Biodegradation of fluoroquinolone antibiotics by laccase enzyme extracted from spent mushroom waste (SMW) of *Pleurotus florida* and its immobilization on biochar derived from SMW: a sustainable approach

A thesis submitted

by

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In partial fulfillment of the requirements for the award of the degree of

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STATEMENT

I, the undersigned, hereby declare that the research embodied in this thesis entitled "**Biodegradation of fluoroquinolone antibiotics by laccase enzyme extracted from spent mushroom waste (SMW) of *Pleurotus florida* and its immobilization on biochar derived from SMW: a sustainable approach**" is the result of experiments carried out at the School of Agro & Rural Technology, Indian Institute of Technology Guwahati, India, under the supervision of Prof. Sudip Mitra.

In keeping with the general practice of reporting scientific observations, due acknowledgments have been made wherever the work described is based on the finding of other's research.

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CERTIFICATE

This is to certify that the work described in this thesis entitled **"Biodegradation of fluoroquinolone antibiotics by laccase enzyme extracted from spent mushroom waste (SMW) of *Pleurotus florida* and its immobilization on biochar derived from SMW: a sustainable approach"** by Anamika Ghose (Roll No. 196154003) for the award of degree of Doctor of Philosophy is an authentic record of the result obtained from the research work carried out under my supervision at the School of Agro & Rural Technology, IIT Guwahati. The work carried out in this thesis has not been submitted elsewhere for a degree.

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Abbreviations

ABTS = 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS)
ANOVA = Analysis of variance
ARBs = Antibiotic resistant bacteria
ARGs = Antibiotic resistant genes
BET = Brunauer-Emmett-Teller
CETP = Common effluent treatment plants
CNT = Carbon nano tubes
COD = Chemical oxygen demand
CTAB = Cetrimonium bromide
DTT = Dithiothreitol
EDCs = Endocrine disrupting chemicals
EDG = Electron donating groups
EDS= Energy-dispersive X-ray spectroscopy
EDTA= Ethylenediamine tetraacetic acid
ET= Electron transfer
EWG= Electron withdrawing groups
FESEM= Field emission scanning electron microscopy
FQs= Fluoroquinolone
FTIR= Fourier-transform infrared spectroscopy
HAT= Hydrogen atom transfer
HBT= 1 hydroxybenzotriazole
HPI= N-hydroxyphthalimide
HPLC= High-performance liquid chromatography
IE= Immobilization efficiency
IY= Immobilization yield
LB= Luria Bertani
LC-MS= Liquid chromatography mass spectrometry
LiP= Lignin peroxidase
MnP= Manganese peroxidase
NH_2= Amine
NHA= N-hydroxyacetalimide
OH= Hydroxyl
OMPs= Organic micropollutants
POPs= Persistent organic pollutants (POPs)
PPCPs= Pharmaceutically persistent personal care products
ROS= Reactive oxygen species
SA= Syringaldehyde
SDS-PAGE= Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMW= Spent mushroom waste
STPs= Sewage treatment plants
TDS = Total dissolved solids

TGA= Thermogravimetric analysis
TNC= Trinuclear cluster
VA= Veratryl alcohol
VLA= Violuric acid
VP= Versatile peroxidase
WRF= White-rot fungi
WWTPs= Wastewater treatment plants
ZOI= Zone of inhibition

Units

µg mL^{-1}= Microgram per millilitre
µl= Microlitre
µM= micromole
cm= Centimetre
cm^{-1}= Wavenumber
°C= Degree centigrade
Df= Dilutions factor
EU= Enzyme dosage
H= Hour
J mol^{-1}= Joule per mole
kDa = Kilo dalton
m^2g^{-1}= Metre square per gram
mg g^{-1}= Milligram per gram
mg mL^{-1}= Milligram per millilitre
mg= Milligram
mL= Millilitre
mM= Millimolar
nm= Nanometre
rpm= Revolutions per minute
Ug^{-1}= Unit per gram
UL^{-1}= Unit per litre
Umg^{-1}= Unit per microgram
UmL^{-1}= Unit per millilitre
U= Unit
w/v= weight by volume

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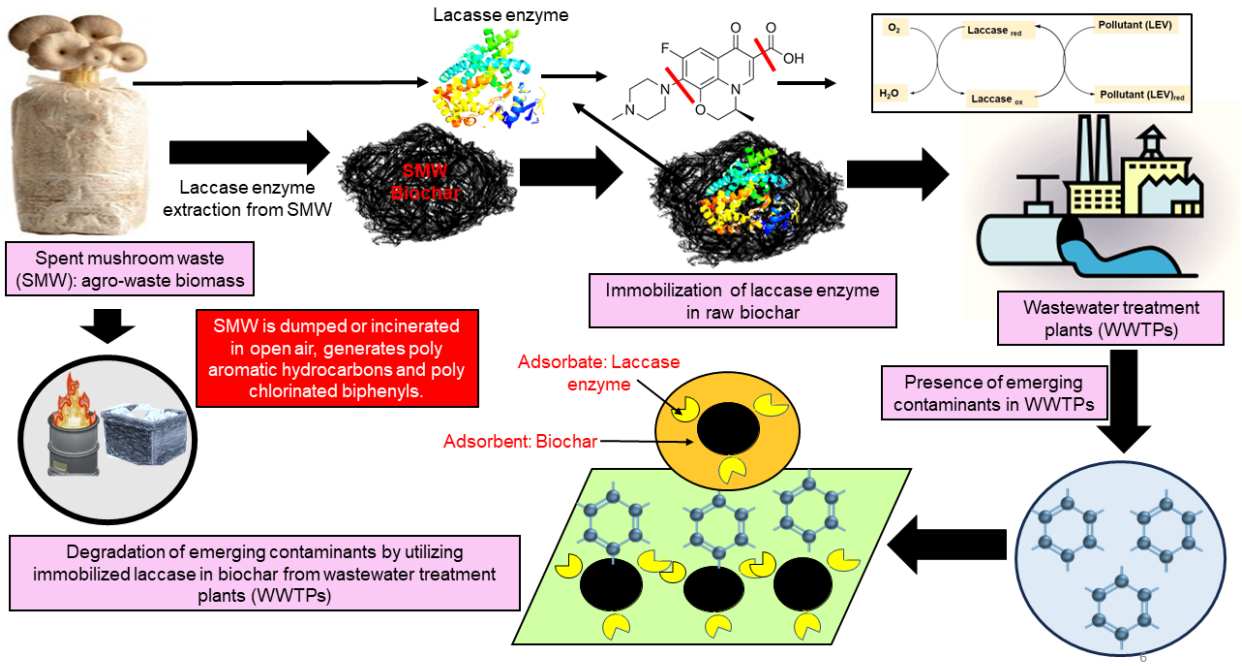
Abstract

It is crucial to develop urgent and innovative strategies for removing persistent organic micropollutants (OMPs) from soil, groundwater, and surface water. Antibiotics commonly found in contaminated soils and wastewater treatment plant effluents are classified as environmentally persistent pharmaceutical pollutants (EPPPs). These pollutants pose serious ecological threats to non-target species. Since conventional wastewater treatment plants (WWTPs) cannot completely eradicate these pollutants, the excessive use of antibiotics has a negative impact on the environment's ability to regenerate and recover. In recent years, various water bodies such as surface water, tap water, groundwater, common wastewater treatment plants, and rivers persist fluoroquinolone antibiotic residues specifically ciprofloxacin, levofloxacin, and norfloxacin. To address this pressing issue, it is essential to develop alternative, sustainable remediation strategies that can effectively degraded theses pollutants. This study explores an innovative approach to antibiotic bioremediation using spent mushroom waste (SMW), a type of agro-waste. SMW, a byproduct of mushroom cultivation, is rich in ligninolytic enzymes like laccase and peroxidase, which facilitate the biodegradation of complex organic pollutants. The tri-nuclear cluster of copper ions in the laccase enzyme has excellent oxidizing capacity for organic and inorganic compounds. However, its low reduction potential limits its ability to oxidize non- phenolic compounds. To address this limitation, small-sized mediators (both synthetic and natural mediators) are used to bind to the enzyme's active site, reducing steric hindrance. By adjusting the reaction conditions like pH, temperature, and mediator concentration, this study aims to optimize laccase output from *Pleurotus florida* SMW.

The biodegradation of OMPs relies heavily on optimal reaction conditions and the presence of susceptible functional groups. This study focuses on the degradation of fluoroquinolone antibiotics with and without a synthetic mediator. By adjusting the parameters mentioned above, we aim to achieve higher levels of degradation. While free enzymes are prone to denaturation in environmental conditions, immobilization is a technique that involves fixing enzymes in a solid support. In this study, a biochar-based technique for removing antibiotics from wastewater by improving the immobilization of enzymes through adsorption was attempted. The research focuses on identifying parameters that can facilitate the process of adsorptive immobilization. Specifically, we explored the immobilization of laccase on raw biochar surface through an adsorption process, taking into account parameters such as pH (3-6), temperature (30-50 ℃), and

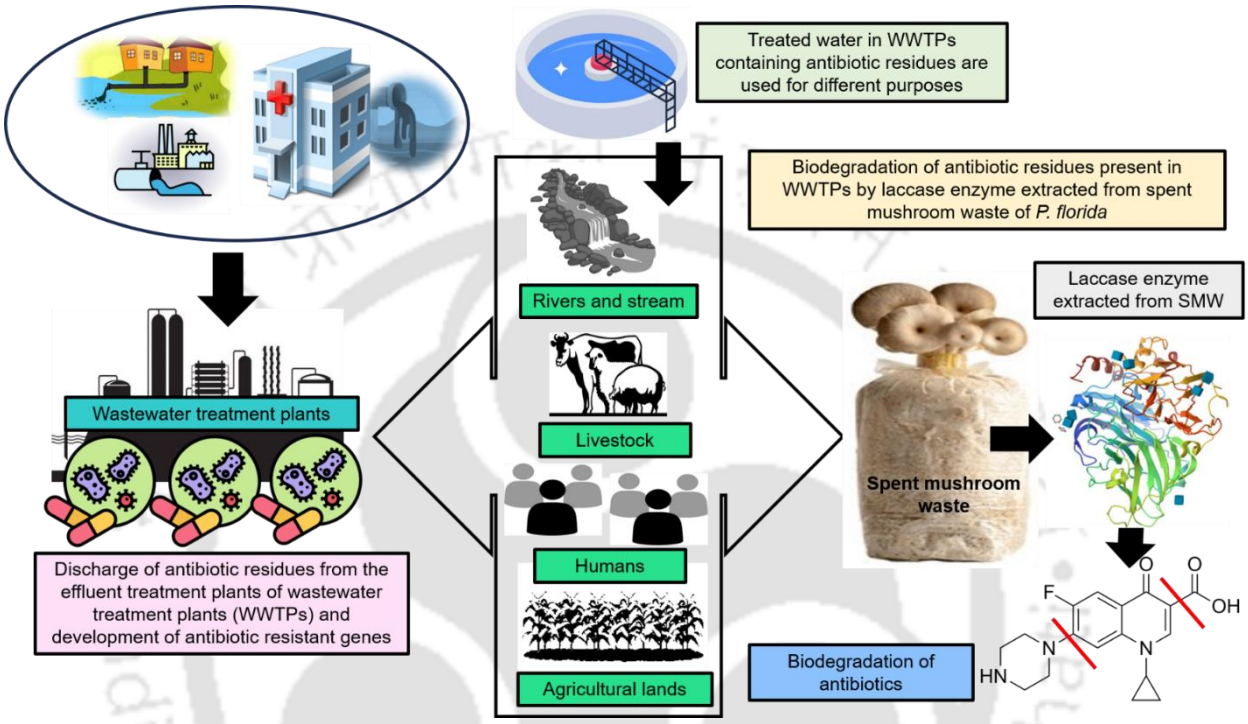
enzyme dosage (1-5 U mL^{-1}). Our findings demonstrate that immobilizing laccase on SMW biochar enhances its productivity and stability. This was achieved at pH 4.5, temperature 30 ℃ and enzyme dosage (5 U mL^{-1}). Additionally, it was observed that the relative activity of laccase immobilized biochar was retained up to 65% after 8 consecutive cycles of 2, 2′-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS). This approach provides a promising alternative to conventional adsorption technologies in WWTPs for OMP remediation. This biochar-immobilized enzyme system provides a sustainable, agro-waste based approach with potential real-world applications in WWTPs, where it could help address the challenges of persistent pollutants, ultimately contributing to environmental restoration and ecological health.

Keywords: Antibiotics; Bioremediation; Fluoroquinolone; Laccase; Organic micropollutants; Pleurotus florida; Spent mushroom waste.



CHAPTER 1

Introduction



"This chapter introduces presence of antibiotic residues in wastewater treatment plants, achieving biodegradation of antibiotic residues by utilizing laccase enzyme extracted from spent mushroom waste (SMW) of Pleurotus florida. It also discusses the specific objectives and structure of the thesis."

1. Introduction

1.1. Background

The pharmaceutical industry has emerged as a significant contributor to ecological crisis due to the production and disposal practices that lead to environmental contamination. Antibiotics, particularly fluoroquinolones, known for their stability and resistance to natural degradation, being detected in water bodies worldwide (Phoon et al., 2020). Their persistence in ecosystems disrupts microbial balance, harms aquatic life, and contributes to a public health crisis by fostering antibiotic-resistant bacteria (Guo et al., 2018). This issue is particularly pronounced in regions where wastewater treatment plants (WWTPs) are inadequately equipped to remove high concentrations of these contaminants, as observed in studies from India, which reveal significant levels of fluoroquinolones like ciprofloxacin in wastewater effluents (Larsson et al., 2007). Consequently, antibiotic resistant genes were found to be significantly higher in pharmaceutical effluent treatment plant (2.3×10^{7} copies mL^{-1}) compared to municipal wastewater (9.50×10^{5} copies mL^{-1}) (Guo et al., 2018). This indicates that current treatment technologies are not adequate to eliminate antibiotic residues from the wastewater treatment plants (WWTPs). Therefore, the problem is acknowledged and a solution is proposed that is environmentally friendly, sustainable, cost-effective and has the potential of biodegradation of antibiotics without producing secondary pollutants.

Ligninolytic enzymes (laccase, versatile peroxidase, manganese peroxidase, and lignin peroxidase) present in spent mushroom waste (SMW) have outspread applications in remediating persistent organic micropollutants (OMPs), antibiotics, heavy metals, inorganic pollutants, dye decolorization, paper pulp delignification and bleaching (Ghose and Mitra, 2022). Laccase is a particularly effective, eco-friendly solution for degrading recalcitrant pollutants due to its ability to catalyze the oxidation of a broad range of organic and inorganic compounds. Although laccase has a low redox potential and cannot oxidize non-phenolic compounds, redox mediators enhance its versatility, acting as electron shuttles that facilitate the detoxification of various recalcitrant substrates (Ghose and Mitra, 2022). The electron-transfer route refers to the process by which electrons are transferred from a donor molecule (the substrate or mediator) to an acceptor molecule (often a part of the enzyme or another chemical species) during a biochemical reaction (Baiocco et al., 2003). Synthetic mediators like ABTS follow an electron-transfer route, while others, such as HBT and violuric acid

(VLA), use hydrogen atom transfer, expanding the range of substrates laccase can degrade (Baiocco et al., 2003, Ghose et al., 2023). However, synthetic mediators carrying nitroxy radicals like 1 hydroxybenzotriazole (HBT), violuric acid (VLA), N-hydroxyphthalimide (HPI) and, N-hydroxyacetalimide (NHA) oxidizes substrate by hydrogen atom transfer action. Concurrently, the imbibe activity of laccase in oxidizing mediators, which diffuses from the laccase enzymatic pocket due to its small size, influences the degradation of substrates most significantly. However, the affinity of laccase specific redox potential is crucial to the substrates electron-withdrawing and electron-donating groups (Guardado et al., 2019). Therefore, the availability of these economical, environmentally friendly mediators ramps up the catalytic action of laccase enzyme in sustainable processes.

However, the practical application of laccase is limited by its stability and reusability. Enzyme immobilization on suitable supports can enhance its activity and resilience to environmental factors such as pH stability, thermal stability, and reusability (Bernal et al., 2018; Wang et al., 2014). Materials such as celite, activated carbon, and charcoal are commonly used as immobilization supports.

Biochar is an emerging carbonaceous material with promising agricultural and environmental uses; it is mostly made from economical biomass leftovers. There has been substantial use of biochar in the remediation of polluted water because of its huge surface area, developed porous structure, and abundance of surface functional groups, which make it an effective and inexpensive adsorbent (Chauhan et al., 2023; Jin et al., 2021). However, to increase surface area of biochar, the surface of biochar is modified with chemical reagents which leads to production cost and leaching out of chemicals (Imam et al., 2021; Pandey et al., 2022). Thus, the need of an hour is to find unmodified or raw biochar for adsorption. Laccase molecules are immobilized onto the surface of raw biochar through van der Waals forces, ionic bonds and hydrogen bonds (Wang et al., 2022). Additionally, the synergistic effect of biochar's adsorptive properties and laccase's catalytic activity enables the degradation of a wide range of recalcitrant pollutants, which may include pharmaceuticals, dyes, and heavy metals (Wu et al., 2019). Therefore, by utilizing biochar as a support material, combines economic feasibility with environmental sustainability, presenting a viable solution for large-scale applications in wastewater treatment. This strategy not only addresses the challenge of antibiotic contamination in pharmaceutical wastewater but also paves the way for

broader applications in environmental remediation, making it a valuable tool in combating pollution from diverse industrial sources.

1.2. Gap of Knowledge:

1. The degraded products generate secondary pollution in conventional and advanced water treatment technologies. Prior to discharge, efficient treatments should be developed to remove degraded products.
2. The concentration of research on understanding the toxicity of degraded products is limited. Hence, more research should be concentrated to study the residual toxicity of the organic micropollutants generated after degradation.
3. Operational stability and applicability of the immobilized system is needed to be studied for potential removal of organic micropollutants from wastewater and to eventually scale them up for industrial applications.
4. The commercial laccase enzyme, made from whole fungal culture cells, is significantly more costly and has lower enzymatic activity. Finding cost-effective enzyme sources is, hence, essential.

1.3. Hypothesis

1. Spent mushroom waste contains extracellular laccase enzyme which may have more enzymatic activity than commercially available pure laccase extracted from whole fungal culture cells.
2. Free laccase enzyme instability can be enhanced by immobilizing it into support material. The immobilization of laccase into support material can enhance its productivity, applicability, reusability, and cost-effectiveness.
3. Carbonaceous material, specifically biochar has wider surface area and porosity. It is therefore hypothesized that the enzyme immobilized in biochar will be an efficient and cost-effective alternative for removal of antibiotics from wastewater.

1.4. Objectives of the study

The key objectives of this study are to:

i. To carry out extraction of laccase enzyme from spent mushroom waste (SMW) of *Pleurotus florida* under various operating conditions and its assay.

ii. To evaluate the potential of laccase enzyme in biodegrading fluoroquinolone antibiotics (ciprofloxacin, levofloxacin and norfloxacin) present in water.

iii. To immobilize laccase onto raw SMW biochar and determine immobilization yield and efficiency.

iv. To assess the efficiency of immobilized laccase in biodegrading fluoroquinolone antibiotics and its operational stability.

1.5. Overview of thesis organization

The thesis is organized into seven different chapters (**Fig. 1.1**). Each objective is purposefully aligned to advance the central goal of developing a sustainable, enzyme-based solution for the degradation of antibiotic residues in wastewater, addressing existing treatment limitations while reducing environmental and ecological risks. The first chapter of the thesis consists of a brief discussion on the problems and challenges for elimination of antibiotic residues from WWTPs. The prevalence of antibiotic residues in groundwater, surface water, and wastewater treatment facilities is a pressing issue, and it suggests that improved treatment technologies fail to address this issue. Enzymatic biodegradation of antibiotic residues is a promising, sustainable, and one-pot solution to the existing problem of WWTPs.

The second chapter covers the detailed review of literature on existence of fluoroquinolone antibiotics in India (surface water, effluent treatment plants and rivers). It also deals with the previous studies, background information, and existing research gaps. However, this study primarily focuses on fluoroquinolone antibiotics, and it is essential for future research to explore the applicability of this method to other classes of antibiotics. The third chapter focuses on the first objectives and details the extraction of enzyme specifically laccase from spent mushroom waste (SMW) of *P. florida*. It also discusses significance of pH, synthetic mediator concentration (ABTS) and solid: solution ratio in obtaining maximum laccase activity. Further, it focuses on stability check (pH, temperature, presence of metal ions and inhibitors) of free laccase enzyme.

Concerning, the problem stated in first chapter (i.e. biodegradation of antibiotics from wastewater treatment plants) fourth chapter deals with achieving maximum degradation of fluoroquinolone antibiotics (ciprofloxacin, levofloxacin and norfloxacin) from synthetically designed wastewater and determining the fate of degraded products generated after degradation mediated by laccase. In addition, this chapter also deals with eco-toxicological assessment of degraded products. Further, in accordance with the problem

discussed in first chapter regarding free laccase enzyme instability at different environmental conditions.

The fifth chapter discusses the significance of immobilizing laccase enzyme in support material that is produced from the SMW of *P. florida* underscoring the need to assess the scalability and cost effectiveness of using biochar for potential industrial applications. The use of SMW and its biochar as materials for antibiotic degradation is a highly sustainable and efficient approach. As an agricultural byproduct, SMW provides an abundant, low-cost source of laccase, converting waste into a valuable resource and minimizing the need for synthetic or expensive enzyme alternatives. When processed into biochar, SMW offers a natural, biodegradable support that enhances enzyme stability and reusability across varied environmental conditions. This approach not only reduces environmental impact but also presents a scalable, eco-friendly solution that surpasses conventional, resource-intensive methods. Moreover, this chapter also deals with obtaining optimal reaction conditions to obtain maximum immobilization efficiency and yield. Further, immobilization of enzymes in support material (raw biochar) was confirmed by instrumental analysis. In the sixth chapter, the degradation of fluoroquinolone antibiotics (ciprofloxacin, levofloxacin and norfloxacin) was obtained by immobilized laccase in the support material (raw biochar). The maximum degradation was achieved by tweaking parameters such as pH, synthetic mediator concentration (ABTS), and temperature. Lastly, the seventh chapter summarizes the objectives and discusses futures prospects. The findings of this study have significant implications for environmental policy, advocating for the integration of sustainable wastewater treatment practices in regulatory frameworks to manage antibiotic residues and reduce environmental contamination. This supports policies aimed at mitigating pharmaceutical pollution and promoting biodegradable materials in treatment processes. In terms of public health, the research emphasizes the need to minimize antibiotic contamination in water systems to combat the spread of antibiotic-resistant bacteria, thereby improving community health and water quality. For industrial applications, the study highlights the potential for wastewater treatment facilities to adopt enzyme-based solutions as a sustainable alternative to conventional methods. This approach not only enhances operational efficiency but also aligns with sustainability goals and regulatory compliance, fostering greener industrial practices.

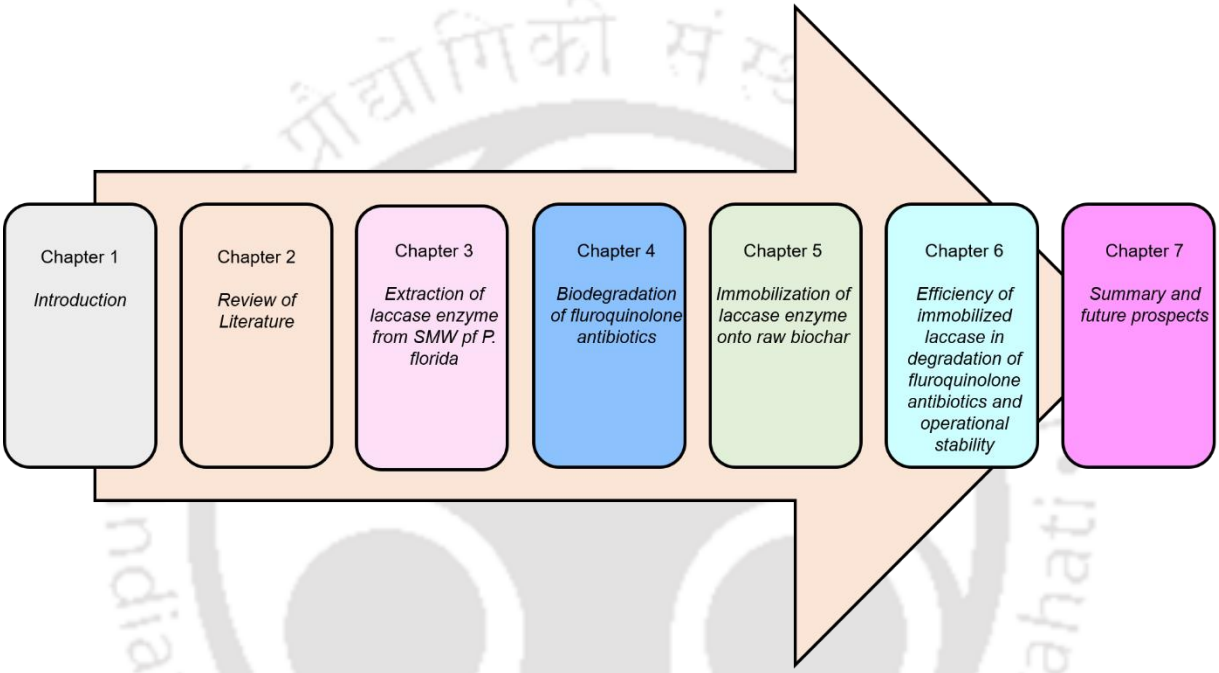


Fig. 1.1. Outline of thesis chapters.

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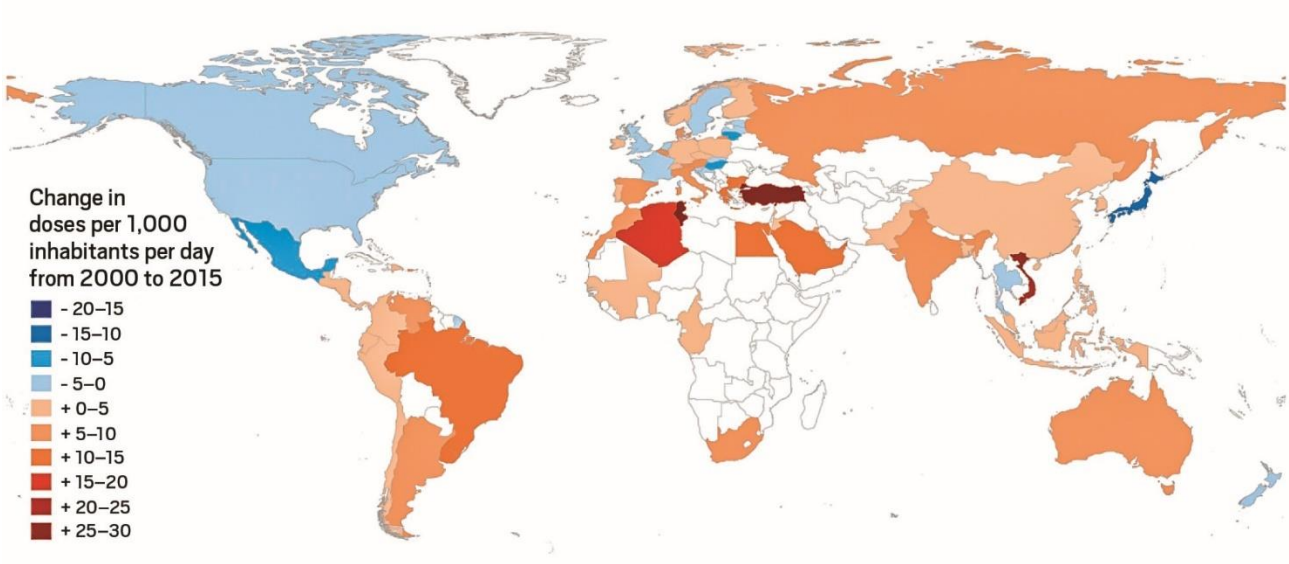
CHAPTER 2

Review of Literature



Consumption of antibiotics at global scale (Klein et al.,2018)

"*This chapter includes a comprehensive and in-depth review of literature on the current status of the antibiotic residues present in the wastewater treatment plant across the world and India. It further intricates the challenges of advanced treatment technologies in the complete removal of antibiotic residues from wastewater treatment plants. This chapter also focuses on a one-pot solution for the biodegradation of antibiotic residues by immobilizing laccase enzyme onto raw biochar and determining its operational stability.*

2. Review of literature

2.1. Emergence of organic micropollutants in wastewater

The global escalation of environmentally emerging organic micropollutants (OMPs) pose an indisputable threat to human lives and aquatic ecosystems. Besides the fact that their presence is ranged ng L^{-1} to µg L^{-1} their implications on vitality, life span, and reproductive success lacked documentation, as sensitive analytical procedures to detect OMPs were previously unavailable (Sathishkumar et al., 2021). They are now recognized as a wide and varied group of chemicals generated from natural and anthropogenic substances that pose significant risks to non-target species, including aquatic animals and humans, engendered by their unpredictable interaction with each other in the wastewater treatment plants (WWTPs) (Sathishkumar et al., 2020). The reason behind the presence of these OMPs in wastewater is lack of discharge regulations and standard effluent criteria (Kanaujiya et al., 2019). In addition, OMPs boost the generation of antibiotic-resistant genes (ARGs) and antibiotic-resistant bacteria (ARBs) in our drinking water and freshwater resources, which pose a grave concern for public health.

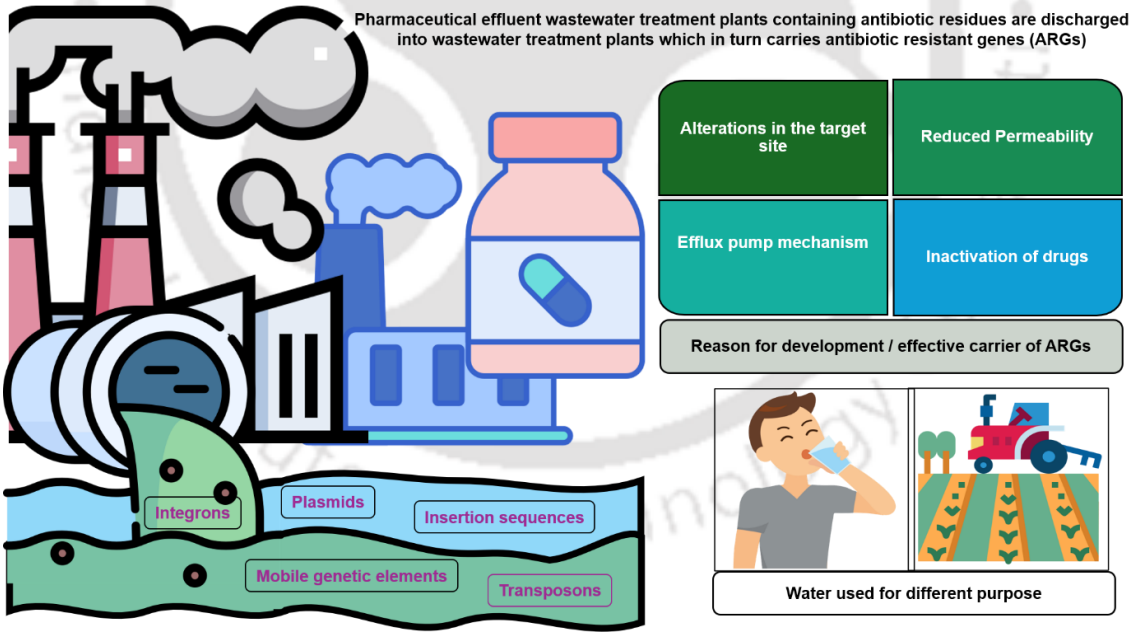


Fig. 2.1. Reason for development of antibiotic resistant genes and antibiotic resistant bacteria in wastewater treatment plants.

In response, several governmental and non-governmental organizations, such as the United States Environmental Protection Agency (USEPA), the European Union (EU), the International Programme on Chemical Safety (IPCS), and the World Health Organization

(WHO), have issued legal directives to address this issue (Kanaujiya et al., 2019). The EU Water Framework Directive 2000/06/CE included 45 priority compounds or groups of compounds, including heavy metals, pesticides, poly aromatic hydrocarbons (PAHs), phthalates, and endocrine disruptors, and mandated their removal from wastewater prior to discharge into the environment in an effort to preserve water resources (Ahmed et al., 2017). Along with that, a watch list of micropollutants, both natural and synthetic, was revealed in the Decision 2015/495/EU on 20 March 2015 (Barbosa et al., 2016). However, countries have adopted regulations for a few OMPs based on the production and usage in their countries, such as bisphenol A, diuron and nonylphenol (Kanaujiya et al., 2019; Yang et al., 2014). However, there are no such directive regulations for pharmaceutically persistent personal care products (PPCPs); the probable reason could be the less known toxicity of these OMPs. Broadly, PPCPs are defined as products related with healthcare or medical purposes utilized both for animal and human (Schumock et al., 2014). Persistent pharmaceuticals are categorized as antibiotics, hormones, analgesics, anti-inflammatory medications, blood lipids regulators, and β-blockers based on their applications and properties. Additionally, sunscreen, insect repellents, fragrances, disinfectants and preservatives are all included in personal care products. The primary sources of these persistent OMPs are derived from agriculture, animal husbandry, and direct discharge (from domestic waste, sewage, and bathing activities) into the water and soil. **Fig. 2.2.** represents different sources and the fate of persistent OMPs.

Antibiotics, are categorized as emerging OMPs by virtue of their across the board unregulated administration in animal husbandry, aquaculture, and even medicine (Yi et al., 2019). The pharmaceutical industry can now mass produce 70% of the world's 4500 chemical compounds that have originally been synthesized biologically. Global antibiotic consumption increased by 65%, from 21.1 to 34.8 billion daily defined doses or (DDDs), and the median antibiotic consumption rate across countries registered an increase of 28% from 2000 to 2015 (Klein et al., 2018). If no changes take place in the existing policy, it has been projected that by 2030 there will be a 200% increase in the consumption of antibiotics, which is higher than the estimated 42 million DDDs in 2015 (Klein et al., 2018). Further, there is a global surge in antibiotic consumption in low-middle income countries with inadequate healthcare, food, education, and living facilities. Whereas, in high-income countries, the increase of antibiotic usage is linked with social and cultural practices, based on prescription and use of antibiotics. The existence of antibiotics is linked to each country's unique sales, practices, regulatory laws, and income levels (Petrovic et al., 2009).

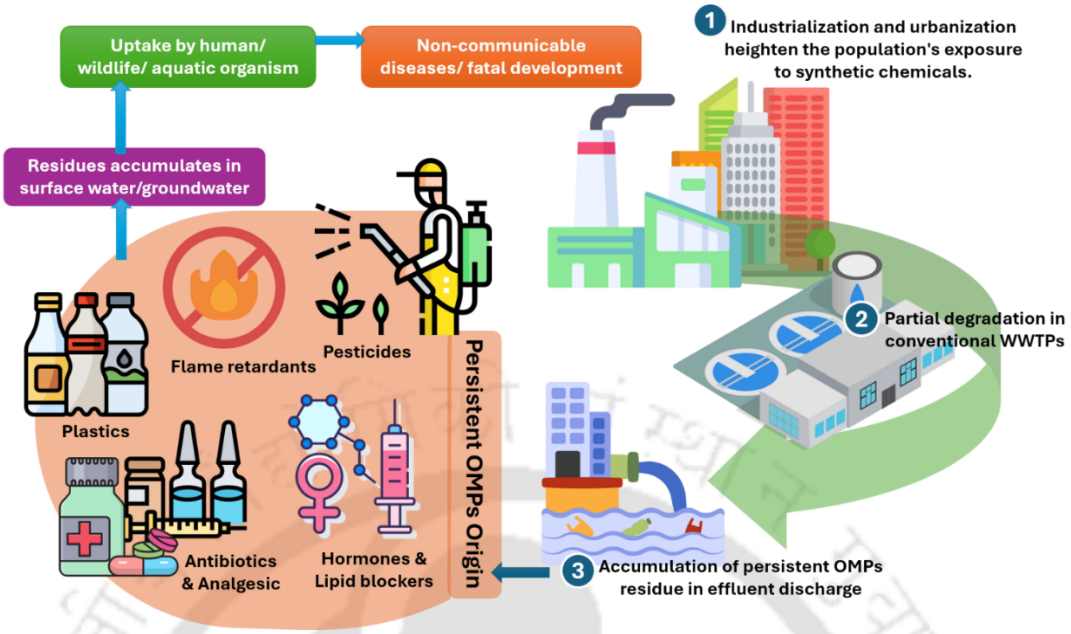


Fig. 2.2. Different sources and the fate of persistent organic micropollutants.

India is one of the world's top pharmaceutical users, ranked 11th globally (Subedi et al., 2015). The Asian pharmaceutical market is rising at 10-15% per year, a rate that is fast outstripping the G7 countries. India's pharmaceutical industry produces 20% of all finished generics worldwide, while China produces 60% of all pharmaceutical products. Though environmental prevalence of antibiotics has been traced from the 1970s, a marked escalation in the aquatic ecosystems, agricultural soils, drinking water, river sediments, and WWTPs effluents, is apparent in the first two decades of the 21st century (Batt et al., 2006). Since the human body does not entirely absorb pharmaceutical compounds, they are excreted as the parent drug, metabolites, or glucuronic or sulphuric acid conjugates; through urine and faeces, forming part of the wastewater discharge (Kovalakova et al., 2020). A few studies have outlined their presence in river water lake water, groundwater, and common effluent treatment plants (CETP) from India (Akiba et al., 2015; Lübbert et al., 2017; Mutiyar and Mittal, 2014; Prabhasankar et al., 2016; Rutgersson et al., 2014; Saxena et al., 2021; Subedi et al., 2017). Thus, in the light of the escalating population in India, uncertain monsoon rains, increased water stress, recycling of treated wastewater, and the medicine consumption pattern, it is critical to garner a holistic understanding of the concentration of pharmaceuticals in Indian wastewater (Mohapatra et al., 2016). Besides, the COVID-19 pandemic has intensified antibiotic pressure on the microbe community. It has also been noted that 72% of hospital patients have received antimicrobial drugs, and

many new antibiotics have been explored to counter the global epidemic (Ghosh et al., 2021). Consequently, COVID-19 has significantly impacted over-the-counter antibiotic access, especially in countries that lack satisfactory health facilities and antibiotic control mechanisms.

2.2. Fluoroquinolone (FQs) antibiotics emerging as OMPs

FQs have recently surfaced as OMPs due to their widespread usage in hospitals, homes, and veterinary uses, as well as their presence in wastewater treatment plants (WWTPs) (Van Doorslaer et al., 2014). FQs antibiotics are broad-spectrum in nature and bioavailability of it have applications in treating alleviating symptoms of urinary tract infections, pneumonia, gastrointestinal illness, and gonococcal infections targeting DNA gyrase and topoisomerase enzymes interfering the DNA replication of bacteria (Mathur et al., 2021; McGregor et al., 2008). The basic structure of FQs include quinolone ring with a fluorine atom at C-6, piperazine at C-7 and a carboxyl group at C-3 (Mathur et al., 2021). Still, the rise of antibiotic-resistant genes (ARGs) is associated with the global upsurge in fluoroquinolone use, which in turn contributes to rising resistance rates. The human microbiome is host to a wide variety of microorganisms, and when antibiotic residues get there, they interact with them. Researchers have reported that antibiotics affect the composition of the microbiome in the gut, which in turn influences resistance in the microbial community rather than in individual bacteria (Mathur et al., 2021). This, in turn, causes the development of bacteria that are resistant to the antibiotics and makes them persist in the human body for years. The primary sources for antibiotics to be introduced into the environment are wastewater treatment plants. Evidence of FQs presence in WWTP effluents, surface water, and other environmental matrices with concentrations ranging from 0.01 to 31,000 $\mu g L^{-1}$ has been reported in research studies. FQs are challenging because of their water solubility and resistant nature; they have a half-life of 100 days and can be found in water bodies at concentrations ranging from 30 g L^{-1} to 130 g L^{-1} (Sengar and Vijayanandan, 2022). Nevertheless, environmental exposure can hasten resistance in microbial communities since up to 70% of FQ antibiotics are excreted unmetabolized. The unaltered form of both ciprofloxacin (40-50%), levofloxacin (71%) and norfloxacin (30-90%) are eliminated through urine and faeces, making it important to introduce them as OMPs (Paredes-Laverde et al., 2018; Thai et al., 2023).

Three FQs residual concentrations specifically ciprofloxacin, levofloxacin and norfloxacin have been detected in Indian river system, treated, surface, ground wastewater

and effluent, sewage treatment plants (Arun et al., 2022; Fick et al., 2009; Gothwal and Shashidhar, 2017; Larsson et al., 2007; Mutiyar and Mittal, 2014; Renganathan et al., 2021; Rutgersson et al., 2014) as shown in **Fig. 2.3.** The tertiary treatment processes involve chlorination, ultraviolet irradiation and sand filtration in FQ levels are reduced whereas in advanced treatment technologies are employed to remove FQs from the system (Mathur et al., 2021). However, FQs presence globally in ground water, common effluent treatment plants, river water, and lakes, indicates towards failure of conventional and advanced treatment technologies. (Castiglioni et al., 2006; Diwan et al., 2018; Ghosh et al., 2009; Golet et al., 2003; Ram and Kumar, 2020; Saxena et al., 2021; Vieno et al., 2007; Xu et al., 2007). Current treatment methods such as adsorption, activated carbon, graphene carbon nano-tubes, electro-chemical treatment, photolysis and biological treatment have been employed. But all these treatments have its own limitations. **Table 2.1.** represent limitation of the existing methods. Thus, cutting-edge treatment methods are required to effectively remove FQs from wastewater while limiting the generation of harmful by-products.

This study addresses these limitations by exploring the use of laccase enzyme from spent mushroom waste as a sustainable solution for degrading fluoroquinolone in wastewater. The main focus of this review is on an innovative strategy to completely remove persistent OMPs using laccase enzyme extracted from spent mushroom waste (SMW) produced by white-rot fungi (*P. florida*). The review examines the current presence of ciprofloxacin, levofloxacin, and norfloxacin in wastewater. It is well known that OMPs are highly persistent in wastewater treatment plants (WWTPs), making their complete removal very challenging. As a result, both emerging and developed countries are resorting to conventional treatments such as ozonation, chlorination, membrane processes, activated carbon filtration, and advanced oxidation processes as well as experimenting with nanomaterials (Cong et al., 2013; Ikehata et al., 2006; Magureanu et al., 2015). The aforementioned physical and chemical treatment technologies generate noxious by-products and carcinogens, that must be eliminated without fail. Therefore, as a viable alternative, lagoon-based WWTPs treatment technology is commonly used in India and many developing countries (Mohapatra et al., 2016). Yet, the lagoon based technology has limitations, and has garnered conflicting reports (Lishman et al., 2006; MacLeod and Wong, 2010); therefore, further validation is required to fully endorse its pharmaceutical elimination potential. Concurrently, insights into the current scenario reveal the removal of persistent OMPs (antibiotics) by the conventional method of WWTPs is inefficient and offers less scope for biodegradability. Against this background, the present review proposes

a cost-effective, sustainable, and innovative strategy of utilizing SMW from white rot fungi (WRF) to biodegrade OMPs from the environment. To summarize, **Table 2.2.** displays various problems caused by PPCPs (antibiotics) and their remediation by utilizing SMW.

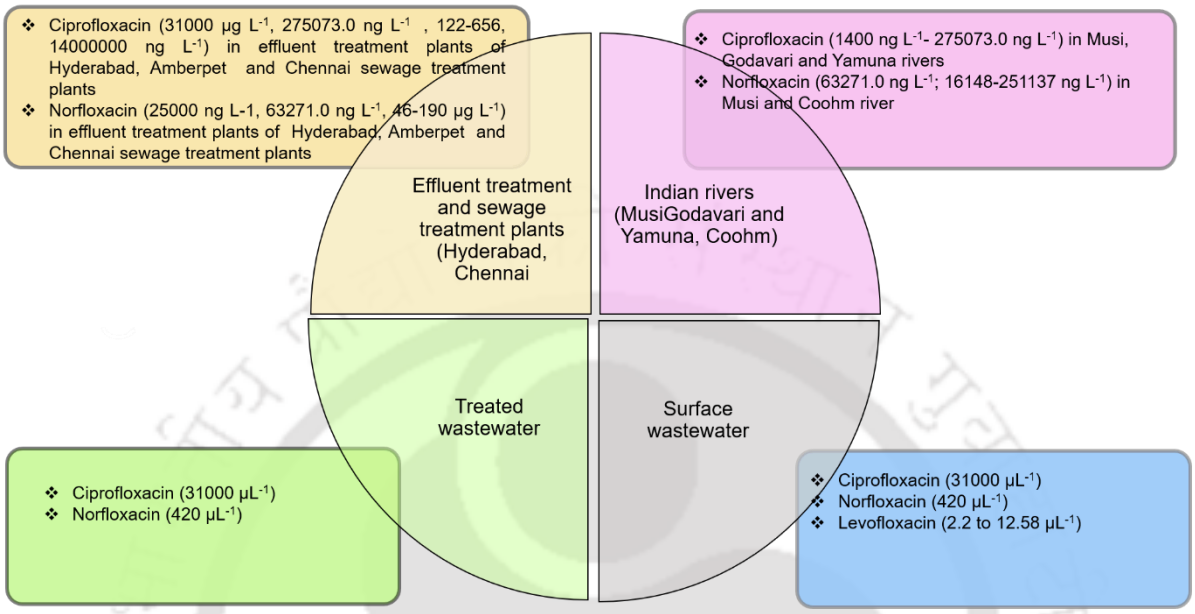


Fig. 2.3. Residual concentrations of three fluoroquinolones (ciprofloxacin, levofloxacin and norfloxacin) detected in Indian river system, treated, surface, ground wastewater and effluent, sewage treatment plants.

