Targeted oxidation of micropollutants in source-separated urine using laccase

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Abstract

Pharmaceutical contamination in source-separated urine is a potential barrier to its safe recycling as fertiliser. Enzymatic oxidation using laccase could provide a mild treatment option to remove pharmaceuticals while preserving nutrients in urine.

This study investigated whether laccase from the fungus *Trametes versicolor* (Turkey tail) could transform selected pharmaceuticals in real pooled human urine and whether the natural redox mediators syringaldehyde and vanillin could enhance treatment. A series of preparatory experiments examined the effects of pH, temperature, urine concentration, acid type, and oxygen supply on laccase activity. Based on these experiments, a mixture of 19 pharmaceuticals was treated with laccase at pH 4.5 and 50 °C under continuous aeration for 24 h, with and without mediators. Changes in parent-compound concentrations were measured by UPLC-MS/MS.

Laccase remained active in unconcentrated urine, although activity declined with increasing urine concentration and during incubation at 50 °C. Continuous aeration was required to prevent oxygen depletion. Sulfamethoxazole showed the clearest treatment response, with a 46% decrease after 24 h, compared with negligible change in the untreated control. Diclofenac showed apparent removal of 50% though control variability was substantial. Three additional compounds (losartan, irbesartan, and furosemide) showed apparent decreases, but anomalous changes in the corresponding controls limited interpretation. Neither syringaldehyde nor vanillin consistently improved pharmaceutical transformation compared with laccase alone.

These findings provide proof-of-concept that laccase treatment can remain active and transform selected pharmaceuticals in real source-separated urine under practically relevant conditions.

Keywords: Enzymatic treatment, Nutrient recovery, Pharmaceuticals, Bioremediation, *Trametes versicolor*

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Abbreviations

Abbreviation	Description
BOKU	Universität für Bodenkultur Wien
COD	Chemical Oxygen Demand
DMP	2,6-Dimethoxyphenol
LMS	Laccase–Mediator Systems
DO	Dissolved Oxygen
LST	Laccase-Syringaldehyde Treatment
LT	Laccase-only Treatment
LVT	Laccase-Vanillin Treatment
PE	Population Equivalent
SLU	Swedish University of Agricultural Sciences
UPLC-MS/MS	Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

1. Introduction

That a valuable resource should be diluted and then discarded in an era where resource recovery is more urgent than ever is, on reflection, not logical. Yet this happens every time a toilet is flushed, and urine enters the mixed wastewater stream. Urine carries the majority of the pharmaceutical residues excreted by the human body (Lienert et al. 2007b) and a disproportionately high concentration of plant-essential nutrients (Friedler et al. 2013). This means in the wastewater matrix it creates a pollution potential but source separated and treated it could become a resource. This chapter presents the background and motivation for the thesis, the research gaps which are addressed, hypothesis and research questions, and structure of the study.

1.1 Background and Motivation

Pharmaceuticals are now recognised as globally distributed environmental contaminants, spanning surface water, groundwater, soil and other matrices (Graumnitz & Jungmann 2021). While the ecological consequences of this contamination are still being characterised, several well-documented cases demonstrate that pharmaceutical residues can cause severe harm to wildlife (Oaks et al. 2004; Kidd et al. 2007). Furthermore, the continuous release of antibiotics into the environment can be a contributing factor in the spread of antimicrobial resistance, a critical global health concern (Wilkinson et al. 2022).

A disproportionate share of the pharmaceutical load in domestic wastewater originates from urine (Lienert et al. 2007b). Source-separating urine could enable targeted interception of one of the most concentrated pharmaceutical streams in domestic wastewater. Although the goal of removal should not be the only incentive behind the implementation of the necessary infrastructure (Lienert et al. 2007b). Urine is also the dominant source of plant-essential nutrients in domestic wastewater. Despite its small volume, it contributes the majority of the nitrogen, phosphorus and potassium present in household wastewater (Friedler et al. 2013). Source separation therefore enables recovery of the most nutrient-rich fraction of domestic wastewater. Recycling source-separated urine as crop fertiliser is gaining momentum as a sustainable strategy to close the nutrient loop and reduce dependence on energy-intensive synthetic fertiliser production (Vinnerås 2025; Mustaffa et al. 2026), and is increasingly being implemented at real scale in public facilities and sport arenas using urine concentration technologies now commercially available (*Sanitation 360* n.d.; *Pee for the Planet: Human Urine as Fertilizer | Oatly* n.d.; *Vunanexus* n.d.).

The pharmaceutical contamination in urine is recognised as one of the key barriers to public and regulatory acceptance of urine-derived fertilisers (Lienert &

Larsen 2010; Mustaffa et al. 2026). Recent regulatory developments, including the EU Urban Wastewater Treatment Directive 2024/3019 (targeting large treatment plants with >100,000 PE) (European Parliament & Council of the European Union 2024), have intensified the need for pharmaceutical removal alongside nutrient recovery. Source separation of urine and subsequent treatment could help to address both objectives simultaneously; by intercepting a concentrated pharmaceutical and nutrient stream before dilution in municipal wastewater and, with that, reducing the total load in the wastewater treatment plants.

Several advanced treatment approaches have been investigated for pharmaceutical removal from urine such as UV light based advanced oxidation (Demissie et al. 2023) and pulsed corona discharge (Petrochenko et al. 2026). The high energy input they typically require, the potential to destroy plant-essential nutrients such as urea and ammonia, or the production of undesirable byproducts motivates interest in milder, more selective treatment options compatible with nutrient recovery.

Laccase, a fungal oxidoreductase enzyme, degrades phenolic and aniline-containing compounds using molecular oxygen as its only co-substrate, and has shown promising pharmaceutical removal in buffer and wastewater systems (Baldrian 2006; Margot et al. 2013; Hultberg et al. 2025).

1.2 Research Gaps

Despite the promising performance of laccase in buffer and wastewater systems, to the best of the author´s knowledge, no study has investigated laccase-mediated treatment of pharmaceuticals in real source-separated urine under conditions relevant to urine storage and handling. Source-separated urine is a highly complex matrix containing over 2,600 confirmed metabolites (Bouatra et al. 2013), several of which are known inhibitors of the laccase produced by the fungi *Trametes versicolor*. Without understanding how this matrix affects laccase activity, it is impossible to predict whether enzymatic treatment is viable in practice or to design effective treatment systems.

Previous laccase studies on pharmaceutical degradation have often been conducted in buffer systems or synthetic matrices, and both Mehaidli et al. (2024) and Petrochenko et al. (2026) demonstrate that synthetic urine is an inadequate proxy for real urine, meaning results from simplified matrices cannot be assumed to transfer to real urine treatment. This raises a fundamental question: does laccase retain sufficient activity in real urine to drive meaningful pharmaceutical degradation? Characterising the specific inhibitory mechanisms is essential not only for understanding this limitation but also for identifying strategies to mitigate it.

Additionally, the performance of natural redox mediators such as syringaldehyde and vanillin, which have been shown to extend the substrate range of laccase to non-phenolic compounds in buffer systems (Margot et al. 2013; Sá et al. 2024), has not been evaluated in urine. As these mediators are themselves organic compounds, their fate and effectiveness in a matrix containing approximately 1.43 g/L of competing organic metabolites remain unknown (Mehaidli et al. 2024). This matters because mediators could represent a pathway to broader pharmaceutical degradation in real urine, but their viability under realistic conditions is currently unresolved. Without this knowledge, it is unclear whether mediator-enhanced laccase treatment is a feasible approach for urine-based systems.

1.3 Hypothesis and Research Questions

Hypothesis:

Laccase from *T. versicolor* will degrade selected pharmaceutical compounds in source-separated human urine under conditions compatible with urine storage and handling, and the addition of natural redox mediators syringaldehyde and vanillin will enhance this degradation compared to laccase treatment alone.

Research Questions (RQ):

RQ1:

Under what pH, temperature, and matrix conditions can laccase activity ≤ 300 U/L[1] be maintained in source-separated urine?

RQ2:

Can laccase degrade selected pharmaceutical compounds in source-separated urine under the established conditions over 24 h?

RQ3:

Does the addition of natural redox mediators syringaldehyde or vanillin affect pharmaceutical degradation in urine compared to laccase alone?

1.4 Structure of Study

This study was conducted as a proof-of-concept investigation in three sequential phases. Firstly, a series of experiments was conducted to assess laccase activity in source-separated urine, addressing the pH, temperature, oxygen, and matrix constraints specific to this system. Secondly, the conditions identified were validated and used to set up the pharmaceutical degradation trial. Thirdly, laccase was applied to a mixture of 19 selected pharmaceuticals in real source-separated urine over 24 h, with and without natural redox mediators, and pharmaceutical removal was quantified using UPLC-MS/M

[1] This activity level was defined as sufficient based on Hultberg et al. 2025, which demonstrated 87% pharmaceutical removal at approximately 300 U/L in tertiary-treated municipal wastewater.

2. Literature

The following chapter will give a brief overview of the theoretical background of this thesis.

2.1 Pharmaceuticals in the Environment

Pharmaceuticals enter the environment through multiple pathways and are now detected worldwide across a range of environmental matrices. This section reviews their occurrence and sources, as well as the environmental and ecological risks they pose.

2.1.1 Occurrence and Sources

Pharmaceuticals have been documented in the environment across all continents and in a wide range of environmental matrices. The "Pharmaceuticals in the Environment" database maintained by the German Environment Agency draws together measured concentrations from over 2,000 publications spanning 89 countries, underlining that pharmaceutical contamination is a global rather than a localised phenomenon (Graumnitz & Jungmann 2021). Urban wastewater is the dominant emission pathway for pharmaceuticals globally, although emissions from industrial production, hospitals, agriculture and aquaculture can be locally important (aus der Beek et al. 2016).

Pharmaceuticals enter the environment not only as parent compounds but also as metabolites, formed either through human metabolism before excretion or through microbial transformation during environmental degradation (Ogata et al. 1985; Sekikawa et al. 1995; Khetan & Collins 2007). Both parent compounds and their metabolites can retain biological activity, yet most environmental risk assessments have historically considered only the parent drug. This is a significant limitation, as metabolites may be more or less toxic than the parent compound, and the scarcity of environmental data on metabolites complicates accurate risk assessment (Celiz et al. 2009).

2.1.2 Environmental and Ecological Risks

Pharmaceuticals are inherently biologically active and often highly potent, and are frequently designed to resist biodegradation, which contributes to their environmental persistence (Khetan & Collins 2007). As a result, they can cause measurable ecological harm even at the low concentrations typically found in the environment.

Several well-documented cases demonstrate effects at the population level. The anti-inflammatory drug diclofenac caused the catastrophic collapse of vulture

populations across the Indian subcontinent, where birds died of renal failure after feeding on carcasses of livestock treated with the drug (Oaks et al. 2004). In aquatic systems, chronic exposure to the synthetic oestrogen ethinylestradiol at environmentally relevant concentrations caused feminisation of male fish and the near-collapse of a wild fathead minnow population in a whole-lake experiment (Kidd et al. 2007).

Beyond these direct toxicological effects, the continuous release of antibiotics into the environment is of particular concern (Wilkinson et al. 2022). At sub-inhibitory concentrations, environmental antibiotics can select for resistant bacterial strains, contributing to the development and spread of antimicrobial resistance (aus der Beek et al. 2016). Antimicrobial resistance is now recognised as one of the major global public health threats of the twenty-first century (World Health Organization 2023). In response to these risks, the regulatory framework is tightening, for example through a new EU directive requiring the removal of pharmaceutical micropollutants from urban wastewater (European Parliament & Council of the European Union 2024).

2.2 Urine as a Key Pharmaceutical Pathway and Case for Source Separation

Source separation of urine offers dual benefits: interception of a concentrated pharmaceutical pathway and recovery of a valuable nutrient resource. Urine, despite representing only approximately 1% of total domestic wastewater volume (Friedler et al. 2013), contains a disproportionately high pharmaceutical load (Lienert et al. 2007b) and carries the majority of plant-essential nutrients excreted in household wastewater (Friedler et al. 2013).

Urine is a complex matrix containing inorganic ions (sodium, potassium, chloride, sulphate, phosphate, ammonium) and over 2,600 confirmed metabolites spanning a wide range of chemical structures and concentrations (Bouatra et al. 2013).

On average, 70% of pharmaceutical active ingredients are excreted via urine as parent compounds or metabolites (Lienert et al. 2007b). Large variability exists between therapeutic groups in urine excretion rates. Some compounds are nearly entirely excreted via urine, such as x-ray contrast media at 94%, while others show lower excretion rates (Lienert et al. 2007a). Therapeutic groups with substantial urine excretion include antiphlogistics (approximately 67%), antiepileptics (approximately 81%), beta blockers (approximately 63%), and antidepressants (approximately 66%) (Lienert et al. 2007a). This concentrated pharmaceutical pathway makes source separation an ideal interception point before dilution in centralised treatment systems.

Beyond pharmaceutical interception, source separation enables simultaneous recovery of plant-essential nutrients that are present in disproportionately high

concentrations. Urine contributes 70 to 90% of nitrogen, 50 to 65% of potassium, and 45 to 65% of phosphorus in domestic wastewater (Mehaidli et al. 2024). In the Swedish context, source-separated urine contains approximately 4 kg nitrogen, 0.4 kg phosphorus, and 0.9 kg potassium per person annually (Vinnerås et al. 2006), representing approximately 75% of total household wastewater nitrogen and approximately 49% of total household phosphorus. This simultaneous recovery of nutrients before dilution and degradation in the wastewater system offers a sustainable pathway to close the nutrient loop and reduce dependence on energy-intensive synthetic fertiliser production. Fresh urine is typically slightly acidic (pH around 6), but composition changes upon storage as urease-producing bacteria catalyse hydrolysis of urea to ammonia, raising pH and altering nitrogen composition (Simha et al. 2023). This process leads to nitrogen loss, making acidification to a pH below 3 necessary for stabilisation and enabling nutrient recovery (Simha et al. 2023).

Source separation is already being implemented at real scale in office buildings, stadiums, and public facilities, with urine concentration technologies producing fertilisers like Aurin and Granurin, which are commercially available (Simha et al. 2023; Vinnerås 2025). The Pee for the Planet project in cooperation with the SLU is currently collecting urine from football supporters at Eleda Stadium in Malmö and transforming it into Granurin fertiliser pellets, using acidification and dehydration technology. The EU Urban Wastewater Treatment Directive 2024/3019 mandates enhanced nutrient removal and 80% removal of specific pharmaceuticals, both of which source separation can address simultaneously by intercepting the concentrated nutrient and pharmaceutical stream before centralised treatment. Infrastructure challenges (particularly the requirement for urine-diverting toilets) represent the primary barrier to large-scale implementation (Vinnerås 2025).

