Potential for Increasing Laccase Activity in White-rot Fungi *Pleurotus ostreatus* in Aquatic Cultivation

For Use in Bioremediation

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Möjligheten att öka lackasaktivitet hos vitrötesvampen Pleurotus ostreatus när den odlas i vatten

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Abstract

This study investigates the enhancement of laccase activity in *Pleurotus ostreatus*, a white-rot fungus, to improve bioremediation of wastewater, addressing the inefficiency of conventional wastewater treatment plants in removing micropollutants. Anthropogenic stress and climate change has intensified environmental pollution and water scarcity, making effective and sustainable treatment methods essential. Laccase is an enzyme with the capability of breaking down a variety of micropollutants, but face challenges in activity decline. This study evaluated the addition of eight substances on laccase activity when pellets of *P. ostreatus* was grown in water. The results demonstrated that apple pomace and coffee grounds significantly increased laccase activity, with apple pomace reaching 98.1 units per litre (U/L) and coffee grounds 67.7 U/L. These organic wastes, rich in nutrients and potential enzyme inducers, not only boost enzyme production but also adhere to circular economy principles by repurposing waste into valuable resources. The findings suggest a sustainable and cost-effective alternative for improving wastewater treatment and highlight the need for further research to optimize substrate conditions and explore practical applications in bioremediation.

Keywords: Laccase, oyster mushroom, *Pleurotus ostreatus*, white-rot fungi, micropollutants, enzymatic biodegradation, bioremediation, mycoremediation

Sammanfattning

Denna studie undersöker hur aktiviteten hos enzymet lackas i svampen *Pleurotus ostreatus* kan ökas för att förbättra rening av avloppsvatten, särskilt när det gäller mikroföroreningar som dagens metoder inte effektivt kan hantera. Antropogen stress och klimatförändringar har ökat mängden föroreningar i miljön och brist på rent vatten, vilket har ökat behovet av effektiva och hållbara behandlingsmetoder. Lackas är ett enzym som kan bryta ned många av dessa föroreningar, men möter utmaningar med sjunkande enzymaktivitet. I denna studie testades åtta olika ämnen för att undersöka deras effekt på lackasaktiviteten när *P. ostreatus* odlades i vatten. Resultaten visade att både äppelrester och kaffesump signifikant ökade lackasaktiviteten, där äppelrester nådde en aktivitet på 98.1 U/L och kaffesump 67.7 U/L. Dessa material är dessutom organiska avfall, vilket innebär att de följer principerna för cirkulär ekonomi genom att omvandla avfall till värdefulla resurser. Dessa upptäckter innebär ett hållbart och kostnadseffektivt alternativ för att öka reningen av avloppsvatten och minska förekomsten av mikroföroreningar, men samtidigt krävs fortsatt forskning för att optimera substratförhållanden och för att undersöka praktiska applikationer inom biosanering.

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Abbreviations

AC	Activated carbon
ANOVA	Analysis of variance
BPA	Bisphenol-A
DMP	2,6-dimethoxyphenol
EDCs	Endocrine disrupting chemicals
GAC	Granulated activated carbon
GB	Glycine betaine
IPCC	Intergovernmental Panel on Climate Change
MPs	Micropollutants
PAC	Powdered activated carbon
PFAS	Perfluoroalkyl and polyfluoroalkyl substances
PPCPs	Pharmaceuticals and personal care products
SCG	Spent coffee grounds
SF	Submerged fermentation
SSF	Solid state fermentation
WRF	White rot fungi
WWTPs	Wastewater treatment plants

1. Introduction

The escalating global concerns regarding environmental pollution and water contamination from anthropogenic stress have fuelled a growing interest in sustainable and efficient remediation techniques. Among these, mycoremediation, utilizing the enzymatic abilities of fungi to degrade or transform contaminants, has emerged as a promising alternative (Mohammadi et al., 2022). Micropollutants such as residues from pharmaceuticals and personal care products (PPCPs), industrial wastes and pesticides are not successfully removed from conventional wastewater treatment plants (WWTPs), causing contamination of water bodies and harming ecosystems (Mohammadi et al., 2022). This study focuses on the potential of enhancing laccase activity, a key enzyme with promising abilities for the degradation of micropollutants, in the white-rot fungus *Pleurotus ostreatus*.

Recent studies have indicated successful laccase-mediated degradation of micropollutants in wastewater but face a challenge in the decline of laccase activity in this environment (Arregui et al., 2019). This study aims to investigate the impact of additional substances to potentially increase laccase activity in *P. ostreatus* when cultivated in water. As laccase have demonstrated the ability to break down pollutants, understanding the factors influencing their activity could provide insights into more effective and sustainable strategies for treating micropollutant contamination in wastewater.

According to a 2022 report by the Intergovernmental Panel on Climate Change (IPCC), half of the world's population experiences water scarcity during certain parts of the year, a situation exacerbated by climate change. Rising sea levels are increasing the salinity of groundwater, while extreme weather events such as floods are contaminating water supplies. Improving water quality is therefore crucial for both human survival and environmental sustainability.

1.1 Aim and research questions

The aim of this thesis was to perform an experimental study on the impact of selected substances on laccase activity when *Pleurotus ostreatus* was cultivated in water.
The substances should be non-toxic, readily available, cost-effective, easy to remove and have low impact on the quality of water regarding nutrient levels and pH.

The specific research questions were:

- Is laccase production by *P. ostreatus* strain M2191 affected by addition of the selected substances when grown in water?
- If so, can any of the substances be considered suitable for addition to water treatment on a larger scale?

1.2 Limitations of the study

The study is performed in connection to on-going projects where pellets of *P. ostreatus* are developed for treatment of micropollutants in water. The experimental study was limited to the white-rot fungi *P. ostreatus* strain M2191 which is the strain used in the on-going work. It has been confirmed that laccase is the dominating enzyme in both solid-state fermentation (SSF) and submerged fermentation (SF) by this strain. Peroxidase activity was therefore not determined. The selected substances were tested in one concentration only.

2. Background

2.1 Micropollutants

Micropollutants (MPs) are molecules of organic and inorganic contaminants, such as pesticides, pharmaceuticals and personal care products (PPCPs), perfluoroalkyl and polyfluoroalkyl substances (PFAS), bisphenol-A (BPA), trace metals and industrial chemicals. Micropollutants are of anthropogenic origin and can be found at trace concentrations in the environment, usually at microgram or nanogram per litre or kilogram (Bertram et al., 2022; Lou et al., 2014). According to the Council of the European Union (2024), over 90% of toxic micropollutants in wastewaters originate from PPCPs.

The removal of MPs is challenging due to their low concentration, which conventional wastewater treatment processes often fail to adequately address, leading to their release back into the environment where they are reintroduced into the food chain. Additionally, the broad spectrum of MPs and the absence of a universal indicator for their detection complicate their removal (Bofill, 2023).

