (■) and without aeration (♦), and in 5 mL scale at pH 4 and 30 °C without the addition of surfactant or mediator (●).

Analysis of FT-IR spectra of lignin treated with and without air supply compared to untreated lignin shows an increase in the relative intensity of the 1708 $cm^{-1}$ band, related to carbonyl groups (Table 5.4, Figure A5.4-e). Furthermore, it is remarkable the decrease in the intensity of the 3650-3250 $cm^{-1}$ bands for lignin treated with *T. versicolor* laccase under aeration, which is more relevant than that of lignin treated under the same conditions in the absence of aeration. The band at 750-850 $cm^{-1}$ is also of interest, because the higher intensity of treated lignin in the presence of aeration may suggest an increase of aromaticity in the final sample, which may be related to a polymerisation process (Figure A5.4-e).

**Table 5.4.** Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at the optimum pH and temperature with and without aeration. Data for untreated lignin are the same as those given in Table 5.3.

| Wavenumber ($cm^{-1}$) | Assignment | Relative intensity[a] | | | | |
|---|---|---|---|---|---|---|
| | | control | no air | air | Δ[b] (%) no air | Δ[b] (%) air |
| 3388 | Phenolic OH + aliphatic OH | 0.72 | 0.63 | 0.57 | -12.2 | -20.1 |
| 2935 | $CH_3$ + $CH_2$ | 0.71 | 0.64 | 0.64 | -10.2 | -10.6 |
| 2841 | $CH_3$ + $OCH_3$ | 0.56 | 0.50 | 0.48 | -10.1 | -12.9 |
| 1708 | C=O (Aromatic-Carbonyl) | 0.38 | 0.44 | 0.43 | +14.9 | +11.2 |
| 1456 | $CH_3$ + $CH_2$ | 1.32 | 1.14 | 1.17 | -14.2 | -11.6 |
| 1213 | Phenolic OH | 1.60 | 1.42 | 1.48 | -10.8 | -7.5 |

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 $cm^{-1}$.

[b] Change of the relative intensity with respect to the control with inactivated laccase; a negative value means a decrease, whereas a positive value means an increase.

### 5.3.6. Optimal conditions

After optimisation of all variables in the organosolv lignin enzymatic transformation experiments, the reaction was carried out in the 150 mL-reactor under the optimal conditions (aeration, pH 4, T 30 °C, addition of Tween-80 and use of HBT). As shown in Figure 5.7 and according to the values of methanol released, it is observed that Tween-80 and HBT are the key parameters to increase methanol release, presenting a greater impact than other elemental variables, such as pH, temperature, or aeration. However, this increase in lignin demethylation is more remarkable due to the synergistic action between HBT and Tween-80. In this case, methanol release reached 363 mg/L, compared to values of 202 and 173 mg/L when both compounds are considered individually. If it is taken into account that methoxyl groups represents 23.94 % of the total weight of organosolv lignin (Lê et al., 2016) and the relation between methoxyl and methanol MW, it can be stablished that the 363 mg/L released in this experiment represents a 14.7 % of demethylation of the original lignin. This value is in the same range that the reported by Wang et al. (2018) in the study of kraft lignin demethylation with *T. versicolor* laccase and HBT during 24 h and working with the same concentration of lignin and also with the addition of Tween-80 as solubilizing agent (19.2 % of

demethylation). It is also remarkable that in the reactor the loss of activity was slightly higher (29.6 %, Table A5.1) than in the previous optimal conditions without aeration (19.8 %), but with a lower enzyme inactivation value than in the experiment with HBT and without addition of Tween-80, which confirms the protective effect of this compound on the enzyme even under aerated conditions.

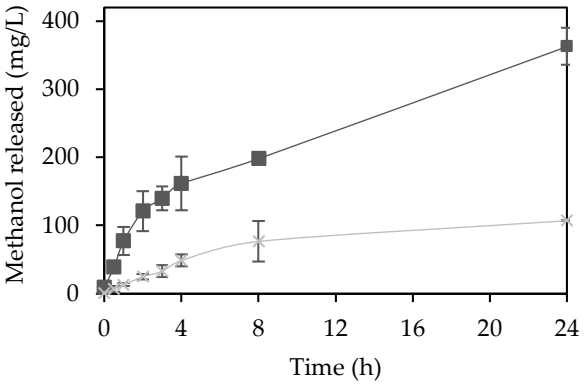



**Figure 5.7.** Comparison of methanol released in 150 mL-reactor-scale experiments: addition of aeration at pH 4 and 30 °C (×) and use of all optimized conditions (pH 4, 30 °C, Tween-80, HBT and aeration) (■).

The FT-IR analysis of the lignin treated in the reactor experiments shows the same trend as that of the lignin obtained in the small-scale optimisation (Figure A5.4-f). The band related to the aromatic carbonyl (1708 $cm^{-1}$) was more intense for the treated lignin, and the decrease of the signals related to the phenolic hydroxyl group (1213 $cm^{-1}$) and methyl/methylene groups (1456 $cm^{-1}$) is also remarkable (Table 5.5). All these results, in addition to the methanol released during the enzymatic treatment, reinforce the hypothesis of a two-step oxidation of the methoxy groups, with an initial transformation into hydroxyl groups, which are then oxidised to carbonyl groups.

**Table 5.5.** Signal assignment and relative intensities in FT-IR spectra of untreated lignin, control (with inactivated laccase) and treated with laccase at optimum pH, temperature, the presence of Tween-80, HBT and aeration.

| Wavenumber ($cm^{-1}$) | Assignment | Relative intensity[a] | | | |
|---|---|---|---|---|---|
| | | untreated | control | treated | Δ[b] (%) |
| 3388 | Phenolic OH + aliphatic OH | 0.74 | 0.64 | 0.60 | -5.9 |
| 2935 | $CH_3 + CH_2$ | 0.71 | 0.99 | 0.92 | -7.2 |
| 2841 | $CH_3 + OCH_3$ | 0.56 | 0.80 | 0.74 | -8.0 |
| 1708 | C=O (Aromatic-Carbonyl) | 0.43 | 0.53 | 0.61 | +16.7 |
| 1456 | $CH_3 + CH_2$ | 1.34 | 1.53 | 1.27 | -17.0 |
| 1213 | Phenolic OH | 1.63 | 1.73 | 1.35 | -22.0 |

[a] Relative intensity was calculated as the ratio of intensity of each band to the intensity of the reference band at 1595 $cm^{-1}$.

[b] Change of the relative intensity with respect to the control with inactivated laccase; a negative value means a decrease, whereas a positive value means an increase.

The SEC results (Figure A5.6) show a slight increase in the MW (791 Da) of the lignin treated under optimal conditions relative to the original lignin (Figure A5.6-a). This polymerization is probably obtained mainly through the formation of aromatic bonds of the hydroxyl group. These phenolic groups can be oxidised by laccase with a corresponding yield of lignin radicals that interact with other radicals to form phenyl-ether or carbonyl-ether bonds, with a corresponding increase in MW (Antúnez-Argüelles et al., 2020). Whereas the lignin backbone, in which most of the aliphatic hydroxyl groups are located, was not degraded by laccase. This preference for aromatic hydroxyl groups over aliphatic groups in laccase has already been described by other studies (Gouveia et al., 2018), including studies using LMS with HBT as a mediator (Nugroho Prasetyo et al., 2010).

It is also noteworthy that in Figure A5.6-b, comparing the control with the inactivated laccase and the lignin treated under optimal conditions, the MW of the control was higher than that of the original lignin, which is attributed to the presence of Tween-80. MALDI-ToF spectra of the original untreated lignin and the modified lignin under the optimal conditions in the reactor experiments are presented as Annexes (Figure A5.7 and Figure A5.8).

Although MALDI-ToF is not a quantitative technique, it can provide information of interest for predicting the architecture of the lignin molecule.

The spectra of modified lignin (Figure A5.8) show the presence of higher dimers, trimers or oligomers with the addition of the lignin unit defined in Figure 5.8 (176 Da). This pattern is indicative of the achievement of lignin polymerization after enzymatic treatment. For example, in the scan range of m/z 300-600 and 600-900 Da a series was characterised starting from the precursor ion 360.96 (m/z), which gives rise to the ions of 536.6, 712.1 and 888.0 (m/z). Another series is that corresponding to 1035.7, 1210.9 and 1686.6 (m/z). The formation of these lignin oligomers could explain the decrease of 177.2 (m/z). The signal of the lignin monomer at 137.2 (m/z), related to guaiacyl units (Saito et al., 2005), also diminished in the treated lignin. Dimers and trimers of this compound, corresponding to signals at 223.2 and 408.8 (m/z), were detected in the enzymatically transformed lignin.

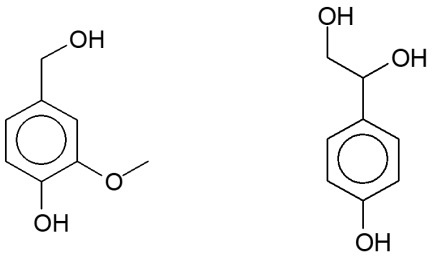



**Figure 5.8.** Possible lignin unit structures based on MALDI-ToF spectra.

It is also important to note the systematic increase in 44 Da for the different m/z signals starting from 744.7 (Figure A5.8 and Figure A5.9) and covering the whole m/z range, which is related to the ethoxy repeat units of Tween-80 present in the control, the treated sample but not in the original untreated lignin (Figure A5.7).

In order to compare the effect of the different parameters, Figure 5.9 summarises the results of methanol release obtained throughout the optimisation experiments.

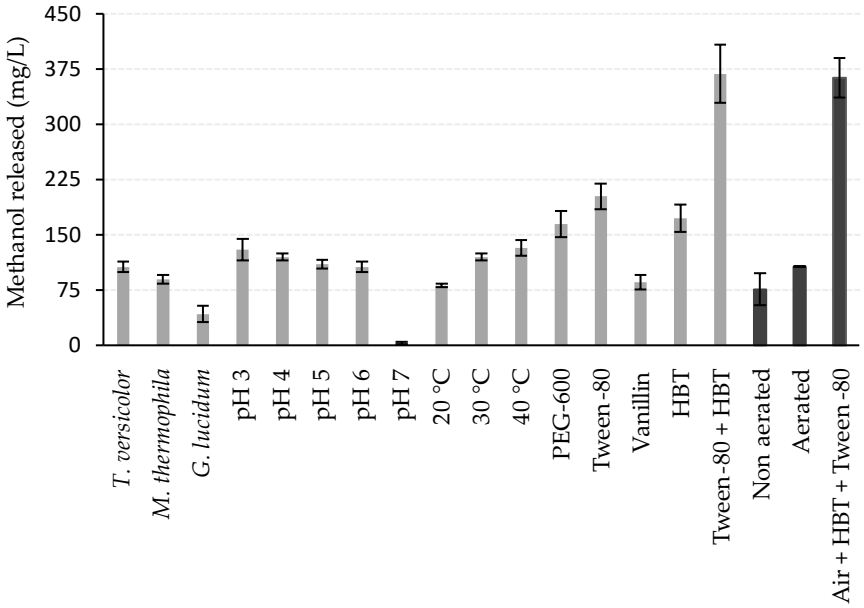



**Figure 5.9.** Comparison of methanol released in all performed experiments: at 5 mL scale (light grey) and at 150 mL-reactor (dark grey).

It is clearly observed that the addition of Tween-80 had a noticeable effect on methanol release, but the combined action of the LMS and the addition of the surfactant seemed to have a synergistic effect, reaching almost double the methanol concentration in the medium. Tween-80 not only acts as a surfactant but is also a source of unsaturated lipids. Böhmer et al. (1998) demonstrated that the laccase/HBT system can catalyse the peroxidation of unsaturated fatty acids. In this mechanism, peroxyl radicals (ROO-) are strong oxidants that propagate lipid peroxidation, allowing the oxidation of recalcitrant compounds (Camarero et al., 2008). Elegir et al. (2005) demonstrated that LMS can oxidise fully methylated non-phenolic lignin substructures, but only in the presence of surface-active agents.

## 5.4. Conclusions

The demethylation of organosolv lignin has been achieved with different fungal laccases, concluding that commercial laccases produced higher lignin demethylation than the enzymatic crude obtained in complex fermentation medium. A demethylated organosolv lignin which may be suitable for use in

the synthesis of bioadhesives was obtained using *T. versicolor* laccase. It was shown that the most relevant parameters for the enzymatic demethylation reaction are those directly involved in the enzyme-substrate contact. In this case, these are the addition of solubilising agents to increase lignin solubility and the use of mediator compounds. The combined action of unsaturated fatty acids and LMS could favor lignin demethylation by lipid peroxidation mechanism. In addition, the optimal operating range of other key parameters in lignin demethylation (such as pH, temperature and aeration) was experimentally determined. The SEC and MALDI analyses revealed that the enzymatic treatment promoted the oligomerisation of lignin.

The feasibility of using enzymatically demethylated lignin in the formulation of bioadhesives is based on the transformation of part of the methoxy groups of the original lignin into hydroxyl groups and finally into carbonyl groups which are more reactive and may involve the formation of reinforced networks. However, although the results obtained show that the enzymatic transformation has improved the reactivity of the lignin, a next step would be to apply the modified lignin for the formulation of bioadhesives and to test its viability as a precursor.

## 5.5. Annexes Chapter 5

**Table A5.1.** Activity loss (%) in enzymatic treatment of organosolv lignin by *T. versicolor* laccase at 5 mL scale under different conditions.

| Evaluated condition | Value | Activity loss (%) |
|---|---|---|
| **pH** | 3 | 74.8 |
| | 4 | 68.6 |
| | 5 | 10.3 |
| | 6 | 14.2 |
| | 7 | 5.9 |
| **Temperature** | 20 °C | 15.4 |
| | 30 °C | 68.6 |
| | 40 °C | 81.0 |
| **Mediators** | Vanillin | 16.4 |
| | HBT | 45.8 |
| **Lignin solubility** | Tween-80 | 5.8 |
| | PEG-600 | 28.0 |
| **Optimum** | 5 mL tube | 19.8 |
| | 150 mL - reactor | 29.6 |

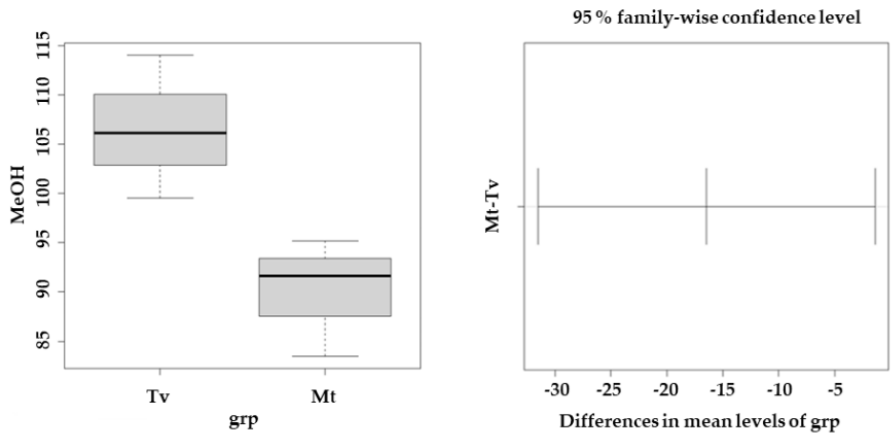



**Figure A5.1.** Statistical analysis (Tukey multiple comparisons, 95 % family-wise level) of methanol released with *M. thermophila* (Mt) and *T. versicolor* (Tv) laccases.

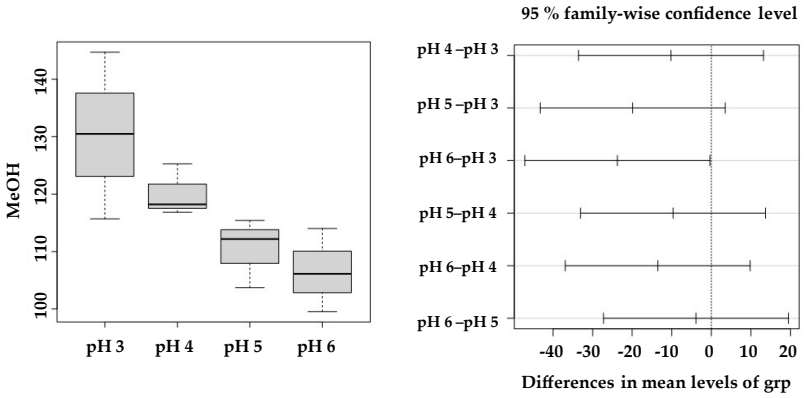



**Figure A5.2.** Statistical analysis (Tukey multiple comparisons, 95 % family-wise level) of methanol released with *T. versicolor* laccase at 30 °C with different acid pH.

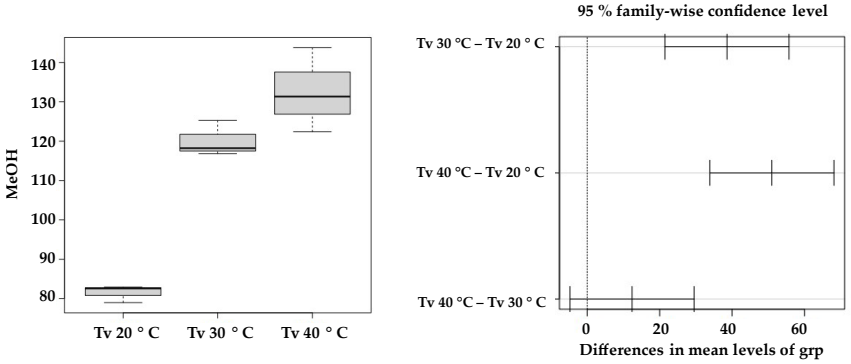



**Figure A5.3**. Statistical analysis (Tukey multiple comparisons, 95 % family-wise level) of methanol released with *T. versicolor* laccase at pH 4 with different temperatures.