While pharmaceutical contamination has historically been cited as a regulatory and acceptance barrier to urine-derived fertilisers (Lienert & Larsen 2010), recent multinational research suggests that consumer acceptance may not be the limiting factor. A survey of 3,763 university respondents across 16 countries found that 59% would be willing to consume food grown with human urine, with 68% accepting urine recycling as a management option overall (Simha et al. 2021). Acceptance was most strongly predicted by cognitive factors (perceptions of risks and benefits) and social norms rather than demographic characteristics. This suggests that communication strategies emphasising the benefits and safety of urine-derived fertilisers, adapted to specific cultural contexts, can effectively increase consumer acceptance.

2.3 Laccase as a Nature-Based Treatment Option

The following section reviews the properties, mechanism, applications and potential challenges of laccase-based treatment in urine.

2.3.1 Enzyme-based wastewater treatment

Enzymatic treatment has emerged as a promising approach for removal of recalcitrant micropollutants, including pharmaceuticals, that conventional wastewater treatment only partially addresses (Naghdi et al. 2018; Ben Younes et al. 2019; Al-Maqdi et al. 2021).

Oxidoreductase enzymes catalyse oxidative transformation of aromatic and phenolic compounds. Pharmaceuticals bearing electron-donating groups such as amine and hydroxyl substituents are readily degraded by fungal oxidoreductases (Naghdi et al. 2018).

Among oxidoreductases, laccase has received increased research attention for pharmaceutical degradation. Laccase catalyses oxidation of phenolic and aniline-containing compounds (Naghdi et al. 2018) and is commercially available from fungal sources (Margot et al. 2013). Its cofactor is molecular oxygen, requiring no additional costly or hazardous electron donors (Naghdi et al. 2018).

2.3.2 Properties and Mechanism

Laccases are multicopper oxidase enzymes that catalyse one-electron oxidation of substrates coupled to four-electron reduction of molecular oxygen to water (Baldrian 2006; Riva 2006). The catalytic centre contains four copper atoms: one T1 copper where substrate oxidation occurs, and a trinuclear T2/T3 cluster where oxygen is reduced to water (Baldrian 2006).

Laccases are produced by various fungi, plants and bacteria. Fungal laccases from species such as *T. versicolor* are most relevant for biotechnological applications due to their high redox potential and commercial availability (Baldrian 2006; Margot et al. 2013).

Laccase activity is strongly influenced by pH (Margot et al. 2013). Fungal laccases typically exhibit pH optima in the acidic range, with optima for phenolic substrates such as DMP generally falling between pH 4.0 and 7.0 (Baldrian 2006). For *T. versicolor* laccase specifically, the pH optimum for DMP oxidation is reported as pH 4.5 to 6.5 (Margot et al. 2013). Activity declines above pH 7 due to hydroxide anion binding at the T2/T3 copper cluster, which interrupts internal electron transfer (Baldrian 2006).

Laccase activity is temperature-dependent, exhibiting a characteristic activity-stability trade-off. The temperature optimum for fungal laccases typically falls between 50 and 70°C (Baldrian 2006), where higher temperatures produce higher initial catalytic rates but accelerate irreversible thermal inactivation. Thermal

inactivation of *T. versicolor* laccase at elevated temperatures is progressive and largely irreversible (Rancaño et al. 2003; Stoilova et al. 2010). Cooling after thermal inactivation does not fully restore activity.

The substrate used in activity assays influences measured thermal stability. For example 2-2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) shows lower affinity for the enzyme than 2,6-dimethoxyphenol (DMP), meaning ABTS-based stability values may not directly apply to DMP-based assays (Han et al. 2005). For industrial and treatment applications, kinetic stability - *i.e.* the irreversible loss of activity over time at a given temperature - is the relevant parameter for assessing enzyme performance (Brissos et al. 2024).

Molecular oxygen is the terminal electron acceptor in laccase catalysis (Margot et al. 2013). Stoichiometric oxygen consumption during treatment means oxygen supply must be maintained for sustained activity. In closed systems without external aeration, laccase rapidly depletes dissolved oxygen, leading to activity limitation (Ortner et al. 2015). Oxygen availability is therefore a critical design consideration for laccase-based treatment systems operating in closed vessels over extended incubation periods.

2.3.3 Applications for Pharmaceutical Degradation and Potential Inhibitors in Urine

Susceptibility to laccase oxidation is primarily determined by molecular structure. Phenolic compounds and compounds containing aniline or other electron-donating functional groups are direct laccase substrates (Gianfreda et al. 1999). The electron-donating groups facilitate one-electron oxidation at the T1 copper centre, converting substrate molecules into reactive radicals that drive further degradation (Baldrian 2006; Sá et al. 2024). Non-phenolic compounds dominated by electron-withdrawing groups are generally resistant to direct laccase oxidation. This structural resistance is well established across multiple compound classes including certain antibiotics, antiepileptics, beta blockers and antidepressants (Stadlmair et al. 2017; Sá et al. 2024). Partial susceptibility is observed for compounds with mixed structural features; compounds containing both phenolic and non-phenolic moieties may show intermediate degradation depending on conditions (Margot et al. 2013).

Purified lab-grade laccase from *T. versicolor* can degrade selected pharmaceuticals including diclofenac and sulfamethoxazole (Alharbi et al. 2019). However redox mediators such as syringaldehyde and vanillin can extend the substrate range of laccase to non-phenolic compounds. Mediators are oxidised by laccase to generate reactive radical species that can in turn oxidise compounds laccase cannot directly attack (Margot et al. 2013; Ashe et al. 2016; Sá et al. 2024). In citrate-phosphate buffer systems, higher removal of sulfamethoxazole was achieved with laccase-syringaldehyde than with laccase only, demonstrating

the potential of mediator enhancement for aniline-containing compounds (Sá et al. 2024).

Fungal pellet systems have demonstrated promise for broad-spectrum pharmaceutical removal. Hultberg et al. (2020) achieved 90% removal of diclofenac and 43 – 73% removal of bicalutamide, lamotrigine and metformin using oyster mushroom (*Pleurotus ostreatus*) colonised substrate at approximately 50 U/L laccase activity within 5 minutes. More recently, Hultberg et al. (2025) demonstrated broad-spectrum pharmaceutical removal of 87% for 33 compounds within 24 hours using *T. versicolor* fungal pellets producing approximately 300 U/L laccase activity in tertiary-treated wastewater. High removal was achieved across multiple compound classes, including compounds generally considered resistant to direct laccase oxidation.

Immobilisation of laccase on carrier materials such as spent mushroom waste biochar has been shown to substantially improve thermal and pH stability compared to free laccase. Immobilised laccase retained over 60% activity up to 75°C, while free laccase dropped to 27% at 65°C (Ghose et al. 2025). Immobilisation therefore represents a promising strategy for sustained laccase treatment at elevated temperatures.

But also free lab grade laccase from *T. versicolor* showed potential of oxidising certain pharmaceutical compounds like diclofenac and sulfamethoxazole (Alharbi et al. 2019).

Real urine presents a substantially more complex matrix than buffer and synthetic urine matrices. Consequently, experiments must be performed in real pooled human urine, as results of buffer or synthetic urine matrices may not be transferable. While Demissie et al. (2023) and Petrochenko et al. (2026) demonstrate this for other oxidation methods, similar effects are expected for laccase.

Several components present at high concentrations in source-separated urine are known inhibitors of *T. versicolor* laccase specifically. Urea causes concentration-dependent unfolding and deactivation (Najafabadipour et al. 2021). Chloride competitively inhibits laccase at the copper active site (Xu 1996), and this inhibition is substantially stronger at lower pH (Raseda et al. 2014). Acetate is a mixed inhibitor of laccase at concentrations of 10–100 mM (Ters et al. 2009). Oxalic and citric acid inhibit laccase in a concentration-dependent manner through copper chelation (Lorenzo et al. 2005).

The need for a pH below 3 for storage as explained in Section 2.2 creates another challenge. This is a pH range at which laccase activity is severely inhibited (Baldrian 2006; Margot et al. 2013), requiring pH adjustment before treatment can proceed. The pH compromise between storage requirement and enzymatic activity represents an operational challenge.

If laccase activity can be maintained in real source-separated urine under these inhibitory and pH-constrained conditions to drive considerable pharmaceutical degradation has never been investigated. This represents the central knowledge gap addressed by the present study and provides the direct motivation for the experimental design described in Section 3.

3. Methodology

This chapter gives a description of the methodology used in this study.

3.1 Materials

The following section gives an overview over the materials used for the experiments.

3.1.1 Chemicals

2,6-Dimethoxyphenol (DMP, Thermo Scientific, 99%) was used as the substrate for laccase activity measurements. Two laccase preparations were used: a commercial-grade laccase powder (Biovencer Healthcare Private Limited) and a lab-grade laccase powder (Sigma-Aldrich, Laccase from *T. versicolor*, ≥0.5 U/mg, source BCCP2912). Acetate buffer (1 M, pH 5.0, Thermo Scientific) was used in all experiments at a working concentration of 100 mM. Sodium hydroxide (NaOH, [1 M and 5 M]) and sulfuric acid (H_2SO_4, 96%, VWR Chemicals) were used for pH adjustment. For the acid comparison experiment, phosphoric acid, oxalic acid dihydrate (VWR, BDH Chemicals), citric acid (monohydrate, Sigma-Aldrich), and acetic acid (99-100%, VWR BDH Chemicals) were additionally used. Methanol (LiChrosolv, hypergrade for UPLC-MS) served as solvent for the pharmaceutical and mediator stock solutions. Vanillin (Thermo Scientific, 99%) and syringaldehyde (Thermo Scientific, 98+%) were used as laccase mediators. Silicone oil (VWR Chemicals) was used as the heating medium in the incubation setup. All solutions were prepared with distilled water unless otherwise stated.

3.1.2 Urine Collection, Preparation and Analysis

Freshly excreted human urine was collected at the Department of Energy and Technology. Because donations were anonymous, only the number of samples and an estimated age range of 20–65 years can be given. Urine was collected in single use sterile HDPE bottles (Avantor HDPE Bottle SQ 500 mL, EP liner cap). For the bulk urine used in all experiments except the acid comparison experiment (Section 3.2.4), 42 samples were collected over three working days. At the end of each collection day, all collected urine was pooled into a 30-litre plastic storage container with a lid. The pooled urine was acidified by adding 96% sulfuric acid to a target pH < 3 and stored at room temperature (20 ± 2 °C) until use. Every addition of collected urine to the storage container was followed by an addition of sulfuric acid to prevent enzymatic hydrolysis of urea (Simha et al. 2023). For the acid comparison experiment (Section 3.2.4), 27 samples were collected on one day and used directly for the experiment without prior storage.

The composition of the pooled bulk urine used in all experiments except the acid comparison experiment was analysed using standard photometric and discrete analyser methods. Chemical Oxygen Demand (COD) was measured using a Thermo Fisher COD Cell Test photometric method. Ammonia, calcium, chloride, potassium, magnesium, phosphate (PO_4), sulfate (SO_4), and urea were measured using a Gallery Discrete Analyser (Thermo Fisher). The composition of the pooled bulk urine is reported in Appendix 8.

3.1.3 Laccase Stock Solution

A defined mass of laccase powder was dissolved in 100 mM acetate buffer at pH 5.0 to prepare stock solutions freshly on the day of each experiment. The commercial-grade laccase (Biovencer Healthcare Private Limited) was prepared at 0.05 g/mL for all experiments. The lab-grade laccase (*T. versicolor*, Sigma-Aldrich) was prepared at 0.01 g/mL for the comparison experiment (Section 3.3.1) and at 0.005 g/mL for the pharmaceutical degradation trial.

Stock solutions were stirred magnetically for 2 h at room temperature (20 ± 2 °C) to ensure dissolution. The commercial-grade laccase solution was additionally filtered through a 0.45 µm syringe filter to remove insoluble material present in the preparation. Filtration was not required for the lab-grade laccase.

Laccase stock solution was measured in distilled water at the beginning and end of Experiments 1 (5 h) and 3 (3.5 h) to confirm no activity loss during the time required to complete each experiment.

Unless stated otherwise, laccase stock solution was added to urine after the urine pH had first been adjusted to 3.9 with NaOH. After enzyme addition, the pH was readjusted to 4.5. This stepwise adjustment was used to avoid direct exposure of the enzyme to the highly acidic stored urine (pH < 3), because Experiment 1 showed that laccase activity was strongly reduced below pH 3.

3.1.4 Pharmaceutical Stock Solution

A pharmaceutical stock solution was prepared in methanol at 10 µg/mL per compound. The solution contained 19 compounds: atenolol, furosemide, irbesartan, carbamazepine, sertraline, trimethoprim, bicalutamide, oxazepam, diclofenac, losartan, bisoprolol, clarithromycin, hydrochlorothiazide (HCTZ), lamotrigine, clindamycin, propranolol, metoprolol, sulfamethoxazole, and venlafaxine. The stock solution was stored at −20°C until use.

3.1.5 Mediator Stock Solutions

Vanillin and syringaldehyde stock solutions were prepared separately by dissolving 0.3 g of each compound in 20 mL methanol, giving a final

concentration of 15 mg/mL. The solutions were stirred gently for 1 h using a magnetic stirrer and prepared fresh on the day of each experiment.

Experimental outline

The work was divided into three parts. Firstly, a series of experiments was conducted to establish conditions for laccase activity in urine (Section 3.2). Secondly, validation experiments were conducted prior to the pharmaceutical degradation trial (Section 3.3). Thirdly, laccase was used to oxidise selected pharmaceuticals in urine under selected conditions (Section 3.4). Methods applied across multiple experiments are described separately. The DMP assay used for all laccase activity measurements is described in Section 3.5, and the dissolved oxygen measurement procedure is described in Section 3.6.

3.2 Laccase Conditions in Urine

Six experiments were conducted to establish suitable conditions for laccase activity in urine. The findings from each experiment informed the conditions used in subsequent experiments, and ultimately, in the pharmaceutical degradation trial. Unless otherwise stated, all experiments used commercial-grade laccase and acidified urine at room temperature (20 ± 2 °C) as described in Section 3.1.2. Based on results of Experiment 1, a pH of 4.5 was used in all experiments in this section. Laccase activity was measured using the DMP assay described in Section 3.5. Samples were protected from light using aluminium foil-covered beakers, amber glass bottles, or amber falcon tubes (CellPro™, 45 mL).