To the best of our knowledge, existing literature discusses the utilization of SMW from different classes of fungi for bioenergy conversion, degradation of heavy metals, dye decolorization, and bioremediation of petroleum conversion (Grimm and Wösten, 2018; Marín-Benito et al., 2016; Okerentugba et al., 2015; Phan and Sabaratnam, 2012). Therefore, the novelty of this review is that it highlights the efficacy of SMW from edible WRF in the degradation of OMPs (antibiotics) by utilizing crude extracellular laccase enzyme. The review subsequently showcases a holistic and sustainable approach for a greener future while providing an expanded perspective on the transition from linear to circular economies.

Table 2.1. Limitations of the existing methods for removal of three FQs from wastewater treatment plants**.**

Antibiotics degradation achieved	Existing treatment technologies	Raw material used	Limitations	Removal efficiency (% and mg g^{-1})_	References
Ciprofloxacin	Adsorption (activated carbon: Physical and chemical process)	Wood, coal and peat, Bamboo-based activated carbon	Chemical activation process involves cost of activating agents, leaching out of chemicals during long run of usage and less operational stability	77%, 63%, 62% and 93%	(Rathi and Kumar, 2021; Wang et al., 2015)
Norfloxacin	Adsorption (activated carbon: Physical and chemical process)	Coal, peat, *Albizia lebbeck* seed pods		69%, 62%, 166.69 mg g^{-1}	(Ahmed and Theydan, 2014; Rathi and Kumar, 2021)
Ciprofloxacin	Adsorption (Carbon- nano tubes)	Single-walled, double-walled, multi-walled and graphitized wall	Secondary pollution due to leaching out of metals, poor separability limits, poor operational feasibility	724, 605, 475, 3821 mg g^{-1}	(Ncibi and Sillanpää, 2015; Peng et al., 2021)
Norfloxacin	Adsorption (Carbon- nano tubes)	Carboxylated multi-walled, hydroxylated		90.30, 76.34 and 57. 55 mg g^{-1}	(Yang et al., 2012)

		multi-walled and graphitized			
Norfloxacin	Metal organic frameworks	PCN-124—stu-Cu, MIL-101 (Cr)-SO3H	Porosity of metal organic frameworks and operational stability	36.61 mg g^{-1}, 400.9 mg g^{-1}	(Titchou et al., 2021; Yang et al., 2012)
Ciprofloxacin	Metal organic frameworks	ZIF-67-SO4, Fe_3O_4/HKUST-1, Magnetic mesoporous carbon materials		2537.5 mg g^{-1}, 538 mg g-1, 868 mg g^{-1}	(Jin et al., 2017)
Levofloxacin	Metal organic frameworks	MIL-100(Fe)		87.34 mg g^{-1}	(Chaturvedi et al., 2020)
Ciprofloxacin and Norfloxacin	Advanced oxidation process	UV/H_2O_2/Fe^{2+} and UV/H_2O_2	Higher generation cost of O_3, poor mass transfer rate and formation of carboxylic acids and aldehydes	100% and 93%	(De la Cruz et al., 2012; Titchou et al., 2021)
Levofloxacin	Advanced oxidation process	Ozonation and TiO_2/UV		97%	(Afonso-Olivares et al., 2016)
Ciprofloxacin	Electro-Fenton catalysis	Mesoporous $MnCo_2O_4$-CF	Limited surface zone of metal compounds	100	(Dao et al., 2023; Mi et al., 2019)

Ciprofloxacin	Graphitized mesoporous carbon-TiO_2 nanocomposite	Adsorption and photocatalysis		100	(Zheng et al., 2018)
Ciprofloxacin	ZnO immobilized on a glass plate	Photocatalysis		98.36	(Gharaghani and Malakootian, 2017)
Norfloxacin	Magnetic cobalt ferrite nanoparticles-GO coupled with peroxymonosulfate	AOP	Higher generation cost of O_3, poor mass transfer rate and formation of carboxylic acids and aldehydes	98	(Chen et al., 2018)
Norfloxacin	Alginate-Fe^{2+}/Fe^{3+} polymer coated Fe_3O_4 magnetic nanoparticles	Fenton catalysis		100	(Niu et al., 2012)
Levofloxacin	TiO_2 nanobelts decorated by bimetallic Au–Pd nanoparticles	Photocatalysis		95	(Chen et al., 2015)

Ciprofloxacin and norfloxacin	White-rot fungi (*I. lacteus, P. tigrinus, D. squalens, T. versicolor and P. ostreatus*)	Fungal degradation	Increases cost of production due to use growth media and inducer, Time consuming degradation process	About 40%,77% and 63% of residual concentration of ciprofloxacin was left in the medium after 14 days of incubation by *T. versicolor, P. tigrinus, D. squalens* and *P. ostreatus*. Similarly, 15% of norfloxacin residual concentration was left in the medium	(Čvančarová et al., 2015)
Ciprofloxacin and norfloxacin	Mycelium consortia of *P. chrysosporium and P. sanguineus.* Ciprofloxacin and norfloxacin degradation were achieved through mycelial adsorption of pure culture and			98.5% ciprofloxacin and 96.4% norfloxacin was degraded after 2 days of incubation by pure culture of *P. sanguineus* whereas 64.5% ciprofloxacin,	(Gao et al., 2018; Purvi et al., 2021)

	co-culture of both the strains				73.2% norfloxacin removal efficiency by *P.chrysosporium* was achieved.	

Table 2.2. Various problems caused by PPCPs (antibiotics) and their remediation by utilizing SMW**.**

Antibiotics	Problems on human exposure	Problems on aquatic animals and wildlife	Sources of exposure	Environmental presence	Enzymatic remediation by SMW	References
Sulfonamides	Nausea, hypersensitivity reactions, hepatoxicity, development of ARGs (*Escherichia coli, Salmonella, Shigella* sp)	Accumulation in the food chain, and development of *sul* resistant gene (*sul 1, sul 2, sul 3*)	Meat and meat products, livestock, contaminated water, inefficient degradation by conventional WWTPs leads to accumulation of residue	Wastewater	SMW of *P. eryngii, P. ostreatus*	(Baran et al., 2011; Pallares-Vega et al., 2019).

Tetracyclines	Joint pain, nephrotoxicity, endocrine disorders, central nervous system disease	Accumulation in the food chain, developmental delay, oxidative stress in fishes and development of *tet* resistant genes	Meat and meat products	Wastewater	SMW of *P.eryngii*	(Chang et al., 2014; Xu, LongyaoOccurrence, fate et al., 2021)
Acetaminoph-en	Hepatoxicity, nausea, induce antibiotic resistance in *Escherichia coli* (Acr-AB-TolC efflux pump)	Delayed hatching, escalation in swimming, delayed developmental functions in fishes	Inefficient degradation by conventional WWTPs leads to accumulation of residue	Wastewater	SMW of *P. eryngii*, *P. ostreatus*, *L. edodes*	(Chang et al., 2018; Erhunmwunse et al., 2021)

2.3. Ligninolytic enzymes from SMW in biodegradation of PPCPs (antibiotics)

Biodegradation is a promising, cost-effective, and environmentally safe solution to sequester emerging toxic contaminants. SMW is the major waste product of the mushroom industry. Since 1978, the global production of cultivated edible mushrooms has escalated from about 1 billion in 1978 to 34 billion in 2013, a remarkable increase of more than 30-fold (Royse et al., 2017) with a reciprocal increase in consumption since 1977. Approximately 2 million tons of SMW being generated, and oyster mushroom accounts for about 1.2 million tons. The fact mushrooms yield copious quantities of waste (Grimm et al., 2021), augurs well for a circular economy. Thus, 5 kg of SMW (about 2 kg of dry weight) are generated from 1 kg of fresh mushrooms Though this massive amount of waste is clearly unqualified for discharge into the environment, they are generally dumped or incinerated in the open air to avoid labor charges and extra efforts by the cultivators. A plethora of poly aromatic hydrocarbons like benzopyrene, hydroquinone, catechol, phenanthrene, and naphthalene are generated during the incineration of lignin-rich wastes from agro-industries and forests. All of these elements trigger a slew of environmental and health issues for humans and animals, including inhibition of DNA synthesis and the induction of malignant tumors in the (lungs, liver, larynx, and cervix). Though SMW is considered as waste, it contains extracellular lignocellulosic enzymes which have wide and potential applications in the degradation of persistent PPCPs like antibiotics, inorganic pollutants such as heavy metals, and dye decolorization (Chang and Chang, 2016; Dao et al., 2019; Lim et al., 2013; Sadiq et al., 2018; Zhou et al., 2020). WRF could play an important role in sequestering these emerging OMPs.

Cultivation of *Pleurotus* sp., the WRF or oyster mushroom varieties has grown significantly in Asia because of low production costs, readily available substrates, and high yielding capacity, contributing to 27% of its global production. *Pleurotus* sp. can be found in temperate and tropical areas and are a high-end delicacy and a well-known edible fungus all over the globe. *Pleurotus* is a genus that encompasses roughly 40 species. *Pleurotus eryngii*, *Pleurotus citrinopileatus*, *Pleurotus flabellatus*, *Pleurotus ostreatus*, *Pleurotus djamor var. roseus*, and *Pleurotus florida* are among the 26 species that are documented as cultivable mushrooms using different types of agro-wastes (Golak-Siwulska et al., 2018). Since *Pleurotus* sp. produces ligninolytic enzymes laccase, manganese peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP), the SMW of WRF has emerged as an intriguing option for bioremediation or biodegradation. The ligninolytic enzymes contained in SMW can be easily extracted, and could assist mushroom cultivators in

lowering its disposal costs. SMW also contains fungal mycelia that secrete many residual enzymes such as hemicellulose, cellulase, protease. The role of extracellular ligninolytic enzymes, particularly laccase from SMW of WRF in the breakdown of antibiotics is covered extensively in this review.

2.4. Mechanism of laccase enzyme

Laccase are found in various species, including plants, bacteria, and fungi; their physiological functions differ depending on the source. Laccase is found both intracellularly and extracellularly in bacteria. Cot A protein from *Bacillus subtilis* is a well-known extracellular bacterial laccase. Though bacterial laccase offers intriguing applications in the breakdown of pollutants such as textile effluents, it is less effective in degrading pharmaceutical wastes from WWTPs, to the best of our knowledge. On the other hand, plant laccase have not been employed in bioremediation (Arregui et al., 2019; Janusz et al., 2020). Meanwhile, a recent analysis found that laccase from *Trametes versicolor* is 20 times more potent than *Streptomyces cyaneus* in degrading micropollutants. This study revealed that fungal laccase is the best emerging alternative for wastewater treatment in a sustainable manner (Margot et al., 2013)

WRF has recently gained a lot of attention as a source of laccase enzyme. Laccase are mainly found in fungi that are ascomycetous or basidiomycetous. The molecular size of monomeric globular proteins of fungal laccase ranges from 60 to 70 KDa, with isoelectric point (pI) of around pH 4.0, and glycosylation levels varying from 10 to 25% and at times even exceeding 30% (Yesilada et al., 2018).

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) employ the copper ions unique redox potential capacity in oxidizing an extensive spectrum of aromatic substrates, while simultaneously reducing molecular oxygen into water (Rahmani et al., 2015). Laccase has the prowess to catalyze the oxidation of polyphenolic, amines, diamines, and a few inorganic compounds (More et al., 2011). The ability of laccase to oxidize substrates is influenced by parameters such as the difference in redox potential between substrate, the T1 site of copper ion, pH of the medium, and the chemical structure of the substrates (Janusz et al., 2020). Laccase has a unique physical structure within which it forms a trinuclear cluster (TNC) with type-3 (T3) copper ions (Cu-II and Cu-III). It also contains one type-1 (Cu-I) copper ion and one type-2 (T2) copper ion. Laccase utilizes four Cu atoms dispersed over three spectroscopically specified sites, to correlate four single-electron oxidations of the reducing substrate with the four-electron reductive cleavage of

the dioxygen bond (More et al., 2011). Laccase can not only be ramped up by using redox mediators (Baiocco et al., 2003), but its versatility is heightened by wide substrate specificity, use of molecular oxygen as an electron acceptor, and generation of water as the sole reaction by-product. However, the action of laccase is restricted to oxidation of the phenolic moiety by the inherently lower redox potential. Therefore, the inability of laccase to oxidize non-phenolic compounds with higher redox potential can be overcome by inclusion of artificial or natural mediators along with laccase. Due to their steric hindrance-induced restriction of complex substrates to the active site, mediators function as an electron shuttle and allow oxidation of complex substrates (Ghose and Mitra, 2022). High sized mediators or with high redox potential do not fit into the enzymatic pocket, and a different mechanistic action validates the oxidation of complex non-phenolic substrate. Artificial mediators like 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) can oxidize non-phenolic compounds via electron-transfer route (Baiocco et al., 2003; Bourbonnais and Paice, 1990).

Other mediators such as nitroso compounds, phenothiazine derivative or triphenylamine, have widely used for the oxidation of phenolic and non-phenolic compounds (Fabbrini et al., 2002). Thus, laccase-mediator systems are appropriate for the oxidative transformation of aromatic compounds, decolorization of industrial dyes, detoxification of contaminants and paper pulp delignification and bleaching. Thus, comprehending the role of mediators is crucial in terms of ideal reaction conditions, substrate specificity, oxidation of substrates, and the inherent variation among the mediators (Parra Guardado et al., 2019). Therefore, the existence of these ecologically favourable mediators, which are readily available, accelerate the utilization of these enzymes in sustainable processes. and laccase appears to be a greener technology for remediation because of its unique mechanistic features.

2.5. Mechanism of degradation of persistent OMPs by laccase

As a result of its unique mechanistic features, extracellular laccase enzyme appears to be a greener technology for remediation. Several factors like pH, temperature, mediators, electron donating or electron withdrawing groups are responsible for the oxidation of OMPs. The degradation of OMPs is influenced on the redox potential of laccase and the presence of strong electron donating functional groups (EDGs) or electron withdrawing functional groups (EWGs) in the substrate. EDGs are functional groups that donate electrons, enhancing the nucleophilicity of the substrate whereas EWGs are functional

groups that decrease the electron density of a molecule by attracting electron density of a molecule by attracting electron towards themselves. Among the functional EDG that are susceptible to oxidation by laccase are hydroxyl (-OH), amines (-NH_2), alkoxy (-RO), alkyl (-R), and acyl (-COR) groups. In contrast, EWG carboxylic (-COOH), amide ($CONR_2$), halogen (-X) and nitro (-NO_2) are resistant to degradation of OMPs, because the electron abstraction produces a steric shield (Yang et al., 2013), consequently the efficiency of the remedial process is influenced by the chemical structure of OMPs. Parra Guardado et al., (2019) highlighted the catalytic action of laccase on EWG and EDG groups and attributed laccase catalytic action to the presence of an EDG group (-OH, -NH_2) and increased effectiveness in degrading OMPs. Thus, the accelerated degradation of refractory micropollutants is dependent on the laccase catalytic system and the existence of highly reactive compounds represented in **Fig. 2.4**.

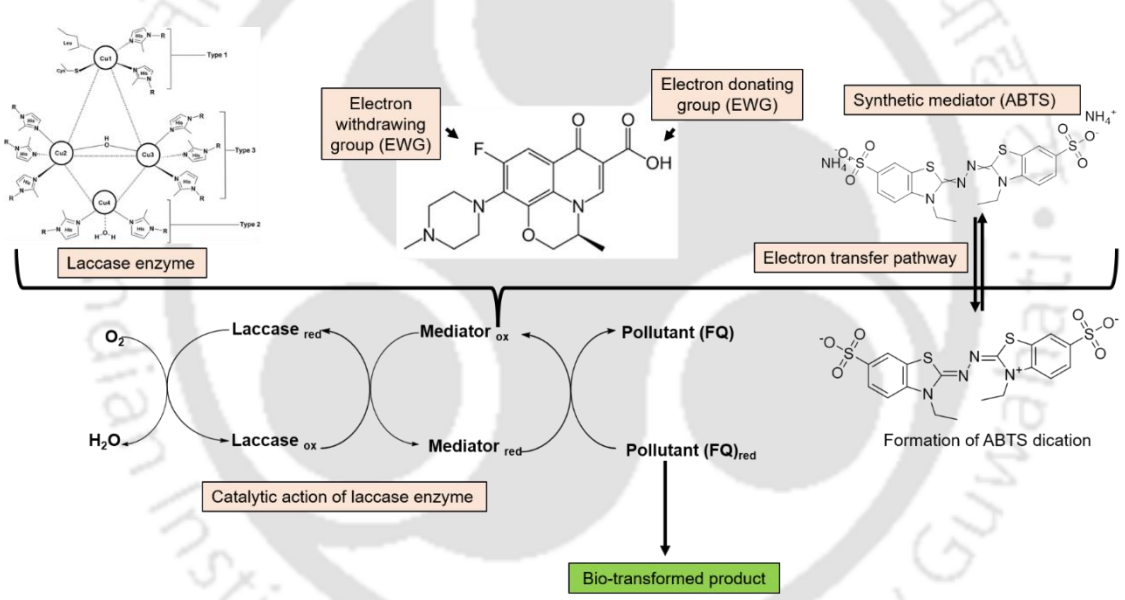


Fig. 2.4. Mechanism of degradation by laccase enzyme in presence of synthetic mediators.

In addition (Lloret et al., 2010; Margot et al., 2015) reported optimal pH 5.5 for the degradation of several micropollutants; underscoring the pivotal role of optimal pH and temperature in enzyme stability and functionality. In the final analysis, the degradation mechanism is completely dependent on the redox mediator-laccase system advocated by (Parra Guardado et al., 2019), which promises degradation in less than 3 h with 100% efficiency. Also, redox mediators discussed earlier (both natural and synthetic) degrade EPPPs and EDCs via electron transfer (ET) or hydrogen atom transfer (HAT) mechanisms.

Contrarily, non-recyclability, poor reaction stability, minimal cost-effective ratio, high processing costs, and sensitivity to severe environmental conditions are all disadvantages of enzymes in free forms. Immobilization is the way forward to overcome these drawbacks and extend the shelf life of the laccase enzyme.

2.6. Immobilization of laccase enzyme onto raw biochar derived from spent mushroom waste of Pleurotus florida

Immobilization of enzyme is a potential approach to overcome the drawback of free laccase. Adsorption processes such as encapsulation and adsorption (physical adsorption), cross-linking and covalent binding (chemical adsorption) have been employed in immobilization of enzyme. Physical adsorption process involves non-covalent interactions (van der Waals forces, hydrogen bonding and hydrophobic interactions) whereas chemical adsorption processes involve covalent bonding between enzyme and support material (Kaur et al., 2021; Pandey et al., 2022; Zou et al., 2023). Immobilization carriers such as nanoparticles, nanofibrous membrane, carbon nano tubes (CNT) and graphene oxide have been reported (Han et al., 2023; Ji et al., 2021; Sharma et al., 2022; Zhao et al., 2023; Zhu et al., 2020; Zou et al., 2023). However, above-mentioned carriers are not suitable support material for immobilization of enzyme due to high production cost, complex fabrication and high operating conditions.

Carbon material derived from biomass is an excellent immobilizing carrier. Biochar, porous solid rich in carbon, is an excellent resource that can be used as a catalyst, immobilizer, or adsorbent support due to its extensive surface area and low porosity (Chauhan et al., 2023; Jin et al., 2021). It is produced by thermal breakdown of feedstock to a moderate temperature in an oxygen-free environment. The surface functional groups on biochar can be easily tuned, making it a promising platform for the adsorptive immobilization of enzymes. Compared to other adsorbents, biochar is a more cost-effective and user-friendly option for eliminating OMPs such as acetaminophen, tetracycline, anthracene, diclofenac and sulfonamide (García-Delgado et al., 2018; Imam et al., 2021; Pandey et al., 2022; Yang et al., 2017) represented in **Table 2.3.** However, biochar adsorption ability is limited by saturation, so regeneration is necessary to maintain its adsorbent capacity (García-Delgado et al., 2018; Wang et al., 2022b). Several research studies have reported immobilization of laccase onto biochar such as pinewood, almond shell, pig manure, rice straw (Imam et al., 2021; Lonappan et al., 2018; Naghdi et al., 2017; Pandey et al., 2022). Despite the fact that, previous studies reported immobilization of

laccase into modified surface of biochar which increases production cost and secondary pollution caused by the use of chemical reagents. To circumvent, this study aimed to immobilize laccase into raw biochar (without activating biochar chemically) through adsorption process derived from SMW of *P. florida*.

Table 2.3. Various methods of immobilization of laccase onto biochar and removal efficiency of OMPs.

OMPs	Type of feedstock used	Surface modification methods of biochar for immobilization of laccase	Removal efficiency	References
Diclofenac	Pine wood biochar, Almond shell biochar, Pig manure biochar	Covalent immobilization and cross-linking with glutaraldehyde, COOH enhance the adsorption of laccase	100% within few hours of reaction	(Lonappan et al., 2018)
Norfloxacin, enrofloxacin and moxifloxacin	Apple branches	Adsorption and cross-linking	93.7%, 65.4% and 77.0% after 48 h reaction	(Zou et al., 2023)
Diclofenac, ciprofloxacin, amoxicillin, carbamazepine	Coconut shell	Commercially available granular activated carbon through adsorption	90% removal after 120 mins of reaction	(Al-sareji et al., 2023)
Anthracene	Rice husk	Mineral acids (H_2SO_4, HNO_3 and HCl)	98% after 24 h of reaction	(Imam et al., 2021)
Malachite green	Pine needles	Weak acid (acetic acid and phosphoric acid) functionalization	85% within 5 h of reaction	(Pandey et al., 2022)

Despite being viewed as a waste by-product, SMW is an ideal feedstock for biochar production due to its high residual nutrient content like nitrogen, phosphorus, organic compounds, and potassium (Zhang and Sun, 2014). A unique attribute of SMW is that it

does not originate from a particular type of agricultural waste. For instance, wood-rotting mushrooms, are grown on forest remnants like sawdust and wood chips, while others are cultivated on diverse agricultural wastes including cotton seed hulls, rice straw, wheat straw, corn stalk, and corn cob. Concurrently, with the carbon sources mentioned, substrate culture often involves the addition of nitrogen sources such as rice bran and wheat bran. Other supplements like lime, gypsum, calcium superphosphate, and calcium carbonate are also added in the substrate (Jin et al., 2021; Yang et al., 2016). Thus, SMW is a complex material that exhibits distinct characteristics of biochar derived from other agricultural wastes after pyrolysis. However, there is a dearth of literature regarding the characteristics of biochar made from pyrolyzing mushrooms at different temperatures or using different waste substrates. Immobilization of enzymes in a firm support is obligatory as it can increase enzyme activity, and stability to other factors like pH, and temperature and increases its reusability (Zhou et al., 2021) as shown in **Fig. 2.5**.

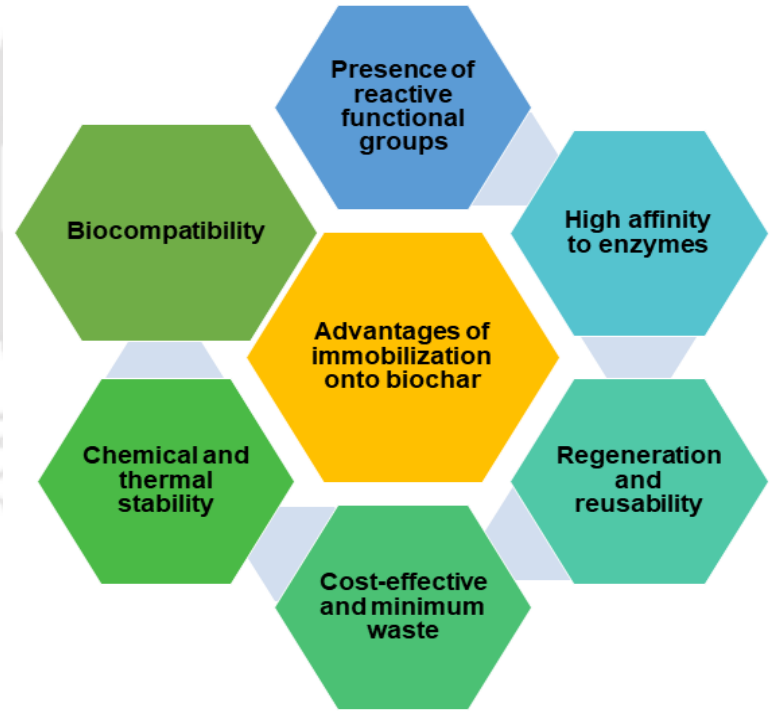


Fig. 2.5. Advantages of enzyme immobilization onto biochar

Biochar exhibits strong physicochemical resistance owing to its electron-accepting (EAC) and electron-donating (EDC) capacities, in addition to its high sorption ability and electron exchange capacity (Naghdi et al., 2017; Prévoteau et al., 2016). Laccase molecules attach to the surface of raw biochar through different types of bonds, including van der Waals forces, hydrogen bonds, and ionic bonds. The reusability and instability of free

laccase, however, are its main practical constraints. (Primožič et al., 2020; Wang et al., 2022a; Zdarta et al., 2019; Zhou et al., 2021). Further, research is needed to determine the practical application of biochar as an immobilization support. One of the main challenges of using biochar as a support for immobilization is the absence of optimized parameters that can lead to the maximum immobilization efficiency (IE). Therefore, to obtain maximum immobilization efficiency of laccase immobilized in raw biochar this study involved tweaking of parameters such as pH, temperature and enzyme dosage (discussed in chapter 5). Biochar serves as a dual benefit i.e. degradation of OMPs and by immobilizing laccase enzyme it increases its stability, productivity and operational stability. In addition, utilizing SMW for immobilization of laccase facilitates transition of linear economy to circular economy.

Therefore, this work stands out from previous studies through its innovative use of raw biochar from spent mushroom waste (SMW) of *P. florida* for laccase immobilization without chemical activation, reducing costs and environmental impact. Unlike traditional enzyme sources, which can be expensive to purify and process, SMW provides a sustainable, low-cost laccase source, supporting waste valorization in the mushroom industry. Additionally, this study uniquely focuses on degrading fluoroquinolone antibiotics, a particularly persistent group of pollutants that contribute to antibiotic resistance, thereby addressing a critical public health issue. In contrast to conventional treatments like chlorination or ozonation, which often yield toxic by-products, this enzymatic approach with SMW-derived laccase offers a safer, eco-friendly alternative. Furthermore, the use of biochar for enzyme immobilization enhances laccase stability and reusability, making it a practical and scalable solution that builds on, yet differs from, existing methods.

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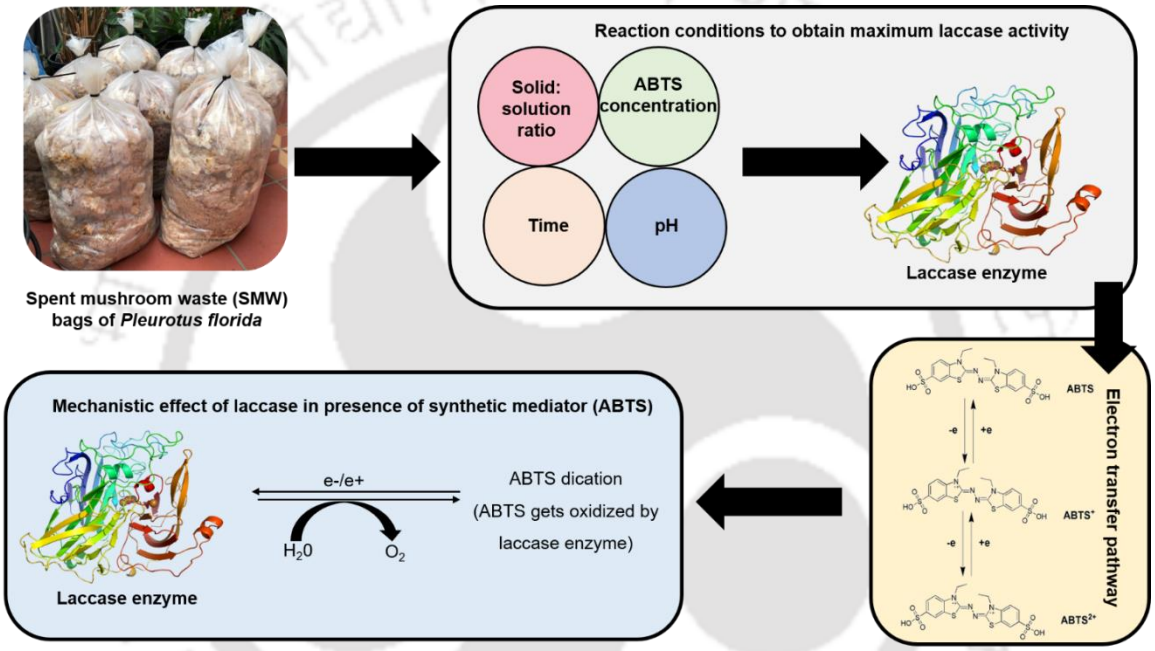
CHAPTER 3

To carry out extraction of laccase enzyme from spent mushroom waste of *Pleurotus florida* under various operating conditions and its assay



"*This chapter deals with obtaining optimal reaction conditions for maximum laccase activity extracted from spent mushroom waste (SMW) of Pleurotus florida.*"

3. To carry out extraction of laccase enzyme from spent mushroom waste of *Pleurotus florida* under various operating conditions and its assay

3.1. Introduction

Spent mushroom waste (SMW) refers to the lignocellulosic waste generated by mushroom production. Thus, the mushroom industry yields an ample amount of waste, accounting for 1.2 million tons of SMW engender from oyster mushrooms. Further, it is estimated that 5kg of waste is generated from 1kg of fresh mushroom (Grimm et al., 2021; Royse et al., 2017). However, despite the fact that this enormous volume of SMW is dumped into the open air or incinerated, resulting in environmental pollution and triggering health issues for animals and humans. Even though SMW is considered waste, it is also rich in nutrients (cellulose, crude protein, dry matter, hemicellulose, calcium, phosphorus, and carbohydrate). The nutrient composition of SMW makes a sustainable substrate and has extensive potential applications such as compost, livestock feed, fertilizer, biogas production, enzyme production, and bioremediation of petroleum hydrocarbon (Mohd Hanafi et al., 2018; Okerentugba et al., 2015; Phan and Sabaratnam, 2012).

Laccase is an excellent, environmentally friendly, and long-lasting solution for cleaning up recalcitrant pollutants. The unique ability of copper ions present in the trinuclear cluster in laccase has prowess to catalyze the oxidation of organic and inorganic compounds. Laccase is found in plants, bacteria, and fungi, and their effective physiological function differs species to species. Contrarily, recent studies reveal that fungal laccases are more potent than bacterial laccase (Arregui et al., 2019). To enhance the mechanistic action of laccase, laccase production was aided by various approaches, including changing the composition of growth media, adding chemical inducers (such as metal ions and aromatics), and tweaking process conditions had a synergistic effect on the production of laccase. However, the lack of an expression system and high production costs have long impeded laccase output. Nonetheless, the aforementioned methods cannot be implemented in the short term and are economically not viable. One such viable option and economically friendly is extracting laccase enzyme from SMW. Evidently, the presence of extracellular laccase in the growth media of fungal culture requires 7-14 days from the day of inoculation. In addition, to maximize the production of, laccase several parameters are tweaked, like the addition of supplements (guaiacol, veratryl alcohol, tryptophan substituents), the addition of inducers (tannic acid, 2, 6-dimethoxyphenol, copper sulphate),

the addition of metal ions, different culture modes (static or shaking). The process of extraction of laccase from fungal culture is time consuming. On the contrary, extraction of laccase enzyme from SMW is way more effective, less time consuming and fewer parameters are needed to be tweaked for effective production of laccase. Research studies revealed that optimizing the SMW of *Pleurotus sajor caju* resulted in maximum laccase production in less than one day by tweaking and optimizing a few parameters like temperature, pH, type of extraction medium, and time of incubation (Singh et al., 2003). Similarly, extraction of laccase using a distinct extraction buffer system from SMW of *P. ostreatus* and *Pleurotus eryngii* has revealed maximum laccase production at an economical time (Lim et al., 2013). However, to the best of existing knowledge, optimization parameters like solid: solution ratio (SS) and varying ABTS concentration have not been studied in SMW *of P. florida*. Hence, recognizing the gap in current research regarding the optimization parameters for laccase extraction from SMW of *P. florida*, this study aims to investigate the influence of specific conditions, particularly solid-to-solution (SS) ratio and varying ABTS concentrations, while maintaining a constant temperature of 30 °C. By optimizing these extraction conditions, we anticipate improvements in enzymatic efficiency, enabling more effective laccase production. The extraction process will involve precise control of operating conditions such as temperature, pH, and incubation time, which are critical for maximizing laccase activity. This cost-effective method of enzyme extraction not only enhances production efficiency but also presents a sustainable solution for waste management, addressing both environmental concerns and the economic feasibility of laccase commercialization.

3.2. Materials and methods

3.2.1. Materials

3.2.1.1. Collection, composition and storage of spent mushroom waste of Pleurotus florida

Spent mushroom waste (SMW) bags of *P. florida* were collected from a mushroom cultivation farm named Phantom in Guwahati, Assam, India. SMW bag substrate comprised of primary substrate (paddy straw), spawn (*P. florida*), and calcium carbonate. No additional additives were added to the substrate bag. The entire cultivation process lasted 90-120 days (till fruiting stage). For this study, SMW bags were collected after 3rd fruiting stage of the mushroom (the stage where bags are not used further for the cultivation of the mushroom) as shown in **Fig. 3.1.** The bags were stored at 4 ℃ for future use, and there was no loss of enzyme activity.

Fig. 3.1. Collection of spent mushroom waste bags of *Pleurotus florida* from mushroom cultivation farm

3.2.1.2. Chemical and reagents

ABTS, Bovine serum albumin, Ammonium sulphate, Dialysis membrane (D7884, 32mm, molecular cut-off kDa ≤1200>2000), commercial laccase enzyme from *Trametes versicolor* (>0.5 U mg^{-1}) were procured from Sigma Aldrich, India. Sodium citrate, citric acid, sodium phosphate monobasic, sodium phosphate dibasic were purchased from Merck, India (>99.5% purity).

3.3. Methods

3.3.1. Optimal reaction conditions for maximum laccase enzyme production from spent mushroom waste of Pleurotus florida

Spent mushroom waste of *Pleurotus florida* bags were used in the extraction of extracellular laccase enzyme. The extraction procedure was followed earlier published in (Economou et al., 2017). Here, in this study with respect to find optimal reaction condition from SMW of *P. florida*, parameters like solid: solution (SS) (1:5, 1:10, 1:25) ratio was dissolved in various buffers of varying pH 50 mM (glycine buffer, pH 3.0; sodium citrate

buffer, pH 4.5; distilled water pH 5.7; phosphate buffer, pH 8.0) and was incubated at 30 ℃ temperatures, followed by constant shaking at 180 rpm. A denser ratio (1:5) maximizes contact between SMW and buffer, potentially enhancing extraction efficiency, while higher ratios like 1:25 allow us to observe the effect of dilution on enzyme recovery (Economou et al., 2017). The experiment was carried out in Erlenmeyer flask. The extracted sample was collected at varying time ranges (3 h, 6 h, 9 h, 12 h) and was assayed for laccase enzymatic assay. The extracts were centrifuged (10000 g, 10 min) and the supernatants were used to study laccase enzymatic assay. ABTS was used as a substrate for laccase enzymatic assay. To determine laccase enzymatic assay different ABTS concentration was used (0.05 mM, 0.2 mM, 0.6 mM and 1 mM). All conditions were tested in triplicate to ensure statistical validity and reproducibility, enabling robust analysis of mean activity values and reliable assessment of variability across trials.

3.3.2. Enzymatic assay

The laccase enzyme assay was measured using a UV-visible spectrophotometer (Thermo Scientific, Genesys 10S, UV-Visible spectrophotometer) using ABTS as a substrate. The reaction mixture volume contained 1000 µl buffers of different pH (3, 4.5, 5.7 and 8), 250 µl of ABTS of varying concentrations (0.05 mM, 0.2 mM, 0.6 mM and 1 mM), 100 µl of extracted laccase, extracted at varying time intervals and was incubated for 15 min at room temperature. The enzymatic assay was measured at 420 nm (ε_{420} = 36,000 $M^{-1}cm^{-1}$). To measure laccase activity, one µmol of ABTS had to be oxidized by the enzyme per minute under test conditions, and the lowry method was used to determine the protein concentration (Waterborg, 2009).

3.3.3. Partial purification of laccase

The extracted laccase was subjected to partial purification. Ammonium sulphate (80%) precipitation was subjected to the supernatant slowly in stirring conditions and was kept for 3-4 h in 4 ℃ for precipitation. After, precipitation the pellet was collected and dialyzed using sodium citrate buffer (pH 4.5) at 4 ℃ for 12 h. The dialyzed supernatant was assayed for laccase enzyme activity and protein assay. Further, samples were lyophilized and stored at -20 ℃ for future use. Further, to obtain laccase bands sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12% (w/v) separating gels and 5% stacking gels along with the low-molecular mass protein marker. The laccase bands were visualized with Coomassie Brilliant Blue RG250. Native-PAGE

was performed with 6% stacking gel and 10% separating gels. In case of native gel, samples were not denatured with SDS and SDS was omitted from separating and stacking gel. To visualize laccase bands native-PAGE gels were stained with 5 mM ABTS solution prepared in sodium citrate buffer (pH 4.5) for 15 min at 30 ℃.

3.3.4. ABTS plate screen assay

Plate assay was performed by following (More et al., 2011). The plate screen assay media consists of 0.5% sterile agarose containing 0.5 mM of ABTS added in sodium citrate buffer of pH 4.5, 0.1 M, and was poured into a petri plate. The gel was punctured, and a partially purified laccase enzyme was added to the wells. In contrast, commercial laccase enzyme from *Trametes versicolor* was employed as a positive control, and autoclaved milli Q water was used as a negative control in the wells. Laccase activity was confirmed by the appearance of greenish-blue color around the wells. **Fig. 3.2.** displays the ABTS plate screen assay.

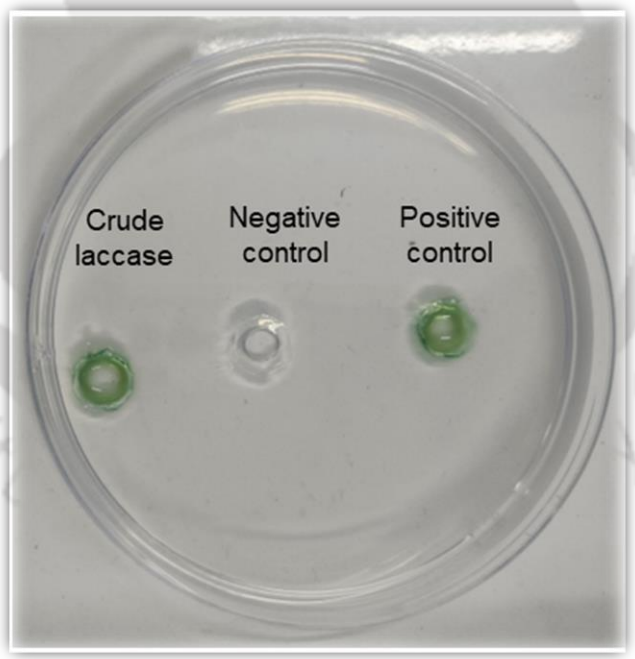


Fig. 3.2. ABTS plate screen assay.

3.3.5. Enzyme stability and characterization

The partially purified laccase enzyme was checked for stability at different pH ranging from (1-10) by using 0.1 M different buffers KCL-HCl buffer (pH 1.0 and 2.0), glycine

buffer (pH 3.0), sodium citrate buffer (pH 4.0, 4.5 and 5.0), phosphate buffer (pH 6.0 and 7.0), tris-HCL buffer (pH 8.0) and carbonate buffer (pH 9.0 and 10.0) for 1 h at temperature 30 ℃. Additionally, the temperature profile of the partially purified enzyme was examined from 4 ℃ to 70 ℃ using substrate ABTS (1mM) for 1 h at a temperature 30 ℃. Further, the stability of the enzyme was checked in metal ions (Fe, Cu, Zn, Mn) at two different concentrations (2 mM and 5 mM) at a temperature of 30 ℃. It was incubated for 30 min at constant shaking. The influence of inhibitors EDTA, sodium azide, dithiothreitol, beta mercaptoethanol and urea were also checked in varying concentrations ranging (2 mM and 10 mM), and surfactants sodium dodecyl sulphate (SDS), cetrimonium bromide (CTAB), tween 80 and triton X-100 in concentrations 0.01% to 1% was studied for 30 min at constant shaking at temperature 30 ℃. Residual activity was calculated using ABTS substrate (1 mM) (Baldrian, 2004; Forootanfar et al., 2011; Liao et al., 2012; Matsuse et al., 1998; More et al., 2011; Niladevi et al., 2008).

3.3.6. Storage stability

The storage stability of partially purified free laccase was evaluated on distinct buffers in the range of pH (1-10, 0.1 M concentration) using KCL-HCL buffer (pH 1.0), glycine buffer (pH 3.0), sodium citrate (pH 4.5), tris-HCL buffer (pH 8.0) and carbonate buffer (pH 10.0) at temperatures (4-45 ℃) for 28 days followed by determination of residual activity (Baldrian, 2004; Liao et al., 2012; More et al., 2011). Residual activity was determined at equal time intervals (1, 7, 14 and 28 days). Further, the stability check was confirmed with commercial laccase (*Trametes versicolor*).

3.3.7. Statistical analysis

All experiments were conducted in triplicates. Analysis of variance (ANOVA) was conducted by employing Duncan multiple range test to determine statistical significance. The analysis of variance was conducted using SPSS statistics software. Statistical significance was determined by *p*-values less than 0.05.

3.4. Results and discussion

3.4.1. Enzymatic activity in spent mushroom waste of Pleurotus florida

The enzymatic activity of extracellular laccase extracted from the SMW of *P. florida* varied based on the extraction time, pH of buffers used for extraction, solid: solution ratio and ABTS concentration used to determine enzymatic activity. The most influential parameters were found to be pH and ABTS concentration. The enzymatic activity descended in magnitude for pH as follows: pH 4.5>pH 3.0> pH 5.7>pH 8.0. The efficiency of the best extraction time is descended in the order of magnitude as follows: 3 h>6 h>9 h>12 h conditions. **Fig. 3.3.** represents the evaluation of laccase enzyme activity at pH 3.0, 4.5, 5.7 and 8.0 at a temperature of 30 ℃.

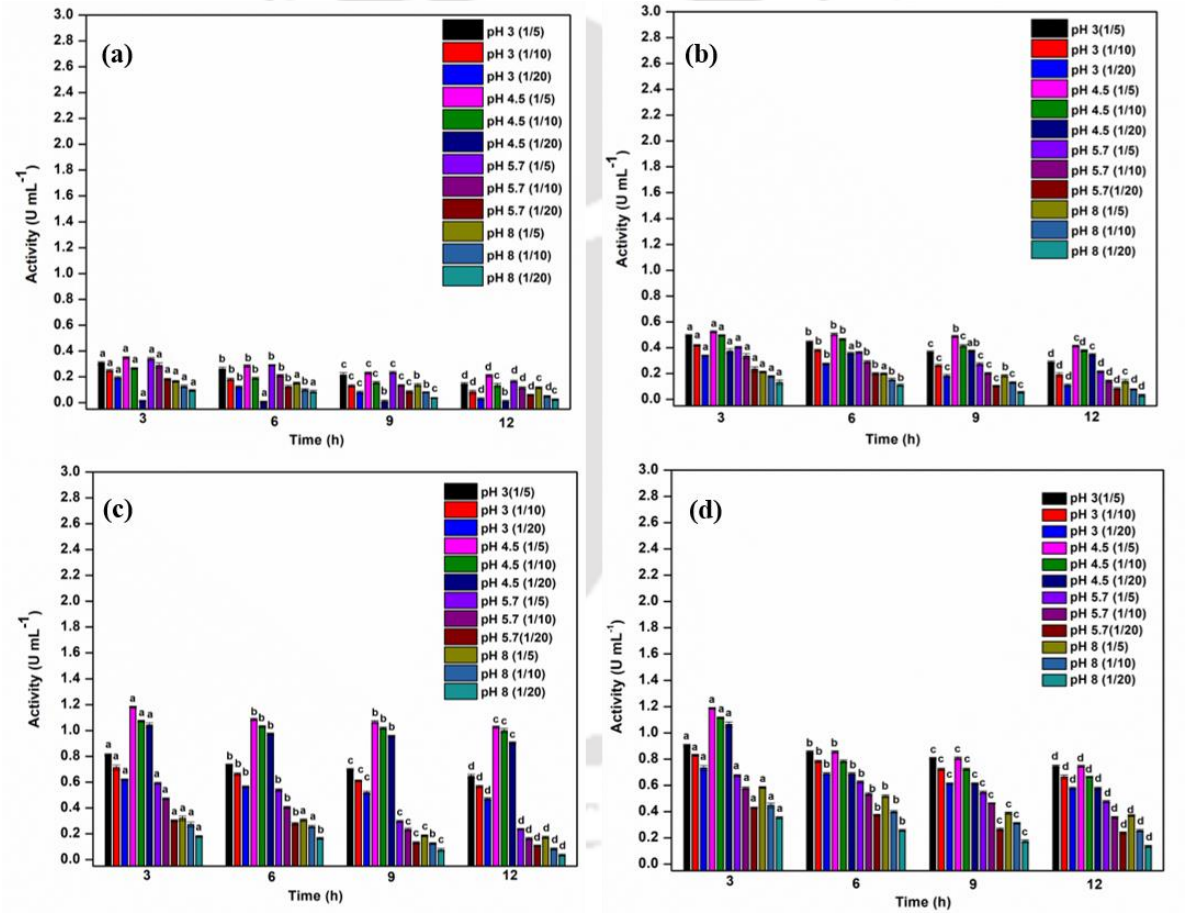


Fig. 3.3. Evaluation of laccase enzyme activity: (a) at pH 3.0, (b) at pH 4.5, (c) at pH 5.7 and (d) at pH 8.0 at a temperature of 30 ℃. Different letters in the column indicate significant difference at $p<0.05$ according to Duncan multiple range test.