There is evidence indicating that even trace amounts of certain pollutants in water and wastewater can pose significant health risks to humans, as well as to plant and animal species, potentially disrupting entire ecosystems (Mohammadi et al., 2022).

Currently, regulations governing the discharge of MPs from WWTPs are lacking (Arregui et al., 2019), and the emergence of new pollutants presents challenges not adequately addressed by existing frameworks (EU, 2024). The European parliament (2024) has set a target for mandatory quaternary treatment of MPs in WWTPs over 150,000 population equivalents (p.e. the average pollution released by one person per day) by 2045. This initiative aims to ensure monitoring and control of chemical pollutants.

Despite restrictions of PFAS over the past 10 years, these substances are still detected in water, wildlife, and humans due to their persistence and bioaccumulative properties (Torres-Farradá et al., 2024). Bioaccumulation occur when a substance is absorbed faster than it can be eliminated (Lou et al., 2014).

2.1.1 Spreading of micropollutants

The occurrence of micropollutants in the aquatic environment has become a significant environmental issue over the last few decades. One of the main ways micropollutants spread is through wastewater discharge. Industrial, agricultural and municipal wastewater often contain micropollutants which are released into surface waters (rivers and lakes), groundwater or soil when wastewater is discharged. This is as the current technologies for wastewater treatment are not designed to remove these types of compounds, but rather target easily degraded carbon and plant nutrients (Lou et al., 2014; Bofill, 2023; Rogowska et al., 2019).

Apart from the natural disposal of PPCPs through household wastewater such as toilets and shower drains, improper disposal of pharmaceuticals and personal care products further contribute to the introduction of micropollutants into the environment (Luo et al., 2014). Additionally, unintentional spreading of pesticides, herbicides and fungicides through agricultural runoff plays a significant role in the spreading of micropollutants (Bertram et al., 2022). Given the variety of pathways, micropollutants can be found in a range of different environments, including freshwater and marine ecosystems, groundwater, runoff, soil and sediment (Bertram et al., 2022).

Micropollutants, once introduced into the environment, tend to persist for a long time due to their slow degradation, leading to bioaccumulation. Moreover, these compounds can degrade or react with other substances, potentially forming more toxic by-products than the original pollutants (Rogowska et al., 2019). Aquatic organisms, including fish and other marine species, absorb micropollutants through their skin and respiratory systems and ingest them through their diet or contact with contaminated sediments. Micropollutants can also accumulate in plants via their roots, stems and foliage. This has been studied particularly in the field of agricultural systems, with concern that commercially grown crops watered with treated wastewater, may accumulate contaminants (Bertram et al., 2022).

Zamri et al. (2021) compiled evidence on the impact of micropollutants, particularly endocrine disrupting chemicals (EDCs), on living organisms. Notable effects include feminisation of male fish and reduced reproductive fitness, thinning of eggshells in birds, reproductive dysfunction in reptiles, and decreased photosynthesis and growth in plants. The health effects of BPA, pesticides, and pharmaceuticals on humans include growth and mental retardation, early puberty, reduced sperm quality, and neurological and carcinogenic effects in infants and children (Kang et al., 2022). BPA, an endocrine disruptor, mimics oestrogen, exacerbating these issues.

2.1.2 Wastewater treatment plants and micropollutants

Wastewater treatment plants (WWTPs) have been identified as a major source of pollution, as micropollutants are only partially removed in conventional wastewater treatment processes (Rogowska et al., 2019). The main stages of conventional treatment occur in several steps: primary, secondary, and tertiary treatment, which could be described as mechanical, biological and chemical treatment respectively. Industrial wastewater also undergoes pre-treatment which uses mechanical processes like filtration and settling to remove varying larger objects depending on the source of wastewater. Primary treatment is similar to pre-treatment in which it uses gravity and continued physical processes to remove solids, as well as oil and grease through skimming or saponification. Secondary treatment, the biological treatment, makes use of microorganisms such as bacteria to break down organic matter, converting it into less harmful substances (Frankel, 2021; Sundin et al., 2017). Secondary treatment is successful in removing some pharmaceuticals like paracetamol and ibuprofen, but fails to remove compounds of other groups of PPCPs and biocides (Rogowska et al., 2019). Tertiary treatment, the chemical process, is used when WWTPs must meet certain criteria of quality. This treatment involves filtration and some form of disinfection, such as chlorination, ultraviolet (UV) disinfection or ozone treatment (Frankel, 2021; Sundin et al., 2017).

According to Pistocchi et al. (2022), current techniques to remove micropollutants primarily include ozonation and/or the use of activated charcoal (AC). Ozonation involves adding ozone (O_3) to water, which can remove contaminants either through direct reactions with the pollutants or by generating reactive molecules like hydroxyl radicals ($\bullet OH$) that further react with the contaminants. This method effectively targets a variety of MPs, improving water quality by mitigating oestrogenicity and other harmful effects, such as antibiotic activity and algal toxicity. However, ozonation can lead to the formation of potentially harmful by-products and may only partially oxidise certain compounds, resulting in potentially hazardous transformation products. Consequently, additional treatment like sand filtration or activated carbon are often needed to remove these residual by-products (Pistocchi et al., 2022).

Due to its high specific surface area, activated carbon (AC) can efficiently adsorbs dissolved MPs through electrostatic interactions. AC is available in two main forms: powdered (PAC) or granular (GAC). PAC can be added during biological treatment, effectively removing MPs and enhancing the energy content of sludge for incineration. However, the use of PAC can complicate sludge management by making it unsuitable for agricultural reuse due to the presence of adsorbed MPs, while also increasing sludge volume by 20-30% (Pistocchi et al., 2022).

Enzyme-based wastewater treatment using laccase from white rot-fungi as a quaternary treatment holds implication for a more sustainable remediation, with the only by-product being water (Arregui et al., 2019).

2.2 Bioremediation with white-rot fungi

Bioremediation is a technique which uses microorganisms such as fungi, bacteria and algae to remove contaminants from water or soil. Enzyme-based bioremediation has been proven to be a promising, economically efficient and environmentally friendly method for removing micropollutants in wastewater without harmful impacts to the environment (Mohammadi et al., 2022).

For fungal bioremediation of wastewater, also known as mycoremediation, enzymatic treatment with laccases and peroxidases is particularly effective. These enzymes offer faster detoxification, lower sludge volume, and adaptability to various conditions, operating efficiently across a wide range of temperatures, pH levels, and salinity. Additionally, they are less inhibited by organic and inorganic compounds commonly found in industrial wastewater (Torres-Farradá et al., 2024).