3.2.1 Experiment 1 - Effect of pH on Laccase Activity

The effect of urine pH on laccase activity was assessed across a pH range of 1 to 11. A bulk urine sample of 120 mL at pH 2.8 was prepared in a beaker covered with aluminium foil and stirred continuously on a magnetic stirrer throughout aliquot preparation. The pH was first lowered to 1.0 by dropwise addition of 0.95 mL of 96% sulfuric acid. Aliquots were then prepared at one-unit pH intervals from pH 1 to pH 11. At each target pH, 10 mL aliquot was removed from the bulk sample, and the pH of the remaining bulk was raised to the next target value by initial dropwise addition of 5 M NaOH, then switching to 1 M NaOH for precise adjustment to reduce the risk of overshooting. Each aliquot received 2 mL of laccase stock solution after which the pH was checked and, if necessary, readjusted to the target value. Enzyme activity was measured immediately in duplicate using the DMP assay before the next aliquot was processed. The dilution caused by NaOH addition differed slightly between samples and was accounted for in the calculations (Appendix 2, Table 1).

3.2.2 Experiment 2 - Effect of Temperature on Laccase Activity

This experiment investigated the effect of temperature on laccase activity and was conducted in two parts.

In Part 1, a single 70 mL bulk urine sample was filtered through a 0.45 µm syringe filter and transferred into an amber glass bottle before laccase addition. This allowed activity to be measured immediately after heating without additional filtration. The bulk sample was prepared at pH 4.5 with 12 mL of laccase stock solution and 0.16 mL NaOH, giving a dilution factor of 1.17. Aliquots were heated to 30, 40, 50, 60, 70, 80, and 90 °C using a block heater (Grant QBD4 Digital Dry Block Heater). Enzyme activity was measured using the DMP assay at each temperature at two time points: immediately after the target temperature was reached and after 5 min of incubation. After each temperature step, incubated aliquots were discarded and fresh aliquots used for the next step to avoid cumulative thermal effects. The bulk urine-laccase mixture was measured at room temperature at the beginning of the experiment, between the 50 °C and 60 °C steps, and at the end of the experiment to confirm that no significant activity loss occurred over the course of the experiment.

In Part 2, heat treatment was tested as a method for stopping enzyme activity. Two aliquots were heated to 90 °C for 10 and 15 min, respectively, stored at room temperature for 24 h, and then analysed for residual activity.

3.2.3 Experiment 3 - Effect of Urine Concentration on Laccase Activity

The effect of urine concentration on laccase activity was assessed at mass concentration factors (CF) of 1, 2, 4, 5, and 10. CF was defined as the initial urine mass divided by the final mass after evaporation. For each target CF, 200 g acidified urine was poured into a glass baking dish and evaporated in a laboratory drying oven at 50 °C until the target final mass was reached. The unconcentrated sample (CF 1) served as a reference and was not evaporated.

After evaporation, 10 mL aliquots were taken and stored in amber falcon tubes. Each sample received 2 mL of laccase stock solution, was adjusted to pH 4.5 with NaOH, and measured in duplicate using the DMP assay at room temperature.

Due to the addition of laccase stock solution and NaOH, the actual CF differed slightly from the target CF for each sample (Appendix 2, Table 2).

3.2.4 Experiment 4 - Effect of Acid Type on Laccase Activity

The effect of acid type used for urine acidification on laccase activity was assessed using five acids: sulfuric (96%, 0.05 mL), phosphoric (3 M, 0.34 mL), oxalic (2 M, 0.32 mL), citric (3 M, 0.30 mL), and acetic acid (96%, 0.48 mL). Fresh urine was collected as described in Section 3.1.2 and divided into five

50 mL aliquots. Each aliquot was acidified with one acid to a target pH of 3.9. All aliquots then received 8 mL of laccase stock solution, the pH was adjusted to 4.5 with NaOH, and activity was measured immediately in duplicate using the DMP assay. The time between enzyme addition, pH adjustment, and measurement was kept as consistent as possible across all samples. The volume of acid and NaOH added and the dilution factor for each sample are provided in Appendix 2, Table 3.

3.2.5 Experiment 5 - Oxygen Depletion by Laccase in Urine

This experiment investigated whether laccase activity causes oxygen depletion in urine. Two samples were prepared sequentially, each containing 70 mL of urine. Sample U I received 12 mL of 100 mM acetate buffer and 0.13 mL of NaOH solution, and sample U II received 12 mL of laccase stock solution and 0.2 mL of NaOH solution, giving a dilution factor of 1.17 for both samples.

A dissolved oxygen meter (Hanna HI2040 Edge) was suspended in the sample, and the contents were stirred until a dissolved oxygen level of approximately 40% was reached before logging was initiated at t = 0 s. Dissolved oxygen was logged continuously at 5 s intervals from t = 0 s to t = 1000 s. The pH was first adjusted to 3.9 with NaOH, after which either laccase stock solution or plain acetate buffer was added at t = 295 s, followed by readjustment of the pH.

3.2.6 Experiment 6 - Evaluation of Aeration Requirements

This experiment evaluated whether active aeration would be needed for longer incubation periods. Four bottles were prepared with 70 mL urine, 12 mL of laccase stock solution and a NaOH addition of 0.1 mL, giving a dilution factor of 1.17 in all four samples. Two bottles (U I and U II) were mixed with a magnetic stirrer and

two (U III and U IV) were actively aerated using an aquarium pump (Rena 301 Aquarium Pump) connected to plastic tubing (Figure 1).

Figure 1. Picture of the experimental setup for Experiment 6 (Evaluation of Aeration Requirements) showing bottles U III and U IV with aeration tubes from an aquarium pump inserted through the bottle caps. Bottles U I and U II (not shown) were mixed using a magnetic stirrer without aeration and served as the stirred control condition.

Dissolved oxygen (DO) was monitored at t = 0, 1, 3, and 5 h in all bottles using the dissolved oxygen meter described in Section 3.9. Once the pH reached 4.5 and the pH probe was removed, the dissolved oxygen probe was immediately inserted and the first DO reading was recorded, defining t = 0 for each bottle. For DO measurements in the aerated bottles, aeration was paused and the bottles were placed on a magnetic stirrer and gently stirred during measurement. The DO value was recorded once the reading had stabilised. Laccase activity was additionally measured in duplicate at t = 0, 2, and 4 h using the DMP assay.

3.3 Validation prior to Pharmaceutical Degradation Trial

Two experiments were conducted prior to the pharmaceutical degradation trial to validate the conditions to be used in the trial. Experiment 7 determined the concentration of lab-grade laccase to achieve a target activity of 300 U/L at 50 °C. The target value of 300 U/L was selected based on Hultberg et al. (2025), who reported this activity level in a system using laccase producing *T. versicolor* fungal pellets, measured activity with the same DMP assay, and achieved significant reduction of multiple pharmaceutical compounds in a wastewater matrix. Several of which were also assessed in the present study.

Experiment 8 validated the final incubation setup and generated a 24 h activity profile under those conditions.

3.3.1 Experiment 7 - Laccase Comparison and Dose Finding

Experiment 7 was divided into two parts. In Part 1, five samples of 30 mL urine at pH 4.5 were prepared. Both laccases were supplied as powders and dissolved in 100 mM acetate buffer to prepare stock solutions as described in Section 3.1.3. Because the two stock solutions differed in mass concentration and enzyme source, their activities per litre could not be assumed to be equivalent. Part 1 therefore aimed at identifying the volume of lab-grade stock required to match the activity delivered by the commercial-grade stock.

The commercial-grade stock (0.05 g/mL) was added at 200 mL/L (6.00 mL per 30 mL sample). The lab-grade stock (0.01 g/mL) was tested at four volumes ranging from 80 to 93 mL/L (2.40 to 2.80 mL per sample). Acetate buffer was added to the lab-grade samples to bring the total liquid addition to 200 mL/L (6.00 mL per sample) across all conditions, ensuring a consistent dilution factor. Activity was measured in duplicate at room temperature (20 ± 2 °C) immediately after preparation using the DMP assay. Sample compositions and dilution factors are provided in Appendix 2, Table 4.

In Part 2, the target activity for the pharmaceutical degradation trial was set at 300 U/L at 50 °C. A fresh lab-grade laccase stock was prepared at a lower concentration (0.1 g in 20 mL acetate buffer) to ensure complete dissolution. Stock activity was measured in duplicate immediately after preparation at both room temperature and 50 °C at 0 and 5 min of incubation using the DMP assay.

Four samples of 8.4 mL urine were prepared, designated U I, UII, UIII and U IV, receiving lab-grade laccase stock volumes of 1.0 (U I), 1.2 (U II), 1.4 (U III), and 1.6 (U IV) mL respectively. Acetate buffer was added to samples U I, U II, and U III to bring the total liquid addition to 1.6 mL across all four samples, ensuring a consistent buffer contribution. All samples received 0.01 mL NaOH, giving a dilution factor of 1.19.

3.3.2 Experiment 8 - Setup Validation and 24-h Activity Profile

This experiment tested the final incubation setup and monitored laccase activity over 24 h under the selected conditions. The experiment was conducted in two parts.

Experiment 8 Part 1

In Part 1, a bulk of 850 mL urine was prepared in a beaker covered with aluminium foil, the pH adjusted to 3.9 with NaOH, and 119 mL of commercial-grade laccase stock solution was added, after which the pH was readjusted to 4.5.

The total NaOH addition was 1.67 mL giving a dilution factor of 1.14. The bulk was then distributed into twelve 100 mL amber glass bottles of 80 mL each and incubated at 50 °C in a silicone oil bath (Büchi Heating Bath B-300 Base). Aeration distribution was assessed by measuring dissolved oxygen in all twelve bottles after 2 and 24 h of aeration using the dissolved oxygen meter (Section 3.6). For activity measurement, aliquots from four bottles were pooled at each time point. Activity was measured at t = 0 at both room temperature (20 ± 2 °C) and 50 °C, and at t = 3 and t = 24 h at 50 °C using the DMP assay. Temperature was recorded across all bottle positions with a temperature logger (testo 176 T2). Significant temperature variation across bottle positions and evaporative losses observed in Part 1 led to the modified incubation setup described below, which was subsequently used in Part 2 and in the pharmaceutical degradation trial.

Oil Bath and Temperature Control

An oil bath filled with silicone oil was used as the incubation vessel. To achieve uniform temperature distribution, a custom plastic tray was constructed from a cut plastic plate as the upper surface and the base of a pipette tip box, cut to 5 cm height, as the support. The tray had holes of 9 mm diameter at regular intervals through both the tray surface and the sides of the base to allow circulation of the silicone oil. This tray was placed on the bottom of the oil bath, elevating the twelve incubation bottles above the base, and arranged in four columns with three bottles per column. An overhead stirrer (OHS 60 Digital) set to 900 rpm was mounted on a metal pole at the back centre of the oil bath, with its stirring rod extending downward through the centre of the aeration block and the plastic tray into the silicone oil, continuously circulating the oil. This setup maintained a temperature range of 48 to 52 °C across all bottle positions (Figure 2).

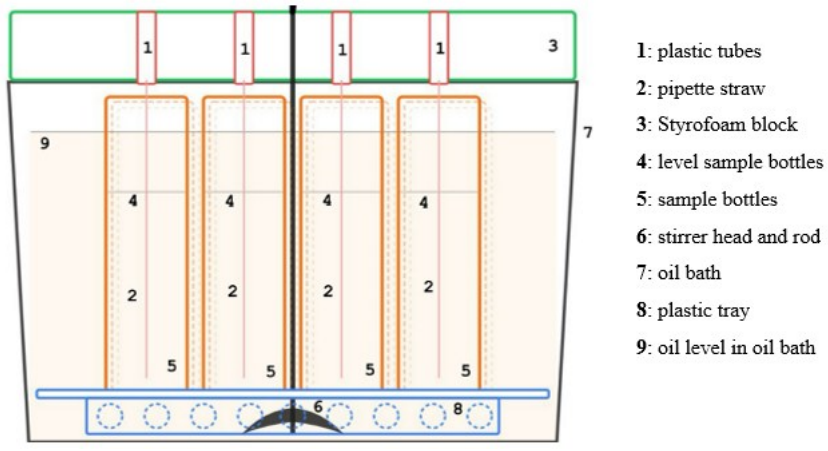

Figure 2. Schematic illustration of the incubation setup used in Experiment 8 Part 2 and the pharmaceutical degradation trial. Twelve 100 mL amber glass bottles were arranged in four columns of three on a custom plastic tray elevated above the base of the silicone oil bath to ensure uniform temperature distribution. Continuous aeration of each bottle

was provided by an aquarium pump via a Styrofoam aeration block resting on the bottle necks. Individual plastic tubes run into the aeration block, with a pipette straw inserted at the base of each tube extending to the bottom of the bottle to ensure air is released at the lowest point and rises through the entire liquid volume. An overhead stirrer mounted at the rear of the oil bath circulates the silicone oil to maintain uniform temperature across all bottle positions.

Aeration System

Continuous aeration was provided by an aquarium pump (Rena 301 Aquarium Pump). Air was delivered from the pump via larger tubing (inner diameter 6 mm, outer diameter 9 mm, approximate) to a T-piece on the main line. One branch of the T-piece was attached with a valve (Gardena Shut-Off Valve 4.6 mm, 3/16") and used as a bypass at to release excess air pressure. The airflow was then split into 12 individual lines via smaller tubing (inner diameter 4 mm, outer diameter 5 mm), each controlled by an individual valve (Gardena Shut-Off Valve 4.6 mm, 3/16"). The 12 lines ran into a custom-built Styrofoam aeration block (21 cm × 17 cm × 6 cm) placed directly on top of the twelve bottle openings, resting on the bottle necks. Inside the aeration block, each line was connected to a pipette straw (VWR 1 mL, PP, NA, 190 mm), which was cut to end just above the base of each bottle, ensuring that air was released at the bottom and rose through the entire liquid volume, providing both oxygenation and mixing. Each bottle was closed with the inner part of its lid, which contained a hole to allow the pipette straw to pass through, and sealed with a layer of aluminium foil to minimise evaporative losses while allowing gas exchange. The aeration block was stabilised by a metal bar fixed across its top surface, attached on both sides to small metal poles positioned outside the oil bath (Figure 3).

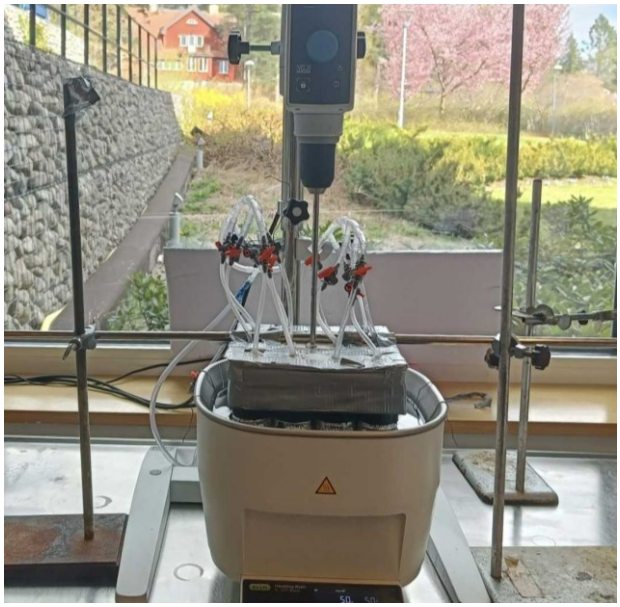

Figure 3. Picture of the incubation setup used in Experiment 8 Part 2 and the pharmaceutical degradation trial, showing the silicone oil bath with the Styrofoam aeration block resting on the bottle necks and the overhead stirrer mounted at the rear. The aeration block distributes air from an aquarium pump to all twelve bottle positions simultaneously via individual valves.