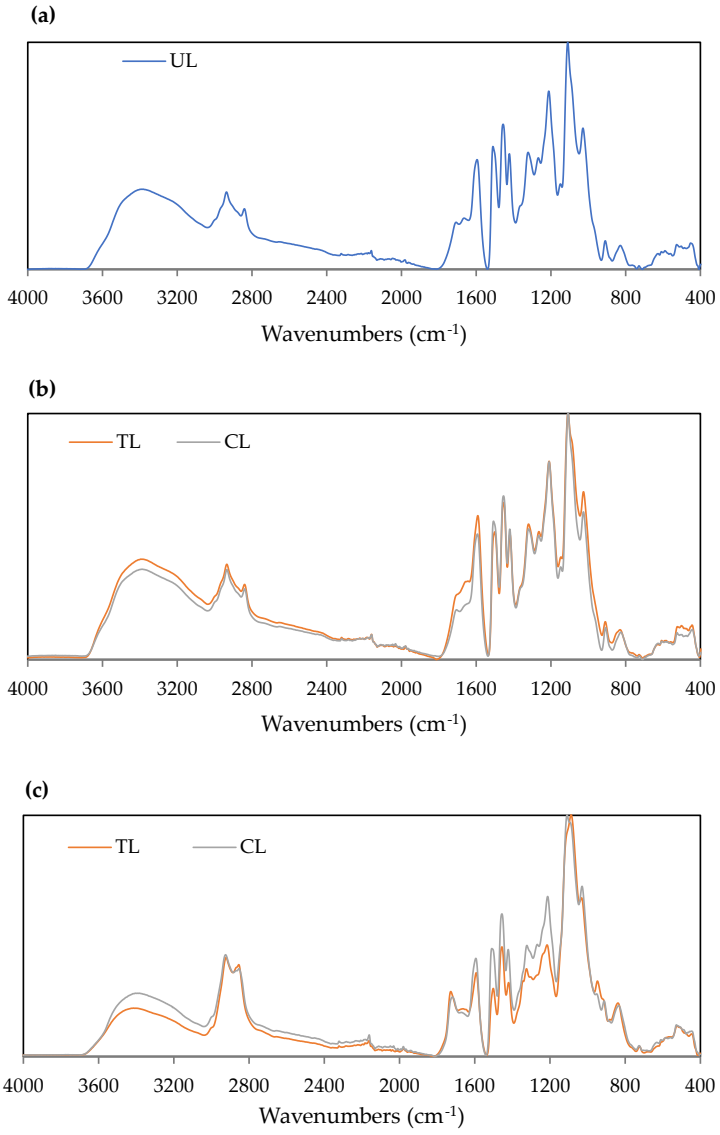



**Figure A5.4.** FT-IR spectra (4000-400 $cm^{-1}$) of: **(a)** untreated organosolv lignin, **(b)** treated lignin after 24 h of enzymatic transformation with *T. versicolor* laccase at pH 4 and 30 °C (TL) and its control with the deactivated enzyme (CL), **(c)** treated lignin after 24 h of enzymatic transformation with *T. versicolor* laccase at pH 4, 30 °C and with addition of Tween-80 (TL) and its control with the deactivated enzyme (CL).

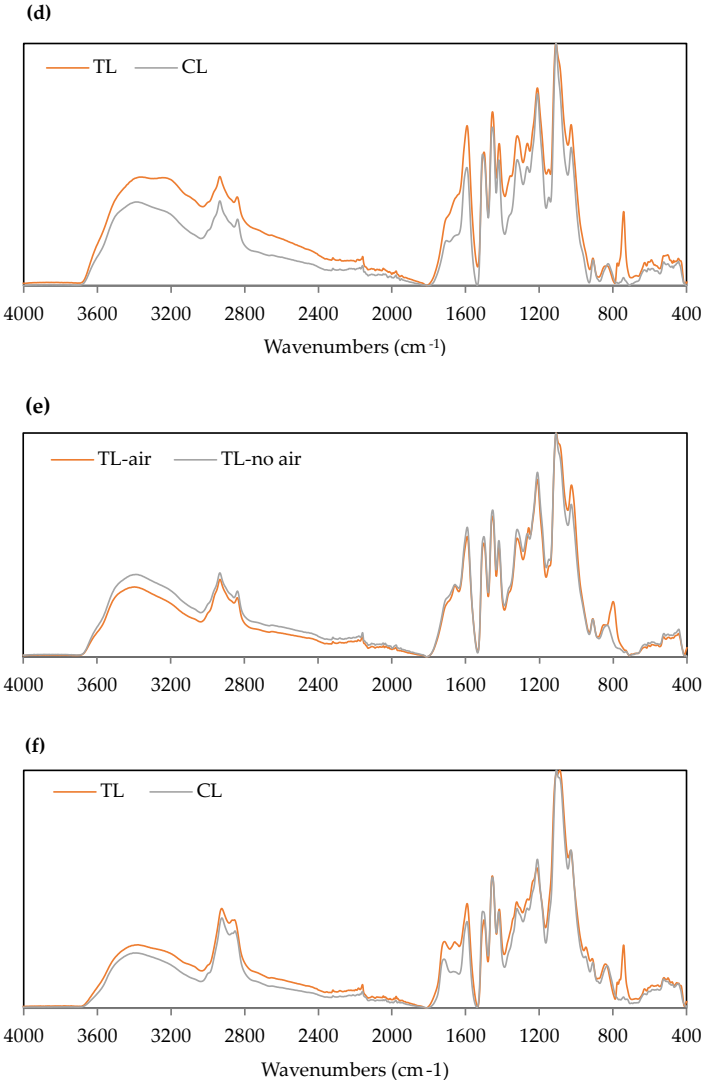



**Figure A5.4-Cont.** FT-IR spectra (4000-400 $cm^{-1}$) of: **(d)** treated lignin after 24 h of enzymatic transformation with *T. versicolor* laccase at pH 4, 30°C and with addition of HBT as mediator (TL) and its control with the deactivated enzyme (CL), **(e)** treated lignin after 24 h of enzymatic transformation with *T. versicolor* laccase at pH 4 and 30 °C and with aeration (TL-air) and without aeration (TL-no air), **(f)** treated lignin after 24 h of enzymatic transformation with *T. versicolor* laccase in the optimal conditions (pH 4, 30 °C, Tween-80, HBT, aeration) at 150 mL reactor (TL) and its control with the deactivated enzyme (CL).

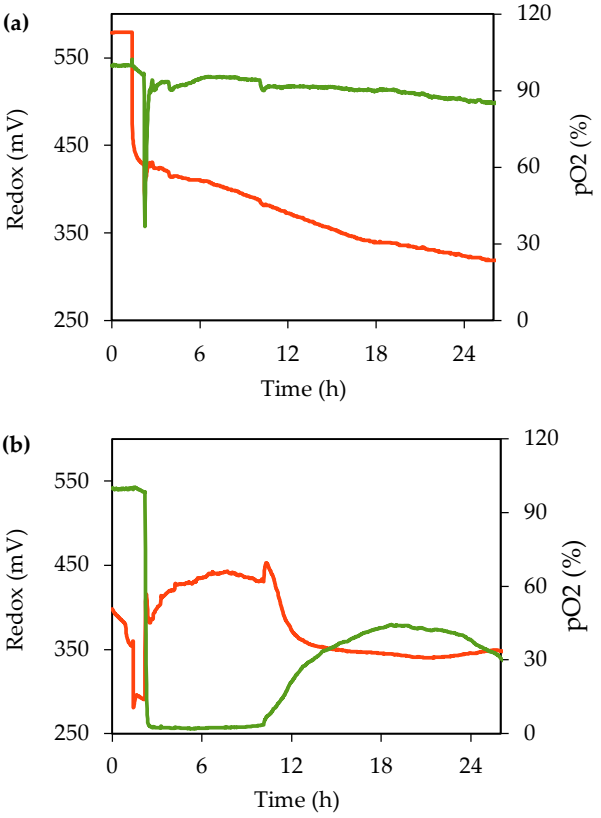



**Figure A5.5.** Redox (orange) and $pO_2$ (green) profiles in experiments at 150 mL-reactor at pH 4 and 30 °C with aeration **(a)** and without aeration **(b)**.

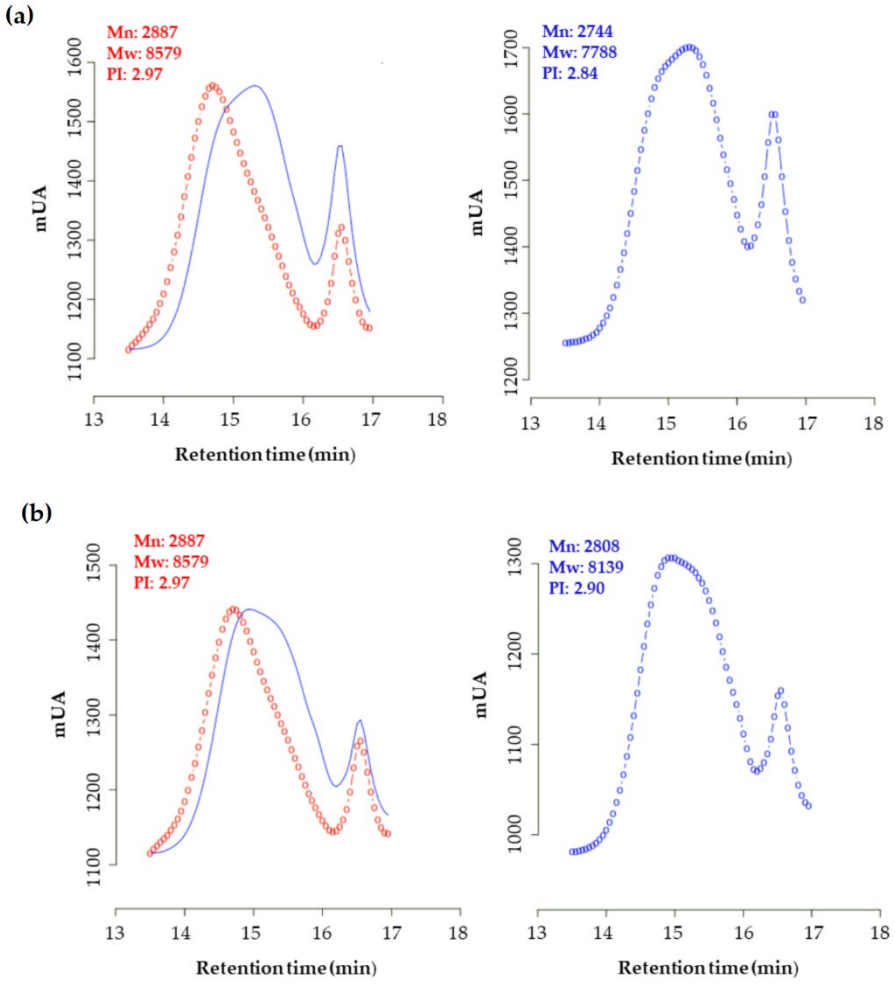



**Figure A5.6.** Comparison of the results obtained by SEC: **(a)** Modified lignin in optimal conditions (red) vs. original lignin (blue); **(b)** Modified lignin in optimal conditions (red) vs. with deactivated laccase (blue).