In the subsequent steps, solid: solution affects measuring the maximum activity of laccase, and the order of magnitude is as follows 1:5> 1:10> 1:20; additionally, ABTS

concentration has a significant effect on determining the maximum activity of laccase. The order of magnitude of ABTS concentration is as follows: 1 mM> 0.6 mM> 0.2 mM> 0.05 mM. These optimized parameters indicate pH 4.5, time 3 h for extraction, solid: solution ratio 1:5 and ABTS concentration 1 mM to be the best conditions to determine maximum laccase activity. This optimized parameter measured laccase activity at around 1.185 U mL^{-1}. However, there was a slowdown in activity at alkaline pH 8.0. Thus, it is suggested that maximum laccase activity can be measured in acidic conditions compared to alkaline.

Research studies revealed acidic conditions to be in favor for recovery of laccase relative to alkaline conditions. In addition, laccase efficiency depends on the pH of the medium, redox mediators and functional groups present in the substrate. An essential factor in the breakdown of pollutants is optimal pH. Thus, pH can influence the enzyme's charge and shape, which are critical for substrate binding and access to the active site (Kumar et al., 2022; Lim et al., 2013; Singh et al., 2003).

Furthermore, ABTS concentration and the mechanism of the laccase-ABTS mediator system have a critical role in measuring laccase activity. Besides, other mediators, HBT and veratryl alcohol (VA) are detrimental to enzyme activity at high concentrations. In contrast, ABTS is the most stable and exhibits no loss of enzyme activity at a high concentration of ABTS (Ashe et al., 2016). In the present study, *P. florida* was stable at a high concentration of ABTS, whereas reports revealed that enzyme-mediator stability depends upon the species of fungi. Khlifi et al., (2010) reported deactivation of laccase enzyme from *Trametes trogii* by syringaldehyde mediator while stabilizing laccase from *Pycnoporus cinnabarinus*. In addition, the stability of ABTS in the solution can be linked with the mediator radical (radical cation or the dication form). The dication form of $ABTS^{++}$ is more stable at acidic pH and this dication form of ABTS causes substrate oxidation in solution (Fabbrini et al., 2002; Messerschmidt, 1997). Unlike other mediators, the ABTS oxidation mechanism follows the electron transfer pathway and forms a stable blue-green radical cation. However, in the present investigation, increasing concentration of ABTS from 0.05-1 mM generated an exponential increase in the laccase activity due to the absorption of $ABTS^{+}$ at 1 mM concentration of ABTS (Johannes and Majcherczyk, 2000). Recent findings also revealed that the lower concentration of ABTS showed decreased absorption in comparison to increased ABTS concentration up to 1-5 mM. Thus, the present investigation corroborates the earlier findings, and *P. florida* showed maximum laccase activity at 1mM concentration of ABTS. This observation indicates a deviation from typical Michaelis-Menten behavior. In standard Michaelis-Menten kinetics, enzyme

activity increases linearly with substrate concentration until it reaches a maximum (Vmax). However, in the case of laccase, the nonlinear response at higher ABTS concentrations suggests possible allosteric interactions or substrate stabilization effects that enhance activity exponentially up to 1 mM. This non-standard behavior further emphasizes the significance of acidic conditions for optimal enzyme efficiency.

Time and solid: solution ratio played a definite role in optimizing optimal conditions for laccase activity. Three hour was the maximum time for attaining maximum crude laccase activity with a solid: solution ratio of 1:5. Laccase activity increases with a higher substrate ratio in the buffer. Literature review revealed that laccase enzyme from SMW of fungi species like *Ganoderma lucidum, P. sajor caju, P. eryngii, P. pulmonarius, Lentinula edodes* for degradation of pollutants (Chang and Chang, 2016; Lau et al., 2003; Liao et al., 2012; Lim et al., 2013) are extracted based on time and solid: solution ratio where with the current finding it can be stated that 3 h is the economic time and 1:5 is the substrate extraction ratio to extract laccase enzyme from *P. florida* at temperature 30 °C. Therefore, we set it as the standard for further experiments with the above obtained optimal conditions for laccase activity.

3.4.2. Partial purification and protein identification

The crude enzyme was purified 1.74-fold following ammonium sulphate precipitation and dialysis yielded 83.54% purification of crude enzyme presented in the tabulated form of partial purification of laccase **(Table 3.1)**. Dialysis was used to eliminate the excess salt. (Kumar et al., 2016) reported 2.67-fold purification of laccase from the liquid culture of *Aspergillus flavus* by partial purification. Likewise, Hublik and Schinner, (2000) also reported a 1.4 fold purification of crude laccase from the liquid culture of *P. ostreatus*.

Table 3.1. Partial purification of laccase

Steps	**Enzyme activity**	**Protein (mg)**	**Specific activity (U mg^{-1})**	**Purification**	**Yield (%)**
Crude enzyme	1185 $\mu mol^{-1}min^{-1}$	30.20	39.23	1	-
Ammonium sulphate precipitation followed by dialysis	990 $\mu mol^{-1}min^{-1}$	14.30	69.23	1.74	83.54

Further, the molecular weight of partially purified laccase extracted from *P. florida* was approximately 45 kDa represented in **Fig. 3.4.** Meanwhile, the partially purified laccase showed major band with ABTS and the molecular weight was estimated to be 45 kDa which appeared to be same size as a result of the SDS-PAGE. However, the results are consistent with the earlier research studies. Purified laccase isolated from *Ganoderma lucidum* was measured 43 kDa (Shrestha et al., 2016). Similarly, purified laccase isolated from *P. ostreatus* (strain 10969) the molecular weight was determined 40 kDa (Liu et al., 2009). Contrarily, the molecular weight of four isozymes of laccase purified from *P. ostreatus* (strain V1884) were estimated 60 and 65 kDa (Mansur et al., 2003). Thereby, partial purification (ammonium sulphate precipitation followed by dialysis) can increase the purification fold of the laccase enzyme and further this partially purified laccase enzyme was used for degradation studies and this partial purification step confirmed that laccase enzyme is the sole responsible enzyme for degradation of fluoroquinolone antibiotics (particularly ciprofloxacin, levofloxacin and norfloxacin) considered for further studies.

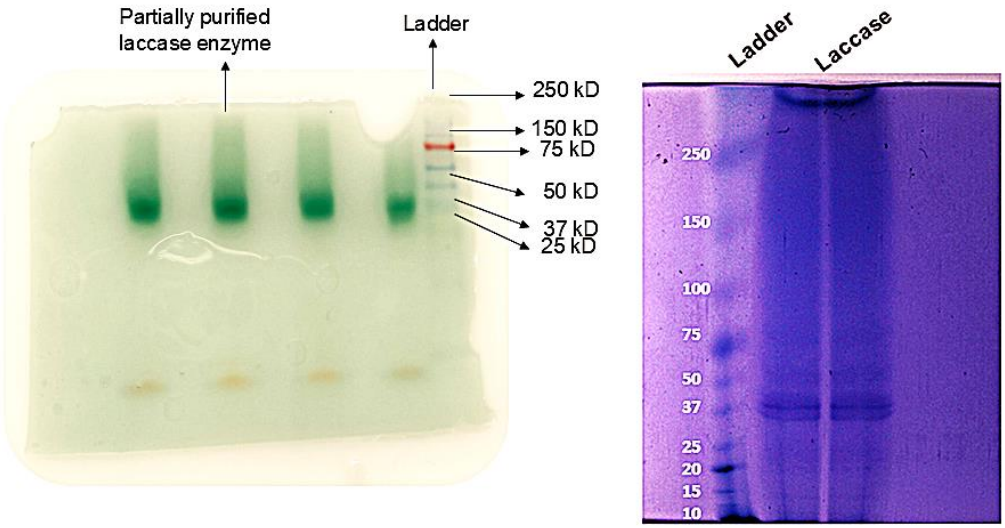


Fig. 3.4. Gel electrophoresis of laccase enzyme extracted from spent mushroom waste of *Pleurotus. florida* (a) Native-PAGE and (b) SDS-PAGE (10%) (Coomassie blue R-250 staining).

3.4.3. Enzyme characterization of partially purified laccase (pH, temperature, inhibitors and metal ions)

The one-step purification process purified crude laccase enzyme. The partially purified laccase enzyme was subjected to check stability and residual activity on free laccase at

distinct buffers in the range of pH (1-10), temperature (4 ℃-70 ℃), different inhibitors ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), sodium azide, beta-mercaptoethanol, urea and metal ions (Fe, Cu, Zn, Mn) **(Table 3.2)**. Residual activity was determined by subtracting maximum activity of laccase with the reduction in activity. Residual activity was determined against control, without any compounds. The control for inhibitors and metal ions was 100%.

3.4.3.1. pH stability

The partially purified laccase stability was checked at a varied pH range (1-10, 0.1M concentration) at 30 ℃ temperature **Table 3.2 (a)**. It was evident that pH influences the stability of the laccase enzyme. The highest laccase activity was observed at pH 4.5, which retained its activity at 100% for 1 h. There was a steep decrease in both activities and the stability of laccase from pH 7-10. The presence of hydroxide ions is notable in the decrease in enzyme activity and stability at higher pH levels. Higher pH levels cause hydroxide ions to bind to the type 1 center of copper ions, which prevents electron transfer and ends the reaction. The laccase enzyme stability and activity trend is observable, showing that laccase activity is higher and more stable at acidic pH levels (Margot et al., 2013). Contrary to alkaline pH, laccase recovery is typically done in acidic environment. Laccase being stable at acidic pH is based on the growing conditions of fungi in the natural environment, and the stability of free laccase varies from species to species. Nonetheless, purified laccase showed 85% stability at 20 ℃ under 8h incubation at acidic pH, whereas the activity of laccase diminished completely at pH 8.0 under 9h incubation (Kumar et al., 2022). Furthermore, earlier studies (Ghobadi Nejad et al., 2019; Kumar et al., 2016; More et al., 2011) also reported maximum stability and activity of laccase at pH (4-5). Therefore, for the application of enzymes commercially, enhancing stability and sustaining the requisite activity level over a long period of time are the two essential considerations.

3.4.3.2. Temperature stability

It was apparent that the laccase enzyme from *P. florida* was stable at pH 4.5, and partially purified laccase temperature stability was studied at pH 4.5 for 1 h at a temperature ranging from 4-70 ℃ represented in **Table 3.2 (b)**. The temperature profile of the laccase enzyme was most stable at 30 ℃, having residual activity of 100%. It is a pretty evident fact that at high temperatures, there is denaturation of protein activity which is perceptible in the present study at a temperature of 70 ℃. At temperature 4 ℃, the temperature is stable and

has a residual activity of 97.41%. Laccase activity was even thermostable from temperatures 45 ℃-65 ℃, as shown in **Table 3.2 (b).** High temperature reactions may be necessary to boost productivity and eliminate microbiological contaminations in industrial processes and biotechnological applications (Dogan and Tari, 2008). To better understand the mechanism of thermal inactivation and devise new methods for increasing enzyme stability, numerous researchers have focused on thermostable enzymes.

Studies reported the thermostability of laccase isolated from *Trametes versicolor* at temperature of 60 ℃ after pre-incubating the enzyme for 60 min, and initial activity was retained till 92% (Omeje et al., 2020; Stoilova et al., 2010). Although, the half- life of laccase is reported below 70 ℃ temperature for 1 h and 80 ℃ for 10 min from *Pycnoporus cinnabarinus* (Sadhasivam et al., 2008). Concerning the present study thermostable nature of the laccase enzyme from *P. florida* hold its residual activity of 83% at 65 ℃. Further, the laccase enzyme isolated from *P. osteratus* showed maximum laccase activity at temperatures 30-35 ℃ (Kumar et al., 2022). However, temperature stability from 30-35 ℃ was reported from *T. hazarinum*, *Aspergillus flavus* and *Phaerochaete chrysoporium* (Ghobadi Nejad et al., 2019; Kumar et al., 2016; Sadhasivam et al., 2008). Likely, we can conclude that 30-35 ℃ is the optimum temperature to obtain maximum laccase activity. On the contrary, laccase isolated from *Ganoderma lucidum* reported maximum activity at 4 ℃ (Liao et al., 2012) temperature stability varies from species to species depending on the source of the organism and the growing environment condition. Because of its robust thermostable nature, laccase enzyme is well known for degrading contaminants from the environment and various industrial processes, making it economically sustainable.

Table 3.2 (a) pH stability of partially purified laccase

pH	Enzyme activity (U ml^{-1})	Relative activity (100%)
1	0.211	17.95%
2	0.309	26.25%
3	0.914	77.52%
4	1.131	95.88%
4.5	1.180	100.00%
5	1.092	92.58%
6	0.782	66.39%
7	0.174	26.79%
8	0.314	14.69%
9	0.042	3.55%
10	0.018	1.55%

Table 3.2 (b) Temperature stability of partially purified laccase

Temperature (°C)	Enzyme activity (U ml^{-1})	Relative activity (%)
4	1.149	97.41
30	1.180	100.00
45	1.101	93.33
55	1.069	90.61
65	0.981	83.13
70	0.541	45.90

3.4.3.3. Effects of inhibitors

In the current findings effect of inhibitors (EDTA, DTT, sodium azide, beta-mercaptoethanol, urea) at two concentrations (2 mM and 10 mM) tabulated in **Table 3.2 (c)**. It was perceptible that EDTA at both 10 mM and 2mM concentration has the highest laccase activity and stability compared to other inhibitors. Reducing agents like beta-mercaptoethanol and dithiothreitol reduce disulfide bonds; therefore, there is an apparent inhibitory effect on laccase activity at 2 mM and 10 mM of beta-mercaptoethanol. Although, triton X-100 and other surfactants retain laccase stability after 8 days of incubation because of the increased interaction between hydrophobic molecules and enzymes (Liao et al., 2012). An earlier study reported an inhibitory effect of DTT at 0.2 mM concentration on laccase stability isolated from *Paraconiothyrium variabile* (Forootanfar et al., 2011). EDTA is a metal chelator that can bind with inorganic prosthetic groups attached to metalloenzymes which moderately inhibit the enzyme at high concentrations (Ghobadi Nejad et al., 2019). Likewise, sodium azide blocks the electron shuttle between the copper centers (T2 and T3), inhibiting laccase activity which agrees with the finding (Sadhasivam et al., 2008). The residual activity of laccase was measured after incubating with inhibitors for 30 min at a temperature of 30 °C, as displayed in **Table 3.2 (c).** However, research studies are compelled by the fact that EDTA has a less inhibiting effect on laccase activity than other inhibitors reported on different species of fungi (Ghobadi Nejad et al., 2019; Kumar et al., 2022; More et al., 2011; Sadhasivam et al., 2008).

3.4.3.4. Effect of metal ions

The metal ions had stimulatory as well as mildly inhibitory effects. Current investigation tested two concentrations of metal ions (Cu^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}), a 2 mM and 5 mM as shown in **Table 3.2 (d)**. Cu and Fe had a stimulatory effect at both concentrations, whereas

in the presence of Mn, there is a decrease in laccase activity. Compared with the current study, there was a decrease in laccase activity at a higher concentration of Cu, which agrees with the studies (Sadhasivam et al., 2008). In addition, the stimulatory effect of Cu in laccase activity is due to binding in the type 2 copper center (Nagai et al., 2002).

Furthermore, earlier findings also reported a decrease in the laccase activity of metal ions (Zn, Fe, Mn) for 7 days at 10 mM concentration. In contrast, Zn (10 mM) was not inhibited by laccase isolated from *Ganoderma lucidum*. The stimulatory effect of Zn and Fe has been reported in fungal species like *Cladosporium cladosporioide* (Ghobadi Nejad et al., 2019; Niladevi et al., 2008). On the contrary, in the earlier study, laccase isolated from *Ganoderma lucidum*, the minimum inhibitory concentration of Fe was found to be at 0.3 mM where only 10% laccase activity was lost despite 50% laccase activity diminished at 0.36% mM due to interference in the electron transport system of laccase (Murugesan et al., 2009). Even though, Ghobadi Nejad et al., (2019) reported 240% stability of laccase at 10 mM concentration of Fe. Following the current study, Fe had a residual of 93% activity at 2 mM concentration and 61% residual activity at 5 mM concentration. Additionally, Zn reported 11.4% inhibition at 5 mM on laccase activity isolated from *Tricoderma harzianum* (Sadhasivam et al., 2008). Similar to the findings activity of purified laccase isolated from *Lentinula edodes* was also inhibited by Zn in the presence of 1 mM concentration Couto et al., (2005) whereas, the current study reports 56% residual activity of laccase at 5 mM concentration. Furthermore, Zn also inhibited laccase isolated from *P.ostreatus*. Mn is a potent inhibitor of laccase and inhibits laccase activity up to 91% at 1mM concentration (Baldrian, 2004). Therefore, from the literature study and current investigation, it can be inferred that the laccase enzyme is less sensitive to Zn and Cu. Thus, the activation or inhibition effect of metal ions on the residual activity of laccase depends entirely on the fungal species. We can infer that the turnover rate of extracellular enzymes depends on the metal ions.

Table 3.2 (c) Effect of inhibitors on partially purified laccase

Inhibitors (2 mM)	**Relative activity (100%)**	**Inhibitors (10 mM)**	**Relative activity (100%)**
EDTA	122.44%	EDTA	88.84%
DTT	7.68%	DTT	1.73%
Sodium azide	14.35%	Sodium azide	5.23%
Beta-mercaptoethanol	1.63%	Beta-mercaptoethanol	0.74%
Urea	58.14%	Urea	9.96%

Table 3.2 (d) Effect of metal ions on partially purified laccase

Metal ions (2 mM)	Relative activity (100%)	Metal ions (5 mM)	Relative activity (100%)
Fe	93.74%	Fe	61.01%
Cu	139.68%	Cu	73.65%
Zn	84.19%	Zn	56.88%
Mn	20.77%	Mn	14.41%

3.4.4. Storage stability of the partially purified laccase extracted from spent mushroom waste of Pleurotus florida.

The storage stability of partially purified free laccase was evaluated on distinct buffers in the range of pH (3-10) and temperature (4-45 ℃). The residual activity was calculated (as mentioned in sub-sub section 3.4.3.) against control, and the control activity was 100%.

3.4.4.1. Storage stability during pH

The pH was a key factor throughout the 28 days of storage, and the results clearly indicated a decline in laccase activity. The relative activity of laccase decreased from 100% to 80.26 % percent during a 28-day storage study at a pH of 4.5. In contrast, the relative activity decreased from 28.69 to 10.58% from day 1 to day 28 at pH 10. At pH 4.5, laccase activity reached its peak, and at pH 10, it was at its lowest for the entire 28-day duration. Around 80%-50% of laccase activity was retained at acidic pH (3-4.5) for up to 28 days, while only 17%-10% was retained at basic pH (8-10) represented in **Fig. 3.5. (a)**. Further, the storage pH stability was confirmed with commercial laccase (*Trametes versicolor*) and it was observed that decline in laccase activity at pH 10 during 28-day duration (data not shown). However, the stability of pH retained at pH 4.5-6 in case of commercial laccase. This clearly, helps us to understand that the laccase extracted from SMW of *P. florida* sp. is stable at acidic pH for longer time. At higher pH level, hydroxide ions bind to the T1 copper ions and inhibits electron transfer, resulting end of reaction (Margot et al., 2013). The stability of laccase at acidic pH is due to the fact that laccase producing mycelia have a lower pH value because of their tendency to grow in acidic environment in natural ecosystem (Espinosa-Ortiz et al., 2022; Kumar et al., 2022). Research conducted by Kumar et al. (2022) revealed that laccase extracted from *P. ostreatus* is most stable at a pH of 4.5, whereas its activity reduces at pH (6-7). Despite the fact that higher laccase production

varies from species to species Ghose et al., (2023), study reported by Niladevi et al. (2007) observed 26.9 U g^{-1} activity of laccase at pH 8.0 extracted from *Streptomyces*.

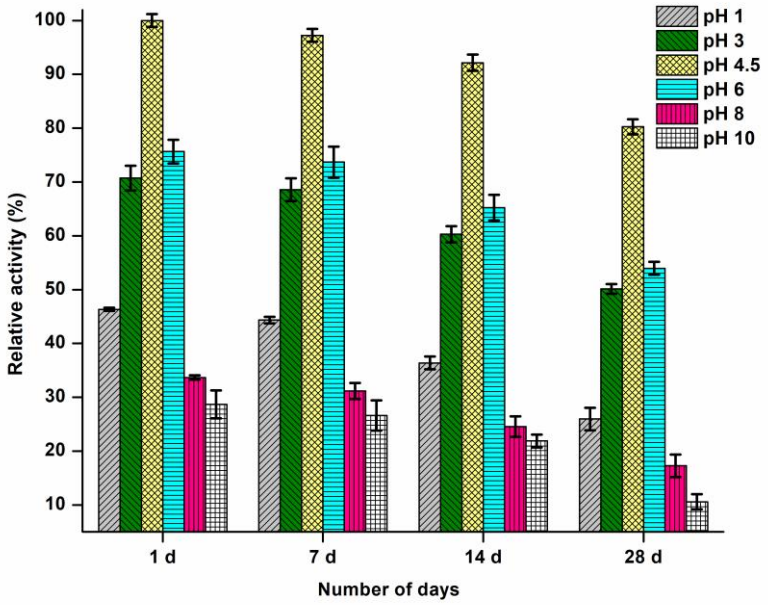


Fig. 3.5 (a) Storage stability of laccase derived from spent mushroom waste of *Pleurotus florida*: pH stability.

Similarly, research by Ghobadi Nejad et al. (2019) observed that at pH 4.0, 100% laccase activity (*Phanerochaete chrysosporium*) was retained after 2 h, while the activity was decreased at pH 9–12. Further, at pH 5, 22.54 U mL^{-1} of laccase activity was achieved after 1 h of incubation, while at pH 6, 17 U mL^{-1} of activity was retained (Kumar et al., 2016). Similarly, other research also reported maximum laccase at acidic pH with ABTS as substrate (More et al., 2011). Thus, results corroborate the findings from previous studies revealing that laccase extracted from *P. florida* is stable at pH 4.5.

3.3.2. Temperature stability during storage

It was evident from the results that denaturation of protein is achieved at higher temperature and previous research studies have reported the same. During 28 days of storage at 45 ℃, the laccase activity decreased, while there was a modest decrease from 14 days onwards at 4, 30, and 37 ℃ represented in **Fig. 3.6. (b)**. Since, pH stability of laccase in this study was maintained at pH 4.5, the samples were kept at pH 4.5 at varying temperature (4-45 ℃).

The results revealed the optimum temperature to maintain maximum laccase activity up to 28 days is from 4 and 30 ℃. Study by Kumar et al. (2022) reported maximum laccase activity for *P. ostreatus* was obtained at temperature 30-35 ℃. Further, other research studies have reported that at temperatures between 20 ℃ and 40 ℃, 100% laccase activity was retained for up to 1 h (Kumar et al., 2016; Sadhasivam et al., 2008). Because of its thermostability, it shows great promise in the realm of industrial biotechnology. Contrarily, *Ganoderma lucidum* reported laccase stability at 4 ℃ (Liao et al., 2012). However, *T. hirsuta* laccase remained stable at 50 °C for 8 h, exhibiting just a 50% decrease in enzyme activity (Navada and Kulal, 2021). Therefore, this study concludes that thermostability of laccase at 45 ℃ retaining 72.15% of the laccase activity till 28 days.

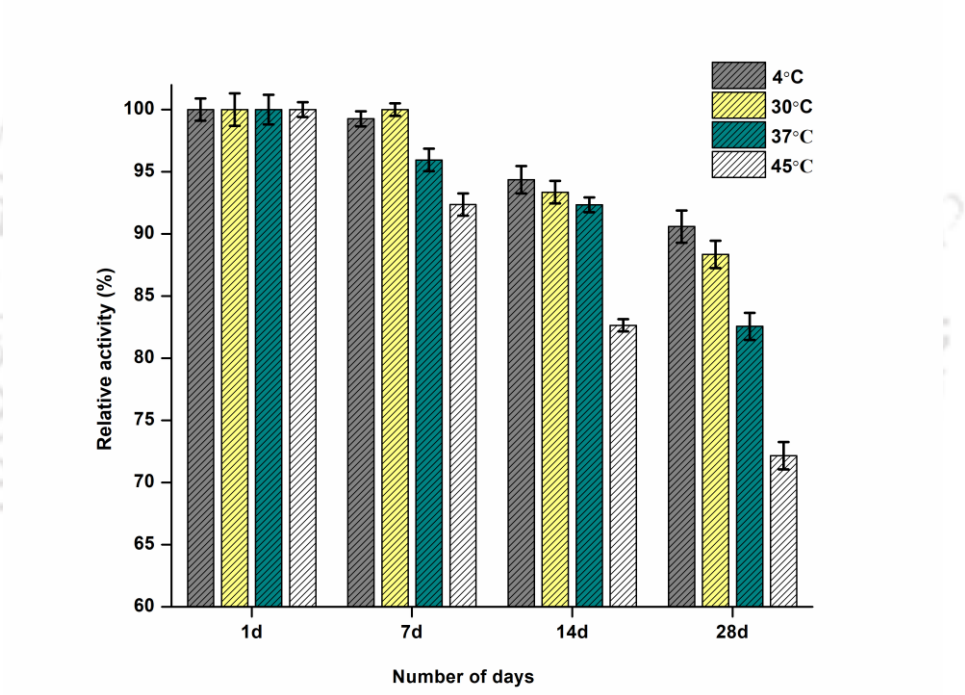


Fig. 3.6 (b) Storage stability of laccase derived from spent mushroom waste of *Pleurotus florida*: temperature stability.

3.4.5. Cost of production

The higher cost associated with the commercial laccase and laccase isolated from fungal culture, laccase extracted from SMW is a much more cost-effective option. Notably, the constraint behind in inclusive use of laccase enzyme from the SMW in the biodegradation of pollutants is the maximum production of laccase enzyme at a particular set of reaction conditions. This study also aimed to demonstrate the differences in the cost of production between commercial laccase and laccase enzyme extracted from SMW of *P. florida*

represented in **Table 3.3 (a-b)**. Earlier studies reported that SMW is less expensive than extracting laccase from fungal culture (Mathur et al., 2021). By employing crude extracellular laccase enzyme from SMW, 67% of the costs can be saved. Thus, the authors anticipate that this optimization of extracellular crude laccase enzyme from SMW will ease the scientific community's work, and further studies will be carried out on laccase enzyme from SMW for biodegradation of OMPs, which will be the cost-effective, sustainable, and one-pot solution.

Table 3.3 (a) Estimated cost of chemical consumption for extraction of crude laccase enzyme from SMW

Chemicals used	**Manufacturer**	**Quantity required for experimental purpose**	**Unit price**	**Cost (INR)**	**Cost (USD)**	**Enzyme activity**
Sodium citrate	Himedia	645.5 mg	1.65	1065.85	13.58	
Citric acid	Himedia	480.5 mg	0.49	235.26	2.99	1.185 U ml^{-1}
ABTS	Sigma	25.7 mg	101.34	2607.51	33.23	
Total				**3908.62**	**49.80**	

Table 3.3 (b) Estimated cost of chemical consumption for commercial enzyme

Chemicals used	**Manufacturer**	**Quantity required for experimental purpose**	**Unit price**	**Cost (INR)**	**Cost (USD)**	**Enzyme activity**
Sodium citrate	Himedia	645.5 mg	1.65	1065.85	13.58	
Citric acid	Himedia	480.5 mg	0.49	235.26	2.99	0.54 U ml^{-1}
ABTS	Sigma	25.7 mg	101.34	2607.51	33.23	
Commercial laccase	Sigma	20 U	455.00	9100.00	115.85	
Total				**13008.62**	**165.65**	

3.5. Conclusions

The results demonstrated that the parameters viz. pH, time, temperature and ABTS influence the activity of the extracellular laccase enzyme. The present investigation revealed that pH 4.5, 1 mM ABTS, and 1:5 solid: solution ratio at temperature 30 ℃ are the optimal reaction conditions for obtaining maximum laccase activity (1.185 U ml^{-1}). Evidently, from the earlier research studies, there were associated constraints for maximizing the laccase activity. Undoubtedly, the robust laccase enzyme has been widely

used in various biodegradation of OMPs. To the best of existing knowledge, laccase extracted from SMW of *P. florida* has been unexplored widely. The ambiguity remains in obtaining proper experimental conditions. Thus, this study beholds the notion of reducing ambiguity by considering the influential parameters to maximize laccase activity. Notably, the laccase enzyme extracted from SMW of *P. florida* is stable at different pH, metal ions, and a wide range of temperatures. Based on the earlier findings, it can be stated that the enzyme stability check differs from species to species.

SMW is likely to become an important agro-waste and an added concern to the existing environmental problems. But, if properly explored, SMW can be a future endeavour in the field of a circular economy. The comparison of cost economics between crude enzymes and commercial enzyme gives us insight that crude enzyme is a more effective and sustainable approach than commercial enzymes. Furthermore, research should be dedicated to utilizing different types of substrates using various mediators at different temperature conditions, increasing reproducibility and minimizing ambiguity. Therefore, as demonstrated by the existing literature, future research should be dedicated towards the utilization of SMW ligninolytic enzymes for biodegradation of recalcitrant organic micropollutants.

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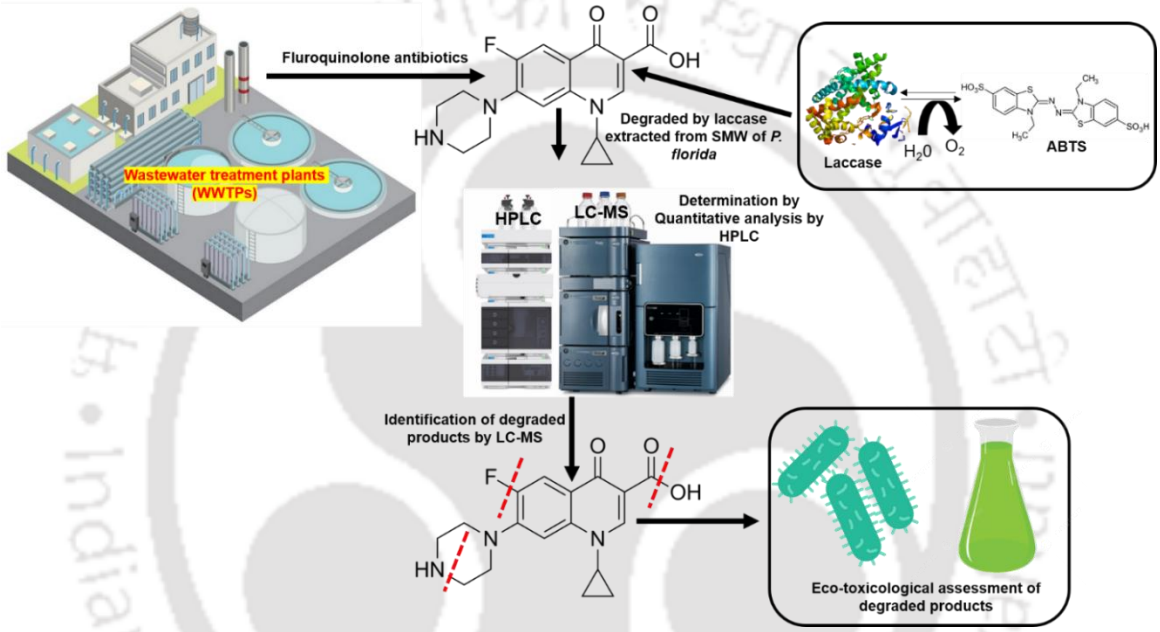
CHAPTER 4

To evaluate the potential of laccase enzyme in biodegrading fluoroquinolone antibiotics (ciprofloxacin, levofloxacin and norfloxacin) present in water.



"*This chapter deals with achieving degradation of fluoroquinolone antibiotics (ciprofloxacin, levofloxacin and norfloxacin) at optimal reaction conditions by employing laccase enzyme extracted from spent mushroom waste of Pleurotus florida. Identification and eco-toxicological assessment of degraded products.*"

4. To evaluate the potential of laccase enzyme in biodegrading fluoroquinolone antibiotics (ciprofloxacin, levofloxacin and norfloxacin) present in water.

4.1. Introduction

The indiscriminate and heedless usage of organic micropollutants (OMPs) has escalated globally. The fundamental cause for its emergence is egregious unapproved usage in humans and livestock. Their usage in metropolitan lifestyles to meet global needs has led to irrefutable damage to the human lives and aquatic ecosystems. Notably, these OMPs are engendered from natural and anthropogenic substances and their interaction with each other in WWTPs and contaminated soil poses risks to non-target species, including human and aquatic animals (Ghose and Mitra, 2022; Sathishkumar et al., 2021).

Among these OMPs, fluoroquinolone antibiotics glared antimicrobial resistant in *E.coli* and *S. pneumoniae* (Seok et al., 2018). Ciprofloxacin, levofloxacin and norfloxacin are broad-spectrum fluoroquinolone antibiotics. Despite its remarkable effectiveness, levofloxacin has been overused, which has led to a decline in the antibiotic's susceptibility to pneumonia. Additionally, it is a crucial medication for bacterial infections that have developed resistance to beta-lactam antibiotics (Endimiani et al., 2005; Maezawa et al., 2013). Studies have reported negative health effects such as nausea, vomiting, diarrhea, teratogenesis, and genotoxicity (Chen and Wang, 2020) due to its presence in WWTPs. They pose a potential threat to aquatic organisms as well (Chen and Wang, 2020). The unaltered form of ciprofloxacin (40-50%), levofloxacin (71%) and norfloxacin (30-90%) are eliminated through urine and faeces, making it important to introduce them as OMPs (McGregor et al., 2008; Paredes-Laverde et al., 2018; Thai et al., 2023). These antibiotics are present globally in ground water, common effluent treatment plants, river water, and lakes, indicating the failure of conventional and advanced treatment technologies (Castiglioni et al., 2006; Diwan et al., 2018; Ghosh et al., 2009; Golet et al., 2003; Ram and Kumar, 2020; Saxena et al., 2021; Vieno et al., 2007; Xu et al., 2007). Several treatment technologies have been employed to remove these antibiotics, such as electrochemical treatment (Cuprys et al., 2020; Pourzamani et al., 2018; Tang et al., 2022), advanced oxidation processes (Huang et al., 2021; Mondal et al., 2018), biological treatment (Hom-Diaz et al., 2017), photolysis (Snowberger et al., 2016; Zou et al., 2023), membrane bioreactor (Becker et al., 2016; Zou et al., 2023), and enzymatic degradation (Liu et al.,

2019). However, these methods have their own limitations, such as the generation of secondary toxic intermediates, higher cost, membrane fouling, and high energy consumption.

The use of SMW as a one-pot solution for managing agro waste and wastewater, while also effectively degrading OMPs like ciprofloxacin, levofloxacin and norfloxacin from WWTPs, is a highly promising approach. This study focuses on exploring this novel method further and aims to contribute to the ongoing efforts towards sustainability and efficient waste management.

SMW is a bulky lignocellulosic waste produced by the mushroom industry. Despite being considered as waste, SMW contains ligninolytic enzymes (peroxidases and laccases), which have the potential to degrade OMPs (Ghose and Mitra, 2022). SMW generates 5 kg of waste from 1 kg of fresh mushroom bags, and due to the lack of regulative policy, these bags are often composted (releases harmful gases such as methane, nitrous oxide, NH_3 and sulphur compounds) or incinerated in the open air. This practice leads in the contamination of soil, water and air which can affect the health of humans and animals (Leong et al., 2022; Sayara and Sánchez, 2021).

Further, studies have reported that whole fungal culture cells can be used to degrade recalcitrant OMPs such as tetracyclines, amoxicillin, sulfonamides, chloramphenicol, (Baran et al., 2021; Migliore et al., 2012; Navada and Kulal, 2019). However, the process can be inefficient for real-time applications due to the extensive time (7-14 days) required for cultivating fungal cultures and the need for optimization of parameters to achieve maximum laccase output and reciprocating OMP degradation (Mathur et al., 2021). Consequently, the expense of production inevitably increases. Various biotechnological approaches have not been realistically adopted due to their prohibitive production costs, extensive processing period of time, inhibitory effects on laccase activity, and slower degradation rates of OMPs (Mathur et al., 2021). However, the laccase buffer extraction process is faster and requires fewer process optimization conditions to achieve maximum laccase production reported in our own studies (Ghose et al., 2023). It's efficient, quick, cheap, and it accelerates up the degrading process without producing any secondary pollutants. Therefore, using laccase extracted from SMW is a great way to improve solid waste and wastewater management.

In light of these findings, there is a knowledge gap regarding the optimal reaction conditions necessary to achieve higher degradation or removal of OMPs. The novelty of this study lies in the fact that no research reports exist achieving degradation for

ciprofloxacin, levofloxacin and norfloxacin by tweaking parameters particularly pH (3-6), ABTS (0.05-1mM) and investigating degradation at two different temperature (30 ℃ and 50 ℃) and further investigating the fate of degraded metabolites for the antibiotics by administering laccase extracted from SMW of *P. florida*. Contrarily, research published by Becker et al., (2016) revealed that harmful byproducts were produced during enzymatic treatment with *Trametes versicolor* suggesting that enzymatic membrane bioreactors, a cutting-edge technique for water purification, are not practicable.

4.2. Materials and methods

4.2.1. Materials

Mushroom farm named Phantom in Guwahati, India, SMW bags of *Pleurotus florida* were procured (as mentioned in Chapter 3, sub-section no: 3.2.1.1). Ciprofloxacin (99.0% purity), levofloxacin (99.0% purity) and norfloxacin (99.0% purity), commercial laccase (*Trametes versicolor*) (>0.5 U mg^{-1}) and ABTS were procured from Sigma Aldrich, India. Sodium citrate, citric acid, sodium phosphate monobasic, and sodium phosphate dibasic were obtained from Merck India (>99.5% purity). Organic solvents such as methanol and formic acid were procured from Himedia, India (HPLC grade, ≥ 99.9%).

4.2.2. Methods

4.2.2.1. Extraction, enzymatic assay, and partial purification of laccase from spent mushroom waste of Pleurotus florida

Pleurotus mushrooms have a biological efficiency of 45.53 to 120.07%, grow in a broad range of temperatures and relative humidity, and can withstand high amounts of carbon dioxide. Results showed that *P. florida* grown on biomasses of wheat straw and wetland plant biomass had a greater number of proximate and biochemical characteristics, according to the studies (Elbagory et al., 2022). A high concentration of protein, both essential and non-essential amino acids, and moisture content are due presence of extracellular enzymes (laccase and peroxidases) in the SMW of *Pleurotus* sp. (Raman et al., 2021). In addition, studies revealed that when compared to *P. ostreatus*, *P. florida* has a greater yield, a shorter growing period, and better biological efficiency (Ahmed et al., 2009; Jafarpour and Eghbalsaeed, 2012). Hence, considering biological characteristics of *P. florida* extracellular laccase was extracted and partially purified from SMW of *P. florida*. In depth-description of extraction, enzymatic assay and partial purification of laccase has been detailed in (Chapter 3) and our own study (Ghose et al., 2023). The activity of laccase

enzyme obtained was 1.185 U mL^{-1} and laccase was purified 1.74-fold and purification yield obtained was 83.54%. Therefore, the extracted partially purified extracellular laccase was employed for studying degradation of ciprofloxacin, levofloxacin and norfloxacin.

4.2.2.2. Biodegradation of ciprofloxacin, levofloxacin and norfloxacin by partially purified laccase enzyme extracted from spent mushroom waste of Pleurotus florida

The degradation study was investigated in artificial wastewater. The artificial wastewater was prepared by the method described by (Zhao et al., 2017). The mixture was autoclaved at 121 ℃ for 15 mins. Prior to enzymatic treatments, physico-chemical parameters like colour, electrical conductivity (EC), pH, total dissolved solids (TDS), and chemical oxygen demand (COD), were assessed **(Table 4.1)**. The COD was determined using APHA protocol represented in **Eq. (4.1)** (Mathur et al., 2021; Sawyer et al., 2003).

$$COD\ (mg\ L^{-1}) = (A - B) \times M \times \frac{8000}{D} \qquad (4.1)$$

where, A= ferrous ammonium sulphate (FAS) used for blank, B= FAS used for sample, M= molarity of FAS, D= ml sample used, 8000 = milliequivalent weight of oxygen*1000 ml^{-1}.

Table 4.1 Artificial wastewater physico-chemical properties.

Parameter	Unit	Value
pH	-	5.8
COD	mg L^{-1}	221.5
TDS	ppm	168.5
Color	-	Milky white

The degradation of ciprofloxacin, levofloxacin, norfloxacin was studied by varying two parameters i.e. pH (3, 4.5 and 6) and ABTS concentration (0.05, 0.5 and 1mM) at two different temperatures 30 ℃ and 50 ℃. Ciprofloxacin and norfloxacin standard solutions were prepared in 0.1M formic acid of varying concentration ranging from 5 µg mL^{-1}, 15 µg mL^{-1}, 25 µg mL^{-1} and 50 µg mL^{-1}. Whereas levofloxacin standard solution was prepared in citrate buffer. The degradation experiment was commenced by using 1.18 units of partially purified laccase enzyme. The volume of total reaction mixture was 4 mL and reaction mixture were kept at shaker incubator for 12 h at constant 180 rpm. The degraded samples were collected at equal time interval (0, 3, 6, 9 and 12 h) and the reaction was terminated by adding methanol (HPLC grade). Additionally, to exclude the possibility of

self-degradation during incubation, appropriate control samples such as positive control (partially purified laccase and ABTS) and negative control (ABTS and ciprofloxacin, levofloxacin and norfloxacin at varied concentration mentioned above) were incubated at 30 ℃ and 50 ℃ to ensure no self-degradation for both the antibiotics. The degradation efficiency was estimated by using **Eq. (4.2)**

$$Degradation\ (\%) = 100 \times \frac{C_0 - C_t}{C_o} \qquad (4.2)$$

where, C_t final concentration (µg mL^{-1}) and C_0 is the initial concentration.

4.2.3. Quantitative analysis by high performance liquid chromatography (HPLC)

The residual concentrations of ciprofloxacin, levofloxacin and norfloxacin was quantitatively determined by high-performance liquid chromatography (HPLC) using an Agilent system (Agilent technologies, Product no: G7114A) consisting quaternary pump system, an automatic sampler, and a column oven. Mobile phase consisted of two eluents phosphoric acid: methanol (65:35) and both the antibiotics were separated in C18 column (250×4.6 mm×5 µm particle size) with a flow rate of 0.5 mL min^{-1} and injection volume 20 µl. Ciprofloxacin, levofloxacin and norfloxacin was detected in UV range of 278 nm, 294 nm and 279 nm.

4.2.4. Identification of degraded or bio-transformed products

The degraded products of ciprofloxacin, levofloxacin and norfloxacin were analyzed by using liquid chromatography mass spectrometry (LC-MS). Waters Acquity ultra-performance liquid chromatography (UPLC) system with Xevo QTof mass spectrometer (Waters corp., Milford, MA, USA) was used to detect the products. Acquity UPLC BEH C18 column (2.1 × 100 mm, particle size 1.7 µm, pore size 130 Å, Waters, #186002352) with BEH C18 Van Guard Pre-Column (2.1 × 5 mm, particle size 1.7 µm, pore size 130 Å, Waters, #186003975) were used for LC. Mobile phase A was purified water containing 0.1% formic acid and mobile phase B was methanol containing 0.1% formic acid. The gradient condition was as follows: 0 min-1 min, 5% B; 1 min-10 min, 5% B to 99.9% B, linear gradient; 10 min – 12 min, 99.9% B; 12 min-15 min, 5% B. Injection volume was 10 µL for all samples. The analytes were ionized by positive ESI. The scan range was 50-1000 *m/z*. The settings for the mass spectrometer were as follows: capillary voltage, 2.5kV; sampling cone, 40.0 (arbitrary value); extraction cone, 4.0 (arbitrary value); source temperature, 125°C; desolvation temperature, 350°C; cone gas flow, 50 Lh^{-1}; and

desolvation gas flow, 1000 Lh^{-1}. The acquired data were analyzed using Masslynx and Markerlynx software (Waters Corp).