Experiment 8 Part 2

In Part 2, the modified incubation setup described above was used with lab-grade laccase at 0.7 g/L. A bulk of 283.8 mL urine was prepared in an amber glass bottle, the pH adjusted to 3.9 with NaOH, and 46.2 mL of lab-grade laccase stock solution was added, after which the pH was readjusted to 4.5. The total NaOH addition was 0.41 mL giving a dilution factor of 1.16.
Four bottles were filled with 80 mL each. Three bottles were placed in the oil bath at 50 °C and the remaining nine positions were filled with bottles containing 80 mL of distilled water to maintain comparable heat distribution and aeration. A fourth bottle was kept at room temperature (20 ± 2 °C) on a magnetic stirrer as a control. Activity was monitored at t = 0, 1.5, 2.5, 4.5, 5.5, 7 and 24 h using the DMP assay. For each measurement a combined sample of 2 mL from each of the three incubation bottles was taken.

A preliminary experiment, conducted under identical conditions, had shown a drop in activity upon removal from the oil bath at t = 14 h. Based on this result, earlier cooling was tested as a way to preserve residual enzyme activity. One bottle was therefore removed from the oil bath at t = 4.5 h placed on a magnetic stirrer and allowed to equilibrate to room temperature (20 ± 2 °C) and the activity was then measured at t= 5.5, 7 and 24 h.

3.4 Pharmaceutical Degradation Trial

The pharmaceutical degradation trial tested the effect of laccase on degradation of selected pharmaceuticals in urine over 24 h, with and without mediators.

3.4.1 Experimental Design

Four conditions were tested, each with three analytical replicates taken from the same bulk solution. Each laccase condition also included a fourth bottle used exclusively for activity measurements during incubation. The experiment was split over two days: Day 1 covered the control condition without laccase, which did not require an activity bottle; Day 2 covered the three laccase-based conditions, each with three analytical replicates and one activity bottle (Table 1). All samples were incubated in 100 mL amber glass bottles containing 80 mL of solution at 50 °C using the incubation setup described in Section 3.3.2. Each sample contained the same volume of urine and the same total volume of acetate buffer. In the laccase conditions, acetate buffer was contributed by the laccase stock solution itself; in the control condition, it was added as plain 100 mM acetate buffer. The same volume of methanol was added to all conditions to account for the solvent introduced with the mediator stock solutions. Thus, the only difference between conditions was the presence or absence of laccase and mediators. The target pharmaceutical concentration in all samples was 50 µg/L.

Table 1. Experimental design of the pharmaceutical degradation trial. Each condition was run with three analytical replicate bottles (Replicates) used for UPLC-MS/MS sampling and, for laccase conditions, one additional dedicated activity bottle (Activity bottle) used exclusively for DMP assay measurements throughout the incubation. The control condition (Day 1) did not require an activity bottle as no laccase was present (not measured NM). All bottles contained 80 mL of solution at the same composition, differing only in the presence or absence of laccase and mediator.

Day	Code	Composition	Replicates	Activity bottle
1	U	Urine + Pharmaceuticals (Control)	3	NM
2	LC	Urine + Pharmaceuticals + Laccase	3	1
2	LV	Urine + Pharmaceuticals + Laccase + Vanillin	3	1
2	LS	Urine + Pharmaceuticals + Laccase + Syringaldehyde	3	1

3.4.2 Day 1 Control

A bulk solution was prepared in a 400 mL amber glass bottle covered with aluminium foil under gentle stirring. It contained 283.8 mL of acidified urine (pH 2.9), 48.62 mL of 100 mM acetate buffer, 0.022 mL of methanol, and 1.25 mL of pharmaceutical stock solution (Section 3.1.4). The pH was adjusted to 4.5 with 0.8 mL NaOH, giving a dilution factor of 1.17. Once homogeneous, 80 mL of the

bulk solution was transferred to each of three 100 mL amber glass bottles. The remaining nine positions in the oil bath were filled with bottles containing 80 mL distilled water to maintain comparable heat and aeration distribution. The bottles were placed in the oil bath, the start time was recorded as t = 0, and aeration was started. Samples of 3 mL were collected from each replicate bottle at t = 0 and t = 24 h and immediately heat inactivated (Section 3.4.4), and was stored at −20 °C until UPLC-MS/MS analysis.

3.4.3 Day 2 Laccase Treatment

A bulk solution was prepared in a 1.2 L beaker covered with aluminium foil under gentle stirring with 851.4 mL of acidified urine (pH 2.75). Then, 138.6 mL of freshly prepared laccase stock solution was added, which also provided the acetate buffer fraction. The pH was adjusted with 2.08 mL NaOH, giving a dilution factor of 1.17. A total of 4.95 mL of the pharmaceutical stock solution (Section 3.1.4) was added, and the bulk was mixed thoroughly. Three initial samples of 3 mL were taken directly from the bulk at t = 0 and immediately heat inactivated (Section 3.4.4).

The bulk was then divided into three 400 mL amber glass bottles with 330 mL each, one for each treatment. Either 0.022 mL of the respective mediator stock solution (Section 3.1.5) or an equal volume of methanol was added to each bottle. Each treatment was then distributed into four 100 mL amber glass bottles of 80 mL each, with bottles 1–3 used for UPLC-MS/MS sampling and bottle 4 reserved for activity measurements only.

Each set of four bottles was placed in the oil bath, and the start time for that treatment was recorded as t = 0. A 10 min interval was used between placing each treatment in the oil bath to provide sufficient time at each sampling point to complete both UPLC-MS/MS sampling and activity measurement for all treatments without overlap. Aeration was started only after all three treatments were placed in the oil bath.

Samples of 3 mL were collected from each replicate bottle at defined time points for each condition. For the laccase-only treatment (LT) samples were collected at t = 0, 1, 6, and 24 h. For the mediator Syringaldehyde (LST) and Vanillin (LVT) treatment, samples were collected at t = 6 and 24 h only. All samples were immediately inactivated upon collection as described in Section 3.4.4. Time points and sample codes are provided in Appendix 4, Table 1.

3.4.4 Sample Inactivation, Storage and UPLC-MS/MS Preparation

To stop enzyme activity and prevent further pharmaceutical degradation after sampling, all samples were placed in a block heater at 90 °C for 15 min immediately after collection.[1] This inactivation method was validated in

[1] Previous tests showed <5% degradation at 90 °C across 40 pharmaceutical compounds, including all 19 compounds analysed in the present study (P. Simha, personal communication, 2026).

Experiment 2 (Section 3.2.2). Upon inactivation, samples were cooled to room temperature (20 ± 2 °C) and stored at −20 °C until UPLC-MS/MS analysis.

The samples were analysed with a DIONEX UltiMate 3000 ultra performance liquid chromatograph (UPLC) system (Thermo Scientific, Waltham, MA, USA) with a triple quadrupole mass spectrometer (MS/MS) (TSQ Quantiva, Thermo Fischer Scientific, Waltham, MA, USA). The data were evaluated with Tracefinder 4.1 (Thermo Fischer Scientific, MA, USA).

For UPLC-MS/MS analysis, frozen samples were thawed and equilibrated to RT, filtered through a 0.45 µm syringe filter, and diluted 1:10 in MilliQ water. An internal standard solution was added to all samples prior to analysis. A procedural control, consisting of untreated acidified urine without enzyme addition spiked with pharmaceutical stock solution at the target concentration of 50 µg/L, was prepared to assess background pharmaceutical concentrations. Two lab blanks consisting of urine from the same bulk, neither heated to 90 °C nor incubated at 50 °C and, with internal standard added, were prepared to assess background signal.

Raw chromatographic data were exported from the UPLC-MS/MS instrument as peak areas for each target pharmaceutical and its corresponding isotopically labelled internal standard. All data processing was performed in Microsoft Excel.

Quantification was based on internal standard normalisation. For each compound in each sample, the peak area of the target analyte was divided by the peak area of its corresponding internal standards to yield a response ratio.

Calibration was performed using a series of standards prepared at eleven concentration levels spanning 0.01 to 500 ng, each containing the same fixed amount of IS. Response ratios were calculated for each calibration level and plotted against the nominal analyte amount. A linear regression was applied to each compound, yielding a compound-specific calibration slope. Sample concentrations in ng/mL were then calculated by dividing the sample response ratio by the calibration slope, accounting for the injection aliquot volume.

For each treatment condition and time point, three analytical replicate bottles were sampled independently. The mean concentration and standard deviation were calculated across the three replicates. One injection in the laccase-vanillin 6 h treatment (BLV3) was identified as a failed injection based on an anomalously low internal standard area, indicative of a blocked needle or failed injection, and was excluded from the calculation of the group mean and standard deviation for that condition.

For compounds measured below the instrument limit of quantification (LOQ) at a given sampling point, a value of one half of the compound-specific LOQ was substituted for data analysis purposes, in accordance with standard practice for non-detected results in environmental analytical chemistry.

Pharmaceutical removal was expressed as the relative change in measured concentration compared to the initial concentration at t = 0 h, calculated as:

$$Relative\ change = \frac{C_t - C_0}{C_0}$$

where C_t is the mean measured concentration at time point t and C_0 is the mean measured concentration at t = 0 h.

A positive value indicates a decrease in concentration relative to the initial value, and a negative value indicates an increase. Results are reported as percentage change by multiplying by 100. The untreated control condition (urine incubated without laccase for 24 h) was included to distinguish laccase-driven removal from abiotic losses or matrix-related concentration changes occurring during incubation

3.5 DMP Assay for Laccase Activity Measurement

Laccase activity was measured using the DMP assay described by Santoyo et al. (2008). The assay is based on the oxidation of 2,6-dimethoxyphenol (DMP), which forms a coloured product with an absorbance maximum at 468 nm. A 10 mM DMP solution was prepared by dissolving 0.154 g of DMP in 100 mL of 100 mM acetate buffer (pH 5.0) and was protected from light until use.

Prior to each measurement session, the spectrophotometer (UV-6300PC Double Beam Spectrophotometer, VWR, 1 cm path length cuvettes) was pre-warmed for 15 min. Sample were filtered through a 0.45 µm syringe filter before measurement. For each assay, 1500 µL DMP solution was pipetted into a 1 cm cuvette, followed by 1350 µL sample. The mixture was homogenised by aspirating and dispensing twice, immediately inserted into the spectrophotometer, and measured. A blank cuvette containing 1500 µL of distilled water and 1350 µL of sample was used to correct for background absorbance at 468 nm using the spectrophotometer's second light beam. Absorbance was recorded at 468 nm in kinetic mode every 0.5 s for 40 s. Only the initial linear part of the absorbance–time curve was used for activity calculation. Each sample was measured in duplicate, and the average value was used for all calculations.

Enzyme activity was calculated as:

$$\frac{U}{L} = \frac{dA/\min \times 2.85\ mL \times 10^6}{49.600\ M^{-1}cm^{-1} \times 1\mu mol \times 1.35\ mL}$$

where the slope (dA/min) was multiplied by 0.0426 to convert absorbance into activity in U/L. One unit (U) was defined as the formation of 1 µmol of oxidation product per minute. The conversion factor included the extinction coefficient (ε = 49,600 M^{-1} cm^{-1}), the reaction volume (V_{total} = 2.85 mL), the sample volume (V_{sample} = 1.35 mL), and the unit conversion from mol to µmol (10^6).

When activity was measured at elevated temperature, both the sample and the DMP solution were equilibrated to the target temperature before the assay. For experiments where samples were heated from room temperature (20 ± 2 °C) to the target temperature, samples were first filtered through a 0.45 µm syringe filter and then placed in the Grant QBD4 Digital Dry Block Heater covered with aluminium foil for UV protection. A temperature logger sensor was suspended in the sample to ensure that the measurement was performed when the target temperature was reached. During the pharmaceutical degradation trial, the block heater was used for enzyme inactivation at 90 °C (Section 3.4.4) and was therefore not available for activity measurement equilibration. Instead, Erlenmeyer flasks filled with silicone oil were pre-heated to 50 °C in an oven and taken out directly before sampling. The sample was then filtered through a 0.45 µm syringe filter directly into a sampling tube covered with aluminium foil suspended in the flask, keeping the sample at 50 °C until measurement. The DMP stock bottle was pre-heated in the same oven.

For samples already incubated at the target temperature, activity measurements were performed directly without additional temperature control, as the heating device was assumed to maintain 50 °C. In all cases, the assay was conducted adjacent to the respective heating device to minimise heat loss between equilibration and measurement.

When activity was measured while samples were being heated to a target temperature, the two replicate measurements are reported separately. When samples were already at the target incubation temperature, duplicate measurements were averaged.

3.6 Dissolved Oxygen Measurement

Dissolved oxygen was measured using a dissolved oxygen meter (Hanna HI2040 Edge). Prior to each experiment, the meter was calibrated according to the manufacturer's instructions. For each measurement, the DO probe was inserted into the sample, which was placed on a magnetic stirrer and gently stirred until a stable reading was obtained.

For samples incubated at elevated temperature in the oil bath, exact dissolved oxygen values could not be obtained. Measurement required stopping aeration, removing the bottle from the oil bath, and placing it on a magnetic stirrer. Because the probe required time to equilibrate to the sample temperature, these reading were unreliable. Waiting for a stable reading could reduce dissolved oxygen due to the interruption of aeration, whereas taking an earlier reading could give inaccurate values because the meter calculates dissolved oxygen saturation based on temperature.

4. Results

This chapter presents the results of Laccase Conditions in Urine experiments (Section 4.1), the laccase dose finding and setup validation (Section 4.2), and the pharmaceutical degradation trial (Section 4.3). The Laccase was always measured with the DMP assay (Section 3.5).

4.1 Laccase Treatment in Urine

Six experiments were conducted to investigate suitable conditions for laccase in urine. The results are presented in the order in which the experiments informed subsequent parameter choices.

4.1.1 Experiment 1 - Effect of pH on Laccase Activity

Laccase activity across a pH range of 1 to 11 is shown in Figure 4. No activity was detected at pH 1 and 2. Activity increased from pH 3 onwards, reaching a broad optimum between pH 5 and 7, with a maximum of 159 U/L at pH 7. Above pH 7, activity declined and was no longer detectable at pH 11 below the assay´s detection limit (10.83 U/L).