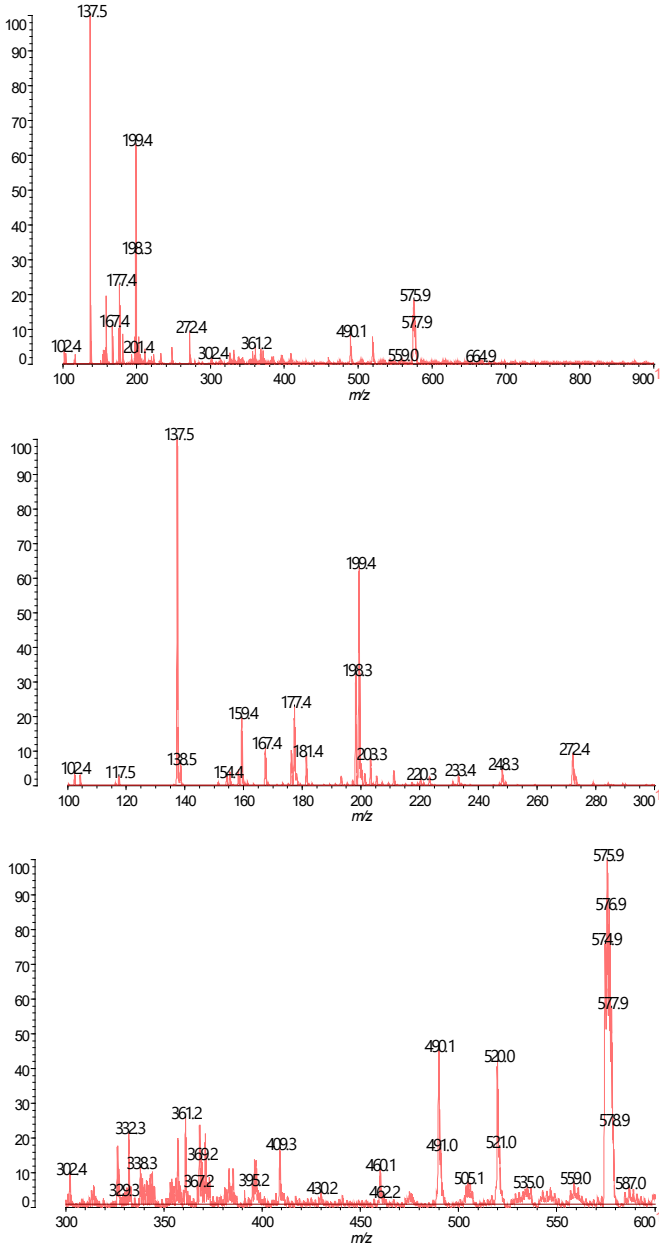



**Figure A5.7.** MALDI-ToF spectra of the original organosolv lignin.

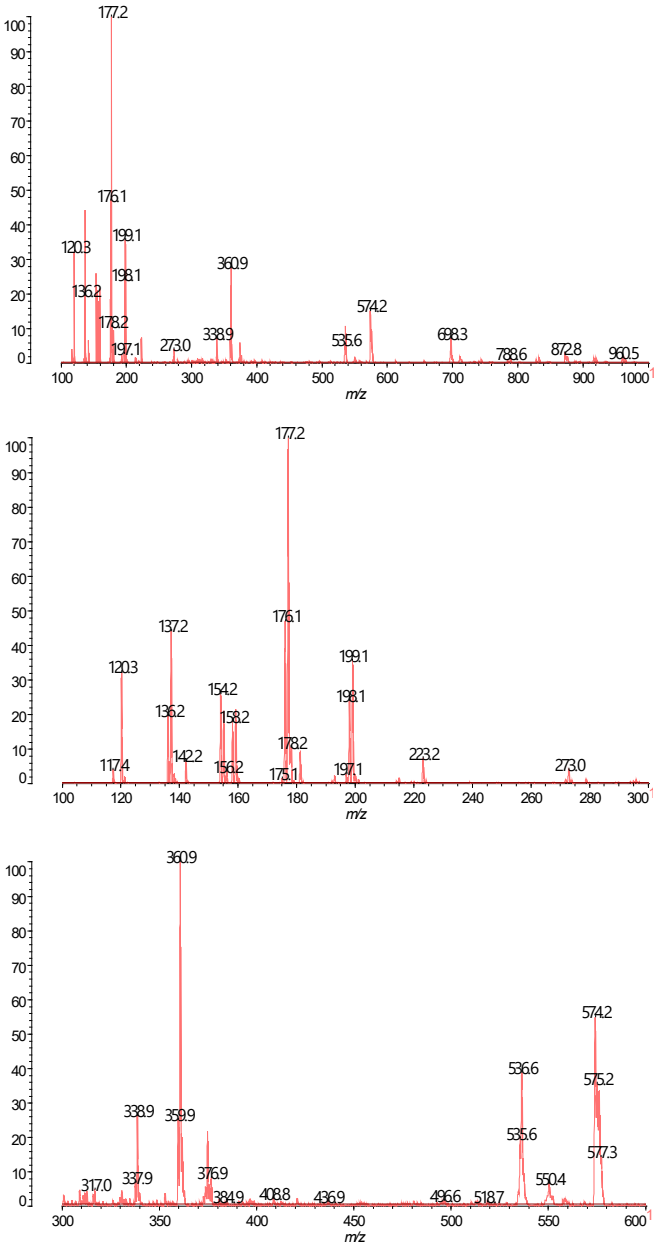



**Figure A5.8.** MALDI-ToF spectra of the lignin modified in optimal conditions at 150 mL-reactor scale.

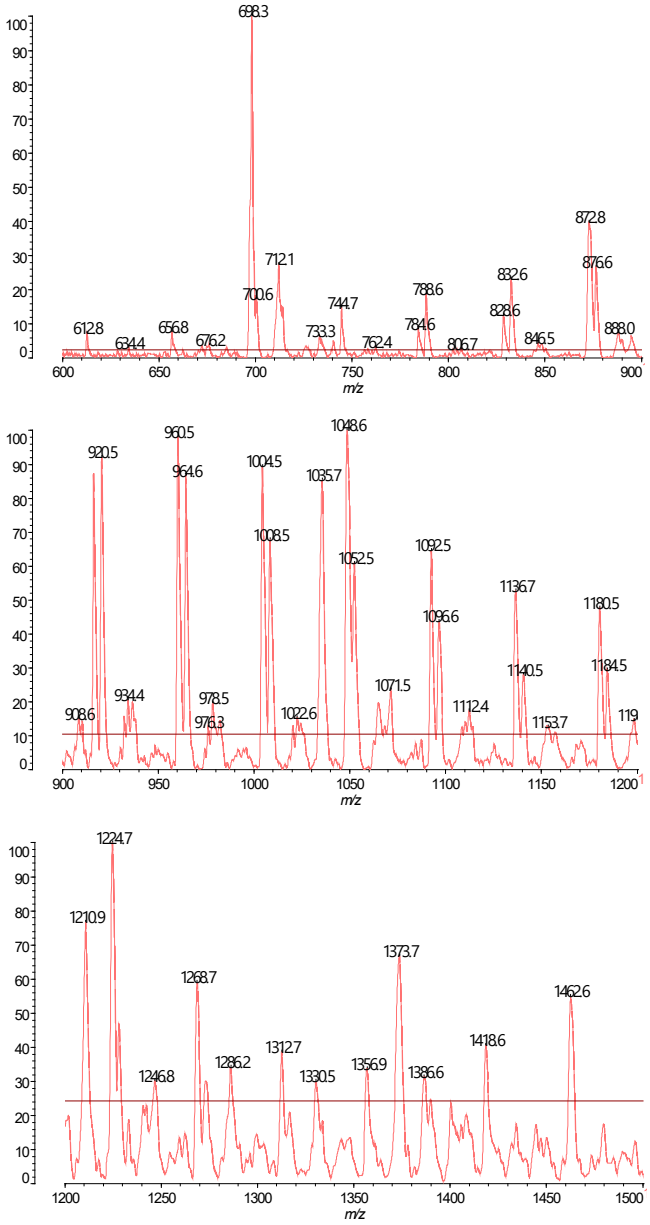



**Figure A5.8.-Cont.** MALDI-ToF spectra of the lignin modified in optimal conditions at 150 mL-reactor scale.

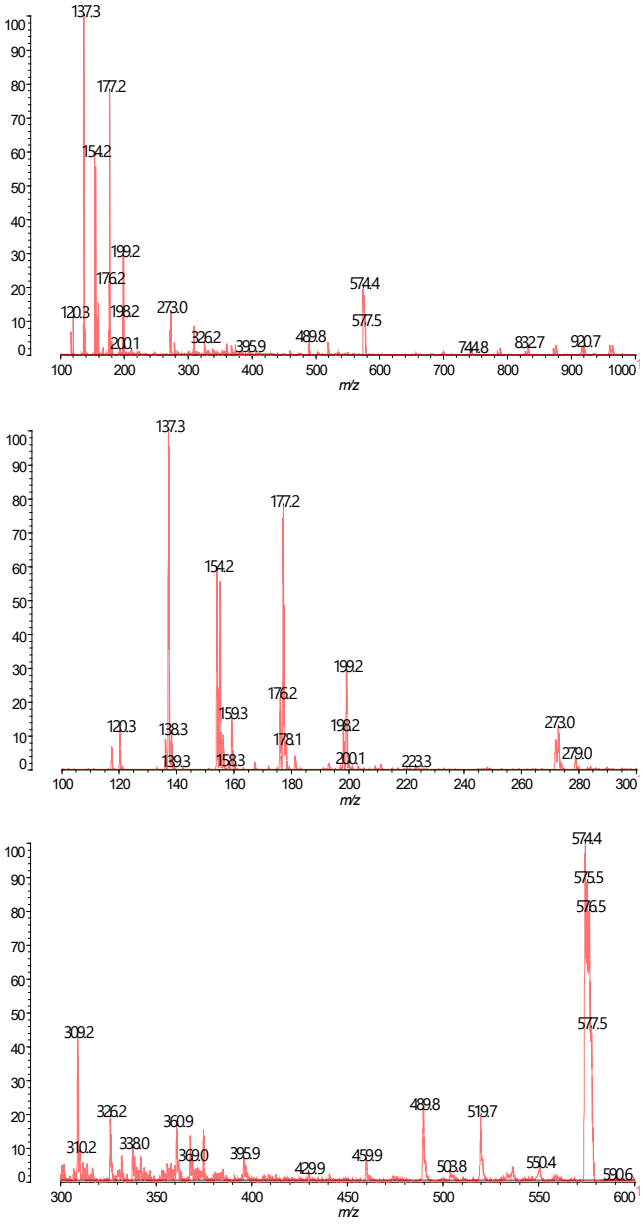



**Figure A5.9.** MALDI-ToF spectra of the control with deactivated enzyme in optimal conditions at 150 mL-reactor scale.

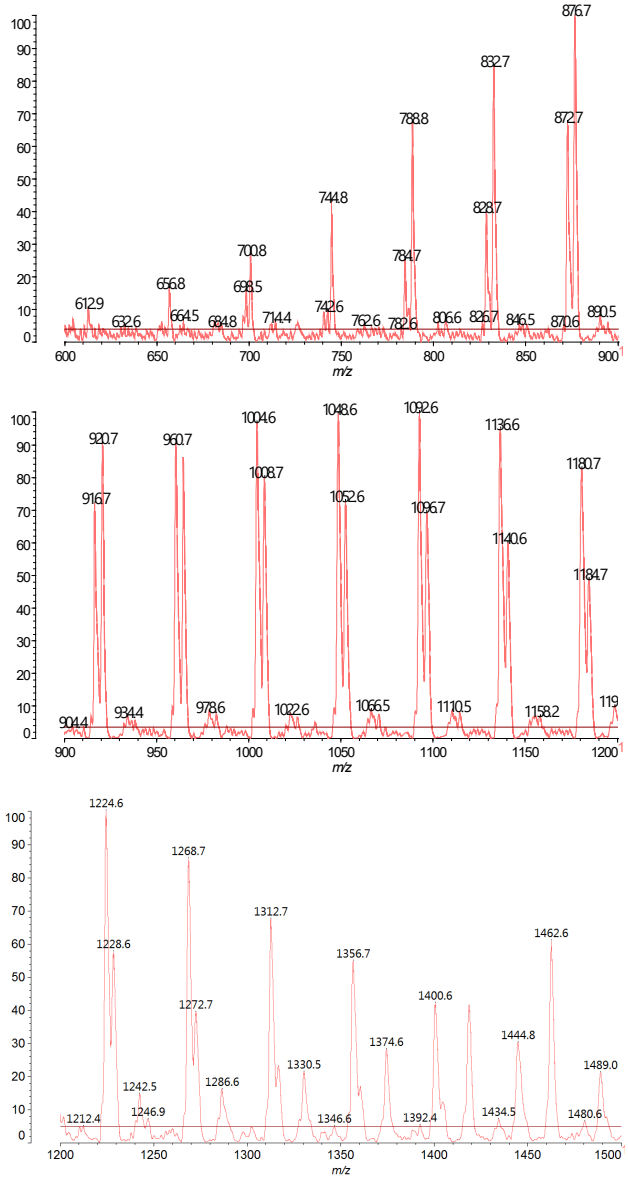



**Figure A5.9.-Cont.** MALDI-ToF spectra of the control with deactivated enzyme in optimal conditions at 150 mL-reactor scale.

# CHAPTER 6

## ENZYMATIC ABATEMENT OF EMERGING CONTAMINANTS

Part of the results of this chapter have already been published as "González-Rodríguez S, Lu-Chau TA, Trueba-Santiso A, Eibes G, Moreira MT. Bundling the removal of emerging contaminants with the production of ligninolytic enzymes from residual streams. Appl Microbiol Biotechnol. 2022 Feb;106(3):1299-1311. https://doi.org/10.1007/s00253-022-11776-7"

Detailed information available at "Manuscripts considered in this thesis: rights, contributions and quality indicators", page 227.

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**SUMMARY**

This chapter addresses the degradation of six emerging contaminants (ECs) with two different enzyme cocktails based on manganese peroxidase (MnP) and unspecific peroxygenase (UPO) to demonstrate the high oxidative capacity of these fungal enzymes. The degradation of several pharmaceuticals and personal care products (PPCPs) (carbamazepine (CBZ), bisphenol A (BPA), estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2)) and the degradation of pyrene as an example of hydrophobic polycyclic aromatic hydrocarbon (PAH) were studied. The most relevant factors influencing enzymatic activity and stability (pH, enzyme dose, co-factor concentration) were evaluated in order to maximise the degradation rate of the pollutants. The enzymatic treatment allowed the complete degradation of 300 μg/L PPCP in less than 1 h for all tested enzymes, with the exception of CBZ, which was degraded up to 30 % after 24 h with the manganese peroxidase (MnP) cocktail produced by *I. lacteus*. Despite these results, it is noted that a significant improvement was achieved compared to previously reported enzymatic treatments for this compound. The degradation of pyrene required longer reaction times, with complete degradation of this compound (100 μg/L) being achieved after 22 h and 3.5 h of treatment with MnP and unspecific peroxygenase (UPO) cocktails, respectively.

After optimisation of EC degradation, the enzymatic reactions were evaluated with radioactively labelled compounds ($^{14}C$) in order to identify possible metabolites obtained during the enzymatic transformation of the selected pollutants (BPA, EE2 and pyrene). Liquid chromatography coupled to mass spectrometry (LC-MS) was used to identify the metabolites produced in the degradation experiments with $^{14}C$-compounds. In addition, mineralisation tests were performed using alkaline traps for the quantification of $CO_2$ released by radioactivity measurements, in order to evaluate whether the enzyme cocktails used were capable of carrying out the mineralisation of the pollutants.

## 6.1. Introduction

### 6.1.1. Emerging contaminants and the role of ligninolytic enzymes as green biocatalysts in their abatement

ECs are in the spotlight due to their negative impact on the environment. There is a substantial literature on the effects of ECs on aquatic organisms such as fish, algae and molluscs (Mehinto et al., 2010; Santos et al., 2010; Vannini et al., 2011). For example, the existence of hormones in the environment has been linked to endocrine disruption in most animals (Li, 2014).

The widespread worldwide use of PPCPs and the generation of PAHs in different industries results in the presence of these compounds and their derivatives in wastewater and natural water resources at concentrations between ng and μg/L (Edokpayi et al., 2016; Tran et al., 2018). This significant presence of ECs in wastewater and natural water courses has led to the search for new processes focused on the degradation of these type of compounds. In this sense, the use of enzymatic treatments offers a sustainable and green alternative to other techniques such as chemical oxidation processes (Heath and Turner, 2022).

Enzymes such as laccases and peroxidases are capable of performing the oxidation of different aromatic and non-aromatic compounds through a radical-catalysed reaction In addition, enzymatic processes have the advantage of avoiding the generation of large amounts of chemical sludge, which is common in chemical treatment processes (Zdarta et al., 2022). Physicochemical characteristics such as acidity, lipophilicity, volatility and adsorption/absorption potential of ECs influence their removal (Luo et al., 2014). For this reason, it is important to evaluate the degradation of different types of compounds by enzymatic treatment, trying to identify those suitable for enzymatic degradation. Factors related to enzymatic activity and stability are crucial in the enzymatic process. In this sense, it is important to evaluate co-factor concentration and/or enzyme dosage and their influence on the degradation efficiency of ECs (Eibes et al., 2006; Taboada-Puig et al., 2016).

Finally, the analysis of the metabolites generated or the effect of the enzymatic treatment on toxicity is key. In this sense, the use of radiolabelled compounds is a versatile tool for monitoring the degradation of ECs and the identification of the metabolites related (Guthi and Therapeutics, 2016).

### 6.1.2. Objectives of this chapter

Given the current scenario of increasing presence of ECs in wastewater and natural water resources, it is crucial to evaluate green technologies to reduce their concentration and toxic effects on the environment. For this reason, the research presented in this chapter aims to evaluate the degradation kinetics of different ECs using different enzyme cocktails and the analysis of their biotransformation under different reaction conditions, including enzyme dose, $H_2O_2$ addition and pH. In addition, a study of the metabolites produced was attempted to understand the metabolic pathways that allow the transformation of the selected ECs.

The present work does not focus on the use of purified enzymes, but on the use of enzyme cocktails with potential application in the degradation of ECs. Under this premise, the objective is to endorse the enzymatic treatment as a competitive technology against other available technologies used in the degradation of ECs due to the simplification of the downstream process and the cost reduction compared to the use of purified enzymes.

## 6.2. Materials and methods

### 6.2.1. Chemicals

CBZ, BPA, E1, E2, EE2, $MnSO_4$, $C_2H_3NaO_2$, $H_2O_2$, 2,6-dimethoxyphenol (DMP) and veratryl alcohol were purchased from Sigma-Aldrich. Citric acid and sodium phosphate were purchased from Panreac. [Ring-$^{14}$C(U)]-BPA (radiochemical purity 98.8 %, 50 mCi/mmol) was purchased from Movarek Inc., [4-$^{14}$C]-EE2 (radiochemical purity >98 %, 51 mCi/mmol) from Schering AG and [4,5,9,10-$^{14}$C]-pyrene (radiochemical purity 100 %, 55 mCi/mmol) from Hartmann Analytic. High flash-point LSL-cocktail for scintillation measurement was purchased from PerkinElmer (Ultima Gold™ XR). Organic solvents used as mobile phase for chromatography analysis were purchased from Panreac and VWR Chemicals.

Commercial UPO (UPO23, ≥25 U/mg protein) purchased from EvoEnzyme was a recombinant enzyme from *Agrocybe aegerita* and expressed in *Pichia pastoris*. MnP cocktail was produced as described in Chapter 3 (section 3.2.2.4), whereas UPO cocktail was obtained from the concentration of the enzyme produced at flask scale (as described in Chapter 4, section 4.2.2.3) in an

Amicon stirred cell (Millipore) using an ultrafiltration membrane with a nominal molecular weight limit of 10 kDa.

### 6.2.2. Experimental design

The degradation of CBZ, BPA, E1, E2 and EE2 by MnP was evaluated under different ranges of $H_2O_2$ (1-10 μM/min) and enzyme doses (50-200 U/L) at pH 4.5 and 20±2 °C. In the removal of these compounds by UPO, the experimental design and modelling were performed by Response Surface Methodology (RSM) with a Box-Behnken model (BBM). The study was designed using three levels (+1, 0, −1) and two factors with three replicates at the center point. As independent variables, pH (4-7) and $H_2O_2$ dose (5-15 μM/h) were selected, while the percentage of degradation at 1 h of reaction was established as a response variable.

The relationships between reaction conditions and evaluated responses (% of degradation of each EC) were fitted to the following second-order polynomial equation (Equation 6.1):

$$y_j=\beta_0+\sum_{i=1}^{3}\beta_i x_i+\sum\sum_{i<j=1}^{3}\beta_{ij}x_i x_j+\sum_{i=1}^{3}\beta_{ii}x_i^2 \qquad \textbf{Eq. 6. 1}$$

y: response variable; $x_i$, $x_j$: independent variables; $\beta_0$, $\beta_i$, $\beta_{ij}$, and $\beta_{ii}$: regression coefficients determined from experimental data

The experimental data were fitted to the equations of the proposed model using the regression analysis function of the data analysis of Microsoft Excel (Microsoft 365) and the quality of the generated model was checked by the coefficient of determination (R2) and the F-test value obtained from the analysis of variance. Multi-response optimisation was carried out with the software STATGRAPHICS Centurion XVI (version 16.1.03) to maximize the degradation rate of E1, E2, EE2 and BPA at 1 h of reaction. The optimal conditions were tested in triplicate, and the measured dependent variables were compared with those predicted by the model.

The experimental conditions tested for hydrophilic ECs removal with UPO were also considered for $^{14}$C-pyrene degradation as a preliminary screening, as these conditions favor the activity or stability of the enzyme.

### 6.2.3. Emerging contaminants degradation

#### *6.2.3.1. Removal with I. lacteus enzyme cocktail*

The enzymatic removal of CBZ, BPA, E1, E2 and EE2 was carried out in batch experiments using the enzyme cocktail produced by *I. lacteus*. These experiments were performed in 100 mL Erlenmeyer flasks sealed with Teflon stoppers, with continuous agitation at 150 rpm (orbital shaker SSM1, Stuart, Stone, UK) and at room temperature (22 ± 2 °C). The reaction mixture (50 mL) was composed of a mixture of the five PPCPs (300 μg/L each), enzymatic crude with MnP activity (50-200 U/L), $MnSO_4$ (1 mM) and sodium malonate (50 mM) at pH 4.5. This pH was selected based on previous work evaluating the degradation of emerging contaminants by peroxidases (Eibes et al., 2011; Méndez-Hernández et al., 2015; Moon and Song, 2012; Taboada-Puig et al., , 2015, 2016).

The reaction was initiated by the continuous addition of $H_2O_2$ (4.3 and 42.9 $H_2O_2$ mM stock) at a rate of 1-10 μM/min (referred to the reaction volume) with a syringe pump (Multi-PhaserTM NE-4000 double syringe pump, New Era Pump Systems Inc) operating at 11.65 μL/min. In addition, controls for each experiment were performed with thermally inactivated enzyme to confirm that $H_2O_2$ cannot degrade PPCPs without enzyme activity. Samples were taken at regular intervals to measure MnP and independent-manganese peroxidase (MiP) activities. The reaction was stopped by adding methanol (50 % v:v) and stored at -20 °C for subsequent analysis of the PPCPs concentration. Each condition was carried out in triplicate.

For the degradation of pyrene, the same experimental design was used as for the degradation of PPCPs but changing the EC concentration to 100 μL/L and the reaction volume to 5 mL. Concentrations of MnP, $MnSO_4$ and sodium malonate were maintained. A GFL 3017 shaker (Burgwedel) was used for continuous stirring of the tubes in which the enzymatic reaction took place at 120 rpm and room temperature (22 ± 2 °C). Samples were analyzed immediately after collection, so no addition of any enzyme activity inhibitor or freezer storage was necessary.

#### *6.2.3.2. Removal with unspecific peroxygenase*

Removal of CBZ, BPA, E1, E2, and EE2 was first conducted with commercial UPO following the experimental design described in section 6.2.2. Once the

optimal conditions were obtained, the concentrated enzyme cocktail produced by *A. aegerita* (described in Chapter 4) and containing UPO and laccase activities, was tested. The enzymatic reactions for the removal of PPCPs were performed at room temperature (22 ± 2 °C) in 12 mL glass tubes placed in an orbital shaker. In this way it is guaranteed a homogeneous reaction medium. The reaction volume was 5 mL with a mixture of the 5 PPCPs (300 μg/L each) and 500 U/L of UPO in citrate-phosphate buffer. Different pHs (4-7) and different $H_2O_2$ doses (5-15 μM/h) added in continuous with a syringe pump (NE-1200 syringe pump, New Era Pump Systems Inc) were evaluated.

Controls without the addition of the enzyme were performed for the maximum $H_2O_2$ dose, in order to evaluate if $H_2O_2$ can be involved in the degradation of the PPCPs. For experiments with *A. aegerita* enzyme cocktail, a control was performed to evaluate only the degradation derived from laccase activity. For this purpose, the experiment was performed under optimal conditions without the addition of $H_2O_2$. During the experiments, UPO activity was measured at regular intervals. In experiments with *A. aegerita* enzymatic cocktail, laccase activity was also monitored. Samples were collected periodically for the subsequent analysis of PPCPs concentration by HPLC. Inactivation of the enzyme was performed by addition of 5 mM sodium azide and samples were stored at -20 °C prior to HPLC analysis.

For pyrene degradation, the same experimental design described for PPCPs degradation was used, but changing the EC concentration to 100 μL/L. The same equipment employed for pyrene degradation with MnP cocktail was used, maintaining the same conditions of agitation and temperature. Samples were immediately analysed by radio-HPLC after withdrawal.

#### *6.2.3.3. Analytical protocols*

Enzyme activities were determined following the protocols described in Chapter 3 (section 3.2.5) and Chapter 4 (section 4.2.4) for MnP, MiP and UPO activity. The concentrations of PPCPs (CBZ, BPA, E1, E2, EE2) were determined by HPLC (Jasco XLC HPLC, Jasco Analitica) equipped with a 3110 MD diode array detector and a reverse phase column (150 × 4.6 mm, particle size: 3 μm) (Gemini, Phenomenex, Jasco Analitica). The operational conditions for PPCPs analysis were: 100 μL injection volume, λ 210 nm, the eluent acetonitrile:$H_2O$ (55:45) and 35 °C. Retention times were 3.3, 4, 4.5, 5

and 5.4 min for CBZ, BPA, E1, E2, EE2, respectively. The quantification limit for all ECs was 10 μg/L. Pyrene concentration was analyzed by radio-HPLC, in the operational conditions explained in section 6.2.4.1. Sample withdrawal and the effect of dilution due to continuous $H_2O_2$ feed were considered when calculating the percentage of degradation.

### 6.2.4. Metabolites analysis

#### *6.2.4.1. Radiochemical analysis*

The monitoring of $^{14}$C-pyrene degradation and the search of possible metabolites of $^{14}$C-BPA, $^{14}$C-E2 and $^{14}$C-pyrene was performed by radio-HPLC, with an Agilent 1100 system in combination with a Ramona Star radio-detector (Elysia Raytest) using a C18 column (150 × 4.6 mm, particle size: 3 μm) (Macherey-Nagel) for $^{14}$C-BPA and $^{14}$C-E2 and a C18 column (125 × 4 mm, particle size: 3 μm) (Macherey-Nagel) for $^{14}$C-pyrene.

Operational conditions for determination of $^{14}$C-BPA and $^{14}$C-EE2 were those described in section 6.2.3.3, with the exception of temperature that was changed to room temperature (20 ± 2 °C). Retention times were 10 min and 14.5 min for $^{14}$C-BPA and $^{14}$C-EE2, respectively. The operational conditions for the determination of pyrene were: 100 μL injection volume, room temperature (20 ± 2 °C), 0.5 mL/min of flow rate and the use of the following eluents: methanol:$H_2O$ (80:20, v:v) (eluent A) and acetonitrile (eluent B). Gradient elution was performed at constant flow of 1.5 mL/min and with the following program: 80 % A and 20 % B for 1.5 min, 100 % B for 4.5 min and 100 % A for 1 min. Data were evaluated using the software Gina Star (Version 6.0, Elysia Raytest, Straubenhardt, Germany). The quantification limits were 10 μg/L for all ECs studied.

