Impact of nitrogen source on laccase activity in water by spawn pellets of *Pleurotus ostreatus* (oyster mushroom) and applications for water treatment

Inoka Sanjeewani Ranamukha Hewage

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Swedish University of Agricultural Sciences, SLU
Faculty of Landscape Architecture, Horticulture and Crop Production Science (LTV)
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Inoka Sanjeewani Ranamukha Hewage

Supervisor: **Malin Hultberg, Swedish University of Agricultural Sciences, Department of Biosystems and Technology**

Examiner: Samar Khalil, Swedish University of Agricultural Sciences, Department of Biosystems and Technology

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Swedish University of Agricultural Sciences

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Department of Biosystems and technology

Abstract

Chemical pollution due to organic micropollutants results in a significant threat to aquatic ecosystems and human health. In addressing this challenge, bioremediation treatments by *Pleurotus* spp. based on oxidoreductase enzymes, including laccases, demonstrate potential for the removal of organic micropollutants from wastewater. This thesis aimed to study a spawn-based method for the production of laccase in water using the white-rot fungi *P. ostreatus* M2191.

The study investigated the impact of two different spawn types, either grain-based or sawdust-based, and four nitrogen sources (ammonium nitrate, urea, yeast extract, wheat bran) on laccase activity in both experimental and literature studies. The research revealed that grain-based spawn resulted in significantly higher laccase activity compared to sawdust-based spawn. Among the nitrogen sources tested, yeast extract followed by wheat bran were the most effective in increasing laccase activity. The results indicated that *P. ostreatus* exhibits lower enzyme activity with inorganic nitrogen sources compared to organic nitrogen sources when subjected to submerged fermentation. The literature study demonstrated the variability in laccase production among *Pleurotus* spp. and strains for different nitrogen sources even when provided at the same concentrations. Thus, detailed studies on specific strains are needed to optimize laccase activity. This study contributes valuable insights toward developing a sustainable method for enzyme-based wastewater treatment by illustrating the influence of spawn type and nitrogen source on laccase activity.

Keywords: Laccase, *Pleurotus ostreatus*, micropollutants, nitrogen, wastewater

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Abbreviations

N	Nitrogen
SmF	Submerged Fermentation
SSF	Solid State Fermentation
TPs	Transformation products
WFD	Water Framework Directive
WRF	White-rot fungi
WWTPs	Wastewater Treatment Plants

1. Introduction

Chemical pollution is considered an increasing threat to the environment, particularly increased with the influences of climate change and population growth. In recent years, the emergence of organic micropollutants in aquatic environments has grown into a global environmental concern. Most of the micropollutants are not eliminated in traditional wastewater treatment plants. As a result, they persist in aquatic ecosystems or transform into new chemical species (Mir-Tutusaus et al. 2018). Therefore, different techniques, including physical, chemical, and biotechnological approaches, have been investigated to either retain or convert these molecules into less harmful forms (Arregui et al. 2019). The use of enzyme-mediated processes for degrading and eliminating pollutants from contaminated water is the focus of the present work and it represents a significant area of intensive research (Mohammadi et al. 2022; Saravanan et al. 2021; Naghdi et al. 2018). It aims to develop eco-friendly and efficient tools to treat and enhance water quality.

2. Background

2.1 Micropollutants

Micropollutants, also known as emerging contaminants, are substances that can be harmful, persistent, and bioaccumulative, and which even at very low concentrations can have an adverse impact on the environment and human health (Mir-Tutusaus et al. 2018; Luo et al. 2014). This diverse group contains a wide range of anthropogenic and naturally occurring substances ranging from pharmaceuticals to ingredients of personal care products, pesticides, endocrine disruptors, and industrial chemicals and are often found in water at low concentrations ranging from a few ng/L to several µg/L (Grandclement et al. 2017; Luo et al. 2014).

Compounding this issue, micropollutants also transform in the environment through various chemical, physical, and biological processes or during the water treatment processes and this results in transformation products (TPs). For instance, during water treatment processes such as ozonation, the parent compounds can react with ozone to form TPs like bromate. Some of these TPs are found to be more abundant in the aquatic environment and even have higher toxicity compared to their parent compounds or may persist even after the primary compound has been completely degraded (Escher et al. 2011).

2.2 Spreading of micropollutants in aquatic environments

Micropollutants are discharged into the water bodies through several pathways including effluents of industrial and municipal wastewater treatment plants (WWTPs), sewer overflows, improper disposal, and diffuse sources such as runoff from agriculture, livestock, and aquaculture fields. As a result, these micropollutants can be detected in surface water, groundwater, and even in drinking water (Hermes et al. 2018). The release of wastewater treatment plant effluent into

surface water is the main cause of micropollutants in surface water. In addition, high rainfall causes combined sewer overflows, resulting in high discharge of micropollutants to the surface water. In general, groundwater is less contaminated with micropollutants than surface water. Sources of micropollutants in groundwater include sewage from septic tanks and sewer systems, interactions between surface and groundwater, infiltration of contaminants from agricultural land, and landfill leachate (Luo et al. 2014).

2.3 EU Water Framework Directive

The EU Water Framework Directive (WFD) is a section of legislation implemented by the European Union in 2000 to establish a framework for the protection and sustainable management of water resources across Europe. One of its key objectives is to set environmental quality standards for priority pollutants in surface water to reduce the risks these substances cause to human health and the aquatic environment (European Commission n.d; EU Water Framework Directive 2000).
The Surface Water Watch List is a monitoring programme established in 2013 based on the WFD to identify and address emerging pollutants or pollution trends that could potentially impact the quality of surface waters throughout the European Union (European Commission n.d; EUR – Lex 2022). Substances on the watch list are required to be monitored by member states for up to four years, at least once a year. The watch list was first released in 2015, and it has been subsequently updated every two years through additions or removals in 2018, 2020, and 2022 (Backhaus 2023; European Commission n.d). This list includes a wide range of substances, including pharmaceuticals, pesticides, industrial chemicals, and personal care products. For instance, in 2022 the commission identified several candidates, including the fungicide azoxystrobin, herbicide diflufenican, and insecticide fipronil, as well as antibiotics clindamycin and ofloxacin, human pharmaceuticals metformin and included them into the list, bringing the total to 26 substances (Backhaus 2023; EUR – Lex 2022).

2.4 Wastewater treatment plants

The WWTPs serve as a primary source containing micropollutants and therefore, ensuring proper treatment of wastewater is essential for preserving water resources. In a conventional WWTP, the treatment procedures include aeration, coagulation, flocculation, sedimentation, filtration and ozone, chemical or ultraviolet disinfection process before it is released into the environment (Krishnan et al.

2021). The primary processes for wastewater treatment consist of grit removal, sedimentation, and filtration. These processes target the removal of suspended solids in WWTPs and are ineffective in removing the majority of micropollutants. The secondary process involves biological treatment methods, and their efficiency is linked to biodegradation and sorption. During this process micropollutants degrade to different extents, resulting in mineralization or incomplete degradation. Due to its high treatment costs tertiary treatment processes are used when high quality discharged water is required for certain purposes (Krishnan et al. 2021; Luo et al. 2014).

2.5 Methods for removal of micropollutants

There are various treatments including physical, chemical, and biological methods for the removal of micropollutants from water sources. Physical methods involve processes where pollutants are not destroyed but trapped or filtered out from wastewater with physical interaction with a moving agent without undergoing chemical changes. Adsorption, absorption, sedimentation, and membrane filtration are considered important methods of physical removal (Khan et al 2022; Kanaujiya et al 2019).

Chemical methods are used alone or in combination with physical methods that have been enhanced by advanced technological improvements, including coagulation-flocculation, ion exchange, heterogeneous photocatalysis, and advanced oxidation processes such as ozonation, electrochemical oxidation, and Fenton oxidation. But still, the implementation of these advanced treatment methods is limited due to economic constraints. Hence, there is a growing demand for alternative, cost-efficient wastewater treatment solutions (Khan et al 2022; Karungamye. 2020).