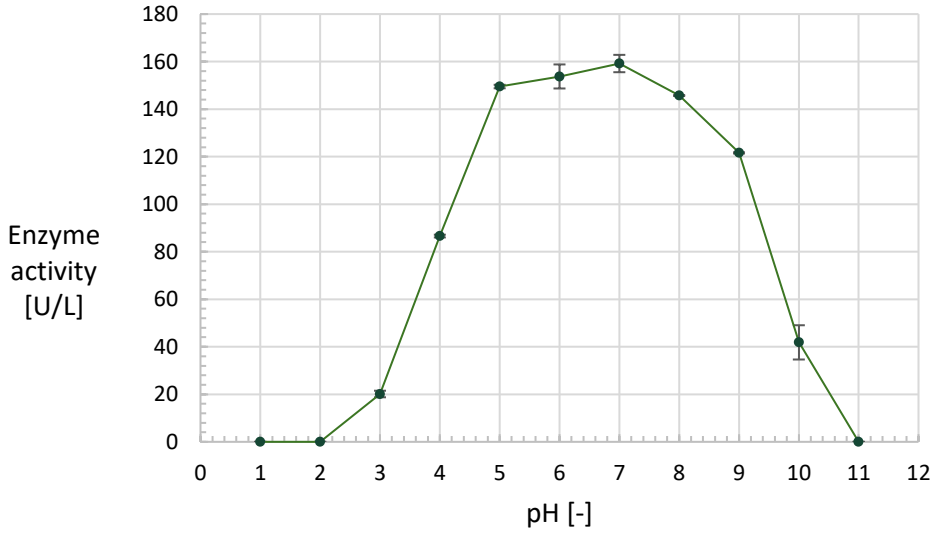

Figure 4. Effect of pH on laccase activity in acid-stabilised urine. Urine initially at pH 2.8 was sequentially adjusted to pH 1–11 using H_2SO_4 or NaOH. Commercial-grade laccase was added to an aliquot collected at each pH, and activity was measured immediately at room temperature using the DMP assay. Points show the mean of duplicate measurements and error bars show the standard deviation.

4.1.2 Experiment 2 - Effect of Temperature on Laccase Activity

Laccase activity at temperatures between 20 and 90 °C is shown in Figure 5. Activity increased with temperature up to 50 °C, where the highest activity of 192 U/L was recorded at 0 min incubation. At 60 °C, activity dropped to 148 U/L and declined sharply above this temperature, becoming undetectable at 80 and 90 °C. At all temperatures, activity measured after 5 min of incubation was lower than at 0 min.

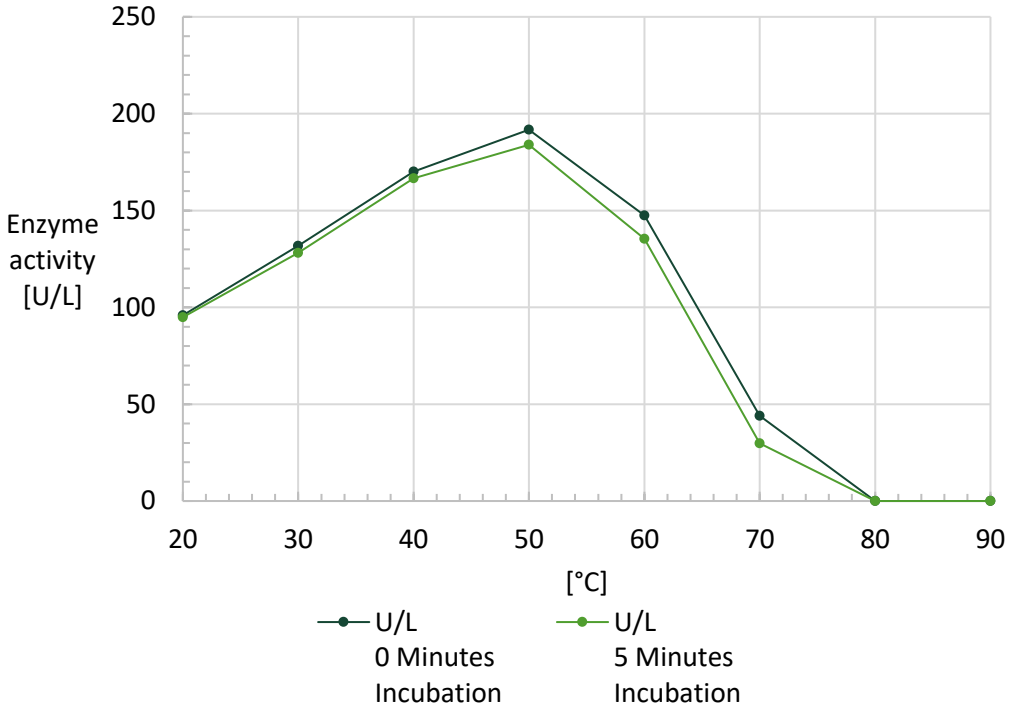

Figure 5. Laccase activity (U/L) measured by the DMP assay in acidified urine (pH 4.5) with commercial-grade laccase (Biovencer Healthcare Private Limited) at temperatures from 20 to 90°C. Activity was measured always after 0 and 5 min of incubation. Six room temperature (20 ± 2°C) control measurements taken at three intervals throughout the experiment were averaged to a single value (mean 95 U/L, standard deviation = 3.2 U/L), which serves as the shared starting point at 20°C for both curves.

No activity was detected in either aliquot after heating at 90 °C for 10 or 15 min and storing at room temperature (20 ± 2 °C) for 24 h, confirming that the heat inactivation method used for sample preservation was effective.

4.1.3 Experiment 3 - Effect of Urine Concentration on Laccase Activity

Laccase activity at mass concentration factors of 1, 2, 4, 5, and 9 is shown in Figure 6. Activity decreased with increasing urine concentration, from 205 U/L at CF 1 to undetectable at CF 9, below the detection limit of the assay (10.83 U/L).

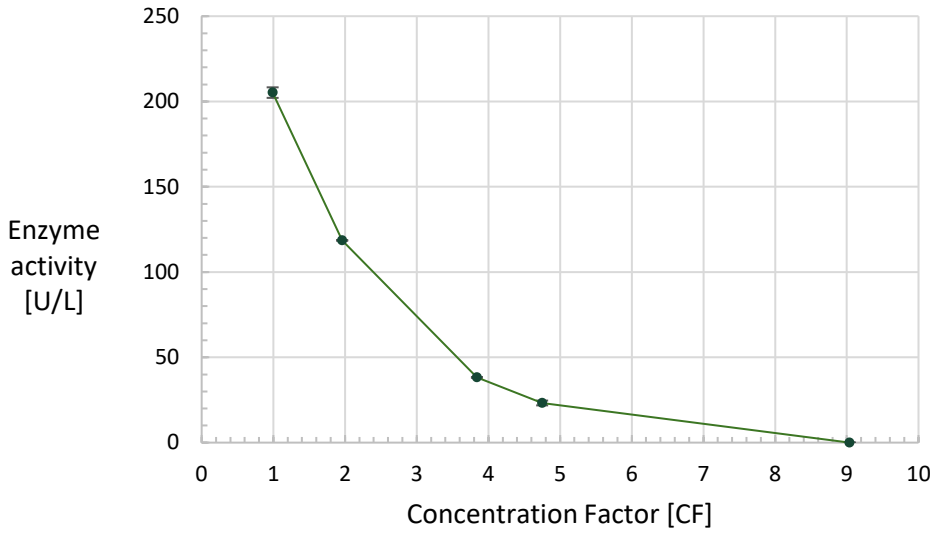

Figure 6. Laccase activity (U/L) measured by the DMP assay at increasing urine concentration factors (CF), where CF is defined as initial urine mass divided by final mass after evaporation at 50°C. Points show the mean of duplicate measurements at each CF, and error bars show the standard deviation.

4.1.4 Experiment 4 - Effect of Acid Type on Laccase Activity

Laccase activity in urine acidified with five different acids is shown in Table 2. Activities were broadly comparable across sulfuric, phosphoric, oxalic, and citric acid, ranging from 130 to 136 U/L. Acetic acid resulted in a notably lower activity of 107 U/L.

Table 2. Effect of acid type on laccase activity in fresh urine. Urine was acidified to pH 3.9 using one of five different acids (sulfuric, phosphoric, oxalic, citric, or acetic acid). All samples received the same laccase stock volume, and the pH was adjusted to 4.5 with NaOH. Values show the mean of duplicate activity measurement; SD indicates standard deviation.

Acid	Sulfuric acid	Phosphoric acid	Oxalic acid	Citric acid	Acetic acid
Activity (U/L)	135	131	136	130	107
SD	1	0.3	10	2.6	0.2

4.1.5 Experiment 5 - Oxygen Depletion by Laccase in Urine

Dissolved oxygen was monitored continuously in two separate incubations: U I served as a control containing urine with acetate buffer (70 mL urine + 12 mL buffer), while U II contained urine with added laccase stock solution (70 mL urine + 12 mL laccase stock) (Figure 7). During the preparation phase prior to t = 295 s, continuous stirring resulted in increasing dissolved oxygen in both bottles.

Immediately following laccase and buffer addition at t = 295 s, the two systems displayed markedly different oxygen dynamics. Dissolved oxygen in U II dropped rapidly from 45.8% to 39.5% within 35 s, with further decline to 31.3% observed by t = 1000 s. The buffer control (U I) showed opposite behaviour: dissolved oxygen rose from 43.6% to 50.2% following buffer addition and then stabilised at approximately 51% for the remainder of the measurement.

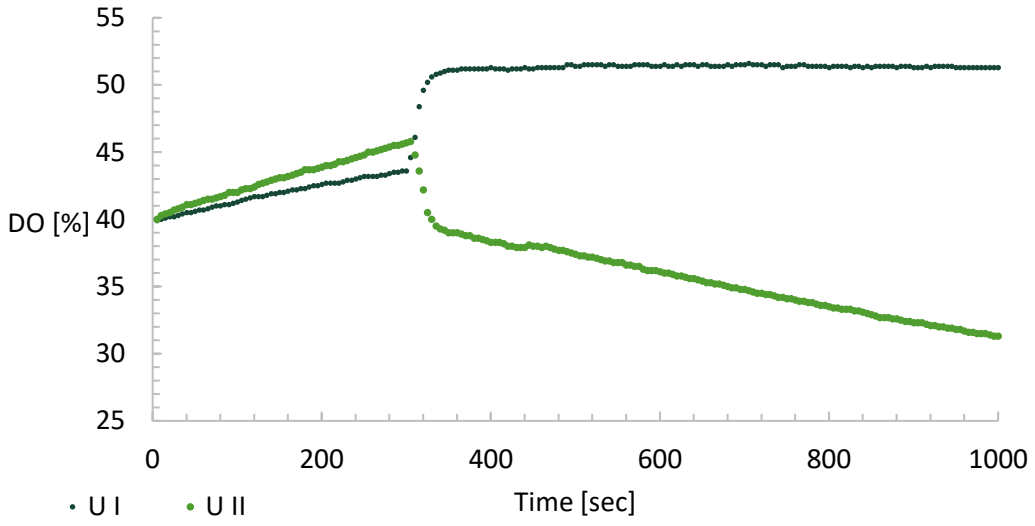

Figure 7. Dissolved oxygen dynamics in urine with and without laccase. Two samples containing 70 mL of acidified pooled urine (initial pH 2.9) were prepared: U I (urine + acetate buffer, control) and U II (urine + laccase stock). At t = 295 s, either plain acetate buffer (U I) or laccase stock (U II) was added. Dissolved oxygen was logged continuously at 5-second intervals throughout. Each point shows a single measurement at 5-second intervals for each condition.

4.1.6 Experiment 6 - Evaluation of Aeration Requirements

Dissolved oxygen and laccase activity were measured in stirred and aerated bottles over 5 h. Laccase stock solution (12 mL) was added to 70 mL of acidified source-separated urine. Dissolved oxygen declined rapidly in the two stirred bottles (U I and U II), falling to near-zero values of 0.8% and 0.7% after 5 h. In contrast, the two aerated bottles (U III and U IV) maintained substantially higher dissolved oxygen levels throughout, with values of 54.0% and 70.9% at 5 h.

Table 3. Effect of aeration on dissolved oxygen maintenance in laccase-treated urine. Four bottles containing 70 mL of acidified urine (pH 4.5) with commercial-grade laccase were incubated: U I and U II were mixed using a magnetic stirrer only (no aeration); U III and U IV were actively aerated using an aquarium pump.

Time (hours)	U I – (DO%) Stirred	U II – (DO%) Stirred	U III – (DO%) Aerated	U IV – (DO%) Aerated
0	26.1	5.4	5.4	5.4
1	39.5	42.9	71.7	81.7
3	10.8	26.9	79.9	82.7
5	0.8	0.7	54.0	70.9

The laccase activity in aliquots taken from all four bottles remained stable over the 4 h measurement period, with no clear difference between stirred and aerated conditions.

4.2 Selection and validation of laccase treatment conditions

The following section presents the results of the validation experiments 7 and 8, which tested the final setup for the pharmaceutical trial experiment.

4.2.1 Experiment 7 - Comparison of laccase preparations and selection of enzyme dose

Laccase activity measured in urine with commercial-grade and lab-grade laccase at varying stock volumes is provided in Appendix 5, Table 1. The commercial-grade laccase stock at 6.00 mL (0.05 g/mL) yielded an activity of 126 U/L at room temperature (20 ± 2 °C). Comparable activities were obtained with lab-grade laccase at stock volumes of 2.40–2.80 mL (0.01 g/mL), confirming that the lab-grade laccase had a considerably higher specific activity than the commercial preparation.

Laccase activity measured at room temperature (20 ± 2 °C) and 50 °C for increasing volumes of lab-grade laccase stock is provided in Appendix 5, Table 2. At 50 °C and 0 min incubation, a stock volume of 1.4 mL yielded 310 U/L and 1.6 mL yielded 323 U/L, both exceeding the target of 300 U/L. Activity declined at all volumes after 5 min of incubation at 50 °C. At room temperature (20 ± 2 °C), activity ranged from 67 to 113 U/L across all stock volumes.

4.2.2 Experiment 8 - Setup Validation and 24-h Activity Profile

In Part 1, dissolved oxygen measurements after 1, 2 and 24 h of aeration confirmed uniform distribution across all twelve bottles, verifying that the aeration system functioned as intended.

In Part 2, laccase activity measured over 24 h at 50 °C and room temperature (20 ± 2 °C) is shown in Figure 8. Activity in the 50 °C bottles peaked at t = 0.5 h with 299 U/L and declined steadily thereafter, falling to 80 U/L at t = 24 h. Activity in the room temperature (20 ± 2 °C) control remained broadly stable during the first seven hours, ranging from 122 to 138 U/L, before declining to 101 U/L at t = 24 h. Activity in the bottle removed from the oil bath and allowed to equilibrate to room temperature (20 ± 2 °C) at t = 4.5 h declined to 61 U/L at t = 5.5 h and remained at approximately 54 U/L at t = 7 and 53 U/L at t = 24 h, considerably lower than both the 50 °C bottles and the room temperature (20 ± 2 °C) control at those time points.