Both fungal mass and enzymes can be used for wastewater treatment. Fungal biomass can be formed into pellets or immobilized on solid supports, while enzymes can be used either free or immobilized. Enzyme immobilization is a preferred method in industrial applications due to its economic feasibility. Immobilization improves enzyme stability against proteolysis (breakdown of proteins/enzymes) and extreme conditions, enhances productivity, and extends shelf life. Techniques for immobilization include chemical bonding, adsorption, microencapsulation, and using various organic and inorganic support materials (Torres-Farradá et al., 2024). In this study, the aim was to create pellets of mycelium and grain that function similarly to immobilized fungal biomass.

2.3 Laccase

Enzymes are a specific type of protein that catalyse biochemical reactions, i.e. they reduce the activation energy required for chemical reactions to occur, thereby accelerating reaction speed. Without enzymes, many chemical reactions would take too long to sustain life. The energy reduction is achieved through stabilisation of the transition state, which refers to an unstable arrangement of atoms and bonds during a reaction and represents the highest potential energy along the reaction coordinate. Enzymes feature specific locations called active sites, which bind to substrates, facilitating the reaction and promoting the efficient formation of products (Lewis & Stone, 2023).

In the context of this study, specifically focusing on laccase enzymes, their role is highlighted in their ability to degrade micropollutants. Unlike many enzymes that commonly exhibit high substrate specificity, meaning the active site only fit specific substrates, laccases are versatile enzymes with low substrate

specificity, making them particularly suitable for the remediation of various micropollutants (Arregui et al., 2019).

Laccase is a multicopper protein belonging to the oxidoreductase group. It utilises molecular oxygen to oxidise itself, a process that enables it to break down the substrate (MPs). After oxidising the MPs, the laccase enzyme is reduced back to its original state and can continue to perform its function, provided there is oxygen available (Mohammadi et al., 2022). Laccases only require oxygen to function and generate water as the sole by-product, which enhances their environmental suitability and reduces toxicity (Arregui et al., 2019). These enzymes are naturally occurring in various organisms such as fungi, plants, bacteria and insects (Mohammadi et al., (2022). Among these, laccases derived from white-rot fungi have been proven to be particularly valuable for water remediation purposes (Arregui et al., 2019). In nature, fungal laccases play a crucial role in decomposing organic matter, specifically lignin.

2.4 Oyster mushroom (*Pleurotus ostreatus)*

Pleurotus ostreatus, commonly known as oyster mushroom, is a widely cultivated white-rot basidiomycete with significant ecological and biotechnological importance. This fungus plays a crucial role in the carbon cycle due to its ability to degrade lignocellulose through enzymes such as peroxidase and laccase, with the latter being particularly effective in breaking down micropollutants, as noted by Arregui et al. (2019). *P. ostreatus* is not only one of the most cultivated edible mushrooms, valued for its high nutritional content (including vitamin C, B-complex, and proteins), but also for its versatility in colonizing a wide range of substrates and thriving in various temperatures (Piska et al., 2016; Nongthombam et al., 2021). Additionally, the spent mushroom substrate (SMS) from cultivation can be repurposed as biofertilizer or for biogas production, contributing to waste minimization (Nongthombam et al., 2021).

Given its large-scale cultivation, spawn of *P. ostreatus* is readily accessible for potential use in bioremediation. One of the key advantages of utilizing a commercially produced mushroom like *P. ostreatus* is the ease of inoculating wastewater with the fungus using grain spawn. Grain spawn, a staple in commercial mushroom production, is non-toxic, widely available, and produced on a large scale (Balan et al., 2022).

For commercial cultivation of fruiting bodies, P. ostreatus is typically grown using solid-state fermentation (SSF), where the fungus develops on solid substrates such as agricultural residues or grains. However, for bioremediation purposes, submerged fermentation (SF) is more applicable. SF involves cultivating microorganisms in a liquid medium, which requires aeration to oxygenate the liquid, distribute nutrients, and prevent the settling of microorganisms.

3. Materials and methods

3.1 Microorganisms and cultivation

The cultivation comprised introducing grain spawn (*Figure 1*) from the white-rot fungal species *Pleurotus ostreatus* strain M2191, obtained from Mycelia BVBA, Belgium. The process involved adding the grain spawn to sterile distilled water in Erlenmeyer glass flasks, maintaining a concentration of 40g/L wet weight. The cultures were incubated on a horizontal orbital shaker (VWR, Advanced 5000 shaker, Radnor, PA, USA) operated at 100 rpm at room temperature (20-22°C) for three days.

Figure 1. Image of grain spawn of Pleurotus ostreatus strain M2191.

3.2 Experimental set-up

Each Erlenmeyer flask contained a volume of 25 mL of the sample which was composed of sterile distilled water and grain spawn. The chosen substances were introduced to the samples at the onset of the experiment, with concentrations specified in *Table 1*. The experimental design included two control groups: one with only grain spawn and one with lignin. The experiment was performed using three replicas of each experimental condition. Aeration was facilitated using a shaking table, leveraging the Erlenmeyer flasks low volume to provide a large surface area for optimal fungal growth.

Following a three-day incubation period on the shaker, pellets composed of grain, mycelium and lignin had developed, as described in detail by Hultberg and Golovko (2020) and shown in *Figure 3*. Subsequently, laccase activity was measured to evaluate the impact of the different substances added to the samples.

Table 1. Substances tested for their impact on laccase production of pellets of P. ostreatus in water with concentration and type of compound is presented.

Substance	**Concentration**	**Type of compound**
Spawn only (control 1)	-	Control
Lignin (control 2)	5 g/L	Natural substrate for *Pleurotus ostreatus*
Glucose	5 g/L	Carbon source
Glycerol	5 g/L	Surfactant, carbon source
Tween-80	15 mg/L	Surfactant, possible carbon source
Ethanol	50 mg/L	Surfactant, carbon source
Glycine betaine	50 mM	Surfactant, carbon and nitrogen source
Coffee grounds	2 g/L	Carbon and nitrogen source, will release phenolic compounds
Gallic acid	10 mg/L	Naturally occurring low-molecular weight phenolic compound
Apple pomace	4 g/L	Carbon source, possible copper source

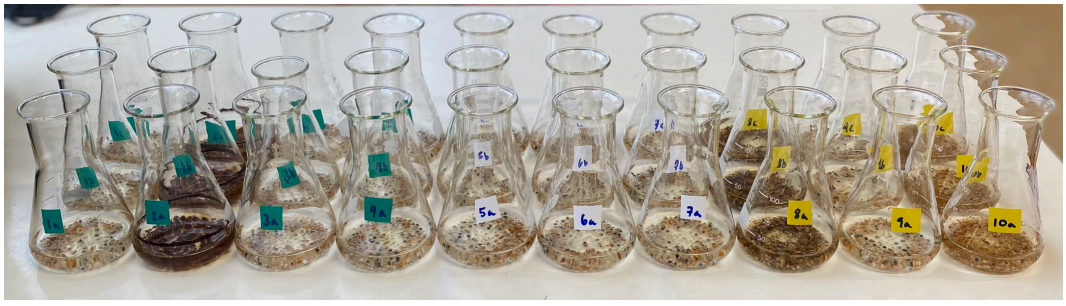

Figure 2. Image of Erlenmeyer flasks after 72 hours of incubation.