Another option is biological treatments, numerous species of bacteria and fungi have been demonstrated to break down micropollutants even when present in high concentrations. However, effective bioreactor systems are essential for industrial sustainability and commercial viability, highlighting the importance of bioprocess development (Kanaujiya et al. 2019).

2.5.1 Bioremediation and mycoremediation

Microorganisms play an important role in various environmental processes such as bioremediation (biological degradation) of organic and inorganic contaminants. Bioremediation is a natural treatment process that uses microorganisms and their enzymes to break down persistent compounds by transforming highly toxic substances into less toxic compounds or complete mineralization, producing inorganic compounds such as CO_2 and H_2O (Sattar et al. 2022; Becerra et al. 2018). Fungi, bacteria, and algae are widely utilized microorganisms in bioremediation.

Some organisms from the kingdom fungi are being utilized for the treatment of water or soil contaminated with micropollutants and this process is known as mycoremediation (Sattar et al. 2022). This eco-friendly approach utilizes the natural abilities of fungi to break down pollutants, providing a promising solution for environmental restoration. An important group of enzymes that are involved in this process are the oxidoreductase enzymes. Due to the low specificity of these enzymes, their use has gained increasing attention in the removal of various pollutants through enzymatic bio-oxidation (Mohammadi et al. 2022).

2.5.2 The bioremediation potential of white rot fungi

White-rot fungi (WRF) of basidiomycetes are crucial in natural bio-oxidation processes in terrestrial environments, primarily targeting lignin in plant structures as their substrate and effectively oxidizing and degrading it (Mohammadi et al. 2022). The WRF typically consists of hyphae surrounded by cell walls and branch periodically, forming a network known as mycelium. Because of their rigid cell walls, WRF releases extracellular enzymes to degrade complex polymers and then absorb the smaller molecules as nutrients (Sattar et al. 2022).

These fungi release lignin-modifying enzymes and other compounds to degrade lignin. These enzymes include laccase and lignin-modifying peroxidases particularly, lignin peroxidase, manganese peroxidase, and versatile peroxidase which act synergistically during lignin degradation (Tutusaus et al. 2018). The lignin degradation process is induced by carbon or nitrogen starvation, and the secretion patterns of enzymes vary among species. Additionally, white-rot fungi consist of the cytochrome P450 system, an intracellular enzymatic system important for degrading various organic micropollutants (Dashtban et al. 2010). Numerous studies indicate that white-rot fungi, including *Pleurotus* spp., *Phanerochaete chrysosporium* and *Trametes versicolor* have the capability to degrade micropollutants (Naghdi et al 2018; Margot et al 2013; Dashtban et al 2010). Among these fungi, *P. ostreatus* (oyster mushrooms) shows strong laccase activity and is easily cultivable on a wide range of natural or synthetic media (Yang

et al.2017; Chiranjeevi et al. 2014). It is also an edible and commonly cultivated species within the mushroom production industry. Thus, equipment and technologies for its production are easily accessible and well- developed (Sanchez 2010).

2.6 Fungal cultivation

Submerged fermentation (SmF) and solid state fermentation (SSF) are two distinct methods used in the cultivation and production of fungi (Lubeck et al. 2022; Adamian et al. 2021). In SmF, microorganisms are cultivated in a liquid medium with appropriate nutrients and oxygen, typically carbohydrates with nitrogen supplements, under aerobic conditions (Adamian et al. 2021). SmF enables easy monitoring of various process parameters such as temperature, dissolved oxygen, pH, and the concentration of water-soluble substrates. Additionally, it facilitates easy mixing of the broth and biomass separation after fermentation. This level of control allows for optimized conditions throughout the fermentation process, resulting in high yields of desired products (Lubeck et al. 2022; Adamian et al. 2021). Although industrial enzyme production often utilizes SmF due to its simple scalability, SmF can be limited by uncontrolled mycelial growth, leading to excessive biomass. This expansion causes an increase in broth viscosity, thereby limiting mass and oxygen transfer, resulting in reduced metabolic rates and enzyme secretion. Therefore, various strategies have been employed to address oxygen and mass transfer limitations (Brijwani et al. 2010).

Fungal production in SSF involves the growth of microorganisms on the inert substrate (synthetic materials) or a natural substrate (organic materials) as solid support in the absence or near absence of a free liquid medium (Brijwani et al. 2010; Gowthaman et al. 2001). A variety of solid substrates can be used, including wheat bran, grains, legumes, spent grains, and other lignocellulosic plant materials (Lubeck et al. 2022). SSF is demonstrated to be suitable for enzyme production by filamentous fungi due to its ability to replicate the natural growth conditions of these fungi (Brijwani et al. 2010). This type of cultivation, SSF, is mainly used to produced the fruiting bodies.

2.7 Laccase

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are obtained from various sources such as plants, fungi, bacteria, and insects (Arregui et al. 2019; Gasser et al. 2014). Especially fungal laccases have received much attention

considering mycoremediation due to their low substrate specificity and capacity to transform diverse compounds such as aminophenols, polyphenols, and polyamines (Gasser et al. 2014). Fungal laccase belongs to the multicopper blue phenoloxidases. The oxidation reactions facilitated by laccases produce free radicals which assist in producing mediators which then can react with a wide range of high-redox potential substrates. Thus, non-enzymatic pathways for oxidative polymerization or depolymerization reactions are created without the direct involvement of enzymes (Dashtban et al. 2010). Since laccases have wide substrate ranges and use only oxygen as the final electron receptor, they play an important role in the bioremediation of micropollutants (Yang et al. 2017).

The production of laccase in fungi is affected by culture conditions, including the type of carbon and nitrogen source, the ratio of carbon to nitrogen, the pH of the fermentation medium, presence of lignocellulosic substances and inducers (Chiranjeevi et al. 2014). In several fungi, laccase production is considered part of secondary metabolism, and synthesis of laccase is induced when there is a lack of carbon or nitrogen. This results in a long production cycle, obstructing industrial laccase production (Yang et al. 2017). However, in some strains nitrogen levels have no impact on laccase activity. But it was also reported that higher laccase production under low carbon-to-nitrogen ratios and other studies demonstrated that high laccase production occurred at high carbon-to-nitrogen ratios. As well as the addition of various supplements, specific compounds such as lignin and some metal ions to the media can significantly increase the production and activity of laccase (Brijwani et al. 2010).

2.8 Nitrogen and fungal metabolism of nitrogen

Nitrogen (N) is present in numerous simple compounds and plays an important role as a fundamental component in complex macromolecules such as proteins and nucleic acids and is essential for the structure and function of all living organisms (Marzluf 1996). About 78% of the atmosphere consists of N_2, but despite its abundance, N is a limiting factor for plant and microbial growth. Therefore, these organisms have control mechanisms to regulate N uptake, assimilation, and utilization (Marzluf 1997).

As nitrogen is an essential requirement for growth, fungi possess the ability to metabolize a wide variety of compounds as nitrogen sources and can produce the necessary catabolic enzymes for different metabolic pathways as needed (Marzluf 2003). Filamentous fungi can use a variety of substances as their only nitrogen sources, but for as long as they are present in the medium, they mostly depend on

energetically favourable nitrogen sources such as glutamine and NH_4^+ (Tudzynski 2014). In the absence of these preferred nitrogen compounds, fungi readily utilize numerous secondary nitrogen sources such as nitrate, proteins, purines, acetamide, and various amino acids (Tudzynski 2014; Wong et al. 2008). Using secondary nitrogen sources is highly regulated in filamentous fungi, and typically requires the synthesis of pathway-specific catabolic enzymes and permeases (Marzluf 1997).