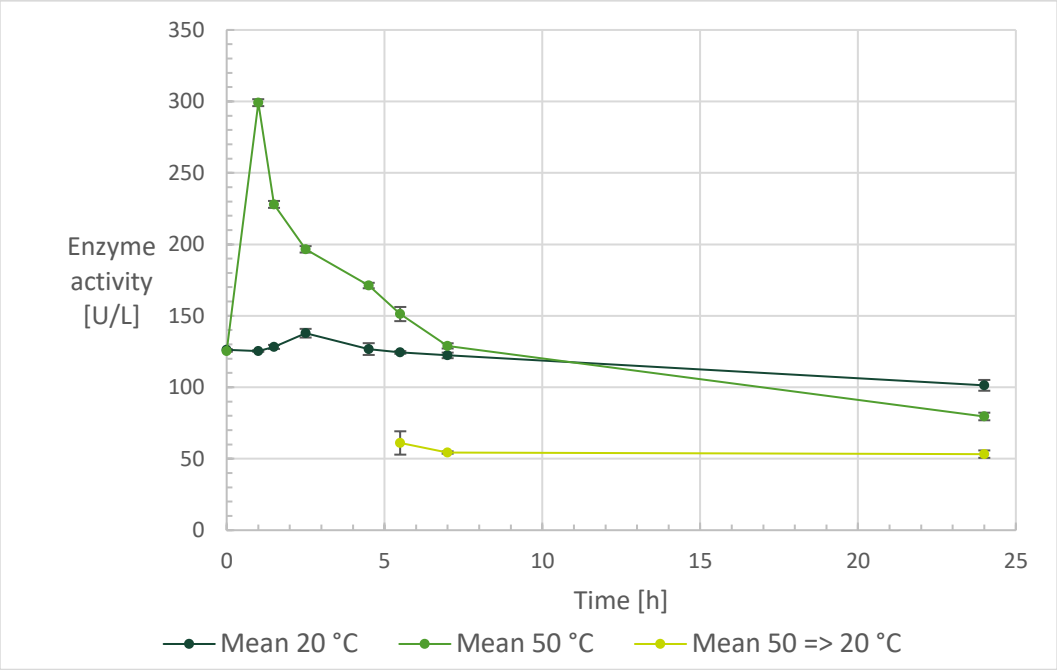

Figure 8. Laccase activity profile over 24 h in acidified urine (pH 4.5) with lab-grade laccase from T. versicolor. *Three bottles were incubated at 50°C in a silicone oil bath; at each time point, a combined sample from all three bottles was measured twice by the DMP assay. Points show the mean of the two measurements and error bars show the standard deviation. One bottle was removed from the oil bath at t = 4.5 h and allowed to cool to room temperature (20 ± 2°C), with activity measured at subsequent time points in the cooled sample. One bottle was maintained at room temperature (20 ± 2°C) throughout as a reference control. At t = 0, activity was measured once in the bulk before distribution into bottles; this value serves as the shared starting point for all treatments.*

4.3 Pharmaceutical Degradation Trial

In the following section, the UPLC-MS/MS results of the pharmaceutical degradation trial are presented. Section 4.3.1 presents the results of the laccase-only treatments and Section 4.3.2 presents the results of the laccase-mediator treatments. Section 4.3.3 presents the laccase activity measured throughout the trial.

The analysis of the pharmaceutical trial should be seen as preliminary results. All data are presented for completeness. The 24 h timepoint is emphasised as the primary endpoint of the treatment duration. Earlier sampling points (1 h and 6 h laccase only and 6 h mediators conditions) are included in the results but are not the focus of the interpretation given the substantial variability in control measurements and the scope of this thesis.

4.3.1 Results of the Laccase-only Treatment

Table 4 presents the relative percentage change in measured response for each of the 19 pharmaceutical compounds following laccase treatment at 1, 6, and 24 h, alongside the untreated control after 24 h, where positive percentages indicate a decrease in relative measured response and negative percentages indicate an increase over the incubation period.

Table 4. Relative percentage change in the measured response of selected pharmaceutical compounds following laccase treatment over incubation periods of 1, 6, and 24 h, and in the untreated control after 24 h. Values are expressed as percentage change relative to the initial concentration at t = 0 h, where positive percentages indicate less compound is detected at each time point, and negative indicate more is detected.

Pharmaceutical compound	LT 1 hr	LT 6 hr	LT 24 hr	Control-24 hr
Bicalutamide	-218%	-407%	-366%	-806%
Clindamycin	-66%	-57%	-43%	-28%
Bisoprolol	-31%	-21%	-18%	-4%
Propranolol	-17%	-19%	-9%	-1%
Venlafaxine	-15%	-12%	-9%	-1%
Metoprolol	-8%	-6%	-7%	3%
Lamotrigine	10%	4%	-7%	4%
Atenolol	-5%	-3%	-1%	4%
Sertraline	5%	0%	9%	19%
Carbamazepine	-7%	0%	13%	-2%
Trimethoprim	3%	14%	14%	3%
Furosemide	-18%	0%	32%	-25%
HCTZ	16%	14%	34%	54%
Losartan	16%	25%	34%	-13%
Irbesartan	8%	29%	36%	-13%
Clarithromycin	21%	33%	39%	35%
Sulfamethoxazole	41%	31%	46%	-2%
Oxazepam	-7%	8%	46%	40%
Diclofenac	-42%	0%	50%	-123%

Pharmaceutical responses differed considerably among compounds and were grouped according to the consistency of the concentration change relative to the

untreated control. Sulfamethoxazole showed the clearest treatment-specific response, decreasing by 46% after 24 h in the laccase treatment while remaining stable in the control. Diclofenac, losartan, irbesartan, and furosemide also decreased in the laccase treatment, but substantial increases in their corresponding controls limited attribution of these changes to laccase. Several other compounds showed either negligible treatment-specific changes or analytically implausible responses and were therefore not interpreted as evidence of transformation.

4.3.2 Results of the Mediator Compositions

Table 5 presents the relative percentage change in measured response for laccase-syringaldehyde (LST) and laccase-vanillin (LVT) treatments at 6 and 24 h, alongside the untreated control after 24 h, where positive percentages indicate a decrease in relative measured response and negative percentages indicate an increase over the incubation period.

Table 5. Relative percentage change in the measured response of selected pharmaceutical compounds following laccase treatment in the presence of syringaldehyde (LST) or vanillin (LVT) as mediators after 6 and 24 h of incubation, and in the untreated control after 24 h. Values are expressed as percentage change relative to the initial concentration at t = 0 h, where positive percentages indicate a decrease in measured response and negative percentages indicate an increase over the incubation period.

	LST 6 hr	LVT 6 hr	LST 24 hr	LVT 24 hr	Control-24 hr
Bicalutamide	-447%	-233%	-415%	-410%	-806%
Clindamycin	-46%	-7%	-37%	-42%	-28%
Bisoprolol	-18%	1%	-17%	-16%	-4%
Propranolol	-13%	6%	-8%	-5%	-1%
Venlafaxine	-13%	-1%	-6%	-7%	-1%
Metoprolol	-22%	-13%	-8%	-12%	3%
Lamotrigine	-20%	-7%	-12%	0%	4%
Atenolol	-6%	1%	-4%	2%	4%
Sertraline	-1%	-6%	3%	3%	19%
Carbamazepine	-16%	-8%	-5%	19%	-2%
Trimethoprim	3%	12%	22%	25%	3%
Furosemide	0%	8%	11%	32%	-25%
HCTZ	10%	25%	17%	29%	54%
Losartan	5%	-2%	14%	21%	-13%
Irbesartan	11%	7%	20%	27%	-13%
Clarithromycin	23%	23%	36%	43%	35%
Sulfamethoxazole	39%	55%	23%	34%	-2%
Oxazepam	21%	3%	36%	45%	40%
Diclofenac	-27%	-7%	55%	62%	-123%

Mediator responses differed considerably among compounds and were constrained by the same control interpretation limitations encountered in the laccase-only treatment. Sulfamethoxazole showed modest responses to both syringaldehyde (23%) and vanillin (34%), compared with laccase alone (46%), suggesting no consistent mediator enhancement. Compounds that showed apparent removal in the laccase-only treatment (diclofenac, losartan, irbesartan, furosemide) displayed similar patterns in the mediator treatments, but substantial control anomalies again limited interpretation of whether mediators enhanced transformation. Several other compounds showed either negligible treatment-specific changes or analytically implausible responses and were therefore not interpreted as evidence of mediator enhancement.

4.3.3 Results of the Activity measurement

Laccase activity at selected time points during the pharmaceutical degradation trial is shown in Table 6. Activity in the laccase-only condition was 226 U/L at t = 1 h, 170 U/L at t = 6 h, and 203 U/L at t = 24 h. Activity in the laccase-syringaldehyde condition was 155 U/L at t = 6 h, declining to 90 U/L at t = 24 h. Activity in the laccase-vanillin condition was 51 U/L at t = 6 h, declining to 24 U/L at t = 24 h. The mediator treatments were not sampled at t = 1 h and therefore no activity measurement is available for that time point.

Table 6. Laccase activity (U/L) measured by the DMP assay at selected time points during the pharmaceutical degradation trial for the laccase-only (LT), laccase-syringaldehyde (LST), and laccase-vanillin (LVT) treatments. Activity was not measured at t = 1 h for the mediator NM = not measured; SD indicates standard deviation

Time h	LT (U/L)	SD	LVT (U/L)	SD	LST (U/L)	SD
1	226	5.7	NM	NM	NM	NM
6	170	2.9	51	1.1	155	4.8
24	203	13.6	24	2.4	90	4.7

5. Discussion

In the following section, the results of this study are discussed in the context of existing literature. Section 5.1 discusses the results of the laccase condition experiments in urine together with the validation experiments conducted prior to the pharmaceutical degradation trial. Section 5.2 discusses the results of the pharmaceutical degradation trial.

5.1 Laccase Conditions in Urine

This section discusses the laccase conditions in Urine in the conducted experiment and compares it with existing literature.

5.1.1 Urine as a complex and challenging matrix

The progressive decline in laccase activity with increasing urine concentration factor observed in Experiment 3 demonstrates that the urine matrix inhibits laccase. Source-separated urine contains over 2,600 confirmed metabolites (Bouatra et al. 2013), several of which are known inhibitors of *T. versicolor* laccase acting through four distinct molecular mechanisms.

Urea causes concentration-dependent unfolding and deactivation of *T. versicolor* laccase through disruption of the protein's tertiary structure (Najafabadipour et al. 2021). Although the urea concentration in the pooled urine was lower than the ranges tested by Najafabadipour et al. (2021), the dose-response relationship makes a minor inhibitory contribution in unconcentrated urine plausible. Chloride, measured at approximately 3800 mg/L in the pooled urine (Appendix 6), inhibits laccase via competitive inhibition at the T2/T3 trinuclear copper cluster, where it blocks the reduction of molecular oxygen to water (Xu 1996). This inhibition has been shown to be substantially stronger at lower pH (Raseda et al. 2014), providing a further rationale for minimising direct enzyme exposure to the highly acidic stored urine during treatment. Acetate acts as a mixed inhibitor of laccase at concentrations of 10 to 100 mM (Ters et al. 2009). The acetic acid acidification in Experiment 4 resulted in total acetate concentrations exceeding this inhibitory range, consistent with the notably lower activity observed in that condition. Oxalic and citric acid inhibit laccase in a concentration-dependent manner through copper chelation (Lorenzo et al. 2005) and are naturally present in urine at concentrations that may contribute to the overall inhibitory burden (Bouatra et al. 2013). In Experiment 4 no additional inhibitory effect was observed for these acids relative to the non-inhibitory reference acids sulfuric and phosphoric acid, suggesting the additional volumes applied for acidification did not substantially increase the overall inhibitory effect of the urine matrix. These naturally occurring organic acids could therefore be

preferable alternatives to sulfuric acid for urine acidification. Simha et al. (2023) demonstrated that they are equally effective for urine stabilisation and nutrient recovery, with higher buffering capacity and better protection against ammonia nitrogen loss, although higher doses are required compared to sulfuric acid.

The simultaneous presence of inhibitors acting through protein unfolding, active site blockage, mixed inhibition and metal chelation means that the combined inhibitory effect of the urine matrix likely exceeds the sum of individual compound effects, as has been demonstrated for other oxidative treatment systems applied to real versus synthetic urine (Demissie et al. 2023; Petrochenko et al. 2026).

Beyond enzyme inhibition, the high organic load of source-separated urine means that laccase encounters a large pool of endogenous oxidisable substrates during treatment. Urine contains phenolic metabolites, indole derivatives and aromatic amines (Mehaidli et al. 2024), all of which are direct laccase substrates. The pharmaceuticals targeted in the degradation trial were present at spiked concentrations of 950 µg/L, negligible compared to approximately 1.43 g/L of endogenous organic metabolites (Mehaidli et al. 2024). This competitive substrate problem was directly demonstrated in Experiment 5, where rapid oxygen depletion occurred in plain acidified urine without any pharmaceutical addition, confirming that endogenous urine organics alone provided sufficient substrate to drive continuous laccase-catalysed oxygen consumption. Endogenous substrates therefore compete directly with pharmaceutical target compounds for the available oxidative capacity of laccase during incubation, consistent with findings from other oxidative treatment approaches in real urine (Demissie et al. 2023; Petrochenko et al. 2026). Whether laccase preferentially oxidises endogenous metabolites or pharmaceutical compounds under these conditions remains unknown. The critical role of aeration in maintaining dissolved oxygen availability was confirmed in Experiment 6, where oxygen depletion in stirred-only conditions showed the need for active aeration to sustain laccase activity.

Despite this inhibitory complexity, sufficient laccase activity was achieved across all experiments when an appropriate enzyme dose was applied. These findings suggest that laccase treatment must be integrated into the early stages of urine processing, before concentration factors become prohibitively high. Treatment should occur at moderate concentration factors where activity remains detectable, and active aeration would be an essential design consideration for any scaled-up laccase treatment system, as demonstrated in Experiments 5 and 6. While activity declined progressively with increasing concentration factor and became undetectable at CF9, treatment could potentially be integrated into the early stages of urine-based fertiliser production where concentration factors remain moderate and elevated drying temperatures may further enhance laccase activity, as discussed in Section 5.1.3.

