

https://dspace.org/

Enzyme-MOFs for biocatalytic degradation of organic pollutants

Dlamini, Mbongiseni Lungelo

2024
https://hdl.handle.net/10539/37461

Downloaded from a DSpace repository. https://dspace.org/

Enzyme-MOFs for biocatalytic degradation of organic pollutants



WITS
UNIVERSITY

Mbongiseni Lungelo Dlamini

A Thesis submitted to the Faculty of Science for the degree of Doctor of Philosophy, University of the Witwatersrand, Johannesburg

JUNE 2023

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Candidate signature:  **Date**: 3 June 2023

ABSTRACT

The widespread use of pharmaceutically active compounds (PhACs) and the incidental discharge of endocrine-disrupting chemicals (EDCs) into surface water has resulted in the detection of such compounds in effluents of wastewater treatment plant effluents (WWTPs). This indicates that such contaminants are highly stable and recalcitrant, defying total elimination by traditional technologies. These compounds have been designated as emerging contaminants, and involuntary exposure to them may have negative consequences for humans and aquatic life. As a result, there is a need to investigate potential strategies for effectively eliminating such contaminants from water. Among the various remediation technologies, enzymatic biotransformation of these organic pollutants by oxidative enzymes is a green, cost-effective, and considerably sustainable approach.

In this study, an *Aspergillus*-based laccase, immobilized on zeolitic imidazolate frameworks (ZIF) was investigated for its potential to biodegrade three model PhACs (carbamazepine (CBZ), diclofenac (DFC), and norfloxacin (NOR)), and dimethyl phthalate (DMP) - a potential endocrine-disrupting chemical. The laccase was immobilized via a facile one-pot biomineralization covalent binding procedure. The success of this attachment was confirmed by Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy analysis, demonstrating peaks and proton shifts attributable to functional groups present in the protein structure of the enzyme. Furthermore, Powder X-Ray Diffraction (PXRD) analysis revealed a preserved crystallographic structure of the ZIF particles after the laccase attachment. Additionally, the characteristic rhombic dodecahedral morphologies of ZIF crystals were retained after enzyme attachment, confirmed by Scanning Electron Microscope (SEM) micrographs, with slightly agglomerated particles.

The immobilized laccase (Lac-ZIF) demonstrated significant resistance to adverse environmental conditions such as extreme pH, elevated temperatures of up to 70 °C (relatively higher deactivation energies (E_d)), and harsh organic solvents in comparison to the free laccase. Also, the Lac-ZIF proved to be quite recyclable retaining high efficiencies (>70%) for up to 5 reuse cycles, as well as remarkable storage stabilities of up to 15 d.

Even though the immobilization of the laccase resulted in diminished affinities for the substrates (analytes) as illustrated by increasing Michaelis constant (K_m) values, the Lac-ZIF still outperformed the free laccase in the removal of all the investigated contaminants. This was attributed to the synergistic adsorption and biodegradation effects provided by the ZIF backbone and the attached laccase enzyme.

For the optimization of operational parameters for DFC and NOR removal, the quadratic response surface methodology (RSM) regression model proved effective and viable with high accuracy as confirmed by experimental results. In this sense, the experimental values (93.5% for DFC and 94.9% for NOR) were found to be very close to the predicted removal efficiencies, 93.9 and 95.1% for DFC and NOR, respectively.

Finally, the synthesized biocatalyst was used to decontaminate the investigated compounds in real river water samples, and it significantly outperformed the free laccase. Due to its remarkable attributes such as thermal and storage stabilities, strong pH resistance, recyclability, and high degradation potential, the Lac-ZIF is a potential candidate for application in greener and more effective wastewater remediation approaches.

DEDICATION

This work is dedicated to the Almighty God, my family, and friends

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my supervisors, Dr Heidi L. Richards and Dr Izak A. Kotzé, for their guidance throughout this journey. Your presence, insights, advice, support, and most importantly your patience made the study simple to tackle. You have instilled in me independence and life lessons that I will forever cherish. Thank you again for your assistance and input in all my manuscripts and the thesis write-up.

I would also like to appreciate the members of the WITS Environmental Analytical Chemistry research group for their warm welcome, friendliness, and continued support, positively impacting the quality of this research. Thank you for sharing your insights on the operation of analytical instruments.

Profound thanks go to the following special people: Mahadi Lesaoana for being the best lab buddy, your support and insights have been really helpful; Kgomotso Maiphetlho, Dr Khathutshelo Netshiongolwe, Khona Maziya, Boitumelo Setati, Malehlogonolo Mphahlele, Odwa Mbanga, Dr Yannick Nuapia, Silindile Ntombela, Dr Sinegugu Khulu, and Nkazimulo Ngwenya for their contribution, academic and moral support to the benefit of the study.

To Dr Eric Morifi and the mass – spectroscopy staff, thank you for your assistance with HPLC-MS instrument operation and data analyses.

To my parents, Mr and Mrs Dlamini, siblings, and friends, thank you for your kindness and support during my studies. Being away from home can be difficult at times, but your assistance has made it easier.

Special thanks to the Council for Scientific and Industrial Research – Department of Science and Innovation (CSIR-DSI) Inter-bursary Support Programme (IBS) for funding this study. Additionally, I am grateful for the financial support from the WITS postgraduate merit award (PMA) throughout the study period.



PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

1. **Mbongiseni Lungelo Dlamini**, Mahadi Lesaoana, Izak Kotzé, Heidi Lynn Richards: Zeolitic imidazolate frameworks as effective crystalline supports for *Aspergillus*-based laccase immobilization for the biocatalytic degradation of Carbamazepine; **Chemosphere, 2022, (311)**

   https://doi.org/10.1016/j.chemosphere.2022.137142

2. **Mbongiseni Lungelo Dlamini**, Mahadi Lesaoana, Izak Kotzé, Heidi Richards: An oxidoreductase enzyme, fungal laccase immobilized on zeolitic imidazolate frameworks for the biocatalytic degradation of an endocrine-disrupting chemical, dimethyl phthalate.

   Journal of Environmental Chemical Engineering, 2023
   https://doi.org/10.1016/j.jece.2023.109810

3. **Mbongiseni Lungelo Dlamini**, Mahadi Lesaoana, Izak A. Kotzé, Heidi Richards: Response surface methodology mediated optimization of diclofenac and norfloxacin biodegradation using laccase immobilized on metal-organic frameworks.

   Manuscript in communication for submission to **Separation and Purification Technology** journal

PRESENTATIONS

1. Title: Laccase immobilization on zeolitic imidazolate frameworks for the synergistic adsorption and biocatalytic degradation of an antibiotic, norfloxacin: **Oral presentation**
   Authors: Mbongiseni L. Dlamini, Mahadi Lesaoana, Heidi Richards, Izak Kotzé

   South African Chemical Institute (SACI) Young Chemists' Symposium (YCS), October 2022

   This presentation relates to chapter 4.3

2. Title: Enhanced synergistic adsorption and biocatalytic degradation of diclofenac by fungal laccase immobilized on zeolitic imidazolate frameworks: **Oral presentation**

Authors: Mbongiseni L. Dlamini, Mahadi Lesaoana, Heidi Richards, Izak Kotzé

Water Institute of Southern Africa (WISA) Conference, September 2022, Sandton

Award: Best student paper and presentation

This presentation relates to chapter 4.3

3. Title: An oxidoreductase enzyme, fungal laccase immobilized on zeolitic imidazolate frameworks for the biocatalytic degradation of an endocrine-disrupting chemical, Dimethyl Phthalate: **Virtual Oral Presentation**
   Authors: Mbongiseni L. Dlamini, Mahadi Lesaoana, Heidi Richards, Izak Kotzé

   13th Wits Cross Faculty Postgraduate Symposium, University of the Witwatersrand

   Award: 3rd Prize Oral Presentation

   This presentation relates to chapter 4.2

4. Title: Zeolitic imidazolate frameworks as effective crystalline supports for *Aspergillus*-based laccase immobilization for the biocatalytic degradation of Carbamazepine: **Oral Presentation**

   Authors: Mbongiseni L. Dlamini, Mahadi Lesaoana, Izak Kotzé, Heidi Richards

   31st Catalysis Society of South Africa (CATSA) November 2021 International virtual Conference, University of KwaZulu-Natal.

   This presentation relates to chapter 4.1

TABLE OF CONTENTS

DECLARATION……...i
ABSTRACT……..….ii
DEDICATION……iv
ACKNOWLEDGEMENTS……...…..v
PUBLICATIONS AND PRESENTATIONS……vi
LIST OF FIGURES…….…….……x
LIST OF TABLES……...……xi
ABBREVIATIONS AND NOMENCLATURE……...……xii

CHAPTER 1 – INTRODUCTION……1
1.1 Background and motivation.....1
1.2 Problem statement.....4
1.3 Novelty and scientific contribution to new knowledge 5
1.4 Aim and objectives 5
1.5 Scope of work 6
1.6 Hypothesis……7
1.7 Thesis structure 7
1.8 References.....8

CHAPTER 2 - LITERATURE REVIEW…….……14
2.1 Introduction.....14
2.2 Emerging water contaminants.....14
2.2.1 Pharmaceutically active compounds (PhACs) 14
2.2.2 Endocrine-disrupting chemicals (EDCs).....16
2.3 Remediation of synthetic organic contaminants 16
2.3.1 Ion exchange 17
2.3.2 Membrane filtration.....17
2.3.3 Advanced oxidation processes (AOPs) 18

2.3.4 Adsorption technology 19
2.3.5 Biocatalytic remediation 21
2.4 Enzymatic degradation of organic pollutants 22
2.4.1 Peroxidases in organic contaminants removal 22
2.4.2 Laccases in organic pollutants removal 24
2.5 Laccase immobilization 27
2.5.1 Metal-organic frameworks (MOFs) for enzyme immobilization 30
2.5.2 Synthesis of MOFs and enzyme-MOFs 30
2.6 Chapter conclusion 34
2.7 References 35
CHAPTER 3 - MATERIALS AND METHODS 51
3.1 Synthesis of ZIF and Lac-ZIF 50
3.2 Operating conditions 50
3.3 Characterization 50
3.3.1 FTIR 51
3.3.2 FE-SEM 51
3.3.3 HR-TEM 52
3.3.4 NMR spectroscopy 53
3.3.4 PXRD 53
CHAPTER 4 - RESULTS AND DISCUSSION 55
4.1 Paper 1 56
4.2 Paper 2 88
4.3 Paper 3 116
CHAPTER 5 - CONCLUSIONS AND RECOMMENDATIONS 145
5.1 General conclusions 145
5.2 Recommendations 146

LIST OF FIGURES

CHAPTER 2

Figure 2. 1: Chemical structures of (a) Carbamazepine (CBZ), (b) Diclofenac (DFC), (c) Norfloxacin (NOR), and (d) Dimethyl phthalate (DMP).....15
Figure 2. 2: The catalytic cycle of heme-peroxidases. RH: organic pollutant; $R^{\bullet}$: radical form of the organic pollutant (Alneyadi et al., 2017).....23
Figure 2. 3: Degradation mechanism of laccase enzyme (Wong, 2009).....25
Figure 2. 4: Approaches for the immobilization of enzymes (Guardado, 2019).....27
Figure 2. 5: Schematic diagram for the formation of MOFs (Silva et al., 2022).....30
Figure 2. 6: MOFs fabrication approaches and their synthesis conditions.....32
Figure 2. 7: ZIF synthesis and pictorial representation of crystals (Ortiz et al., 2014).....33

CHAPTER 3

Figure 3. 1: FTIR analysis illustration (Griffiths and Chalmers, 2006).....51
Figure 3. 2: Schematic representation of SEM (Hafner, 2007).....52
Figure 3. 3: Schematic diagram for TEM (Lagrange, 2016).....53

LIST OF TABLES

CHAPTER 2

Table 2. 1. Advantages and drawbacks of conventional technologies for organic pollutants decontamination. 20
Table 2. 2. Application of laccase in PhACs degradation. 26
Table 2. 3. Advantages and drawbacks of immobilization approaches. 28
Table 2. 4. Immobilization supports for laccase. 29

ABBREVIATIONS AND NOMENCLATURE

•OH	Hydroxyl radicals
2-MIm	2-methylmidazole
ABTS	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
ADHD	Attention-deficit hyperactivity disorder
ANOVA	Analysis of variance
AOPs	Advanced oxidation processes
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared
CBZ	Carbamazepine
CCF	Central Composite Face
CPO	Chloroperoxidase
DF	Degrees of freedom
DFC	Diclofenac
DMP	Dimethyl phthalate
DO	Dissolved oxygen
EDCs	Endocrine-disrupting Chemicals
FE-SEM	Field Emission Scanning Electron Microscopy
H_2O_2	Hydrogen peroxide
HBT	1-hydroxybenzotriazole
HPI	N-hydroxyphtalamide
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HR-TEM	High-Resolution Transmission Electron Microscopy
Lac	Laccase
LC-MS	Liquid chromatography – Mass Spectroscopy
LOD	Limit of detection
LOQ	Limit of quantification
MIL	Materials of Institute Lavoisier
MNPs	Magnetic nanoparticles
MOF	Metal-organic framework
MS	Mean squares
NMR	Nuclear Magnetic Resonance

NOR	Norfloxacin
NSAID	Nonsteroidal anti-inflammatory drug
OPP	Orange peel peroxidase
PA	Polyamide
PAHs	Polycyclic aromatic hydrocarbons
PCN	Porous coordination network
PES	Polyether sulfone
PhACs	Pharmaceutically Active Compounds
PSF	Polysulfone
PVDF	Polyvinylidene difluoride
PXRD	Powder X-ray Diffraction
qTOF/MS	Quadrupole time-of-flight/Mass Spectrometer
RSD	Relative standard deviation
RSM	Response surface methodology
SBP	Soybean peroxidase
SD	Standard deviation
SS	Sum of squares
SYR	Syringaldehyde
TDS	Total dissolved solids
TEMPO	2,2’,6,6’-Tetramethylpiperidine-N-oxyl
UV-vis	Ultraviolet-visible
VA	Violuric acid
WWTPs	Wastewater treatment plants
ZIF	Zeolitic Imidazolate Framework

CHAPTER 1
INTRODUCTION

1.1 Background and motivation

Water of high quality is vital for human health and the natural ecological balance. However, the detection of emerging contaminants with tenacious and bio-accumulative properties in recent years has raised concerns on the potential risk to water systems, owing to their possible detrimental effects on the health of humankind and the environment. These are typically compounds originating from pharmaceuticals, detergents, complexing agents, hormones, and personal care products among others (Deblonde, Cossu-leguille & Hartemann, 2011; Sorensen et al., 2014; Katsigiannis et al., 2015; Cobas et al., 2016). In this sense, these anthropogenic land pollutants are usually leached into rivers and streams by water runoff, joining the diverse anthropogenic pollutants that are directly deposited into such waterways and subsequently transported into oceans and seas.