2.9 Aim and research objective

Micropollutants in wastewater result in significant environmental challenges due to their persistence and potential harm to aquatic ecosystems and human health. Conventional treatment methods are often insufficient, and advanced technological methods are economically constrained, Therefore, there is a need to develop innovative, efficient, and sustainable methods to address this issue. In this context, Laccase, an enzyme from white-rot fungi, that can degrade a wide range of organic micropollutants, becomes interesting making it valuable for wastewater treatment. This study is part of an effort to develop a method where laccase is produced by living fungi directly in water.

The aim of this thesis was to study a spawn-based method for the production of laccase in water using the white-rot fungi *P. ostreatus*. The long-term goal is to develop a mycoremediation strategy which can be applied for wastewater treatment. Two different types of spawn were evaluated, commercial grain spawn as well as a spawn based on sawdust. Kraft lignin was used as an inducer of laccase activity. Specific focus was on the impact of nitrogen source on the laccase activity.

The specific research questions were:

1. Which impact does the type of spawn (grain-based or sawdust-based) have on laccase activity by *P. ostreatus* M2191 when grown in water?
2. Is laccase production by *P. ostreatus* strain M2191 affected by addition of the selected compounds ammonium nitrate, urea, yeast extract and wheat bran when grown in water?

2.10 Limitations of the study

The experimental work was performed with strain *P. ostreatus* M2191 and the obtained results applies currently only to this strain. As previously mentioned, this study was performed in order to develop a mycoremediation strategy suitable for wastewater treatment. The nitrogen concentrations which were applied in this

study, 1 and 10 mM (14 and 140 mg/L), were selected based on levels of nitrogen which can be expected in effluent wastewater in Swedish wastewater plants.

3. Material and methods

3.1 Literature study

To understand the impact of nitrogen source and concentration on the production of laccase in water by the white-rot fungus *Pleurotus ostreatus*, a comprehensive literature study was conducted. Information was collected from multiple search engines, with a primary focus on Google Scholar and Web of Science. The used keywords were laccase/laccases; *Pleurotus*; nitrogen; water.

3.2 Experimental work performed in the study

3.2.1 Microorganism and cultivation

Grain spawn of the white-rot fungal species *Pleurotus ostreatus* M2191 was obtained from Mycelia BVBA, Belgium. The C/N ratio of the grain spawn has been determined in previous work as approximately 10:1. Spawn based on sawdust (Birch, 2-4 mm) was produced by inoculation of 3% (w/w) of the grain spawn in sterilized sawdust with a moisture content of 65%. The inoculated sawdust was cultivated for 12 days before use in experiments. Sawdust has been reported to have a C/N ratio ranging between 200-400:1 (Nihlstrand, 2013). Thus, the fungi can be expected to have access to considerably more nitrogen when produced in grain compared to sawdust.

The spawn was added to sterile distilled water (40 g/L wet weight) cultivated in Erlenmeyer glass flasks on a horizontal orbital shaker (VWR, Advanced 5000 Shaker, Radnor, PA, USA) operated at 100 rpm at room temperature (20-22 °C). For some treatments, kraft lignin (Sigma-Aldrich 370959) was added at start in a concentration of 5 g/L (dry weight, dw) in order to induce the laccase production. The spawn will develop into pellets composed of the grain and mycelium growing out of the grain.

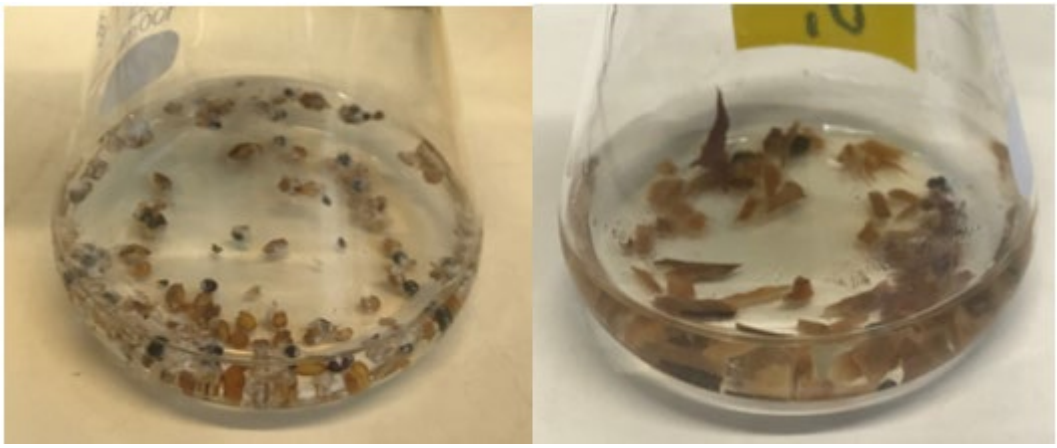

Figure 1.Left: P. ostreatus M2191 grain spawn cultivated in Erlenmeyer flask. Right: P. ostreatus M2191 spawn based on sawdust cultivated in Erlenmeyer flask.

3.2.2 Experimental set-up

The Erlenmeyer flasks each contained 25 mL of the sample, consisting of sterile distilled water and grain spawn, with or without the addition of lignin. The experiment was performed with three replicates. At start, different nitrogen sources were added to the samples. The nitrogen sources were ammonium nitrate (NH_4NO_3), urea ($CO(NH_2)_2$), yeast extract, and wheat bran. Nitrogen source was introduced at concentrations of 1 mM and 10 mM, and each experiment included a control without the addition of any nitrogen source. The nitrogen sources, including ammonium nitrate, urea, and yeast extract, were dissolved in water and subjected to filter sterilization. The yeast extract contained approximately 9.8% nitrogen based on its dry weight (dw). The stock solutions were prepared at concentrations of 0.5 M (40 g/L) for ammonium nitrate, 0.5 M (30 g/L) for urea, and 140 g/L for yeast extract. To maintain consistency, it was approximated that the nitrogen concentration in each of these stock solutions was 1 M (14 g/L). To achieve a nitrogen concentration of 1 mM in the samples (with a sample volume of 25 ml), 25 µl of the stock solution was added. For a concentration of 10 mM, 250 µl of the stock solution was added. The nitrogen content in the solid nitrogen source, wheat bran, has been determined as 1.8% in previous work. For flasks with a nitrogen concentration of 1 mM, 20 mg of wheat bran was added. For those with a 10 mM nitrogen concentration, 200 mg of wheat bran was added. Following 3 days of incubation on the shaker, pellets containing spawn, mycelium, and lignin had formed and the laccase activity was measured.

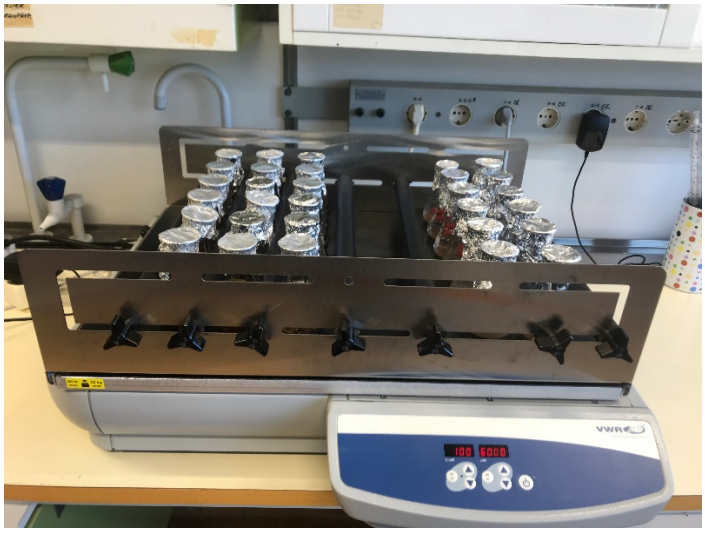

Figure 2.Erlenmeyer glass flasks with spawn on horizontal orbital shaker (VWR, Advanced 5000 Shaker, Radnor, PA, USA) operated at 100 rpm

3.2.3 Enzyme analysis

Laccase activity was analyzed colorimetrically by detecting the oxidation product 2,6-dimethoxyphenol (DMP), following the method described by Parenti et al. (2013), which utilized a molar extinction coefficient (ε468) of 49,600 M^{-1} cm^{-1} for DMP. The reaction mixture comprised 0.45 mL of diluted sample and 0.5 mL of 10 mM 2,6-dimethoxyphenol (DMP) in 100 mM acetate buffer (pH 5). Absorbance readings were taken at 468 nm. Enzyme activity was quantified, with one unit (U) defined as the production of 1 µmol of product per minute.