4.2.5. Eco-toxicological assessment

4.2.5.1. Residual anti-bacterial activity assay

The degraded products that are formed after degradation of antibiotics is of high concern to confirm the anti-bacterial activity to determine the toxicity of the formed products. The residual anti-bacterial activities of degraded products and antibiotics were determined by agar-well diffusion method both in gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*E. coli*). Luria Bertani (LB) agar was prepared and autoclaved at 121 ℃. The LB agar plates were made and bacterial culture was spread evenly in the plate with the help of sterile glass spreader. Further, using cork borer wells were made of 6mm. 100 µL of degraded sample of the corresponding antibiotics and positive control sample (ciprofloxacin and norfloxacin of concentration 5 µg mL^{-1}) were placed in each well. Antibacterial activity was evaluated by calculating zone of inhibition (ZOI). Diameters of ZOIs were measured and compared with the positive control. The autoclaved milli-Q water was used as a negative control. All the experiments were performed in triplicates to minimize errors and the average values if the measurements were recorded. **Eq. 4.3** represents zone of inhibition for antimicrobial activity (ZOI)

$$\textit{Zone of Inhibition (ZOI)} = 100 - \frac{(ZOI\ control - ZOI\ sample)}{ZOI\ control} \times 100 \qquad (4.3)$$

4.2.5.2. Algal toxicity test of degraded products

The algal toxicity of degraded products was investigated on algal strain *Scenedesmus abundans.* This strain was acquired from the Integrated Bioprocessing lab (Biosciences and Bioengineering department) IIT Guwahati. Microalgae culture was maintained with the following composition of media followed from (Mahesh et al., 2019). The algal toxicity test was conducted in an Erlenmeyer flask with sterile media containing 1% algal suspension and 2 mL of antibiotic sample (degraded by laccase) had been added in the solution. The total reaction volume was 50 mL. The flask was kept for shaking continuously at 150 rpm and exposed to white light for 7 days at 27 ℃. The toxicity test was tested on 15 µg mL^{-1} degraded sample as well as on the standard (positive control). An appropriate control sample containing only media and 1% microalgae was also cultivated in the incubator. Additionally, cultures treated with potassium dichromate at a dosage of 4 mg

mL^{-1} were used as negative control. Potassium dichromate has been reported to suppress the growth of algae (Sun et al., 2021). Microalgal culture with OD_{680} of 0.1-0.7 was centrifuged at 13000 rpm for 10 min. Lysis of cell pellet was done with 1.5 ml of methanol by incubating in a water bath at 45 ℃ for 30 min. Then the sample was centrifuged at 13000 rpm for 10 min and absorbance at 480 nm, 652 nm, 665 nm and 680 nm was measured using a visible spectrophotometer (Thermo Fisher) (Mahesh et al., 2019). Dry cell weight (Rai and Gupta, 2017; Xiong et al., 2017a), chlorophyll-a (Mahesh et al., 2019) and chlorophyll-b (Mahesh et al., 2019) were quantified after cultivation in the degraded as well as control samples according to the following **Eq. (4.4)**, **(4.5)** and **(4.6).**

$$Dry\ cell\ weight\ (g\ L^{-1}) = \frac{mass\ of\ culture}{volume} \quad (4.4)$$

$$Chlorophyll\ a\ (mg\ L^{-1}) = 16.5169A_{665} - 8.0962A_{652} \quad (4.5)$$

$$Chlorophyll\ b\ (mg\ L^{-1}) = 36.92A_{652} - 9.28A_{665} \quad (4.6)$$

4.2.6. Statistical analysis

All experiments were conducted in triplicates. Analysis of variance (ANOVA) was conducted by employing Duncan test to determine statistical significance. The analysis of variance was conducted using SPSS statistics software. Statistical significance was determined by *p*-values less than 0.05.

4.3. Results and discussion

The degradation of ciprofloxacin, levofloxacin and norfloxacin was obtained at 3 h incubating the sample at varying pH (3-6), ABTS concentration (0.05-1 mM) at two different temperature 30 and 50 ℃. Notably, levofloxacin degradation was achieved both in presence and absence of ABTS whereas low degradation was observed for ciprofloxacin and norfloxacin in absence of ABTS (data not shown). The present degradation study was carried out to understand the influence and mechanistic effect of biodegradation in the presence and absence of a mediator**.** Therefore, degradation for ciprofloxacin and norfloxacin was studied in presence of ABTS and levofloxacin studied both in presence and absence of laccase-ABTS system. The optimal reaction conditions were achieved at temperature 30 ℃, pH 4.5 and ABTS concentration of 0.05 mM. **Fig. 4.1** represents surface and contour plot analysis for ciprofloxacin, levofloxacin and norfloxacin; these results are in agreement with the experimental values, and the results indicate that the reaction

conditions with the highest degradation attained were around pH 4.5, 0.05-0.6 mM ABTS, and 30 ℃. The quadratic regression equation was obtained from the experimental data for ciprofloxacin, levofloxacin and norfloxacin represented in **Eq. (4.7), (4.8)** and **(4.9)** and ANOVA table tabulated in **Table 4.2. (a-c)** confirms the significance of quadratic equation (p value < 0.05) obtained from MINITAB 18 (version 1.0) software**.**

$$\textit{Ciprofloxacin degradation (\%)} = 98.60 - 1.122\, tempearature + 7.90\, pH + 2.61\, ABTS - 0.916\, pH \times pH - 20.33\, ABTS \times ABTS + 0.0165\, tempearture \times pH - 0.034\, tempearture \times ABTS + 0.0861\, pH \times ABTS \quad (4.7)$$

$$\textit{Norfloxacin degradation (\%)} = 75.3 - 1.233\, tempearature + 19.88\, pH + 2.06\, ABTS - 2.428\, pH \times pH + 1.43\, ABTS \times ABTS + 0.0284\, tempearture \times pH - 0.156\, tempearture \times ABTS - 1.029\, pH \times ABTS \quad (4.8)$$

$$\textit{Levofloxacin degradation (\%)} = 62.85 - 0.089\, temperature + 9.25\, pH + 16.34\, ABTS - 0.880\, pH \times pH - 26.73\, ABTS \times ABTS - 0.0378\, temperature \times pH - 0.3086\, temperature \times ABTS + 0.937\, pH \times ABTS \quad (4.9)$$

4.3.1. Laccase degradation of ciprofloxacin antibiotic

The degradation experiment commenced on artificial wastewater revealed that maximum degradation of ciprofloxacin (5 µg mL^{-1}, 15 µg mL^{-1}, 25 µg mL^{-1} and 50 µg mL^{-1}) were achieved under the following reaction condition: temperature 30 ℃, pH 4.5 and ABTS concentration 0.05mM. Ciprofloxacin (5 µg mL^{-1}), 86.21% degradation was achieved at pH 4.5, temperature 30 ℃ and 0.05 mM ABTS concentration whereas at pH 3, 83.21% degradation were achieved. Similarly, at pH 8 and 0.05 mM ABTS concentration 72.57% degradation were achieved. Following 15, 25 and 50 µg mL^{-1} degradation of 84.49%, 81.91% and 75.94% was achieved at pH 4.5, 0.05 mM ABTS concentration and at 30 ℃. Notably, decrease in degradation have been observed at pH 3 and pH 8 along with increase in ABTS concentration (0.5 mM and 1mM). At pH 3, 1 mM ABTS concentration, 5 µg mL^{-1} ciprofloxacin concentration, degradation of 67.49% was attained. Similarly, at pH 8, 1 mM ABTS concentration 64.26% of degradation was achieved. At temperature 30 ℃, a decrease in degradation was observed for 15, 25, and 50 µg mL^{-1}. For 50 µg mL^{-1}, the degradation was found to be lowest at pH 8, 1 mM ABTS concentration at 30 ℃.

Degradation of ciprofloxacin was also noted at 50 ℃. Correspondingly, at pH 4.5, 0.05 mM ABTS concentration degradation of 63.93% was achieved for 5 µg mL^{-1} whereas at pH 3 and pH 8, 0.05mM ABTS concentration 57.17% and 55.23% degradation were observed. Likewise, 61.16%, 59.25 and 47.55% of degradation was observed for 15, 25 and 50 µg mL^{-1}. The degradation rate decreased as the ABTS concentration increased from 0.6 mM to 1 mM, regardless of the temperature. At 50 ℃ and 1 mM ABTS concentration, the degradation of 23.36% was attained for 50 µg mL^{-1} as shown in **Fig. 4.2.**

4.3.2. Laccase degradation of levofloxacin antibiotic

Levofloxacin degradation was achieved at 3 h both in laccase-ABTS system and in absence of ABTS at 30 ℃ and 50 ℃. However, in laccase-ABTS system, different concentrations of ABTS played a significant role in degradation. Notably, it was observed that at higher concentrations of ABTS degradation rate decreases. The optimal concentration of ABTS to produce maximal degradation was determined to be 0.05 mM in the present investigation as shown in **Fig. 4.3 (a-j).** In presence of ABTS, 0.05 mM ABTS concentration, 30 ℃ and pH 4.5 80.23% degradation efficiency was observed whereas at 0.6 mM ABTS 73.31% degradation was observed for 5 µg mL^{-1}. Similarly at pH 3 and pH 8, 0.05 mM ABTS concentration 76.21% and 70.46% of degradation was observed. At 1mM ABTS concentration at pH 4.5 (63.17%), pH 3 (60.49%) and pH 8 (57.58%) of degradation was achieved. Further for 15, 25 and 50 ug mL^{-1}, 0.6 mM ABTS and at pH 4.5 the degradation of 78.40%, 75.81% and 67.58% was achieved whereas at pH 8, 62.15%, 53.17% and 46.20% was achieved at 30 ℃. Likewise at temperature 50 ℃, pH 4.5 and 0.05 mM ABTS, 75.21%, 70.42, 66.70 and 60.58% degradation were achieved at 5, 15, 25 and 50 µg mL^{-1} whereas at 1mM ABTS, 50.17%, 46.47%, 39.44% and 35.35% of degradation was attained. At 0.6 mM, degradation efficiency of 68.17%, 65.56%, 60.68% and 56.61% was achievable. The lowest degradation efficiency (25.45%) was observed at pH 8, 1mM ABTS concentration at 50 ℃ as represented in **Fig. 4.3 (a-e)**

Moreover, levofloxacin degradation efficiency was observed in absence of laccase-ABTS system at both 30 and 50 ℃ at above-mentioned concentration. The highest degradation efficiency (82.23%) was achieved at pH 4.5 and temperature 30 ℃ whereas lowest degradation efficiency (30.32%) was achieved at 50 ℃ and at pH 8. The results evidence us with the fact that functional groups present in the levofloxacin structure are susceptible towards laccase degradation in absence of laccase-ABTS system. In absence of ABTS, pH played a decisive role in attaining maximum and minimum degradation

efficiency. At pH 8 decrease in degradation rate was observed for 5 µg mL^{-1} (74.23%), 15 µg mL^{-1} (70.92%), 25 µg mL^{-1} (65.45) and 50 µg mL^{-1} (57.48%) at 30 ℃ whereas at pH 3, 76.21% (5 µg mL^{-1}), 74.31% (15 µg mL^{-1}), 70.19% (25 µg mL^{-1}) and 64.56% (50 µg mL^{-1}) degradation was attained. Similar trend in decrease of degradation have been observed at temperature 50 ℃ represented in **Fig. 4.3 (f-j)**.

4.3.3. Laccase degradation of norfloxacin antibiotic

The degradation attained for norfloxacin at pH 4.5, 0.05 mM ABTS concentration at 30 ℃ was 83.27%. Similarly, like other two fluoroquinolone antibiotic the maximum degradation efficiency of norfloxacin was achieved at pH 4.5, 0.05 mM ABTS concentration.

At pH 3, 0.05 mM ABTS, 5 µg mL^{-1}, 79.56% of degradation was achieved whereas at pH 8, 70.56% degradation was achieved. Degradation of 77.26%, 72.63% and 63.54% was achieved for 15, 25 and 50 µg mL^{-1}. However, at pH 8, 0.05 mM ABTS concentration degradation of 70.56%, 67.24%, 63.18% and 57.26% was observed for 15, 25 and 50 µg mL^{-1} at 30 ℃. The remarkable decrease in degradation was observed at 0.6 mM and 1 mM ABTS concentration. At pH 4.5, 0.6 mM ABTS concentration, 5 µg mL^{-1}, 77.24% degradation was achieved whereas at pH 3, 74.23% and pH 8, 70.56% degradation was observed. Likewise, for 5,15, 25 and 50 µg mL^{-1} at pH 8, 0.6 mM ABTS degradation of 67.23%, 64.56%, 60.12% and 53.65% was achieved. However, for 1 mM ABTS concentration decline in degradation was observed at pH 3, 4.5 and 6. Norfloxacin degradation was also investigated at 50 ℃. The maximum degradation of 58.93% was achieved at pH 4.5 and 0.05 mM ABTS concentration whereas lowest degradation (28.20%) was achieved at pH 8, 1 mM ABTS concentration. At 50 ℃, a similar trend of degradation was observed as shown in **Fig. 4.4.** Degradation rate decreased at 50 ℃ with increasing ABTS concentration.

Results showed that degradation of 54.96% at pH 3 and 50.24% at pH 8, with 0.05 mM ABTS concentration was attained. Likewise, at pH 3 and pH 8, degradation decrease at concentrations of 15, 25, and 50 µg mL^{-1} of norfloxacin as well.

Therefore, results apparently revealed that pH and ABTS were crucial in attaining optimum degradation efficiency for three fluoroquinolone antibiotics. The significant impact of pH has been observed in achieving maximum degradation rate. Since the binding of the substrate to the active site of the enzyme is affected by the change in the charge and structure of the enzyme caused by the pH, we found that at irrespective of temperature at 30 ℃ and 50 ℃, the degradation efficiency decreased. This is because pH plays a critical

factor in laccase and substrate properties (Guardado et al., 2019). The T1 site of copper present in laccase is responsible for ionizable state, regardless the degradation rate also depends on the ionization and physical state of the compound (Margot et al., 2013; Xu, 1997). Prior research studies reported degradation of aniline and phenolic compounds by laccase achieved at pH (5-6) depends on the redox potential difference between the compounds and laccase (Margot et al., 2013). However, at higher pH, hydroxide ions binds with the T2/T3 copper ions thus resulting in the inhibition of electron transfer (Joshi et al., 2000; Xu, 1997). Contrarily, studies reported by Kumar et al. (2022) and Lim et al. (2013) evidenced us with the fact that laccase enzyme is more stable at acidic pH rather than in alkaline conditions. Consistent with the previous research, in this study antibiotics degraded more rapidly at pH 4.5, and it also provided evidence that laccase isolated from SMW of *P. florida* remains stable at pH 4.5 (Ghose et al., 2023).

Besides, pH, ABTS concentrations also plays a decisive role in achieving higher degradation efficiency and at higher concentration of ABTS decrease in degradation efficiency was observed. The probable reason could be saturation effect of mediator (Navada and Kulal, 2019). Previous research studies have also reported that at higher concentration of ABTS (3,5 and 10 mM), degradation of chloramphenicol was not achieved by *Trametes hirusta* whereas, at 0.25 mM ABTS 82% degradation of chloramphenicol was achieved (Navada and Kulal, 2019). Correspondingly studies reported by Margot et al. (2013) speculate that at higher concentration of ABTS, reactive radical cation of ABTS are formed that interact with each other, rather than with the pollutants which leads to decrease in degradation rate at higher concentration of mediator. $ABTS^{2+}$ radical bind in the enzymatic pocket of laccase and forms laccase-ABTS system and hence oxidation of pollutant takes place in few hours. Further, ABTS employment resulted in 84.96% degradation of ciprofloxacin, 85.82% degradation of norfloxacin and 100% degradation of sulfamethaxzole was achieved at 12 h and 72 h of incubation by the fungal cell of *Phanerochaete chrysosporuium* and *P. sanguineus* (Gao et al., 2018). Contrarily, research studies revealed degradation of carbamazepine (Tran et al., 2010), clofibric acid (Tran et al., 2010), diclofenac (Zhang and Geißen, 2010) and estrone (Lloret et al., 2010) were not achieved after addition of ABTS. Nevertheless, the catalytic properties of mediator are pollutant specific.

Nevertheless, the degradation rate of pollutants is also implied by electron donating groups (EDGs) and electron withdrawing groups (EWGs). Here in this study, ciprofloxacin contains both EDG hydroxyl (-OH), amine ($-NH_2$) and alkyl group and EWG carboxylic

(COOH) and halogen (fluorine) (Parra Guardado et al., 2019). The combined effect of both EWG and EDG leads to higher removal of pollutants in presence of mediator. Research studies reported combined effects of EWG and EDG of pollutants such as naproxen (Marco-Urrea et al., 2010; Rodarte-Morales et al., 2011), ibuprofen (Tran et al., 2010), sulfonamide (Accinelli et al., 2010) and diclofenac (Rodarte-Morales et al., 2011; Tran et al., 2010) helped to achieve 80-100% degradation of concentration ranging from 0.01 mg L^{-1} to 10 mg L^{-1}. Similarly, due to presence of EDG (-OH) group in diclofenac (Ruiz-Dueñas et al., 2008) and amoxicillin (Parra Guardado et al., 2019) the degradation rate was achieved in 15 min to 1 h of incubation. However, the aromatic ring of norfloxacin has an EWG group (-COOH) and a halogen (fluorine), and the combined effects of the two groups led to an 83.27% degradation efficiency. Furthermore, the likely explanation of levofloxacin degradation in absence of laccase-mediator system is derived by the fact that levofloxacin has strong EDG (hydroxyl) and EWG (fluorine group), which facilitates its degradation within 3 h of incubation. Contrarily, research studies reported low degradation rate of pollutants containing both EWG and EDG such as fluoxetine (Eibes et al., 2011), diuron, atrazine, and terbuthylazine (Bending et al., 2002). The likely explanation could be the absence of strong EDG (phenolic or aromatic amine groups) and presence of strong EWG (-Cl) (Torres-Duarte et al., 2009; Yang et al., 2013).

Therefore, the fundamental findings from this study are that irrespective of the presence of ABTS, laccase has its own potential to degrade levofloxacin antibiotic because of the presence of susceptible functional groups and degradation efficiency is achieved at an economical time, which supports the notion in cost reduction where the use of mediator can be eliminated. Additionally, the degradation efficiency achieved at 50 °C points towards thermotolerance of laccase extracted from SMW of *P. florida*. However, this study postulates that optimal experimental conditions particularly, temperature, mediator concentration, reaction durations, and pH influence the degradation efficiency. Furthermore, the reason behind variations in degradation efficiency depends on the chemical constituents present in pharmaceutical compounds.

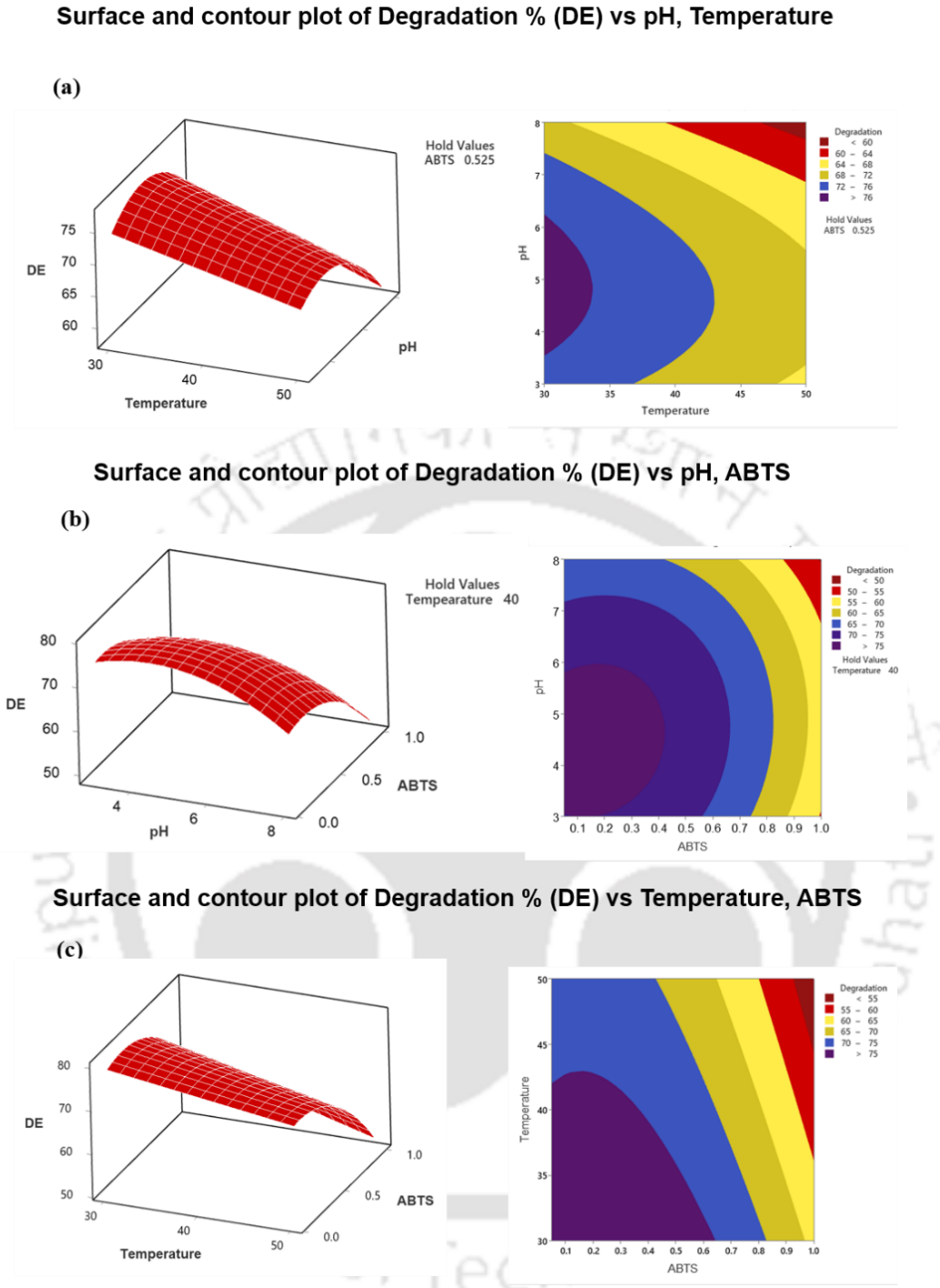


Fig. 4.1 (a) Surface and contour plots for ciprofloxacin at temperature 30 ℃, pH 4.5 and ABTS (0.05 mM concentration): (a) Degradation (%) vs pH, temperature; (b) Degradation (%) vs pH, ABTS; (c) Degradation (%) vs temperature, ABTS.

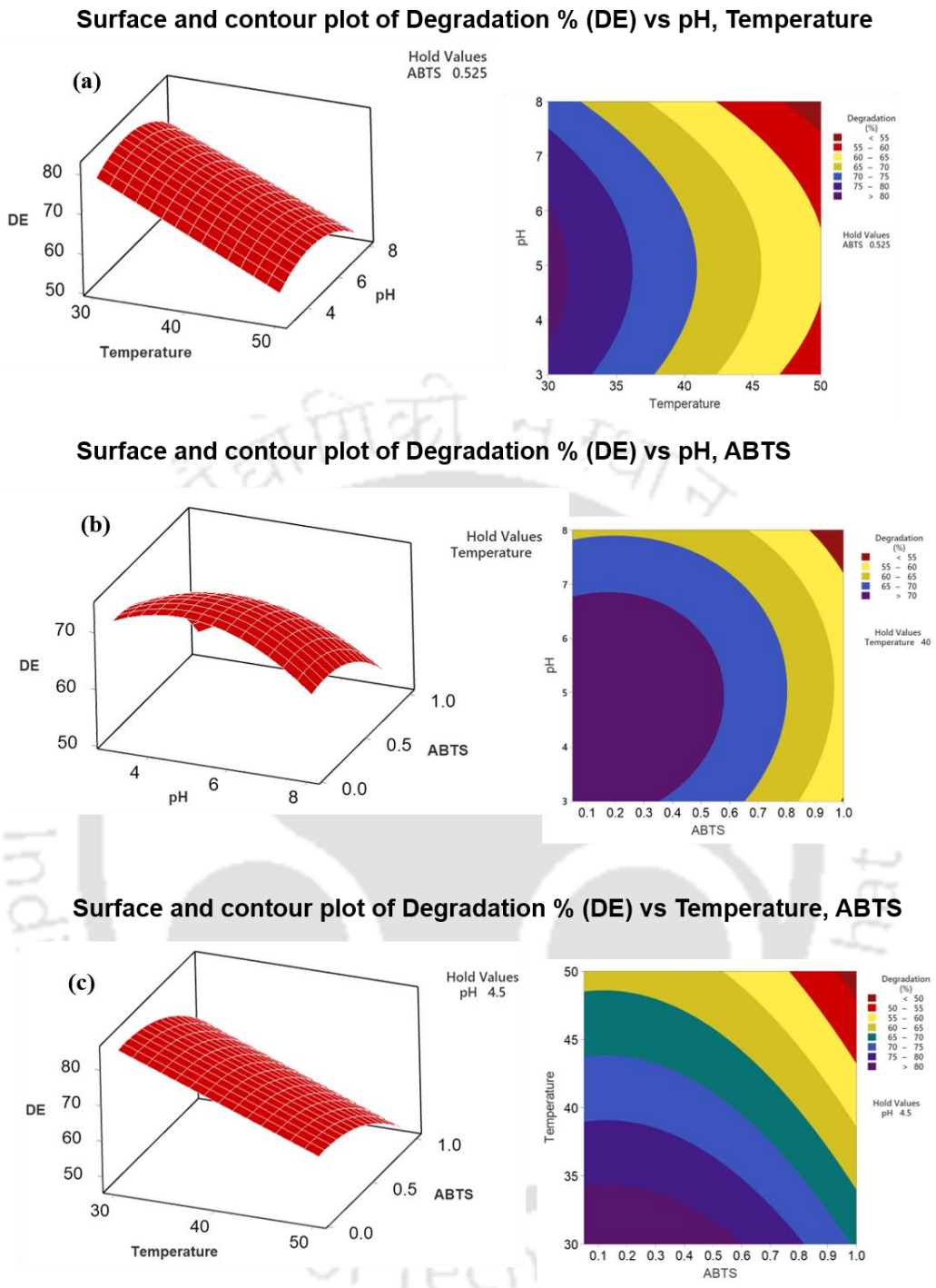


Fig. 4.1 (b) Surface and contour plots for levofloxacin at temperature 30, pH 4.5 and ABTS (0.05 mM concentration): (a) Degradation (%) vs pH, temperature; (b) Degradation (%) vs pH, ABTS; (c) Degradation (%) vs temperature, ABTS.

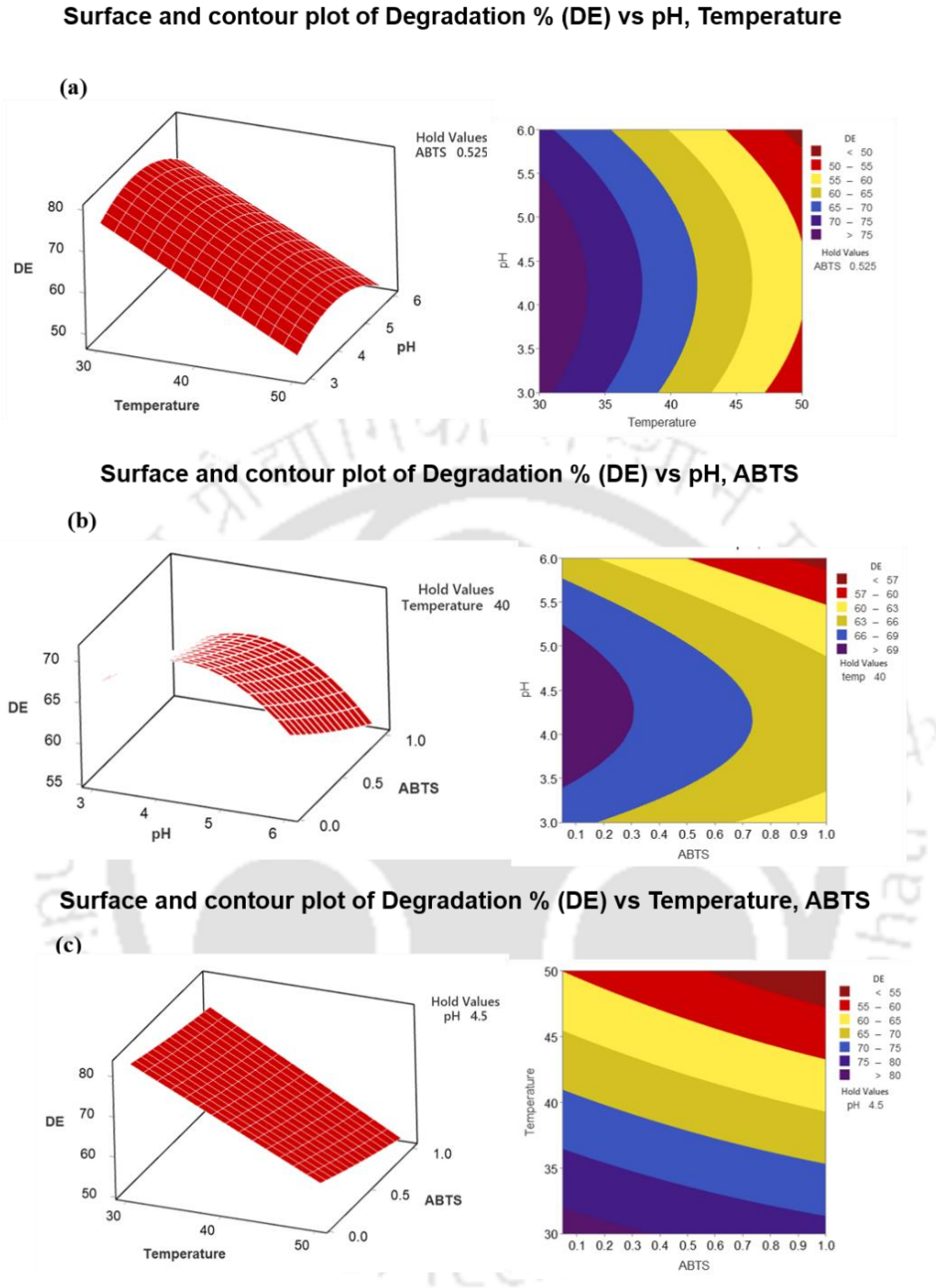


Fig. 4.1 (c) Surface and contour plots for norfloxacin at temperature 30, pH 4.5 and ABTS (0.05 mM concentration): (a) Degradation (%) vs pH, temperature; (b) Degradation (%) vs pH, ABTS; (c) Degradation (%) vs temperature, ABTS.

Table 4.2 (a) Ciprofloxacin degradation study using Analysis of Variance (ANOVA).

Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	***P-Value***
Model	8	2942.35	367.79	64.36	0.000
Linear	3	2508.54	836.18	146.32	0.000
Temperature	1	1849.13	1849.13	323.57	0.000
pH	1	9.60	9.60	1.68	0.227
ABTS	1	648.37	648.37	113.46	0.000
Square	2	167.22	83.61	14.63	0.001
pH*pH	1	87.85	87.85	15.37	0.004
ABTS*ABTS	1	79.36	79.36	13.89	0.005
2-Way Interaction	3	11.34	3.78	0.66	0.596
Temperature*pH	1	2.14	2.14	0.38	0.555
Temperature*ABTS	1	0.32	0.32	0.06	0.818
pH*ABTS	1	8.88	8.88	1.55	0.244
Error	9	51.43	5.71		
Total	17	2993.78			

DF: Degrees of freedom, Adj SS; Adjusted sum of square, Adj MS: Adjusted mean square, F-value: Fisher test value, p-value: probability value.

Table 4.2 (b) Levofloxacin degradation study using Analysis of Variance (ANOVA).

Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Model	8	1795.09	224.386	100.92	0.000
Linear	3	1476.02	492.005	221.29	0.000
Temperature	1	368.44	368.441	165.72	0.000
pH	1	155.29	155.290	69.85	0.000
ABTS	1	944.69	944.695	424.90	0.000
Square	2	218.13	109.066	49.06	0.000
pH*pH	1	80.97	80.967	36.42	0.000
ABTS*ABTS	1	137.17	137.166	61.69	0.000
2-Way Interaction	3	47.76	15.921	7.16	0.009
Temperature*pH	1	11.26	11.260	5.06	0.051
Temperature*ABTS	1	25.99	25.994	11.69	0.008
pH*ABTS	1	10.51	10.509	4.73	0.058
Error	9	20.01	2.223		
Total	17	1815.10			

DF: Degrees of freedom, Adj SS; Adjusted sum of square, Adj MS: Adjusted mean square, F-value: Fisher test value, p-value: probability value.

Table 4.2 (c) Norfloxacin degradation study using Analysis of Variance (ANOVA).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	8	2857.63	357.20	101.79	0.000
Linear	3	2730.05	910.02	259.32	0.000
temp	1	2534.37	2534.37	722.19	0.000
pH	1	50.71	50.71	14.45	0.004
ABTS	1	144.98	144.98	41.31	0.000
Square	2	119.81	59.90	17.07	0.001
pH*pH	1	119.39	119.39	34.02	0.000
ABTS*ABTS	1	0.41	0.41	0.12	0.739
2-Way Interaction	3	13.10	4.37	1.24	0.350
temp*pH	1	2.18	2.18	0.62	0.450
temp*ABTS	1	6.61	6.61	1.88	0.203
pH*ABTS	1	4.30	4.30	1.23	0.297
Error	9	31.58	3.51		
Total	17	2889.21			

DF: Degrees of freedom, Adj SS; Adjusted sum of square, Adj MS: Adjusted mean square, F-value: Fisher test value, p-value: probability value.

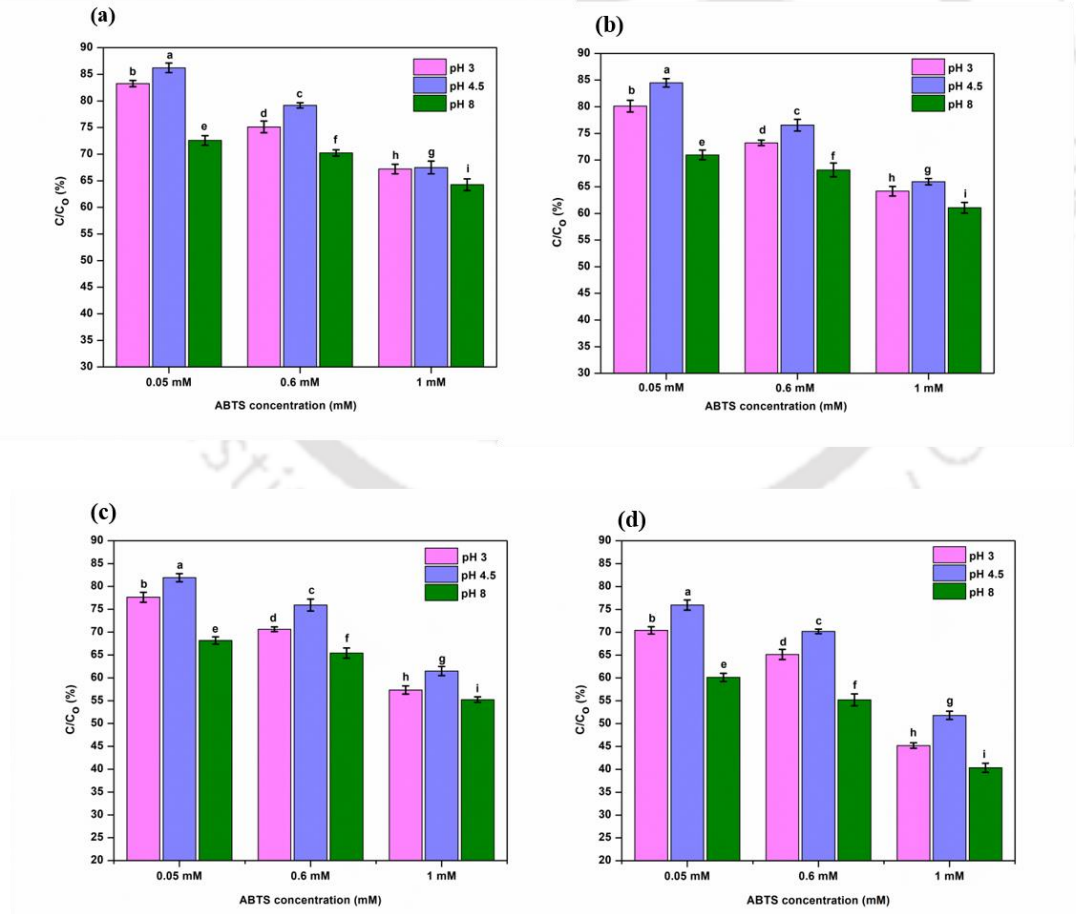


Fig. 4.2. (a-d) Degradation of ciprofloxacin at temperature 30 ℃ and 50 ℃, pH (3-6) and

ABTS concentration (0.05- 1 mM): (a) 5 µg mL^{-1} at 30 ℃, (b)15 µg mL^{-1} at 30 ℃, (c) 25 µg mL^{-1} at 30 ℃, (d) 50 µg mL^{-1} at 30 ℃.

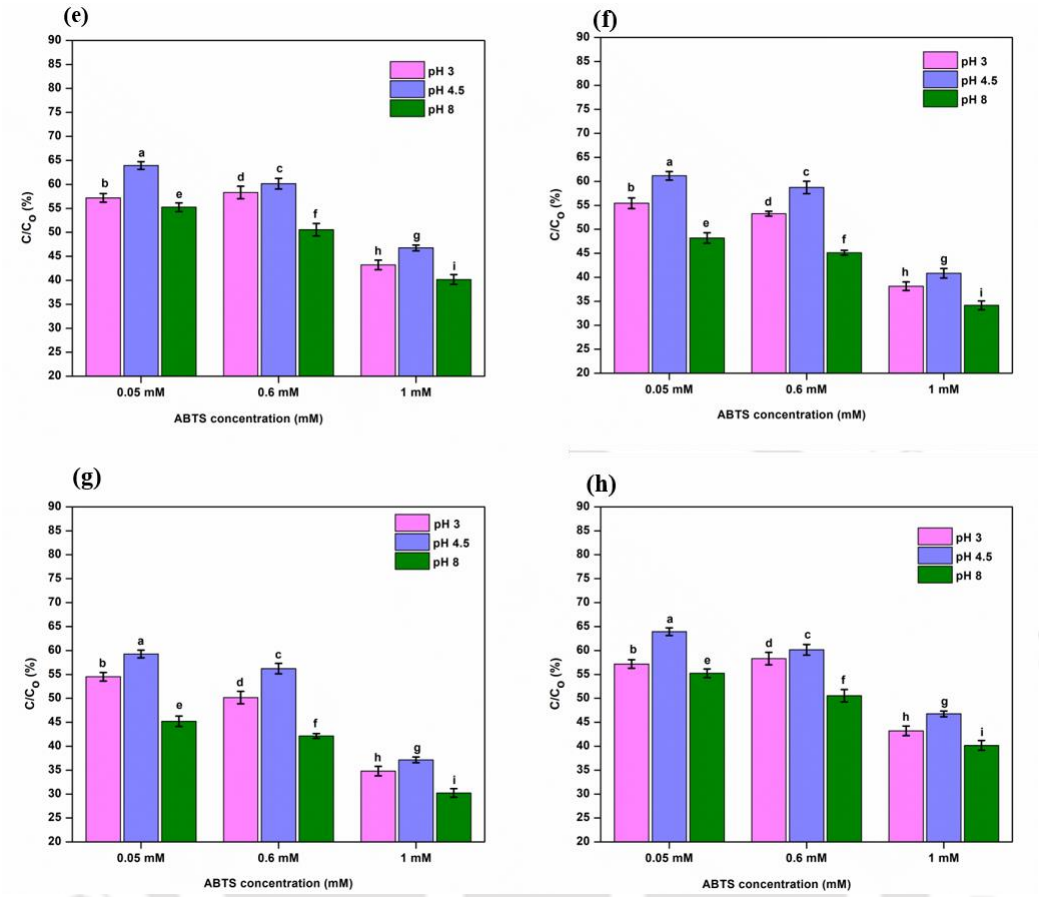


Fig. 4.2. (a-h) Degradation of ciprofloxacin at temperature 30 ℃ and 50 ℃, pH (3-6) and ABTS concentration (0.05- 1 mM): (e) 5 µg mL^{-1} at 50 ℃, (f) 15 µg mL^{-1} at 50 ℃, (g) 25 µg mL^{-1} at 50 ℃ and (h) 50 µg mL^{-1} at 50 ℃. Different letters in the column indicate significant difference at $p<0.05$ according to Duncan multiple range test.

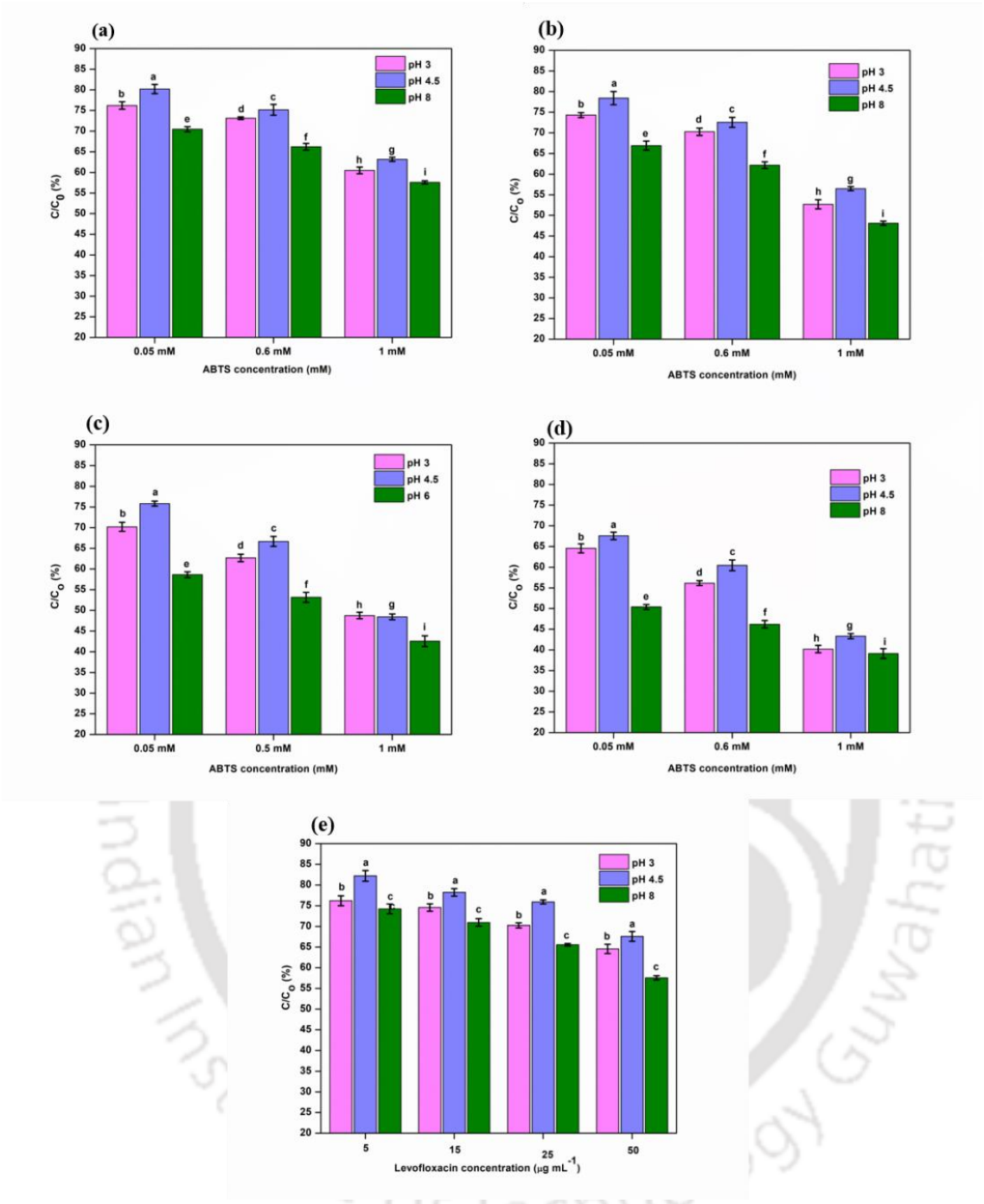


Fig. 4.3. (a-e) Degradation of levofloxacin at temperature 30 ℃, pH (3-6) and in the presence of ABTS concentration (0.05- 1 mM): (a) 5 µg mL^{-1} at 30 ℃, (b) 15 µg mL^{-1} at 30 ℃, (c) 25 µg mL^{-1} at 30 ℃, and (d) 50 µg mL^{-1} at 30 ℃ and (e) levofloxacin degradation in absence of ABTS at 30 ℃, Different letters in the column indicate significant difference at $p<0.05$ according to Duncan multiple range test.