Among the various categories of emerging contaminants, pharmaceutically active compounds (PhACs) (e.g., carbamazepine, diclofenac, norfloxacin, etc.) are frequently detected in aquatic systems attributable to their widespread use, because of the rapidly increasing global population, as well as their non-biodegradable and recalcitrant nature. Unmetabolized pharmaceuticals and their byproducts are consistently discharged into waterways, along with raw wastewater and effluents from wastewater treatment plants (WWTPs). Consequently, traces of various pharmaceutical chemicals (such as analgesics, antibiotics, anti-epileptics, antidepressants, and more) have been found in sewages, surface, and groundwater systems (Ersan et al., 2017; Barrios-Estrada et al., 2018; Fang et al., 2021; García et al., 2021). Even though these compounds may be detected at rather low concentrations (0.01 – 10 mg L^{-1}) in the environment, they may, however, be biologically active at these concentrations and can induce long-term effects as a result of constant and extended exposure, since they are developed to exhibit biological impacts even at minimal concentrations (Méndez et al., 2017; Naghdi et al., 2018). These long-term impacts include alterations of the endocrine system, carcinogenic effects, the emergence of resistant bacterial strains, as well as potentially toxic effects on natural microbial ecosystems (Alharbi et al., 2019; Primožič et al., 2020; George et al., 2022).

Another widely detected class of emerging wastewater contaminants includes endocrine-disrupting chemicals (EDCs) such as phthalates. These are pollutants that originate from

leached waste from plastic manufacturing sites, as well as the use and disposal of such plastic substances into the environment (Yao et al., 2018; Wang et al., 2020; Feng et al., 2022). Phthalates are primarily used as vinyl softeners in plastic synthesis to improve the plastic's flexibility. (Julinová & Slavík, 2012; Zaki & Shoeib, 2018). They are also used in cosmetic products, lubricants, and food packaging coatings (Alhaddad et al., 2021). Exposure to leached phthalates may have detrimental effects on the body's endocrine system, resulting in adverse reproductive, neurological, developmental, and immunological effects in both humans and wildlife (Science, Widel & Oszczudlowski, 2017). Due to these adverse effects, it is imperative to develop effective techniques for the decontamination of such pollutants from water.

The constant detection of PhACs and EDCs, as well as their metabolites in water systems, suggests that typical WWTPs are inefficient at removing these substances. Although some traces of these pollutants can be decontaminated using traditional wastewater treatment systems, typically through physicochemical adhesion on adsorbent materials, a significant amount of these compounds may persist in treated effluents. As a result, effective treatment alternatives must be developed to allow for the effective and total elimination of the pollutants. Accordingly, physicochemical, microbiological, and advanced oxidation processes have been explored for the remediation of wastewater containing these organic pollutants, yielding promising results (Petrie, Barden & Kasprzyk-hordern, 2014; García-Morales et al., 2018; Kudlek, 2018; Joseph et al., 2019; Wu, Chen & Wei, 2020). However, these techniques may be effective only for removing certain types of pollutants, with additional drawbacks such as costly operation, variable efficiencies, and the potential production of hazardous metabolites, restricting their successful application (Alshabib & Onaizi, 2019; Das et al., 2021; Gadupudi et al., 2021).

The use of enzymes such as oxidoreductase enzymes, in the biotransformation of organic pollutants, is a potential alternative to traditional water remediation techniques. Enzymes, in general, are capable of catalyzing specific reactions under moderate operating conditions over a wide range of concentrations, resulting in low energy input and a relatively eco-friendly process (Etesami, 2017; Saravanan et al., 2021). Several types of bacterial and fungal enzymes have been defined as having the potential to oxidize hazardous pollutants. As a result, white rot fungal cultures (WRF) and their lignin catalyzing enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, have recently drawn considerable interest from researchers for their capacity to catalyze the biodegradation of a broad spectrum of toxic contaminants (Lueangjaroenkit et al., 2019; Pylypchuk et al., 2020; Rath et al., 2022).

Particularly interesting, laccases are multicopper oxidative enzymes that catalyze the single electron oxidation of a variety of phenolic and aromatic compounds while simultaneously reducing molecular oxygen to water (Sharma, Dangi & Shukla, 2018). In comparison to peroxidases, they have been extensively studied in recent years due to their apparent stability (pH, temperature, storage) and the requirement of only oxygen as a co-substrate, making them appealing and suitable for bioremediation applications (Wu, Chen & Wei, 2020; Unuofin, Moloantoa & Khetsha, 2022; Zhou, Zhang & Cai, 2022). As a result, research has shown that laccases have a high capacity to remediate wastewater containing various kinds of organic contaminants such as dyes, polycyclic aromatic hydrocarbons, phenols, pesticides, and pharmaceuticals among others (Dai et al., 2016; Tahmasbi et al., 2016; Bagewadi, Mulla & Ninnekar, 2017; Jia et al., 2019; Zdarta et al., 2020; Ameri et al., 2021; Chen et al., 2022; Deng et al., 2022).

However, the biocatalyst's reliability across processes and its recycling and reuse potential ought to be improved to enhance the efficacy of enzyme utilization in bioremediation processes. To accomplish this, the enzyme is commonly integrated into insoluble supports, resulting in stable linkages between the enzyme and the support material with subsequent enhancement in stabilities toward potentially inhibitory external environments (Knedel et al., 2019; Deng et al., 2022). Accordingly, different immobilization supports have been explored including inorganic materials like carbon nanotubes (Jun et al., 2019), organic polymers such as chitosan (Jaiswal, Pandey & Dwivedi, 2016), cellulose (Sathishkumar et al., 2014), polyacrylonitrile (PAN) beads (Catapane et al., 2013), polyaniline nanofibers (Kim et al., 2016), and hybrid/composite materials including copper alginate beads (Le et al., 2016), cellulose nanofiber/alginate hydrogel (Zhang et al., 2022), etc.

Metal-organic frameworks (MOFs), such as the zeolitic imidazolate framework (ZIF), have lately become popular as appealing organic-inorganic porous hybrid support matrices for enzyme immobilization. They have special features such as ultra-high and well-defined porous structures with tunable pore sizes, a large hierarchical surface area, crystalline nature, and configurable organic ligands (Nadar & Rathod, 2018; Ahmad et al., 2020; Du et al., 2022). Owing to their high affinity for enzymes, MOFs have been investigated as potential platforms for oxidative enzyme immobilization and subsequent application in the degradation of

pollutants using enzymes like peroxidase and lipases, etc (Vaidya, Nadar & Rathod, 2020; Lou et al., 2023).

1.2 Problem statement

The increasing detection of PhACs and EDCs in the aquatic environment is a global concern, particularly concerning the potentially harmful effects these compounds may have on aquatic life and humankind. These include compounds such as carbamazepine, diclofenac, norfloxacin, and phthalates (or phthalic esters), and they may be found in surface waterways as intact molecules or metabolites excreta (Barrios-estrada et al., 2018; Pylypchuk et al., 2020; Higgins, Siddiqui & Kumar, 2022; Morovati et al., 2022). Their high stability and refractory nature make these compounds persist in WWTPs resisting complete elimination by conventional water treatment technologies. This results in the inevitable discharge of such pollutants into surface water (Chen, Zhuan & Wang, 2021; de Carvalho Filho et al., 2022; Mkaddem et al., 2022).

Due to the persistent detection of the toxic pollutants of different origins and their associated detrimental effects on aquatic life and humans, there is a need to develop efficient and highly stable biocatalyst composites and systems for an eco-friendly process, aiding complete biotransformation of such pollutants to less or non-toxic compounds. Although oxidative enzymes such as laccases, peroxidases, and lipases have been explored for the biodegradation of these pollutants (Bilal, Iqbal & Barceló, 2019; Nadar & Rathod, 2020; Sadeghzadeh et al., 2020), the use of soluble enzyme molecules limits their practical application in wastewater treatment. This is predominantly owing to their low stabilities and recyclability potential, probable enzyme leakage during the degradation procedure, and high sensitivity to denaturing agents (Sheldon & van Pelt, 2013; Gasser & Ammann, 2014; Lassouane et al., 2019). Therefore, to overcome these drawbacks, immobilization of the enzyme on appropriate stable supports has proven effective (Primožič et al., 2020).

The immobilization procedure is a crucial factor for the optimum degradation efficiency of the system. It must, therefore, be carefully selected considering the features of the support, the enzyme's characteristics, and the anticipated application process. Among the various immobilization approaches available, covalent binding, typically via a biomineralization procedure, appears to be the most appropriate for application in wastewater treatments, as it necessitates the formation of network linkages between the enzymes and the support, effectively improving the stability and recyclability of the enzyme, while lowering its leakage (Kashe et al., 2019; Nadar, Vaidya & Rathod, 2020; Li et al., 2021). Therefore, the need to

further investigate the covalent binding procedure for laccase attachment onto stable and highly efficient hybrid materials with subsequent application in organic pollutants decontamination is of critical importance.

1.3 Novelty and scientific contribution to new knowledge

The purpose of this study was to develop an effective, environmentally friendly, and highly stable biocatalyst for the degradation of recalcitrant emerging organic contaminants in synthetic water and application in real water remediation. Accordingly, zeolitic imidazolate framework (ZIF) was used as a stable crystalline support for laccase immobilization via a facile one-pot biomineralization procedure. This newly developed enzyme-MOF referred to as Lac-ZIF, was used to effectively biodegrade PhACs such as carbamazepine, diclofenac, and norfloxacin as well as the ubiquitous plasticizer, dimethyl phthalate. To the best of our knowledge, there is no evidence of reports for such rapidly synthesized ZIF in the presence of laccase, especially for the application in biodegradation of the investigated emerging contaminants.

The Lac-ZIF exhibited remarkable decontamination efficiency with high recyclability and reuse potential in comparison to other reported biocatalyst composites. This activity enhancement was due to mutual adsorption and biodegradation effects provided by the ZIF crystals and the immobilized laccase molecules. Therefore, this study contributes new insights into the development of novel support materials for laccase enzymes, with synergistic performances, and this may capture the interest of water treatment companies, remediation scientists, and environmental scientists. This newly developed biocatalyst may be scaled up to use in continuous-flow fixed-bed column studies, with a potential application in municipal and industrial wastewater remediation. Furthermore, this research can spark the interest of various research institutions that are extensively investigating biodegradation approaches for organic pollutants in water.

1.4 Aim and objectives

This study aimed at evaluating the potential of laccase immobilized on zeolitic imidazolate framework (ZIF) as an effective biocatalytic enzyme-matrix for the biodegradation of pharmaceutically active compounds and phthalates.

This aim was achieved by addressing the following objectives:

1. Synthesizing water-stable zeolitic imidazolate frameworks and immobilizing laccase enzymes via a one-pot mineralization procedure.
2. Characterizing the synthesized Laccase-ZIF (Lac-ZIF) composite using Fourier Transform Infrared Spectroscopy (FTIR), Powder X-Ray Diffraction (PXRD), Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM), and Nuclear Magnetic Resonance (NMR) spectroscopy.
3. Evaluating the potential of the synthesized biocatalytic composite in the biodegradation of carbamazepine, diclofenac, norfloxacin, and dimethyl phthalate.
4. Assessing experimental variables such as solution pH and concentration, biocatalyst dosage, contact time, temperature, storage, and solvent stability as well as the recyclability and re-use potential of the biocatalytic material.

1.5 Scope of work

The scope of the study entailed the fabrication of enzyme-MOFs, particularly laccase immobilized on zeolitic imidazolate framework (ZIF) via a single-pot synthetic route. This approach enables covalent binding of the laccase's protein structure onto the ultra-porous ZIF crystals. The covalent binding immobilization procedure is intended to produce stable and recyclable biocatalysts, capable of withstanding harsh environmental conditions and preventing enzyme leaching during the application process. The synthesized Lac-ZIF material was effectively applied in the synergistic adsorption and biodegradation of PhACs and EDCs such as carbamazepine, diclofenac, norfloxacin, and dimethyl phthalate in batch reaction mode. Mutualistic effects of experimental variables for DFC and NOR removal were studied and optimized using response surface methodology (RSM) (chapter 4.3).

In this study, all experiments were conducted in batch reaction mode. However, it would be interesting to use the biocatalyst in continuous-flow fixed-bed column studies. This approach provides better insights into the development of a treatment plant for the practical application of such techniques. Accordingly, in column studies, parameters such as the material exhausting rate (determining the replacement rate of the material) and the volume of water treated at the breakthrough point may be evaluated. The breakthrough point refers to the time at which a significant proportion of the influent concentration (analyte) is detected from the effluent.

Moreover, it is well documented that the addition of redox mediators which act as electron donors during the biocatalytic process may enhance the biodegradation rate of enzymatic processes. However, the long-term use of redox mediators may increase the process's operating

costs due to the need for constant addition of the mediators. The mediators may also contribute to a further environmental issue by potentially increasing the toxicity of the treated effluent. As a result, immobilization of the laccase was viewed as a trade-off for improved enzyme performance over systems employing redox mediators.

1.6 Hypothesis

This study presents a facile and environmentally friendly approach to effective biodegradation of persistent organic compounds in water. The hypothesis 'Immobilization of laccase onto zeolitic imidazolate frameworks improves the enzyme's performance in the degradation of selected PhACs and EDCs' was set. The chosen model pollutants are potential substrates of laccases and can readily adsorb onto the ZIF's surface. This is anticipated at increasing the chances for the synergistic adsorption of the pollutants and subsequent biodegradation offered by the immobilized laccase. Furthermore, the attachment of the laccase onto the ZIF is envisaged at enhancing the stability of the biocatalyst resulting in improved thermal, storage and extreme pH conditions stability, as well as enhanced recyclability and re-use potential of the biocatalyst.

1.7 Thesis structure

The thesis is structured as follows:

Chapter 1 presents the background and motivation of the study as well as the problem statement. Additionally, the research aims and objectives, scope, and limitations of the study are outlined in this chapter.

Chapter 2 outlines the literature and theoretical background of emerging water contaminants and the conventional techniques for removing such compounds in water. The drawbacks associated with these methods are also discussed. Furthermore, the rationale for investigating bioremediation options is presented, and so are the different immobilization procedures.

Chapter 3 briefly describes the materials and methodologies used in this study. The instruments used are also included in this chapter. However, further details for such methods are given in each of the papers presented in chapter 4.

Chapter 4 presents the results and discussion of the study in the form of papers. These papers are either published or under review for publication.

Chapter 5 outlines and provides general conclusions on the outputs of the study, and further presents the recommendations for consideration in future.

1.8 References

Ahmad, R., Shanahan, J., Rizaldo, S., Kissel, D.S. & Stone, K.L. 2020. Co-immobilization of an enzyme system on a metal-organic framework to produce a more effective biocatalyst. *Catalysts*. 10(5). DOI: 10.3390/catal10050499.