5.1.2 pH Optimisation and the Preservation-Activity Compromise

The results of Experiment 1 demonstrated that laccase activity was undetectable below pH 3 with the DMP assay and increased progressively from pH 3 onwards, reaching a broad optimum between pH 5 and 7 with a maximum of 159 U/L at pH 7. This optimum presents a direct conflict with the requirement to store urine at pH < 3. Low pH storage inhibits urease-catalysed hydrolysis of urea to ammonia, preserving the nitrogen composition essential for subsequent nutrient recovery (Simha et al. 2023). Raising the pH to activate laccase treatment inevitably compromises this urease inhibition, creating a fundamental trade-off between maintaining storage-compatible conditions (deactivating urease) and enabling enzymatic pharmaceutical degradation (activating laccase).

The observed pH optimum of pH 5 to 7 is consistent with the broad range reported for DMP oxidation by fungal laccases (Baldrian 2006). As the commercial-grade laccase used in Experiment 1 was of unspecified fungal origin, a direct comparison with species-specific literature values is not possible.

A working pH of 4.5 was selected as a compromise between the urease inhibition requirement (pH ≤ 3) and the laccase activity requirement (pH ≥ 4.5). At pH 4.5, the measured activity of 87 U/L represented approximately 55% of the maximum activity recorded at pH 7, indicating a meaningful but acceptable reduction in enzymatic capacity. Margot et al. (2013) reports a pH optimum of 4.5 to 6.5 for *T. versicolor* laccase, confirming that pH 4.5 is within the active range for the enzyme used in the pharmaceutical degradation trial. While at the same time it is under the urease optimum pH range where only a residual activity is left (Simha 2021).

The stepwise pH adjustment was applied to minimise direct enzyme exposure to the highly acidic stored urine. Both the activity data from Experiment 1 and the inhibition mechanisms discussed in Section 5.1.1 provided rationale for avoiding prolonged enzyme exposure below pH 4.5.

An alternative stabilisation method with electrochemical H_2O_2 synthesis (Arve et al. 2024) could address this pH compromise. With this method, the pH would be maintained closer to neutral while still preventing urea hydrolysis. This could be preferable for the application of laccase and would make the pH adjustment in this study unnecessary.

5.1.3 Temperature Selection and Thermal Inactivation Trade-off

Laccase activity measured using the DMP assay increased with temperature up to 50°C, where the highest initial activity (192 U/L) was recorded. This pattern is consistent with the broad temperature optima commonly reported for fungal laccase (Baldrian 2006). A working temperature of 50°C was selected for the

pharmaceutical degradation trial because it achieved the target activity of 300 U/L with a manageable volume of laccase stock, and because operating at elevated temperature may provide an additional benefit in the context of urine-based fertiliser production, where drying temperatures could enhance laccase activity during early treatment stages.

However, a critical trade-off emerged: activity declined rapidly above 50°C, and at 50°C itself, activity measured after 5 min was lower than at 0 min, indicating progressive thermal inactivation during incubation. Experiment 8 Part 2 confirmed this pattern using the actual *T. versicolor* laccase preparation applied in the pharmaceutical degradation trial. Cumulative enzymatic exposure over 24 h remained greater at 50°C than after cooling to room temperature (20 ± 2°C) at t = 4.5 h, confirming that the higher initial activity at 50°C did not translate into sustained activity over the full incubation period. This observation agrees with previous reports for *T. versicolor* laccase (Rancaño et al. 2003; Stoilova et al. 2010; Margot et al. 2013).

A limitation of Experiment 2 is that it used commercial Biovencer laccase rather than the *T. versicolor* preparation applied in the pharmaceutical trial. Experiment 8 Part 2 addresses this uncertainty by directly measuring the activity profile of the trial enzyme under the selected incubation conditions and is therefore more relevant for assessing performance in the pharmaceutical degradation trial. Thermal stability and measured activity vary substantially between different enzyme preparations and assay methods (Rancaño et al. 2003; Han et al. 2005; Stoilova et al. 2010), underscoring the importance of empirically characterising the specific enzyme preparation under the actual assay conditions used in the study.

Whether the declining laccase activity over the 24 h incubation was sufficient to drive degradation of resistant pharmaceutical compounds is discussed in Section 5.2.

5.2 Pharmaceutical Degradation Trial

In the following section, the results of the pharmaceutical degradation trial are discussed. Section 5.2.1 addresses laccase activity dynamics during the 24 h incubation, and Section 5.2.2 presents analysis of pharmaceutical removal across the 19 compounds tested.

5.2.1 Laccase Activity During the Trial

The pharmaceutical degradation trial targeted a laccase activity of 300 U/L, based on Hultberg et al. (2025), who demonstrated 87% removal of multiple pharmaceuticals at this activity level in tertiary-treated wastewater. In that wastewater, the initial COD was 31.6 mg/L, and the total pharmaceutical load was 16 µg/L, significantly lower than in the current study with 950 µg/L and an initial

COD of 8800 mg/L. This substantially higher pharmaceutical concentration and organic load may contribute to a lower degradation efficiency. Due to dilution concerns, the laccase stock could not be increased further without excessively diluting the urine matrix, and therefore the activity level of 300 U/L was used for the pharmaceutical degradation trial.

Although activity at t = 1 h was measured at 226 U/L, the enzyme dose likely achieved activity around 300 U/L at t = 0 or shortly after, when it reached the target temperature of 50°C. Experiment 8 Part 2 supported this estimate, showing a comparable decrease in activity. Activity then declined progressively to 170 U/L at t = 6 h, indicating that most oxidative capacity was delivered during the first hours of incubation. This early-phase activity decline is consistent with the thermal inactivation profile established in Experiment 8 Part 2, which demonstrated irreversible inactivation of *T. versicolor* laccase at 50°C over time.

The apparent increase in activity at t = 24 h to 203 U/L should not be interpreted as enzyme recovery. This increase reflects an evaporation artefact due to water loss during the 24 h incubation at 50°C, which concentrated the remaining enzyme and produced artificially elevated measured activity.

Both mediator conditions (laccase-syringaldehyde and laccase-vanillin) showed substantially lower measured laccase activity than the laccase-only treatment across all time points. The mechanism driving this activity reduction cannot be resolved from the present data, as the complex treatment matrix (source-separated urine at 50°C) differs substantially from the buffer systems in which mediator effects have been previously characterised (Ashe et al. 2016). Whether the mediators were directly inactivating laccase, being preferentially oxidised themselves, or driving alternative reaction pathways remains unclear.

Whether 226 U/L at t = 1 h was sufficient to drive meaningful degradation over 24 h is compound-dependent and is discussed in Section 5.2.2.

5.2.2 Pharmaceutical Degradation Results

Interpretation of pharmaceutical removal was constrained by two factors. The untreated control condition was conducted on Day 1 and the laccase treatment on Day 2, and substantial variability was observed in several control measurements, meaning observed changes should be interpreted as apparent parent compound concentration changes rather than definitive removal. And more important only parent compounds were analysed; transformation products were not characterised. Decreases in measured parent compound concentration indicate biotransformation but do not demonstrate mineralisation or confirm that degradation products are non-toxic or non-persistent (Hultberg et al. 2025). These limitations constrain conclusions regarding the true environmental benefit of the treatment.

Sulfamethoxazole showed the clearest treatment response. Its measured concentration decreased by 46% after 24 h in the laccase treatment, while the

untreated control remained essentially unchanged at -2%, representing the most stable and interpretable result across all compounds tested. Literature confirms that laccase from *T. versicolor* can oxidise sulfamethoxazole (Alharbi et al. 2019), validating that our observed apparent transformation is realistic.

Diclofenac showed apparent transformation of 50% in laccase treatment although it is important to mention the high change in the control of -123%. Alharbi et al. (2019) showed oxidation by laccase from *T. versicolor*. Other examples show the oxidation potential through laccase; Ben Younes et al. (2019) reported laccase-mediated degradation with detoxifying capacity, and (Hultberg et al. 2025) achieved high removal using colonised mushroom substrate. The lower removal efficiency in this study may reflect competitive inhibition from the complex organic composition of source-separated urine.

Losartan, irbesartan, and furosemide also showed lower measured concentrations after laccase treatment (34%, 36%, and 32% removal, respectively), compared to substantial apparent increases in the untreated control conditions (-13%, -13%, and -25%, respectively). The anomalous control values constrain interpretation of these results. A common mechanism may explain both the control increases and the apparent laccase-driven removal: several pharmaceuticals are excreted predominantly as phase II conjugates (glucuronides and sulfates) rather than free parent compounds, and these conjugates can undergo hydrolysis at elevated temperature to regenerate the parent compound during the 24 h incubation at 50°C.

Diclofenac exemplifies this mechanism. Diclofenac is excreted predominantly as glucuronide (DCF-G) and sulfate conjugates, with little or no free unchanged diclofenac in urine (FDA 2009). Lee et al. (2012) demonstrated that DCF-G readily deconjugates to parent diclofenac through both microbial pathways and abiotic hydrolysis. During the 24 h control incubation at 50°C, thermal hydrolysis of DCF-G could regenerate free parent diclofenac, explaining the observed -123% apparent increase. The 50% removal observed in the laccase treatment likely reflects laccase-driven degradation of both the regenerated parent compound and the conjugate species themselves.

Furosemide showed a similar pattern: 32% removal in the laccase condition versus -25% in the control. Furosemide undergoes glucuronidation as a major metabolic pathway, with glucuronide conjugates present in urine alongside the parent compound (Ogata et al. 1985). Sekikawa et al. (1995) demonstrated that furosemide glucuronide is unstable in solution and undergoes thermal hydrolysis to regenerate parent furosemide. The apparent increase in the control likely reflects this hydrolysis pathway during the 24 h incubation at 50°C. Laccase treatment resulted in 32% apparent removal, though literature specifically addressing laccase-furosemide interaction is limited. This result warrants further

investigation to establish the robustness of furosemide degradation by laccase in real urine.

Losartan and irbesartan showed comparable removal in the laccase treatment (34% and 36% respectively) despite similarly anomalous control values. In contrast, Hultberg et al. (2025) observed > 87 % removal of these compounds using *T. versicolor* pellets in municipal wastewater. The substantially lower removal efficiency in this study may reflect differences in treatment conditions, including enzyme source, activity level, the higher total pharmaceutical concentration and the competitive substrate effects of the urine matrix described in Section 5.1.1 compared to the matrix used by Hultberg et al. (2025).

5.3 Outlook

The results establish that laccase can degrade selected pharmaceuticals in real source-separated urine under storage-compatible conditions but also reveal significant practical constraints to the approach. Most critically, laccase activity declined sharply with increasing urine concentration, suggesting that treatment is feasible only at early concentration stages (CF 1–2) before inhibitor burdens become prohibitive. At these early stages, most oxidative capacity is delivered during the first 6 h of incubation at 50°C, indicating that laccase treatment functions as a short-term, time-limited process rather than a prolonged standalone method. Successful application requires three operational prerequisites: active aeration to maintain dissolved oxygen availability, pH adjustment from storage pH (< 3) to working pH (4.5), and a sufficient enzyme dose to achieve meaningful removal.

Within the context of source-separated urine processing for fertiliser production (such as the Pee for the Planet initiative), laccase treatment is most plausibly positioned as an early-stage in the concentration step, applied immediately following urine acidification and during the initial stages of concentration and drying. At this point in the treatment cascade, inhibitor concentrations remain manageable, and temperature increases from subsequent drying steps may even enhance laccase activity. Treatment at this stage offers dual benefits: pharmaceutical removal occurs while nutrients are preserved for recovery, and the process duration aligns naturally with the timeline of early concentration stages rather than requiring a separate extended treatment step.

Laccase alone is unlikely to provide a complete pharmaceutical removal solution. This study demonstrates removal of some compounds, but non-phenolic classes remain resistant. The endogenous substrate competition within real urine (1.43 g/L of organics versus 950 µg/L pharmaceuticals) means that laccase's oxidative capacity is substantially consumed by matrix components, limiting its effectiveness against target contaminants. Laccase treatment should therefore be conceived as a partial removal step contributing to broader pharmaceutical

management within an integrated treatment approach. Whether this partial removal, combined with other methods such as activated carbon adsorption or subsequent advanced oxidation, can be combined with fertiliser production remains to be established.

Three factors will determine whether laccase treatment becomes practically viable for commercial urine processing systems. Firstly, the cost-benefit ratio relative to competing methods must be favourable; enzyme costs, aeration requirements, and operational complexity must be offset by avoided costs of alternative treatment approaches. Secondly, regulatory acceptance of partially transformed pharmaceutical products is essential; if degradation products are identified as equally or more persistent than parent compounds, treatment provides no environmental benefit. Thirdly, technical feasibility at scale must be demonstrated; whether aeration can be maintained effectively in large-volume systems, whether the pH-adjustment requirement is operationally practical, and whether enzyme stability can be extended beyond 6 h of activity remain open questions.

There are some research priorities emergent from this work. The most urgent one is understanding laccase selectivity in urine. A metabolomics study should clarify whether laccase preferentially oxidises endogenous organics in urine versus exogenous pharmaceutical compounds, as this selectivity directly determines the practical feasibility of pharmaceutical removal under the high organic load conditions documented in this study. Also important is the identification and toxicity assessment of transformation products formed during laccase oxidation, using bioassays to determine whether degradation products pose reduced environmental and health risk compared to parent compounds. Also the enzyme stability must be enhanced through immobilisation on carrier materials (biochar, polymers) or through fungal pellet systems that continuously produce laccase and complementary ligninolytic enzymes *in situ*, overcoming both the thermal inactivation observed here and the limited substrate range of laccase alone. And finally, the integration of laccase treatment into realistic urine-processing workflows, such as within the Pee for the Planet drying and concentration systems, is essential to bridge the gap between laboratory proof-of-concept and practical implementation at scale.

6. Conclusion

This study represents the first systematic investigation of laccase-mediated treatment of real source-separated urine. The research successfully demonstrates proof-of-concept that laccase from *T. versicolor* can maintain sufficient activity and potentially transform selected pharmaceuticals in real source separated urine under storage compatible conditions for fertiliser production.

Laccase activity could be maintained at pH 4.5, 50°C with continuous aeration. Treatment was feasible at early concentration stages (CF 1-2) before inhibitor burdens became prohibitive.

Sulfamethoxazole showed the clearest treatment response of an apparent transformation of 46% with the control remaining stable at -2%. Diclofenac also showed a high transformation of 50% though the control had a high change during the 24 h incubation of -124%. For both compounds literature confirms that laccase from *T. versicolor* can oxidise these (Alharbi et al. 2019). Three additional compounds (losartan, irbesartan, furosemide) showed apparent removal with anomalous controls, and literature specifically addressing laccase oxidation of these compounds with purified laccase is lacking.

The addition of syringaldehyde or vanillin did not consistently enhance pharmaceutical degradation under the conditions tested, contradicting the original hypothesis. Whether alternative mediator concentrations, timing of addition, or different urine treatment conditions could yield more favourable results remains unknown and needs further investigation.