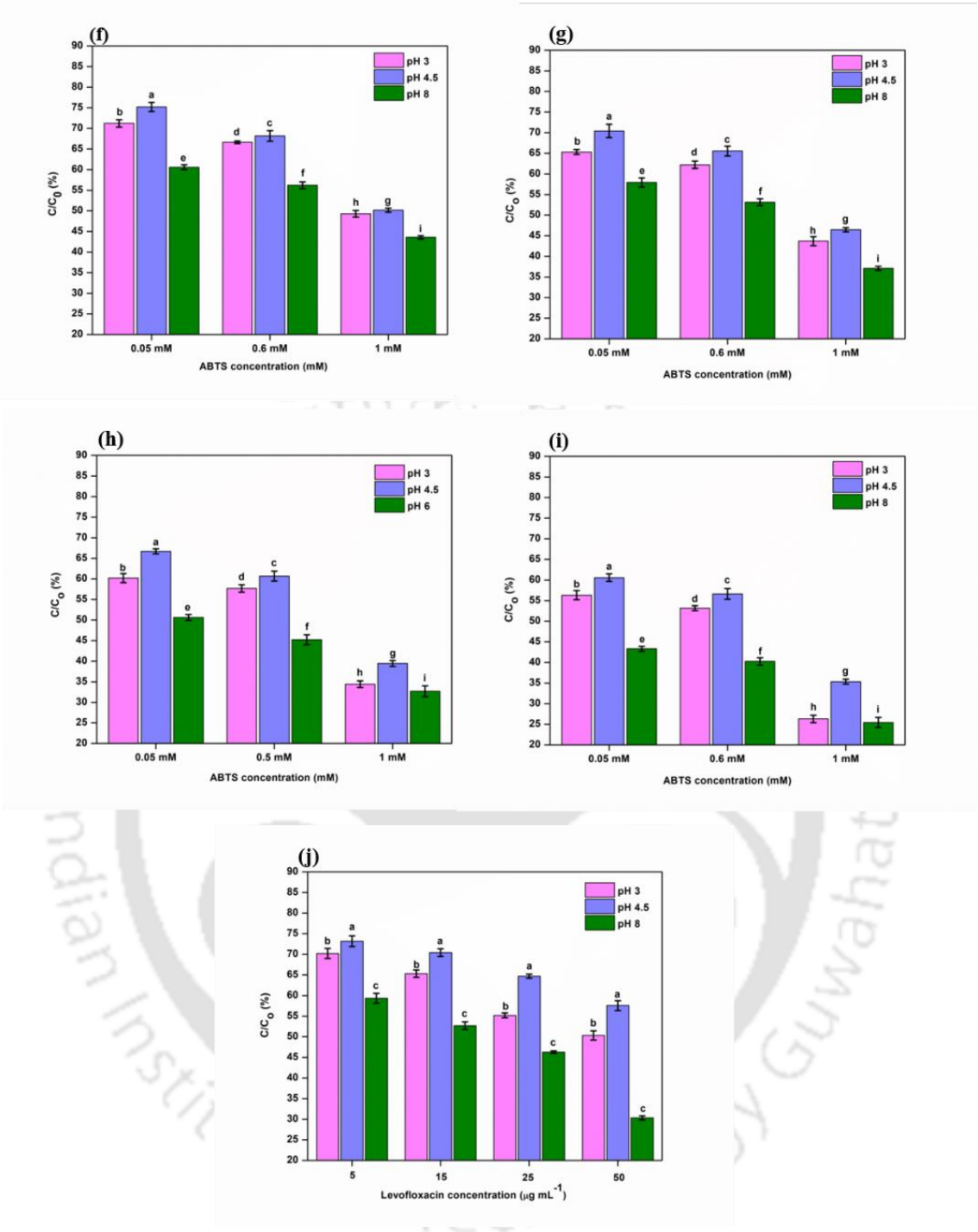


Fig. 4.3. (f-j) Degradation of levofloxacin at temperature 50 ℃, pH (3-6) and in the presence of ABTS concentration (0.05- 1 mM): (f) 5 µg mL^{-1} at 50 ℃, (g) 15 µg mL^{-1} at 50 ℃, (h) 25 µg mL^{-1} at 50 ℃, (i) 50 µg mL^{-1} at 50 ℃, (j) levofloxacin degradation in absence of ABTS at 50 ℃, Different letters in the column indicate significant difference at $p<0.05$ according to Duncan multiple range test**.**

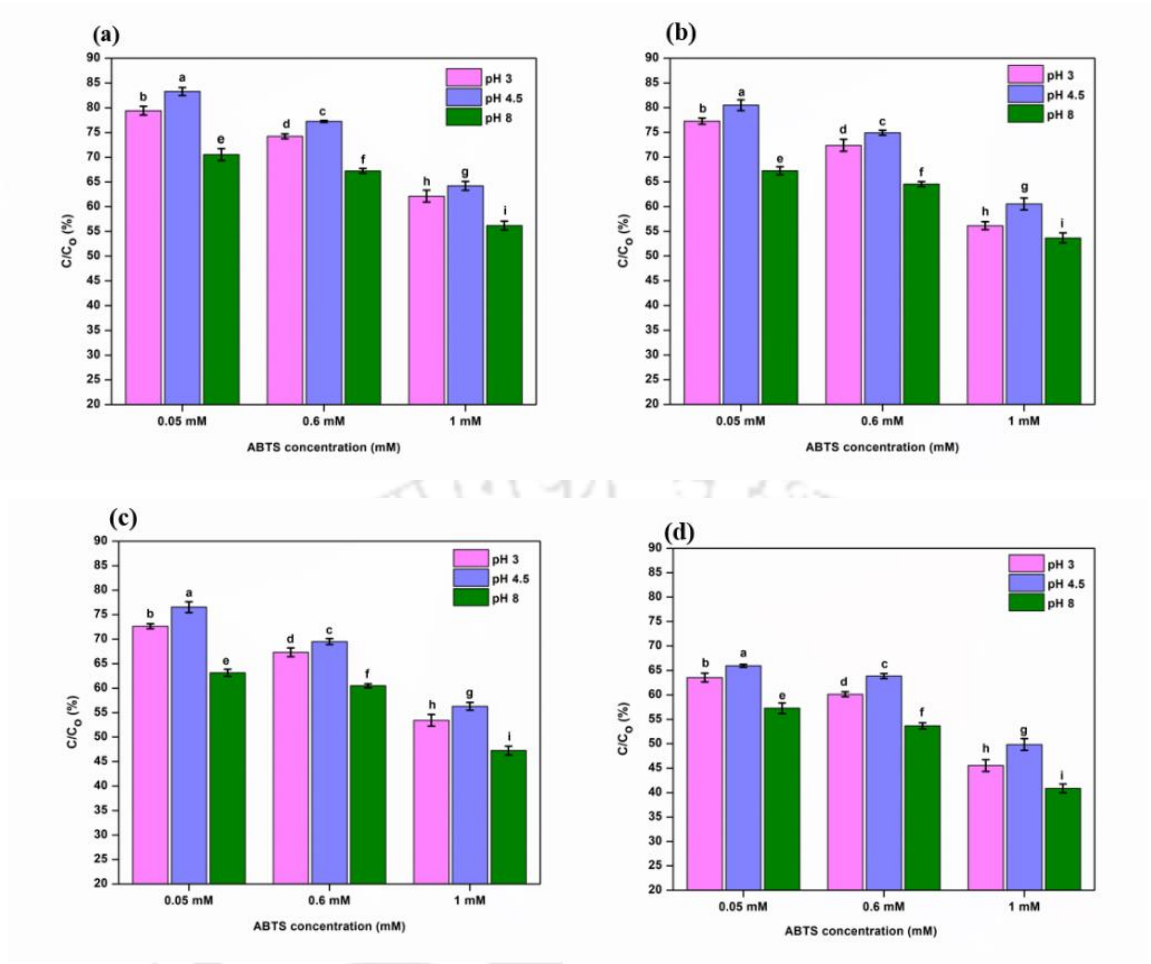


Fig. 4.4 (a-d) Degradation of norfloxacin at temperature 30 ℃ and 50 ℃, pH (3-6) and ABTS concentration (0.05- 1 mM): (a) 5 µg mL^{-1} at 30 ℃, (b) 15 µg mL^{-1} at 30 ℃, (c) 25 µg mL^{-1} at 30 ℃, (d) 50 µg mL^{-1} at 30 ℃. Different letters in the column indicate significant difference at $p<0.05$ according to Duncan multiple range test.

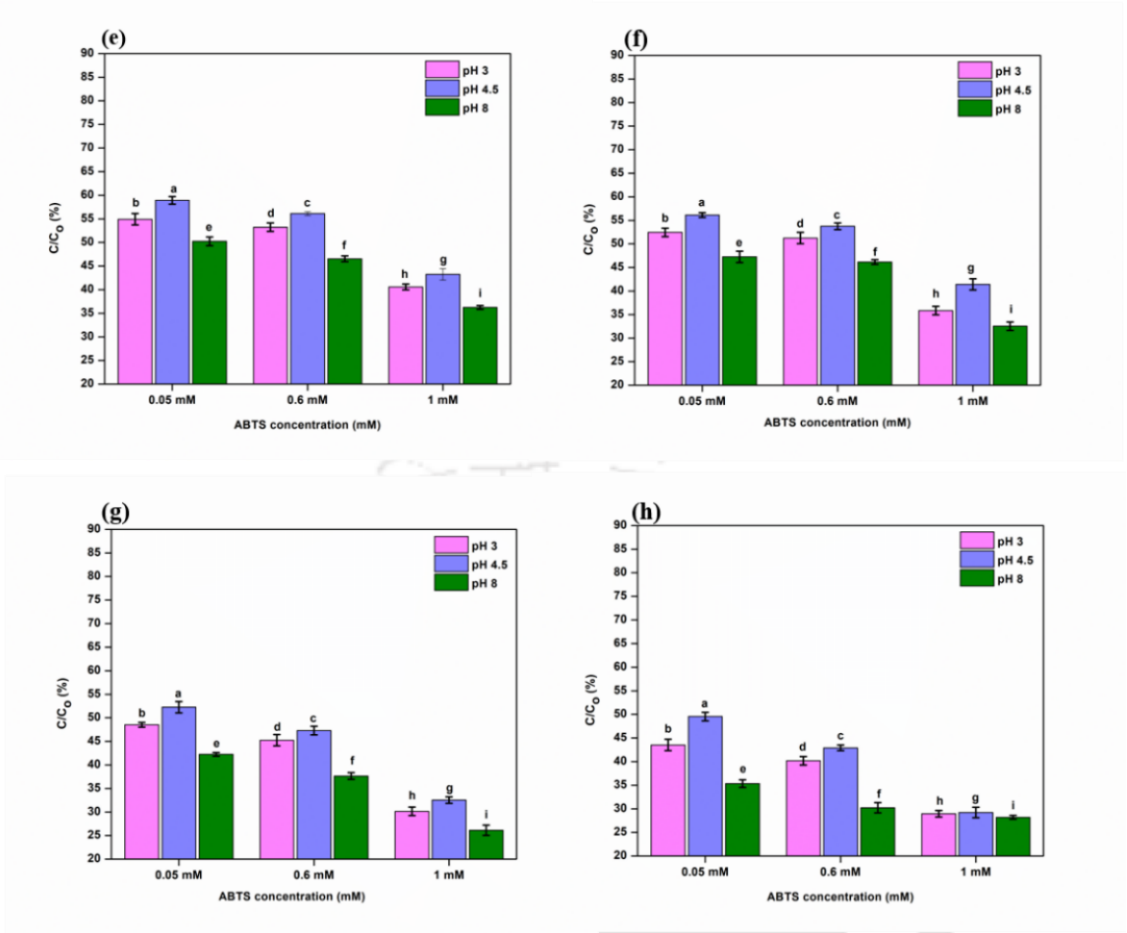


Fig. 4.4 (e-h) Degradation of norfloxacin at temperature 30 ℃ and 50 ℃, pH (3-6) and ABTS concentration (0.05- 1 mM): (e) 5 µg mL^{-1} at 50 ℃, (f) 15 µg mL^{-1} at 50 ℃, (g) 25 µg mL^{-1} at 50 ℃ and (h) 50 µg mL^{-1} at 50 ℃. Different letters in the column indicate significant difference at $p<0.05$ according to Duncan multiple range test.

4.4.1. Identification of degraded products and possible degradation pathway

The degraded intermediates of ciprofloxacin, levofloxacin and norfloxacin were identified using LC-MS. The samples were incubated at pH 4.5, 0.05 mM ABTS concentration, and 30 ℃, with a sample concentration of 15 µg mL^{-1} for 0, 3, 6, 9, and 24 hours. A degradation pathway has been proposed for the 3 h degraded sample, no further valid degradation pathway was observed. Both ciprofloxacin and norfloxacin shared a common degradation pathway that involves defluorination, decarboxylation, dealkylation, and net loss of C_2H_2 at the piperazinyl substituent (Čvančarová et al., 2015; Parshikov et al., 2001; Prieto et al., 2011). In addition, HPLC chromatogram and mass spectra of the degraded products are represented in **appendix A**.

4.4.4.1. Ciprofloxacin

Ciprofloxacin (*m/z* 332), parent compound could be transformed by decarboxylation and product ions C1 (*m/z* 288) is formed. Protonated metabolite from the parent CIP is formed at *m/z* 306 (C2), and product ion C3 (*m/z* 266). A net loss of C_2H_2 from the piperazine group and dealkylation (removal of cyclopropyl group) results in the formation of product ion C2 and C3. Further, loss of C_2H_5N fragment results in the formation of C4 (*m/z* 263). Defluorination of the C4 leads to the formation of product ion C5 *m/z* 245 and further decarboxylation and dealkylation of C4 resulted in the formation of product ion C6 (*m/z* 161) represented in **Fig. 4.4 (a) and Table 4.3 (a)**. Consequently, study by Prieto et al. (2011), occurrence of *m/z* 288 product ion from the parent ciprofloxacin was formed due to decarboxylation. Čvančarová et al. (2015), Prieto et al. (2011), and Wetzstein et al. (1999) observed that whole fungal culture cells and the ciprofloxacin degradation by ozonation both produce the C2 product ion, which is known as desethylene ciprofloxacin. This is formed due to net loss of C_2H_2 from the piperazinyl moiety. According to Čvančarová et al. (2015) and Prieto et al. (2011), the product ion *m/z* 306 loses a C_2H_5N fragment, resulting in the formation of product ion *m/z* 263. The breakage of the C-F bond (defluorination) leads to the formation of the ion at *m/z* 245. Another possible mechanism by which the *m/z* 245 ion is produced is by the photocatalytic degradation of ciprofloxacin, which leads to ring cleavage and the loss of primary amine nitrogen (An et al., 2010; Paul et al., 2010). Prior investigations employing purified laccase isolated from *Trametes versicolor* suggested the formation of the fragmentation ion as a result of the removal of the cyclopropyl group from ciprofloxacin (Fujihiro et al., 2009; Li and Hu, 2018; Prieto et al., 2011; Yang et al., 2016). However, molecule's toxicity is determined by alterations to the quinolone structure, F-molecule and N-piperazine ring moiety, therefore previous studies reports less toxicity of ciprofloxacin fragments than a ciprofloxacin parent molecule (Li and Hu, 2018; Shen et al., 1989; Zhang et al., 2015). Therefore, degradation of ciprofloxacin by whole fungal culture (*Trametes versicolor*, *I. lacteus*, *P.ostreatus* and *P. tigrinus*), photolysis, and ozonation have all been documented for obtaining ciprofloxacin intermediates at *m/z* 288, 306, 263, and 245. Hence, this study presented evidence of ciprofloxacin degradation by partially purified extracellular laccase isolated from SMW of *P. florida*, with product ions observed at m/z 161 and 266.

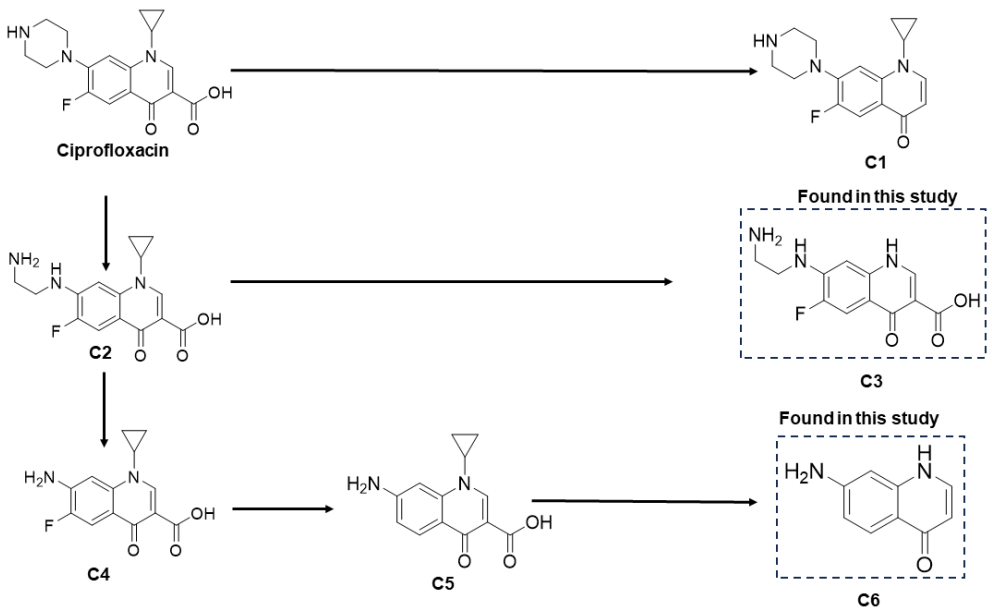


Fig. 4.4 (a) Ciprofloxacin proposed degradation pathway by laccase derived from spent mushroom waste of *Pleurotus florida.*

Table 4.3 (a) Degradation products detected by LC-MS of ciprofloxacin depicting formula, *m/z* and the individual product ion were compared with literature. The product ion marked with asterisk are found in this study.

Product ion	**Formula**	**$[M^+H^+]$**	**Current study**	**From literature**
C1	$C_{16}H_{19}FN_3O$	288	-	(Prieto et al., 2011)
C2	$C_{15}H_{17}FN_3O_3$	306	-	(Čvančarová et al., 2015; Prieto et al., 2011; Wetzstein et al., 1999)
C3*	$C_{12}H_{13}FN_3O_3$	266	SMW of *P. florida*	**This study**
C4	$C_{13}H_{12}FN_2O_3$	263	-	(Čvančarová et al., 2015; Prieto et al., 2011)
C5	$C_{13}H_{11}NO_4$	245	-	(An et al., 2010; Paul et al., 2010)
C6*	C_9HN_2O	161	SMW of *P. florida*	**This study**

4.4.4.2. Levofloxacin

The presence of additional oxygen in the parent compound (*m/z* 363) forms product ion L1 at *m/z* 378. L1 undergoes decarboxylation and forms product ion L2 at *m/z* 317. Further, defluorination of L2 leads to the formation of product ion L3 at *m/z* 300. Removal of C_4H_9N forms L3 form product ion at L4 represented in **Fig. 4.4 (b) and Table 4.3 (b)**. Levofloxacin degradation through daylight infusion Czyrski et al. (2019) confirmed the formation of the product ion N-oxide or hydroxy derivative (*m/z* 378) as an effect of addition of an oxygen molecule to the parent levofloxacin. In contrast to hydrogen, methyl groups are clearly stronger at donating electrons, which results in the formation of N-oxide. This similar phenomena were studied by (Devi and Chandrasekhar, 2009; Wang et al., 2012). Nevertheless, there have been reports of mechanisms such as dehalogenation, piperazine ring removal and fluorine group substitution of degradation of levofloxacin from wastewater and untreated water (Sturini et al., 2015). In photo-induced levofloxacin degradation decarboxylation, defluorination, and piperazinyl dealkylation were observed (Ge et al., 2010). Decarboxylation by *S. obliqus* enzyme results in a comparable structure L2, as previously described by (Xiong et al., 2017b). In addition, (Tang et al., 2012) noted the loss of C_4H_9N, analogic fragmentation ion. Therefore, we propose that the defluorination and loss of C_4H_9N from the piperazine moiety is responsible for the generation of the m/z 229 product ions in this investigation, in accordance with the existing literature.

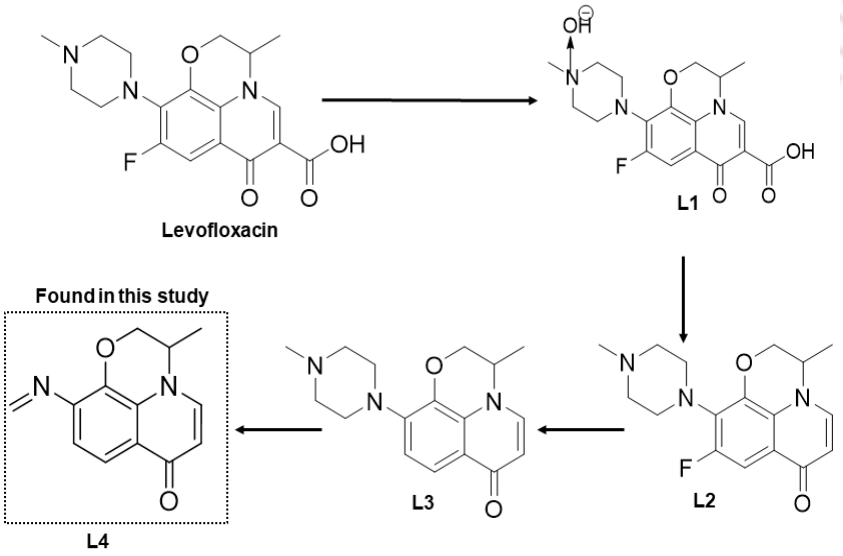


Fig. 4.4 (b) Levofloxacin proposed degradation pathways by laccase derived from spent mushroom waste of *Pleurotus florida*.

Table 4.3 (b) Degradation products detected by LC-MS of levofloxacin depicting formula, *m/z* and the individual product ion were compared with literature. The product ion marked with asterisk are found in this study.

Product ion	Formula	$[M^+H^+]$	Current study	From literature
L1	$C_{18}H_{21}FN_3O_5$	378	-	(Czyrski et al., 2019; Devi and Chandrasekhar, 2009; Wang et al., 2012)
L2	$C_{17}H_{20}FN_3O_2$	317	-	(Tang et al., 2012; Xiong et al., 2017b)
L3*	$C_{17}H_{21}FN_3O_2$	299	SMW of *P. florida*	**This study**

4.4.4.3. Norfloxacin

Norfloxacin (*m/z* 320), the degraded products ions are formed at *m/z* 294, 250 and 164 out of which *m/z* 164 was not reported in literature represented in **Fig. 4.4 (c) and Table 4.3 (c)**. The proposed degradation pathway is in consistent with (Čvančarová et al., 2015; Prieto et al., 2011). Product ion N1 (*m/z* 294), which involves the loss of ethylene (C_2H_2) at the piperazinyl substituent. Further, with a loss of C_2H_5N fragment, product ion N2 formed at *m/z* 250. Product ion N3 (*m/z* 164) is formed due to decarboxylation, dealkylation and deamination. Previous studies have reported occurrence of *m/z* 294 and *m/z* 250 by following same mechanism (Čvančarová et al., 2015; Parshikov et al., 2001; Prieto et al., 2011). Loss of the C_2H_2 group from the piperazine substituent supported the norfloxacin breakdown by white rot fungus (*Trametes versicolor*, *P. ostreatus*, *I. lacteus*, and *P. tigrinus*), confirming that the degradation mechanism followed by whole fungal culture cells and enzymatic degradation (laccase) is similar. Correspondingly, study by Zou et al. (2023) reported loss of C_2H_5N fragment and ethylene group by laccase immobilized biochar which further validates our laccase mediated norfloxacin degradation pathway and the degradation products formed in our study. However, degradation of norfloxacin by ionizing radiation followed the similar pathway and m/z 250 occurred due to piperazinyl ring opening of the parent norfloxacin (Chen and Wang, 2020). Therefore, from the

literature and this study results confirmed that piperazinyl ring substituent, decarboxylation and dealkylation are the common breakdown points for norfloxacin

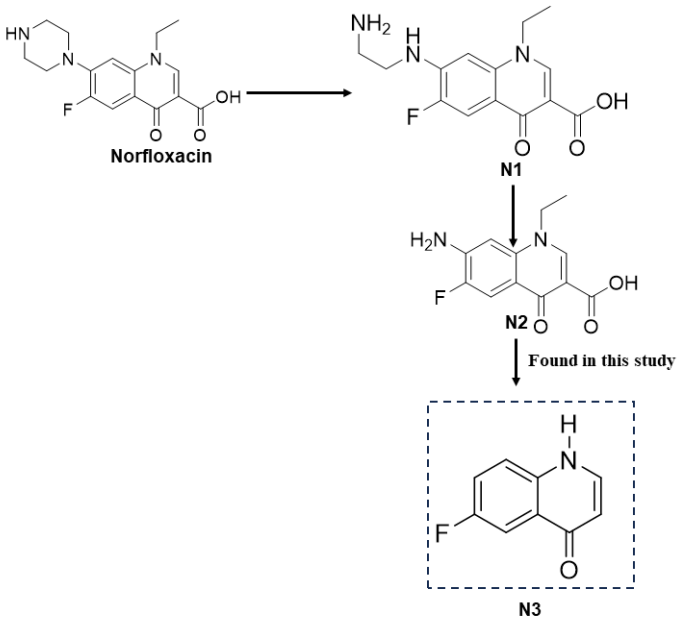


Fig. 4.4 (c) Norfloxacin proposed degradation pathway by laccase derived from spent mushroom waste of *Pleurotus florida*.

Table 4.3 (c) Degradation products detected by LC-MS of norfloxacin depicting formula, *m/z* and the individual product ion were compared with literature. The product ion marked with asterisk are found in this study.

Product ion	**Formula**	**$[M^+H^+]$**	**Produced from this study**	**Produced from literature**
N1	$C_{14}H_{17}FN_3O_3$	294	-	(Čvančarová et al., 2015; Parshikov et al., 2001; Prieto et al., 2011)
N2	$C_{12}H_{12}FN_2O_3$	250	-	(Čvančarová et al., 2015; Prieto et al., 2011; Zou et al., 2023)
N3*	C_9H_6FNO	164	SMW of *P. florida*	**This study**

4.5.1. Toxicity of degraded products

4.5.1.1. Residual anti-bacterial activity

The residual anti-bacterial activity of enzymatically treated ciprofloxacin, levofloxacin and norfloxacin degraded products were determined by two test organism *E. coli* (gram negative) and *S. aureus (*gram negative*)*. Antibiotics could retain antimicrobial activity in their fragmented ions after breakdown, which is why it is necessary to determine residual anti-bacterial activity. The untreated antibiotics were kept as controls and the residual activity of treated antibiotics were measured with respect to these controls. The percentage of anti-microbial activity was measured. The ZOI diameters are presented in appendix **Table A.1 (a-b).** Hence, toxic effects of degraded product produced from three fluoroquinolone antibiotics could only be ascertained by this experiment. Therefore, research into the biological effect of the breakdown products is necessary in order to lessen bacterial resistance. The harmful effects of a degraded product following treatment have received very little attention in the scientific literature. Based on the results obtained, there is a notable disparity in the residual anti-bacterial activity of antibiotics between the control and degraded sample when tested against the test organism (*E. coli* and *S. aureus*).

In comparison to the control, ciprofloxacin degraded by laccase extracted from SMW of *P. florida* resulted in a 34.52 % reduction in *E. coli* activity. A 25.42% reduction in activity was also noted against *S. aureus*. Similarly, the anti-microbial activity of laccase mediated norfloxacin degraded products against *E. coli* decreased by 37.60% and *S. aureus* by 38.38%. In case of levofloxacin, 30.77% of anti-microbial activity was observed against *E. coli* and 33.63% of anti-microbial activity was measured against *S. aureus* represented in **Fig. 4.5.**

Decarboxylation, piperazine ring removal and defluorination all contribute to a decrease in the anti-microbial activity of the parent compounds ciprofloxacin, levofloxacin and norfloxacin. Photocatalysis, ionizing radiation, ozonation, and gamma radiation all follow the same degradation pathway, and prior research has reported that norfloxacin antibacterial activity against *S. aureus* decreases under this above-mentioned mechanism.

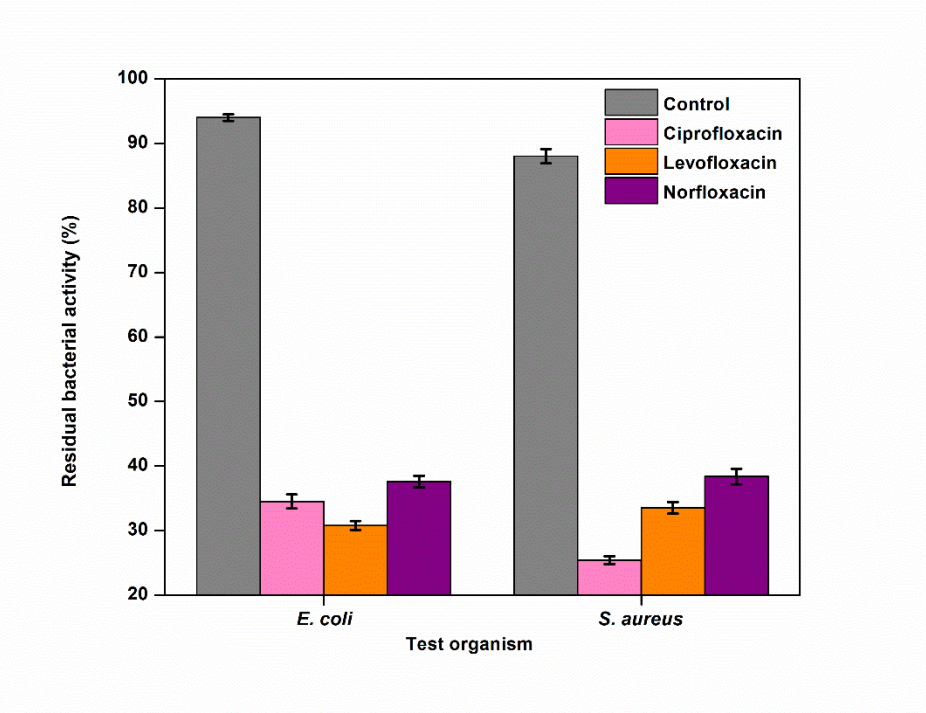


Fig. 4.5. Residual anti-bacterial test between degraded sample (ciprofloxacin, levofloxacin norfloxacin) by using laccase derived from spent mushroom waste against the test organism (*E. coli* and *S. aureus*).

In addition, regardless of the presence of an ethyl group in norfloxacin, the cyclopropyl group of ciprofloxacin is more susceptible for degradation by laccase and the laccase-mediator system, and hence degraded products formed in ciprofloxacin showed less inhibitory action for both the test organism in comparison to norfloxacin (Prieto et al., 2011). Prevailing research indicates that green algae, daphnids, *Vibrio fischeri*, and fish has lower toxicity towards ciprofloxacin degraded products reported in earlier studies (Li et al., 2020). The structural alterations in the piperazine and quinolone groups could be the cause of the reduced antibacterial activity; this is because degradation products produced following piperazine ring transformation have been proposed to bind with the DNA of the topoisomerase enzyme of bacteria. Further, breakdown of ciprofloxacin and norfloxacin by the enzymatic remediation of whole fungal culture of *Trametes verscicolor*, *P. ostreatus*, *I. lacteus* and *P. tigrinus* showed significant reduction in anti-microbial activity (Čvančarová et al., 2015). Additionally, research reports revealed growth inhibition of levofloxacin treated with osmolyte treated laccase. The degraded products formed following defluorination, decarboxylation and deamination showed growth inhibition against *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* revealing non-toxic nature of degraded

products produced from levofloxacin (Najafabadipour et al., 2021). Therefore, degradation of three fluoroquinolone antibiotics by laccase is an encouraging method to facilitate antibiotic removal from wastewater, according to the results and the literature. The resultant products exhibited less anti-microbial activity compared to the parent compounds

4.5.5.2. Algal toxicity test

Microalgae are crucial indicators of water quality as they share the extremity of the aquatic food chain. Reactive oxygen species' equilibrium in the cell was disrupted as a result of the microalgae's regular exposure to and accumulation of OMPs. Over accumulation of reactive oxygen species (ROS) in the cell can lead to structural and functional damage to the cell, which inhibits the growth of algal culture (Xiong et al., 2017a). Despite the fact that, a decrease in chlorophyll (chl *a and b*) is an indicator of pollutant toxicity. Herein, the algal toxicity test was carried out to validate that the degraded products achieved after the degradation are non-toxic to the environment.

There was a profound difference observed in terms of dry cell weight and chlorophyll content in the degraded sample in comparison to the undegraded sample. Cultured algal cells with degraded ciprofloxacin measured chl *a* and chl *b* contents of 7.85 and 7.56 mg 50 mL^{-1}, respectively, compared to 3.26 and 2.74 mg 50 mL^{-1} in undegraded cells. Levofloxacin degraded sample resulted in chl *a* and chl *b* values of 6.86 and 6.18 mg 50 mL^{-1} respectively, while the undegraded algal cells measured 3.15 and 3.76 mg 50 mL^{-1}. In case of norfloxacin degraded sample chl *a* and chl *b* content measured 8.89 and 8.18 mg 50 mL^{-1} whereas undegraded norfloxacin measured 2.17 and 2.64 mg 50 mL^{-1} as shown in **Fig. 4.6. (a-c)**. Simultaneously, increase in dry cell weight for ciprofloxacin, levofloxacin and norfloxacin was measured and it was observed that 60% increase in dry cell weight of degraded antibiotic sample as compared to undegraded antibiotic cell. For degraded ciprofloxacin, levofloxacin, and norfloxacin, the dry cell weight values were 2.56, 1.66, and 1.466 mg 50 mL^{-1}, respectively. In contrast, for undegraded ciprofloxacin, levofloxacin, and norfloxacin, the values were 0.0595, 0.0695, and 0.06 mg 50 mL^{-1}, respectively represented in **Fig. 4.6 (d).** Therefore, this toxicity test suggests that the degraded sample contains no secondary pollution or that the degraded products which are formed do not inhibit the algal growth and does not interfere with the photosystem II chain. Contrarily, studies reported impeded algal growth in the presence of levofloxacin ranging from 1-10 mg L^{-1} on various algal species like *Dunalellia tertiolecta*, *Scenedesmus obliquus, Chlorella vulgaris, Pseudokirchneriella subcapitata , Synechocystis* sp. (Pan et

al., 2009; Tsiaka et al., 2013; J.-Q. Xiong et al., 2017). Probably, inhibition is likely exerted by preventing protochlorophyll production. Studies also revealed that the levofloxacin antibiotic was effective in inhibiting cyanobacterial growth (*Synechocystis* sp.) (Pan et al., 2009). The inhibition impeded photosystem II and oxygen evolution. Among lomefloxacin, clinafloxacin, ciprofloxacin, ofl0xacin, and enrofloxacin, levofloxacin was found to be the most toxic to *Microcystis aeruginosa.* However, levofloxacin was fourth most toxic reported in *Pseudokirchneriella subcapitata* (Robinson et al., 2005).

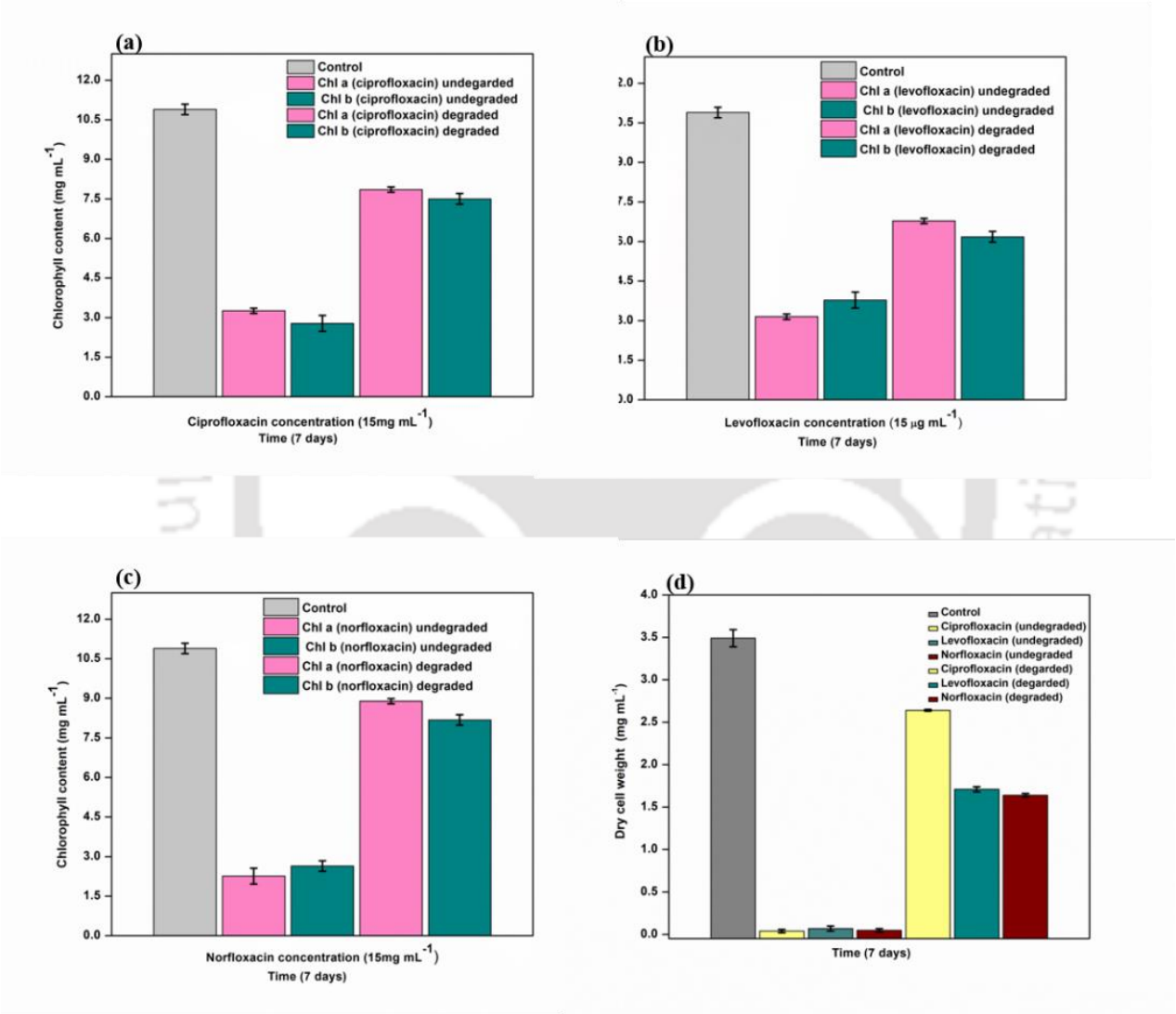


Fig. 4.6. Algal toxicity test of three fluoroquinolone antibiotics: (a) ciprofloxacin chlorophyll content, (b) levofloxacin chlorophyll content, (c) norfloxacin chlorophyll content and (d) dry cell weight of ciprofloxacin, levofloxacin and norfloxacin.

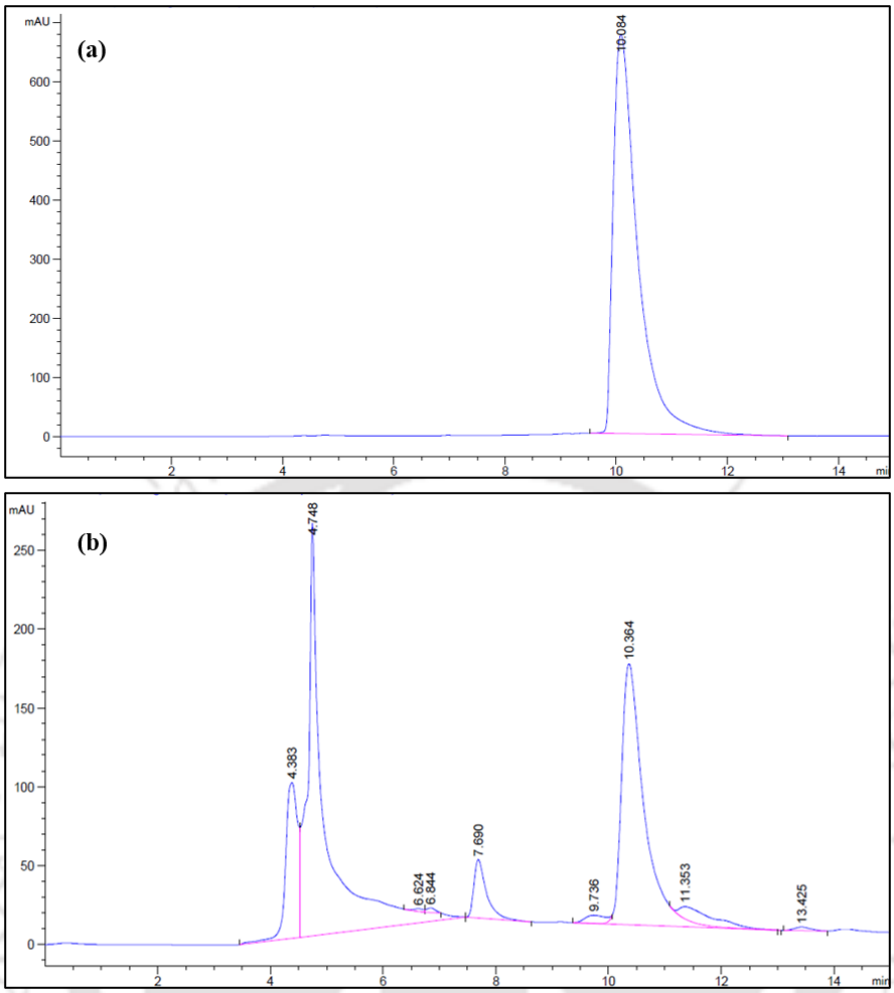
Additionally, investigations have revealed that cyanobacteria, which are aquatic photoautotrophs, are impacted by ciprofloxacin concentrations of 10.2 µg L^{-1}. *L. minor*, a higher plant, is remarkably sensitive to cyanobacteria (Ebert et al., 2011). Similarly,

exposure of norfloxacin to cyanobacteria and green algae (*Scenedesmus quadricauda*) resulted in decrease in biomass, chl *a* and soluble protein content (Li et al., 2021). Thus, laccase mediated degradation of three fluoroquinolone antibiotics resulted in significant growth of algal cells as compared to undegraded antibiotics. Consequently, sewage treatment plants (STPs) should include safeguards to protect the aquatic organism population in the vicinity. Evolutionary divergence, translocation, absorption, and metabolism all contribute to the fact that different antibiotics will affect algal cells differently.

4.4. Conclusions

The results evident us with the fact that to achieve higher degradation of OMPs optimal reaction condition is required. In this study, at pH 4.5, temperature 30 ℃ and ABTS concentration 0.05 mM highest degradation was achieved for the fluoroquinolone antibiotics at time 3 h. Despite the fact that pH and ABTS played a decisive role for achieving maximum degradation. Regardless of fungal culture, photocatalytic degradation, or ozonation, the degradation pathways of ciprofloxacin, levofloxacin and norfloxacin were equivalent (piperazinyl substituent removal, decarboxylation, and defluorination). This allowed to confirm that fluoroquinolone antibiotics have similar degradation sites for enzymatic degradation as well. Further, the residual anti-bacterial test of three laccase mediated degraded fluoroquinolone antibiotics confirmed growth of *E. coli* and *S. aureus* and as compared to control (undegraded sample) where bacterial growth was inhibited. In addition, increase in the chl *a, b* content and dry cell weight of the algal cells proved the fact that the parent compound of ciprofloxacin, levofloxacin and norfloxacin were toxic to the aquatic organism as compared to the degraded products formed after laccase- mediated degradation. Therefore, laccase isolated from SMW of *P. florida* helps to improve wastewater quality, as this study additionally investigated the toxicity test of the degraded products, proving that they are non-toxic in nature. Hence, this study sheds light on the possibility of extracting laccase from agro-waste (SMW), which decreases waste accumulation and has the added benefit of potentially degrading OMPs. However, using whole fungal culture is an expensive and time-consuming procedure. With buffer extraction laccase, OMP degradation from wastewater can be achieved more efficiently and economically. Therefore, SMW offers two advantages: cost-effectiveness and waste management in addition to recycling and reusing materials.

Appendix:



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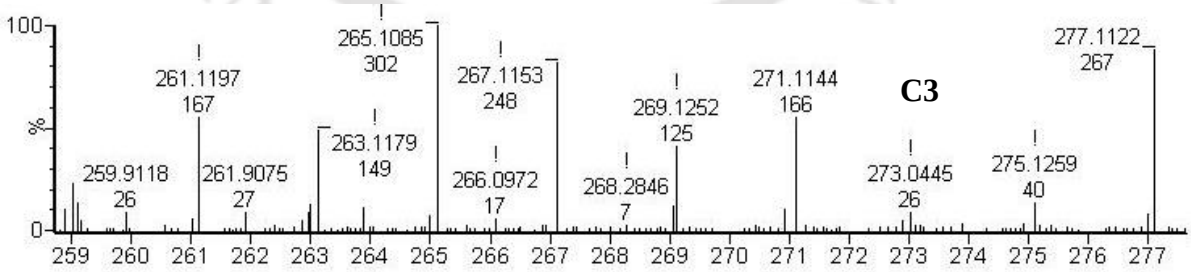

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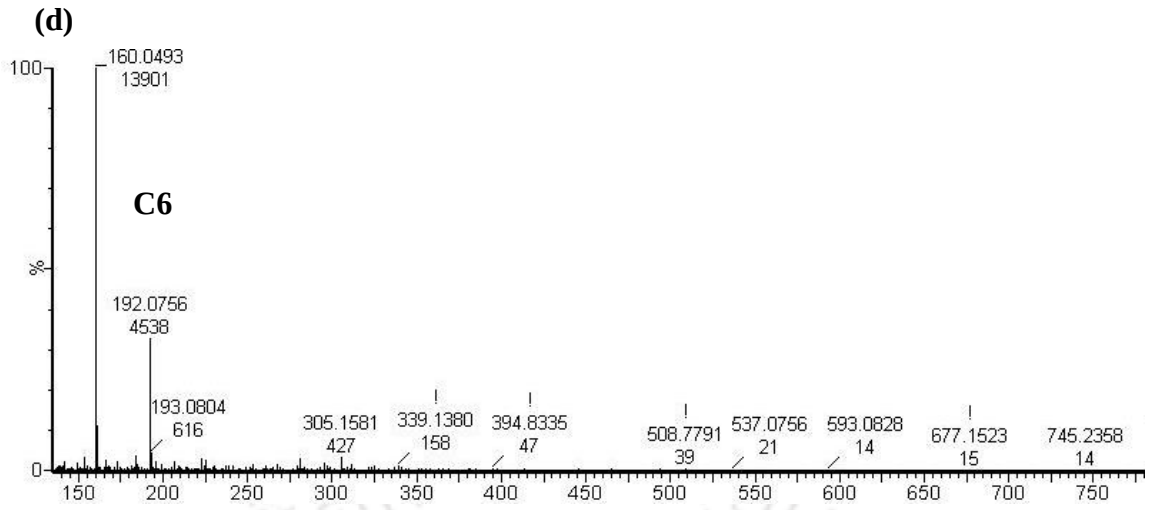
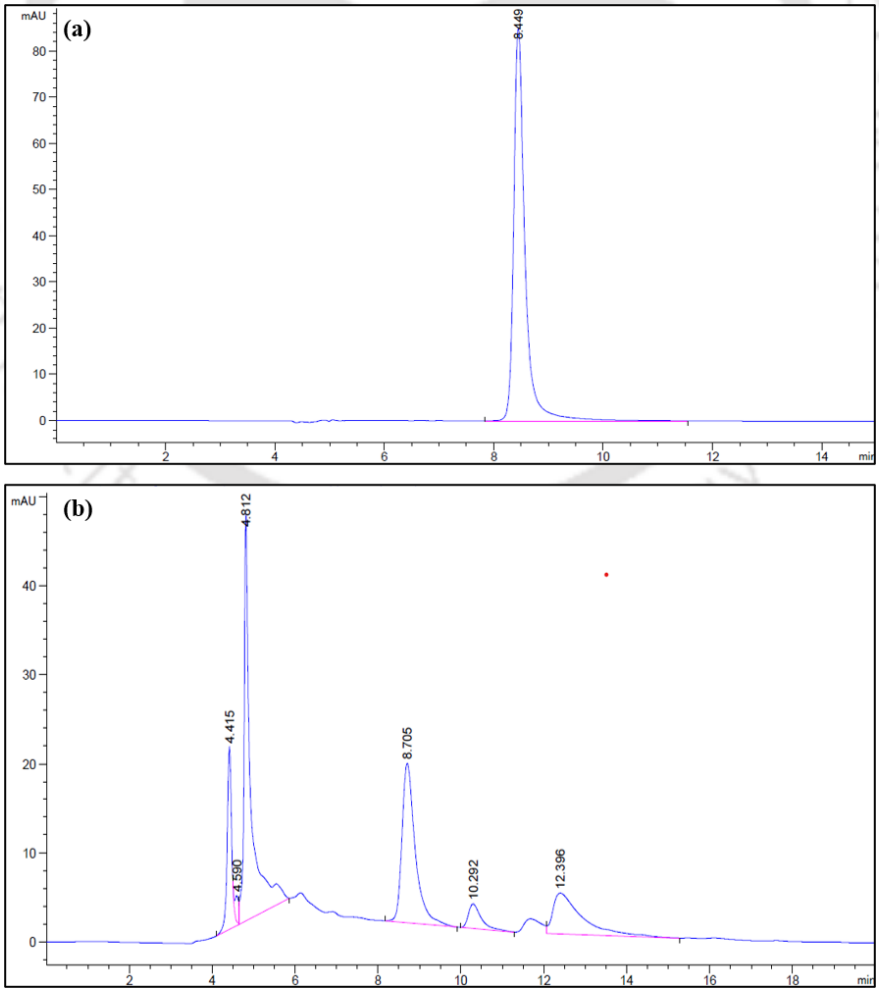


Fig. A1. Quantitative analysis of ciprofloxacin: (a) HPLC chromatogram for standard ciprofloxacin sample, (b) HPLC chromatogram for degraded ciprofloxacin sample by laccase enzyme after 3 h, (c) Mass spectra of C3 degraded product, (d) Mass spectra of C6 degraded product.