Alhaddad, F.A., Abu-Dieyeh, M., Da'ana, D., Helaleh, M. & Al-Ghouti, M.A. 2021. Occurrence and removal characteristics of phthalate esters from bottled drinking water using silver modified roasted date pits. *Journal of Environmental Health Science and Engineering*. 19(1):733–751. DOI: 10.1007/s40201-021-00642-9.

Alharbi, S.K., Nghiem, L.D., van de Merwe, J.P., Leusch, F.D.L., Asif, M.B., Hai, F.I. & Price, W.E. 2019. Degradation of diclofenac, trimethoprim, carbamazepine, and sulfamethoxazole by laccase from Trametes versicolor: Transformation products and toxicity of treated effluent. *Biocatalysis and Biotransformation*. 37(6):399–408. DOI: 10.1080/10242422.2019.1580268.

Alshabib, M. & Onaizi, S.A. 2019. A review on phenolic wastewater remediation using homogeneous and heterogeneous enzymatic processes: Current status and potential challenges. *Separation and Purification Technology*. 219(October 2018):186–207. DOI: 10.1016/j.seppur.2019.03.028.

Ameri, A., Taghizadeh, T., Talebian-Kiakalaieh, A., Forootanfar, H., Mojtabavi, S., Jahandar, H., Tarighi, S. & Faramarzi, M.A. 2021. Bio-removal of phenol by the immobilized laccase on the fabricated parent and hierarchical NaY and ZSM-5 zeolites. *Journal of the Taiwan Institute of Chemical Engineers*. 120:300–312. DOI: 10.1016/j.jtice.2021.03.016.

Bagewadi, Z.K., Mulla, S.I. & Ninnekar, H.Z. 2017. Purification and immobilization of laccase from Trichoderma harzianum strain HZN10 and its application in dye decolorization. *Journal of Genetic Engineering and Biotechnology*. 15(1):139–150. DOI: 10.1016/j.jgeb.2017.01.007.

Barrios-estrada, C., Rostro-alanis, M.D.J., Muñoz-gutiérrez, B.D., Iqbal, M.N., Kannan, S. & Parra-saldívar, R. 2018. Emergent contaminants : Endocrine disruptors and their laccase-assisted degradation – A review. *Science of the Total Environment*. 612:1516–1531.

Barrios-Estrada, C., de Jesús Rostro-Alanis, M., Muñoz-Gutiérrez, B.D., Iqbal, H.M.N., Kannan, S. & Parra-Saldívar, R. 2018. Emergent contaminants: Endocrine disruptors and their laccase-assisted degradation – A review. *Science of the Total Environment*. 612:1516–1531. DOI: 10.1016/j.scitotenv.2017.09.013.

Bilal, M., Iqbal, M.N. & Barceló, D. 2019. Mitigation of bisphenol A using an array of laccase-based robust bio-catalytic cues – A review. *Science of the Total Environment*. 689:160–177. DOI: 10.1016/j.scitotenv.2019.06.403.

de Carvalho Filho, J.A.A., da Cruz, H.M., Fernandes, B.S., Motteran, F., de Paiva, A.L.R. & Pereira Cabral, J.J. da S. 2022. Efficiency of the bank filtration technique for diclofenac removal: A review. *Environmental Pollution*. 300(January). DOI: 10.1016/j.envpol.2022.118916.

Catapane, M., Nicolucci, C., Menale, C., Mita, L., Rossi, S., Mita, D.G. & Diano, N. 2013. Enzymatic removal of estrogenic activity of nonylphenol and octylphenol aqueous solutions

by immobilized laccase from Trametes versicolor. *Journal of Hazardous Materials*. 248–249(1):337–346. DOI: 10.1016/j.jhazmat.2013.01.031.

Chen, X., Zhuan, R. & Wang, J. 2021. Assessment of degradation characteristic and mineralization efficiency of norfloxacin by ionizing radiation combined with Fenton-like oxidation. *Journal of Hazardous Materials*. 404(PA):124172. DOI: 10.1016/j.jhazmat.2020.124172.

Chen, Z., Yao, J., Šolević Knudsen, T., Ma, B., Liu, B., Li, H., Zhu, X., Zhao, C., et al. 2022. Degradation of novel mineral flotation reagent 8-hydroxyquinoline by superparamagnetic immobilized laccase: Effect, mechanism and toxicity evaluation. *Chemical Engineering Journal*. 432(September 2021). DOI: 10.1016/j.cej.2021.134239.

Cobas, M., Danko, A.S., Pazos, M. & Sanrom??n, M.A. 2016. Removal of metal and organic pollutants from wastewater by a sequential selective technique. *Bioresource Technology*. 213:2–10. DOI: 10.1016/j.biortech.2016.02.036.

Dai, Y., Yao, J., Song, Y., Wang, S. & Yuan, Y. 2016. Enhanced adsorption and degradation of phenolic pollutants in water by carbon nanotube modified laccase-carrying electrospun fibrous membranes. *Environmental Science: Nano*. 3(4):857–868. DOI: 10.1039/c6en00148c.

Das, M.T., Kumar, S.S., Ghosh, P., Shah, G., Malyan, S.K., Bajar, S., Thakur, I.S. & Singh, L. 2021. Remediation strategies for mitigation of phthalate pollution: Challenges and future perspectives. *Journal of Hazardous Materials*. 409(November 2020):124496. DOI: 10.1016/j.jhazmat.2020.124496.

Deblonde, T., Cossu-leguille, C. & Hartemann, P. 2011. Emerging pollutants in wastewater : A review of the literature. *International Journal of Hygiene and Environmental Health*. 214(6):442–448. DOI: 10.1016/j.ijheh.2011.08.002.

Deng, J., Wang, H., Zhan, H., Wu, C., Huang, Y., Yang, B., Mosa, A. & Ling, W. 2022. Catalyzed degradation of polycyclic aromatic hydrocarbons by recoverable magnetic chitosan immobilized laccase from Trametes versicolor. *Chemosphere*. 301(April):134753. DOI: 10.1016/j.chemosphere.2022.134753.

Du, Y., Jia, X., Zhong, L., Jiao, Y., Zhang, Z., Wang, Z., Feng, Y., Bilal, M., et al. 2022. Metal-organic frameworks with different dimensionalities: An ideal host platform for enzyme@MOF composites. *Coordination Chemistry Reviews*. 454:214327. DOI: 10.1016/j.ccr.2021.214327.

Ersan, G., Apul, O.G., Perreault, F. & Karanfil, T. 2017. Adsorption of organic contaminants by graphene nanosheets: A review. *Water Research*. 126:385–398. DOI: 10.1016/j.watres.2017.08.010.

Etesami, H. 2017. Role of Various Enzymes of Microbes in Bioremediation. *Environmental Science and Engineering*. 8(February).

Fang, L., Miao, Y., Wei, D., Zhang, Y. & Zhou, Y. 2021. Efficient removal of norfloxacin in water using magnetic molecularly imprinted polymer. *Chemosphere*. 262. DOI: 10.1016/j.chemosphere.2020.128032.

Feng, Y.L., Singh, R., Chao, A. & Li, Y. 2022. Diagnostic Fragmentation Pathways for Identification of Phthalate Metabolites in Nontargeted Analysis Studies. *Journal of the American Society for Mass Spectrometry*. 33(6):981–995. DOI: 10.1021/jasms.2c00052.

Gadupudi, C.K., Rice, L., Xiao, L. & Kantamaneni, K. 2021. Endocrine Disrupting Compounds Removal Methods from Wastewater in the United Kingdom: A Review. *Sci*.

3(1):11. DOI: 10.3390/sci3010011.

García-Morales, R., García-García, A., Orona-Navar, C., Osma, J.F., Nigam, K.D.P. & Ornelas-Soto, N. 2018. Biotransformation of emerging pollutants in groundwater by laccase from P. sanguineus CS43 immobilized onto titania nanoparticles. *Journal of Environmental Chemical Engineering*. 6(1):710–717. DOI: 10.1016/j.jece.2017.12.006.

García, M.A., Cristofoletti, R., Abrahamsson, B., Groot, D.W., Parr, A., Polli, J.E., Mehta, M., Shah, V.P., et al. 2021. Biowaiver Monograph for Immediate-Release Solid Oral Dosage Forms: Carbamazepine. *Journal of Pharmaceutical Sciences*. 110(5):1935–1947. DOI: 10.1016/j.xphs.2021.02.019.

Gasser, C.A. & Ammann, E.M. 2014. Laccases to take on the challenge of emerging organic contaminants in wastewater. (October). DOI: 10.1007/s00253-014-6177-6.

George, J., Rajendran, D.S., Venkataraman, S., Rathankumar, A.K., Saikia, K., Muthusamy, S., Singh, I., Singh, I., et al. 2022. Insolubilization of Tramates versicolor laccase as cross-linked enzyme aggregates for the remediation of trace organic contaminants from municipal wastewater. *Environmental Research*. 209(January):112882. DOI: 10.1016/j.envres.2022.112882.

Higgins, P., Siddiqui, S.H. & Kumar, R. 2022. Design of novel graphene oxide/halloysite nanotube@polyaniline nanohybrid for the removal of diclofenac sodium from aqueous solution. *Environmental Nanotechnology, Monitoring and Management*. 17(December 2021):100628. DOI: 10.1016/j.enmm.2021.100628.

Jaiswal, N., Pandey, V.P. & Dwivedi, U.N. 2016. Immobilization of papaya laccase in chitosan led to improved multipronged stability and dye discoloration. *International Journal of Biological Macromolecules*. 86:288–295. DOI: 10.1016/j.ijbiomac.2016.01.079.

Jia, Y., Chen, Y., Luo, J. & Hu, Y. 2019. Immobilization of laccase onto meso-MIL-53(Al) via physical adsorption for the catalytic conversion of triclosan. *Ecotoxicology and Environmental Safety*. 184(September):109670. DOI: 10.1016/j.ecoenv.2019.109670.

Joseph, L., Jun, B.M., Jang, M., Park, C.M., Muñoz-Senmache, J.C., Hernández-Maldonado, A.J., Heyden, A., Yu, M., et al. 2019. Removal of contaminants of emerging concern by metal-organic framework nanoadsorbents: A review. *Chemical Engineering Journal*. 369(March):928–946. DOI: 10.1016/j.cej.2019.03.173.

Julinová, M. & Slavík, R. 2012. Removal of phthalates from aqueous solution by different adsorbents: A short review. *Journal of Environmental Management*. 94(1):13–24. DOI: 10.1016/j.jenvman.2011.09.006.

Jun, L.Y., Mubarak, N.M., Yon, L.S., Bing, C.H., Khalid, M., Jagadish, P. & Abdullah, E.C. 2019. Immobilization of Peroxidase on Functionalized MWCNTs-Buckypaper/Polyvinyl alcohol Nanocomposite Membrane. *Scientific Reports*. 9(1):2215. DOI: 10.1038/s41598-019-39621-4.

Kashe, S., Mehdi, S., Mohammad, N., Kashefi, S., Borghei, S.M. & Mahmoodi, N.M. 2019. Covalently immobilized laccase onto graphene oxide nanosheets : Preparation, characterization, and biodegradation of azo dyes in colored wastewater. *Journal of Molecular Liquids*. 276:153–162. DOI: 10.1016/j.molliq.2018.11.156.

Katsigiannis, A., Noutsopoulos, C., Mantziaras, J. & Gioldasi, M. 2015. Removal of emerging pollutants through Granular Activated Carbon. *Chemical Engineering Journal*. 280:49–57.

DOI: 10.1016/j.cej.2015.05.109.

Kim, J.J.H., Hong, S.G., Sun, H.J., Ha, S. & Kim, J.J.H. 2016. Precipitated and chemically-crosslinked laccase over polyaniline nanofiber for high performance phenol sensing. *Chemosphere*. 143:142–147. DOI: 10.1016/j.chemosphere.2015.08.011.

Knedel, T.O., Ricklefs, E., Schlüsener, C., Urlacher, V.B. & Janiak, C. 2019. Laccase Encapsulation in ZIF-8 Metal-Organic Framework Shows Stability Enhancement and Substrate Selectivity. *ChemistryOpen*. 8(11):1337–1344. DOI: 10.1002/open.201900146.

Kudlek, E. 2018. Decomposition of contaminants of emerging concern in advanced oxidation processes. *Water (Switzerland)*. 10(7). DOI: 10.3390/w10070955.

Lassouane, F., Aït-Amar, H., Amrani, S. & Rodriguez-couto, S. 2019. A promising laccase immobilization approach for Bisphenol A removal from aqueous solutions. *Bioresource Technology*. 271(August 2018):360–367. DOI: 10.1016/j.biortech.2018.09.129.

Le, T.T., Murugesan, K., Lee, C.S., Vu, C.H., Chang, Y.S. & Jeon, J.R. 2016. Degradation of synthetic pollutants in real wastewater using laccase encapsulated in core-shell magnetic copper alginate beads. *Bioresource Technology*. 216:203–210. DOI: 10.1016/j.biortech.2016.05.077.

Li, D., Cheng, Y., Zuo, H., Zhang, W., Pan, G., Fu, Y. & Wei, Q. 2021. Dual-functional biocatalytic membrane containing laccase-embedded metal-organic frameworks for detection and degradation of phenolic pollutant. *Journal of Colloid and Interface Science*. 603:771–782. DOI: 10.1016/j.jcis.2021.06.155.

Lou, X., Zhi, F., Sun, X., Wang, F., Hou, X., Lv, C. & Hu, Q. 2023. Construction of co-immobilized laccase and mediator based on MOFs membrane for enhancing organic pollutants removal. *Chemical Engineering Journal*. 451(July 2022). DOI: 10.1016/j.cej.2022.138080.

Lueangjaroenkit, P., Teerapatsakul, C., Sakka, K., Sakka, M., Kimura, T., Kunitake, E. & Chitradon, L. 2019. Two Manganese Peroxidases and a Laccase of Trametes polyzona KU-RNW027 with Novel Properties for Dye and Pharmaceutical Product Degradation in Redox Mediator-Free System. *Mycobiology*. 47(2):217–229. DOI: 10.1080/12298093.2019.1589900.

Méndez, E., González-Fuentes, M.A., Rebollar-Perez, G., Méndez-Albores, A. & Torres, E. 2017. Emerging pollutant treatments in wastewater: Cases of antibiotics and hormones. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*. 52(3):235–253. DOI: 10.1080/10934529.2016.1253391.

Mkaddem, H., Rosales, E., Pazos, M., Ben Amor, H., Sanromán, M.A. & Meijide, J. 2022. Anti-inflammatory drug diclofenac removal by a synthesized MgAl layered double hydroxide. *Journal of Molecular Liquids*. 359:119207. DOI: 10.1016/j.molliq.2022.119207.

Morovati, R., Hoseini, M., Azhdarpoor, A., Dehghani, M., Baghapour, M.A. & Yousefinejad, S. 2022. Removal of Diclofenac Sodium from Wastewater in Microbial Fuel Cell by Anode Modified with MnCo2O4. *Sustainability*. 14(21):13907. DOI: 10.3390/su142113907.