Metabolites obtained in $^{14}$C-pyrene degradation by MnP and UPO were isolated using radio-TLC before LC-MS analysis. Initially, 1 mL of the final reaction medium was deposited on silica gel plate (200 × 200 mm, 0.25 mm, Macherey-Nagel) using a semiautomatic sample application device (Linomat 5, CAMAG). Solvent system for metabolites separation was methanol/$H_2O$/acetonitrile (64:16:20). Qualitative analysis was based on the radioactivity determined using a Rita Star radio-TLC scanner (Elysia Raytest). Data were evaluated using an instrument specific software (Gina Star TLC 6).

#### *6.2.4.2. Liquid chromatography coupled to mass spectrometry*

After performing the isolation of $^{14}C$-metabolites obtained during the enzymatic treatment by radio-TLC, the metabolite spot was scratched from the TLC plate and dissolved with 2 mL of methanol. The solution was then centrifuged for 5 min at 4000 rpm to separate the silica particles and recover the supernatant. This centrifugation step was performed 2-3 times until a clarified supernatant was obtained. Once the silica was completely removed from the sample, the vials were bubbled with nitrogen gas to promote evaporation of the methanol and reduce the sample volume to 1 mL.

Final samples were analysed by LC-MS (Agilent 1200 series) equipped with a C18 column for PAH analysis (125 × 4 mm, particle size: 3 μm) (Macherey-Nagel). The operational conditions for $^{14}C$-pyrene metabolites analysis were the following: 50 μL injection volume, and eluents and gradient program were the same used in radio-HPLC for pyrene analysis, but changing flow rate to 0.5 mL/min. This LC system was connected to a Thermo Scientific LTQ XL Iontrap mass spectrometer with an ESI source operated at positive mode and under the following conditions: capillary voltage, 82.5 V, drying gas ($N_2$) 5 L/min and gas temperature 250 °C. Full scan mode was used to identify the possible metabolites.

#### *6.2.4.3. Mineralisation assays*

$CO_2$ released during enzymatic treatment of $^{14}C$-labelled compounds was determined using an alkali trap with 250 μL KOH (1 M) that was inserted in the reaction medium. In order to avoid the contamination of alkali solution with the medium, the reaction volume was decreased to 3 mL. The $CO_2$ stream was absorbed by the KOH solution, which could be analysed later by scintillation measurement to relate the radioactivity of KOH solution with the proportion of $^{14}C$ adsorbed in relation to that present in reaction medium at the beginning of enzymatic treatment. Scintillation measurement was performed using an automatic liquid scintillation counter (Hidex 600 SL, Hidex). For this analysis, 100 μL of sample was mixed with 2 mL of scintillation cocktail and measured for 10 min. This allows an indirect analysis of the percentage of $^{14}C$-compound that was mineralised.

## 6.3. Results and discussion

### 6.3.1. Pharmaceutical and personal care products degradation

#### *6.3.1.1. Removal with manganese peroxidase cocktail*

Enzymatic removal of CBZ, BPA, E1, E2 and EE2 was carried out in batch experiments using the enzyme cocktail produced by *I. lacteus*. Structure and characteristics of these model PPCPs are shown in Table 1.5. Different enzyme activities and $H_2O_2$ concentrations were evaluated, trying to identify the optimal conditions to ensure the highest removal of PPCPs.

None of the controls carried out with inactivated enzyme showed degradation of the compounds (data not shown), which rules out the possibility that $H_2O_2$ alone could transform the PPCPs. Hormones and BPA were completely biodegraded under all the conditions studied. As shown in Figure 6.1, the fastest degradation was achieved with the highest enzyme activity (200 U/L) and the addition of 10 μM/min $H_2O_2$. Under these conditions, the total removal of these compounds was achieved in less than one hour. Biodegradation of CBZ was studied over 24 h, with the maximum degradation between 12.5 (lowest $H_2O_2$ supply) and 28.8 % (highest $H_2O_2$ supply and highest enzyme activity).

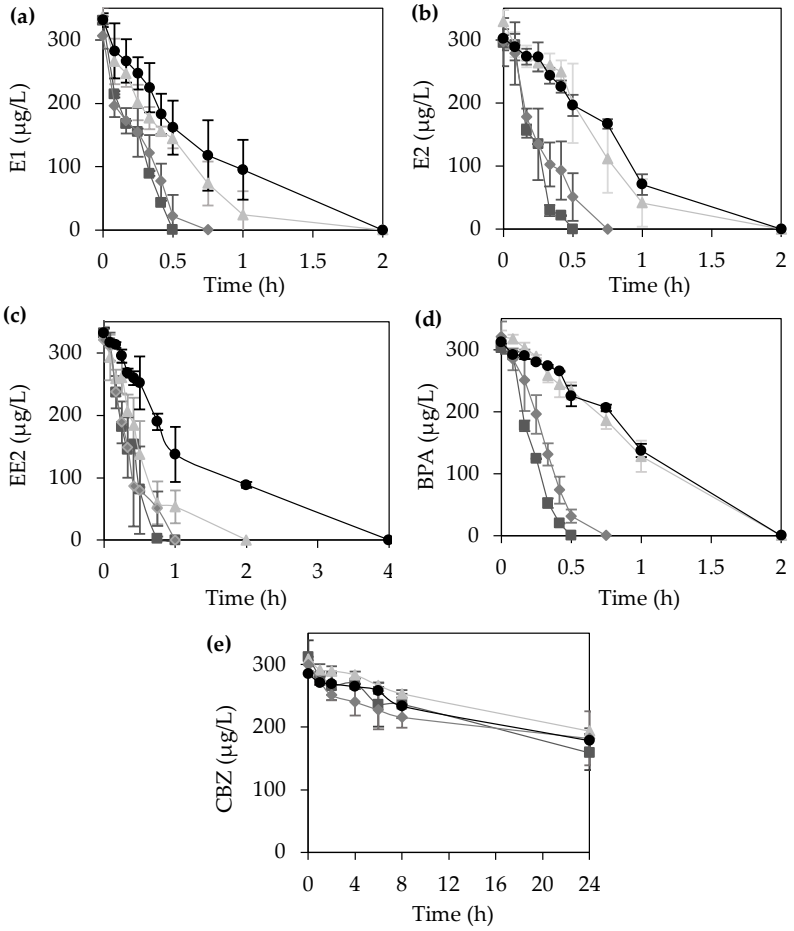


**Figure 6.1.** Time course of PPCPs removal: **(a)** E1 **(b)** E2 **(c)** EE2 **(d)** BPA **(e)** CBZ in experiments with 200 U/L MnP and 10 µM/min $H_2O_2$ (■), 200 U/L MnP and 1 µM/min $H_2O_2$ (▲), 50 U/L MnP and 10 µM/min $H_2O_2$ (♦), 50 U/L MnP and 1 µM/min $H_2O_2$ (●).

Table 6.1 shows the apparent first-order kinetic constants for each PPCP studied. These results show that higher concentrations of $H_2O_2$ improved the biodegradation for all the compounds studied. In fact, the highest apparent kinetic constants (> 2 $h^{-1}$) corresponded to experiments with 10 µM/min $H_2O_2$. Comparing experiments with the same $H_2O_2$ addition rate, decreasing the initial activity from 200 U/L to 50 U/L resulted in decreased apparent kinetic constant values (from 8 % to 55 % lower values). It is also remarkable the high

stability of the enzyme, since the decrease in MnP and MiP activities after 24 h was always lower than 15 %.

The enzymatic cocktail produced is considered a suitable biocatalyst for the degradation of the PPCPs. BPA and hormones were degraded in less than one hour for the highest dose of $H_2O_2$. This shows that $H_2O_2$ concentration is a key factor for enzymatic activity, since when a lower dose was used, twice as much time was needed to obtain complete degradation of these compounds. This correlation between the $H_2O_2$ dose and the improvement of the degradation kinetics of aromatic pollutants with peroxidases has been reported in different studies (Alneyadi et al., 2017; Eibes et al., 2011).

However, $H_2O_2$ concentration should be a parameter to control, since an excess of hydrogen peroxide can lead to irreversible damage of the heme group, leading to enzyme inactivation (Valderrama et al., 2002). Apparently, the feed rates used in the present work did not lead to an excess of $H_2O_2$, since activity losses of less than 13 % were measured after 24 h of reaction. Therefore, the enzyme cocktail could be reused in consecutive cycles or even in continuous process, using an ultrafiltration membrane to retain the enzyme in the reactor (Lloret et al., 2013).

The degradation profiles were similar for BPA, E1 and E2, comparing the same conditions. However, in the case of EE2, despite having a similar structure to the other two hormones, the degradation in some experiments was slower. This could be due to the presence of an ethinyl group in its structure (Table 1.5), which may result in EE2 being more resistant to degradation (Nejedly and Klimes, 2017).

**Table 6.1.** Results of PPCPs degradation by *I. lacteus* cocktail with different enzyme dose and $H_2O_2$ concentration.

| Compound | Initial activity (U/L) | | 24 h activity (U/L) | | $H_2O_2$ (μM/min) | Time (h) | Degradation (%) | k[a] (1/h) | $r^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | MnP | MiP | MnP | MiP | | | | | |
| **E1** | 193 ± 1 | 123 ± 7 | 168 ± 3 | 121 ± 12 | 10 | 0.5 | 100 | 3.5 | 0.95 |
| | 202.± 8 | 143 ± 11 | 188 ± 4 | 134 ± 8 | 1 | 2 | 100 | 1.9 | 0.98 |
| | 48 ± 3 | 30 ± 2 | 48 ± 2 | 26 ± 1 | 10 | 0.75 | 100 | 2.5 | 0.93 |
| | 45 ± 2 | 29 ± 7 | 43 ± 7 | 27 ± 3 | 1 | 2 | 100 | 1.1 | 0.97 |
| **E2** | 193 ± 1 | 123 ± 7 | 168 ± 3 | 121 ± 12 | 10 | 0.5 | 100 | 6.4 | 0.82 |
| | 202 ± 8 | 143 ± 11 | 188 ± 4 | 134 ± 8 | 1 | 2 | 100 | 0.70 | 0.90 |
| | 48 ±3 | 30 ± 2 | 48 ± 2 | 26 ± 1 | 10 | 0.75 | 100 | 3.4 | 0.97 |
| | 45 ± 2 | 29 ± 7 | 43 ± 7 | 27 ± 3 | 1 | 2 | 100 | 0.59 | 0.91 |
| **EE2** | 193± 1 | 123 ± 7 | 168 ± 3 | 121 ± 12 | 10 | 1 | 100 | 2.6 | 0.97 |
| | 202 ± 8 | 143 ± 11 | 188 ± 4 | 134 ± 8 | 1 | 2 | 100 | 1.3 | 0.92 |
| | 48 ± 3 | 30 ± 2 | 48 ± 2 | 26 ± 1 | 10 | 1 | 100 | 2.4 | 0.96 |
| | 45 ± 2 | 30 ± 7 | 43 ± 7 | 27 ± 3 | 1 | 4 | 100 | 0.59 | 0.92 |
| **BPA** | 193 ± 1 | 123 ± 7 | 168 ± 3 | 121 ± 12 | 10 | 0.5 | 100 | 5.2 | 0.91 |
| | 202 ± 8 | 143 ± 11 | 188 ± 4 | 134 ± 8 | 1 | 2 | 100 | 0.63 | 0.90 |
| | 48 ± 3 | 30 ± 2.0 | 48 ± 2 | 26 ± 1 | 10 | 0.75 | 100 | 2.60 | 0.93 |
| | 45 ± 2 | 29 ± 7 | 43 ± 7 | 27 ± 3 | 1 | 2 | 100 | 0.37 | 0.93 |
| **CBZ** | 193 ± 1 | 123 ± 7 | 168 ± 3 | 121 ± 12 | 10 | 24 | 28.8 | 0.03 | 0.96 |
| | 202 ± 8 | 143 ± 11 | 188 ± 4 | 134 ± 8 | 1 | 24 | 12.5 | 0.02 | 0.99 |
| | 48 ± 3 | 30 ± 2.0 | 48 ± 2 | 26 ± 1 | 10 | 24 | 15.1 | 0.02 | 0.83 |
| | 45 ± 2 | 29 ± 7 | 43 ± 7 | 27 ± 3 | 1 | 24 | 12.5 | 0.02 | 0.98 |

[a] Apparent kinetic constant according to first-order kinetics.

Although the antiepileptic CBZ was more recalcitrant than the other compounds, the highest apparent kinetic constant was obtained with the highest addition of peroxide and enzyme. It might be interesting to evaluate degradation at even higher concentrations, although Eibes et al. (2011) reported that there was no significant removal of CBZ upon enzymatic treatment with peroxidases, even increasing the initial versatile peroxidase (VP) activity to 1000 U/L.

In the case of CBZ, longer reaction times are required since a partial degradation of 30 % was achieved after 24 h. To our knowledge, this is the first work evaluating CBZ degradation with crude MnP. Studies performed with VP and lignin peroxidase failed to achieve CBZ degradation or was less than 10 % for reaction times between 2 and 7 h (Eibes et al., 2011; Zhang and Geißen, 2010). The higher degradation achieved in the present study may be due to the presence of dye-decolorizing peroxidase (DyP) and short MnP in the enzyme cocktail, which can act on the same substrates as the main MnP. In this regard, it has been reported that some DyP from white rot fungus (WRF) may also have catalytic efficiency in the oxidation of $Mn^{2+}$ at pH 4.5 (Fernández-Fueyo et al., 2015), which could have a slight impact on the degradation of CBZ. Furthermore, although the catalytic efficiency towards $Mn^{2+}$ of short MnP may be lower than that of other MnPs, it is able to oxidise other substrates (such as ABTS and different phenols) (Fernández-Fueyo et al., 2015).

To achieve complete removal of CBZ, increasing the reaction time and enzyme dose could lead to better removal values of the target compound. If complete removal is not possible with this strategy, enzymatic treatment could be combined with other techniques, such as photocatalytic treatment. In this regard, Calza et al. (2016) found a synergistic effect of photocatalysis and soybean peroxidase (SBP), obtaining a complete degradation of CBZ within 60 min, whereas SBP alone was not effective. However, advanced oxidation processes can result in the transformation of CBZ into compounds with higher toxicity potential (Mohapatra et al., 2014), so any degradation process has to be supported by lower environmental impact and toxicity.

#### *6.3.1.2. Removal with unspecific peroxygenase*

Commercial UPO was used for the removal of CBZ, BPA, E1, E2 and EE2 at 5 mL scale, modifying pH (4-7) and $H_2O_2$ dose (5-15 μM/h) and with a fixed

enzyme activity of 500 U/L of UPO. Control samples were performed in the conditions with higher $H_2O_2$ dose (15 μM/h) and without enzyme addition. In the control experiments, no degradation of the ECs studied was observed (data not shown). In the case of the enzymatic transformation of CBZ, no degradation was observed at any of the pH and $H_2O_2$ dosage conditions evaluated, so this compound, although present in the reaction mixture, was excluded from the figures in this section.

Figure 6.2 shows the response surfaces obtained for each PPCP (E1, E2, EE2 and BPA) as a function of the two independent variables selected (pH and $H_2O_2$ dose). In general, for all the PPCPs studied, the highest removal was achieved at pH values of 4 and 7 with an intermediate $H_2O_2$ dose (around 10 μM/h) , with the exception of EE2, which required a lower $H_2O_2$ dose, close to 5 μM/h. Degradation percentages higher than 100 % are related to those conditions where the pollutants were completely removed in reaction periods shorter than 1 h (Figure 6.3-Figure 6.5).

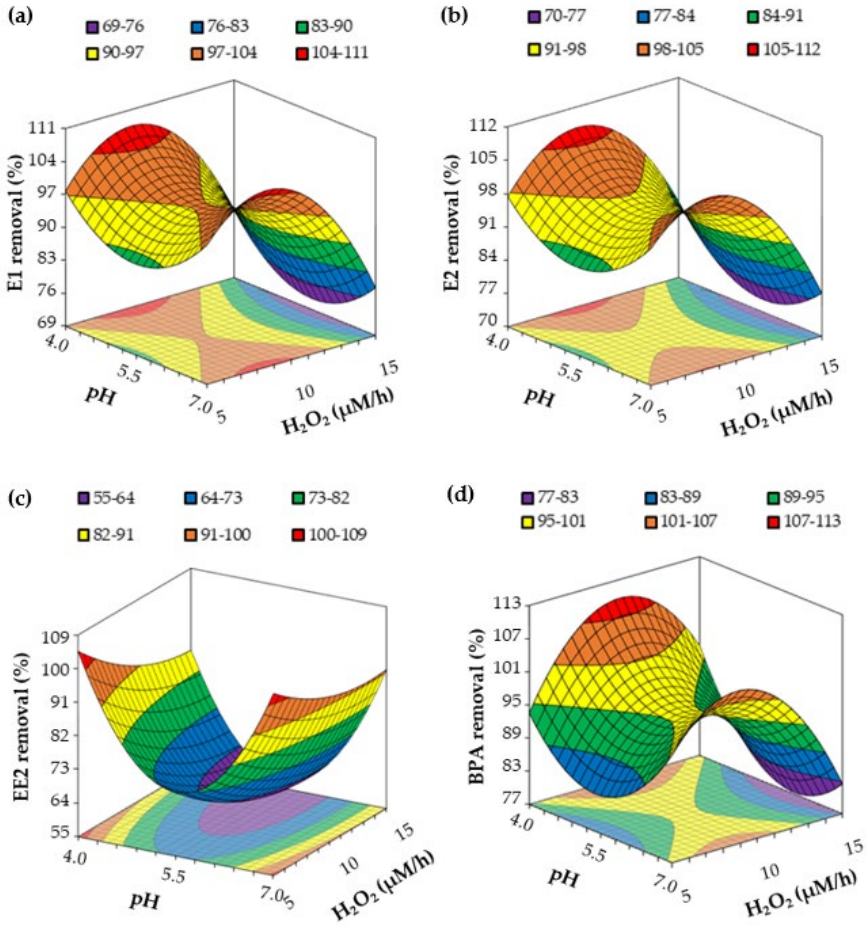


**Figure 6.2.** Response surfaces for PPCPs removal in 1 h reaction: **(a)** E1, **(b)** E2, **(c)** EE2 and **(d)** BPA.

The fit of the experimental data to the proposed model was evaluated. Table 6.2 shows the regression coefficients obtained for Equation 6.1 of each dependent variable. Attending to the coefficients of determination, the model fits well to EE2 biotransformation ($R^2 = 0.91$), but for the rest of the PPCPs studied, the strength of the model was poorer, resulting in a $R^2$ of only 0.71 for BPA.