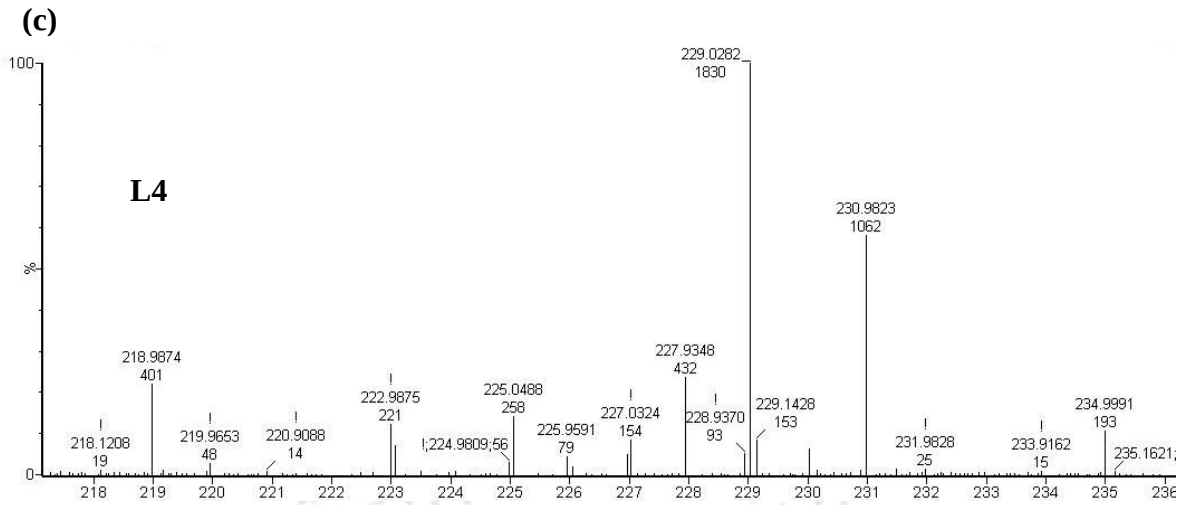
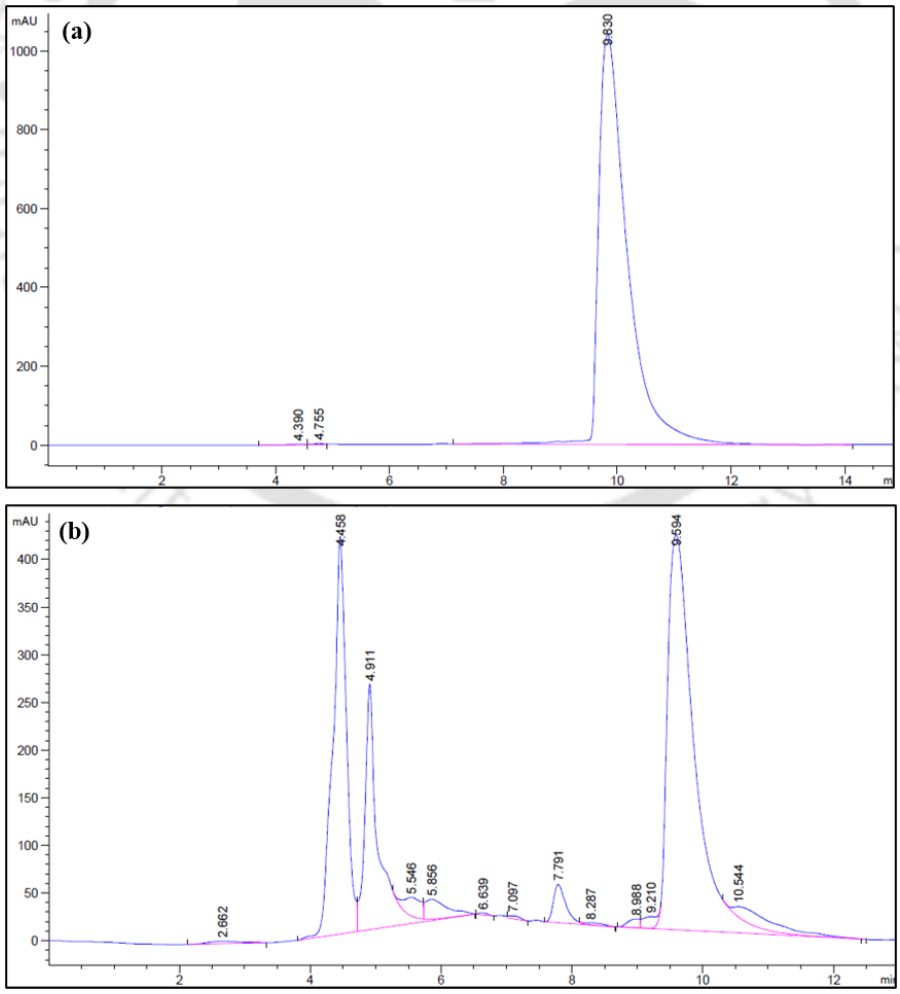


Fig. A2. Quantitative analysis of levofloxacin: (a) HPLC chromatogram for standard levofloxacin sample, (b) HPLC chromatogram for degraded levofloxacin sample by laccase enzyme after 3 h, (c) Mass spectra of L4 degraded product.



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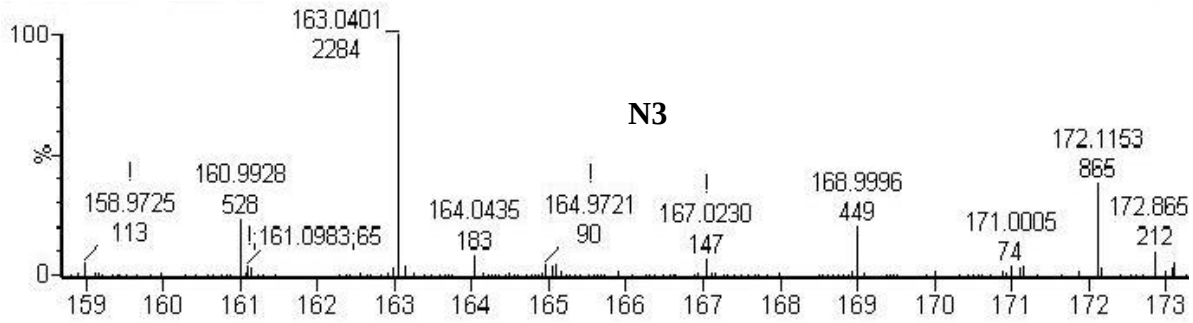

Fig. A3. Quantitative analysis of norfloxacin: (a) HPLC chromatogram for standard norfloxacin sample, (b) HPLC chromatogram for degraded norfloxacin sample by laccase enzyme after 3 h, (c) Mass spectra of N3 degraded product.

Table A.1. (a) Anti-bacterial activity of fluoroquinolone antibiotics and their degraded products tested against *E. coli*. Data is represented as average of triplicate values.

Antibiotic	**Antibiotic (control)**	**Zone of inhibition (in cm) Degraded products tested after treatment with laccase obtained from SMW of *P. florida***
Ciprofloxacin	3.34±0.1	1.13±0.2
Levofloxacin	3.70±0.3	1.10±0.3
Norfloxacin	2.49±0.1	0.62±0.3

Table A.1. (b) Anti-bacterial activity of fluoroquinolone antibiotics and their degraded products tested against *Staphylococcus aureus*. Data is represented as average of triplicate values.

Antibiotic	**Antibiotic (control)**	**Zone of inhibition (in cm) Degraded products tested after treatment with laccase obtained from SMW of *P. florida***
Ciprofloxacin	2.49±0.1	0.81±0.2
Levofloxacin	3.14±0.3	1.23±0.3
Norfloxacin	3.01±0.1	1.14±0.1

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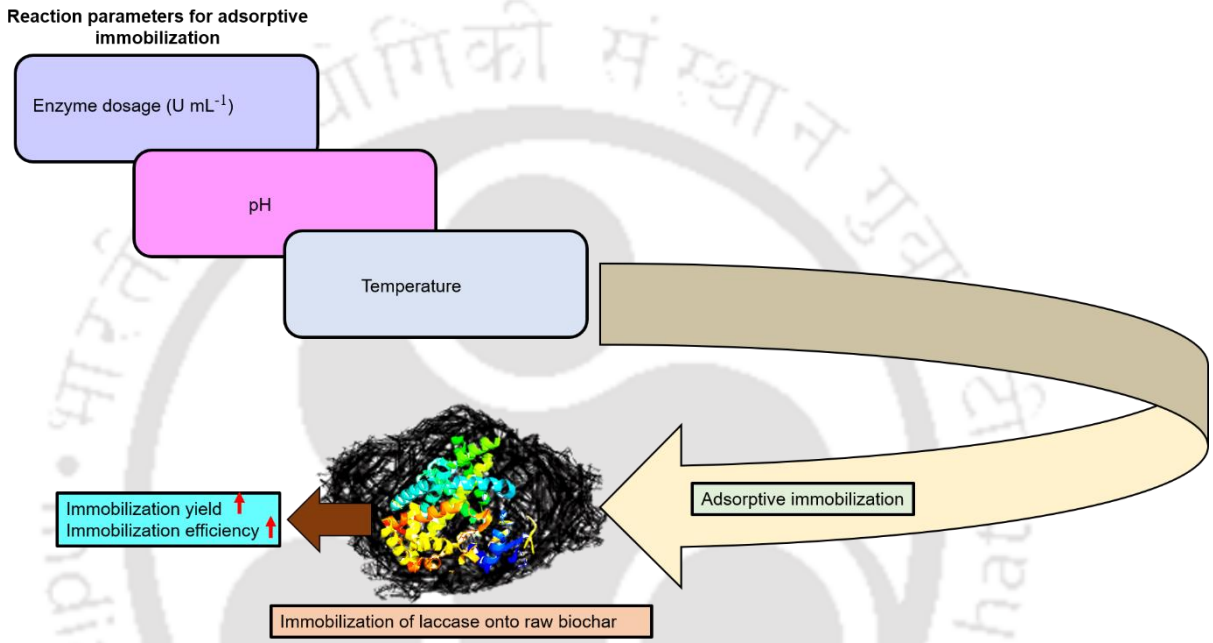
CHAPTER 5

To immobilize laccase onto raw spent mushroom biochar and determine immobilization yield and efficiency.



"*This chapter deals with obtaining optimal reaction conditions for adsorptive immobilization of laccase onto raw biochar (derived from spent mushroom waste of P. florida). This chapter also elucidates significance of reaction parameters in obtaining maximum immobilization yield and efficiency"*

5. To immobilize laccase onto raw spent mushroom biochar and determine immobilization yield and efficiency.

5.1. Introduction

The synthetic organic compounds i.e. OMPs that are used to fulfill basic human needs contribute to accumulating in wastewater. However, research reports revealed contamination of drinking water with metabolites of OMPs (Couto et al., 2019; Mompelat et al., 2011; Rivera-Utrilla et al., 2013; Sang et al., 2022; Vulliet and Cren-Olivé, 2011). Evidence suggests that existing wastewater treatment methods do not adequately remove OMPs metabolites. The prevalence of fluoroquinolone antibiotics, specifically ciprofloxacin, levofloxacin and norfloxacin, have been found in wastewater, river water, and common effluent treatment plants around the world, according to research studies (Castiglioni et al., 2006; Diwan et al., 2018; Ghosh et al., 2009; Saxena et al., 2021). Because of their bioavailability, these two broad-spectrum antibiotics are introduced as potential OMPs. Treatment technologies such as electrochemical treatment (Cuprys et al., 2020), advanced oxidation processes (Huang et al., 2021; Mondal et al., 2018), photolysis (Snowberger et al., 2016) and enzymatic degradation (Liu et al., 2019) have been employed to remove these antibiotics. Despite the fact that, these treatment technologies come with their own limitations such as increased cost, high energy consumption and generation of secondary pollutants. Thus, there is a growing need to find an economically viable and innovative method that can address the urgent need for a removal of OMPs from wastewater for sustainable future.

To achieve this, it is important to optimize the process of immobilizing enzymes through adsorption to develop a biochar-based remediation method for removing OMPs from wastewater. The process needs to be optimized to achieve maximum adsorptive immobilization efficiency at specific temperature, pH and enzyme dosage. This helps in reducing ambiguity and finding the optimal condition which requires fewer characterizations, fewer experimental runs, and is cost-effective while having better adsorption immobilization capacity. The primary method for degrading OMPs is through adsorption and degradation processes.

Biochar is becoming increasingly popular due to its numerous advantages such as having a larger surface area, high porosity, surface functional groups, low cost, and being produced from waste materials (Jin et al., 2021; Masrura et al., 2023; Zhang et al., 2023). Biochar is

a better and more cost-effective choice for immobilization compared to activated carbon as its production costs are lower (Masrura et al., 2023). Biochar is also suitable for the adsorptive immobilization of enzymes as its surface functional groups can be easily adjusted. Therefore, biochar is frequently used for the breakdown of organic micropollutants (OMPs) (García-Delgado et al., 2018; Imam et al., 2021; Pandey et al., 2022; Tomar et al., 2023; Yang et al., 2017). However, research is going on to improve its adsorption capacity, which may increase its production costs and lead to more pollution (García-Delgado et al., 2018; Wang et al., 2022b; Zhang et al., 2023). Nonetheless, raw biochar is preferred for immobilization since it is economical to produce and safer for the environment (no surface modification means no chemical leaching after repetitive use). Studies have revealed that SMW has the ability to adsorb micropollutants on account of its aromaticity and wide surface area, making it a promising feedstock for the production of raw biochar (Wu et al., 2019). Hence, it's important to note that the type of feedstock used for producing biochar affects its adsorption capacity, and its capacity eventually decreases over multiple uses (Ding et al., 2014; Suman et al., 2017; Wu et al., 2019). Thus, it's essential to regenerate biochar to maintain its adsorption capacity.

This study aims to manage SMW biomass by producing biochar and immobilizing partially purified laccase, extracted from SMW, onto the surface of biochar through an adsorption process. Previous research studies have utilized modified biochar surfaces to immobilize laccase ((Daâssi et al., 2014; Imam et al., 2021; Lassouane et al., 2019; (Nguyen et al., 2016; Taghizadeh et al., 2020). However, this approach has resulted in increased costs. On the other hand, the use of biochar alone leads to saturation in the degradation of OMPs. Therefore, developing an immobilized system for biochar will improve its adsorption ability and enhance the degradation of OMPs through enzymatic action such as laccase. This will allow for better and longer-lasting applications in real-time scenarios.

5.2. Materials and methods

5.2.1. Materials

SMW bags (*Pleurotus florida*), were collected from a mushroom farm in Guwahati, India (as mentioned in (Chapter 3, sub-section no: 3.2.1.1.) and all the other chemical reagents purchased mentioned in (Chapter 3, sub-section no: 3.2.2.).

5.2.2. Methods

5.2.2.1. Preparation of raw biochar from spent mushroom waste of Pleurotus florida

In this study, the SMW of *P. florida* was dried at 105 ℃ in a hot air oven for 24 h to remove moisture. The dried waste was then subjected to slow pyrolysis at 500 ℃ for 60 minutes under a limited oxygen environment. As reported by Jin et al. (2021), the BET surface area and yield percentage of different SMW were measured at different temperatures (350 ℃, 400 ℃, 500 ℃, and 600 ℃). The results revealed *P. ostreatus* achieved the highest yield at 500 ℃ (61.27%) with BET surface area 103.3 m^2g^{-1}. Pyrolysis temperature has less influence on the ash content than the material type (Jin et al., 2021) i.e. the type of material used to make the main substrate. Stable aromatic structures, lower H/C molar ratio, and a reduction in acidic-functional groups can create a negative charge (Cao et al., 2019; Jin et al., 2021; Liu et al., 2016). This is achieved by reducing the polar content of the functional group at higher pyrolysis temperatures, which in turn enhances its adsorption ability. Recent study by Jin et al., (2021) found that at higher temperatures, *P. ostreatus* had lower functional groups and a lower H/C molar ratio. However, we would like to emphasize that *P. ostreatus* and *P. florida* are part of the same family of *Pleurotus* species. Therefore, higher surface area and yield reported by Jin et al., (2021) on *P. ostreatus* substrate at 500 ℃. Our study focused on the characteristics of biochar developed at 500 ℃, and SMW of *P. florida* was prepared at 500 ℃ for the experiment. Therefore, raw biochar (without surface modification) was employed to immobilize laccase enzyme and degrade levofloxacin antibiotic from wastewater. The pyrolysis yield achieved for SMW of *P. florida* in this study was 52.61%.

5.2.2.2. Adsorptive immobilization process on SMW raw biochar

Raw biochar produced from SMW was immobilized using partially purified extracellular laccase enzyme (extraction and partial purification of laccase enzymes has been discussed in *Chapter 3, Sub-section no: 3.3.1. and 3.3.3*.) through an adsorption method. In this study, no chemical was used to treat the SMW biochar or to functionalize it chemically. The optimization of laccase immobilization was performed by using 5 different dosages of the enzyme (0.5, 1, 3, 5, and 8 U mL^{-1}) for a duration of 5 h (1, 2, 3, 4 and 5 h) and 10 mg of laccase immobilized biochar was mixed with the varying dosages of enzyme. The process of adsorption was carried out by mixing biochar with partially purified laccase enzyme for 5 hours in a shaker incubator. This was done at a temperature of 30 ℃ and a speed of 150 rpm. The aim was to ensure complete adsorption as described by (Lonappan et al., 2018b).

Once the immobilization was successful, the laccase-biochar mixture was centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed and analyzed to measure the remaining activity. The immobilized laccase was washed with sodium citrate buffer (pH 4.5) to avoid any non-specific binding. After that, the laccase immobilized biochar was dried at room temperature and stored at -20 ℃ for further use.

To better understand the process of laccase binding to biochar, adsorption isotherms were mapped out. Langmuir, Freundlich, and Temkin isotherms were plotted to investigate the interaction between adsorbent and adsorbate at equilibrium. The equations of the three models were provided in **Eq. (5.1)** represents the Langmuir isotherm model, **(5.2)** represents the Freundlich isotherm model, and **(5.3)** represents the Temkin model. These models were linearized to achieve equilibrium ((Inyinbor et al., 2016).

$$1/q_e = 1/q_m K_L C_e + 1/q_m \quad (5.1)$$

where, C_e = equilibrium concentration of adsorbate (U L^{-1}); q_m = maximum amount of energy required to form a monolayer (U g^{-1}) and K_L = Langmuir constant related to adsorption energy (L U^{-1})

$$\log q_e = \log K_f + \frac{1}{n} \log C_e \quad (5.2)$$

where, q_e= adsorption capacity at equilibrium (U g^{-1}); K_F= Freundlich constant (U g^{-1}) (U $L^{-1})^{1/n}$; C_e = equilibrium concentration of adsorbate (U L^{-1}) and *1/n* = degree of adsorption or the favourability factor of adsorption

$$q_e = B \ln A + B \ln C_e \quad (5.3)$$

where, q_e= amount of adsorbate adsorbed at equilibrium (mg g^{-1}); C_e = concentration of adsorbate in solution at equilibrium (mg L^{-1}); B= RT/b, b is the Temkin constant (J mol^{-1}); T= absolute temperature; R= gas constant and A= Temkin isotherm constant (L g^{-1})

Langmuir linearized plot determines the value of q_m and K_L from the plot of C_e/ q_e versus C_e which explains adsorption at distinct sites. However, the linearized plot of Freundlich which extrapolates K_F and *1/n* from the plot of log q_e versus log c_e, demonstrates sorption variation with temperature. Additionally, the linearized plot of Temkin determines heat sorption distributed non-uniformly.

5.2.2.3. Adsorption kinetics

Pseudo-first-order kinetics and pseudo-second-order kinetics were used to fit the experimental data of laccase loading (0.5, 1, 3, 5, and 8 U mL^{-1}). The R^2 value of both the

kinetic models were used to justify the fit. **Eq. (5.4)** and **(5.5)** represents pseudo- first and second order kinetic model provided in SI.

$$\ln(q_e - q_t) = \ln q_e - K_1 t \quad (5.4)$$

where, q_e= amount of enzyme adsorbed (U g^{-1}) at equilibrium; q_t= amount of enzyme adsorbed (U g^{-1}) at that instantaneous time t (min); K_1= pseudo-first order rate constant (min^{-1})

$$\frac{t}{q_t} = \frac{1}{K_{2q_e^2}} + \frac{t}{q_e} \quad (5.5)$$

where, K_2= pseudo-second order rate constant (g U^{-1} min^{-1}); q= adsorbed amount (U g^{-1}) and q_e= adsorption capacity at equilibrium (U g^{-1})

5.2.2.4. Enzymatic assay, immobilization yield and immobilization efficiency of laccase immobilized biochar.

To determine the activity of immobilized and free laccase enzymes, an enzymatic assay was conducted using ABTS. For immobilized laccase (10 mg), a mixture of 1mL sodium citrate buffer (pH 4.5) and 0.1 M ABTS (250 µl) was incubated for 15 minutes at room temperature. For free laccase, the mixture contained 100µl of laccase, 0.1M ABTS (250 µl), and 1mL of sodium citrate buffer. The change in absorbance due to oxidation of ABTS was measured at 420 nm using a UV spectrophotometer. The activity of immobilized laccase enzyme was calculated using **Eq. (5.6)** and expressed in U g^{-1}.

$$\frac{U}{g} = \frac{abs\ min^{-1} \times df \times R_v \times cf}{\in \times M_{carrier}} \quad (5.6)$$

where, *U/g*= Unit per gram (amount of enzyme needed to oxidize 1 µM ABTS per min per unit); *abs* min^{-1} = absorbance per minute; *df* = dilution factor; R_v = reaction volume (L); C_f = conversion factor for mole to micromole concentration-10^6; $\in$ = molar extinction coefficient= $3.6*10^{-4} M^{-1}$ cm^{-1} and $M_{carrier}$ = mass of biochar on which enzyme was immobilized (g).

The immobilization yield (IY) was calculated in **Eq. (5.7)**

$$IY\ (\%) = \frac{Immobilized\ activity}{Starting\ activity} \times 100 \quad (5.7)$$

The residual activity (left in the supernatant after immobilization) subtracted by the initial activity of the enzyme determined immobilized activity.

The immobilization efficiency (IE) was calculated using **Eq. (5.8):**

$$IE\ (\%) = \frac{Immobilized\ activity}{Activity\ of\ the\ free\ laccase} \times 100 \qquad (5.8)$$

5.2.2.5. Characterization of laccase immobilized biochar

The study monitored the functional groups before and after laccase enzyme immobilization using Fourier transform infrared (FT-IR) spectrometer (Thermo Scientific, Genesys 10S, UV-Visible spectrophotometer) spectral analysis. Additionally, field emission scanning electron microscopy (FESEM; ZEISS SIGMA-HDVP, Carl Zeiss NTS, Cambridge, UK) was conducted for surface morphology characterization. The elemental analysis of raw biochar and immobilized laccase was determined by energy-dispersive X-ray spectroscopy (EDS). The biochar samples, both before and after immobilization, were placed on a copper-containing conductive adhesive surface and coated with gold. Furthermore, the specific surface area of pyrolyzed SMW biochar was analyzed using the Brunauer-Emmett-Teller (BET) technique The adsorption-desorption isotherms of N_2 at 77K were used to estimate porosity, and the surface area was determined by an automatic surface area pore analyzer. The samples were degassed at 423K (Autosorb-1, Quantachrome, USA, iSORB-HP- single station). To investigate the thermal endurance of biochar, free laccase, and laccase immobilized biochar, the study used thermogravimetric analysis (TGA). The analysis was conducted in a nitrogen flow of 80 mL min^{-1}, with a rate of 10 ℃ min^{-1}, from 25 ℃ to 1207 ℃.

5.2.2.5. Stability of the adsorbed-immobilized laccase into raw biochar

The stability of laccase immobilized biochar was evaluated at various pH levels ranging from 3 to 10. This was done using different buffers such as glycine buffers (pH 3), sodium citrate buffer (pH 4.5), phosphate buffer (pH 6), tris-HCL buffer (pH 8) and carbonate buffer (pH 10) for a period of 7 days at 30 ℃. The temperature profile was also evaluated for 3 h, ranging from 4°C to 75°C. Additionally, the stimulatory and inhibitory effects of metal ions were examined. Metal ions (Mg, Cu, Zn and Mn) were examined at 10 mM concentration for 1 week at a temperature of 30 ℃. The effect of inhibitors such as EDTA, urea, and dithiothreitol was investigated at 10 mM concentration for 1 week. All the

experiments were performed in triplicate and residual activity was calculated using ABTS substrate (1 mM).

5.2.2.6. Statistical analysis

To evaluate statistical significance, the Duncan test was used to do analysis of variance (ANOVA). SPSS statistical software was used to conduct the analysis of variance. Results with *p*-value lower than 0.05 were considered statistically significant and all the experiments were conducted in triplicates.

5.3. Results and discussion

5.3.1. Adsorptive immobilization of laccase onto SMW raw biochar

The immobilized activity at pH 4.5 for laccase dosage (0.5, 1, 3, 5 and 8 U mL^{-1}) was investigated at a temperature 30 ℃. The enzymatic assay, immobilization yield and immobilization efficiency calculation are mentioned in section 2.2.5. The immobilization yield increased with respect to enzyme dosage. Laccase dosage of 5 U g^{-1} resulted in the highest immobilization yield of 58.42% in this study, however with a laccase dosage of 8 U g^{-1}, there was no such increase in immobilization yield (56.12%). The lowest immobilization yield was observed at a laccase dosage of 0.5 U g^{-1} with immobilization yield of 30%. Correspondingly, highest immobilization efficiency was achieved at a laccase dosage of 5 U g^{-1} (65.03%) and lowest immobilization efficiency was achieved at a laccase dosage of 0.5 U g^{-1} (42.23%), whereas no such increase in immobilization efficiency was observed at a laccase dosage of 8 U g^{-1} as shown in **Fig. 5.1**. According to research by Lassouane et al., (2019) immobilization load and yield both increased as the concentration of enzyme loading increased. However, in agreement with this study further no such increase in immobilization yield and load was observed at higher concentration of enzyme dosage. Similar phenomena were observed by Sanlıer et al., (2013) with 94% efficiency at 25 mg mL^{-1} of laccase dosage and 69% efficiency at 50 mg mL^{-1}.

These phenomena can be explained by the increased interactions between the laccase molecules and biochar, eventually leading to better adsorption with high immobilization yield and efficiency. Baysal et al., (2014) reported an increase in adsorption with increased enzyme concentration of α-amylase onto chitosan nanocomposite. Concurrently, studies by (Lassen and Malmsten, 1996; Nakanishi et al., 2001) explained probable reason could be selective and competitive adsorption or hydrophobic and electrostatic interaction between biochar and laccase. Therefore, these studies evidenced the fact that enzyme activity

beyond a threshold level will not enhance the adsorption capacity which validate the current study in support of the investigation done by (Lonappan et al., 2018a).

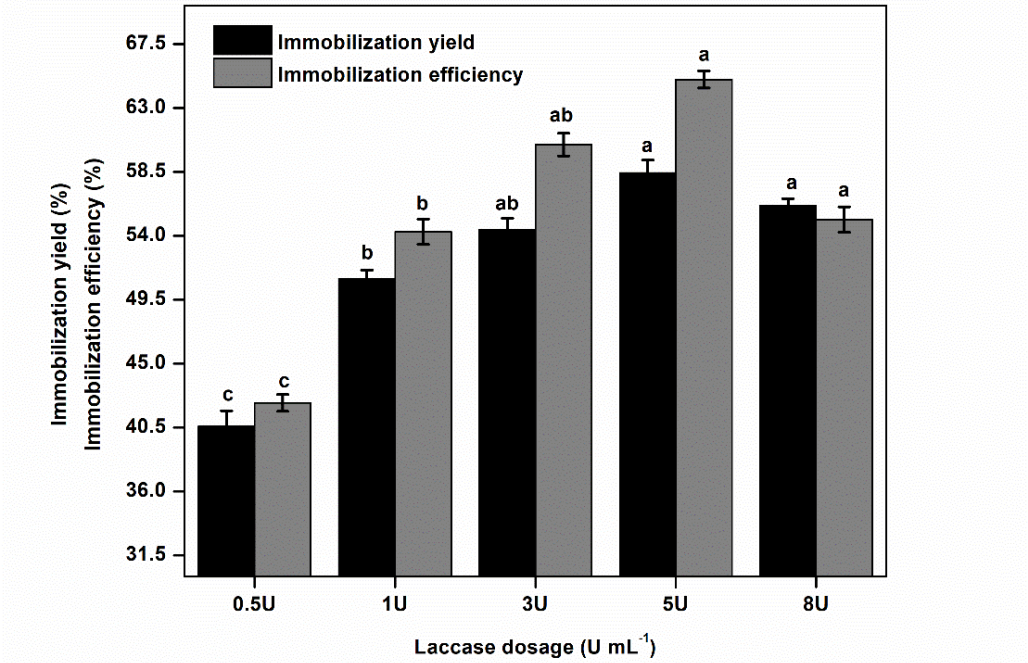


Fig. 5.1. Effect of laccase dosage at determined at pH 4.5 and temperature 30 °C. Different letters in the column indicate significant difference at $p<0.05$ according to Duncan multiple range test.

Nevertheless, pH plays a decisive role in the immobilization of enzymes onto biochar. Enzyme stability is a crucial factor in its immobilization on any support material, and this stability is largely influenced by the enzyme's pH due to the molecular makeup and ionization action of the enzyme. Research studies evidenced the fact that laccase enzyme is more stable in acidic conditions rather than in alkaline conditions (Kumar et al., 2022; Lim et al., 2013). Immobilization of laccase enzyme in rice straw biochar, pine wood biochar, pig manure biochar, almond shell biochar, wood biochar and pine needles biochar were optimized in varied pH ranges ranging from (2-8). The results revealed higher immobilization yield could be attained from pH (3-6). The rationale behind this could be the denaturation of the enzyme both at higher acidic conditions or alkaline conditions also, stability depends upon the isoelectric point of enzyme (Imam et al., 2021; Li et al., 2018; Lonappan et al., 2018a; Pandey et al., 2022; Xu et al., 2014). Further, pH 4.5 for laccase-immobilization was chosen as per the earlier study done by (Ghose et al., 2023).

To achieve maximum laccase output during immobilization, the concentration of ABTS and pH levels play a crucial role along with enzyme dosage. Previous studies have shown that higher concentrations of synthetic mediators can have harmful effects on enzyme activity, but ABTS does not cause any loss of enzyme activity at higher concentrations. In this study, 1mM concentration of ABTS was used to determine the immobilization yield and efficiency. ABTS works by oxidizing the substrate via an electron transfer pathway while in its dication state, which is more stable at acidic pH. The findings of our study align with earlier reports (Johannes and Majcherczyk, 2000; Mitra et al., 2021), which suggest that high immobilized yields and efficiency can be obtained by using 1mM ABTS concentration at a temperature of 30 ℃.

The yield of immobilization depends on several factors like enzyme dosage (laccase), pH, and ABTS concentration. Reusability and stability of the support material also play a crucial role in promoting circularity in the economy. However, by taking into account these factors, laccase immobilized biochar can be effectively utilized in real-time applications.

5.3.2. Adsorption isotherm and kinetics

The Langmuir, Freundlich, and Temkin isotherm models were used to compare experimental results of laccase immobilization on SMW raw biochar and determine the adsorption isotherm as shown in **Fig. 5.2**. Langmuir linear plot of C_e/q_e versus C_e determines the value of q_m and K_m constant with R^2 value of 0.95. The linear plot of $log\ q_e$ versus $log\ C_e$ determines the Freundlich constant K_F and n with linear coefficient value of 0.90. Similarly, binding constant K_T and isotherm constant b_T is determined by the linear plot q_e versus $ln\ (Ce)$ with R^2 value of 0.90 represented in (**Table 5.1)**. The Langmuir model showed a stronger linear correlation than the Freundlich and Temkin models. The K_L value of Langmuir <1 favors monolayer adsorption of adsorbent and adsorbate at temperature 30 ℃ with 5 U g^{-1} enzyme loading. Concurrently, the value $1/n$ (1.025) suggests homogeneous adsorption through physisorption or chemisorption.

Two kinetic models were evaluated for the adsorption process: linearized pseudo-first-order and pseudo-second-order models were fitted to the experimental data. The kinetic constant K_1 and theoretical q_e of pseudo-first-order were calculated from the plot of $log(q_e-q_t)$ versus t (data of pseudo-first-order kinetics did not match experimental equilibrium and calculated equilibrium). Furthermore, the pseudo-second-order constant K_2 and theoretical q_e were determined from the plot of t/q_t versus t. At 30 ℃ the pseudo-second-order represented R^2 value 0.92 (data of enzyme loading 5 U g^{-1} is shown), indicating that this

form of the model best fits the experimental data. Also, the experimental and calculated q_e is in close approximation, suggests chemisorption is the behavior of the adsorption process in accordance with previous studies (Ho and McKay, 1999; Imam et al., 2021; Inyinbor et al., 2016) represented in **Table 5.2 (a-b).**

Table 5.1 Adsorption isotherms using Langmuir, Freundlich, Temkin at 30 ℃.

Langmuir isotherm			**Freundlich isotherm**			**Temkin isotherm**			
q_m (U g^{-1})	K_L (U L^{-1})	R^2	K_F (U g^{-1}) (U $L)^{1/n}$	1/n	R^2	B	A (L g^{-1})	b (J mol^{-1})	R^2
111.11	0.013	0.95	0.27	1.02	0.90	40.44	0.038	62.28	0.90

Table 5.2 (a) Pseudo-first-order kinetic data for laccase adsorption over SMW raw biochar.

Enzyme initial loading (U g^{-1})	**Experimental equilibrium (q_e) (U g^{-1})**	**Calculated equilibrium (q_e) (U g^{-1})**	**K1 (g U^{-1} min^{-1})**	**R^2**
5	176	215	0.0143	0.91

Table 5.2 (b) Pseudo-second-order kinetic data for laccase adsorption over SMW raw biochar.

Enzyme initial loading (U g^{-1})	**Experimental equilibrium (q_e) (U g^{-1})**	**Calculated equilibrium (q_e) (U g^{-1})**	**K2 (g U^{-1} min^{-1})**	**R^2**
5	183	185	0.0143	0.92

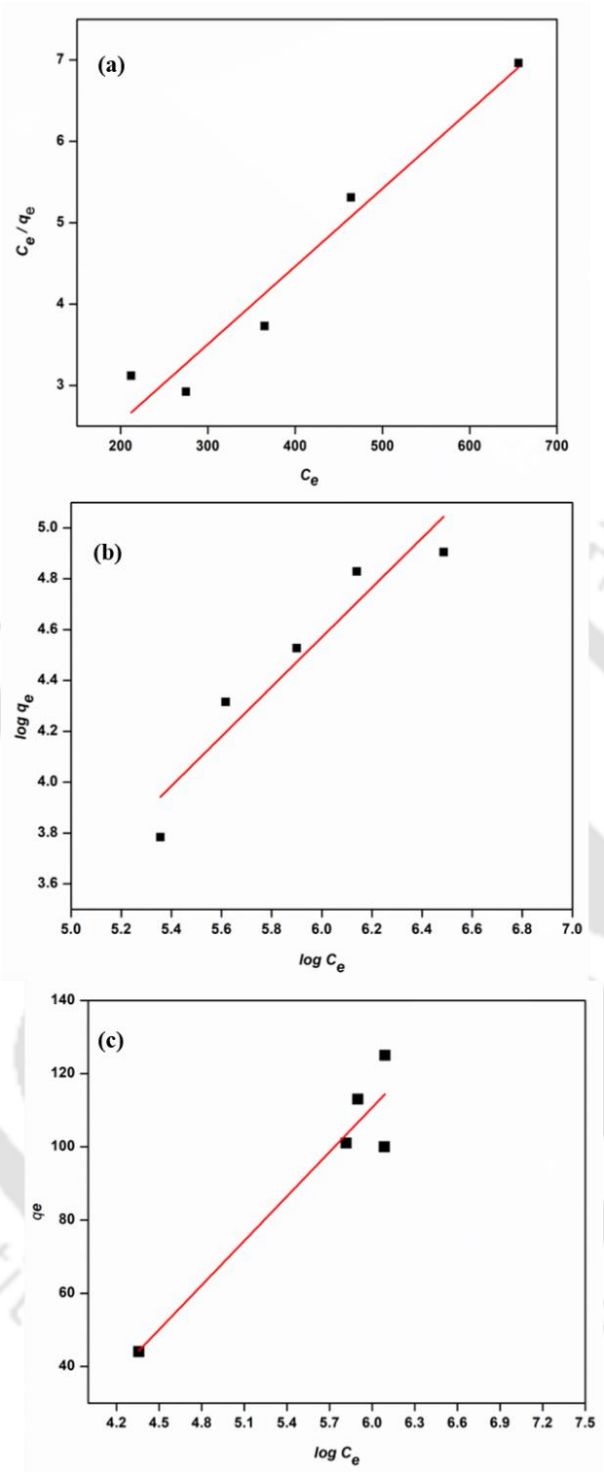


Fig. 5.2. Adsorption isotherm plots of laccase immobilized on raw biochar fitted linearly with (a) Langmuir isotherm; (b) Freundlich isotherm and (c) Temkin isotherm.

5.3.3. Characterization analysis of adsorbed-immobilized laccase onto raw biochar

The comparison between the precise peak positions before, and after immobilization of laccase enzyme in raw biochar and free laccase confirms immobilization in raw biochar depicted in **Fig. 5.3.** The peaks observed in SMW raw biochar indicate the presence of functional groups belonging to hemicellulose, cellulose, and lignin. As per the study reported by Al-Adhami et al., (2002) hemicellulose exhibits vibrations of C=O carbonyl and acetyl groups in the fingerprint region between 1800 and 600 cm^{-1}, C=C coniferyl alcohol, and C-O-C asymmetric stretching in both hemicellulose and lignin. Specific peaks are also visible at 1427.75 cm^{-1} (syringyl and guaiacyl), 898 cm^{-1} (C-H deformations in amorphous cellulose), and 1515 cm^{-1} (aromatic vibrations in lignin) respectively. The sharp peak detected at 1050 cm^{-1} indicates the vibration of the hydroxyl group (O-H) (Wang et al., 2021). The degradation of lignin and hemicellulose is confirmed by the narrowing of the band above the fingerprint region, which lies between 1736 cm^{-1} and 1515 cm^{-1}. Besides, the diminution of bands found over the region between 1427 cm^{-1} and 898 cm^{-1} confirms cellulose degradation (Corrêa et al., 2016; Imam et al., 2021). However, the strong signal at 1628.17 cm^{-1}, indicates amide stretching in laccase immobilized biochar. Similarly, the broad peak stretch between 3500 cm^{-1} and 3000 cm^{-1} is the vibration peak of the intermolecular hydrogen bond of multi-molecule association (Wang et al., 2021). The N-H stretching vibration at 3330.23 cm^{-1} confirms laccase immobilization in biochar as shown in **Fig. 5.3.** The designated peaks in confirm earlier research studies (Imam et al., 2021; Lonappan et al., 2018a; Mathur et al., 2021).

The images in **Fig. 5.4** (a-b) reveal that pyrolyzed biochar, when not immobilized, forms a porous structure. This structure is created due to the release of volatile organic materials from the lignocellulosic skeleton during pyrolysis, which causes distinctive pore-formation, shrinkage, and breakdown of the material structure (Xiong et al., 2017). According to previous studies, pyrolysis at higher temperatures results in rough surface areas, cracks, cavities, and debris, which in turn increases the number of adsorption sites and enlarges the surface area (Fattahi et al., 2015). **Fig. 5.4** (c-d) clearly shows immobilized laccase present on the surface of biochar and the planar surface of nodules (Wang et al., 2021) along with smoothening of the biochar surface (Pandey et al., 2022; Zhang et al., 2020). Further, the increase in %N content in laccase immobilized biochar suggests immobilization of laccase onto raw biochar (Pandey et al., 2022; Uygun, 2013) represented in **Fig. 5.5**. and **Table 5.3. (a-b).**

The thermal stability of immobilized laccase, raw biochar, and free laccase were investigated using TGA analysis. The analysis was conducted with a constant heating rate of 10 ℃ min^{-1}, from 100 ℃ to 1027 ℃ under N_2 environment. The results of the analysis are shown in **Fig. 5.6.** A significant reduction in weight was observed in the free laccase at temperatures ranging from 60 to 150 ℃. This weight loss may be due to the structural breakdown of the enzymatic laccase and the removal of structural water at high temperatures. However, when subjected to high temperature, the raw biochar and immobilized laccase experienced less deformation and maintained maximum stability up to 600 ℃. The weight loss in both cases was linear and at 1207 ℃, 14% stability remained. There are two distinct stages to the weight reduction process when using raw biochar or immobilized laccase. During the initial stage, when the temperature ranges from 60 ℃ to 200 ℃, the weight loss may be attributed to water evaporation (Abdul et al., 2017). However, immobilized laccase biochar is responsible for the weight loss when the temperature exceeds 300 ℃. This may explain why immobilized laccase achieves a greater amount of weight loss (Paul and Londhe, 2019). The results we obtained and the literature prompted us to conclude that raw biochar immobilized laccase is thermally stable.

The surface area of the immobilized laccase and raw biochar were analyzed by using BET raw biochar had a surface area of 60.206 m^2 g^{-1} when subjected to 500 ℃, whereas, surface area of adsorbed immobilized laccase was 20.193 m^2 g^{-1}. BET plot of N_2 adsorption at a temperature of 77k with pore diameter 8.68 nm and pore volume 0.246 cm^3 g^{-1} depicted in **Fig. 5.7.** This indicates adsorptive immobilization of laccase enzyme in raw biochar. It is important to note that the pore volume increases as pyrolysis temperature rises (Wu et al., 2019). Moreover, higher pyrolysis temperatures lead to the formation of microporous structures in the biochar. This is probable due to the evaporation and breakdown of volatile organic components (Wang et al., 2017). However, the higher amount of lignin, hemicellulose, and cellulose in SMW of *P. florida* contributes to the formation of non-homogeneous surfaces and microporous structures of biochar (Wu et al., 2019). Therefore, for effective adsorptive immobilization, biochar produced at higher pyrolysis temperatures is necessary because it increases the surface area, which in turn contributes to more active sites (Wang et al., 2017).