3.3 Substances tested

The substances tested in the experiment were selected based on several key-criteria: non-toxicity, availability, cost-effectiveness, environmental safety, and minimal impact on quality parameters such as pH and nutrient levels.

Lignolytic enzyme production, including laccase, can be significantly influenced by the presence of various organic compounds. These include carbon and nitrogen sources, as well as lignin-related phenolic derivatives and aromatic compounds, which are known to act as inducers of enzyme activity (Parenti et al., 2013; de Souza et al., 2004). Furthermore, essential micronutrients such as copper are known to induce laccase gene transcription, significantly enhancing enzyme activity in fungi (Palmieri et al., 2000). Concentrations were based on previous research and levels below toxicity and are specified in *Table 1.*

Lignin

In this experiment, lignin was used as a second control given that it is the substrate for which *P. ostreatus* grows naturally. Lignin, a complex organic polymer found in the cell walls of plants, has a structure that is difficult to degrade (Torres-Farradá et al., 2024). The primary role of lignolytic enzymes, such as laccase in *P. ostreatus,* is to break down lignin. The decomposition of lignin allows the mushroom to access cellulose and hemicellulose, which are essential carbon sources. However, lignin's use in bioremediation is limited by the phenomenon of brownification, where the breakdown products of lignin contribute to the discoloration of water (Blanchet et al., 2022) which can be seen in *Figure 3*. Due to limited understanding of the environmental consequences of brownification, lignin was used only as a second control to provide a baseline comparison for assessing the effectiveness of other tested substances in enhancing laccase activity.

Figure 3. Image of pellets composed by grain, mycelium and lignin.

Glucose

Glucose ($C_6H_{12}O_6$), a monosaccharide and essential energy source for many organisms (PubChem, n.d.b), was used as a carbon source in this experiment. Composed of carbon atoms, glucose was expected to supply readily metabolized carbon, potentially boosting the overall metabolic rate of *P. ostreatus* and leading to enhanced synthesis of laccase enzymes. Glucose met the criteria of being a non-toxic, readily available, and cost-effective carbon source. Additionally, as a simple sugar, glucose was expected to have minimal effect on water quality (PubChem, n.d.b).

Glycerol

Like glucose, glycerol was used as a carbon source for fungal metabolism. Glycerol, also known as glycerine, is a simple polyol compound (containing multiple hydroxyl (-OH) groups) with the molecular formula $C_3H_8O_3$. It is a colourless, odourless, viscous, non-toxic liquid (PubChem, n.d.e) and a common by-product of soap production and biofuel manufacturing, widely used in food, pharmaceuticals, and cosmetics due to its humectant properties (PubChem, n.d.e). These properties are known to alter cell membrane permeability, which could potentially enhance nutrient uptake and enzyme excretion. This was expected to lead to higher extracellular laccase activity, as the enzyme could be more efficiently secreted into the surrounding medium.

Tween-80

Tween-80 (Polysorbate 80) was chosen due to its properties as a non-ionic surfactant and emulsifier, known for stabilizing emulsions and increasing the solubility of various compounds (PubChem, n.d.f). Surfactants, including Tween-80, have been shown to modify the permeability of fungal cells membranes, facilitating increased oxygen exchange and extracellular transport of compounds, which enhance enzyme production.

Tween-80 has been reported to stimulate the activity of lignolytic enzymes in various fungal species. Usha et al. (2014) specifically highlighted Tween-80's role in stimulating the production of enzymes in the white rot fungus *Stereum Ostrea*. El-Batal et al. (2015) observed high laccase concentrations during SSF of *P. ostreatus* in the presence of Tween-80. Furthermore, Teodoro et al. (2018) reported a significant increase in laccase activity in *Pleurotus sajor-caju* cultures supplemented with Tween-80, with activity levels up to 50 times higher than the control. However, the increase in laccase activity was only observed in the presence of a carbon source, suggesting that Tween-80 primarily acts as a facilitator of nutrient absorption rather than as a direct carbon source.

Tween-80 was selected for its food-grade status and low ecotoxicity, making it suitable for applications where environmental impact is a concern (Usha et al., 2014).

Ethanol

Ethanol (C_2H_5OH) was chosen for its versatility as a simple alcohol widely used in various applications. Ethanol's molecular structure allows it to mix with both water and organic compounds, making it an effective solvent and surfactant (PubChem, n.d.c). In this experiment, ethanol was used as both a surfactant and a carbon source. Various microorganisms, including fungi, can utilize ethanol as a carbon source for growth and metabolism (Shaw, 2011), potentially leading to increased biomass and enzyme production.

Ethanol disrupts cell membranes by increasing permeability, which facilitates nutrient uptake and waste excretion. At higher concentrations, ethanol acts as a disinfectant by denaturing proteins and dissolving lipids in cell membranes, leading to cell lysis. Therefore, ethanol concentration was a critical factor. The concentration used in this experiment, 50 mg/L, was below levels associated with ecotoxicity (Shaw, 2011).

Ethanol is rapidly biodegraded in soil, surface water, and groundwater (Shaw, 2011). Should ethanol be used as an inducer for laccase in bioremediation of wastewater, it would be readily biodegradable by microorganisms present in wastewater treatment systems, breaking it down into carbon dioxide and water (Shaw, 2011).

Glycine betaine

Glycine betaine (GB) is a by-product of processing sugar beets and can function as a possible surfactant, carbon-, and nitrogen source. GB is a naturally occurring compound found in plants, often produced in response to stress conditions like drought and salinity. It is commonly used as a humectant in personal care products due to its hygroscopic properties, meaning it can adsorb moisture from the air (Clendennen & Boaz, 2019).

The addition of GB could potentially increase laccase activity by simulating stress conditions that induce laccase production. GBs properties as an osmoprotectant may help maintain cell stability and enhance metabolic processes, leading to higher laccase production. The surfactant properties of GB may facilitate interaction between the fungi and its environment. Furthermore, its role as a carbon and nitrogen source can support the nutritional needs of *P. ostreatus*, possibly resulting in improved enzyme synthesis and overall laccase activity.

Additionally, GB is biodegradable with low ecotoxicity (Clendennen & Boaz, 2019), making it an environmentally friendly option for enhancing laccase activity in wastewater treatment plants.

Coffee grounds

Spent coffee grounds (SCG) are composed primarily of cellulose and hemicellulose, which account for roughly 50% of their content. Lignin and proteins constitute around 20% each, with the remainder consisting of oil, phenolic

compounds, minerals, caffeine and tannins (McNutt & He, 2019). Lignin and phenolic compounds are well known inducers for laccase production (Parenti et al., 2013; de Souza et al., 2004), suggesting that SCG could be used for enhancing laccase activity.