Laccase is a promising candidate for systems seeking to address pharmaceutical contamination in urine recycling, provided that future research resolves the product safety question and demonstrates practical scalability.

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Popular science summary

That a valuable resource should be diluted and then discarded in an era where resource recovery is more urgent than ever is, on reflection, not logical. Yet this happens every time a toilet is flushed, and urine enters the mixed wastewater stream. The recovery of this resource creates challenges that need to be addressed. For example, urine carries most of the pharmaceutical residues excreted by the human body, and these must be dealt with.

This study investigated whether laccase, an enzyme produced by fungi such as Turkey tail (*Trametes versicolor)*, could help remove pharmaceutical micropollutants from real, source-separated human urine. Unlike many conventional treatment methods, enzymatic treatment could offer a milder approach that preserves valuable nutrients.

The study first examined how pH, temperature, urine concentration, acidification, and oxygen availability (oxygen is the only cofactor which the enzyme needs to degrade pharmaceuticals) affect laccase activity. Then a mixture of 19 pharmaceuticals was dosed into urine and treated with laccase.

The results demonstrated that multiple compounds were transformed by laccase, providing proof-of-concept that enzymatic treatment is viable in real urine. Sulfamethoxazole, for example, showed 46% removal after 24 hours of treatment, while several other pharmaceuticals achieved 32–50%. This also identified which pharmaceutical structures are susceptible to laccase oxidation, guiding selection of candidates for future optimisation studies.

Further research is needed to improve enzyme stability, reduce treatment requirements, and test the process at larger scales. In the long term, enzymatic treatment could contribute to safer urine recycling by combining pharmaceutical removal with nutrient recovery.

Appendix 1 Certificate of Analysis Laccase

Biovencer

Biovencer Healthcare Private Limited

(Leading Contract Manufacturer of Advance Nutraceutical Products - GMP & ISO Certified)

Reg. Office - K-230, Site-V, Industrial Area, Kasna, Greater Noida, G.B. Nagar, Uttar Pradesh 201308

CIN - U74999UP2016PTC087050, **GSTIN -** 09AAGCB9232J1ZE, **PAN -** AAGCB9232J

CERTIFICATE OF ANALYSIS

Product Name	**Laccase Enzyme Powder**	**Mfg Date**	**Aug 2024**
Date	**02/11/2024**	**Exp Date**	**36 months**
Batch number	**BI/24/LCE116**	**Packing**	**Pouch**

Details Of Analysis report

Items	Specification	Results	Qualified
Physical & Chemical Analysis			
Description	White Powder, Slight fermentation	White Powder, Slight fermentation	Qualified
pH	4 – 7	5.15	Qualified
Activity			
Laccase Enzyme activity	≥2000 U/g	Complies	Qualified
Microbiology test			
Aerobic bacterial count cfu/g Escherichia coli cfu/g(ml) Salmonella 25 g 25(ml)	≤ 5.0 X 10^1 ≤ 10 Not detectable	≤ 5.0 X 10^2 ≤ 10 Negative	Qualified
Heavy metals			
Lead Arsenic	≤ 2.0 ≤2.0	Complies Complies	Qualified

Declaration: Products complies with above specification.
Instructions:

1) During the process, the product must be kept under controlled conditions
2) The shelf life of finished product is dependent on excipient, process and storage conditions.

	Prepared By	Verified By	Checked by
Name	AMIT KUMAR	SUNIL PAL	PUSHPA ADHIKARI
Signature			
Date	11/2024	11/2024	11/2024

Manufacturing Units

K-230, Site-V, Industrial Area, Kasna, Greater Noida, G.B. Nagar, Uttar Pradesh 201308 | K-232, Site-V, Industrial Area, Kasna, Greater Noida, G.B. Nagar, Uttar Pradesh 201308 | Plot No. - 114, NSEZ, Phase-II, Noida, UP - 201305 | Plot No. : G-14, Site-V, Industrial Area, Kasna, Greater Noida, G.B. Nagar, Uttar Pradesh 201308 | Plot No. - 181, NSEZ, Phase-II, Noida, UP - 201305 | K-436, Site-V, Industrial Area, Kasna, Greater Noida, G.B. Nagar, Uttar Pradesh 201308

Contact No. : +91-9650418220, +91- 120-234 1080, **Email :** info@biovencer.com, **Website :** www.biovencer.com

Appendix 2 Dilution Factors and Sample Compositions

Table 1. Sample codes, target pH values, and dilution factors for Experiment 1 (pH optimisation). Each sample consisted of acidified pooled urine adjusted to the target pH with sulfuric acid (pH 1) or NaOH (pH 2–11), followed by addition of commercial-grade laccase stock solution (Biovencer Healthcare Private Limited). Dilution factors reflect the volume of laccase stock and NaOH added relative to the total sample volume.

Sample Code	Composition	Dilution Factor
I	Urine with laccase stock (pH 1)	1.20
II	Urine with laccase stock (pH 2)	1.22
III	Urine with laccase stock (pH 3)	1.23
IV	Urine with laccase stock (pH 4)	1.24
V	Urine with laccase stock (pH 5)	1.24
VI	Urine with laccase stock (pH 6)	1.24
VII	Urine with laccase stock (pH 7)	1.25
VIII	Urine with laccase stock (pH 8)	1.26
IX	Urine with laccase stock (pH 9)	1.25
X	Urine with laccase stock (pH 10)	1.26
XI	Urine with laccase stock (pH 11)	1.26

Table 2. Sample codes, actual concentration factors (CF), and measured laccase activity (U/L) for Experiment 3 (concentration factor experiment). Acidified pooled urine was evaporated at 50 °C to the target CF before addition of commercial-grade laccase stock solution (Biovencer Healthcare Private Limited) and pH adjustment to 4.5 with NaOH. Distilled water controls (B I and B II) were measured at the start and end of the experiment to confirm stability of the laccase stock. Actual CF values are lower than nominal target values due to dilution by laccase stock and NaOH addition. Activity was measured at room temperature by the DMP assay.

Sample	Composition	Actual CF	Activity U/L
B I	Distilled water + laccase stock (start control)	–	301
U I	Acidified urine + laccase stock	0.99	205
U II	Acidified urine + laccase stock	1.96	119
U III	Acidified urine + laccase stock	3.84	38
U IV	Acidified urine + laccase stock	4.75	23
U V	Acidified urine + laccase stock	9.04	0.00
B II	Distilled water + laccase stock (end control)	–	301

Table 3. Sample codes, acid type, acid concentration, volume of acid added, and measured laccase activity (U/L) for Experiment 4 (acid type comparison). Each sample consisted of 30 mL acidified pooled urine adjusted to pH 3.9 using the indicated acid, followed by addition of commercial-grade laccase stock solution (Biovencer Healthcare

Private Limited) and readjustment to pH 4.5 with NaOH. Activity was measured at room temperature by the DMP assay.

Sample	Acid	Concentration	Volume (mL)	Activity (U/L)
U I	Sulfuric acid	99%	0.05	134
U II	Phosphoric acid	3 M solution	0.34	131
U III	Oxalic acid	2 M solution	0.32	136
U IV	Citric acid	3 M solution	0.30	130
U V	Acetic acid	96%	0.48	107

Table 4. Sample codes, laccase type, volumes of urine, laccase stock, acetate buffer, and NaOH added, and resulting dilution factors for Experiment 6 Part 1 (laccase type and stock volume comparison). All samples contained 30 mL of acidified pooled urine at pH 4.5. Commercial-grade laccase (Biovencer Healthcare Private Limited) and lab-grade laccase from T. versicolor (Sigma-Aldrich) were compared at a total added volume of 6 mL (laccase stock plus acetate buffer) to achieve a consistent dilution factor of 1.2 across all samples.

Sample Code	Laccase Type	Urine (mL)	Stock added (mL)	Acetate Buffer added (mL)	NaOH added (mL)	Dilution Factor
U I	(commercial-grade)	30	6	0	0.07	1.2
U II	(lab-grade)	30	2.40	3.60	0.06	1.2
U III	(lab-grade)	30	2.70	3.30	0.06	1.2
U IV	(lab-grade)	30	2.75	3.25	0.07	1.2
U V	(lab-grade)	30	2.80	3.25	0.07	1.2

Appendix 3 Preliminary Activity Drop Experiment

A preliminary experiment was conducted under identical conditions to Experiment 7 Part 2 to assess whether cooling a bottle from 50 °C to room temperature (20 ± 2 °C) could preserve residual enzyme activity. A bulk of 283.8 mL urine was prepared with 46.2 mL of lab-grade laccase stock solution and a total NaOH addition of 0.41 mL, giving a dilution factor of 1.16. One bottle was removed from the oil bath at t = 14 h and allowed to equilibrate to RT. Activity was measured at t = 16 and t = 19 h in the cooled bottle.

Table 1. Laccase activity (U/L) measured by the DMP assay over 24 h in the preliminary activity drop experiment. One bottle was incubated at 50 °C throughout; a second bottle was removed from the oil bath at t = 14 h and allowed to equilibrate to room temperature (50 °C cooled). A room temperature control bottle was maintained throughout. Two activity measurements were taken at each time point for the 50 °C condition and are reported separately. No measurements were taken in the cooled bottle before t = 16 h, and no measurements were taken in the 50 °C bottles after t = 14 h before the final t = 24 h reading.

Time (h)	20 °C Control (U/L)	50°C first Measurement (U/L)	50°C second Measurement (U/L)	50°C cooled (U/L)
0	116			
1	115	282	271	
2	124	276	262	
3	124	216	228	
4	127	191	207	
14	95	87	87	
16				21
19				21
24	100	62	63	

Appendix 4 Sampling Time Points and Sample Codes

Table 1. Sampling time points and sample codes for all conditions in the pharmaceutical degradation trial. Samples were collected at t = 0, 1, 6, and 24 h, where applicable. Numbers in parentheses (1–3) denote the three analytical replicate bottles per condition. A NM (not measured) indicates that no sample was collected at that time point for that condition.

Condition	t = 0 h Code = -	t = 1 h Code = A	t = 6 h Code = B	t = 24 h Code = C
Control (Day 1)	U	NM	NM	CU (1-3)
Code = U	(1-3)			
Laccase only (Day 2)	L	A L	B L	C L
Code = L	(1-3)	(1-3)	(1-3)	(1-3)
Laccase + Vanillin (Day 2)	NM	NM	B LV	C LV
Code = LV			(1-3)	(1-3)
Laccase + Syringaldehyde (Day 2)	NM	NM	B LS	C LS
Code = LS			(1-3)	(1-3)

Appendix 5 Laccase Activity Comparison (Experiment 7)

Table 1. Laccase activity (U/L) measured at room temperature by the DMP assay for commercial-grade (Biovencer Healthcare Private Limited) and lab-grade (Trametes versicolor, Sigma-Aldrich) laccase at varying stock volumes added to 30 mL of acidified pooled urine (pH 4.5). Acetate buffer was added to lab-grade samples to equalise the total added volume to 6 mL across all samples.

Sample	Enzyme Source	Stock Vol. Added (mL)	Acetate Buffer Added (mL)	NaOH Addition (mL)	Dilution factor	Activity (U/L)
U I	Commercial (Biovencer)	6	0	0.07	1.2	126
U II	Lab-grade (Sigma-Aldrich)	2.4	3.6	0.06	1.2	98
U III	Lab-grade (Sigma-Aldrich)	2.7	3.3	0.06	1.2	115
U IV	Lab-grade (Sigma-Aldrich)	2.75	3.25	0.07	1.2	111
U V	Lab-grade (Sigma-Aldrich)	2.8	3.2	0.07	1.2	133

Table 2. Laccase activity (U/L) measured at room temperature (20 ± 2 °C) and at 50 °C (0 and 5 min incubation) for increasing volumes of lab-grade laccase stock (Trametes versicolor, Sigma-Aldrich) added to 30 mL of acidified pooled urine (pH 4.5) in Experiment 6 Part 2. Activity was measured by the DMP assay.

Sample	Stock Vol. Added (mL)	(20 ± 2 °C) (U/L)	50 °C 0 min (U/L)	50 °C 5 min (U/L)
U I	1	67	242	223
U II	1.2	80	274	257
U III	1.4	97	310	286
U IV	1.6	113	323	298

Appendix 6 Analysis of Urine

Table 1. Individual measurements of selected physicochemical parameters in the pooled source-separated urine used in this study. Ammonia, calcium, chloride, potassium, magnesium, phosphate (PO_4), sulphate (SO_4), and urea were measured in triplicate (M-1, M-2, M-3) using a Gallery Discrete Analyser (Thermo Fisher). COD was measured in duplicate using a Thermo Fisher COD Cell Test photometric method. A dash (-) indicates that the measurement could not be obtained. All values in mg/L.

Measurement	Test Name	Compound	mg/L
M-1	Ammonia PS	Ammonia	234.5
M-2	Ammonia PS	Ammonia	254.4
M-3	Ammonia PS	Ammonia	251.7
M-1	Ca PS New	Calcium	98.6
M-2	Ca PS New	Calcium	94.0
M-3	Ca PS New	Calcium	96.6
M-1	ChloridePS	Chloride	3825.6
M-2	ChloridePS	Chloride	3741.2
M-3	ChloridePS	Chloride	3848.1
M-1	K-TEST	Potassium	2408.7
M-2	K-TEST	Potassium	2338.3
M-3	K-TEST	Potassium	2327.9
M-1	Mg PS	Magnesium	48.8
M-2	Mg PS	Magnesium	45.9
M-3	Mg PS	Magnesium	49.3
M-1	PO4 PS	Phosphate (PO_4)	457.9
M-2	PO4 PS	Phosphate (PO_4)	430.1
M-3	PO4 PS	Phosphate (PO_4)	450.1
M-1	SO4 PS	Sulphate (SO_4)	2589.0
M-2	SO4 PS	Sulphate (SO_4)	2517.3
M-3	SO4 PS	Sulphate (SO_4)	2512.2
M-1	UreaPS	Urea	10017.5
M-2	UreaPS	Urea	9900.4
M-3	UreaPS	Urea	-

Table 2. Summary statistics for the physicochemical composition of the pooled source-separated urine. Values are the mean and sample standard deviation of triplicate measurements (n = 3), except for urea (n=2).

Compound	Average mg/L	Std. dev.
Ammonia PS	246.9	8.8
Calcium	96.4	1.9
Chloride	3805.0	46.0
Potassium	2358.3	35.9
Magnesium	48.0	1.5
PO4	446.0	11.7
SO4	2539.5	35.1
Urea	9959.0	58.6

Chemical oxygen demand (COD) of the pooled urine was measured in duplicate using a Thermo Fisher COD Cell Test photometric method, yielding values of 8940 mg/L and 8660 mg/L (mean 8800 mg/L).

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