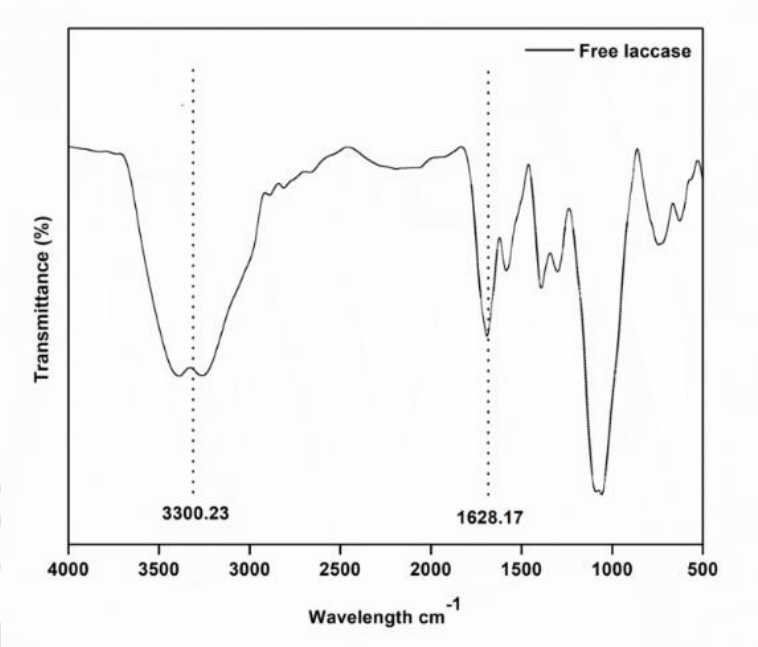


Fig. 5.3. FTIR spectra of before and after immobilization of laccase in spent mushroom waste raw biochar: (a) before immobilization.

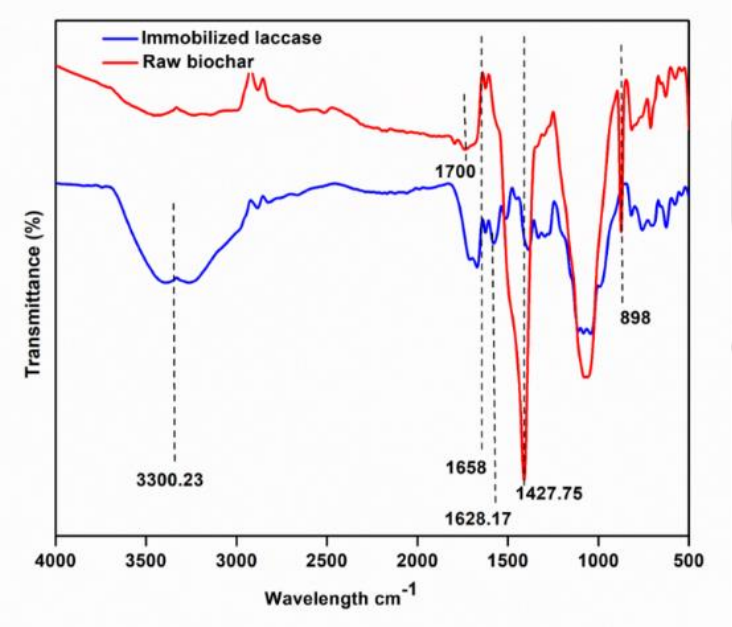


Fig. 5.3. FTIR spectra of before and after immobilization of laccase in spent mushroom waste raw biochar: (b) after immobilization

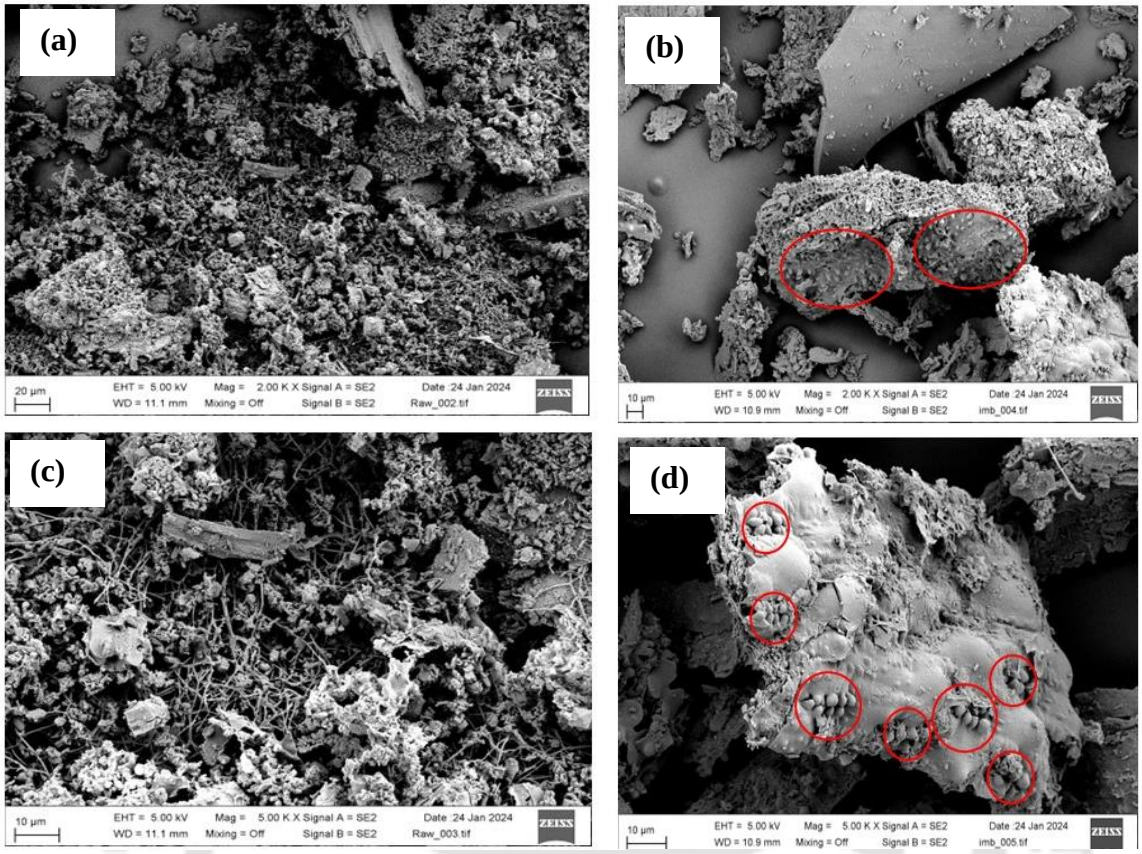


Fig. 5.4. FESEM images, before and after immobilization of laccase in raw biochar: raw biochar image before immobilization (a) at magnification 2 kx and (b) at magnification 5 kx and (c) after immobilization at magnification 2 kx and (d) at magnification 5 kx.

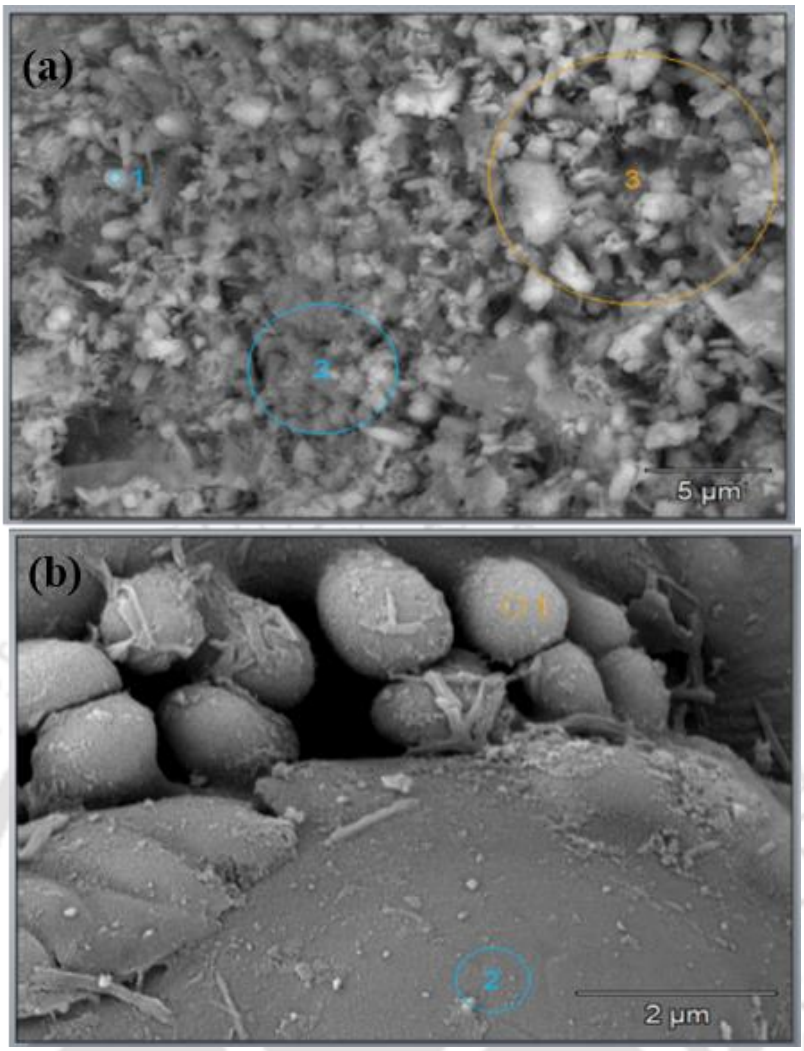


Fig. 5.5. Energy-dispersive X-ray spectroscopy analysis of raw biochar and laccase immobilized biochar: (a) planar area and (b) nodule

Table 5.3 (a) Elemental analysis of raw biochar

Element	Point 1 (Weight %)	Point 2 (Weight %)	Point 3 (Weight %)
C K	27.1	28.8	28.8
N K	**0.1**	**1.6**	**1.4**
O K	46.4	36.7	38.2
Na K	0.1	0.1	0.1
Mg K	0.6	0.6	0.6
Al K	0.3	0.8	0.5
Si K	17.3	9.6	11.4
S K	ND	0.4	0.3
Cl K	0.2	0.2	0.2
K K	1.3	1.9	1.6
Ca K	6.3	18.4	16.3
Mn K	0.4	0.7	0.6
Fe K	ND	0.3	ND
	100.0	100.0	100.0

Table 5.3 (b) Elemental analysis of laccase immobilized biochar

Element	Point1 (Weight %)	Point 1 (Weight %)
C K	21.4	16.1
N K	**2.6**	**1.6**
O K	35.8	34.4
F K	2.4	ND
Na K	2.3	3.0
Mg K	0.2	0.2
Al K	0.1	0.1
Si K	21.1	28.8
K K	0.2	0.3
Ca K	0.6	0.8
Mn K	ND	0.1
Cu K	0.5	0.4
Au M	12.7	14.2
	100.0	100.0

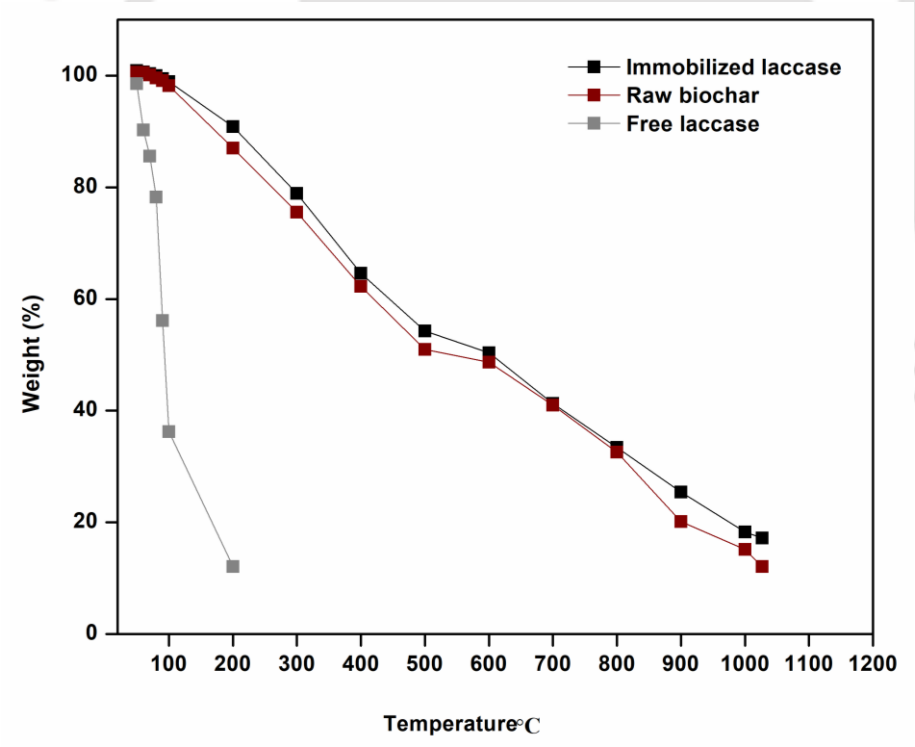


Fig. 5.6. Thermogravimetric analysis of free laccase, immobilized laccase and raw biochar.

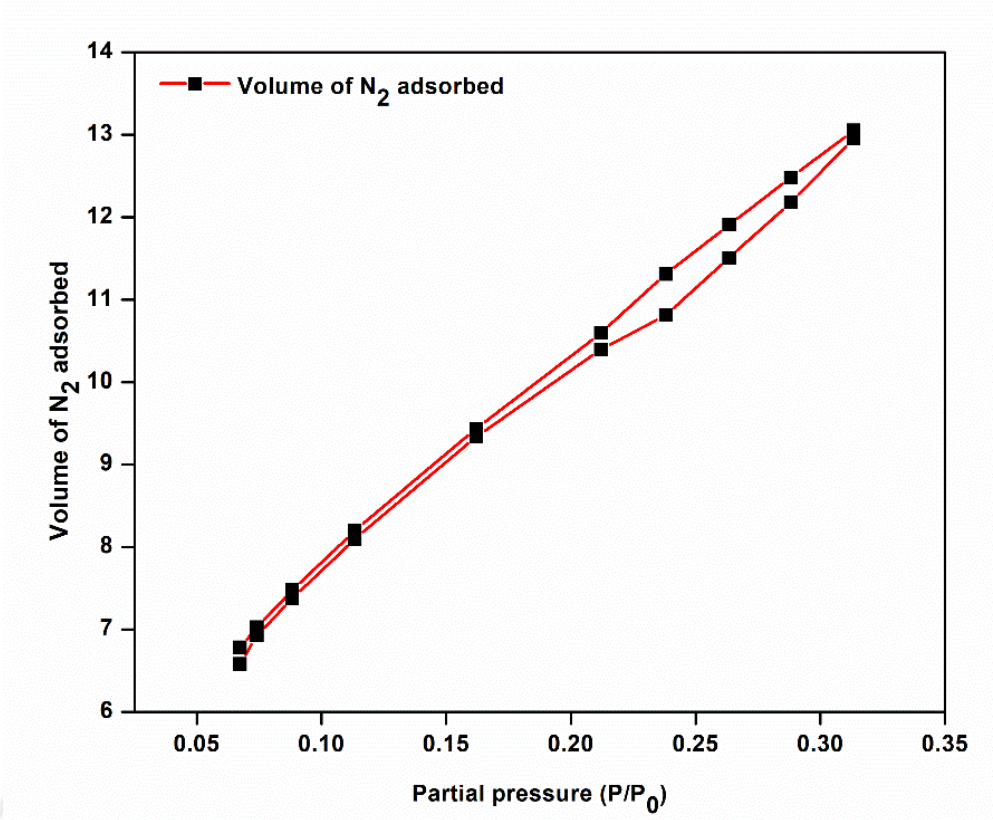


Fig.5.7. Brunauer-Emmett-Teller plot of N_2 adsorption at a temperature of 77k.

5.3.4. Stability checks of the adsorbed-immobilized laccase onto raw biochar and free laccase.

5.3.4.1. pH stability

The study evaluated the persistence of adsorbed immobilized laccase in raw biochar and free laccase at different pH levels (ranging from 3 to 10 with a concentration of 0.2 M), over a period of 7 days at a temperature of 30°C. The results showed that the pH-dependence of both free laccase and adsorbed-immobilized laccase was significant. The highest residual activity for laccase immobilized biochar was observed at pH 4.5 (100%), but it decreased significantly at higher pH levels (at pH 10, only 16% relative activity was observed). On the other hand, the free laccase enzyme exhibited a substantial drop-in laccase activity at pH 4.5 (69.74%), as shown in **Table 5.4 (a).** Additionally, the results indicate that immobilized laccase retains over 60% of its laccase activity at pH 6, which is significant for the degradation of pollutants at both acidic and basic pH. The stability of laccase was improved by immobilization, which made it impervious to inactivation of laccase activity for 7 days and prevented its decline at higher pH levels. In its free form,

laccase activity decreases after 8 h of incubation at 20 ℃ reported by Kumar et al. (2022) due to the ionization of amino acids, which affects the enzyme's structure and activity (Wang et al., 2022a). The ideal pH for laccase was found to be 4.5, which is consistent with other research studies (Bourbonnais and Paice, 1992; Collins et al., 1998; Lonappan et al., 2018b). The presence of hydroxide ions at higher pH levels binds with the type 1 copper ions, impeding the transfer of electrons and terminating the reaction, which causes a decline in laccase activity (Catapane et al., 2013). Similar research studies (Catapane et al., 2013; Ghobadi Nejad et al., 2019; Kumar et al., 2016; Leonowicz et al., 1988; Lonappan et al., 2018b) revealed that the highest laccase activity is observed at acidic conditions rather than in alkaline conditions. However, laccase stability also depends on the mycelium growth conditions in a natural ecosystem, therefore, different species of fungi have different pH stability (Ghose et al., 2023). The study's results indicate that the optimal pH stability of laccase extracted from *P. florida* is at pH 4.5.

Table 5.4 (a) pH stability of laccase immobilized biochar.

pH	**Immobilized laccase**		**Free laccase**	
	Enzyme activity (U mL^{-1})	**Relative activity (100%)**	**Enzyme activity (U mL^{-1})**	**Relative activity (100%)**
3	1.76	74.60%	1.27	54.20
4.5	2.36	100.34%	1.64	1.64
6	1.52	64.77%	0.80	34.03
8	1.26	53.58%	0.54	22.90
10	0.62	26.69%	0.34	14.79

5.3.4.2. Temperature stability

This study examines the effect of temperature on both immobilized and free laccase enzymes, ranging from 4 ℃ to 75 ℃. Free laccase enzyme activity reduced to 17% at 65 ℃ due to protein denaturation at higher temperatures. However, immobilized laccase enzyme activity remained over 60% in this temperature range represented in supporting information **Table 5.4 (b)**. Loss of activity for free laccase enzyme can be attributed to protein denaturation at higher temperatures; thus, the rationale for immobilizing laccase enzyme in support material is to boost its stability and tolerance to higher temperatures. However, previous research studies revealed immobilized laccase tolerance at higher temperatures immobilized in different support material as well as biochar produced from the almond shell, pig manure and pine wood (Bayramoğlu et al., 2008; Cabana et al., 2007;

Imam et al., 2021; Leonowicz et al., 1988; Nguyen et al., 2016). In this study, the immobilized laccase enzyme retained more than 50% of its relative activity at 75 ℃, which is potentially due to increased substrate diffusion at high temperatures (Lonappan et al., 2018a; Naghdi et al., 2018; Zhang et al., 2020). Previous research has shown that the free form of laccase has the highest relative activity between 30 ℃ and 40 ℃, which is consistent with the results of this study. However, the choice of biochar feedstock determines its tolerance to higher temperatures (Lonappan et al., 2018b).

Table 5.4 (b) Temperature stability of laccase immobilized biochar.

Temperature	**Immobilized laccase**		**Free laccase**	
	Enzyme activity (U mL^{-1})	**Relative activity (100%)**	**Enzyme activity (U mL^{-1})**	**Relative activity (100%)**
4	2.04	86.63	1.322	56.03
20	2.05	87.28	1.09	46.48
30	2.37	100.64	0.70	70.04
40	2.17	92.35	0.51	51.54
50	1.90	80.72	0.39	39.91
65	1.59	67.73	0.17	17.06
75	1.29	54.81	0.14	14.00

5.3.4.3. Effect of metal ions and inhibitors

We investigated the effect of metal ions and inhibitors on free and immobilized laccase over a period of one week at 30 ℃. Metal ions including Cu, Zn, Mg, and Mn at a concentration of 10 mM showed both stimulatory and inhibitory effects. According to **Table 5.4 (c)** as shown in supporting information, the order of stimulatory effect of metal ions on immobilized and free laccase is Cu>Zn>Mg>Mn. The binding of laccase to the type 2 copper core is responsible for the stimulatory action of Cu on laccase activity (Niladevi et al., 2008). Previous studies also suggest that copper has a stimulating influence as a catalytic oxidant (Lu et al., 2012). Furthermore, the inhibitory effect of zinc in the free form of laccase has been documented in various fungal species like *Ganoderma lucidum*, *Cladosporium cladosporioide*, *Trichoderma harzanium*, *Lentinula edodes* and *P. ostreatus* (Baldrian, 2004; Forootanfar et al., 2011; Naghdi et al., 2018). In addition, Mn being a potent inhibitor at 1 mM concentration inhibits the relative activity of laccase up to 91% (Liao et al., 2012). The results of the current study have shown that, similar to earlier studies, at a concentration of 10 mM, the relative activity of laccase in the presence of Mn is only 17%. However, when laccase is immobilized, its relative activity is retained up to

27%. This means that biochar, acting as a support substance, protects laccase from losing its structural integrity even in the presence of Mn. Additionally, in this study, the relative activity of immobilized laccase in the presence of Mg was retained up to 71%. A previous study found that laccase isolated from *Trametes versicolor* and immobilized in barium alginate beads retained laccase activity up to 80% when incubated at 25 ℃. In contrast, at a 1 mM concentration of Mg, a 38% inhibitory effect was observed (Latif et al., 2022).

Table 5.4 (c) Effect of metal ions of laccase immobilized biochar.

Metal ions	**Immobilized laccase**		**Free laccase**	
	Enzyme activity (U mL^{-1})	**Relative activity (100%)**	**Enzyme activity (U mL^{-1})**	**Relative activity (100%)**
Mg	1.69	71.64	1.28	54.64
Cu	2.28	96.90	0.76	76.50
Zn	1.78	75.72	0.65	65.52
Mn	0.65	27.67	0.17	17.47

The effect of inhibitors (EDTA, Urea and DTT) concentration 10 mM was studied at 30 ℃ for 1 week for both immobilized and free laccase. Based on the results presented in supporting information **Table 5.4 (d)** inhibition strength can be ranked as follows: EDTA < Urea < DTT.

Table 5.4 (d) Effect of inhibitors of laccase immobilized biochar.

Inhibitors	**Immobilized laccase**		**Free laccase**	
	Enzyme activity (U mL^{-1})	**Relative activity (100%)**	**Enzyme activity (U mL^{-1})**	**Relative activity (100%)**
EDTA	2.02	85.99	1.30	55.39
DTT	0.30	13.05	0.22	9.72
Urea	0.76	32.91	0.29	12.51

When immobilized laccase was exposed to EDTA, the enzyme retained up to 85% of its activity. Interestingly, the presence of EDTA allowed other inhibitors such as Triton X-100 and surfactants to sustain their laccase activity for up to 8 days. This was possibly due to the increased contact between hydrophobic molecules (Liao et al., 2012). EDTA's mild

inhibition of enzyme activity at high concentrations is likely because, as a metal chelator, it binds with the inorganic prosthetic groups associated with metalloenzymes, rendering it more resilient at higher concentrations (Naghdi et al., 2018). DTT breaks the disulfide bonds of enzyme. At lower concentrations of 0.2 mM, there is a 90% inhibitory effect in laccase activity isolated from *Paraconiothyrium variabile* in the presence of DTT (Forootanfar et al., 2011). In this study, both immobilized and free forms of laccase showed a reduction in activity up to 30% in the presence of DTT.

Therefore, immobilized biochar could be utilized in real-time applications for the degradation of emerging OMPs from wastewater, as it acts as a support material for adsorptive immobilization of laccase enzyme and thus, increases the relative activity of laccase in various environmental conditions. This, in turn, prolongs the shelf life of the immobilized product.

5.4. Conclusions

This study highlights that pH, temperature and enzyme dosage are paramount factors to achieve maximum adsorptive immobilization yield. Undoubtedly, the maximum immobilization yield obtained at pH 4.5, temperature 30 ℃ and enzyme dosage (5 U g^{-1}) can be considered in future studies for adsorptive immobilization in the support material. Despite, the fact that fewer variations may occur due to extraction of laccase enzyme from different species of fungi. These features of immobilization and support material promise sustainability as well as cost-effectiveness. Therefore, immobilized laccase onto raw biochar highlights the real- time application to be achieved by utilizing adsorption-immobilization process as using raw biochar reduces the load of chemical leaching and secondary pollution, and higher degradation efficiency of pollutants can be achieved. The findings contribute to the development of scalable enzyme-based solutions for treating wastewater, offering a viable approach to mitigate the impact of organic micropollutants in the environment. Ultimately, this innovative method can play a crucial role in advancing sustainable wastewater treatment practices and improving overall environmental health.

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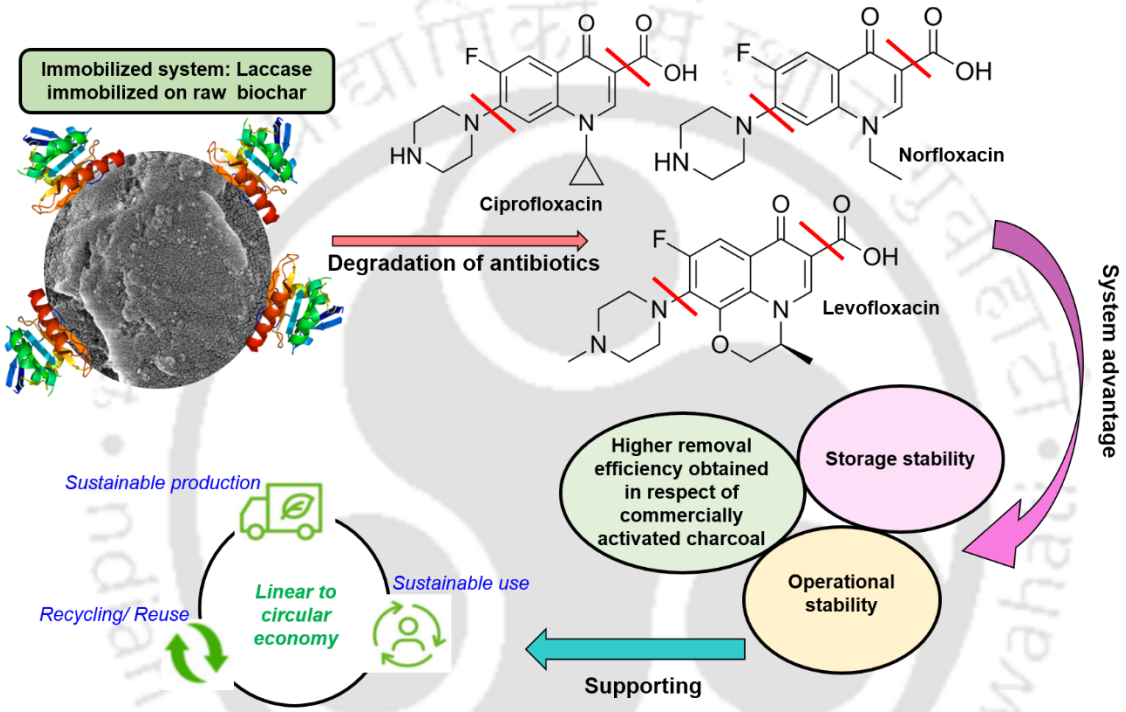
CHAPTER 6

To assess the efficiency of immobilized laccase in biodegrading fluoroquinolone antibiotics and its operational stability.



"This chapter deals with the developed immobilized system i.e. laccase immobilized onto raw biochar produced from spent mushroom waste of Pleurotus florida. This immobilized system has great potential in degrading fluoroquinolone antibiotics. Enhanced storage stability, operational stability, and removal efficiency were all achieved by the immobilized system."

6. To assess the efficiency of immobilized laccase in biodegrading fluoroquinolone antibiotics and its operational stability.

6.1. Introduction

The adsorptive immobilization process relies on two factors: the enzyme-support affinity and the surface area. Covalent bonding and physical binding are the two main methods for immobilizing enzymes on solid substrates (Lonappan et al., 2018a). The most practical and cost-effective approach is typically physical binding through adsorption, which encompasses ionic interactions, hydrogen bonding, and van der Waals forces which do not alter the enzyme's original structure (Jesionowski et al., 2014). This cutting-edge approach preserves the enzyme's activity by avoiding alterations to its active areas. Active functional groups on both the enzymes and the support material are necessary for adsorption to take place. Contrarily, support material lacking functional sites, are altered by using 'chemical modifiers' that pertains enzyme-support material interactions (Kaur et al., 2021). However, using chemical reagents to alter the surfaces of support material leads to secondary pollution and leaching out of chemicals (Wu et al., 2019). Therefore, there is a need of an hour to find unmodified support material. Recent advancements in immobilization have led to the successful use of carbonaceous materials (biochar) and nano-particles for immobilization (Ge et al., 2009).

Carbonaceous materials are derived from agricultural wastes and forestry residues. For immobilization, agricultural wastes have been potential feedstock for biochar production and immobilization of enzymes (Fatma et al., 2018). The benefits of immobilized enzymes, in contrast to free enzymes, are recoverable and reusable upon reaction completion. The enzyme can be easily purified via immobilization. Also, unlike free enzymes, immobilized enzymes are stable. Further, immobilization of enzymes can prevent enzyme leeching, higher operational stability, and enhances activity of the reaction. Hence, enzyme immobilization has significant importance in industries (Imam et al., 2021; Lonappan et al., 2018a, 2018b).

The lignocellulosic nature of SMW, which has a large surface area and numerous microspores is enhanced when it is pyrolyzed into biochar, and the resulting biochar is composed of a wide variety of organic compounds (Wu et al., 2019). Thus, SMW is a potential raw material for biochar production and immobilization of laccase enzyme. Laccase enzyme has been immobilized in biochar derived from pine wood, pig manure,

maple wood, almond shell and rice straw for degradation of OMPs such as anthracene, diclofenac, sulfonamides, and pesticides (Imam et al., 2021; Li et al., 2018; Lonappan et al., 2018b; Pandey et al., 2022). This study aims to produce biochar from SMW without chemical alteration of its surface. The study also seeks to immobilize laccase enzyme through the adsorption method (as discussed in Chapter 5) and to remediate fluoroquinolones antibiotic specifically ciprofloxacin, levofloxacin and norfloxacin in wastewater.

Ciprofloxacin, levofloxacin and norfloxacin are persistent organic pollutant (POPs) due to its discharge into wastewater which has negative impacts on algae and promotes the growth of resistant bacteria (Xiong et al., 2017). Though ciprofloxacin, levofloxacin and norfloxacin has been found to be effective against diseases its residues have been detected in surface water, tap water, and domestic wastewater (Diwan et al., 2018; Ghosh et al., 2009; Saxena et al., 2021). This indicates that both conventional and advanced wastewater treatment technologies have been unsuccessful in removing the medication residues. Nevertheless, research studies mark the presence of fluoroquinolone antibiotics from the secondary effluent of biologically treated wastewater (Van Doorslaer et al., 2014). Ciprofloxacin, levofloxacin, and norfloxacin constitute 80% of fluoroquinolone antibiotics found in secondary effluent water (Van Doorslaer et al., 2014). Several researchers have pointed to the deconjugation of fluoroquinolone metabolites in raw influent as the likely cause of the increased concentration of secondary effluent during water treatment (Plósz et al., 2010; Verlicchi et al., 2012). Thus, criteria such as the kind of treatment process, the composition of the influents, and parameters such as solid retention time, hydraulic retention time, and temperature affect the efficiency of fluoroquinolone removal (Gao et al., 2012; Tang et al., 2012). Recent treatment technologies such as microfiltration, AOPs and ultrafiltration have been employed in the tertiary treatment process for fluoroquinolone removal resulting in negligible-55% contribution of microfiltration and ultrafiltration from secondary effluents (Li et al., 2013). Additionally, studies reported 17% of fluoroquinolone antibiotics are removed using tertiary sand filtration (Batt et al., 2007). Granular activated carbon is said to have a comparable effect for the elimination of ciprofloxacin (18%) and levofloxacin (30%) (Yang et al., 2011).

In consideration, with above mentioned failure of treatment technologies. This study is focused on degradation of fluoroquinolone antibiotics by immobilizing laccase in raw biochar derived from SMW of *P. florida* through adsorptive immobilization (discussed in Chapter 5) and then utilizes the same to degrade ciprofloxacin, levofloxacin and

norfloxacin antibiotics in wastewater. The results of Chapter 5 demonstrate that the optimized conditions for immobilization (pH 4.5, 30℃ and an enzyme dosage of 5 U $g^{-}1$) are not only optimal for achieving maximum immobilization yield but also critical for enhancing degradation efficiency. Immobilization under these conditions helps maintain the enzyme's active structure and stability, which are essential for sustained degradation activity. The stability of the immobilized enzyme allows for repeated use in real-time applications, making it more resilient to environmental stresses often encountered in wastewater treatment. By ensuring high adsorptive capacity and stable enzyme activity, this immobilized laccase-biochar system supports both efficient pollutant degradation and prolonged operational durability. Therefore, this engineered immobilized system has a dual role: adsorption by the biochar and degradation by the enzymatic action of immobilized laccase. The application of biochar for OMPs remediation could attain higher removal efficiency with this multi-purpose immobilized system.

6.2. Material and methods

6.2.1. Materials

SMW bags (*Pleurotus florida*), were collected from a mushroom farm in Guwahati, India (as mentioned in (Chapter 3, sub-section no: 3.2.1.1.) and all the other chemical reagents purchased mentioned in (Chapter 3, sub-section no: 3.2.2.).

6.2.2. Methods

6.2.2.1. Preparation of raw biochar from spent mushroom waste of Pleurotus florida and adsorptive immobilization of laccase on spent mushroom waste raw biochar

The process of preparing raw biochar from SMW of *P. florida* is thoroughly described in Chapter 5 (Sub-sub-sub section 5.2.2.1.) and further adsorptive immobilization of laccase on SMW raw biochar has been detailed in Chapter 5 (Sub-sub-sub section 5.2.2.2). The immobilized laccase on raw biochar as obtained by experimental procedure detailed in Chapter 5 was further utilized to degrade ciprofloxacin, levofloxacin and norfloxacin antibiotics from artificial wastewater in this study.

6.2.2.2. Degradation studies of ciprofloxacin, levofloxacin and norfloxacin by using laccase immobilized biochar

The degradation experiment was commenced in artificial wastewater using 10 mg of immobilized biochar. The artificial wastewater was prepared following (Bracklow et al.,

2007; Taştan and Dönmez, 2015; Zhao et al., 2017). Preceding to enzymatic treatments, physicochemical parameters of artificial wastewater, including pH, total dissolved solids (TDS), and COD, were measured and listed in **(**Chapter 4 and Table 4.1).

Laccase immobilized biochar (5 U g^{-1}) obtained by adsorptive immobilization by tweaking parameters such as pH, enzyme dosage and temperature (as discussed in **Chapter 5**) of 10 mg was used for the degradation of ciprofloxacin, levofloxacin and norfloxacin at different concentration 5 µg mL^{-1}, 15 µg mL^{-1}, 25 µg mL^{-1} and 50 µg mL^{-1} and degradation experiment was carried out in the presence and absence of synthetic mediator ABTS at 30 ℃ and 50 ℃. The standard solution of ciprofloxacin and norfloxacin were prepared in 0.1 M formic acid whereas levofloxacin standard solution was prepared in 0.1 M sodium citrate buffer. The reaction mixture consists of laccase immobilized biochar (5 U g^{-1}), 500 µL of ABTS (0.05 mM), 3 mL of artificial wastewater and respective antibiotics concentration were added according to the above-mentioned concentration. The reaction was maintained in a shaker incubator (180 rpm) at 30 °C. The samples were collected at equal time intervals 0, 3, 6, 9 and 24 h and an equal amount of HPLC grade methanol was added to terminate the reaction.

6.2.2.3. Quantitative analysis of three fluoroquinolone antibiotics degraded by laccase immobilized biochar by HPLC

High performance liquid chromatography (HPLC) was used quantitatively to analyze the removal efficiency of ciprofloxacin, levofloxacin and norfloxacin. HPLC method to quantify was developed in Agilent system, C18 column (250 × 4.6 mm× 5 µm) eluted using a mixture of 0.1% phosphoric acid: methanol (65:35) with a flow rate of 0.5 ml min^{-1} and injection volume 20 µl. The detector was set at 279, 294 and 278 nm. Appropriate control samples were also incubated to ensure adsorption with catalytic activity and no self-degradation. Further, in order to determine if the degradation of ciprofloxacin, levofloxacin and norfloxacin is mediated by laccase-ABTS, control samples containing both (laccase and antibiotics) or (ABTS and antibiotics) were also incubated at 30 ℃ and 50 ℃, respectively. The residual activity was calculated by following Eq. (6.1). Further, to ensure degradation of levofloxacin by the enzymatic action of immobilized biochar and not by adsorption, immobilized laccase biochar was thermally inactivated at 80 ℃ for 5 h and degradation experiment was carried out at above-mentioned dilutions of levofloxacin in artificial wastewater.

$$Degradation\ (\%) = 100 \times \frac{C_0 - C_t}{C_o} \quad (6.1)$$

where, C_t final concentration of levofloxacin (µg mL^{-1}) and C_0 indicates initial concentration of levofloxacin.

6.2.2.4. Adsorption ability of raw biochar for degradation of three fluoroquinolone antibiotics

The significant individual role of laccase and raw biochar immobilized together for degradation of three fluoroquinolone antibiotics was understood by this experiment. To ensure that, immobilized laccase in raw biochar was thermally inactivated at 80 °C for 5 h and adsorption of ciprofloxacin, levofloxacin and norfloxacin in raw biochar experiment was carried out at 15 µg mL^{-1} concentration in artificial wastewater. This experiment helped us to understand the adsorption capacity of raw biochar to degrade three fluoroquinolone antibiotics from artificial wastewater.

6.2.2.5. Operational cycle of laccase immobilized biochar

The experiment aimed to assess the reusability or operational stability of the immobilized laccase by measuring its enzyme activity for 8 ABTS oxidation cycles. Section 2.2.5 outlined the methods used to determine laccase activity. After each cycle, the immobilized laccase was washed with a sodium citrate buffer (pH 4.5, 0.1mM sodium citrate) to remove the substrate. The continuity of the process was maintained using ABTS. To evaluate the reusability, relative activity was calculated by measuring enzyme activity after each cycle and comparing it to its activity after the first cycle. Laccase activity was initially set at 100%.

6.2.2.6. Storage stability of immobilized laccase onto raw biochar and free enzyme

The storage stability of immobilized laccase and free enzyme was studied at pH 4.5 (sodium citrate buffer) and temperature 30 °C for 14 days. The relative activity of immobilized laccase and free enzyme was checked at 1 mM ABTS concentration. The reaction volume for free laccase enzyme consists of 100 µl of laccase, 250 µl of ABTS and 1000 µl of sodium citrate buffer. On the other hand, the reaction volume for immobilized laccase contained 5 mg of immobilized laccase onto raw biochar, 250 µl of ABTS and 1000 µl of sodium citrate buffer.

6.2.2.7. Adsorption efficiency of three fluoroquinolone antibiotics by commercially available activated charcoal (comparative study with immobilized laccase onto raw biochar)

This study was conducted to determine the adsorption efficiencies for three fluoroquinolone antibiotics by commercially available activated charcoal (procured from Hi-media). The experiment was employed by applying 10 mg of activated charcoal in synthetic wastewater containing 5, 15, 25 and 50 µg mL^{-1} concentration of three fluoroquinolone antibiotics and was incubated at following time intervals 3 h, 6 h, 9 h and 12 h at 30 ℃. The samples were taken out at respective time intervals and removal efficiency was analyzed by HPLC and calculated by the following formula mentioned in chapter 4 (sub-sub section: 4.2.2. and Eq. 4.2.)

6.2.2.8. Statistical analysis

To evaluate statistical significance, the Duncan test was used to do analysis of variance (ANOVA). SPSS statistical software was used to conduct the analysis of variance. Results with *p*-value lower than 0.05 were considered statistically significant and all the experiments were conducted in triplicates.

6.3. Results and discussion

6.3.1. Degradation of ciprofloxacin, levofloxacin and norfloxacin by laccase immobilized raw biochar

The maximum IE and IY achieved at pH 4.5, temperature 30℃ and EU of 5 U g^{-1} (obtained results in Chapter 5 as discussed) was taken into account for degradation of ciprofloxacin, levofloxacin and norfloxacin antibiotic in artificial wastewater at temperature 30 ℃ and 50 ℃. The degradation experiment was commenced both in presence (0.05 mM ABTS) and absence of ABTS. ABTS concentration (0.05 mM) was considered as the optimal reaction condition study was performed in Chapter 3. ABTS is a synthetic mediator that oxidize substrate via electron transfer pathway and radical form of ABTS is responsible to bind in the enzyme pocket (Ghose et al., 2023). Degradation achieved at 50℃ evident the fact that immobilization of laccase in a support material increases its productivity and thermostability. **Figure 6.1.** represents degradation of ciprofloxacin, levofloxacin and norfloxacin by laccase immobilized raw biochar both in presence and absence of laccase-ABTS system.

In case of ciprofloxacin degradation achieved at 30 ℃ for 5, 15 and 25 µg mL^{-1} were 96.44%, 96.20% and 93.12% whereas for 50 µg mL^{-1}, 85.69% of degradation was attained in 3 h. Similarly, at 50℃ for 5, 15, 25 and 50 µg mL^{-1} 73.44%, 70.25%, 68.15%, 57.15% of degradation was achieved. Similarly, highest degradation of levofloxacin achieved by laccase immobilized biochar were achieved at 5 µg mL^{-1} (92.44%), 15 µg mL^{-1} (92.22%), 25 µg mL^{-1} (88.12%) and 50 µg mL^{-1}(83.69%) at 30 ℃ in presence of ABTS whereas at 50 ℃, 72.12%, 71.16%, 68.56% and 64.36% were achieved for 5, 15, 25 and 50 µg mL^{-1}. In absence of ABTS degradation of 91.27%, 90.54%, 84.36% and 80.16% were achieved of the above-mentioned antibiotic concentration. At 50 ℃ 69.15%, 68.98%, 65.16% and 61.23% of levofloxacin degradation was achieved.

Norfloxacin followed the similar trend and highest degradation was achieved for 5 µg mL^{-1} was 92.44% at 30 ℃ and lowest degradation 55.39% was achieved for 50 µg mL^{-1} at 50 ℃. Similarly, studies reported in absence of ABTS, 16.3% of ciprofloxacin was bio transformed by purified laccase within 20 h of incubation. Whereas no such biotransformation of norfloxacin was observed (Parra Guardado et al., 2019). Mediators both natural and synthetic increases the oxidative potential of laccase by producing radical species. Further, the degradation values obtained with laccase-ABTS system explains probable reason of ciprofloxacin achieving higher degradation then norfloxacin is due to the chemical constituents of both the antibiotics.

The cyclopropyl group of ciprofloxacin is more susceptible towards degradation rather than presence of ethyl group in norfloxacin (Prieto et al., 2011). Degradation of sulfamethoxazole in absence of laccase-ABTS system was observed after 2 h of reaction by laccase extracted from *Trametes versicolor* and 3 h of reaction by laccase extracted from MVL. Whereas amoxicillin is degraded in 15 min by laccase-ABTS system (laccase extracted from *Trametes versicolor*) (Parra Guardado et al., 2019). The dependence of laccase on ABTS for degradation of sulfamethoxazole was reported by (Parra Guardado et al., 2019). Therefore, these studies highlight the fact that functional groups present in the substrate is responsible for the reactivity of mediators (Baiocco et al., 2003). As sulfamethoxazole contains phenylamine in its structure and amoxicillin contains phenol group (Parra Guardado et al., 2019). Similarly, both ciprofloxacin and norfloxacin contains phenol group (EDG substituents).

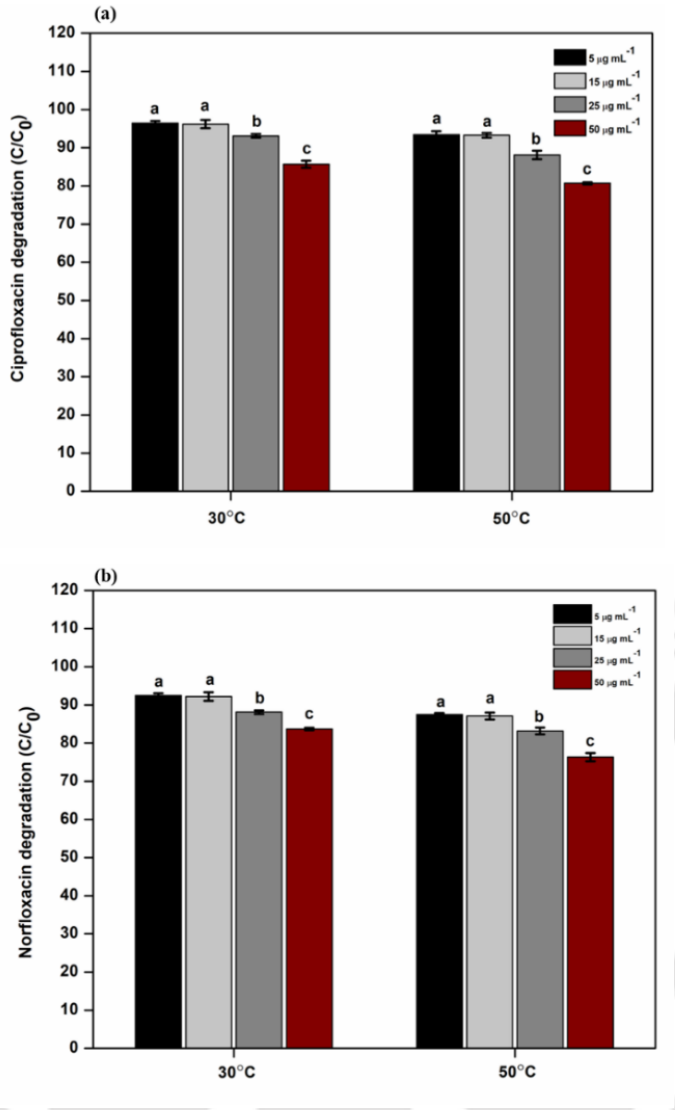


Fig. 6.1. Degradation of fluoroquinolone antibiotics by laccase immobilized biochar at temperature 30 ℃ and 50 ℃, pH 4.5, 0.05 mM ABTS concentration and antibiotic concentration 5 µg mL^{-1}, 15 µg mL-^{1}, 25 µg mL^{-1} and 50 µg mL^{-1} (a) ciprofloxacin degradation at 0.05 mM ABTS concentration; (b) norfloxacin degradation at 0.05 mM ABTS concentration.