Nadar, S.S. & Rathod, V.K. 2018. Magnetic-metal organic framework (magnetic-MOF): A novel platform for enzyme immobilization and nanozyme applications. *International Journal of Biological Macromolecules*. 120(xxxx):2293–2302. DOI: 10.1016/j.ijbiomac.2018.08.126.

Nadar, S.S. & Rathod, V.K. 2020. Immobilization of proline activated lipase within metal organic framework (MOF). *International Journal of Biological Macromolecules*. 152:1108–1112. DOI: 10.1016/j.ijbiomac.2019.10.199.

Nadar, S.S., Vaidya, L. & Rathod, V.K. 2020. Enzyme embedded metal organic framework (enzyme–MOF): De novo approaches for immobilization. *International Journal of Biological Macromolecules*. 149:861–876. DOI: 10.1016/j.ijbiomac.2020.01.240.

Naghdi, M., Taheran, M., Brar, S.K., Kermanshahi-pour, A., Verma, M. & Surampalli, R.Y. 2018. Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes. *Environmental Pollution*. 234:190–213. DOI: 10.1016/j.envpol.2017.11.060.

Petrie, B., Barden, R. & Kasprzyk-hordern, B. 2014. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Research*. 72(0):3–27. DOI: 10.1016/j.watres.2014.08.053.

Primožič, M., Kravanja, G., Knez, Ž., Crnjac, A. & Leitgeb, M. 2020. Immobilized laccase in the form of (magnetic) cross-linked enzyme aggregates for sustainable diclofenac (bio)degradation. *Journal of Cleaner Production*. 275. DOI: 10.1016/j.jclepro.2020.124121.

Pylypchuk, I. V., Daniel, G., Kessler, V.G. & Seisenbaeva, G.A. 2020. Removal of diclofenac, paracetamol, and carbamazepine from model aqueous solutions by magnetic sol–gel encapsulated horseradish peroxidase and lignin peroxidase composites. *Nanomaterials*. 10(2). DOI: 10.3390/nano10020282.

Rath, S., Paul, M., Behera, H.K. & Thatoi, H. 2022. Response surface methodology mediated optimization of Lignin peroxidase from Bacillus mycoides isolated from Simlipal Biosphere Reserve, Odisha, India. *Journal of Genetic Engineering and Biotechnology*. 20(1). DOI: 10.1186/s43141-021-00284-2.

Sadeghzadeh, S., Ghobadi Nejad, Z., Ghasemi, S., Khafaji, M. & Borghei, S.M. 2020. Removal of bisphenol A in aqueous solution using magnetic cross-linked laccase aggregates from Trametes hirsuta. *Bioresource Technology*. 306(March):123169. DOI: 10.1016/j.biortech.2020.123169.

Saravanan, A., Kumar, P.S., Vo, D.V.N., Jeevanantham, S., Karishma, S. & Yaashikaa, P.R. 2021. A review on catalytic-enzyme degradation of toxic environmental pollutants: Microbial enzymes. *Journal of Hazardous Materials*. 419(June):126451. DOI: 10.1016/j.jhazmat.2021.126451.

Sathishkumar, P., Kamala-Kannan, S., Cho, M., Kim, J.S., Hadibarata, T., Salim, M.R. & Oh, B.T. 2014. Laccase immobilization on cellulose nanofiber: The catalytic efficiency and recyclic application for simulated dye effluent treatment. *Journal of Molecular Catalysis B: Enzymatic*. 100:111–120. DOI: 10.1016/j.molcatb.2013.12.008.

Science, A., Widel, D. & Oszczudlowski, J. 2017. Removal of selected phthalates from aqueous solution by mesoporous- ordered carbon adsorbent. (October). DOI: 10.1177/0263617417708675.

Sharma, B., Dangi, A.K. & Shukla, P. 2018. Contemporary enzyme based technologies for bioremediation: A review. *Journal of Environmental Management*. 210:10–22. DOI: 10.1016/j.jenvman.2017.12.075.

Sheldon, R.A. & van Pelt, S. 2013. Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews*. 42(15):6223–6235. DOI: 10.1039/c3cs60075k.

Sorensen, J.P.R., Lapworth, D.J., Nkhuwa, D.C.W., Stuart, M.E., Gooddy, D.C., Bell, R.A.,

Chirwa, M., Kabika, J., et al. 2014. Emerging contaminants in urban groundwater sources in Africa. *Water Research*. 72:51–63. DOI: 10.1016/j.watres.2014.08.002.

Tahmasbi, H., Khoshayand, M.R., Bozorgi-Koushalshahi, M., Heidary, M., Ghazi-Khansari, M. & Faramarzi, M.A. 2016. Biocatalytic conversion and detoxification of imipramine by the laccase-mediated system. *International Biodeterioration and Biodegradation*. 108:1–8. DOI: 10.1016/j.ibiod.2015.11.029.

Unuofin, J.O., Moloantoa, K.M. & Khetsha, Z.P. 2022. The biobleaching potential of laccase produced from mandarin peelings : Impetus for a circular bio-based economy in textile biofinishing. *Arabian Journal of Chemistry*. 15(12):104305. DOI: 10.1016/j.arabjc.2022.104305.

Vaidya, L.B., Nadar, S.S. & Rathod, V.K. 2020. Metal-organic frameworks (MOFs) for enzyme immobilization. *Metal-Organic Frameworks for Biomedical Applications*. (January, 1):491–523. DOI: 10.1016/B978-0-12-816984-1.00024-X.

Wang, X., Ding, Y., Dionysiou, D.D., Liu, C., Tong, Y., Gao, J., Fang, G. & Zhou, D. 2020. Efficient activation of peroxymonosulfate by copper sulfide for diethyl phthalate degradation: Performance, radical generation and mechanism. *Science of the Total Environment*. 749:142387. DOI: 10.1016/j.scitotenv.2020.142387.

Wu, Y., Chen, Y. & Wei, N. 2020. Biocatalytic properties of cell surface display laccase for degradation of emerging contaminant acetaminophen in water reclamation. *Biotechnology and Bioengineering*. 117(2):342–353. DOI: 10.1002/bit.27214.

Yao, C., Wang, J., Chan, P. & Feng, Y.L. 2018. A novel non-targeted screening method for urinary exposure biomarker discovery of phthalates using liquid chromatography-mass spectrometry. *Analytical Methods*. 10(9):959–967. DOI: 10.1039/c7ay02941a.

Zaki, G. & Shoeib, T. 2018. Concentrations of several phthalates contaminants in Egyptian bottled water: Effects of storage conditions and estimate of human exposure. *Science of the Total Environment*. 618:142–150. DOI: 10.1016/j.scitotenv.2017.10.337.

Zdarta, J., Jankowska, K., Bachosz, K., Kijeńska-Gawrońska, E., Zgoła-Grześkowiak, A., Kaczorek, E. & Jesionowski, T. 2020. A promising laccase immobilization using electrospun materials for biocatalytic degradation of tetracycline: Effect of process conditions and catalytic pathways. *Catalysis Today*. 348(May 2019):127–136. DOI: 10.1016/j.cattod.2019.08.042.

Zhang, F., Lian, M., Alhadhrami, A., Huang, M., Li, B., Mersal, G.A.M., Ibrahim, M.M. & Xu, M. 2022. Laccase immobilized on functionalized cellulose nanofiber/alginate composite hydrogel for efficient bisphenol A degradation from polluted water. *Advanced Composites and Hybrid Materials*. (0123456789). DOI: 10.1007/s42114-022-00476-5.

Zhou, W., Zhang, W. & Cai, Y. 2022. Enzyme-enhanced adsorption of laccase immobilized graphene oxide for micro-pollutant removal. *Separation and Purification Technology*. 294(April):121178. DOI: 10.1016/j.seppur.2022.121178.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

This chapter provides a comprehensive review of the literature on the sources and toxicological effects of emerging water contaminants. The traditional approaches for eradicating organic contaminants from aqueous solutions, including the use of biocatalytic materials, are addressed in this chapter. Furthermore, the various approaches for enzyme immobilization and different types of insoluble supports for the enzymes are discussed, along with the rationale for using metal-organic frameworks.

2.2 Emerging water contaminants

The incidence of PhACs and EDCs in the environment is a worldwide concern, and the hazardous effects linked to the exposure to these compounds have led to their classification as emerging environmental contaminants. These are predominantly anthropogenic substances that are either novel (newly synthesized) or previously undetected substances that are increasingly being detected above anticipated concentration levels and are presumed to have adverse environmental consequences and impacts to humankind (Cobas et al., 2015; Sorensen et al., 2015; Moodley, Birungi & Ndungu, 2016). Among the various emerging contaminants, PhACs and EDCs have captivated the attention of numerous investigators as contributory compounds to freshwater system pollution. Chronic exposure to some PhACs may induce endocrine disruptions, therefore, a number of these PhACs may be classified under EDCs.

2.2.1 Pharmaceutically active compounds (PhACs)

PhACs include a wide spectrum of medicinal compounds principally used as prescription and non-prescription drugs. Among the various PhACs, there is an increasing use of synthetic drugs such as antibiotics, analgesics, and anti-inflammatory medications for the relief of mild and highly infectious diseases in humans and animals, and these have been detected in raw wastewater and WWTPs (Al-Kindi et al., 2021; Chen et al., 2021; Tegegne et al., 2021; Hu et al., 2022). These include compounds such as carbamazepine (CBZ), diclofenac (DFC), and norfloxacin (NOR) drugs, which are used as anticonvulsant and analgesic drugs, anti-

inflammatory, and antibiotic medicines, respectively. The chemical structures of the PhACs are shown in Figure 2.1 (a)-(c)

Carbamazepine ($C_{15}H_{12}N_2O$) is an anticonvulsant and mood-stabilizing tricyclic compound that is effective in treating partial seizures and/or epilepsy (Tolou-Ghamari et al., 2013). CBZ has been widely used in the management of disorders such as attention-deficit hyperactivity disorder (ADHD), bipolar, and post-traumatic stress disorder among others (García et al., 2021; Strakos, 2021).

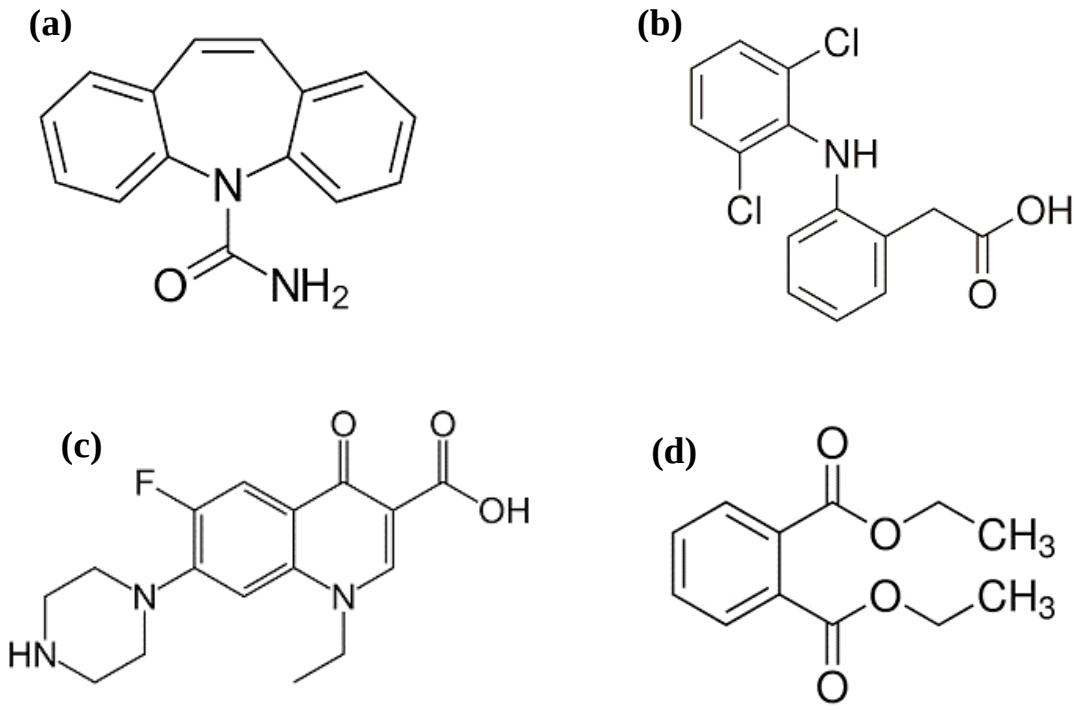


Figure 2. 1: Chemical structures of (a) Carbamazepine (CBZ), (b) Diclofenac (DFC), (c) Norfloxacin (NOR), and (d) Dimethyl phthalate (DMP).

Another common and widespread pollutant in wastewater is diclofenac (DFC) (2-[2-(2,6-dichloroanilino) phenyl] acetic acid), a nonsteroidal anti-inflammatory drug (NSAID) commonly used to relieve pain or reduce inflammation related to injuries and conditions such as osteoarthritis, rheumatoid arthritis, and migraine (Husin et al., 2021; Feng et al., 2022). Also, fluoroquinolone antibiotics such as norfloxacin (NOR) ($C_{16}H_{18}FN_3O_3$) are extensively used due to their broad-spectrum antibacterial properties (Paredes-Laverde, Silva-Agredo & Torres-Palma, 2018; Chen et al., 2021). Since these pharmaceuticals may be partially metabolized, a greater proportion of the medicines consumed may be excreted into the waterways as unaltered compounds or other active metabolites, leading to increased antimicrobial resistance in aquatic

life as well as inhibition of denitrifying bacterial growth associated with the antibiotics (Huang et al., 2022; Jiang et al., 2023; Ta et al., 2023). Furthermore, inadvertent exposure to these PhACs, such as CBZ and DFC, usually through surface water runoff and irrigation systems, has been shown to have negative effects on the receiving biota, including probable carcinogenic effects and alteration of aquatic creatures' normal physiological functions (Laera et al., 2011; Pylypchuk et al., 2020; Al-Mashaqbeh et al., 2021; Prasetya & Li, 2022).

2.2.2 Endocrine-disrupting chemicals (EDCs)

EDCs are compounds that are capable of causing functional alterations in an organism's endocrine system, affecting the formulation, secretion, transport, and binding action of natural hormones in the organism that regulate homeostasis, reproduction, and proper development of the organism (Becker et al., 2017; Canle, Fernández Pérez & Santaballa, 2017; Gao et al., 2020; Mak & Gu, 2021). Due to their relatively low cost, phthalates such as dimethyl phthalate (DMP) are globally regarded as essential industrial chemicals with a variety of commercial applications, including solvents, plasticizers as well as a coating in cosmetics, food packaging, and pharmaceutical products (Jedynak, Wideł & Oszczudłowski, 2017; Zeng et al., 2022). Phthalates as plasticizers are typically not covalently bound to the chemical structure of the final product, as a result, the inevitable leaching of these compounds into environmental water increases the exposure potential to humankind, resulting in carcinogenic, endocrine disruptions, mutagenic and genotoxic effects among others (Zaki & Shoeib, 2018; Liu et al., 2022).