3.2.4 Statistical analysis

All experiments were conducted with three replicates, and the mean values along with their corresponding standard deviations are reported. The data were analyzed by analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was determined at a significance level of $p<0.05$. The statistical analysis was conducted using Minitab version 19.

4. Results

4.1 Literature study

4.1.1 Effect of nitrogen source and concentration on laccase activity in *Pleurotus* spp. under submerged fermentation

In the literature study on the effect of nitrogen source on laccase activity in various *Pleurotus* spp. during submerged fermentation, 11 papers were found. The findings presented in these papers are compiled in Table 1. The species that have mostly been studied are *P. ostreatus* and *P. sajor-caju*. Both inorganic nitrogen sources (ammonium sulphate, ammonium nitrate, ammonium tartrate, ammonium phosphate, ammonium chloride sodium nitrate and potassium nitrate) and organic nitrogen sources have been applied. The organic nitrogen sources include peptone, yeast extract, beer yeast, urea, malt extract, casein extract, soy extract/protein and different amino acids. Different units are applied to nitrogen concentration (mg/L and mM) and to enzyme activity (U/L (or mL), nmol/ml and U/mg dry weight fungal biomass). It should also be pointed out that different methods have been used for measuring laccase activity. Thus, absolute values cannot be compared but the focus is on summarizing the trends.

In several of the studies presented in Table 1, the nitrogen concentrations have been the same but the source of nitrogen have been varied. Based on this setup Hou et al. (2004), Bettin et al. (2008), Stajic et al. (2006), Mikiashvili et al. (2006), Zhu et al. (2016), Elsayed et al. (2012) and Khan et al. (2016) demonstrated variable effects of different nitrogen sources when applied at the same concentration. In the study by Stajic et al. (2006), when nitrate and ammonium were used as inorganic N sources at the same concentrations, nitrate N sources resulted in lower laccase activity compared to ammonium N sources. However, studies by Mikiashvili et al. (2006) and Khan et al. (2016) show different results, indicating that certain nitrate N sources such as sodium nitrate result in higher laccase activity than ammonium N sources. The majority of studies indicate that when inorganic and organic

nitrogen sources are applied at the same concentrations, organic N sources result in higher laccase activity compared to inorganic N sources.

In these studies, various *Pleurotus* spp. are tested for laccase activity, and their behaviours vary. When the laccase activity of various *Pleurotus* spp. and strains was examined using the same concentration of different nitrogen sources in experiments by Stajic et al. (2006) and Mikiashvili et al. (2006), *P. eryngi* exhibited lower laccase activity for both inorganic and organic nitrogen sources compared to the other tested *Pleurotus* spp.

In the study by Kaal et al. (1995), the effect of both inorganic and organic nitrogen source on laccase activity in *P. ostreatus* was investigated. The results showed that when NH_4^+-N was used as the nitrogen source, there was an approximately threefold increase in laccase activity when the concentration increased. Using an organic nitrogen source (peptone) at the same concentration resulted in similar laccase activity. Also in the study by Elsayed et al (2012), the organic nitrogen sources performed on similar or lower level compared to the control in which nitrogen was applied as ammonium. However, in a majority of the studies where the nitrogen levels can be compared, increasing laccase activity was observed when using an organic nitrogen source (Hou et al. 2004; Duran-Sequeda et al. 2021; Mikiashvili et al. 2006; Zhu et al. 2016; Khan et al. 2016).

In most studies (Khan et al. 2016; Stajic et al. 2006; Mikiashvili et al. 2006; Hou et al. 2004), peptone exhibits higher laccase activity compared to yeast extract and casein hydrolysate, although the latter two also demonstrate considerable laccase activity among the tested organic nitrogen sources. Overall, urea performed poorly compared to the other organic nitrogen sources (Khan et al. 2016; Zhu et al. 2016; Elsayed et al 2012; Hou et al. 2004).

Also, the concept of using the same nitrogen source but in increasing concentrations have been tested. Of the inorganic nitrogen source, mostly ammonium has been applied in these experiments (Duran-Sequeda et al. 2021; Khan et al. 2016; Patrick et al. 2011; Stajic et al. 2006). In three of these studies (Duran-Sequeda et al. 2021; Patrick et al. 2011; Stajic et al. 2006) it was shown that increasing levels of ammonium did not result in increasing laccase activity. For the study by Kaal et al. (1995), only two levels of ammonium concentration were applied and increasing concentration resulted in increasing laccase activity. Of the organic nitrogen sources, Zhu et al. (2016) and Stajic et al. (2006) demonstrated increasing laccase activity when the levels of the nitrogen source were increased (yeast extract and peptone). Bettin et al. (2008) applied increasing concentrations of casein and observed a slight decrease in laccase activity in the highest concentration (2%).

Table 1. Effect of nitrogen source and concentration on laccase activity in Pleurotus spp. under submerged fermentation

Pleurotus spp	Tested nitrogen source	Nitrogen concentration	Laccase activity	Reference
P. ostreatus (CIMW 4.92)	NH_4+-N	2.2 mM	1.6 nmol/ml	Kaal et al. 1995
		56.0 mM	5.8 nmol/ml	
	peptone N	56.0 mM	5.9 nmol/ml	
P. ostreatus strain 32	Urea	0.5 g/L	Approximately*90 U/ml	Hou et al. 2004
	$(NH_4)_2SO_4$	0.5 g/L	Approximately*70 U/ml	
	Ammonium tartrate	0.5 g/L	Approximately*95 U/ml	
	Peptone	0.5 g/L	Approximately*143 U/ml	
	yeast extract	0.5 g/L	Approximately*125 U/ml	
P. sajor – caju Pl- 27	NH_4NO_3	2.6 mM	255 U/mg dry weight fungal biomass	Fu et al. 1996
		26 mM	235 U/mg dry weight fungal biomass	
P. sajor-caju PS-2001	Pure casein	0.5 g/L	5.62 ± 1.12 U / mL	Bettin et al. 2008
		1.0 g/L	6.60 ±1.46 U / mL	
		1.5 g/L	15.40 ± 0.73 U / mL	
		2.0 g/L	12.22 ± 1.12 U / mL	
	meat peptone	1.0 g/L	0.24 - 1.22 U / mL	
	beer yeast	1.0 g/L		
	soy extract	1.0 g/L		
	isolated soy protein	1.0 g/L		
P. ostreatus ANDES-F515	$(NH_4)_2SO_4$	1.0 g/L	6.35 ± 3.07 U/L	Durán-Sequeda et al. 2021
		10.0 g/L	1.74 ± 0.15 U/L	
	yeast extract	5.0 g/L	104 ± 10.1 U/L	