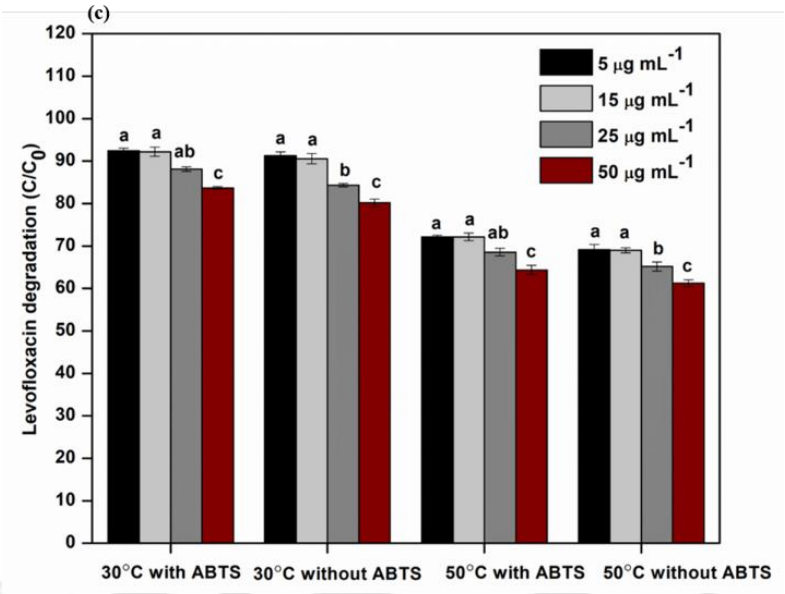


Fig. 6.1. Degradation of fluoroquinolone antibiotics by laccase immobilized biochar at temperature 30 ℃ and 50 ℃, pH 4.5, 0.05 mM ABTS concentration and antibiotic concentration 5 µg mL^{-1}, 15 µg mL^{-1}, 25 µg mL^{-1} and 50 µg mL^{-1} (c) levofloxacin degradation both in presence and absence of 0.05 mM ABTS concentration.

Despite the presence of functional groups, higher electrochemical potential of pollutants is contributing factor towards degradation (Bourbonnais et al., 1998; Parra Guardado et al., 2019). Overall, the degradation of OMPs depends on many contributing factors such as chemical structure of the pollutant, types of mediators, concentration of mediator and redox potential of laccase extracted or isolated from different species of fungi (Ashe et al., 2016). Study by Lloret et al., (2013) observed that ABTS to be the most efficient mediator compared to the N-OH type mediators HBT and syringaldehyde (SA). It is also noted that ABTS is the only mediator that acts as an electron- shuttle which ensures that ABTS is not being used or being decomposed in the reaction (Ashe et al., 2016; Huang et al., 2022). Whereas, other mediators such as HBT, SA, vanillin (VAN) and N-hydroxyphthalimide (HPI) works on the mechanism of hydrogen atom transfer (Ashe et al., 2016). Notably, the laccase-ABTS system works better with benzyl alcohols than with benzylic ether. Conversely, HBT and violuric acid (VA) are more efficient in degrading methyl veratryl ether (Baiocco et al., 2003; Camarero et al., 2005). Therefore, in consistent with previous studies, in this study no significant degradation values were obtained in absence of laccase-ABTS system for both ciprofloxacin and norfloxacin (data not shown). Whereas,

levofloxacin degradation was observed both in presence and absence of laccase-ABTS system. Similar, to the studies degradation of recalcitrant pollutants amoxicillin was observed in absence of ABTS.

Nevertheless, immobilization of laccase into biochar overcomes the limitations of using purified laccase. Research studies reported degradation of many OMPs such as anthracene, diclofenac, carbamazepine, enrofloxacin, norfloxacin, ciprofloxacin and bisphenol A, 90% degradation was achieved in less than 24 h by immobilizing laccase in biochar (Imam et al., 2021; Latif et al., 2022; Lonappan et al., 2018b; Naghdi et al., 2017; Zou et al., 2023). Thus, in agreement with the previous studies (Imam et al., 2021; Pandey et al., 2022; Wang et al., 2022; Zhang et al., 2020) degradation of OMPs such as malachite green, tetracyclines, chlortetracycline, bisphenol A, anthracene from an aqueous solution was achieved by immobilizing laccase onto biochar from rice straw, pig manure, almond shell, and pine wood. However, while the aforementioned OMPs degraded by altering the biochar surface, other studies reported degradation using purified laccase derived from fungal cultures (Gao et al., 2018; Mathur et al., 2021; Prieto et al., 2011; Zhang et al., 2018) which is more expensive and necessitate more stringent conditions for achieving higher degradation. Therefore, using laccase immobilized in raw biochar, which is derived from SMW, is a reliable, profitable, and sustainable way to treat environmental contaminants in water sources. This claim is supported by few previous studies (Lu et al., 2020; Taheran et al., 2017; Wang et al., 2021). The fact that SMW biochar is so effective at breaking down recalcitrant OMPs is that it is not made from a single type of agricultural waste, unlike other biochar produced from other feedstocks. SMW, which comprises the mycelium and other degradation products, is a multi-faceted substance with distinct characteristics when contrasted with the usual agricultural wastes produced from pyrolysis (Jin et al., 2021).

Additionally, laccase immobilized in raw biochar, provides higher degradation efficiency, more operationally friendly, and is cost-effective. Furthermore, using SMW for extraction of laccase and biochar production supports reuse and recycling of waste. Based on these findings, laccase immobilized biochar is significant in degrading fluoroquinolone antibiotics from wastewater, even in the absence of a synthetic mediator. This suggests that the chemical constituents of the compound are primarily responsible for the degradation. However, in case of levofloxacin successful degradation achieved both in presence and absence of ABTS by laccase immobilized biochar at 30 ℃ and 50 ℃ indicates that laccase inserted inside as support material increases its stability and productivity and ensure higher

degradation in comparison to free laccase enzyme as discussed in chapter 3 with the probable reason.

6.3.2. Adsorption efficiency of raw biochar for levofloxacin degradation

This experiment was conducted in order to understand the role of biochar and immobilized laccase in degradation of three fluoroquinolone antibiotics. Antibiotic concentration 15 µg mL^{-1} of three fluoroquinolone antibiotic was taken and 10 mg of laccase immobilized biochar was taken where laccase was thermally inactivated performed at 80 ℃ for 5 h. **Figure. 6.2** shows that raw biochar has an adsorption efficiency of 40-32% of ciprofloxacin, levofloxacin and norfloxacin. Studies by Pandey et al., (2022) reported malachite green degradation by laccase immobilized in biochar and adsorption of degraded products by biochar. It was also observed that adsorption process resulted into mass transfer of malachite green to the active site of enzyme. Hence, catalytic action of enzyme and adsorption process of biochar resulted in 85% removal of malachite green within 5 h of the reaction. Similarly, studies by (Pandey et al., 2022; Zhang et al., 2020) have revealed the dual role of an immobilized system in achieving higher degradation efficiency of bisphenol A. Laccase immobilized in magnetic nano-particles degrade 85% of bisphenol A (25 mg mL^{-1}) within 75 min of reaction. Further, it was speculated that for degradation of bisphenol A, thermally inactivated laccase in magnetic nanoparticles bisphenol A (25 mg mL^{-1}) was adsorbed 40%. Thus, enzymatic conversion plays a dominating role in degradation of OMPs. In consistent with previous studies, in this study inactivated laccase resulted in lesser degradation with respect to immobilized laccase in raw biochar carried out at time 3 h. Hence, this experiment rules out the individual contribution of raw biochar and laccase involved in degradation of three fluoroquinolone antibiotics.

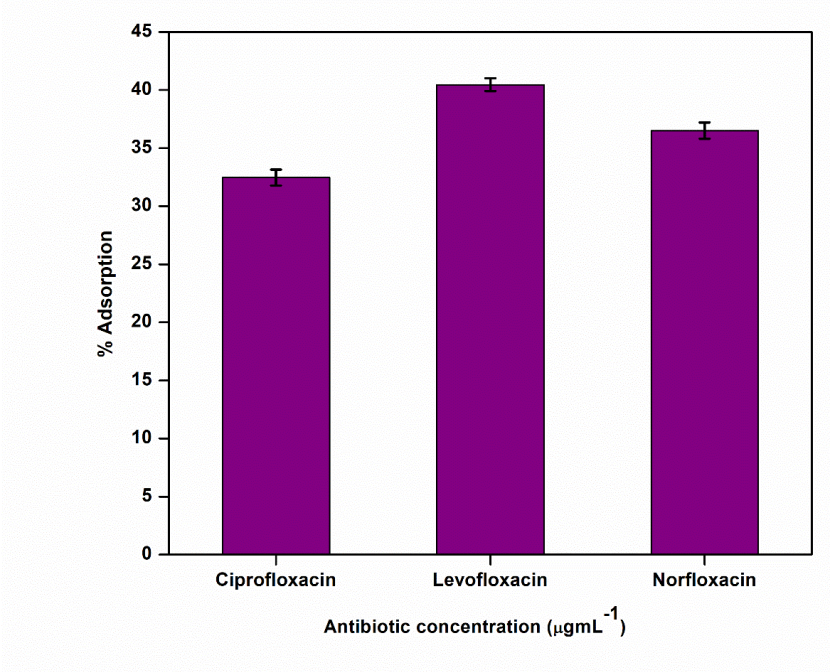


Fig. 6.2. Degradation profile of three fluoroquinolone antibiotics by thermally inactivated laccase in raw biochar at 30°C, pH 4.5 and time 3 h.

6.3.3. Operational stability of laccase immobilized onto raw biochar

The laccase immobilized in raw biochar showed stable catalytic activity over multiple reaction cycles. The results of a determination of the operational stability cycles, displayed in **(Fig. 6.3)** indicated that there was a 65% retention rate of catalytic activity after 8 consecutive cycles. There was a slight decline of 3.68% in activity after 4 cycles, which could be due to the enzyme being washed away during the constant washing process. Earlier Zhang et al. (2014) found a similar 70% loss of catalytic activity after 10 oxidation cycles, while laccase immobilized in kaolinite Wen et al. (2019) observed a 50% decrease in laccase activity after a 2 reaction cycles and concluded that physical adsorptive immobilization results into weak bonds between enzyme and support material. However, rice straw biochar surface functionalized by mineral acids, immobilized laccase resulted in 60% of laccase activity after 6 consecutive reaction cycle (Imam et al., 2021). Further, laccase immobilized in magnetic nanoparticles resulted in 85% reusability for bisphenol A degradation after 7 reaction cycles. Contrarily, covalent immobilization of laccase onto micro-biochar produced from pine wood, pig manure and almond shell retained 40% of laccase relative activity after 4 cycles of repeated use for diclofenac removal (Lonappan et al., 2018b).

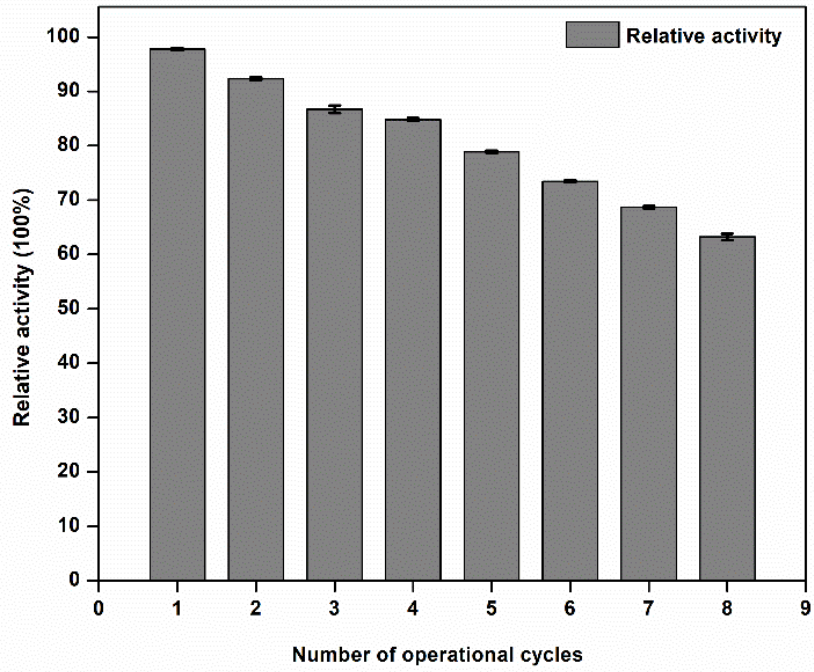


Fig. 6.3. Operational stability of laccase immobilized onto raw biochar for 8 ABTS oxidation cycle.

Laccase immobilized in avocado seed biochar retained operational stability after 7 reaction cycles for the removal of acetaminophen (da Silva et al., 2022). While, laccase immobilized in pine needle biochar surface modified by weak acids resulted in 53% laccase activity after 6 reaction cycles for malachite green removal (Pandey et al., 2022). In accordance with previous studies, in this study laccase immobilized onto raw biochar produced from SMW of *P. florida* through adsorptive immobilization resulted in chemisorption and adsorption process between raw biochar and laccase enzyme. The residue of enzymes such as protease, hemicellulose, and manganese peroxidase and organic substances contribute for the abundance of functional groups in SMW biochar and numerous microporous structures formation may have contributed to the operational stability of immobilized laccase in biochar (Wu et al., 2019; Zhang and Sun, 2014). Thus, in addition to increasing enzyme stability, laccase immobilization enables its reuse, which in turn reduces processing costs. This is a critical parameter for assessing the biocatalytic system's feasibility for industrial use.

6.3.4. Storage stability of the laccase immobilized onto raw biochar

Denaturation of protein bonds during long-term storage is a typical phenomenon, and previous research has suggested that immobilization improves the stability of laccase regardless of its free form. The optimal reaction condition to obtain maximum laccase

activity i.e. pH 4.5 and 30 ℃ was thoroughly discussed in chapter 3. Based on that optimal reaction conditions the stability check for immobilized and free form of laccase was performed at pH 4.5 and at temperature 30 ℃. The enzymatic assay of laccase for both immobilized and free form was determined at 1 mM ABTS concentration (optimized in Chapter 3). The nature of bonding between adsorbate and adsorbent has a notable influence on retaining laccase activity for long-time storage.

In the previous results pine wood biochar covalently bonded with laccase molecules retained laccase activity up to 85% (Lonappan et al., 2018b) whereas, laccase adsorbed in barium alginate beads could retain its storage stability up to 68.64% for 7 days (Latif et al., 2022). Concurrently, the laccase enzyme covalently bonded with chitosan beads retained its 90% activity at 4 ℃ for four weeks whereas the free form of laccase could retain only 47.5% activity (Bilal et al., 2019). Laccase immobilized in pine wood biochar could retains its maximum activity at pH 8 and pH 4 whereas free enzyme could not retain its activity. Immobilization of laccase provides wider pH range to retain its activity (Pandey et al., 2022; Suganthi et al., 2018). Another benefit reported in previous studies is that immobilization of laccase provides wider temperature profile stability. Free enzyme activity optimum activity was obtained at 35 ℃ and its activity (14.5%) was decreased after increasing temperature (70 ℃) whereas immobilized laccase activity was optimum activity was obtained at 45℃ (45% relative activity) (Pandey et al., 2022). Similarly, studies by Taheran et al., (2017), shift in temperature profile was observed in laccase immobilized on polyacrylonitrile-biochar composites. This shift in temperature profile indicates robustness and structural rigidity of the laccase after immobilization (Rouhani et al., 2020).

Correspondingly, this study results revealed that, compared to free laccase, which was able to retain up to 51% of its relative activity, laccase immobilized in raw biochar using an adsorption method retained a relative activity of 72% shown in (**Fig. 6.4.)**. The probable explanation for the loss of enzyme activity during storage could be oxidation and immobilizing laccase in the support material could prevent or minimize the loss (Pandey et al., 2022). Laccase immobilized in support material exhibited improved storage ability than free laccase enzyme which is susceptible towards reduction of its relative activity. Therefore, this experiment emphasizes the significance of the immobilization of enzymes in support material, as this has been proven useful in preserving enzyme integrity in extreme environmental circumstances, thereby lending credence to the concept of harnessing enzymes in industrial domains.

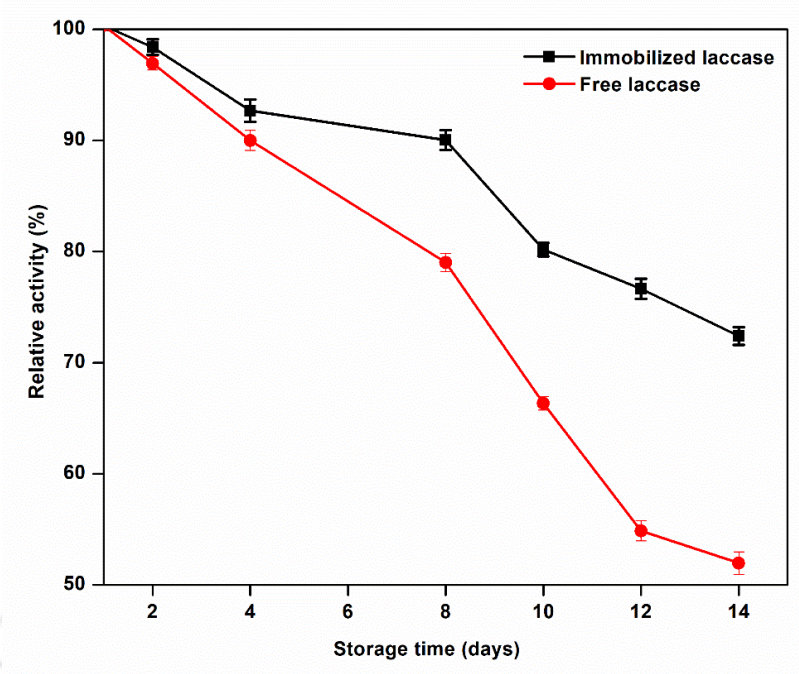


Fig. 6.4 Storage stability of free laccase and immobilized laccase enzyme on raw biochar at temperature 30 ℃ and pH 4.5 for 14 days.

6.3.5. Adsorption efficiency of three fluoroquinolone antibiotics by activated charcoal

This experiment was conducted to determine the efficiency of commercially available activated charcoal in adsorption of ciprofloxacin, levofloxacin and norfloxacin. Activated charcoal (10 mg) was employed in synthetic wastewater at 5, 15, 25 and 50 µg mL^{-1} concentration of three fluoroquinolone antibiotics. The adsorption efficiency of three fluoroquinolone antibiotics was studied at time 3 h, 6 h, 9 h, and 12 h and was incubated at temperature 30 ℃. The maximum adsorption efficiency of three fluoroquinolone antibiotics was observed at time 9 h (70.29% for ciprofloxacin, 68.26% for levofloxacin and 68.96% for norfloxacin) and after 12 h of incubation (70.10% for ciprofloxacin, 67.96% for levofloxacin and 68.12% for norfloxacin) no change in adsorption efficiency was observed. Consequently, maximum adsorption was observed at antibiotic concentration of 5 µg mL^{-1} whereas at 25 and 50 µg mL^{-1} there was decrease in adsorption capacity represented in **Fig. 6.5.**

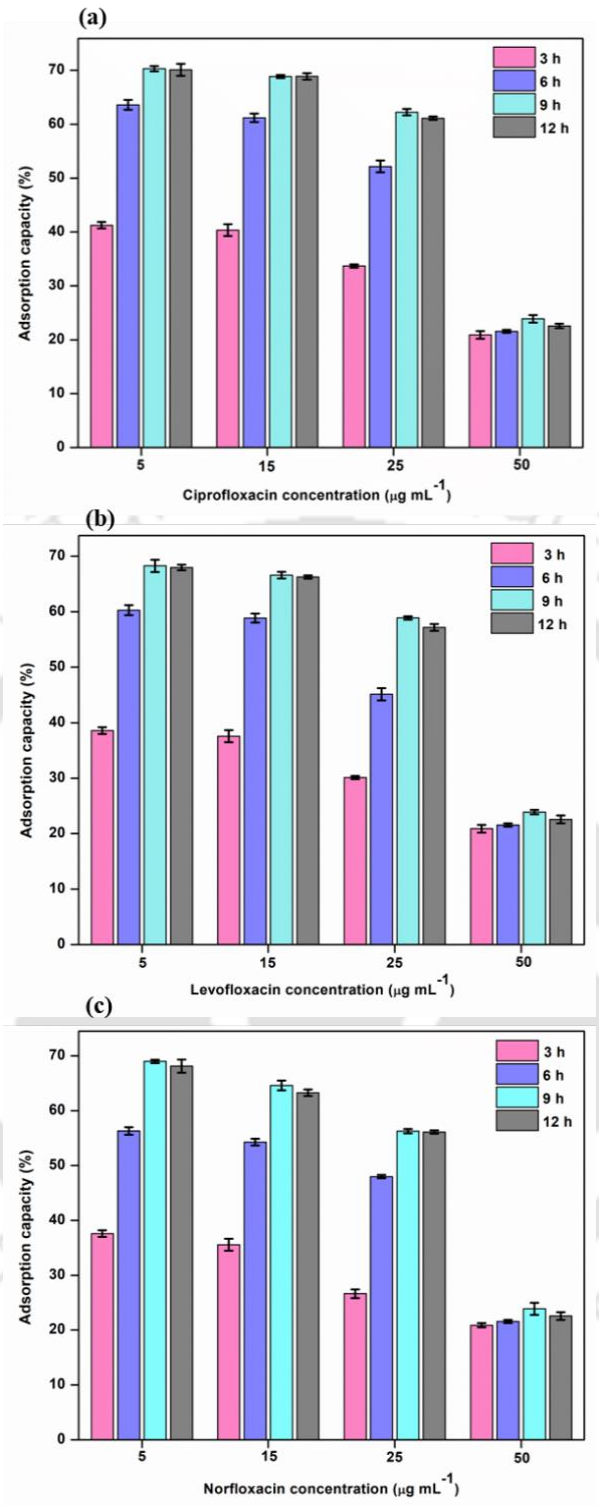


Fig. 6.5. Adsorption efficiency of fluoroquinolone antibiotics by activated charcoal in synthetic wastewater at temperature.

Adsorption efficiency of activated charcoal is influenced by pore size, polar functional groups present on the surface of activated charcoal and mechanistic interactions between adsorbate and adsorbent (hydrogen bonding, hydrophobic interactions, and electron-donor

acceptor formations) (Jeirani et al., 2017). The adsorption mechanism of activated carbon works for degradation of organic pollutants such as paracetamol and atrazine on the mechanism of hydrogen bonding. Paracetamol containing O-H are more susceptible for adsorption than atrazine containing N-H group (Jeirani et al., 2017; Lladó et al., 2015). Thereby, with possible explanation provided in the literature, in this study maximum adsorption for ciprofloxacin was speculated because of the presence of susceptible O-H group and two proton binding sites (amine and carboxyl) (Igwegbe et al., 2021). However, the efficiency of activated carbon is limited, because of its operational stability after repeated use, increase in cost of production due to chemical reagents used for functionalization of its surface area and removal of recalcitrant pollutants at low concentrations due to poor pore size diameter and pore blockage (Igwegbe et al., 2021).

Therefore, laccase immobilized biochar is way more effective for removal of organic pollutants from WWTPs as because laccase immobilized biochar overcomes all the limitations of the activated charcoal such as operational stability after repeated use, no surface modification of biochar is required (minimizes production cost), wider surface area and pore diameter, adsorption process involves chemisorption, no secondary pollution due to leaching out of chemicals and removal of organic pollutants at higher concentrations as because laccase immobilized biochar works in the principle of degradation rather than adsorption of organic pollutants.

6.4. Conclusions

This study highlights that laccase immobilized in raw biochar has promising potential in degradation of fluoroquinolone antibiotics. Immobilized laccase overcomes the limitations of free enzymes in terms of storage stability, operational use, and productivity. The ability of immobilized laccase in degradation of fluoroquinolone antibiotics has been determined both in presence and absence of ABTS. Degradation of levofloxacin in absence of ABTS indicates the fact that degradation of pollutants is determined by the susceptibility of the chemical constituents. Also, degradation achieved at 50 ℃ emphasizes thermostability of the raw biochar and also laccase derived from SMW of *P. florida*. Further, comparison of laccase immobilized biochar with commercially activated charcoal suggests that laccase immobilized biochar is a one stop solution for degradation of pollutants and overcomes all the limitations of activated charcoal such as operational stability, cost of production and removal mechanism. Therefore, immobilized system serves with dual benefits: adsorption as well as degradation. Contarily, the scalability of laccase immobilization on biochar is

promising but requires further optimization for industrial application. The adsorption-based technique is cost-effective and sustainable, particularly with biochar from agricultural waste. However, for large-scale use, consistent biochar quality, enzyme loading, and uniform adsorption conditions are essential to maintain efficiency. Continuous-flow adaptation, enzyme reusability, and resilience under industrial conditions also need refinement. With these adjustments, this method could become a viable, scalable solution for wastewater treatment.

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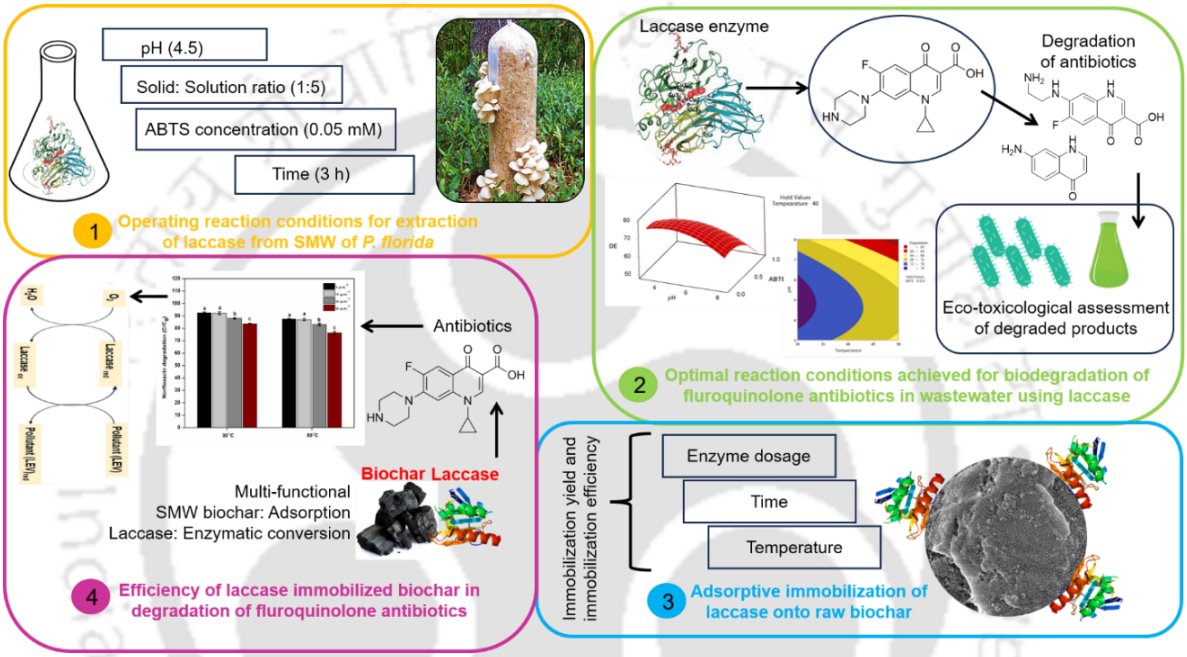
CHAPTER 7

Summary, conclusions, and future recommendations



"This chapter discusses the challenges experienced and recommendations provided for advancing in light of the outcomes of the current study. Further, it compiles the most significant findings from the current study."

7.1. Thesis summary and conclusions

The presence of antibiotic residues in the WWTPs indicates towards the inadequacy of conventional and traditional treatment technologies. Wastewater treatment plants include an elevated level of bacteria, nutrients, and oxygen, all of which create a perfect condition for transferring ARGs, producing new ARBs, and establishing breeding grounds for resistant bacteria. The alarming rise in antibiotic residues in water bodies calls attention to the fact that there is a serious lack of antibiotic monitoring studies in WWTPs around the world, which means that no regulations or mitigation strategies are in existence. Discharge of antibiotics in original or unmetabolized from pharmaceutical firms, food, livestock production, aquaculture, homes and hospitals is the entry route for antibiotic residues to enter WWTPs. The growing concern for remediation of fluoroquinolone antibiotic residues from WWTPs is the need of an hour due to its recalcitrant nature and poor solubility in water. Emergence of fluoroquinolone-resistant bacteria includes strains of *E. coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Salmonella* spp, and *Streptococcus* spp. As a further issue, the high expense of treating diseases linked to antibiotic resistance is a major concern. There are various initiatives aimed at eliminating fluoroquinolone antibiotics from wastewater because of this. Biological treatment process can mostly eliminate the discharged antibiotic in WWTPs by means of adsorption and degradation. For this reason, detailed literature review has been done in **Chapter 2** discusses the limitations of existing treatment technologies in removal of three fluoroquinolone antibiotics from wastewater.

Literature review discusses the potential of extracellular laccase extracted from SMW in remediation of organic pollutants. Additionally, in the same chapter, detailed review addresses the shortcomings of free laccase enzyme in achieving maximum degradation and its stability as free enzymes are susceptible to denaturation in environmental conditions. Therefore, it discusses the importance of immobilization of laccase enzyme in the support material. Nevertheless, detailed review addresses the significance of utilizing carbonaceous material (biochar) for immobilizing laccase enzyme which increase its operational stability and productivity. Hence, based on the literature review and existing gaps this study comprehensive goals was set out to evaluate the potential of extracellular laccase extracted from SMW of *P. florida* in biodegradation of three fluoroquinolone (ciprofloxacin, levofloxacin and norfloxacin) antibiotics from wastewater.

Chapter 3 introduced the potential lignocellulosic material SMW, which is rich in ligninolytic enzymes, in particular laccase. To maximize laccase activity, the extraction procedure of laccase from SMW bags was optimized, taking four parameters into account: pH, temperature, ABTS concentration, and solid: solution ratio. Further, this chapter experiment was conducted for partial purification of laccase by ammonium sulphate precipitation followed by dialysis. Partially purified laccase bands were visible on Native-gel and SDS gel electrophoresis. In addition, the stability check of free laccase enzyme was carried out at different pH, temperature in presence of metal ions and inhibitors and storage stability of laccase enzyme was studied. Nevertheless, comparative analysis of cost of production of commercial enzyme and laccase enzyme extracted from SMW of *P. florida* was done to circumvent the high production costs and extended processing times associated with commercial laccase enzyme. Hence, the results of chapter 3 conclude that at pH 4.5, temperature 30 ℃, 1 mM ABTS concentration, 1:5 solid: solution ratio and time 3 h showed best laccase activity. Partially purified laccase bands were observed at 45 kDa which is consistent with previous studies. Lastly, the relative activity and storage stability of laccase enzyme at different pH, temperature, and in presence of metal ions and inhibitors highlights the sturdiness and effectiveness of extracellular laccase extracted from SMW of *P. florida*. Thus, the cost of production of laccase enzyme in comparison with commercial laccase enzyme suggests the utilization of agro-waste for extraction of laccase enzyme and also signifies transition of linear economy to circular economy and waste reduction in the environment.

In continuation of the work carried out in chapter 3, the partially purified laccase was utilized to study biodegradation of three fluoroquinolone antibiotics i.e. ciprofloxacin, levofloxacin norfloxacin from the artificial wastewater. To achieve higher degradation efficiency, parameters such as: pH and ABTS concentration was tweaked and was studied at two different temperature 30 ℃ and 50 ℃. It was observed that at pH 4.5 and 0.05 mM ABTS concentration and at temperature 30 ℃ maximum degradation efficiency was observed for all three fluoroquinolone antibiotics. However, degradation was observed both in the presence and absence of ABTS. The results speculated that in absence of ABTS degradation of levofloxacin was achieved. The degradation efficiency of organic pollutants is dependent on the EDG and EWG groups, optimal pH and temperature conditions. In addition, degradation achieved at 50 ℃ indicates that laccase extracted from SMW of *P. florida* is thermostable in nature. Further, the degraded products formed after degradation by laccase was studied for all three antibiotics and it was speculated that decarboxylation,

depiperylzation and defluorination are mechanisms followed for breakdown of all three fluoroquinolone antibiotics. To understand the toxicity nature of the degraded products, eco-toxicological assessment (bacterial and algal toxicity test) was performed. Based on this eco-toxicological assessment it can be concluded that the degraded products are non-toxic in nature and fluoroquinolone degraded by laccase can be released into the environment. Therefore, **Chapter 4** spotlights with the paramount findings that with optimum reaction conditions maximum degradation efficiency of antibiotics can be achieved and the degraded products obtained after laccase degradation are non-toxic in nature and concludes that laccase mediated degradation can be achieved at lesser time in compared with whole fungal culture cells.

Chapter 5 delves into the importance of immobilizing enzymes in support materials, drawing on Chapter 4's findings about free enzyme sensitivity and environmental denaturation. Adsorptive immobilization is the method to immobilize laccase enzyme in the support materials. Carbonaceous material, raw biochar (produced from SMW of *P. florida*) is considered for immobilization in this study, as because surface modification of support materials with chemical reagents leads to leaching out of chemicals after repeated use. To obtain, maximum adsorptive immobilization, this study elaborates the importance of immobilization yield and immobilization efficiency in order to achieve maximum degradation efficiency of OMPs. The maximum IY and IE was achieved by tweaking parameters such as: enzyme dosage and time and immobilization study was conducted at 30 ℃ and 50 ℃. It was found that at enzyme dosage of 5 U g^{-1} maximum IE (56.12%) and IY (65. 03%) was observed at time 5 h. The lowest IY and IE was observed at 0.5 U g^{-1} and at 8 U g^{-1} no further increase in IY and IE was observed which suggests saturation of laccase molecules onto the surface of raw biochar. Subsequently, adsorption isotherm and kinetics studies were performed. The results indicates that adsorption of laccase molecules onto surface of raw biochar is monolayer adsorption and chemisorption is involved. The immobilization of laccase onto surface of raw biochar was confirmed by analytical instruments (FTIR, BET, FESEM-EDS and TGA). Nevertheless, the stability of laccase immobilized onto surface of raw biochar was studied on varying pH, temperature, presence of metal ions and inhibitors and compared with the stability of free laccase Thereby, chapter 4 illustrates with the findings that optimal reaction condition is significant to achieve maximum adsorptive immobilization and maximum adsorptive immobilization helps to attain higher degradation efficiency of OMPs. The wider stability achieved for laccase immobilized in raw biochar signifies that productivity of free enzyme can be enhanced by

immobilizing it into support materials. Hence, a single-source approach for the removal of OMPs from the aqueous system could involve SMW to extract laccase enzyme and produce biochar, which will satisfy the sustainable goals and directs us towards greener future.

Laccase immobilized biochar obtained in Chapter 5, was employed for degradation of ciprofloxacin, levofloxacin and norfloxacin in artificial wastewater at varying concentration (5-50 µg mL^{-1}) as described in **Chapter 6.** This chapter discusses the potential of laccase immobilized biochar in achieving higher degradation both in presence and absence of ABTS at temperature 30 ℃ and 50 ℃. Ciprofloxacin and norfloxacin degradation were achieved at both 30 ℃ and 50 ℃ in presence of 0.05 mM ABTS concentration at time 3 h. Whereas levofloxacin degradation was achieved both in presence and absence of ABTS at time 3 h. As discussed in chapter 3, despite the susceptibility of functional groups of OMPs involved in degradation, oxidation-reduction potential of contributes in achieving higher degradation. Considering this mechanism, higher degradation of ciprofloxacin was achieved. Further, the operational stability of laccase immobilized biochar up to 8 cycles and attaining 65% of relative activity signifies that laccase immobilized biochar can be repetitively used for degradation of OMPs. Nevertheless, experiments were conducted to rule out the individual role of laccase and raw biochar and it was observed that raw biochar contributes 32-40% in degradation of OMPs and no degradation was achieved at higher concentration of all three antibiotics. This indicates towards saturation of raw biochar.

The efficacy of laccase immobilized biochar produces in this study was compared with the commercially available activated charcoal in synthetic wastewater. The results revealed that 72-65% adsorption of all three fluoroquinolone antibiotics was achieved at time 12 h and further adsorption of fluoroquinolone antibiotics was not achieved. Activated charcoal could not degrade antibiotics at higher concentration. Therefore, laccase immobilized biochar is much more efficient in removal of antibiotics from wastewater in terms of removal process and time. As degradation mechanism is involved by laccase immobilized biochar for removal of fluoroquinolone antibiotics whereas adsorption process in involved for activated charcoal which limits the efficacy of activated charcoal. Thereby, immobilized laccase is an excellent approach for biodegradation of OMPs. The utilization of agro waste such as SMW for degradation is also a significant advancement towards a more environmentally friendly future and an economy that is more equitable.

7.2. Implications for wastewater treatment practices and environmental policy

This study introduces an innovative approach to wastewater treatment, enhancing the degradation of persistent antibiotic residues particularly fluoroquinolones, which conventional treatment methods often struggle to fully remove. By immobilizing laccase enzymes on biochar produced from agricultural waste, such as SMW, this method allows treatment plants to effectively target and degrade fluoroquinolone antibiotics, reducing the risk of antibiotic resistance propagation through treated effluent (Zhou et al., 2022). This approach can improve the overall effectiveness of wastewater treatment plants, ensuring cleaner effluent release and mitigating environmental contamination risks in aquatic ecosystems (Xiong et al., 2017; Zhang et al., 2019). One of the significant benefits of this method is its emphasis on sustainability and cost-efficiency. Using biochar derived from agro-waste as a support material for enzyme immobilization provides an affordable and renewable alternative to conventional adsorbents like activated carbon. This not only reduces operational costs but also aligns with circular economy principles, where waste materials are repurposed into valuable resources, minimizing both resource consumption and waste generation (Ahmad et al., 2014). Moreover, this biochar-immobilized laccase technology can be implemented in existing treatment infrastructure with minimal modifications, making it feasible for a broad range of facilities, from small-scale to larger municipal plants (Singh et al., 2018).

Further, the laccase-biochar complex demonstrates significant durability and reusability, maintaining stable enzyme activity across multiple treatment cycles. This stability enables treatment plants to achieve high degradation rates with fewer resources and reduced frequency of replacement, a major advantage in sustainable operations (Ji et al., 2021). Such consistency in performance reduces resource use and waste generation, supporting environmentally sustainable practices within wastewater management (Borrelli et al., 2018).

This method also has implications for regulatory compliance. With evolving standards around water quality, particularly regarding pharmaceutical residues, many countries are imposing stricter discharge limits for antibiotics and other emerging pollutants. Laccase-immobilized biochar offers wastewater treatment facilities an advanced solution to meet current regulatory standards and prepare for potential future regulations that may mandate more stringent removal targets (Wang et al., 2020). Proactively adopting such bioremediation strategies could position facilities to remain compliant, avoiding regulatory

penalties and contributing to public health objectives by minimizing antibiotic pollution (Wang et al., 2020).

On a broader scale, this approach aligns well with environmental policies focused on sustainable waste management, pollution reduction, and resource recovery. By using agricultural waste to produce biochar, wastewater treatment plants directly support policy goals related to waste minimization and sustainable resource utilization (Zhang et al., 2020). This approach provides a model for how various sectors can collaborate to repurpose waste streams, turning them into resources for environmental applications, which aligns with circular economy principles and the global shift towards sustainable development (Kumar et al., 2018). Policies that incentivize such green technologies could further encourage the adoption of this method, promoting a shift towards more sustainable, resilient wastewater treatment practices.

In conclusion, this study presents a viable, scalable method for enhancing antibiotic removal from wastewater while supporting environmental sustainability. By integrating biochar-immobilized laccase degradation, treatment facilities can achieve more efficient pollutant removal, aligning with current environmental and regulatory standards. This approach contributes to a greener future in water management and positions wastewater treatment plants at the forefront of environmental responsibility.

7.3. Future recommendations:

This thesis underlines the importance of removal of fluoroquinolone antibiotics from wastewater. The underline existing problem was achieved by utilizing laccase enzyme extracted from SMW of *P. florida* in this study and further immobilized system is introduced in this thesis which overcomes stability of free laccase enzyme, better productivity, and higher operational stability. However, prospects are highlighted for efficient degradation of antibiotic residues from the wastewater:

- **Diversify enzyme extraction**: The biodegradation studies carried out in this thesis involves single use of enzyme i.e. laccase enzyme from SMW of *P. florida*. It would be interesting to extract various ligninolytic enzymes from SMW bags and optimize the reaction conditions to obtain maximum laccase output.

- **Develop multi-enzyme system**: Investigation of a mixture of ligninolytic enzymes optimized at one particular reaction condition should be achieved to obtain maximum degradation efficiency of OMPs.
- **Address real wastewater complexity**: Since real wastewater contains admixture of antibiotic residues, investigation should be carried out to obtain higher degradation efficiency of admixtures of antibiotic residues. In addition, more research should be focused in removing antibiotic resistant genes and antibiotic resistant bacteria from the real wastewater during the tertiary treatment process.
- **Investigate laccase-biochar binding mechanism:** Innovate chemical free immobilization methods: More immobilized system should be developed without using chemical reagents for surface modifications to establish no further generation of secondary pollutants by the immobilized system. Laccase immobilized in raw biochar mechanism should be further investigated in terms of enzyme-biochar binding sites, surface-functions interactions and structural changes of laccase involved during immobilization.
- **Perform comprehensive life-cycle assessment (LCA):** Last but not the least life cycle assessment of the immobilized system should be carried out for its large-scale production, effective degradation, storage, and transportation.

By implementing these targeted recommendations, future research can significantly enhance the effectiveness of enzyme-based wastewater treatment methods, providing practical solutions for managing antibiotic contamination in real-world applications."

Annexure-1

List of publications in peer reviewed journals from the thesis work:

- **Ghose, A**., Mitra, S., 2022. Spent waste from edible mushrooms offers innovative strategies for the remediation of persistent organic micropollutants: a review. ***Environmental pollution***, *305*, 119285.
- **Ghose, A**., Gupta D., V., Rangan, L., Mitra, S., 2023. Optimization of laccase enzyme extraction from spent mushroom waste of *Pleurotus florida* through ANN-PSO modelling: an ecofriendly and economical approach. ***Environmental research***. *222*, 115345.
- **Ghose, A**., V., Gupta D., Kimoto, H., Takashima, S., Harlin., E.W., SS., Ueda, H., Koketsu, M., Rangan, L., Mitra, S., 2024. Micropollutants (ciprofloxacin and norfloxacin) remediation from wastewater through laccase derived from spent mushroom waste: fate, toxicity, and degradation. ***Journal of Environmental Management***. *366*,121587.

Other collaborative work published during PhD:

- Mitra, S., **Ghose, A**., Gujre, N., Senthilkumar, S., Borah, P., Paul, A., & Rangan, L. (2021). A review on environmental and socioeconomic perspectives of three promising biofuel plants *Jatropha curcas*, *Pongamia pinnata* and *Mesua ferrea*. ***Biomass and Bioenergy***. *151*, 106173.
- Sahu, S., Rajbonshi, M. P., Gujre, N., Gupta, M. K., Shelke, R. G., **Ghose, A**., Rangan, L., Pakshiranjan K., Mitra, S. (2022). Bacterial strains found in the soils of a municipal solid waste dumping site facilitated phosphate solubilization along with cadmium remediation. ***Chemosphere***. *287*, 132320.

Manuscripts under preparation:

- **Ghose, A**., V., Gupta D., Das, A., Leskinen, J., Navarro, V.C., Rangan, L., Mitra, S., 2024. Laccase immobilized engineered biochar derived from spent mushroom waste facilitates remediation of levofloxacin antibiotic in wastewater.
- **Ghose, A**., Gupta D., V., Rangan, L., Mitra, S., 2023. Engineered raw biochar derived from spent mushroom waste to degrade emerging micropollutants: An optimization study via Box-Behnken design and artificial neural network modelling.

Conference attended / proceedings

- **Ghose, A**., Rangan, L., Mitra, S. (2021, December). Biodegradation of persistent organic micropollutants (OMPs) by the ligninolytic enzymes present in spent waste of edible mushroom 'A green approach towards a circular economy in India.' **Poster presented** at the International conference on Biotechnology for Resource Efficiency, Energy, Environment, Chemicals and Health (BREECH-2021), Dehradun, India.
- **Ghose, A**., Rangan, L., Mitra, S. (2022, December). Laccase enzyme from edible spent mushroom waste (SMW) offers innovative strategy in degradation of levofloxacin antibiotic: a sustainable approach for environmental issues. **Flash talk and poster presentation** at International conference on Biotechnology, Sustainable, Bioresources and Bioeconomy (BSBB-2022) held at Indian Institute of Technology, Guwahati, India
- **Ghose, A**., V., Gupta D., Kimoto, H., Takashima, S., Koketsu, M., Rangan, L., Mitra, S. (2024, March). Laccase enzyme from edible spent mushroom waste offers innovative strategy in degradation of ciprofloxacin and norfloxacin antibiotic: fate, removal and toxicity. **Poster** presented at Japan-NER Bioeconomic Technology Cooperation Symposium 2024 held at Indian Institute of Technology, Guwahati, India