**Table 6.2.** Regression coefficients and statistical parameters measuring the correlation of the models.

| Coefficient | E1 or $y_1$ (%) | E2 or $y_2$ (%) | EE2 or $y_3$ (%) | BPA or $y_4$ (%) |
|---|---|---|---|---|
| Intercept ($\beta_0$) | 96.08 | 96.18 | 60.76 | 95.54 |
| pH ($\beta_1$) | -0.84 | -1.40 | 1.02 | -2.90 |
| $H_2O_2$ ($\beta_2$) | 8.44 | 7.75 | 7.27 | 2.67 |
| pH·$H_2O_2$ ($\beta_{12}$) | 1.26 | 2.10 | -1.54 | 4.35 |
| $pH^2$ ($\beta_{11}$) | 9.81 | 9.56 | 32.10 | 11.16 |
| $H_2O_2^2$ ($\beta_{22}$) | -16.19 | -15.48 | 4.05 | -14.39 |
| $R^2$ | 0.83 | 0.83 | 0.91 | 0.71 |

Figure 6.3 shows the degradation profiles for experiments at pH 4 and different $H_2O_2$ doses. Experiments with $H_2O_2$ doses of 10 and 15 μM/h presented similar degradations rates for BPA and EE2, whereas degradation of hormones E1 and E2 was more favorable considering a $H_2O_2$ dose of 10 μM/h.

As described for MnP, high level of $H_2O_2$ can lead to UPO oxidative inactivation (Beltrán-Nogal et al., 2022). Only a few studies evaluated catalytic reactions based on UPO as biocatalyst and an external $H_2O_2$ supply (Bormann et al., 2020; Karich et al., 2017), so it is difficult to estimate the $H_2O_2$ concentration that maximises UPO performance avoiding its oxidative inactivation, because this rate also depends on the type to substrate. Most reported studies dealing with UPO catalysis employ *in situ* generation of $H_2O_2$, either by enzymatic generation, a photocatalytic system or electrochemical reactions, to ensure a progressive addition of this co-factor and avoid high concentrations in the medium that may affect enzyme activity (Burek et al., 2019; Karmee et al., 2009; Kiebist et al., 2017). However, although these systems allow control of $H_2O_2$ levels in the reaction medium, they can also come with several drawbacks, such as the requirement of high pressures and temperatures, reliable pH control, or increased power consumption (Freakley et al., 2019). The lower dose of $H_2O_2$ (5 μM/h) seems to not be sufficient for the concentration of enzyme considered, since under these conditions the removal of all PPCPs studied required between 1-2 h of enzymatic reaction.

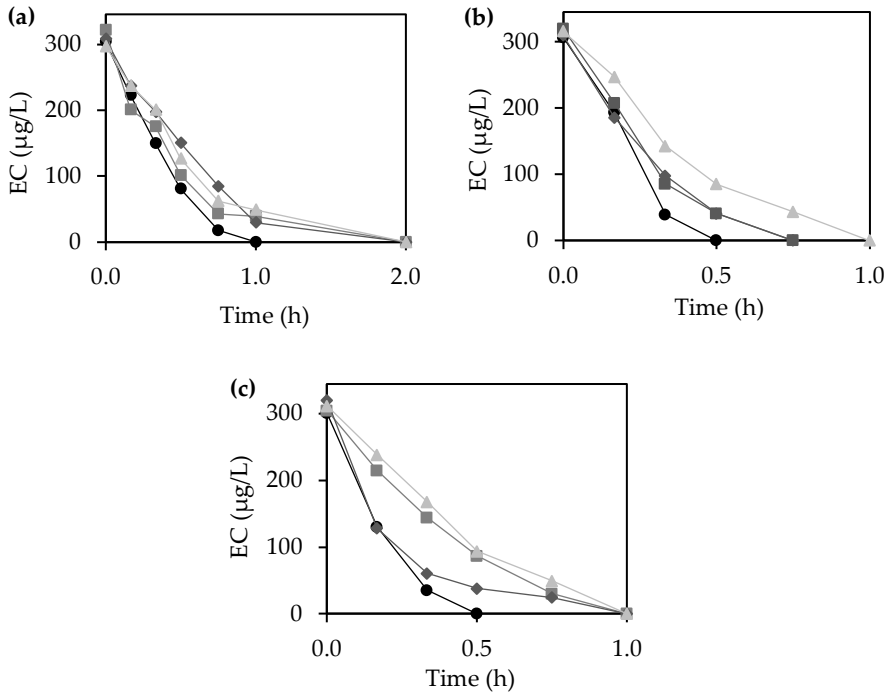


**Figure 6.3.** Time course of removal for the target pollutants: E1 (■) E2 (♦) EE2 (▲) and BPA (•) in experiments at pH 4 with 500 U/L of UPO and $H_2O_2$ dose of **(a)** 5 µM/h, **(b)** 10 µM/h and **(c)** 15 µM/h.

Removal of PPCPs at pH 5.5 (Figure 6.4) required longer times of treatment in comparison with the degradation at pH 4. For the lowest and highest $H_2O_2$ the complete removal of BPA, E1, E2 and EE2 was achieved after 2 h of reaction, whereas with a dose of 10 µM/h of $H_2O_2$ all contaminants were completely removed in 1 h, with the exception of EE2, which required 2 h of treatment.

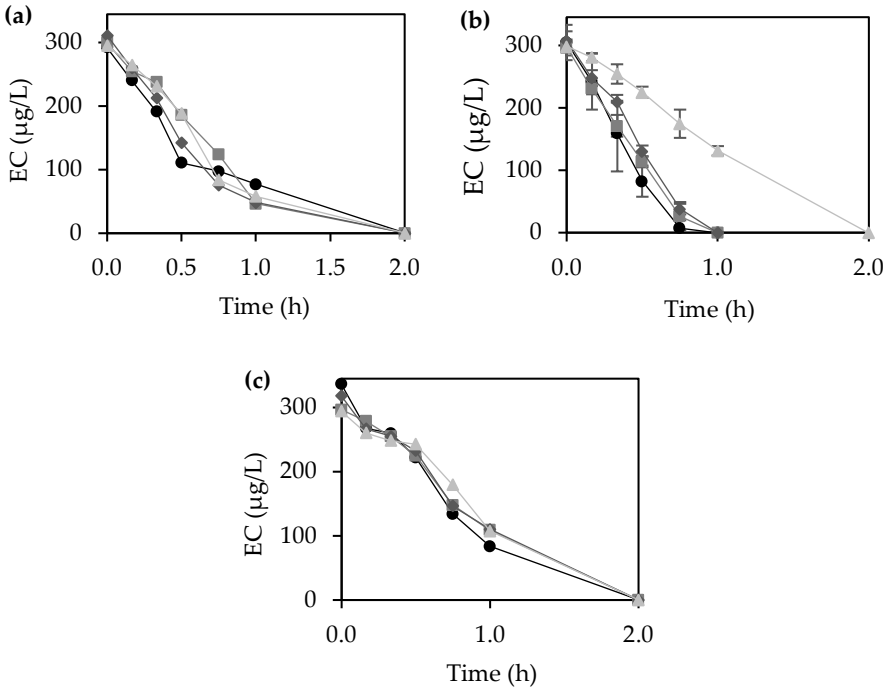


**Figure 6.4.** Time course of removal for the target pollutants: E1 (■) E2 (♦) EE2 (▲) and BPA (•) in experiments at pH 5.5 with 500 U/L of UPO and $H_2O_2$ dose of **(a)** 5 µM/h, **(b)** 10 µM/h and **(c)** 15 µM/h.

Regarding the degradation of PPCPs at neutral pH, Figure 6.5 shows the degradation profiles with UPO at pH 7. The highest dose of $H_2O_2$ resulted in complete removal of ECs in the shortest time reaction. In this sense, removal of BPA took 0.5 h, as observed at pH 4, but the other contaminants were completely removed after 0.75 h of treatment at pH 7, while it required 1 h at pH 4. Nevertheless, pH 4 seems to favor biotransformation for the other $H_2O_2$ doses tested.

UPO activity is highly dependent on the pH value of the reaction medium, which directly influences the removal performance of PPCPs. In general, acidic pH values improve peroxidative activity, whereas neutral pH values enhance peroxygenase activity. Kimani (2019) studied the effect of different pH (3-10) on peroxygenase activity of *Psathyrella aberdarensis* UPOs at 25 °C, finding that neutral pH favor the maintenance of activity, with only 10-15 % of activity loss after 8 h of incubation. This agrees with the trend observed

here, where neutral pH values favor UPO stability (Table 6.3, Table 6.4 and Table 6.5). Experiments at pH 7 and 5.5 showed a loss of activity of 10.6 % after 2 h of reaction, whereas at pH 4 the loss of UPO activity achieved values near to 100 %. In addition, the lowest $H_2O_2$ dose is associated with a low loss of activity.

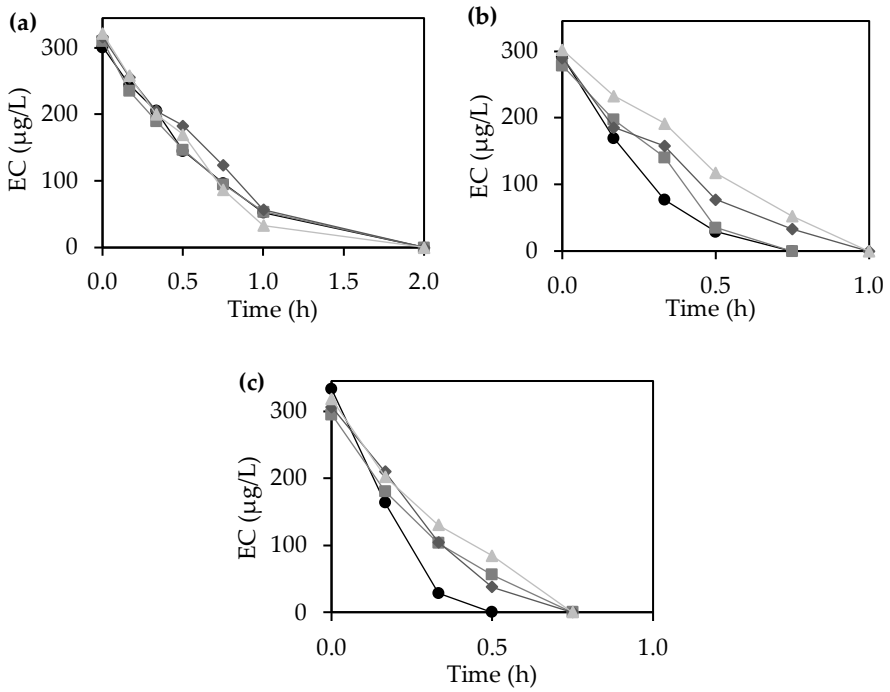


**Figure 6.5.** Time course of removal for the target pollutants: E1 (■) E2 (♦) EE2 (▲) and BPA (•) in experiments at pH 7 with 500 U/L of UPO and $H_2O_2$ dose of **(a)** 5 µM/h, **(b)** 10 µM/h and **(c)** 15 µM/h.

The time required for complete BPA degradation with MnP and UPO was shorter than that needed for the rest of PPCPs studied. Probably, the higher affinity is related to BPA structure, which is formed by two monohydroxylated aromatic rings (Table 1.5). The O-substituents of aromatic rings can lead to an increase of reactivity due to electron delocalisation. In the case of hormones, their structure only present one monohydroxylated aromatic ring in comparison with BPA structure.

Table 6.3, Table 6.4 and Table 6.5 present the kinetic constant values obtained with hydrogen peroxide doses of 5, 10 and 15 μM/h, respectively. Experiments under pH 4 and 7 led to the highest kinetic constant values, which agrees with the shorter time required for complete removal of PPCPs studied. This trend is especially noteworthy at medium a maximum $H_2O_2$ doses, so it can be concluded that concentrations of 5 μM/h or lower can negatively affect the performance of UPO as biocatalyst.

Moreover, at pH 4 experiments, although the kinetic constant was high, two different trends in the slope of the removal course figures were observed (Figure 6.3). This is probably linked to the activity loss observed after 2 h of reaction at pH 4, in comparison to the higher enzymatic stability in more basic pH values.

**Table 6.3.** Kinetic constants for complete PPCPs removal at different pH and a $H_2O_2$ dose of 5 μM/h.

| Compound | Initial UPO (U/L) | UPO 2 h (U/L) | pH | Time (h) | k[a] (1/h) | $r^2$ |
|---|---|---|---|---|---|---|
| **E1** | 490 | 95 | 4 | 2 | 2.1 | 0.90 |
| | 558 | 558 | 5.5 | 2 | 0.91 | 0.95 |
| | 564 | 549 | 7 | 2 | 1.5 | 0.99 |
| **E2** | 490 | 95 | 4 | 2 | 1.4 | 0.99 |
| | 558 | 558 | 5.5 | 2 | 1.5 | 0.96 |
| | 564 | 549 | 7 | 2 | 1.1 | 0.98 |
| **EE2** | 490 | 95 | 4 | 2 | 1.6 | 0.99 |
| | 558 | 558 | 5.5 | 2 | 0.89 | 0.98 |
| | 564 | 549 | 7 | 2 | 1.3 | 0.99 |
| **BPA** | 490 | 95 | 4 | 1 | 2.6 | 0.98 |
| | 558 | 558 | 5.5 | 2 | 1.9 | 0.93 |
| | 564 | 549 | 7 | 2 | 1.4 | 0.98 |

[a] Apparent kinetic constant according to first-order kinetics.

**Table 6.4.** Kinetic constants for complete ECs removal at different pH and a $H_2O_2$ dose of 10 μM/h.

| Compound | Initial UPO (U/L) | UPO 2 h (U/L) | pH | Time (h) | k[a] (1/h) | $r^2$ |
|---|---|---|---|---|---|---|
| **E1** | 516 | 2 | 4 | 0.75 | 4.3 | 0.98 |
| | 537 ± 10 | 480± 9 | 5.5 | 1 | 1.9 | 0.99 |
| | 524 | 484 | 7 | 0.75 | 4.0 | 0.87 |
| **E2** | 516 | 2 | 4 | 1 | 4.0 | 0.99 |
| | 537 ± 10 | 480± 9 | 5.5 | 1 | 1.6 | 0.94 |
| | 524 | 484 | 7 | 1 | 2.5 | 0.94 |
| **EE2** | 516 | 2 | 4 | 1 | 2.7 | 0.98 |
| | 537 ± 10 | 480± 9 | 5.5 | 2 | 0.57 | 0.98 |
| | 524 | 484 | 7 | 1 | 1.8 | 0.96 |
| **BPA** | 516 | 2 | 4 | 0.75 | 6.2 | 0.91 |
| | 537 ± 10 | 480± 9 | 5.5 | 1 | 2.6 | 0.95 |
| | 524 | 484 | 7 | 0.75 | 4.6 | 0.99 |

[a] Apparent kinetic constant according to first-order kinetics.

**Table 6.5.** Kinetic constants for complete ECs removal at different pH and a $H_2O_2$ dose of 15 μM/h.

| Compound | Initial UPO (U/L) | UPO 2 h (U/L) | pH | Time (h) | k[a] (1/h) | $r^2$ |
|---|---|---|---|---|---|---|
| **E1** | 504 | 37 | 4 | 1 | 2.5 | 0.99 |
| | 558 | 506 | 5.5 | 2 | 0.55 | 0.98 |
| | 524 | 472 | 7 | 0.75 | 3.3 | 0.99 |
| **E2** | 504 | 37 | 4 | 1 | 4.3 | 0.98 |
| | 558 | 506 | 5.5 | 2 | 0.59 | 0.94 |
| | 524 | 472 | 7 | 0.75 | 4.2 | 0.96 |
| **EE2** | 504 | 37 | 4 | 1 | 2.4 | 0.97 |
| | 558 | 506 | 5.5 | 2 | 0.37 | 0.88 |
| | 524 | 472 | 7 | 0.75 | 2.7 | 0.99 |
| **BPA** | 504 | 37 | 4 | 0.5 | 6.4 | 0.98 |
| | 558 | 506 | 5.5 | 2 | 0.37 | 0.93 |
| | 524 | 472 | 7 | 0.5 | 7.4 | 0.94 |

[a] Apparent kinetic constant according to first-order kinetics.

In view of the obtained kinetic constants, it can be concluded that the conditions that maximise ECs kinetics were pH 4 and a dose of 10 μM/h $H_2O_2$ for E1, pH 4 and 15 μM/h $H_2O_2$ for E2 and pH 7 and 10 μM/h $H_2O_2$ for BPA. In the case of EE2, conditions of pH 7 and 15 μM/h $H_2O_2$ and pH 4 and 10 μM/h $H_2O_2$ provided similar kinetic constants.

To maximise the degradation of the ECs studied, the optimal degradation conditions were calculated based on the percentage of removal at 1 h of reaction. Data processing in StatGraphics showed that the optimal $H_2O_2$ dose to increase the percentage degradation of the ECs was 14 μM/h and pH 7. The total elimination results obtained in the experimental evaluation of these optimal conditions agreed to those predicted (100-102 %).

As observed in Figure 6.6, the complete degradation of all PPCPs was achieved in 45 min of reaction, and BPA once again showed the highest kinetic constant (Table 6.6), which is double those obtained for the rest of the PPCPs studied. Furthermore, in relation to the results obtained in the initial evaluation of the degradation of the PPCPs, the optimum conditions led to an improvement of all the kinetic constants, especially for EE2, with an increase of 40 % in comparison with the kinetic constant previously achieved (in pH 7 and with 15 μM/h $H_2O_2$).

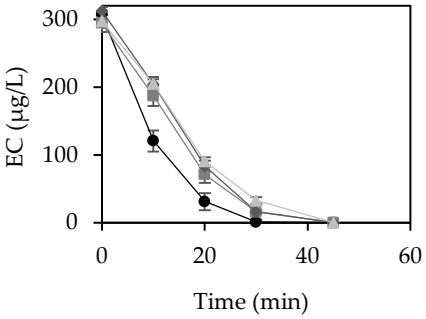


**Figure 6.6.** Time course of removal for the target pollutants: E1 (■) E2 (♦) EE2 (▲) and BPA (•) in optimal conditions (pH 7 and 14 μM/h) with commercial UPO.

**Table 6.6.** Kinetic constants for complete PPCPs removal in optimal conditions (pH 7, 14 μM/h $H_2O_2$) with commercial UPO.

| Compound | Initial UPO (U/L) | UPO 2 h (U/L) | Time (h) | $k^a$ (1/h) | $r^2$ |
|---|---|---|---|---|---|
| **E1** | 521 ± 8 | 448 ± 61 | 0.75 | 5.8 | 0.96 |
| **E2** | 521 ± 8 | 448 ± 61 | 0.75 | 5.9 | 0.93 |
| **EE2** | 521 ± 8 | 448 ± 61 | 0.75 | 4.5 | 0.96 |
| **BPA** | 521 ± 8 | 448 ± 61 | 0.75 | 11.7 | 0.90 |

[a] Apparent kinetic constant according to first-order kinetics.