2.3 Remediation of synthetic organic contaminants

Due to the global concerns about these emerging organic pollutants contaminating water, as well as the toxicology and persistence of these compounds in wastewater, approaches to mitigate pollution in water systems have been proposed and investigated. The conventional technologies for organic pollutants removal comprise membrane separation, ion exchange, chemical oxidation, adsorption, and biodegradation processes among others (Khalid et al., 2017; Jiménez et al., 2018; Han et al., 2019; Routoula & Patwardhan, 2020; Othman et al., 2021).

2.3.1 Ion exchange

Ion exchange is a passive reversible technique that enables ions in an external solution to exchange with electrostatically bound ions within a solid matrix (Gupta et al., 2012). This technique is widely used in water remediation as it facilitates the interchange of toxic ions with non-lethal ions from an insoluble ion exchanger usually referred to as an ion exchange resin (Zhang et al., 2011; Lu & Astruc, 2018). Typical traditional ion exchangers contain sulfonic acid ($–SO_3H$), carboxylic acid ($-CO_2H$), quaternary ammonium groups ($-CH_2N(CH_3)_3$), as well as amine ($-NH_2$), and imine (=NH) groups (Wawrzkiewicz & Hubicki, 2015). In a study by Devaisy et al. (2023), the impact of an ion-exchange resin (Purolite®A502PS) was investigated in a micro-filtration–ion exchange hybrid system to remove organics such as carbamazepine, sulfamethoxazole, paracetamol and diclofenac from a reverse osmosis concentrate. The removal of these organics was accomplished through two mechanisms. The first mechanism involves an electrostatic interaction between the resin and the carboxylic acid moieties in the organic compound, which is enabled by a counter ion release from the resin surface (quarternary amine groups) in exchange for the ionic pollutant. This is summarized in (equation 2.1), where R represents the ionic organic compound.

$$Resin - NMe_3^+Cl^- + R^- \leftrightarrow Resin - NMe_3^+R^- + Cl^- \quad (2.1)$$

The second mechanism involves physical adsorption due to van der Waals forces between the resin's polymeric backbone and non-ionic moieties of the organic compounds. In another study, (Conte et al., 2015), a hydrophobic macroporous resin (Purolite® A520E) proved effective in removing polyfluorinated organic micropollutants with enhanced kinetics and high equilibrium removal capacities. Also, the resins used in this study presented appreciable recyclability using dilute solvents such as NH_4Cl and NH_4OH. The use of similar resins has been explored in many other studies with significantly high efficiencies (Rahmani & Mohseni, 2017; Kaleta, Papciak & Puszkarewicz, 2018; Fontanals, Borrull & Marcé, 2020), however, this technique has drawbacks such as the inefficient non-ionic compound extraction and the need to integrate the ion exchange resins with other techniques like membrane filtration to augment removal efficiencies.

2.3.2 Membrane filtration

Membrane filtration is amongst the notably efficient and extensively employed technologies in water treatment. This technique has gained popularity due to its notable characteristics, which include ease of operation, the potential to scale up (Liu et al., 2013), and its reliability and

effectiveness in pollutant rejection. It also presents benefits associated with the flexibility offered by a variety of membrane materials such as polymeric materials (polysulfone (PSF), polyamide (PA), polyether sulfone (PES), polyvinylidene difluoride (PVDF), etc), ceramics (silica, titania, etc) and metallic materials (gold, silver, palladium, etc.) Different membrane separation procedures for organic pollutants removal have been explored including ultrafiltration, microfiltration, and reverse osmosis among others (Fu & Wang, 2011). Ultrafiltration is the most frequently employed method in water remediation, with reasonably high pollutant removal efficiencies. These are typically pressure-driven techniques that use low transmembrane pressures to remove soluble and colloidal compounds (Ajenifuja et al., 2016; Elrasheedy, Nady & Bassyouni, 2019; Esfahani et al., 2019). Membrane separation techniques, however, have drawbacks such as fouling during the long-term operation of pressure-driven membrane processes. This elevates operating and maintenance costs due to insufficient recoveries, deterioration of permeate quality, decreased service time, and consequently premature membrane replacements. On the other hand, the application of non-pressure-driven membranes is inhibited by low permeation challenges (Huang, Ras & Tian, 2018; Esfahani et al., 2019).

2.3.3 Advanced oxidation processes (AOPs)

Due to their ease of use and replicability, advanced oxidation processes (AOPs) have gained popularity in the decontamination of organic pollutants in recent years (Kudlek, 2018; Martínez et al., 2018). These techniques involve the use of highly reactive species to degrade organic contaminants via oxidation to less toxic compounds with subsequent mineralization of the by-products to CO_2 and H_2O under suitable conditions. Usually, reactive molecular species such as hydrogen peroxide (H_2O_2), sulfate ($SO4^{\bullet-}$), and hydroxyl radicals (•OH) are employed (Oluwole, Omotola & Olatunji, 2020; Tian et al., 2021). Examples of AOPs include heterogeneous photocatalysis and Fenton and Fenton-like processes. In the latter process, H_2O_2 is degraded to produce •OH which readily reacts with organic compounds. This reaction is catalyzed by ferrous iron (FeII). Fenton processes, however, may be limited in the practical application of water remediation the FeII may affect the production of •OH. Also, some compounds in the polluted water can react with the •OH, reducing the presence of radicals in the solution (Das et al., 2021; Ghosh & Sahu, 2022).

In the case of heterogeneous photocatalysis, semiconductor catalysts such as Fe_2O_3, ZnO, TiO_2, Ag_2O, and ZnS have been explored in the photocatalytic degradation of numerous organic

contaminants (Oller, Malato & Sánchez-pérez, 2011; Bethi et al., 2016; Kudlek, 2018; Martínez et al., 2018; Sharma & Feng, 2019; Liang et al., 2021; Tian et al., 2021; Pan et al., 2022). Even though photocatalytic processes can oxidize a variety of organics to biodegradable compounds, the catalysts used may undergo corrosion under illumination in aqueous solutions thus reducing the degradation efficiency of pollutants.

2.3.4 Adsorption technology

Adsorption is a surface phenomenon that involves the adhesion of compounds and/or ions (adsorbate) onto a material's surface (adsorbent) (Salimpour Abkenar, Malek & Mazaheri, 2015). This technique has been identified as the most intriguing and practically applicable approach for the abatement of numerous water contaminants (Boeykens et al., 2016; Chaukura, Murimba & Gwenzi, 2017; Zhou et al., 2023). The interacting forces between adsorbent and adsorbate determine the type of adsorption taking place in a system. This can be categorized as physisorption and chemisorption (Luo et al., 2020; Husin et al., 2021). For physisorption processes, van der Waals interactions are predominant, whilst, in chemisorption, covalent bonds may be formed between the sorbent and sorbate (Gusmão et al., 2014; Mkaddem et al., 2022). Other interactions include electrostatic interactions, hydrogen bonding, and $\pi - \pi$ interactions (Khan et al., 2015; Chen et al., 2017).

The application of adsorption in water remediation is supported by the technique’s relatively easy operation, limited secondary product formation, and most significantly the extensive availability of sorbents. These comprise carboniferous materials like activated carbon (Reddy et al., 2016; Salman, Rasheed & Ismaeel, 2023), clays and/or minerals (Grassi et al., 2012), agricultural waste (Anastopoulos & Kyzas, 2014) and nano-structured polymers (Merlini et al., 2014; Male, Srinivasan & Singu, 2015; Fang et al., 2021) among others. One of the prime advantages of adsorption is the convenience of modifying adsorbents with functional groups that may enhance the adsorption capacity, thermal stability of the material, as well as recyclability potential of the adsorbent. However, like all techniques, this technology may have drawbacks associated with low adsorption capacities of some materials, and conformational destruction of the adsorbent during reuse, consequently affecting the long-term usage of the adsorbent as well as the generation of secondary pollution after desorption procedures (Berhane et al., 2016; Liang et al., 2021).

The summarized advantages and shortcomings of the conventional methods for organic pollutants decontamination are shown in Table 2. 1.

Table 2. 1. Advantages and drawbacks of conventional technologies for organic pollutants decontamination.

Method	Advantages	Drawbacks
Ion exchange	• High selectivity • Recyclable sorbent • Effective for inorganic contaminant removal	• High operation and maintenance costs • Low efficiency at low pH • Generates vastly concentrated sludge • Low efficiency for highly concentrated ions • Ineffective for non-ionic compounds
Membrane filtration	• Simple combination with other procedures • High functionalization and/or modification potential • Produce high-quality treated effluent • Environmentally friendly	• Low contaminant selectivity • Subject to fouling • Limited membrane lifespan • Elevated pressures and restricted applicability in large volume treatment
Advanced oxidation processes (AOPs)	• Rapid procedure • Applicable for the treatment of a broad-spectrum of contaminants • Limited secondary pollution	• May use toxic reagents such as H_2O_2 • Ill-health issues due to oxidants additions in reaction media • Several multi-reactions may be required • High operational costs
Adsorption technology	• Effective in water remediation • Availability of numerous adsorbent materials • Rapid and simple process	• Generation of toxic waste due to spent adsorbents • Low adsorption capacity for some adsorbents • Potential efficiency loss when recycled

The shortcomings associated with the discussed conventional approaches for contaminant elimination have intrigued the need to explore efficient and cost-effective techniques for water treatment. In this sense, biocatalytic degradation systems have emerged as notably the most promising advances in organic wastewater remediation, owing to their eco-friendliness, enhanced efficiency, cost-effectiveness, and limited secondary pollution (Alessandretti et al., 2021; Prasetya & Li, 2022; Crespí Sánchez, Turnes Palomino & Palomino Cabello, 2023; Li et al., 2023).

2.3.5 Biocatalytic remediation

Biocatalytic degradation of organic contaminants, which involves the use of living organisms or their enzymes to biochemically transform organic contaminants into less toxic compounds, is an eco-friendly alternative approach to wastewater remediation. This water treatment method requires limited energy, proceeds in mild environments, and results in less or no toxic metabolites (Asif et al., 2017). Additionally, bioremediation techniques have considerable specificity, sensitivity, and efficiencies, which enhances the potential use of the method in the eradication of low concentrations of organic contaminants, contrary to other physical and chemical techniques (Wang et al., 2017; Amobonye et al., 2021).

A key factor in the use of microorganisms for organic pollutants degradation is their adaptability and activity toward the target compounds. In this sense, various microorganisms have been explored to isolate the potent candidates for the efficient degradation of target pollutants (Hwang, Choi & Song, 2008; Spina et al., 2018; Vikrant et al., 2018; Gu, 2021). The widely explored microorganisms include bacteria, fungi, protozoa, etc. (Mbachu, Chukwura & Mbachu, 2020; Mir & Gulfishan, 2020; Adedeji et al., 2022). Since several factors influence microorganism activity, it is essential to comprehend the catalytic pathways or mechanisms and responsible degradation enzymes to determine crucial factors for optimum organic contaminant removal.

2.3.5.1 Principle of microbial degradation of organic pollutants

Organic pollutants can be degraded and transformed by microorganisms under both aerobic and anaerobic conditions (Stadlmair et al., 2018; Amobonye et al., 2021). However, aerobic conditions lead to the quickest and most complete biodegradation of a majority of organic pollutants. The immediate intracellular attack of an organic pollutant is an oxidative process, followed by an enzymatic process usually initiated by oxygenases and peroxidases to activate and incorporate molecular oxygen (Xu et al., 2015; Lueangjaroenkit et al., 2019; Leng et al.,

2023). For this study, emphasis and focus will be on the enzymatic biodegradation processes of organic pollutants in aqueous solutions.

2.4 Enzymatic degradation of organic pollutants

Oxidoreductase enzymes, such as laccases and peroxidases, have been studied extensively in the elimination of organic contaminants including polychlorinated biphenyls, chlorophenols, and polycyclic aromatic hydrocarbons (PAHs) (Anasonye et al., 2014; Chen et al., 2015; Asif et al., 2017), due to their flexibility, natural abundance, and availability of multiple active sites (Varga et al., 2019). Furthermore, these enzymes can catalyze reactions with high specificity while consuming little energy and producing no toxic waste (Varga et al., 2019).

2.4.1 Peroxidases in organic contaminants removal

Peroxidases are oxidoreductase enzymes that are abundant in nature, particularly in microbes and plants. These enzymes can catalyze the oxidation of a vast array of substrates in the presence of hydrogen peroxide (H_2O_2) (Alneyadi et al., 2017). Peroxidases including horseradish (HRP), lignin, soybean (SBP), manganese, and chloroperoxidase (CPO) have been widely used to break down different classes of organic contaminants (Husain, 2010; Coconi-Linares et al., 2015; Alneyadi & Ashraf, 2016). These peroxidases belong to a superfamily called heme-dependent peroxidases (E.C. 1.11.1) with a preserved catalytic cycle displayed in Figure 2. 2.