Kang et al. (2022) investigated the use of coffee waste as an adsorbent for removing pollutants from wastewater, demonstrating that it is a promising material for wastewater treatment. Rather than investigating coffee waste as a possible source for enzyme production, their study focused on the adsorption potential of coffee waste, emphasising its porous structure and functional groups that enable it to bind heavy metals and dyes.

The coffee industry generates approximately 4 tonnes of coffee waste annually (Kang et al., 2022). This substantial amount of waste presents an opportunity for an environmentally and economically efficient source for laccase production.

Gallic acid

Gallic acid is a naturally occurring low-molecular weight phenolic compound with the molecular formula $C_7H_6O_5$ (PubChem, n.d.d).

Gallic acid could work as an inducer for laccase because it acts as a redox mediator. Laccases can oxidize non-phenolic compounds with the help of redox mediators, which include natural compounds such as gallic acid. By facilitating electron transfer, gallic acid enhances laccase's ability to oxidize a broader range of substrates present in lignocellulosic materials, thereby boosting the enzyme's activity and effectiveness (Torres-Farradá et al., 2024).

Apple pomace

Apple pomace was used as both a carbon and copper source in this experiment. Apples are rich in carbohydrates, fibre and minerals, including copper at a concentration of 1.1 mg/L (Park et al., 2014). Given that apples are among one of the most consumed fruits globally, utilizing waste from apple products helps reduce disposal costs and minimizes environmental impact.

In a study by Palmieri et al. (2000), the addition of $CuSO_4$ to culture broth of *P. ostreatus* resulted in a substantial increase in laccase activity. However, $CuSO_4$ is water soluble and poses an environmental hazard to aquatic organisms, with potential for bioaccumulation (PubChem, n.d.a). To use copper as an inducer for laccase activity for bioremediation, it must be at a low concentration that is non-toxic to the fungi and other organisms and safe for environmental release. Apple pomace could provide a natural and less toxic alternative source of copper.

Park et al. (2014) demonstrated that apple pomace could effectively induce laccase activity. The concentration used in this experiment, 2.5% (w/v), was based on the optimal concentration identified in Park et al. (2014) which enhanced laccase activity without exhibiting toxicity to the fungi or the environment.

3.4 Enzyme analysis

Laccase activity was determined colorimetrically by detecting the oxidation product 2,6-dimethoxyphenol (DMP, ε468=49,600 M-1 cm-1), using a spectrophotometer, as described by Parenti et al. (2013). 50 µl of supernatant was diluted in 0.4 mL of sterile distilled water in cuvettes before 0.5 mL of DMP in 100 mM acetate buffer (pH 5) was added. Absorbance was measured at 468nm twice: initially after one minute, and then again after another minute. The laccase activity was estimated as the difference between these two readings, with one unit (U) representing the formation of 1µmol of product per minute.

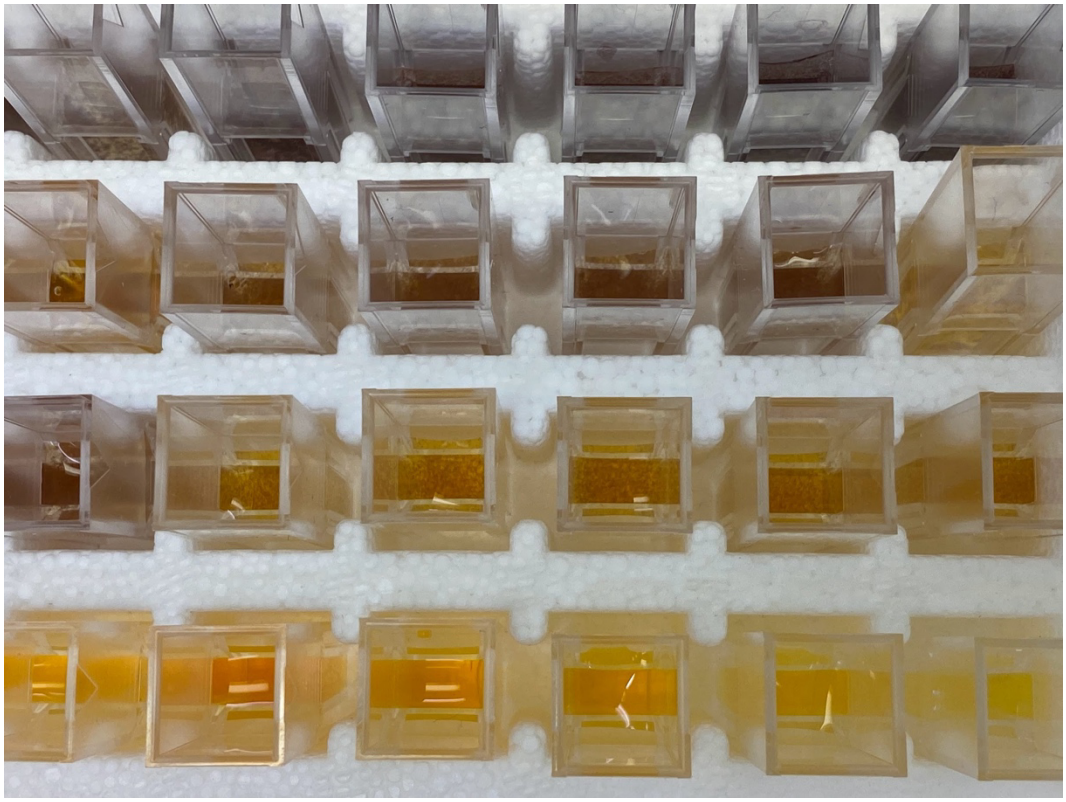

Figure 4. Image of cuvettes after addition of DMP and buffer. The solutions darken as they oxidise.

3.5 Statistical analysis

All experiments were established with three replicates and mean and standard deviation for these are reported in *Table 2*. The data were analysed by analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant at $p<0.05$ using Rstudio, version 2023.12.1 + 402.

4. Results

4.1 Results of experimental study

The results of the experimental study, including mean laccase activity, standard deviation, and p-values for each substance when tested against control 1, are summarised in *Table 2*. All data presented are means of triplicates.

Table 2. Results of laccase activity by substances tested, with mean laccase activity (U/L) and p-value for each substance when tested against control 1.