Upon testing the experimental model for the degradation of PPCPs and the optimal values to maximise the degradation of the compounds, the efficiency

of the cocktail produced by *A. aegerita* was tested under the optimal conditions of pH 7 and 14 $H_2O_2$ μM/h (Figure 6.7 and Table 6.7). As the enzymatic cocktail produced by *A. aegerita* also shows laccase activity, a control in absence of hydrogen peroxide, and then, lacking UPO activity, was carried out (Figure 6.7-b and data in grey in Table 6.7).

The time required to achieve complete degradation of BPA and hormones was similar to that required in experiments with commercial enzyme. In the case of BPA, the time required for its complete elimination was even reduced to 30 min (45 min with commercial UPO). Hence, the use of the enzymatic cocktail produced by *A. aegeri*ta may be more efficient in the elimination of the PPCPs than the commercial enzyme, probably due to the joint action of UPO and laccase. The control experiment with only laccase acting as biocatalyst, showed that 2 h were required for the complete elimination of all contaminants (Figure 6.7-b).

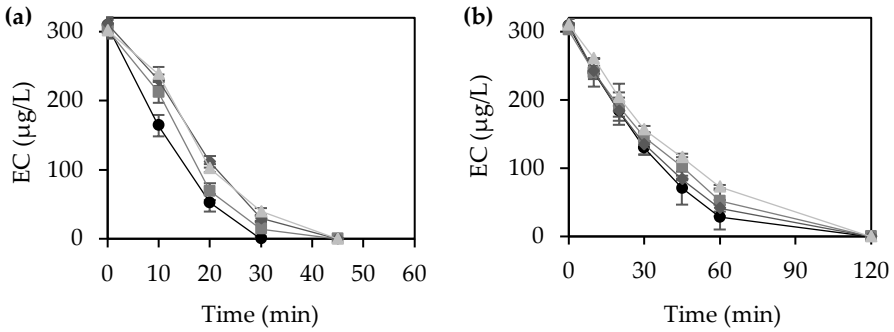


**Figure 6.7.** Time course of removal for the target pollutants: E1 (■) E2 (♦) EE2 (▲) and BPA (•) in optimal conditions (pH 7 and 14 $H_2O_2$ μM/h) with UPO enzyme cocktail produced by *A. aegerita*. **(a)** degradation with joint effect of UPO and laccase **(b)** degradation derived from laccase activity (experiment without $H_2O_2$ supply).

**Table 6.7.** Kinetic constants for complete PPCPs removal in optimal conditions (pH 7 and 14 $H_2O_2$ μM/h) with enzyme cocktail produced by *A. aegerita* (in black data of experiment with $H_2O_2$ supply and in grey data of experiments without $H_2O_2$ supply).

| Compound | Initial UPO (U/L) | UPO 2 h (U/L) | Initial lac (U/L) | Lac 2 h (U/L) | Time (h) | k[a] (1/h) | $r^2$ |
|---|---|---|---|---|---|---|---|
| **E1** | 499 ± 53 | 438 ± 18 | 358 ± 32 | 371 ± 42 | 0.5 | 6.2 | 0.93 |
| | 510 ± 22 | 524 ± 34 | 381 ± 22 | 413 ± 19 | 2 | 1.5 | 0.99 |
| **E2** | 499 ± 53 | 438 ± 18 | 358 ± 32 | 371 ± 42 | 0.75 | 4.7 | 0.92 |
| | 510 ± 22 | 524 ± 34 | 381 ± 22 | 413 ± 19 | 2 | 1.7 | 0.99 |
| **EE2** | 499 ± 53 | 438 ± 18 | 358 ± 32 | 371 ± 42 | 0.75 | 4.2 | 0.95 |
| | 510 ± 22 | 524 ± 34 | 381 ± 22 | 413 ± 19 | 2 | 1.4 | 0.99 |
| **BPA** | 499 ± 53 | 438 ± 18 | 358 ± 32 | 371 ± 42 | 0.75 | 5.3 | 0.97 |
| | 510 ± 22 | 524 ± 34 | 381 ± 22 | 413 ± 19 | 2 | 1.6 | 0.99 |

[a] Apparent kinetic constant according to first-order kinetics.

Considering that the degradation rates under optimal conditions were similar for commercial and laboratory-produced UPO, it can be concluded that the use of this enzyme cocktail is comparable to the use of commercial enzymes.

### *6.3.1.3. Metabolites identification*

Batch experiments were performed with $^{14}C$-labelled BPA and EE2 trying to identify the metabolites obtained during enzymatic treatment with MnP and UPO. Nevertheless, although the complete degradation of the original compound was observed after 1 h of enzymatic treatment, it was not detected any radioactive signal at the end of the experiment that could be related to a transformation product (Figure A6.1 to Figure A6.4).

In addition, different mineralisation assays were addressed to study if the enzymatic treatment allows the transformation of the target contaminants in $CO_2$. Mineralisation of contaminants is of particular relevance because it is the best way to ensure a reduction in the toxicity derived from these compounds. In general, it is assumed that the degradation of an EC leads to a reduction in toxicity, but the transformation products may be more toxic than the parent compound (Lambropoulou and Nollet, 2014).

Taking into account that $CO_2$ generated from $^{14}C$-labelled compounds will be formed by $^{14}C$ atoms, it is possible to follow the $CO_2$ released during enzymatic treatment through the absorption of this compound on alkali solutions. Then, the final radioactivity is measured and related with the amount of original compound transformed into $CO_2$. As observed in Table

6.8, the enzymatic treatment of BPA and EE2 allowed a limited percentage of mineralisation. The enzymatic treatment with UPO led to mineralisation percentages quite low (around 0.5 % for both compounds). However, treatment of BPA with MnP resulted in a mineralisation degree near to 4 %.

**Table 6.8.** BPA and EE2 mineralisation in enzymatic treatment with *I. lacteus* enzyme cocktail.

| Time (h) | | 1 | 3 | 6.5 |
|---|---|---|---|---|
| **BPA mineralisation (%)*** | MnP cocktail | 0.22 ± 0.02 | 1.56 ± 0.05 | 3.88 ± 0.05 |
| | Commercial UPO | 0.10 ± 0.01 | 0.23 ± 0.01 | 0.53 ± 0.09 |
| **EE2 mineralisation (%)*** | MnP cocktail | 0.24 ± 0.04 | 0.81 ± 0.01 | 2.64 ± 0.17 |
| | Commercial UPO | 0.07 ± 0.01 | 0.19 ± 0.02 | 0.51 ± 0.03 |

*Calculated by relating the radioactivity measured in the KOH trap to the initial radioactivity of the medium.

Although high yields of mineralisation of these compounds was reported with more aggressive treatments as electrochemical process or combination of electrocatalysts and Phenton-process (Machado et al., 2022; Murugananthan et al., 2008), no reports about enzymatic mineralisation were found. However, several studies speculate about the possible mineralisation of BPA into $H_2O$ and $CO_2$ based on the metabolites obtained during enzymatic treatment (Gassara et al., 2013b; Wang et al., 2022). So, in this sense, this is the first study that verifies that biodegradation of BPA and EE2 with fungal enzymes can achieve partial mineralisation of these compounds. Nevertheless, more research is necessary to elucidate if this treatment can lead to a complete mineralisation of the parental compounds when considering longer reaction times.

### 6.3.2. Degradation of a model polycyclic aromatic hydrocarbon

#### *6.3.2.1. Removal with manganese peroxidase*

With the aim of evaluating the potential of enzymes in the treatment of PAHs, pyrene was used as a model compound. Table 1.5 shows the main characteristics of this PAH, which, compared to the compounds studied previously, has a lower solubility in water.

In the evaluation of the enzymatic treatment based on the *I. lacteus* enzyme cocktail, the same strategy of optimisation followed for PPCPs transformation

was considered. Figure 6.8 shows the concentration profile obtained for different MnP doses (50-200 U/L) with the addition of different $H_2O_2$ concentrations (1-10 μM/min). Although the complete removal of pyrene required 22 h for all tested conditions, the degradation seems to be faster in experiments at higher $H_2O_2$ concentration. This fact was verified with the determination of the kinetic constants, which are shown in Table 6.9. Doses of 10 μM/min $H_2O_2$ led to kinetic constant values that almost duplicate the values obtained using 1 μM/min $H_2O_2$. Nevertheless, it is noteworthy that, contrary to results obtained for PPCPs, the combination that achieves a faster pyrene degradation is that with the lowest enzyme dose tested. As shown in Table 6.9, the loss of enzyme activity was similar in experiments with 200 and 50 U/L of MnP, so the differences in kinetic constants at different MnP doses and maximum $H_2O_2$ dose cannot be explained by a major deactivation of the enzyme in experiments at 200 U/L.

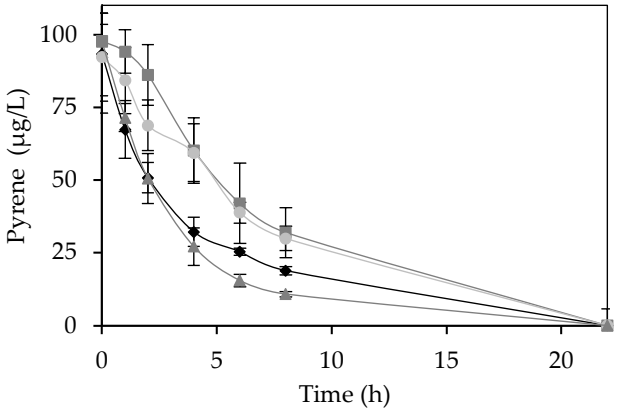


**Figure 6.8**. Time course of pyrene removal with *I. lacteus* enzyme cocktail using 200 U/L and 10 μM/min $H_2O_2$ (♦), 50 U/L and 10 μM/min $H_2O_2$ (▲), 200 U/L and 1 μM/min $H_2O_2$ (■) and 50 U/L and 1 μM/min $H_2O_2$ (●).

**Table 6.9.** Kinetic constants for complete pyrene removal with *I. lacteus* enzyme cocktail at different enzyme dose and $H_2O_2$ concentration.

| Initial activity (U/L) | 22 h activity (U/L) | $H_2O_2$ (μM/min) | Time (h) | k[a] (1/h) | $r^2$ |
|---|---|---|---|---|---|
| 191.3 ± 5.0 | 79.1 ± 7.4 | 10 | 22 | 0.195 | 0.91 |
| 50.4 ± 2.0 | 20.0 ± 2.9 | 10 | 22 | 0.282 | 0.93 |
| 198.6 ± 7.5 | 149.7 ± 17.9 | 1 | 22 | 0.149 | 0.90 |
| 51.4 ± 5.4 | 44.8 ± 11.8 | 1 | 22 | 0.143 | 0.93 |

[a] Apparent kinetic constant according to first-order kinetics.

Most of the studies dealing with enzymatic degradation of pyrene involves *in vivo* assays, where the removal of the compound is evaluated during the growth of different fungi in a contaminated soil (Hong et al., 2010; Imam et al., 2022). In some of these studies, the complete degradation of 100 mg/L of pyrene required several weeks (Hong et al., 2010). Among the few studies evaluating the degradation of pyrene with isolated enzymes, Eibes et al. (2006) evaluated pyrene degradation at different MnP doses and reported a kinetic constant of 0.04 $h^{-1}$ with a MnP activity of 1310 U/L.

#### *6.3.2.2. Removal with unspecific peroxygenase*

Pyrene removal with commercial UPO was evaluated at the same conditions tested for PPCPs degradation. As observed in Figure 6.9, complete removal of pyrene after 8 h of treatment was achieved with all tested $H_2O_2$ doses at pH 4 and 7, whereas at pH 5.5 none of the concentrations tested allowed the complete degradation of pyrene in this reaction period.

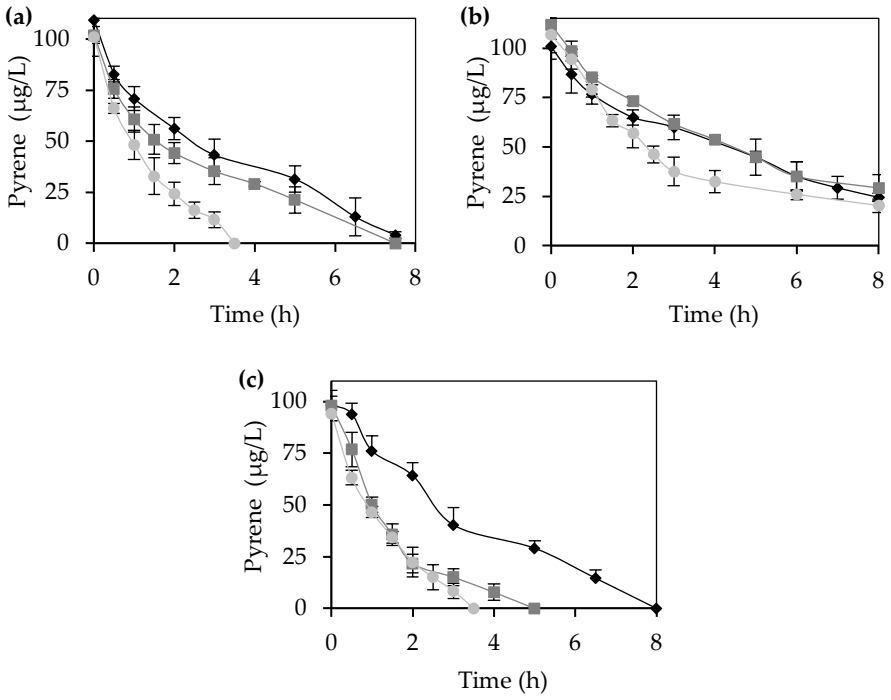


**Figure 6.9**. Time course of pyrene removal with commercial UPO using a $H_2O_2$ dose of 5 (♦), 10 (■) and 15 (●) µM/h at. **(a)** pH 4, **(b)** pH 5.5 and **(c)** pH 7.

The maximum $H_2O_2$ dose allowed the degradation of 100 µg/L of pyrene in 3.5 h at pH 4 and 7, and even if complete removal was not achieved at pH 5.5, the experiments with $H_2O_2$ dose of 15 µM/h presented the higher kinetic constant at this pH (Table 6.10). The increase in the kinetic constant with maximum $H_2O_2$ dose at pH 4 and 7 is noteworthy, being more than twice that obtained for the lowest $H_2O_2$ concentration evaluated.

In comparison with MnP pyrene degradation, the results obtained employing UPO shows that this enzyme is more efficient for pyrene degradation. However, as seen for the degradation of pyrene with MnP, the reaction conforms to first-order kinetics only in the first few hours.

**Table 6.10.** Conditions and results of pyrene degradation with commercial UPO at different pH and $H_2O_2$ concentration.

| Initial activity (U/L) | 8 h activity (U/L) | pH | $H_2O_2$ (μM/min) | Time (h) | Deg (%) | k[a] (1/h) | $r^2$ |
|---|---|---|---|---|---|---|---|
| 511.3 ± 11.1 | 513.5 ± 18.7 | 4 | 5 | 7.5 | 100 | 0.29 | 0.96 |
| 502.3 ± 28.4 | 374.7 ± 68.7 | 4 | 10 | 7.5 | 100 | 0.29 | 0.97 |
| 501.6 ± 22.5 | 390.3 ± 14.2 | 4 | 15 | 3.5 | 100 | 0.71 | 0.99 |
| 520.4 ± 23.8 | 436.9 ± 23.5 | 5.5 | 5 | 8 | 75.7 | 0.17 | 0.99 |
| 495.5 ± 8.4 | 430.7 ± 42.1 | 5.5 | 10 | 8 | 73.9 | 0.17 | 0.99 |
| 523.9 ± 17.9 | 471.5 ± 3.1 | 5.5 | 15 | 8 | 81 | 0.21 | 0.93 |
| 511.1 ± 12.1 | 506.8 ± 2.2 | 7 | 5 | 8 | 100 | 0.29 | 0.98 |
| 515.8 ± 32.5 | 501.0 ± 14.7 | 7 | 10 | 5 | 100 | 0.64 | 0.99 |
| 513.9 ± 19.7 | 511.5 ± 18.8 | 7 | 15 | 3.5 | 100 | 0.78 | 0.99 |

[a] Apparent kinetic constant according to first-order kinetics.

Few studies have evaluated the degradation of pyrene with UPOs, where the initial concentration of the compound was more than 200 times higher than the concentration employed in this study (0.1-1 mM versus 0.5 μM) and organic solvents were added to reaction medium to allow complete solubilisation of pyrene (Aranda et al., 2010; Karich et al., 2017). Aranda et al. (2010) have reported a degradation of 0.45 mM of pyrene after 8 h of reaction at pH 7, with a dose of 1000 U/L of UPO from *A. aegerita* (AaP). Nevertheless, this study also evaluated the use of an UPO from *Coprinellus radians* (CrP) in the same conditions, reporting a degradation rate considerably lower than the achieved with AaP (13 % versus 45 % of degradation for CrP and AaP, respectively). This suggests that performance of pyrene transformation highly depends on the type of UPO employed, as well of the conditions of solubility and $H_2O_2$ concentration in the reaction medium, as in the study of Aranda et al. (2010), a concentration of 5 mM $H_2O_2$ was employed, in addition to the use of Tween-20 in order to increase substrate solubility.

### *6.3.2.3. Metabolites identification*

After enzymatic treatment of $^{14}$C-pyrene with both enzymes in the conditions maximising the degradation rates, it was detected a radioactivity signal by radio-HPLC attributed to transformation products (Figure 6.10 and Figure 6.11). Nevertheless, for pyrene transformation with UPO the final signal was very weak, which is related to a very low concentration of the metabolite detected.

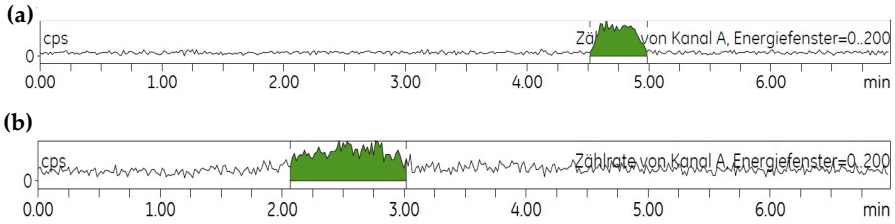


**Figure 6.10.** Radio-HPLC chromatograms of samples of $^{14}$C-pyrene transformation with *I. lacteus* cocktail: **(a)** initial sample (0 h) and **(b)** final sample (22 h).

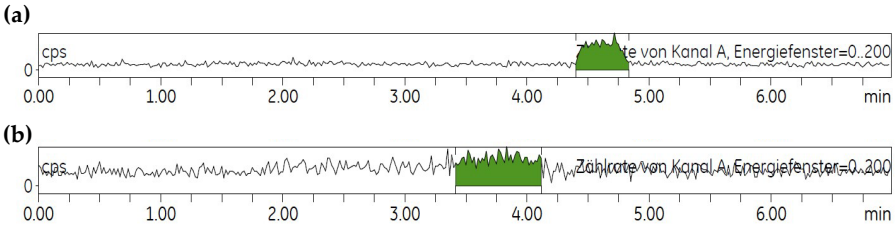


**Figure 6.11.** Radio-HPLC chromatograms of samples of $^{14}$C-pyrene transformation with commercial UPO: **(a)** initial sample (0 h) **(b)** final sample (8 h).