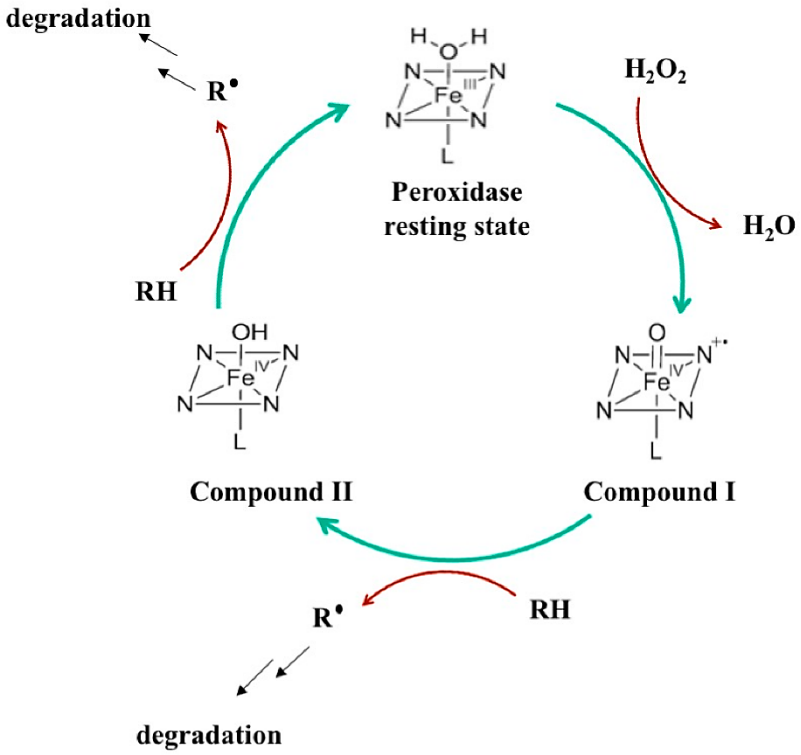


Figure 2. 2: The catalytic cycle of heme-peroxidases. RH: organic pollutant; R•: radical form of the organic pollutant (Alneyadi et al., 2017)

In this cycle, the resting state of the enzyme (heme iron in $Fe(III)^{+}$ state) reacts with H_2O_2 to form a Fe ($IV^{+\bullet}$) cation radical form of the enzyme (compound I), which further reacts with the contaminant (RH) to form Fe (IV^{+}) form of the enzyme (compound II) and a contaminant radical. The cycle proceeds by further reaction of compound II with another contaminant molecule to assume the enzyme's state and further generation of another contaminant radical. The highly reactive contaminant radicals can then react with other species in the reaction medium allowing further degradation (Alneyadi et al., 2017). With this general mechanism of peroxidase activity, the enzymes have been applied in the biotransformation of various organic contaminants with remarkable effectiveness (Husain, 2010; Xu et al., 2015; Li et al., 2016; Lueangjaroenkit et al., 2019; Ladole et al., 2020). For example, Leng et al. (2023), reported the successful catalytic transformation of norfloxacin using horseradish peroxidase in the presence of mediators, through defluorination and piperazinyl ring oxidation. In another study, Pylypchuk et al. (2020), encapsulated horseradish and lignin peroxidase composites were employed in the degradation of three PhACs (diclofenac, paracetamol, and carbamazepine) resulting in satisfactory removal efficiencies with a recyclability potential of up to 20 days. However, the requirement of H_2O_2, an environmentally hazardous compound, for ideal peroxidase activity limits their practical application in water treatment (Alshabib & Onaizi,

2019). Therefore, better options have been explored in this regard, and laccases are desirable replacements for peroxidases as they only require a harmless and generally accessible oxidant, molecular oxygen, to initiate a reaction (Lueangjaroenkit et al., 2019; Wu, Chen & Wei, 2020).

2.4.2 Laccases in organic pollutants removal

Laccases (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) are multicopper oxidases that can catalytically biodegrade a diverse spectrum of phenolic/non-phenolic compounds as well as recalcitrant environmental contaminants (Catapane et al., 2013; Bagewadi, Mulla & Ninnekar, 2017). Laccases are employed in a variety of biotechnological applications due to their high sensitivity, including paper pulverizing, bio-bleaching, textile refining, dye degradation, and chemical synthesis, and they have been investigated for environmental water remediation (Luisa et al., 2019; Varga et al., 2019; Primožič et al., 2020). The general laccase degradation mechanism is presented in Figure 2. 3. The mechanism of action is achieved by the four cupric sites of the enzyme denoted T1, T2, and T3 spectroscopic sites (Wong, 2009). An initial reaction is initiated by the T1 cupric ion, where the reducing substrate is oxidized by a single electron, followed by regeneration of the resting state of the enzyme via the reduction of molecular oxygen to water. This stage is enhanced by the involvement of a tri-cupric cluster composed of one T2 and two T3 cupric ions (Rodríguez-Delgado et al., 2015).

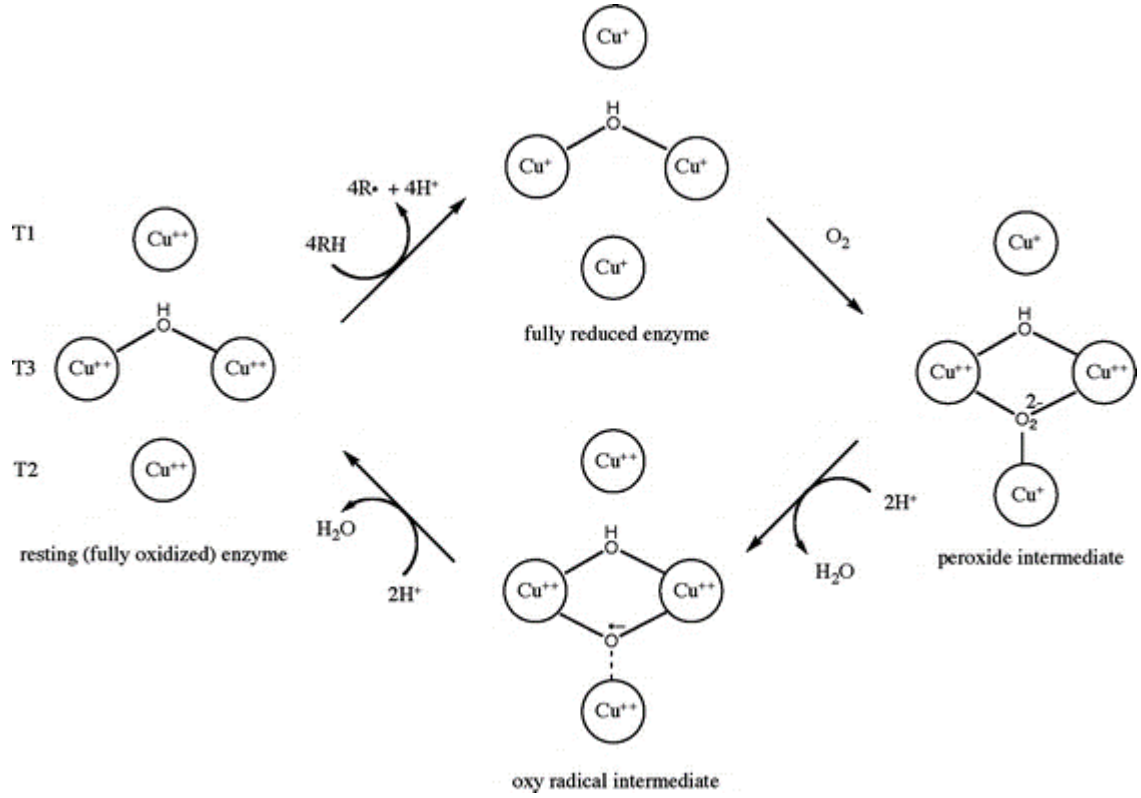


Figure 2. 3: Degradation mechanism of laccase enzyme (Wong, 2009)

The laccase enzymes' proposed mechanism of action has resulted in the enzyme being investigated for the decontamination of various organic pollutants, including PhACs and EDCs, with significantly high reaction rates and efficiencies. In this sense, the use of laccase in the biotransformation of PhACs is summarized in Table 2. 2.

Several studies reveal high removal (>90%) efficiencies of pollutants such as acetaminophen, imipramine, and ketoconazole where refined laccase from *Pleurotus ostreatus*, *Paraconiothyrium variable*, *Trametes versicolor* and *Pleurotus ostreatus* were used (Margot et al., 2013; Guo, Zhu & Li, 2014; Tahmasbi et al., 2016; Yousefi-Ahmadipour et al., 2016; Navada & Kulal, 2019). These results demonstrate the capability of laccase to effectively biodegrade varieties of organic contaminants. However, the majority of these studies were conducted employing artificial water and small reaction media, making it challenging to predict the efficiency of the enzymes in real-world wastewater applications. Only a few studies explored the application of enzymes in real wastewaters. For instance, Lloret et al. (2013) investigated the degradation of estrogenic compounds present in real wastewaters using a laccase-based membrane bioreactor, yielding high efficiencies. Also, a study by Sidy et al. (2013) revealed remarkable removal efficiencies of acetaminophen using cross-linked laccase and tyrosinase aggregates in municipal and hospital wastewaters in batch mode. However,

critical considerations must be made for the potential application of the enzymes at a larger scale, hence the need for further investigations of these procedures. This is due to the biocatalyst's non-reusability, low stability, and the possibility of inactivation due to environmental conditions (Bilal et al., 2017; George et al., 2022). To alleviate these restrictions, innovative treatment approaches have been developed that include redox mediator additions in the reaction media and heterogeneous enzymatic reactions in which the enzyme is immobilized on/within suitable insoluble supports.

Table 2. 2. Application of laccase in PhACs degradation.

Source	Contaminant	Treatment medium	Removal rate /efficiency (%)	Reference
Pleurotus ostreatus	Diclofenac	Artificial water	40 after 24 h	(Stadlmair et al., 2017)
	Acetaminophen	Artificial water	100 in 20 min	
Trametes versicolor	Diclofenac	Aqueous solution	70 after 24 h	(Nguyen et al., 2014)
	Naproxen	Aqueous solution	78 after 24 h	
	Primidone	Aqueous solution	40 after 24 h	
Trametes versicolor	Carbamazepine	Artificial water	30	(Naghdi et al., 2018a)
Trametes versicolor	Ketoconazole	Synthetic water	98	(Yousefi-Ahmadipour et al., 2016)
Trametes hirsuta	Chloramphenicol	Synthetic water	100	(Navada & Kulal, 2019)
Pycnoporus sanguineus	Sulfamethoxazole	Synthetic water	52 after 24 h	(Navada & Kulal, 2019)
Paraconiothyrium variable	Imipramine	Aqueous solution	99	(Tahmasbi et al., 2016)

The use of laccase-mediator systems using redox mediators like 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), violuric acid (VA), syringaldehyde (SYR), N-hydroxyphtalamide (HPI), and 2,2',6,6'-Tetramethylpiperidine-N-oxyl (TEMPO) among others, have been explored and proven effective in organic pollutant removal (Ashe et al., 2016; Ji et al., 2016; Naghdi et al., 2018a; Parra Guardado et al., 2019; Bankole et al., 2021; Leng et al., 2023). However, using laccase-mediator processes in long-term procedures is restricted owing to the requirement for frequent addition of mediators, consequently raising the operational expenses and renders the procedure

further challenging to use (Taheran et al., 2017; Thadathil et al., 2022). Furthermore, as demonstrated by SYR in the degradation of diclofenac, naproxen, and primidone, the mediator may potentially exacerbate the effluent's toxicity (Nguyen et al., 2014), further creating another environmental problem. In this context, enzyme immobilization is the next best alternative to the enhancement of enzyme activity, stability, and recyclability of the biocatalyst. The following sections will discuss this technique in detail.

2.5 Laccase immobilization

Enzyme immobilization is the process of attaching an enzyme to a matrix or support material, resulting in an insoluble biocatalytic composite with preserved catalytic activity (Taheran et al., 2017; Barrios-Estrada et al., 2018). The immobilization procedure results in the stabilization of the protein conformation of the biocatalyst with subsequently enhanced thermal, pH, and storage stabilities, as well as the recovery and recycling potential of the catalytic composite (Kashe et al., 2019; Zofair et al., 2022).

Enzyme immobilization is typically classified into three different approaches: cross-linking; binding to a carrier (adsorption or covalent); and entrapment/encapsulation as shown in Figure 2. 4.

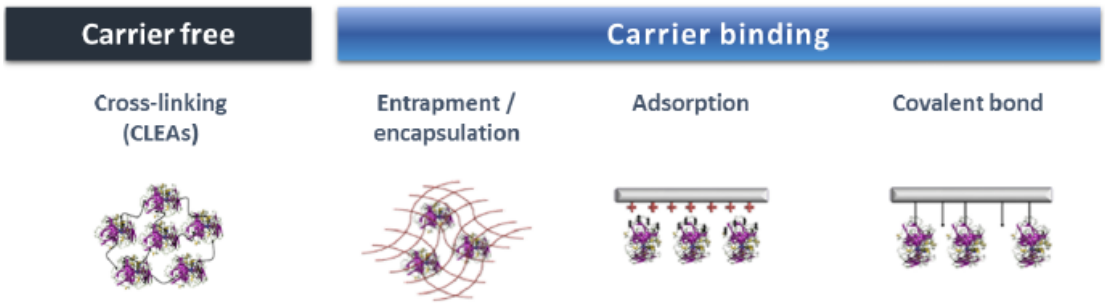


Figure 2. 4: Approaches for the immobilization of enzymes (Guardado, 2019)

For the adsorption immobilization approach, interactions via hydrogen bonds, hydrophobic and ionic interactions occur, and this preserves the protein's structure resulting in high catalytic activity (Zdarta et al., 2018a; Zhang et al., 2022). The covalent binding procedure, on the other hand, is determined by the interaction of functional groups between the enzyme and supporting material (usually -SH, -NH_2, and -OH groups). As a result, strong chemical bonds are formed, considerably increasing the enzyme's stability, and reducing prospects of enzyme leaching (Stine, 2017; Zdarta et al., 2018b). For the encapsulation and entrapment approaches, the enzyme is confined within the polymeric spheres and retained within the grid or fibers of the

supporting substance (Liang et al., 2016; Knedel et al., 2019; Wang et al., 2020), which prevents conformational alterations of the enzyme. Cross linked enzymes are as a result of intermolecular linkages formed between the enzyme molecules, usually initiated by bifunctional compounds like carbodiimides in the absence of a solid support material (Kim et al., 2016; Mehta et al., 2016). The advantages and drawbacks of the immobilization approaches are presented in Table 2. 3.

Table 2. 3. Advantages and drawbacks of immobilization approaches.

Technique	Interaction strength	Advantages	Drawbacks
Adsorption	Weak	Simple and Inexpensive Recyclable support	Prone to enzyme leaching Low efficacies
Covalent binding	Strong	Feasible for industrial applications Strong and stable interactions Easy manipulation Maintained enzyme quantity Multipoint enzyme attachment Variety of supports available	Possible modification of enzyme structure in the active site Competitive inhibition challenges
Entrapment	Weak/strong	Quick approach No enzyme structural alteration	Enzyme leaching Limited commercial application Low pore diffusion rates
Encapsulation	Weak	Cost-effective No enzyme modification	Low concentration of enzymes
Cross-linking	Strong	Carrier or support free High interaction strength	Complicated process High quantities of enzyme required Structural alteration of enzyme

The immobilization technique is normally not solely chosen according to the features of the respective method, but additionally considering the nature of the biocatalyst to be immobilized and the enzymatic process in which the immobilized enzyme will be used. Accordingly, as illustrated in Table 2. 1, covalent binding appears to be the ideal approach for laccase immobilization for application in the remediation of wastewater and water as it produces more stable biocatalysts in comparison to the other immobilization strategies. In this sense, the biocatalyst's stability is a key factor for such applications since the enzymes are subjected to potentially harsh environmental conditions (Bilal, Iqbal & Barceló, 2019; Knedel et al., 2019).

Various support materials have been explored for the immobilization of enzymes, and these are typically divided into inorganic (e.g., clay, silica, activated carbon, etc.), organic (polyamide, chitosan etc.) and hybrid materials (chitosan-silica, metal organic frameworks, etc.) (Meryam Sardar, 2015; Barrios-estrada et al., 2018; Lei et al., 2018). Some of the most recent support materials are given in Table 2. 4.

Table 2. 4. Immobilization supports for laccase.