Substance	Mean Laccase (U/L)	Std	p-value
Spawn only (control 1)	47.76	1.45	-
Lignin (control 2)	330.07	5.65	0.00
Glucose	48.78	2.82	1.00
Glycerol	53.63	1.33	0.98
Tween-80	49.03	6.91	1.00
Ethanol	43.80	16.88	1.00
Glycine betaine	40.09	4.31	0.91
Coffee grounds	67.67	1.35	0.04
Gallic acid	50.31	3.43	1.00
Apple pomace	98.06	5.52	0.00

The highest laccase activity was observed in control 2 with the addition of lignin which reached 330.1 units per litre (U/L). Apple pomace resulted in a significant increase in laccase activity, reaching 98.1 U/L, more than double that of the control (47.8 U/L). Coffee grounds also showed an increase in activity compared to control 1, reaching 67.7 U/L. The other substances tested did not show significant differences in laccase activity when tested against control 1. Additionally, ethanol and glycine betaine showed a lower enzyme activity than control 1.

Statistical analysis indicated that the addition of apple pomace led to a highly significant increase in laccase activity ($p = 0.00$). Addition of coffee grounds also resulted in a statistically significant increase, with a p-value of 0.04, falling under

the $p<0.05$ threshold. ANOVA results revealed statistically significant difference among the group means at the 95% confidence level ($p<0.05$). Post-hoc analysis using Tukey's HSD confirmed that both apple pomace and coffee grounds differed significantly from the control, whereas other substances did not show a significant difference at the 95% confidence level.

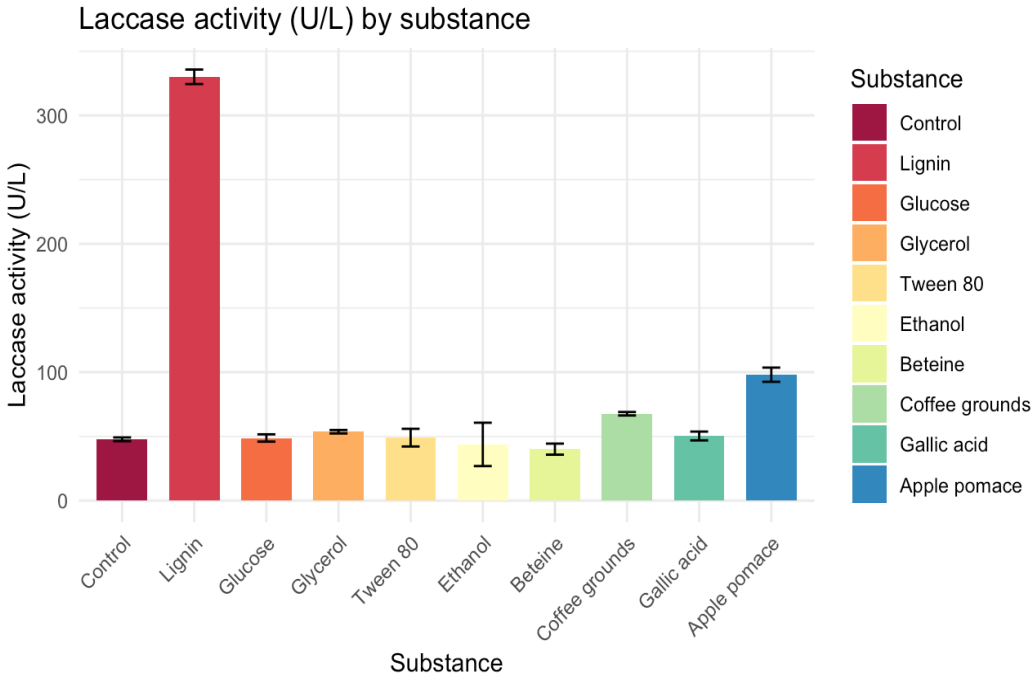

Figure 5. Diagram of results of laccase activity (Unit/Litre) by substance tested, with error bars.

The diagram in *Figure 5* illustrates the laccase activity measured in units per litre across the tested substances. Error bars are included to indicate the standard deviation of the triplicate measurements, providing a visual representation of the variability within each group.

From the diagram, it is evident that the addition of lignin resulted in the highest laccase activity, significantly surpassing all other substances tested. Apple pomace demonstrated the second-highest activity, with a notable increase compared to the control, as indicated by the gap between the bars. Coffee grounds also showed an increase in laccase activity relative to the control, though to a lesser extent than apple pomace.

The error bars highlight the precision of the measurements, with apple pomace and coffee grounds showing relatively tight error bars, suggesting consistent results across replicates, in contrast to ethanol which had a high standard deviation.

The visual data aligns with the statistical analysis, reinforcing that apple pomace and coffee grounds are the only substances that significantly increased laccase activity compared to the control.

5. Discussion

This study aimed to investigate the impact of selected substances on the laccase activity of *Pleurotus ostreatus* when cultivated in water, with possible application to bioremediation. The findings provide valuable insight into the potential for enhancing laccase activity through the addition of organic waste materials, potentially advancing bioremediation practices for treating micropollutant contamination in wastewater.

Apple pomace and coffee grounds were the only substances found to enhance laccase activity in *P. ostreatus* significantly (besides lignin). Apple pomace is rich in carbohydrates and minerals (Park et al., 2014), while coffee grounds contain organic and phenolic compounds (McNutt & He, 2019) that act as inducers for laccase activity (Parenti et al., 2013; de Souza et al., 2004).

In contrast, ethanol and glycine betaine showed lower laccase activity compared to the control. Ethanol, despite being a carbon source, can adversely affect cellular processes and enzyme stability at certain concentrations, suggesting that the concentration used was too high. Glycine betaine, known for its role as an osmoprotectant, may support fungal viability, but did not directly stimulate laccase production.

This study did not measure mycelial growth which could be another factor in interpreting the results. While glucose and other carbon sources likely support mycelial growth, they may not significantly influence laccase production (Isanapong et al., 2017). It is possible that these carbon sources primarily promote fungal biomass rather than enzyme production. Future studies should include assessment of mycelial growth alongside enzyme activity to better understand the relationship between carbon sources and laccase production.

In the study by Teodoro et al. (2018), it was suggested that the presence of a carbon source is essential for maximising the beneficial effects of Tween-80 on laccase production. Tween-80 acts as a facilitator, enhancing enzyme production when paired with an appropriate carbon source. This combination allows Tween-80 to fulfil its role as a facilitator rather than a nutrient provider and could explain the low activity in the experiment.

An important consideration in applying fungal bioremediation to wastewater treatment is the need for aeration. *P. ostreatus* is an obligate aerobe, requiring adequate oxygen for optimal growth and enzyme production (Pointing, 2001). Ensuring proper aeration in wastewater treatment systems is an issue crucial to maintaining effective fungal activity and maximizing laccase output. Another aspect not addressed in this study is the removal of fungal pellets post-remediation, which also warrants further investigation.

Apple pomace demonstrated a significant increase in laccase activity, indicating the presence of potential inducers such as phenolic compounds and copper. However, without detailed compositional analysis, such as mass spectrometry, the exact components responsible for this effect remain speculative.