		10.0 g/L	2317 ± 787.7 U/L	
P. eryngi strain 616	Peptone	0.5%	9.4 ± 1.2 U/L	Stajic et al. 2006
	Casein hydrolysate	0.5%	6.1 ± 0.6 U/L	
	Corn steep liquor	0.8%	57.0 ± 8.5 U/L	
	KNO_3	30 mM	8.30 ± 0.08 U/L	
	NH_4Cl	30 mM	46.0 ± 1.4 U/L	
	$(NH_4)_2SO_4$	30 mM	100.9 ± 5.3 U/L	
	$NH_4H_2PO_4$	30 mM	22.3 ± 0.9 U/L	
	NH_4NO_3	30 mM	9.4 ± 1.2 U/L	
P. ostreatus strain 493	Peptone	0.5%	396.6 ± 48.4 U/L	
	Casein hydrolysate	0.5%	284.3 ± 3.7 U/L	
	Corn steep liquor	0.8%	239.8 ± 8.4 U/L	
	KNO_3	30 mM	198.8 ± 7.1 U/L	
	NH_4Cl	30 mM	412.7 ± 11.6 U/L	
	$(NH_4)_2SO_4$	30 mM	445 ± 31 U/L	
	$NH_4H_2PO_4$	30 mM	304.6 ± 8.2 U/L	
	NH_4NO_3	30 mM	175.4 ± 5.7 U/L	
P. ostreatus strain 493	Peptone	0.5%	466.2 ± 1.1 U/L	
	Casein hydrolysate	0.5%	292.0 ± 7.8 U/L	
	Corn steep liquor	0.8%	260.3 ± 8.3 U/L	
	KNO_3	30 mM	307.2 ± 18.2 U/L	
	NH_4Cl	30 mM	501 ± 124 U/L	

	$(NH_4)_2SO_4$	30 mM	357.6 ± 60.7 U/L	
	$NH_4H_2PO_4$	30 mM	388.9 ± 0.8 U/L	
	NH_4NO_3	30 mM	332 ± 9 U/L	
P. ostreatus strain 494	Peptone	0.5%	352.4 ± 21.3 U/L	
	Casein hydrolysate	0.5%	368.6 ± 22.6 U/L	
	Corn steep liquor	0.8%	273.3 ± 16.7 U/L	
	KNO_3	30 mM	257 ± 22 U/L	
	NH_4Cl	30 mM	441.0 ± 11.6 U/L	
	$(NH_4)_2SO_4$	30 mM	335.8 ± 1.9 U/L	
	$NH_4H_2PO_4$	30 mM	310.2 ± 32.6 U/L	
	NH_4NO_3	30 mM	281.4 ± 24.3 U/L	
*P. ostreatu*s strain 493	$(NH_4)_2SO_4$	20mM	345.2 ± 1.2 U/L	
		40mM	445.9 ± 0.3 U/L	
		60mM	284.5 ± 0.6 U/L	
P. ostreatus strain 494	Peptone	0.2%	383.5 ± 1.9 U/L	
		1 %	388.7 ± 1.4 U/L	
		2%	449.7 ± 1.4 U/L	
P. pulmonarius strain 572	NH_4NO_3	20mM	384 ± 2 U/L	
		40mM	282.7 ± 1.2 U/L	
		60mM	222.6 ± 1.7 U/L	
P. ostreatus 98	control		139±10.4 U/L	Mikiashvili et al. 2006

	NH_4NO_3	30 mM	84± 9.0 U/L	
	$NaNO_3$	30 mM	136±9 U/L	
	NH_4Cl	30 mM	81±8.0 U/L	
	$NH_4H_2PO_4$	30 mM	82±7.2 U/L	
	$(NH_4)_2SO_4$	30 mM	32±6.1 U/L	
	peptone	30 mM	443±29.0 U/L	
	casein hydrolysate	30 mM	401±21.2 U/L	
	corn steep liquor	30 mM	337±15.9 U/L	
P. ostreatus 108	control		132±1.2 U/L	
	NH_4NO_3	30 mM	79±1.0 U/L	
	$NaNO_3$	30 mM	43±1.3 U/L	
	NH_4Cl	30 mM	81±5.1 U/L	
	$NH_4H_2PO_4$	30 mM	3±0.2 U/L	
	$(NH_4)_2SO_4$	30 mM	9±1.2 U/L	
	peptone	30 mM	490±8.7 U/L	
	casein hydrolysate	30 mM	430±12.3 U/L	
	corn steep liquor	30 mM	164±5.3 U/L	
P. ostreatus (ACCC 52857)		control	Approximately*60 U/mL	Zhu et al. 2016
	$(NH_4)_2SO_4$	1%	Approximately*58 U/mL	
	NH_4NO_3	1%	Approximately*75 U/mL	
	urea	1%	Approximately*90 U/mL	
	yeast extract	1%	353.33±20.5 U/mL	
	peptone	1%	198.53 ± 13.58 U/mL	

	malt extract	1%	165.67 ±19.74 U/mL	
	yeast extract	control	20.14 ± 8.11 U/mL	
		0.5%	151.67 ±23.79 U/mL	
		1%	331.67 ± 40.00 U/mL	
		2%	379.87 ± 47.14 U/mL	
P. ostreatus ARC280	$(NH_4)_2SO_4$ (control)	2 g/L	431.33 U/mL	Elsayed et al. 2012
	$NaNO_3$	2 g/L	216.42 U/mL	
	NH_4Cl	2 g/L	229.83 U/mL	
	urea	2 g/L	10.67 U/mL	
	L (+) asparagine	2 g/L	99.30 U/mL	
	L (+) glutamine	2 g/L	243.30 U/mL	
	L- alanine	2 g/L	213.33 U/mL	
	DL- alanine	2 g/L	174.00 U/mL	
	L- arginine	2 g/L	358.33 U/mL	
	L- tryptophan	2 g/L	135.83 U/mL	
P. sajor-caju (MTCC 141)	control		198±4.45 U/L	Khan et al. 2016
	$NaNO_3$	75mM	328±6.32 U/L	
	$(NH_4)_2SO_4$	75mM	216±4.83 U/L	
	NH_4Cl	75mM	218±4.84 U/L	
	urea	75mM	295±5.60 U/L	
	$(NH_4)_2HPO_4$	75mM	456±8.24 U/L	
	peptone	75mM	491±9.02 U/L	
	glutamic acid	75mM	433±7.72 U/L	

	glycine alanine	75mM 75mM	422±7.45 U/L 409±7.11 U/L	
P. sajor-caju	Ammonium tartrate	2.7 mM 5.4 mM 10.9 mM 16.3 mM 21.7 mM 24.4 mM 27.1 mM	Approximately*10.5U/mL Approximately*13.5U/mL Approximately* 2.0 U/mL Approximately* 5.0 U/mL Approximately* 5.0 U/mL Approximately* 4.9 U/mL Approximately* 3.0 U/mL	Patrick et al. 2011

*Values from graph

4.1.2 Effect of nitrogen source and concentration on laccase activity in *Pleurotus* spp. under solid state fermentation

In the literature study on the effect of nitrogen source and concentration on laccase activity in various *Pleurotus* spp., Kachlishvili et al. (2005) and Elisashvili et al. (2008) demonstrated variable effects of different nitrogen sources when applied at the same concentration (Table 2). Three *Pleurotus* spp (*P. ostreatus, P. dryinus*, and *P. tuberregium*) have been studied with three inorganic nitrogen sources (potassium nitrate, ammonium sulphate, ammonium nitrate), and one organic nitrogen source (peptone). Both nitrogen sources were tested with two different substrates, beech tree leaves and wheat straw substrate. In both studies, the use of inorganic nitrogen sources with beech tree leaves substrate resulted in either higher laccase activity or no significant difference compared to the control, except for potassium nitrate in *P. ostreatus*, which exhibited lower laccase activity. Similarly, in the wheat straw substrate, the inorganic nitrogen sources showed the same result.

Although organic nitrogen sources have resulted in higher laccase activity compared to inorganic nitrogen sources under SmF, literature study shows that in solid-state fermentation (SSF) with both substrates (beech tree leaves and wheat straw), the organic nitrogen source (peptone) resulted in either less laccase activity or no significant difference compared to the inorganic nitrogen sources. However, *P. dryinus* on the beech tree leaves substrate exhibited higher laccase activity. In most experiments, peptone showed higher laccase activity compared to the control (Kachlishvili et al. 2005).