TLC of final samples was performed in order to separate the metabolites present at the end of enzymatic treatment with both enzymes. Data from the scan of TLC plates is shown in Figure 6.12, where is observed one metabolite for the degradation with MnP and three metabolites for degradation with UPO. Nevertheless, the metabolite signal seems to be clearer in the case of MnP treatment. The signal in the spot position (30 cm) is a consequence of the scratching of silica coating during the spraying of the sample, that prevented migration of part of the metabolites.

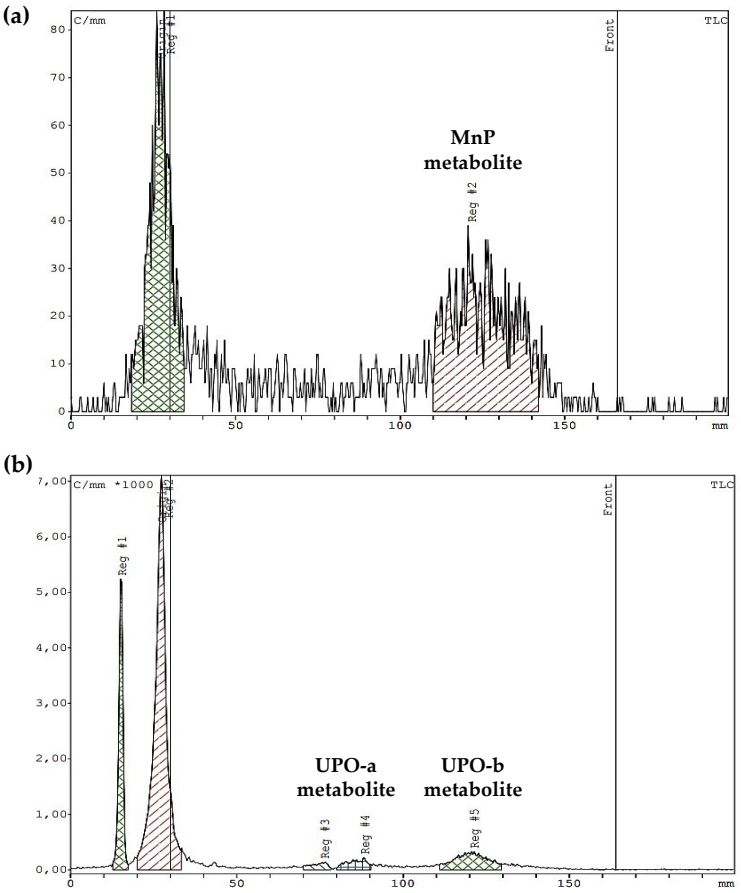


**Figure 6.12.** Chromatograms from radio-TLC scan of **(a)** TLC plate of final sample (22 h) of pyrene degradation by MnP (signal related to metabolite in red) and **(b)** TLC plate of final sample (8 h) of pyrene degradation by UPO (signal related to metabolites in blue and green).

For both final samples, a radioactivity signal was observed at position 120 mm of TLC plate, which suggest that both transformation products, from MnP and UPO, could be the same. In order to identify these metabolites, the compounds separated in TLC were resuspended in methanol and analysed by LC-MS. Since TLC of final samples of pyrene degradation with UPO showed other weaker signals between 70-90 mm, it was prepared one sample with metabolites between these positions (designed as UPO-a metabolite) and another sample with metabolite at 120 mm of TLC plate (UPO-b metabolite).

The LC-MS chromatogram of MnP metabolite (Figure 6.13-a) shows two main signals (2.94 and 7.86 min), whose mass spectra are showed in Figure 6.13-b,c. Although only one signal is visible in radio-TLC of MnP metabolites isolation, it presents a radioactive signal from 110 to 140 cm, so it is possible that the radioactivity signals of both metabolites appear overlapping. Attending to mass spectra, both peaks present molecular ions with a m/z near to 600 and taking into account the limitation of the equipment to this maximum m/z value it is not possible confirm if the signal of 600 m/z corresponds to the metabolite molecules or to one of its possible fragments. However, this high m/z values allows us to confirm that the enzymatic treatment of pyrene with MnP leads to a polymerisation process. Other studies have reported the 1-hydroxypyrene as main product of pyrene transformation with MnP (Eibes et al., 2006). Nevertheless, in the study presented in this chapter, the final samples are taken at longer times of treatment than in the study of Eibes et al. (2006), which can imply a polymerisation of initial transformation products.

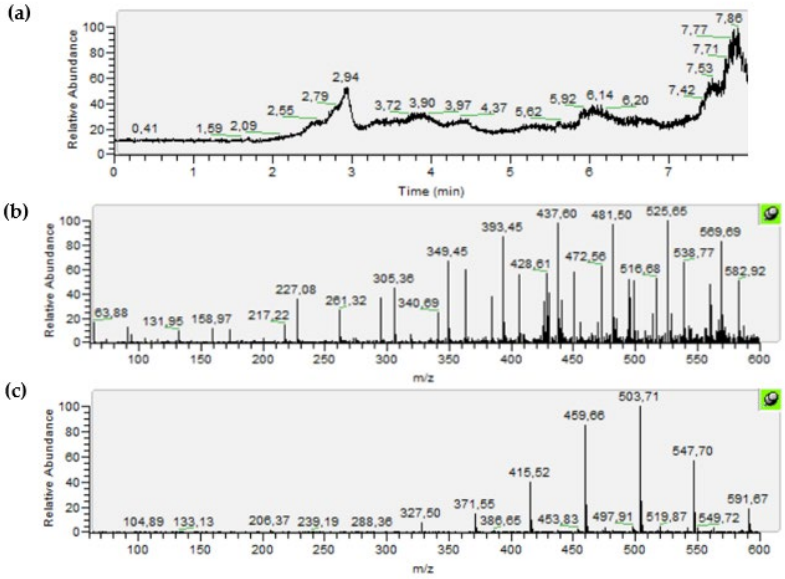


**Figure 6.13.** LC chromatogram and mass spectra for signal related to pyrene metabolite obtained after isolation by TLC: **(a)** LC chromatogram from MnP metabolite analysis, **(b)** mass spectrum of peak at 2.94 min, **(c)** mass spectrum of peak at 7.86 min.

LC-MS results for UPO metabolites (Figure 6.14) also shows a polymerization tendence with enzymatic treatment. Both metabolites show a single main peak, which, as in the analysis of the metabolites obtained with the MnP, have m/z signals around 600 m/z in mass spectra. Again, it is not possible to conclude whether these signals correspond to the molecular ion, so the structure of the final metabolites cannot be deduced.

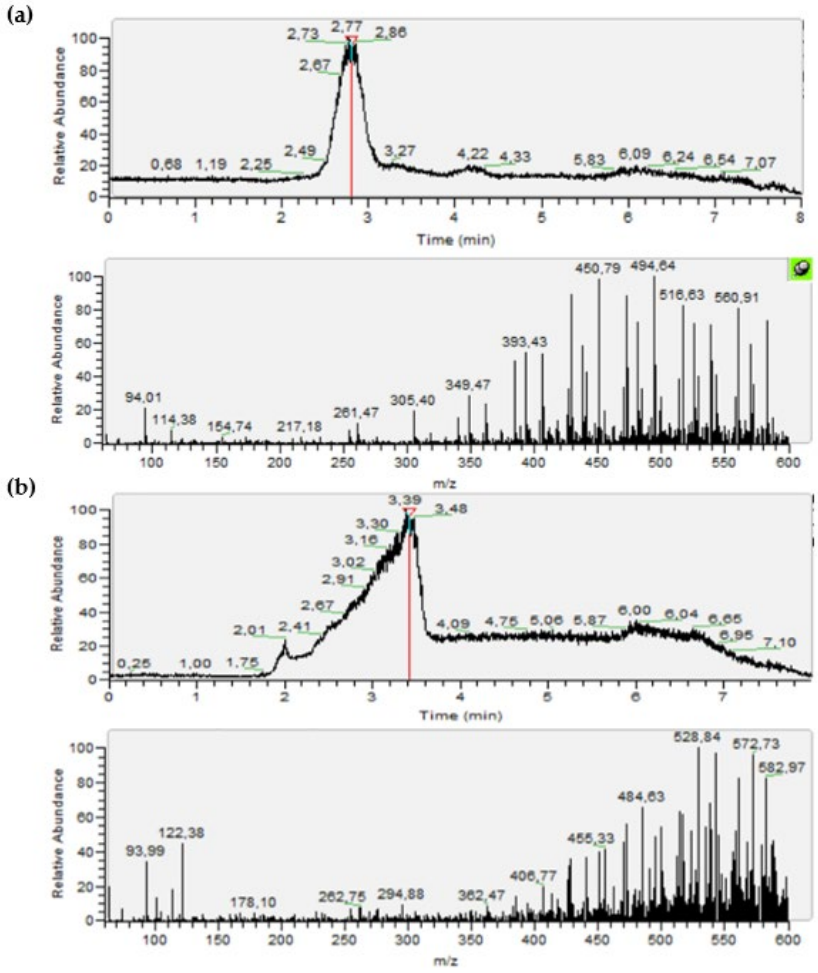


**Figure 6.14.** LC chromatogram and mass spectrum for signal related to pyrene metabolite (showed with red line) obtained for UPO metabolites analysis: **(a)** metabolites detected between 70-90 mm in radio-TLC and **(b)** metabolite detected at 120 mm in radio-TLC.

But it is important to notice that UPO metabolites samples correspond to 8 h of enzymatic reaction, when at 3.5 h all pyrene was completely degraded, so as in the case of MnP it is possible that initial metabolites were hydroxylated forms that are transformed into polymeric compounds.

In addition to the identification of metabolites through LC-MS, mineralisation assays were also performed, since considering the initial and final values of radioactivity observed in the radio-HPLC chromatograms (Figure 6.10 and Figure 6.11), not all the pyrene was transformed into the detected metabolites. Nevertheless, no $CO_2$ released was detected (data not shown), which indicates that even though pyrene may be being transformed to other undetected metabolites, these are not being degraded to their elemental compounds. This also supports the results of LC-MS that indicates that enzymatic treatment entails polymerisation reactions.

## 6.4. Conclusions

Enzymatic cocktails produced from residual streams with mainly MnP and UPO activity are suitable for the degradation of ECs, including PPCPs and PAHs. Complete removal of typical concentrations of these compounds present in wastewater was achieved for all contaminants tested, with the exception of CBZ, where 30 % degradation was achieved after 24 h of treatment with the MnP cocktail. Although this degradation is substantially lower than that achieved for the rest of the ECs, it represents an improvement in the removal of this compound with the enzymatic treatments reported to date.

Moreover, the use of $^{14}C$-labelled compounds has shown that enzymatic treatment with both enzymes leads to the polymerisation of pyrene, which may be related to the large treatment times required to transform all of the initial pyrene. In relation to the transformation of PPCPs, it was found that MnP and UPO are able to mineralise a small amount of BPA and EE2.

## 6.5. Annexes Chapter 6

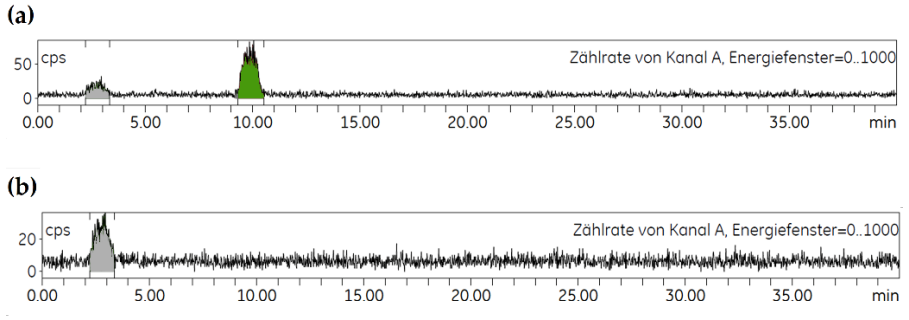


**Figure A6.1.** Radio-HPLC chromatograms of $^{14}$C-BPA degradation in optimal conditions with enzyme cocktail MnP at **(a)** 0 h and **(b)** 1 h. In green BPA signal and in grey column contamination.

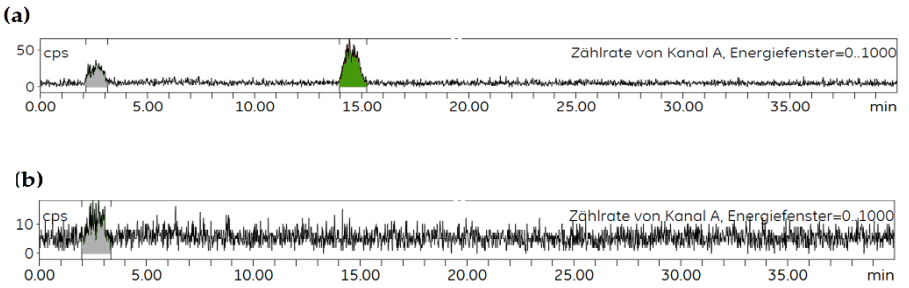


**Figure A6.2.** Radio-HPLC chromatograms of $^{14}$C-EE2 degradation in optimal conditions with enzyme cocktail MnP at **(a)** 0 h and **(b)** 1 h. In green EE2 signal and in grey column contamination.

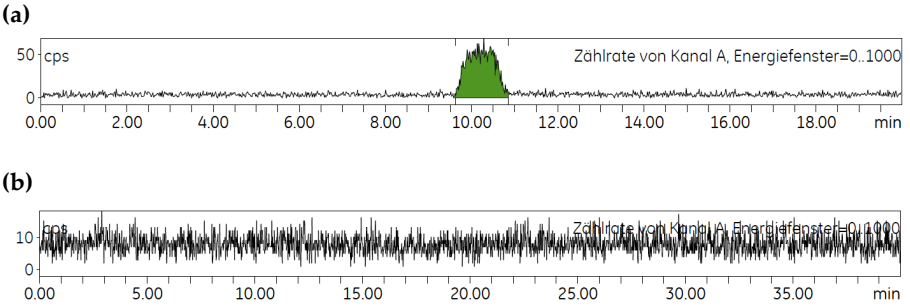


**Figure A6.3.** Radio-HPLC chromatograms of $^{14}$C-BPA degradation in optimal conditions with commercial UPO at **(a)** 0 h and **(b)** 1 h. BPA signal in green.

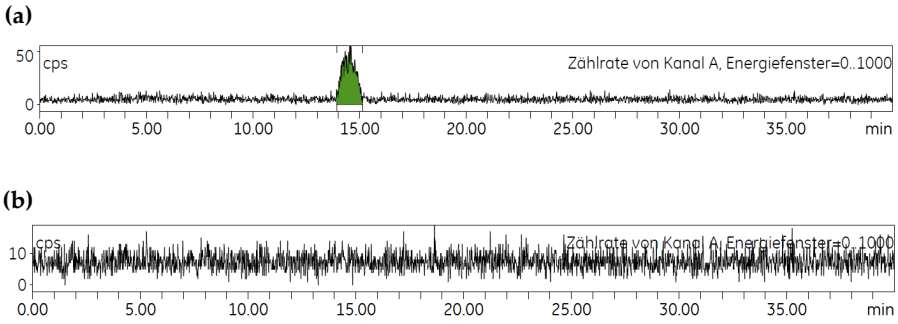


**Figure A6.4.** Radio-HPLC chromatograms of $^{14}$C-EE2 degradation in optimal conditions with commercial UPO at **(a)** 0 h and **(b)** 1 h. EE2 signal in green.

# CHAPTER 7

## GENERAL DISCUSSION AND CONCLUSIONS

## 7.1. Detailed findings of the Thesis

Ligninolytic enzymes (LEs) are versatile biocatalysts that can be applied to a wide range of biotechnological applications. The main reason for the development and implementation of enzymatic treatments in different biotechnology processes is related to the need of sustainable technologies (da Silva Vilar et al., 2022). However, biocatalysis is still not competitive with other conventional processes due to the costs associated with enzyme production. In this sense, the valorisation of agro-industrial waste streams for LEs production is presented as a promising alternative, due to the suitable composition of these wastes for LEs expression and represents an opportunity for integrating circular economy benefits (Isikhuemhen et al., 2009; Khonkhaeng and Cherdthong, 2019).

Although the evaluation of commercial or purified LEs was performed for several biotechnology process (Becker et al., 2017; Kurniawati and Nicell, 2008; Ullrich and Hofrichter, 2005), research focused on the use of enzyme cocktails is less widespread. Studying the performance of crude enzyme extracts allows a better understanding of the synergistic effects of different enzymes. In addition, enzyme cocktails have several advantages over purified enzymes, such as the simplification of downstream steps in enzyme production, which could translate into a significant reduction in production cost. However, to demonstrate the feasibility of enzyme cocktails for biotechnological purposes, the optimisation of different factors related to enzymatic activity is crucial.

This thesis aims to provide different strategies for obtaining LEs through the valorisation of various agro-industrial waste streams, as well as the evaluation of the potential of the obtained enzyme cocktails as biocatalysts in different biotechnological applications, such as the modification of a natural polymer and the degradation of emerging contaminants (ECs). All tested waste streams resulted in the production of LEs of interest, being the highest production titers in submerged fermentations. In this regard, optimisation of different factors involved in fungal growth and enzyme expression is key to improve enzyme production. In addition, most of the enzyme cocktails obtained proved to be suitable biocatalysts in the degradation of emerging pollutants, while for organosolv lignin activation, commercial enzymes achieved better results than the enzyme cocktails.

### 7.1.1. Production of ligninolytic enzymes through the valorisation of agro-industrial wastes

**Chapter 2** demonstrated the suitability of a wood industry waste as a nutrient source to produce an enzyme cocktail by *G. lucidum* with laccase and MnP activities. The presence of these enzymes in the obtained cocktail was confirmed by proteomic analysis. SSF and SmF allowed enzyme production, but higher enzyme activity was attained for the latter, in addition to the reduction of the time required for enzyme expression. Scaling up SmF to a 4 L reactor led to a reduction in enzyme activity probably due to the addition of antifoam or damage to the fungal structure by agitation that affected its growth. However, at reactor scale the time required to reach maximum enzyme activities was less than at flask scale. In addition, the presence of laccase in the concentrated enzyme cocktail obtained could be verified by proteomic analysis.

The use of wheat straw in the formulation of a fermentation medium for MnP production using *I. lacteus* was evaluated in **Chapter 3**. An enzyme cocktail with MnP and MiP activities was obtained in SSF and SmF, although higher enzyme titers were achieved in SmF. In liquid medium, the formulation of a solution containing wheat straw extract (WSE) and nitrogen source were key to enhance enzyme production, achieving the best results with a diluted extract (1:4) and the addition of peptone. In addition, it was demonstrated that higher enzyme titers can be achieved in a WSE-based medium compared to conventional media. Proteomic analysis allowed the identification of other enzymes present in the enzyme cocktail, including DyP and dehydrogenase, which may represent an enzyme cocktail with greater potential for use in applications of different types. Also relevant is the presence of Mn(II)-independent activity in the enzyme cocktail in addition to the main Mn(II)-dependent activity, which has been scarcely described in the literature for enzyme cocktails of *I. lacteus*.