The enhanced laccase activity observed with apple pomace and coffee grounds may be due to their complex composition, which includes a mix of various compounds acting synergistically. This contrasts with the simpler compositions of other tested substances, such as single carbon sources or surfactants. The results suggest that a combination of nutrient sources and surfactants could yield improved outcomes, similar to those achieved with apple pomace and coffee grounds. While these substrates showed promising results, they did not match the activity levels achieved with lignin. However, lignin's application is limited due to brownification, which complicates its use in wastewater treatment (Blanchet et al., 2022).

From an economic and environmental perspective, utilizing apple pomace and coffee grounds for laccase production is advantageous. These substrates are inexpensive, readily available, and align with circular economy principles by converting waste into valuable resources (Park et al., 2014; Isanapong et al., 2017). Their use not only reduces waste but also promotes sustainable wastewater treatment practices, making them suitable for large-scale applications.

The findings of this study open several avenues for future research. Investigating different combinations and concentrations of substances, varying incubation times, and testing other strains of white-rot fungi could optimize laccase production further. Additionally, assessing the long-term stability and efficacy of these substrates in real wastewater scenarios is crucial.

The promising increase in laccase activity with apple pomace and coffee grounds hold potential for more sustainable bioremediation strategies in wastewater treatment applications. These compounds could enhance the degradation of various micropollutants, including pharmaceuticals and personal care products, which are difficult to remove through conventional methods. Integrating *P. ostreatus* in conjunction with these organic wastes into existing wastewater treatment processes could improve overall efficiency and sustainability.

5.1 Conclusion

The specific research questions were:

- Is laccase production by *P. ostreatus* strain M2191 affected by addition of the selected substances when grown in water?
- If so, can any of the substances be considered suitable for addition to water treatment on a larger scale?

From the substances tested in this experiment, apple pomace and coffee grounds had an additive effect on the enzymatic activity recovered from the supernatants in the samples of mycelium, grain, distilled water and tested substances. These findings highlight the potential for these organic waste materials to boost enzyme activity, which is promising for advancing bioremediation techniques.

Apple pomace and coffee grounds not only proved effective in increasing laccase activity, but also represent economically and environmentally sustainable options for wastewater treatment. Their use could offer a cost-effective solution for enhancing wastewater treatment efficiency while supporting circular economy principles. Further research is warranted to explore the full potential of these substances and to optimize their application in practical bioremediation scenarios.

References

Arregui, L., Ayala, M., Gómez-Gil, X., Gutiérrez-Soto, G., Hernández-Luna, C.E., Herrera de los Santos, M., Levin, L., Rojo-Domínguez, A., Romero-Martínez, D., Saparrat, M.C.N., Trujillo-Roldán, M.A. & Valdez-Cruz, N.A. (2019). Laccases: structure, function, and potential application in water bioremediation. *Microbial Cell Factories*, 18 (1), 200. https://doi.org/10.1186/s12934-019-1248-0

Balan, V., Zhu, W., Krishnamoorthy, H., Benhaddou, D., Mowrer, J., Husain, H. & Eskandari, A. (2022). Challenges and opportunities in producing high-quality edible mushrooms from lignocellulosic biomass in a small scale. *Applied Microbiology and Biotechnology*, 106 (4), 1355–1374. https://doi.org/10.1007/s00253-021-11749-2

Bertram, M.G., Martin, J.M., Wong, B.B.M. & Brodin, T. (2022). Micropollutants. *Current Biology*, 32 (1), R17–R19. https://doi.org/10.1016/j.cub.2021.11.038

Blanchet, C.C., Arzel, C., Davranche, A., Kahilainen, K.K., Secondi, J., Taipale, S., Lindberg, H., Loehr, J., Manninen-Johansen, S., Sundell, J., Maanan, M. & Nummi, P. (2022). Ecology and extent of freshwater browning - What we know and what should be studied next in the context of global change. *Science of The Total Environment*, 812, 152420. https://doi.org/10.1016/j.scitotenv.2021.152420

Bofill, J. (2023). Microplastics and Micropollutants in Water. https://www.eib.org/attachments/lucalli/20230042_microplastics_and_micropollutants_in_water_en.pdf [2024-01-26]

Clendennen, S.K. & Boaz, N.W. (2019). Chapter 14 - Betaine Amphoteric Surfactants—Synthesis, Properties, and Applications. I: Hayes, D.G., Solaiman, D.K.Y., & Ashby, R.D. (red.) *Biobased Surfactants (Second Edition)*. AOCS Press. 447–469. https://doi.org/10.1016/B978-0-12-812705-6.00014-9

El-Batal, A.I., ElKenawy, N.M., Yassin, A.S. & Amin, M.A. (2015). Laccase production by Pleurotus ostreatus and its application in synthesis of gold nanoparticles. *Biotechnology Reports*, 5, 31–39. https://doi.org/10.1016/j.btre.2014.11.001

European Parliament (2024). *New EU rules to improve urban wastewater treatment and reuse | News | European Parliament*. https://www.europarl.europa.eu/news/en/press-room/20240408IPR20307/new-eu-rules-to-improve-urban-wastewater-treatment-and-reuse [2024-07-31]

Frankel, T. (2021). *How Do Wastewater Treatment Plants Work? SSI Aeration*. https://www.ssiaeration.com/how-do-wastewater-treatment-plants-work/ [2024-02-13]

Hultberg, M., Ahrens, L. & Golovko, O. (2020). Use of lignocellulosic substrate colonized by oyster mushroom (*Pleurotus ostreatus*) for removal of organic micropollutants from water. *Journal of Environmental Management*, 272, 111087. https://doi.org/10.1016/j.jenvman.2020.111087

IPCC (2022). Fact sheet - Food and Water. https://www.ipcc.ch/report/ar6/wg2/downloads/outreach/IPCC_AR6_WGII_FactSheet_FoodAndWater.pdf

Isanapong, J., Krailoekpaiboon, T., Noiniyom, W. & Panchal, S. (u.å.). Utilization of Organic Wastes for Laccase Production by Pleurotus ostreatus. https://doi.org/10.14416/j.ijast.2017.08.003

Kang, L.-L., Zeng, Y.-N., Wang, Y.-T., Li, J.-G., Wang, F.-P., Wang, Y.-J., Yu, Q., Wang, X.-M., Ji, R., Gao, D. & Fang, Z. (2022). Removal of pollutants from wastewater using coffee waste as adsorbent: A review. *Journal of Water Process Engineering*, 49, 103178. https://doi.org/10.1016/j.jwpe.2022.103178

Lewis, T. & Stone, W.L. (2024). Biochemistry, Proteins Enzymes. I: *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK554481/ [2024-02-06]

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S. & Wang, X.C. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of The Total Environment*, 473–474, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065