Table 2. Effect of nitrogen source and concentration on laccase activity in Pleurotus spp. under solid state fermentation

Pleurotus spp	Tested nitrogen source	Nitrogen concentration	Laccase activity	Reference
P. dryinus IBB 903	(Beech tree leaves substrate)			Kachlishvili et al. 2005
	control		5.6±0.4 U/g	
	KNO_3	20mM	6.2±0.6 U/g	
	$(NH_4)_2SO_4$	20mM	8.4±0.6 U/g	
	NH_4NO_3	20mM	10.0±0.9 U/g	
	peptone	20mM	12.4±1.3 U/g	
	(Wheat straw substrate)			
	control		3.5±0.4 U/g	
	KNO_3	20mM	3.9±0.4 U/g	
	$(NH_4)_2SO_4$	20mM	4.4±0.4 U/g	
	NH_4NO_3	20mM	4.1±0.3 U/g	
	peptone	20mM	4.2±0.3 U/g	
	(Beech tree leaves substrate)			
P. tuberregium IBB 624	control		3.4±0.3 U/g	
	KNO_3	20mM	5.1±0.4 U/g	
	$(NH_4)_2SO_4$	20mM	3.5±0.4 U/g	
	NH_4NO_3	20mM	4.6±0.4 U/g	
	peptone	20mM	3.8±0.4 U/g	

	(Wheat straw substrate)			
	control		1.9±0.2 U/g	
	KNO_3	20mM	2.7±0.3 U/g	
	$(NH_4)_2SO_4$	20mM	2.6±0.3 U/g	
	NH_4NO_3	20mM	3.0±0.3 U/g	
	peptone	20mM	2.4±0.2 U/g	
P. ostreatus 2191	(Beech tree leaves substrate)			Elisashvili et al. 2008
	control		281 ± 35 U/L	
	KNO_3	20mM	234 ± 21 U/L	
	$(NH_4)_2SO_4$	20mM	329 ± 31 U/L	
	NH_4NO_3	20mM	336 ± 29 U/L	
	peptone	20mM	252 ± 28 U/L	
	(Wheat straw substrate)			
	control		311± 36 U/L	
	KNO_3	20mM	275± 34 U/L	
	$(NH_4)_2SO_4$	20mM	551 ± 42 U/L	
	NH_4NO_3	20mM	293 ± 25 U/L	
	peptone	20mM	357 ± 29 U/L	

4.2 Experimental study

In the first experiment, the control had a laccase activity of 15.4±3.6 U/L. A significant increase was observed after the addition of 10 mM urea which resulted in almost 3 times higher (43.3±2.8 U/L) laccase activity (Table 3). However, the addition of ammonium nitrate did not result in a significant impact on laccase activity at concentrations of both 1 mM and 10 mM.

The addition of lignin in the second experiment resulted in a considerable increase in laccase activity of 290.5±13.4 U/L in the control, compared to the treatment with grain spawn-only. The laccase activity was further increased to 364.5±37.1 U/L with the addition of 10 mM urea, marking a substantial 125% increase relative to the control. For ammonium nitrate, 1 mM concentration resulted in a slight increase in laccase activity to 305.2±24.7 U/L while 10 mM ammonium nitrate resulted in no significant difference compared to the control.

Table 3.The impact of the nitrogen sources urea and ammonium nitrate on laccase activity when grain spawn only (experiment 1) and grain spawn with lignin (experiment 2) were applied. Mean±std, n=3

Nitrogen source	Nitrogen conc.	Enzyme activity (U/L)	Relative to control (%)
Control E1 (grain spawn only)		15.4±3.6a*	100
Urea	1 mM	14.0±3.5a	91
	10 mM	43.3±2.8b	281
Ammonium nitrate	1 mM	16.6±1.8a	108
	10 mM	14.9±3.7a	97
Control E2 (with lignin)		290.5±13.4a	100
Urea	1 mM	270.7±13.3a	93
	10 mM	364.5±37.1b	125
Ammonium nitrate	1 mM	305.2±24.7ab	105
	10 mM	240.1±25.8a	83

*Values within a column, for each treatment, followed by different letters indicate significant difference compared to the control.

In the third experiment where the control had a laccase activity of 54.0±5.9 U/L, the addition of yeast extract at 10 mM resulted in an increased laccase activity of 267.5±19.0 U/L, which is approximately five times (495%) higher (Table 4). For wheat bran supplementation at a concentration of 10 mM, the laccase activity increased to 206.9±32.4 U/L, marking a 383% increase compared to the control.

Yeast extract and wheat bran at 1 mM concentration did not result in a significant difference compared to the control.

With the addition of lignin in experiment 4, the control showed a laccase activity of 446.3±43.1 U/L, which was higher than that of the control with grain spawn only. Upon addition of yeast extract, laccase activity increased slightly to 491.3±28.5 U/L at 1 mM concentration and significantly increased to 524.9±28.9 U/L at 10 mM concentration. Conversely, treatment with wheat bran 1mM showed a significant increase in laccase activity to 510.8±26.1 U/L, while with 10 mM wheat bran, there was no significant difference compared to the control.

Table 4. The impact of the nitrogen sources yeast extract and wheat bran on laccase activity when grain spawn only (experiment 3) and grain spawn with lignin (experiment 4) were applied. Mean±std, n=3

Nitrogen source	Nitrogen conc.	Enzyme activity (U/L)	Relative to control (%)
Control E3 (grain spawn only)		54.0±5.9a*	100
Yeast extract	1 mM	64.9±7.2a	120
	10 mM	267.5±19.0c	495
Wheat bran	1 mM	95.5±31.7a	177
	10 mM	206.9±32.4b	383
Control E4 (with lignin)		446.3±43.1ab	100
Yeast extract	1 mM	491.3±28.5ab	110
	10 mM	524.9±28.9b	118
Wheat bran	1 mM	510.8±26.1b	114
	10 mM	415.3±37.9a	93

*Values within a column, for each treatment, followed by different letters indicate significant difference compared to the control.

In the fifth experiment which was based spawn of sawdust, the control had a laccase activity of 4.6±0.4 U/L (Table 5). Treatment with urea at 1 mM and 10 mM concentrations resulted in laccase activity of 4.4±0.2 U/L and 5.6±0.6 U/L respectively with no significant difference observed. Ammonium nitrate at concentrations of 1 mM and 10 mM showed a significant decrease in laccase activity of 3.1±0.3 U/L and 3.2±0.5 U/L, respectively, corresponding to 67% and 69% of the control.

In the sixth experiment, the control with lignin exhibited laccase activity of 8.3±1.5 U/L, slightly higher compared to the control without lignin. In the treatment with urea, laccase activity significantly increased to 17.6±1.5 U/L at 10 mM concentration, which resulted in doubling relative to the control. Similarly, ammonium nitrate at both concentrations resulted in comparable laccase activity,

with values of 9.6±6.6 U/L and 9.3±3.6 U/L at 1 mM and 10 mM, respectively, corresponding to 115% and 112% relative to the control.

Table 5. The impact of the nitrogen sources urea and ammonium nitrate on laccase activity when spawn based on sawdust with and without lignin were applied. Mean±std, n=3

Nitrogen source	Nitrogen conc.	Enzyme activity (U/L)	Relative to control (%)
Control E5 (sawdust spawn only)		4.6±0.4a*	100
Urea	1 mM	4.4±0.2a	96
	10 mM	5.6±0.6a	122
Ammonium nitrate	1 mM	3.1±0.3b	67
	10 mM	3.2±0.5b	69
Control E6 (with lignin)		8.3±1.5ab	100
Urea	1 mM	7.0±1.1a	84
	10 mM	17.6±1.5b	212
Ammonium nitrate	1 mM	9.6±6.6ab	115
	10 mM	9.3±3.6ab	112

*Values within a column, for each treatment, followed by different letters indicate significant difference compared to the control.

In the seventh experiment, the control exhibited a laccase activity of 3.1±0.6 U/L. Treatment with yeast extract at both 1 mM and 10 mM concentrations resulted in no significant difference in laccase activity compared to the control (Table 6). Wheat bran treatment at 1 mM resulted in a laccase activity of 5.3±0.6 U/L, with no significant difference compared to the control. Remarkably, at a concentration of 10 mM, wheat bran treatment showed a significant increase in laccase activity to 25.6±4.0 U/L, which is approximately eight times higher than the control.