In **Chapter 4**, by-products of bioethanol synthesis were confirmed as suitable substrates for the growth of *A. aegerita* and the production of an enzyme cocktail with UPO and laccase activity in solid and submerged fermentations due to their high protein content. The highest titer of UPO production was achieved in SmF, where the percentage of vinasse influenced enzyme production. Scaling up this fermentation to a 4 L bioreactor allowed reducing the production time, reaching concentrations similar to those obtained in

flasks. Proteomic analysis corroborated the presence of UPO, laccase, DyP, storage proteins and enzymes related to production and control of reactive oxygen species (ROS), as well as changes in the metabolism of complex sugars and nitrogen sources during submerged fermentation.

Finally, LCA methodology was applied to study the environmental impacts associated with the reactor operation carried out in **Chapters 3 and 4**, confirming that the fermentation step contributed to a considerable share of the environmental impacts, especially due to energy consumption.

### 7.1.2. Application of ligninolytic enzymes to environmental purposes

**Chapter 5** describes the demethylation of organosolv lignin by the enzyme cocktail obtained in **Chapter 2** and two commercial laccases. A preliminary screening showed a higher degree of demethylation with commercial enzymes, so the optimisation of the enzymatic modification of lignin was performed with *T. versicolor* laccase. It was shown that the most relevant parameters for the enzymatic demethylation reaction were those directly involved in the enzyme-substrate interaction. Specifically, the addition of solubilising agents to increase lignin solubility and the use of mediator compounds were the most relevant factors to increase the demethylation yield. However, other parameters such as pH, temperature and aeration also influenced the enzymatic reaction. The final modified lignin obtained seems to be suitable as a precursor for the synthesis of bioadhesives, since the enzymatic treatment allows a demethylation process in which part of the methoxy groups were transformed into hydroxyl groups and finally into carbonyl groups which are more reactive and may involve the formation of reinforced networks. In fact, SEC and MALDI analysis showed that the final lignin presented a higher molecular weight, which is related to its oligomerisation.

The potential of enzyme cocktails was also tested in the abatement of ECs in **Chapter 6**. The degradation of several pharmaceutical and personal care products (PPCPs), including BPA, E1, E2, EE2 and CBZ and the polycyclic aromatic hydrocarbon (PAH) pyrene was performed with the enzyme cocktails produced as well as with a commercial UPO. The results of the enzymatic treatments in which several factors were optimised (pH, enzymatic dose and $H_2O_2$ concentration), showed a complete elimination of the studied ECs with the exception of carbamazepine, from which only 30 % degradation

was achieved after 24 h of treatment with MnP cocktail. However, this degradation represents an improvement of the enzymatic treatment efficiency compared to that achieved in other studies with peroxidases. In addition, the study of transformation products of BPA, EE2 and pyrene using their $^{14}C$-labeled forms showed that enzymatic treatment with MnP and UPO leads to a small mineralisation of BPA and EE2 and polymerization of pyrene.

## 7.2. Research gaps and future perspectives

The use of waste streams as suitable substrates for LEs production was demonstrated in Chapters 2, 3 and 4 due to their lignin content in the case of laccase and MnP production and their high nitrogen content in the case of UPO production. Submerged fermentations allowed higher enzyme production compared to solid-state fermentation. However, the use of more simple waste streams or the alternative of solid-state fermentation could reduce some problems such as contamination by competitive microorganisms. The use of vinasse for the production of UPO with *A. aegerita* in Chapter 4 resulted in contamination by yeast at reactor scale when a typical sterilisation step was used. In this regard, the use of residues that are not derived from processes employing microorganisms might be a better option to avoid this disadvantage, which also leads to increased energy consumption due to the more extreme sterilisation step required. In addition, the evaluation of other reactor configurations could also be interesting to make more effective use of energy. In addition, the study of the environmental impacts generated by larger scale production processes is necessary to have more realistic data based on a possible industrial scale application, where factors such as those related to energy consumption could be decreased by optimising the use of the equipment. In this sense, performing an economic analysis of enzyme cocktail production could be of interest to discuss the feasibility of using enzyme crudes instead of commercial or purified enzymes.

The complexity in the composition of the enzyme cocktail produced with *G. lucidum* in Chapter 2 made it unsuitable for application to the degradation of ECs, due to the interference of some compounds present in the enzyme cocktail in the HPLC analysis. Therefore, future research should focus on the production of cocktail enzymes taking into account the potential application of the enzyme cocktail.

In relation to the improvement of enzyme production, in the case of UPO, a more complete proteomic study during the whole fermentation process could help to elucidate the optimal conditions for the expression of this enzyme, with a subsequent reduction of production times or the selection of more suitable substrates for its induction. Although heterologous expression allows higher titers of enzyme production, it is important to study the production of enzyme cocktails using homologous hosts, since the different enzymes present in these cocktails may have a synergistic effect that increases the potential of these biocatalysts.

The application of the enzyme cocktails obtained in the lignin activation discussed in Chapter 5 showed that the commercial enzymes were more effective in the demethylation of organosolv lignin. The simplification of the fermentation medium or the use of fungal strains that produce laccases with high redox potential, as is the case of *T. versicolor* laccase, are some of the strategies that could be followed to achieve more competitive enzyme cocktails against commercial or purified enzymes. In addition, the modified lignin obtained after enzymatic treatment should be tested in bioadhesive formulations to confirm its viability as a precursor.

Regarding the use of enzyme cocktails for the degradation of ECs, although the reduction of most of the emerging contaminants was achieved with most of the enzyme cocktails obtained, it could be interesting to study the enzymatic reactions with higher initial doses of ECs to evaluate the effect of the increased substrate on the enzymatic biotransformation yield. Especially, in the case of carbamazepine, increasing the enzyme dose or reaction time together with the use of a mediator could improve the degradation of this pollutant by MnP.

Finally, a more complete evaluation on the mineralisation of bisphenol A and EE2 with MnP could be interesting, since the results obtained suggest that longer reaction times could increase the percentage of mineralisation. Moreover, the study of the enzymatic transformation of pyrene in the presence of some chelating agents such as ascorbic acid that prevents the oxidative coupling of phenolic products could be interesting to determine the primary transformation products obtained after the polymerisation step.

## 7.3. General outcome of the Thesis

In view of the above findings and the future perspectives derived from them, it is possible to conclude that the research addressed in this Thesis confirms that agro-industrial wastes can be used as substrates for fungal growth and LEs expression with various fungi. In case of producing laccase or MnP, the content of lignin in the substrate should be considered, as well as other possible inducers, such as metals or ethanol. When producing UPO, a medium containing high concentrations of complex nitrogen is crucial. Submerged fermentation led to the highest enzyme production values in the shortest fermentation time for all enzymes studied compared to solid-state fermentation. Furthermore, supplementation of agro-industrial waste-based fermentation media leads to higher enzyme production in most fermentations, so the addition of external sources of nitrogen or glucose should be considered in the use of agro-industrial wastes as main substrates for LEs production. However, despite the higher production of enzymes in the submerged state, the use of solid fermentations for LEs production should not be ruled out, since the risk of contamination is reduced, and ultrafiltration as downstream processing could be avoided.

Regarding the application of enzyme cocktails, the results obtained were mixed. It has been shown that the selection of purified enzymes or enzyme cocktails will depend on the process in which they are to be used as biocatalysts. In this regard, organosolv lignin modification resulted in higher demethylation values when commercial enzymes were used. The commercial laccase from *T. versicolor* was the best candidate to perform the transformation of this natural polymer, although the optimisation of different factors involved in the enzymatic activity is essential to enhance the degree of demethylation. However, the suitability of different enzyme cocktails as biocatalysts for ECs reduction was found. Once again, the study of different factors influencing enzymatic activity was fundamental to optimise the degradation reactions, and some of the cocktails used achieved better performances than commercial enzymes.

# MANUSCRIPTS CONSIDERED IN THIS THESIS: RIGHTS, CONTRIBUTIONS AND QUALITY INDICATORS

## Bundling the removal of emerging contaminants with the production of ligninolytic enzymes from residual streams

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**Specific contribution to the publication**

Sandra González Rodríguez performed the experiments, analysed the data and prepared the original draft of the article.

**Quality indicator:**

The journal Applied Microbiology and Biotechnology, where Chapter 3 and Chapter 6 were partially published, currently presents an impact factor of 5.560 (2021 Journal Citation Reports), a CiteScore index of 8.8 (calculated through Scopus 18/04/2023) and next positions in the different categories according to Scimago: Q1 in "Biotechnology", "Applied Microbiology and Biotechnology" and "Medicine (miscellaneous)" categories - 0.99 SJR 2021 calculated by Scimago. Data for the year the article was published is not available yet.

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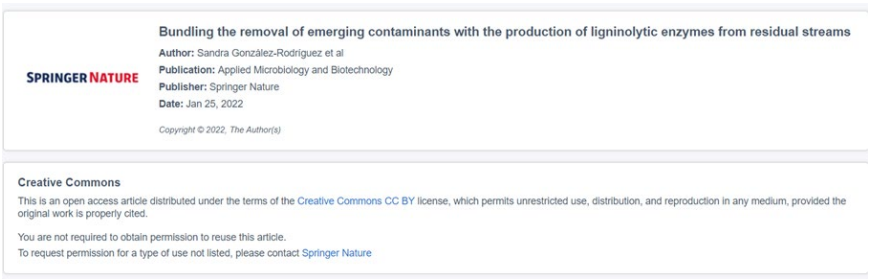

## Functionalisation of organosolv lignin by enzymatic demethylation for bioadhesive formulation

González-Rodríguez, S.[1], Lu-Chau, T.A.[1], Chen, X.[2], Eibes, G.[1], Pizzi, A.[2], Feijoo, G.[1], Moreira M.T.[1]

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Sandra González Rodríguez designed and performed the experiments, analysed the data and prepared the original draft of the article.

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The journal Industrial Crops and Products, where Chapter 5 was published, currently presents an impact factor of 6.449 (2021 Journal Citation Reports), a CiteScore index of 9.6 (calculated through Scopus 18/04/2023) and next positions in the different categories according to Scimago: Q1 in "Agronomy and Crop Science" – 0.987 SJR 2021 calculated by Scimago. Data for the year the article was published is not available yet.

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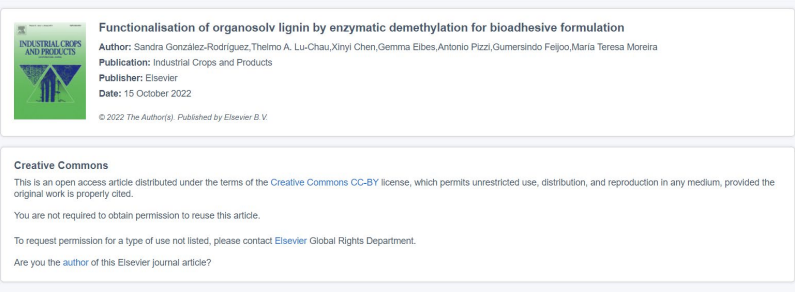

## Valorization of bioethanol by-products to produce unspecific peroxygenase with *Agrocybe aegerita*: Technological and proteomic perspectives

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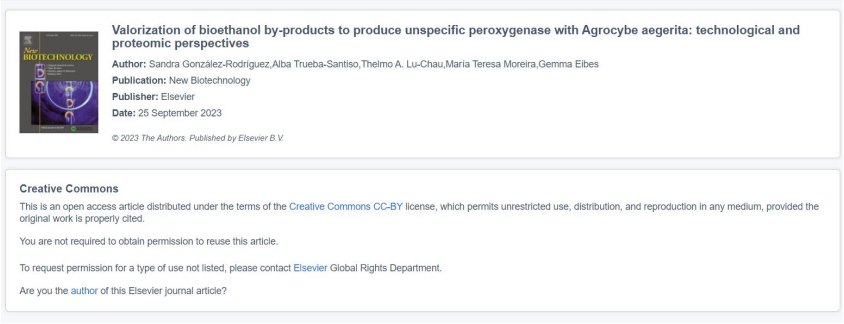

Valorization of bioethanol by-products to produce unspecific peroxygenase with Agrocybe aegerita: technological and proteomic perspectives

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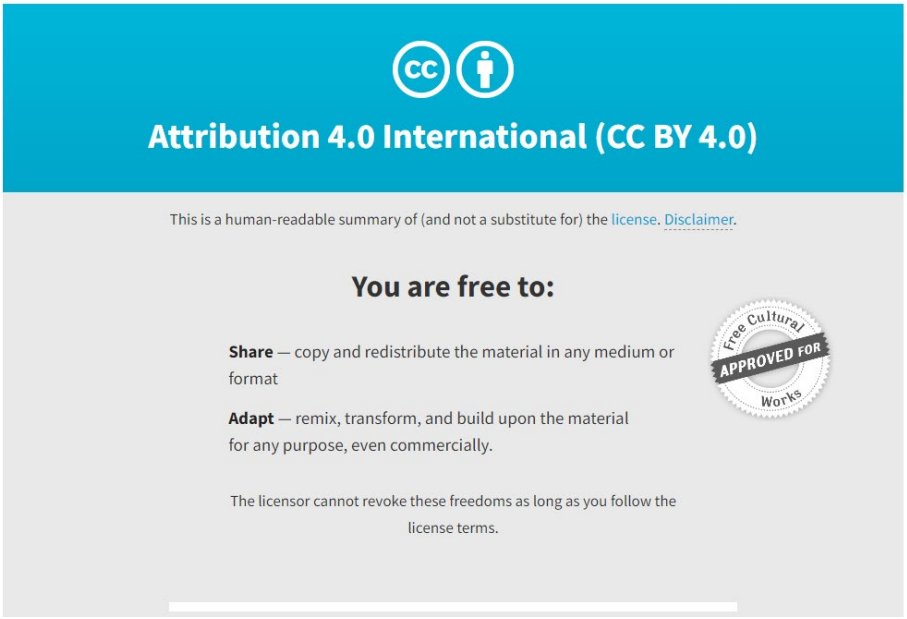

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# LIST OF PUBLICATIONS

**PEER-REVIEWED JOURNALS**

Arias, A., González-García, S., **González-Rodríguez, S.**, Feijoo, G., Moreira, M.T., 2020. Cradle-to-gate Life Cycle Assessment of bio-adhesives for the wood panel industry. A comparison with petrochemical alternatives. Sci. Total Environ. 738, 140357. https://doi.org/10.1016/j.scitotenv.2020.140357

Arias, A., **González-Rodríguez, S.**, Vetroni Barros, M., Salvador, R., de Francisco, A.C., Moro Piekarski, C., Moreira, M.T., 2021. Recent developments in bio-based adhesives from renewable natural resources. J. Clean. Prod. 314. https://doi.org/10.1016/j.jclepro.2021.127892

Conde, J.J., **González-Rodríguez, S.**, Chen, X., Lu-Chau, T.A., Eibes, G., Pizzi, A., Moreira, M.T., 2023. Electrochemical oxidation of lignin for the simultaneous production of bioadhesive precursors and value-added chemicals. Biomass and Bioenergy 169. https://doi.org/10.1016/j.biombioe.2022.106693

**González-Rodríguez, S.**, Arias, A., Feijoo, G., Moreira, M.T., 2022. Modelling and environmental profile associated with the valorization of wheat straw as carbon source in the biotechnological production of manganese peroxidase. Sustain. 14. https://doi.org/10.3390/su14084842

**González-Rodríguez, S.**, Lu-Chau, T.A., Chen, X., Eibes, G., Pizzi, A., Feijoo, G., Moreira, M.T., 2022. Functionalisation of organosolv lignin by enzymatic demethylation for bioadhesive formulation. Ind. Crops Prod. 186, 115253. https://doi.org/10.1016/j.indcrop.2022.115253

**González-Rodríguez, S.**, Lu-Chau, T.A., Trueba-Santiso, A., Eibes, G., Moreira, M.T., 2022. Bundling the removal of emerging contaminants with the production of ligninolytic enzymes from residual streams. Appl. Microbiol. Biotechnol. 106, 1299–1311. https://doi.org/10.1007/s00253-022-11776-7

Pallín, M.Á., **González-Rodríguez, S.**, Eibes, G., López-Abelairas, M., Moreira, M.T., Lema, J.M., Lú-Chau, T.A., 2022. Towards industrial application of fungal pretreatment in 2G biorefinery: scale-up of solid-state fermentation of wheat straw. Biomass Convers. Biorefinery. https://doi.org/10.1007/s13399-022-02319-1

**González-Rodríguez, S.**, Trueba-Santiso, A., Lu-Chau, T.A., Moreira, M.T., Eibes, G., 2023. Valorization of bioethanol by-products to produce unspecific peroxygenase with *Agrocybe aegerita*: Technological and proteomic perspectives. N Biotechnol. 76, 63-71. https://doi.org/10.1016/j.nbt.2023.05.001

**CONGRESS PROCEEDINGS**

***Oral contributions***

**González-Rodríguez, S.,** Martínez. A., Pérez-Rodríguez, N., Lu-Chau, T.A., Eibes, G., Moreira, M.T. Revalorización de diferentes residuos lignocelulósicos para la producción de enzimas ligninolíticas aplicables a la síntesis de precursores de bioadhesivos. *IV Simposio de Investigación en Tecnologías Ambientales.* Santiago de Compostela (Spain), 2020.

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**González-Rodríguez, S.,** Lu-Chau, T.A., Moreira, M.T., Eibes, G. Removal of emerging contaminants with unspecific peroxygenases (UPOs). *European Biotechnology Congress 2022 (EUROBIOTECH 2022).* Prague (Czech Republic), 2022.

***Poster contributions***

**González-Rodríguez, S.,** Lu-Chau, T.A., Trueba-Santiso, A., Eibes, G., Moreira, M.T. The oxidative potential of the enzyme cocktail of *Irpex lacteus* as a biocatalytic tool for the transformation of emerging pollutants. *Ibero-American congress on Biotechnology (III BioIberoamerica)*. Braga (Portugal), 2022.

**González-Rodríguez, S.,** Trueba-Santiso, A., Lu-Chau, T.A., Moreira, M.T., Eibes, G. Production of unspecific peroxygenase (UPO) from *Agrocybe aegerita* using residues from bioethanol production. *Ibero-American congress on Biotechnology (III BioIberoamerica)*. Braga (Portugal), 2022.

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**PROJECTS**

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Fungal enzymes are considered attractive biocatalysts because of their versatility and ability to catalyse multiple oxidation reactions. Their use in biotechnological ields has been widely explored in recent years, demonstrating that they can offer different advantages compared to conventional technologies. However, in view of their application, it is necessary to advance in the improvement of enzyme production. The present PhD Thesis focuses on the production of ligninolytic enzymes by valorisation of different agro-industrial wastes. The results obtained show the importance of integrating waste streams as main substrates for fungal fermentation media and the great potential of enzyme cocktails in environmental biotechnology processes.