McNutt, J. & He, Q. (Sophia) (2019). Spent coffee grounds: A review on current utilization. *Journal of Industrial and Engineering Chemistry*, 71, 78–88. https://doi.org/10.1016/j.jiec.2018.11.054

Meza, J.C., Auria, R., Lomascolo, A., Sigoillot, J.-C. & Casalot, L. (2007). Role of ethanol on growth, laccase production and protease activity in *Pycnoporus cinnabarinus* ss3. *Enzyme and Microbial Technology*, 41 (1), 162–168. https://doi.org/10.1016/j.enzmictec.2006.12.018

Mohammadi, S.A., Najafi, H., Zolgharnian, S., Sharifian, S. & Asasian-Kolur, N. (2022). Biological oxidation methods for the removal of organic and inorganic contaminants from wastewater: A comprehensive review. *Science of The Total Environment*, 843, 157026. https://doi.org/10.1016/j.scitotenv.2022.157026

Nongthombam, J., Kumar, A. & Patidar, S. (2021). A REVIEW ON STUDY OF GROWTH AND CULTIVATION OF OYSTER MUSHROOM. *Plant Cell Biotechnology and Molecular Biology*, 22, 55–65

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B. & Sannia, G. (2000). Copper Induction of Laccase Isoenzymes in the Ligninolytic Fungus Pleurotus ostreatus. *Applied and Environmental Microbiology*, 66 (3), 920–924

Parenti, A., Muguerza, E., Redin Iroz, A., Omarini, A., Conde, E., Alfaro, M., Castanera, R., Santoyo, F., Ramírez, L. & Pisabarro, A.G. (2013). Induction of laccase activity in the white rot fungus Pleurotus ostreatus using water polluted with wheat straw extracts. *Bioresource Technology*, 133, 142–149. https://doi.org/10.1016/j.biortech.2013.01.072

Park, Y.-J., Yoon, D.-E., Kim, H.-I., Kwon, O.-C., Yoo, Y.-B., Kong, W.-S. & Lee, C.-S. (2014). Overproduction of Laccase by the White-Rot Fungus Pleurotus ostreatus Using Apple Pomace as Inducer. Mycobiology, 42 (2), 193. https://doi.org/10.5941/MYCO.2014.42.2.193

Piska, K., Ziaja, K. & Muszynska, B. (2016). Edible mushroom pleurotus ostreatus (Oyster mushroom) – Its dietary significance and biological activity. Acta scientiarum Polonorum. Hortorum cultus = Ogrodnictwo, 16, 151–161

Pistocchi, A., Andersen, H.R., Bertanza, G., Brander, A., Choubert, J.M., Cimbritz, M., Drewes, J.E., Koehler, C., Krampe, J., Launay, M., Nielsen, P.H., Obermaier, N., Stanev, S. & Thornberg, D. (2022). Treatment of micropollutants in wastewater: Balancing effectiveness, costs and implications. *Science of The Total Environment*, 850, 157593. https://doi.org/10.1016/j.scitotenv.2022.157593

Pointing, S. (2001). Feasibility of bioremediation by white-rot fungi. *Applied Microbiology and Biotechnology*, 57 (1–2), 20–33. https://doi.org/10.1007/s002530100745

PubChem (n.d.a). *Copper Sulfate*. https://pubchem.ncbi.nlm.nih.gov/compound/24462 [2024-07-12]

PubChem (n.d.b). *D-Glucose*. https://pubchem.ncbi.nlm.nih.gov/compound/5793 [2024-07-12]

PubChem (n.d.c). *Ethanol*. https://pubchem.ncbi.nlm.nih.gov/compound/702 [2024-07-16]

PubChem (n.d.d). Gallic Acid. https://pubchem.ncbi.nlm.nih.gov/compound/370 [2024-07-17]

PubChem (n.d.e). *Glycerin*. https://pubchem.ncbi.nlm.nih.gov/compound/753 [2024-07-16]

PubChem (n.d.f). *Polysorbate 80*. https://pubchem.ncbi.nlm.nih.gov/compound/Polysorbate-80 [2024-07-16]

Rogowska, J., Cieszynska-Semenowicz, M., Ratajczyk, W. & Wolska, L. (2020). Micropollutants in treated wastewater. *Ambio*, 49 (2), 487–503. https://doi.org/10.1007/s13280-019-01219-5

Shaw. 2011. Large volume ethanol spills - environmental impacts and response options. Commonwealth of Massachusetts. Department of environmental protection. 72 pp. https://www.mass.gov/files/documents/2016/09/us/ethanol-spill-impacts-and-response-7-11_44776_56452.pdf [2024-07-23]

de Souza, C.G.M., Tychanowicz, G.K., de Souza, D.F. & Peralta, R.M. (2004). Production of laccase isoforms by Pleurotus pulmonarius in response to presence of phenolic and aromatic compounds. *Journal of Basic Microbiology*, 44 (2), 129–136. https://doi.org/10.1002/jobm.200310365

Sundin, A.M., Linderholm, L., Hedlund, B., Bly Joyce, K. & Klingspor, K. (2017). *Avancerad rening av avloppsvatten för avskiljning av läkemedelsrester och andra oönskade ämnen*. *Naturvårdsverket*. https://www.naturvardsverket.se/globalassets/media/publikationer-pdf/6700/978-91-620-6766-3.pdf [2024-02-13]

Teodoro, T.S., Oliveira, F.D., Poffo, C., Braga, L.P., Arbigaus, A., Rampinelli, J.R., Wisbeck, E., Bonatti-Chaves, M. & Furlan, S.A. (2018). The influence of Tween 80 on laccase production by Pleurotus sajor-caju and the efficiency of crude enzyme broth in the removal of bisphenol-A. *Arquivos do Instituto Biológico*, 85 (0). https://doi.org/10.1590/1808-1657001022017

Torres-Farradá, G., Thijs, S., Rineau, F., Guerra, G. & Vangronsveld, J. (2024). White Rot Fungi as Tools for the Bioremediation of Xenobiotics: A Review. *Journal of Fungi*, 10 (3), 167. https://doi.org/10.3390/jof10030167

Usha, K.Y., Praveen, K. & Reddy, B.R. (2014). Enhanced Production of Ligninolytic Enzymes by a Mushroom Stereum ostrea. *Biotechnology Research International*, 2014 (1), 815495. https://doi.org/10.1155/2014/815495

Wastewater treatment (2024). *Council of the European Union*. https://www.consilium.europa.eu/en/policies/wastewater-treatment/ [2024-02-08]

Zamri, M.F.M.A., Bahru, R., Suja', F., Shamsuddin, A.H., Pramanik, S.K. & Fattah, I.M.R. (2021). Treatment strategies for enhancing the removal of endocrine-disrupting chemicals in water and wastewater systems. *Journal of Water Process Engineering*, 41, 102017. https://doi.org/10.1016/j.jwpe.2021.102017

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