In the eighth experiment with the addition of lignin, the control had a laccase activity of 5.6±0.6 U/L. Upon treatment with yeast extract, laccase activity showed no significant difference at either 1 mM or 10 mM concentrations, with values of 5.7±1 U/L and 16.8±1.9 U/L, respectively.
Although wheat bran treatment with 1 mM exhibited no significant difference relative to the control (9.1±1.5 U/L), a remarkable increase was observed after the addition of 10 mM wheat bran which resulted in almost 24 times higher (134.3±21.5 U/L) laccase activity.

Table 6. The impact of the nitrogen sources yeast extract and wheat bran on laccase activity when spawn based on sawdust with and without lignin. Mean±std, n=3

Nitrogen source	Nitrogen conc.	Enzyme activity (U/L)	Relative to control (%)
Control E7 (sawdust spawn only)		3.1±0.6a*	100
Yeast extract	1 mM	2.6±0.2a	84
	10 mM	3.6±0.4a	116
Wheat bran	1 mM	5.3±0.6a	171
	10 mM	25.6±4.0b	826
Control E8 (with lignin)		5.6±0.6a	100
Yeast extract	1 mM	5.7±1a	102
	10 mM	16.8±1.9a	300
Wheat bran	1 mM	9.1±1.5a	163
	10 mM	134.3±21.5b	2398

*Values within a column, for each treatment, followed by different letters indicate significant difference compared to the control.

The laccase activity measured in the controls of eight different experiments are presented in Table 7. The laccase activity of the controls varied in the experiments. The spawn based on sawdust resulted in considerable less laccase activity in the water compared to spawn based on grain. For the experiments performed with grain spawn there was strong increase in laccase activity when lignin was added. In the experiments performed with sawdust spawn this increase was smaller.

Table 7. The laccase activity (U/L) in the eight controls included in this study. The controls were not amended with a nitrogen source but distilled water, spawn (grain or sawdust) and with or without lignin. Mean±std, n=3

Control (without lignin)	Enzyme activity (U/L)	Control (with lignin)	Enzyme activity (U/L)
Exp 1 (grain)	15.8±3.6	Exp 2 (grain)	290.5±13.4
Exp 3 (grain)	53.9±5.9	Exp 4 (grain)	446.3±43.1
Exp 5 (sawdust)	4.8±0.8	Exp 6 (sawdust)	8.3±1.5
Exp 7 (sawdust)	3.1±0.6	Exp 8 (sawdust)	5.6±0.6

5. Discussion

5.1 Impact of spawn type on laccase activity by *P. ostreatus* M2191 grown in water

From the results of the experiment comparing two types of spawns, it was observed that grain-based spawn of *P. ostreatus* M2191 exhibited better laccase activity, compared to sawdust-based spawn, when grown in water. The laccase activity measured in the controls (Table 7) clearly demonstrate that the spawn based on grain resulted in significantly higher laccase activity compared to spawn based on sawdust. When lignin was added as an inducer of laccase to both types of spawns, there was a significant increase in laccase production in grain-based spawn, whereas this increase was much smaller with sawdust spawn. All tested nitrogen sources (ammonium nitrate, urea, yeast extract, wheat bran) resulted in higher laccase activity with grain-based spawn compared to sawdust-based spawn. This result was consistent even without addition of lignin. However, it should be noted that the highest concentration of wheat bran exhibited considerable laccase activity with sawdust-based spawn (Table 6).

This finding can most likely be explained by the fact that the nutrient composition of the substrate significantly influences enzyme production by white rot fungi (Martani et al. 2017; Zhu et al. 2016). Grains are rich in nutrients (Mbogoh et al. 2011) and probabaly provide a more accessible substrate compared to sawdust, allowing for better utilization of nutrients by the mycelium. This enhanced nutrient uptake can support higher rates of enzyme production (Baldrian 2006; Singh et al. 2018). Also, the higher surface area and pore of substrates promote optimal mycelium growth rates, potentially contributing to the results observed with grain spawn (Hoa and Wang 2015; Tinoco et al. 2011).

There are four major spawn types available for use in mushroom cultivation: sawdust spawn, grain spawn, stick spawn, and liquid spawn. Among these, sawdust spawn is the most commonly used due to its low cost, easy preparation, and

minimal equipment investment. The use of grain spawn is less widespread due to the high cost and high contamination rate (Zhang et al. 2019).
When comparing the price of the two types of spawn, grain spawn are slightly more expensive than sawdust spawn. The cost of 1 L of grain spawn is approximately 10.00 euros. Despite this cost, grain spawn offers advantages in laccase production due to its nutrient rich composition and better substrate accessibility. Using lignin as an inducer would be an additional cost, but since it is sourced as a waste product from the paper milling industry, it serves as a cost-effective alternative. In the context of wastewater treatment for micropollutant removal, both grain spawn and lignin are considered low cost inputs when compared to other advanced treatment methods.
However, when considering a spawn-based method for the production of laccase in water, our study clearly shows that grain-based spawn is more suitable.

5.2 Effect of nitrogen source and concentration on laccase activity

Laccase production is greatly influenced by the nature and concentration of the nitrogen source in the growth medium. Nitrogen sources have been frequently reported to affect laccase activity in *P. ostreatus* and other white-rot fungi (Durán-Sequeda et al. 2021). Our study is of interest as conflicting reports exist in the literature, with some fungi exhibiting better laccase activity in nitrogen-rich media, while others show higher production under nitrogen-limited conditions (Khan et al. 2016).

From the findings of this study, it is evident that different nitrogen sources (ammonium nitrate, urea, yeast extract, wheat bran) have varying effects on laccase production in *P. ostreatus* M2191 under SmF. Yeast extract, followed by wheat bran, appeared to be the best nitrogen sources for laccase activity by *P. ostreatus* M2191. With grain-based spawn, both yeast extract and wheat bran at a high concentration (10 mM) increased laccase activity (Table 4), although also 1 mM wheat bran showed high laccase production with lignin. In addition, wheat bran exhibited considerable laccase activity with sawdust-based spawn (Table 6). Thus, this nitrogen source should be further explored for optimization of laccase activity of spawn pellets in water.

The addition of ammonium nitrate did not result in a significant effect on laccase production in *P. ostreatus* M2191 in either concentration, with or without lignin, in both types of spawn. For urea, laccase activity in *P. ostreatus* showed a significant increase with the highest concentrations, whereas the low concentrations of urea

had no effect. However, compared to other organic nitrogen sources, urea performed poorly in laccase production in the experiment. Compared to organic nitrogen compounds, *P. ostreatus* resulted in less enzyme activity with supplementation of the medium by inorganic nitrogen sources. This study clearly shows the complexity of the impact of nitrogen sources on laccase production. Nitrogen is an element, but when added as a nutrient source it is never added alone. This is particularly applicable to the organic nitrogen sources, which also act as a carbon source. Addition of an additional carbon source will enhance fungal growth which of course may impact the production of enzymes. In the present study, mycelial growth was not measured and this could be considered a weakness. From table 1 it can be seen that this approach, to determine enzyme activity in relation to the amount of fungal biomass, has been used by Fu et al. (1996). However, for the present study we decided to not take this approach as our main goal was to optimize laccase activity in water irrespectively of fungal biomass production.