Support material	Target pollutant	Removal efficiency (%)	Reference
Cellulose nanofiber/ alginate	Bisphenol A	98.7	(Zhang et al., 2022)
Polyacrylonitrile-biochar	Chlortetracycline	58.3	(Taheran et al., 2017)
Polyimide aerogels	Carbamazepine	76	(Simón-Herrero et al., 2019)
Cellulose beads	Indole	99.7	(Yaohua et al., 2019)
meso-MIL-53(Al)	Triclosan	95.2	(Jia et al., 2019)
Fe_3O_4@ZIF-8	Indigo carmine	100	(Wang et al., 2019)
Titania nanoparticles	Diclofenac	>90	(García-Morales et al., 2018)
Fe_3O_4-NH_2@MIL-101(Cr)	2,4-dichlorophenol	87	(Wu et al., 2019)

Fe_3O_4-MNPs-MOF	Methylene blue, crystal violet	95	(Ladole et al., 2020)
Bimetallic CoCu-MOF	Congo red	95	(Li et al., 2022)
Cu-alginate beads	Bisphenol A	96.1	(Latif et al., 2022)

NB: MIL - Materials of Institute Lavoisier, MNPs – Magnetic nanoparticles

Out of various support materials, metal-organic frameworks (MOFs) have gained prominence as promising new porous materials for enzyme immobilization. The following sections will discuss the synthesis approaches for MOFs and their application in enzyme immobilization.

2.5.1 Metal-organic frameworks (MOFs) for enzyme immobilization

MOFs are porous crystalline organic-inorganic hybrid materials with a structure similar to zeolites but with greater flexibility (Sharma & Feng, 2019). These materials are formed through the coordination of metal ions (Zn, Cr, Co, etc.) and multidentate ligands to form tri-dimensional conformations (Furukawa et al., 2013). They are distinguished by features such as structural stability, low densities, crystalline structure, flexible organic ligand, and high porosities (Yuan et al., 2022). A schematic diagram for the formation of MOF is given in Figure 2. 5.

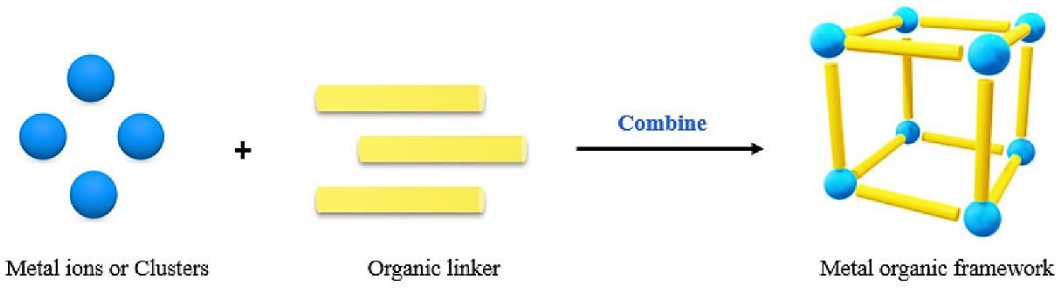


Figure 2. 5: Schematic diagram for the formation of MOFs (Silva et al., 2022)

2.5.2 Synthesis of MOFs and enzyme-MOFs

Various synthetic routes for metal-organic frameworks have been reported including solvothermal, microwave-assisted synthesis, slow evaporation/direct precipitation, electrochemical, sonochemical, and mechanochemical synthesis techniques (Tranchemontagne, Hunt & Yaghi, 2008; Lyu et al., 2014; Yang et al., 2015; Gascón et al., 2017; Kumar et al., 2017; Sargazi, Afzali & Mostafavi, 2018; Bedia et al., 2019; Chen et al.,

2019; Nadar & Rathod, 2019; Rajkumar et al., 2019). The most prevalent approach is solvothermal, but slow evaporation is among the most convenient routes for MOF synthesis since it requires no external energy source (Bedia et al., 2019). The slow evaporation technique is, however, impeded by the long synthesis period which usually takes several hours to days. The microwave-based technique has been applied when using chemically inert metal ions to improve the crystallinity of MOFs, by homogeneously elevating the temperature in a localized zone. The electrochemical and mechanochemical methods have also been suggested to offer additional degrees of freedom in the synthesis of MOFs (Mehta et al., 2016; Bedia et al., 2019). The most applicable route for MOF synthesis is through self-assembly. This technique offers options for morphology control and chances of studying the kinetics of the growth of the particles (Mehta et al., 2016; Hu et al., 2018; Sargazi, Afzali & Mostafavi, 2018; Bedia et al., 2019; Mahmoodi et al., 2019). Figure 2. 6 summarizes the different approaches for metal-organic frameworks fabrication.

Owing to these inherent properties, MOFs have been widely investigated as effective support materials for enzyme immobilization. The MOFs' large hierarchical surface area and exceptional porosity enhance the loading potential of enzymes (Dias & Petit, 2015; Zhu et al., 2017). Furthermore, MOFs form strong linkages between their organic counterparts and enzymes reducing the chances of enzyme leaching during applications. Moreover, enzyme-MOF matrices have proven effective in environmental remediation with exceptional catalytic performances in terms of stability (pH and thermal stability), specificity, storage stability, and recyclability, owing to the shielding of the enzymes by the highly-ordered frameworks (Kumar et al., 2017; Liang et al., 2020; Ulu, 2020; Hoseinzadeh et al., 2021).

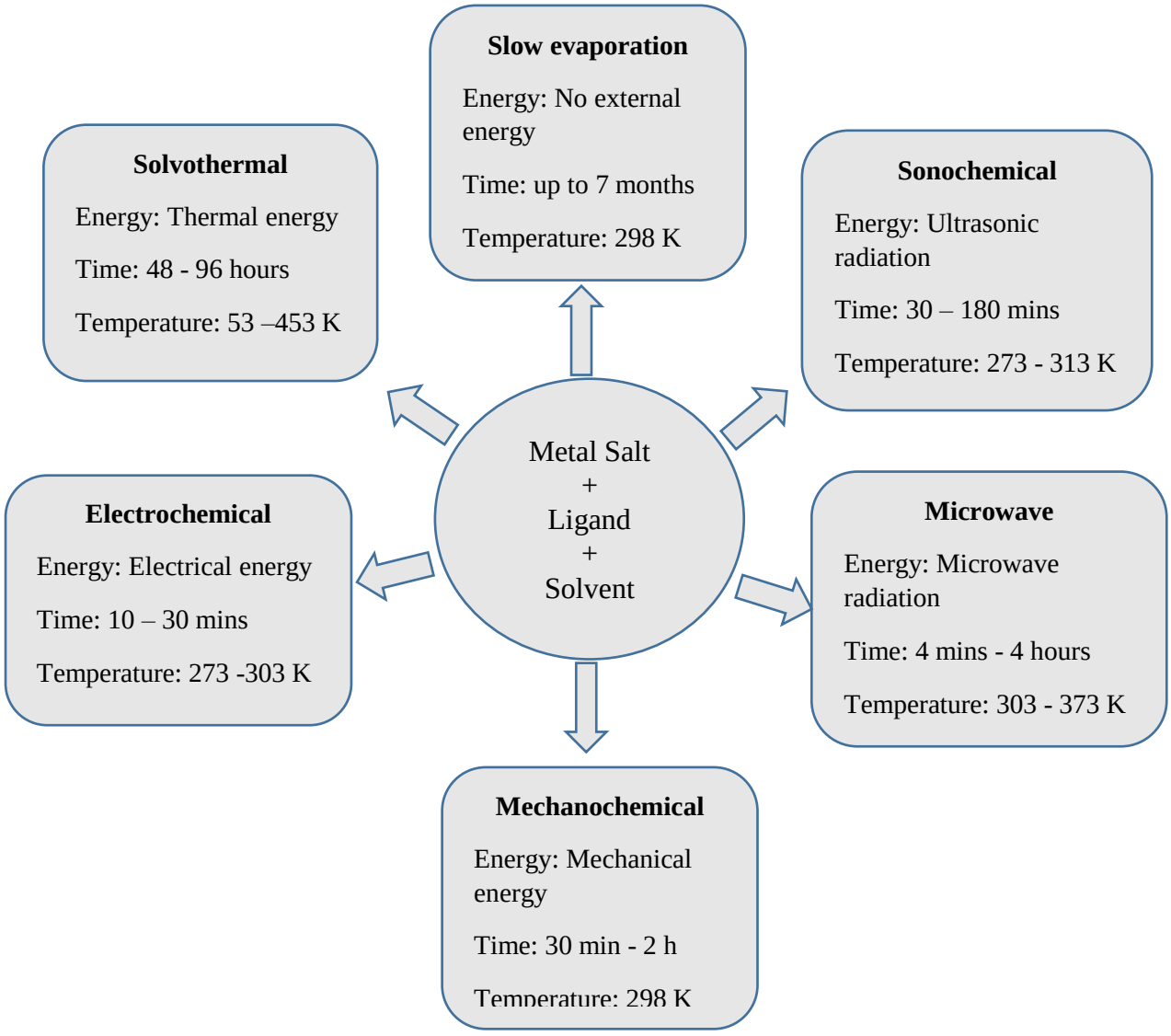


Figure 2. 6: MOFs fabrication approaches and their synthesis conditions

In general, the enzyme can be introduced into MOFs via two approaches: de novo (biomineralization of enzyme and MOF in one pot) and attaching onto pre-synthesized MOFs (adsorption, encapsulation, and covalent binding), as discussed in the laccase immobilization section. Even though both approaches show an increase in catalytic properties such as efficiency, activity, and stability, the pre-synthesized MOF based immobilization technique always suffers from lower yield and stabilities. Additionally, the pre-synthesized MOF based immobilization process involves a series of synthesis steps that may be complicated and labor-intensive (Jia et al., 2019; Knedel et al., 2019).

Different kinds of MOFs have been explored for the immobilization of oxidoreductase enzymes including meso-MIL-53(Al), UiO-66, zeolitic imidazolate framework (ZIF), MIL-53, MIL-125, MIL-101, porous coordination network (PCN-222(Fe)) among others, and ZIF-8 has gained popularity as a potential candidate for enzyme immobilization (Xia et al., 2020; Silva et al., 2022)

ZIFs are a type of MOF that is formed by self-assembly of M-Im-M (where M stands for Zn or Co cation, and Im is an imidazolate linker). ZIFs' diversified conformations are comparable to those of traditional aluminosilicate zeolites, where Zn^{2+} ions usually serve as the equivalent of silicon and imidazolate anions form connections that resemble the function of oxygen in zeolites, with a metal-imidazole-metal (M-Im-M) angle of 145° (Khan, 2015; Terban et al., 2018; Wang et al., 2022). A pictorial representation of the typical ZIF synthesis route is illustrated in figure 2.7.

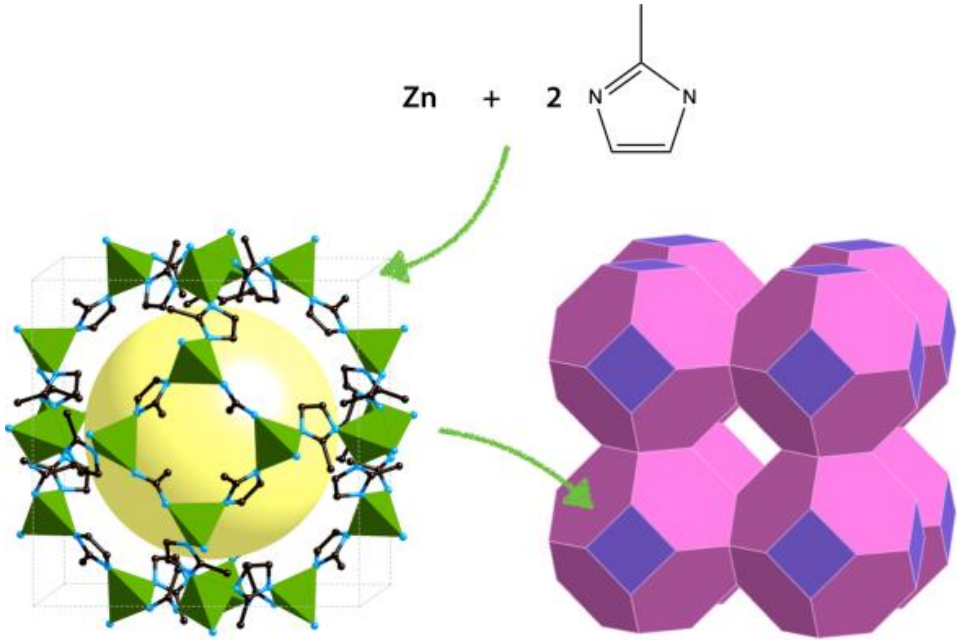


Figure 2. 7: ZIF synthesis and pictorial representation of crystals (Ortiz et al., 2014)

In this sense, ZIFs possess zeolite-like topologies with high zeolite structural resemblances. The tunable pore structure, structural flexibility, and chemical modification of the inner surfaces of the framework, as well as the chemical and thermal endurance of the ZIFs, make them suitable for application in a variety of processes.

In enzyme immobilization, ZIF has been used either with just the enzyme or a composite with specific functional groups. *C. rugose* lipase, for example, was encapsulated within a ZIF-67 hybrid framework. The Lipase@ZIF-67 composites significantly improved the enzyme's storage stability, thermal stability, and reusability. This reliable lipase@ZIF-67 composite, in particular, demonstrated excellent enzymatic performance even after 6 cycles on the complicated solvent-free transesterification of soy protein to biodiesel (Rafiei et al., 2018). In another study, lipase was encapsulated within ZIF-8 via a facile one-step biomineralization technique, and it exhibited improved thermal stability (3.2 folds) compared to the free lipase (Nadar & Rathod, 2018). Furthermore, the immobilized lipase retained 54% residual activity after 7 reuse cycles. Laccase (*C. glutamicum*) was encapsulated in ZIF-8 and it resulted in improved thermal (up to 70°C) and chemical stability (Knedel et al., 2019). In a different study by Habimana et al. (Habimana et al., 2022), laccase was immobilized onto a ZIF-8@MWNT for dyes' biodegradation from aqueous solutions. The performance of the biocatalyst composite improved significantly with removal efficiencies of 97.4 and 95.6% for Eriochrome black T and Acid red 88, respectively. The biocomposite also maintained 68% residual activity after 10 recycles. Also, Salgaonkar, Nadar, and Rathod (2019), investigated the performance of orange peel peroxidase (OPP-MOF) prepared via a single-pot biomineralization procedure, the biocatalyst presented a 2.1-fold improvement in thermal stability and maintained 48% relative activity after 6 cycles of reuse. The presented literature provides a clear indication of ZIF particles' ability to be used as support inorganic-organic hybrid material for enzymes with significant improvement in crucial features such as temperature and storage stabilities, as well as enhanced activities and recyclability potential.