Our experimental results were compared with literature study findings, which focused on the effect of nitrogen sources and concentration on laccase production by *Pleurotu*s spp. Similar with our study, for SmF (Table 1) the literature study showed variable effects of different nitrogen sources when applied at the same concentration or when increased in concentration. In correspondence with the experimental result, the data reported in the literature showed that increasing the ammonium nitrate concentration did not result in increasing laccase activity (Stajic et al. 2006, Fu et al. 1996) and the addition of ammonium nitrate did not significantly impact laccase activity compared to the control (Zhu et al. 2016; Elsayed et al. 2012; Mikiashvili et al. 2006). When urea is used as a nitrogen source, the literature study demonstrates that it yields poor results compared to other organic nitrogen sources, in line with the experimental findings (Khan et al. 2016; Zhu et al. 2016; Elsayed et al. 2012; Hou et al. 2004). On the other hand, *Pleurotus* spp. exhibited high laccase activity when yeast extract was used as a nitrogen source. Thus, both the experiment and literature study reveal that an increase in concentration of this nitrogen source resulted in increased laccase activity (Durán-Sequeda et al. 2021; Zhu et al. 2016). The findings indicate that *Pleurotus* spp. will be able to produce higher laccase activity when organic nitrogen sources are used, compared to inorganic nitrogen sources.

In the reviewed literature on SSF, the organic nitrogen source (peptone), either resulted in lower laccase activity or did not show a significant difference compared to the inorganic nitrogen sources. However, under the SSF conditions, the fungi is exposed to a more complex substrate (leaves, sawdust, wheat bran etc) which contain organic nitrogen sources in variable concentrations. It is therefore more difficult to make a conclusion based on the data which are compiled in Table 2.

Also, fewer studies were found on this topic, laccase activity in relation to nitrogen source during SSF. This is most likely due to the fact that when SSF is applied to *Pleurotus* spp. the aim is generally to produce fruiting bodies. Thus, optimizing enzyme activity is not the main goal of these type of studies.

The literature study also indicated that the species and strains within the *Pleurotus* genus behave differently considering the impact of nitrogen sources laccase production. These organisms exhibit varying responses to different nitrogen sources, even when supplied at the same concentrations (Stajic et al. 2006; Mikiashvili et al. 2006; Kachlishvili et al. 2005). Thus, optimization of laccase activity should be studied at strain level. However, it seems reasonable to conclude that organic nitrogen sources are preferable compared to inorganic nitrogen sources when considering SmF. In future work, it would be of interest to determine if the increased laccase activity is due to increased fungal biomass or if it is due to a specific effect on enzyme production.

5.3 Application of *Pleurotus ostreatus* for wastewater treatment

The oyster mushroom (*Pleurotus ostreatus*) is one of the widely cultivated mushrooms and is the second most commonly grown worldwide. Thus, this species is well-known and safe to use for large-scale production. In addition, substrates for its cultivation is widely available and the cost for these are low (Aditya et al. 2024). Therefore, utilizing *Pleurotus ostreatus* for wastewater treatment could be a viable approach. Incorporating colonized substrate into wastewater treatment methods, such as biofilm systems or filters, would be a potential way to enhance wastewater treatment efficiency considering removal of micropollutants. However, in this type of systems a fast decrease in enzyme activity have been observed (Hultberg and Golovko 2024). The work performed within this thesis therefore aims to explore a different approach where materials (spawn) and knowledge from the mushroom production industry is used to have a continuous laccase production directly in water.

Considering development of an enzyme-based wastewater treatment with spawn of *P. ostreatus*, this study indicate that the presence of organic nitrogen sources is preferable. Wastewater can be categorized into four distinct types: domestic wastewater discharged from households and commercial institutions and similar facilities, industrial wastewater primarily from industrial processes, agricultural water from farming activities, and rainwater runoff from impermeable surfaces. In

wastewater, nitrogen can originate from various sources depending on the type of wastewater (Crini et al. 2018).

In the wastewater, total nitrogen is present in both organic and inorganic forms. Inorganic nitrogen commonly found in wastewater, exists in the form of ammonia (NH_3), nitrate (NO_3^-), and nitrite (NO_2^-). These inorganic nitrogen compounds may originate from various sources, including urine, industrial processes, agricultural runoff, and atmospheric deposition (Bagherzadeh et al. 2021). Organic nitrogen in wastewater is mainly available in two forms, dissolved organic nitrogen and particulate organic nitrogen (Berman and Bronk 2003). The increasing use of nitrification and denitrification techniques in wastewater treatment results in the presence of dissolved organic nitrogen as the primary nitrogen form within the wastewater effluent (Pehlivanoglu-Mantas and Sedlak 2008). Amino acids, urea, nucleic acids, humic and fulvic substances are the major dissolved organic nitrogen compounds widely available in the WWTPs (Zheng et al. 2021).

Thus, not only the complexity of strain variations needs to be considered but also a wide range of different water with variable nitrogen will impact the fungal enzyme production. The present study was set-up with effluent wastewater from a municipal WWTP in mind. In this context, it is evident that amending the water with lignin will impact water quality and that it is not relevant to add an organic nitrogen source to the water. One possibility would be to search for other compounds besides lignin that induce laccase production. Another option which can be explored in future studies is to consider integration of the spawn-based pellets earlier in the wastewater treatment process. This will create a situation where the content of organic nitrogen can be expected to be high while the lignin will be removed during the treatment process. However, this will expose the fungus to a challenging environment and its willingness to grow in different types of wastewater needs to be studied.

5.4 Conclusion

The experimental and literature study investigates the impact of grain-based and sawdust-based spawn on laccase activity and explores the influence of selected nitrogen sources (ammonium nitrate, urea, yeast extract, wheat bran) on laccase production by *P. ostreatus* M2191 when grown in water. It was shown that grain-based spawn exhibited better laccase activity compared to sawdust-based spawn. Yeast extract followed by wheat bran was found to be the most effective nitrogen

source for enhancing laccase activity in *P. ostreatus* M2191. The experimental results and existing literature indicate that *P. ostreatus* exhibits lower enzyme activity with inorganic nitrogen sources compared to organic nitrogen sources in SmF. The research highlights the variability in laccase production among different *Pleurotus* species and strains, emphasizing that the optimization of laccase activity should be studied at the strain level. Further exploration of *P. ostreatus* for its potential in bioremediation could yield sustainable solutions to water pollution challenges caused by micropollutants.

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Popular science summary

Micropollutants are organic contaminants, eg. pharmaceuticals, pesticides and personal care products, which are present in the environment in low concentrations as a result of human activities. The increase of these type of compounds in our water systems has become a major global concern, with traditional wastewater treatment plants struggling to effectively remove them. Therefore alternative techniques, such as bioremediation, which involve microorganisms and their enzymes to break down harmful compounds into less toxic forms will be increasingly important. White-rot fungi including *Pleurotus* spp, play an important role in bioremediation due to their production of enzymes with ability to degrade micropollutants. Fungal laccases are one example of a group of enzymes which are very efficient in degradation of micropollutants. Laccases have received much attention for their potential in mycoremediation, which means bioremediation performed by fungi. The main reason for interest in laccases is because of their low substrate specificity, thus they act upon a wide range of compounds, and their capacity in transforming various compounds into non-toxic metabolites.

Hence, the experimental and literature study performed in this thesis aimed to explore a spawn-based method for producing laccase in water using the white-rot fungus *P. ostreatus*. The study aimed to investigate how the type of spawn (grain-based or sawdust-based) impact on laccase activity, as well as how laccase production is influenced by the addition of selected compounds, ammonium nitrate, urea, yeast extract, and wheat bran by *P. ostreatus* M2191 when grown in water.
The results of the study showed that grain-based spawn exhibited better laccase activity compared to sawdust-based spawn. Yeast extract, followed by wheat bran, resulted as the most effective nitrogen source for enhancing laccase activity in *P. ostreatus* M2191. Both the experiment and the literature confirmed that *P. ostreatus* showed lower enzyme activity with inorganic nitrogen sources compared to organic nitrogen compounds. The study also found that different types of *Pleurotus* species and strains affect laccase production differently, even with the same amounts of nitrogen sources. The findings of this study contribute to a better understanding of how different spawn types and nitrogen sources affect laccase production in *Pleurotus* spp. and provide valuable insights for the development of more efficient and sustainable wastewater treatment strategies.

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