2.6 Chapter conclusion

In this chapter, conventional methods for organic pollutants removal were discussed. From this, emphasis was given to biocatalytic remediation of PhACs and EDCs. Furthermore, several approaches for the improvement of enzymatic processes were discussed. The enzyme immobilization technique presented a feasible and highly effective method for the enhancement of catalytic activity, thermal and storage stabilities as well as the recyclability of the biocatalyst.

Out of the vast potential materials for enzyme immobilization, metal-organic frameworks, specifically zeolitic imidazolate frameworks demonstrated appreciable features in support of the improvement of the bioremediation process and probable application of such enzyme-ZIF matrices in organic pollutant decontamination.

The following chapter gives a discussion of the materials and procedures employed in this study.

2.7 References

Adedeji, J.A., Tetteh, E.K., Opoku Amankwa, M., Asante-Sackey, D., Ofori-Frimpong, S., Armah, E.K., Rathilal, S., Mohammadi, A.H., et al. 2022. Microbial Bioremediation and Biodegradation of Petroleum Products—A Mini Review. *Applied Sciences (Switzerland)*. 12(23). DOI: 10.3390/app122312212.

Ajenifuja, E., Ajao, J.A., Alayande, S.O., Bakare, M.K., Taleatu, B.A., Oladele, E. & Ajayi, B. 2016. Synthesis and Characterization of Pure Aluminosilicate Ceramic Membranes for Water Remediation. 2(May):594–607.

Al-Kindi, G.Y., Al Ani, F.H., Al-Bidri, N.K. & Alhaidri, H.A. 2021. Diclofenac removal from wastewater by activated carbon. *IOP Conference Series: Earth and Environmental Science*. 779(1). DOI: 10.1088/1755-1315/779/1/012091.

Al-Mashaqbeh, O.A., Snow, D.D., Alsafadi, D.A., Alsalhi, L.Z. & Bartelt-Hunt, S.L. 2021. Removal of carbamazepine onto modified zeolitic tuff in different water matrices: Batch and continuous flow experiments. *Water (Switzerland)*. 13(8). DOI: 10.3390/w13081084.

Alessandretti, I., Rigueto, C.V.T., Nazari, M.T., Rosseto, M. & Dettmer, A. 2021. Removal of diclofenac from wastewater: A comprehensive review of detection, characteristics and tertiary treatment techniques. *Journal of Environmental Chemical Engineering*. 9(6):106743. DOI: 10.1016/j.jece.2021.106743.

Alneyadi, A.H. & Ashraf, S.S. 2016. Differential enzymatic degradation of thiazole pollutants by two different peroxidases - A comparative study. *Chemical Engineering Journal*. 303:529–538. DOI: 10.1016/j.cej.2016.06.017.

Alneyadi, A.H., Shah, I., Abuqamar, S.F. & Ashraf, S.S. 2017. Differential degradation and detoxification of an aromatic pollutant by two different peroxidases. *Biomolecules*. 7(1):1–18. DOI: 10.3390/biom7010031.

Alshabib, M. & Onaizi, S.A. 2019. A review on phenolic wastewater remediation using homogeneous and heterogeneous enzymatic processes: Current status and potential challenges. *Separation and Purification Technology*. 219(October 2018):186–207. DOI: 10.1016/j.seppur.2019.03.028.

Amobonye, A., Bhagwat, P., Singh, S. & Pillai, S. 2021. Plastic biodegradation: Frontline microbes and their enzymes. *Science of the Total Environment*. 759:143536. DOI: 10.1016/j.scitotenv.2020.143536.

Anasonye, F., Winquist, E., Kluczek-Turpeinen, B., Räsänen, M., Salonen, K., Steffen, K.T. & Tuomela, M. 2014. Fungal enzyme production and biodegradation of polychlorinated

dibenzo-p-dioxins and dibenzofurans in contaminated sawmill soil. *Chemosphere*. 110:85–90. DOI: 10.1016/j.chemosphere.2014.03.079.

Anastopoulos, I. & Kyzas, G.Z. 2014. Agricultural peels for dye adsorption: A review of recent literature. *Journal of Molecular Liquids*. 200(PB):381–389. DOI: 10.1016/j.molliq.2014.11.006.

Ashe, B., Nguyen, L.N., Hai, F.I., Lee, D.J., van de Merwe, J.P., Leusch, F.D.L., Price, W.E. & Nghiem, L.D. 2016. Impacts of redox-mediator type on trace organic contaminants degradation by laccase: Degradation efficiency, laccase stability and effluent toxicity. *International Biodeterioration and Biodegradation*. 113:169–176. DOI: 10.1016/j.ibiod.2016.04.027.

Asif, M.B., Hai, F.I., Singh, L., Price, W.E. & Nghiem, L.D. 2017. Degradation of Pharmaceuticals and Personal Care Products by White-Rot Fungi—a Critical Review. *Current Pollution Reports*. 3(2):88–103. DOI: 10.1007/s40726-017-0049-5.

Bagewadi, Z.K., Mulla, S.I. & Ninnekar, H.Z. 2017. Purification and immobilization of laccase from Trichoderma harzianum strain HZN10 and its application in dye decolorization. *Journal of Genetic Engineering and Biotechnology*. 15(1):139–150. DOI: 10.1016/j.jgeb.2017.01.007.

Bankole, P.O., Semple, K.T., Jeon, B.H. & Govindwar, S.P. 2021. Impact of redox-mediators in the degradation of olsalazine by marine-derived fungus, Aspergillus aculeatus strain bpo2: Response surface methodology, laccase stability and kinetics. *Ecotoxicology and Environmental Safety*. 208(September 2020):111742. DOI: 10.1016/j.ecoenv.2020.111742.

Barrios-estrada, C., Rostro-alanis, M.D.J., Muñoz-gutiérrez, B.D., Iqbal, M.N., Kannan, S. & Parra-saldívar, R. 2018. Emergent contaminants : Endocrine disruptors and their laccase-assisted degradation – A review. *Science of the Total Environment*. 612:1516–1531.

Barrios-Estrada, C., Rostro-Alanis, M. de J., Parra, A.L., Belleville, M.P., Sanchez-Marcano, J., Iqbal, H.M.N. & Parra-Saldívar, R. 2018. Potentialities of active membranes with immobilized laccase for Bisphenol A degradation. *International Journal of Biological Macromolecules*. 108:837–844. DOI: 10.1016/j.ijbiomac.2017.10.177.

Becker, D., Rodriguez-Mozaz, S., Insa, S., Schoevaart, R., Barceló, D., De Cazes, M., Belleville, M.P., Sanchez-Marcano, J., et al. 2017. Removal of Endocrine Disrupting Chemicals in Wastewater by Enzymatic Treatment with Fungal Laccases. *Organic Process Research and Development*. 21(4):480–491. DOI: 10.1021/acs.oprd.6b00361.

Bedia, J., Muelas-Ramos, V., Peñas-Garzón, M., Gómez-Avilés, A., Rodríguez, J.J. & Belver, C. 2019. A review on the synthesis and characterization of metal organic frameworks for photocatalytic water purification. *Catalysts*. 9(1):52. DOI: 10.3390/catal9010052.

Berhane, T.M., Levy, J., Krekeler, M.P.S. & Danielson, N.D. 2016. Adsorption of bisphenol A and ciprofloxacin by palygorskite-montmorillonite: Effect of granule size, solution chemistry and temperature. *Applied Clay Science*. 132–133:518–527. DOI: 10.1016/j.clay.2016.07.023.

Bethi, B., Sonawane, S.H., Bhanvase, B.A. & Gumfekar, S.P. 2016. Nanomaterials-based advanced oxidation processes for wastewater treatment: A review. *Chemical Engineering and Processing: Process Intensification*. 109:178–189. DOI: 10.1016/j.cep.2016.08.016.

Bilal, M., Asgher, M., Iqbal, H.M.N., Hu, H. & Zhang, X. 2017. Bio-based degradation of emerging endocrine-disrupting and dye-based pollutants using cross-linked enzyme

aggregates. *Environmental Science and Pollution Research*. 24(8):7035–7041. DOI: 10.1007/s11356-017-8369-y.

Bilal, M., Iqbal, M.N. & Barceló, D. 2019. Mitigation of bisphenol A using an array of laccase-based robust bio-catalytic cues – A review. *Science of the Total Environment*. 689:160–177. DOI: 10.1016/j.scitotenv.2019.06.403.

Boeykens, S.P., Piol, M.N., Samudio Legal, L., Saralegui, A.B. & Vázquez, C. 2016. Eutrophication decrease: Phosphate adsorption processes in presence of nitrates. *Journal of Environmental Management*. 1–8. DOI: 10.1016/j.jenvman.2017.05.026.

Canle, M., Fernández Pérez, M.I. & Santaballa, J.A. 2017. *Photocatalyzed degradation/abatement of endocrine disruptors*. V. 6. Elsevier B.V. DOI: 10.1016/j.cogsc.2017.06.008.

Catapane, M., Nicolucci, C., Menale, C., Mita, L., Rossi, S., Mita, D.G. & Diano, N. 2013. Enzymatic removal of estrogenic activity of nonylphenol and octylphenol aqueous solutions by immobilized laccase from Trametes versicolor. *Journal of Hazardous Materials*. 248–249(1):337–346. DOI: 10.1016/j.jhazmat.2013.01.031.

Chaukura, N., Murimba, E.C. & Gwenzi, W. 2017. Synthesis, characterisation and methyl orange adsorption capacity of ferric oxide–biochar nano-composites derived from pulp and paper sludge. *Applied Water Science*. 7(5):2175–2186. DOI: 10.1007/s13201-016-0392-5.

Chen, J., Zhang, D., Zhang, H., Ghosh, S. & Pan, B. 2017. Fast and slow adsorption of carbamazepine on biochar as affected by carbon structure and mineral composition. *Science of the Total Environment*. 579:598–605. DOI: 10.1016/j.scitotenv.2016.11.052.

Chen, L., Huang, F., Zhang, C., Zhang, J., Liu, F. & Guan, X. 2021. Effects of norfloxacin on nitrate reduction and dynamic denitrifying enzymes activities in groundwater. *Environmental Pollution*. 273:116492. DOI: 10.1016/j.envpol.2021.116492.

Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D. & Zhang, J. 2015. Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. *Biotechnology Advances*. 33(6):745–755. DOI: 10.1016/j.biotechadv.2015.05.003.

Chen, Z., Wang, X., Islamoglu, T. & Farha, O.K. 2019. Green Synthesis of a Functionalized Zirconium-Based Metal–Organic Framework for Water and Ethanol Adsorption. *Inorganics*. 7(5):56. DOI: 10.3390/inorganics7050056.

Cobas, M., Danko, A.S., Pazos, M. & Sanrom??n, M.A. 2015. Removal of metal and organic pollutants from wastewater by a sequential selective technique. *Bioresource Technology*. 213:2–10. DOI: 10.1016/j.biortech.2016.02.036.

Coconi-Linares, N., Ortiz-Vázquez, E., Fernández, F., Loske, A.M. & Gómez-Lim, M.A. 2015. Recombinant expression of four oxidoreductases in Phanerochaete chrysosporium improves degradation of phenolic and non-phenolic substrates. *Journal of Biotechnology*. 209:76–84. DOI: 10.1016/j.jbiotec.2015.06.401.

Conte, L., Falletti, L., Zaggia, A. & Milan, M. 2015. Polyfluorinated organic micropollutants removal from water by ion exchange and adsorption. *Chemical Engineering Transactions*. 43:2257–2262. DOI: 10.3303/CET1543377.

Crespí Sánchez, N., Turnes Palomino, G. & Palomino Cabello, C. 2023. Sulfonic-functionalized MIL-100-Fe MOF for the removal of diclofenac from water. *Microporous and*

Mesoporous Materials. 348(October 2022). DOI: 10.1016/j.micromeso.2022.112398.

Das, M.T., Kumar, S.S., Ghosh, P., Shah, G., Malyan, S.K., Bajar, S., Thakur, I.S. & Singh, L. 2021. Remediation strategies for mitigation of phthalate pollution: Challenges and future perspectives. *Journal of Hazardous Materials*. 409(November 2020):124496. DOI: 10.1016/j.jhazmat.2020.124496.

Devaisy, S., Kandasamy, J., Aryal, R., Johir, M.A.H., Ratnaweera, H. & Vigneswaran, S. 2023. Removal of Organics with Ion-Exchange Resins (IEX) from Reverse Osmosis Concentrate. *Membranes*. 13(2):136. DOI: 10.3390/membranes13020136.

Dias, E.M. & Petit, C. 2015. Towards the use of metal-organic frameworks for water reuse: A review of the recent advances in the field of organic pollutants removal and degradation and the next steps in the field. *Journal of Materials Chemistry A*. 3(45):22484–22506. DOI: 10.1039/c5ta05440k.

Elrasheedy, A., Nady, N. & Bassyouni, M. 2019. Matrix Membranes : Review on Applications in Water Purification. *Membranes*. 9(7):1–31.

Esfahani, M.R., Aktij, S.A., Dabaghian, Z., Firouzjaei, M.D., Rahimpour, A., Eke, J., Escobar, I.C., Abolhassani, M., et al. 2019. Nanocomposite membranes for water separation and purification: Fabrication, modification, and applications. *Separation and Purification Technology*. 213(December 2018):465–499. DOI: 10.1016/j.seppur.2018.12.050.

Ezumi, M. & Todokoro, H. 1997. Scanning electron microscope. *US Patent 5,608,218*. 1–21. Available: http://www.google.com/patents/US5872358.

Fang, L., Miao, Y., Wei, D., Zhang, Y. & Zhou, Y. 2021. Efficient removal of norfloxacin in water using magnetic molecularly imprinted polymer. *Chemosphere*. 262. DOI: 10.1016/j.chemosphere.2020.128032.

Feng, Y., Li, Z., Long, Y., Suo, N., Wang, Z. & Qiu, L. 2022. Electro/magnetic superposition effects on diclofenac degradation: Removal performance, kinetics, community structure and synergistic mechanism. *Environmental Pollution*. 292(PA):118357. DOI: 10.1016/j.envpol.2021.118357.

Fontanals, N., Borrull, F. & Marcé, R.M. 2020. Overview of mixed-mode ion-exchange materials in the extraction of organic compounds. *Analytica Chimica Acta*. 1117:89–107. DOI: 10.1016/j.aca.2020.03.053.

Fu, F. & Wang, Q. 2011. Removal of heavy metal ions from wastewaters: A review. *Journal of Environmental Management*. 92(3):407–418. DOI: 10.1016/j.jenvman.2010.11.011.

Furukawa, H., Cordova, K.E., O'Keeffe, M. & Yaghi, O.M. 2013. The chemistry and applications of metal-organic frameworks. *Science*. 341(6149). DOI: 10.1126/science.1230444.

Gao, X., Kang, S., Xiong, R. & Chen, M. 2020. Environment-friendly removal methods for endocrine disrupting chemicals. *Sustainability (Switzerland)*. 12(18):1–16. DOI: 10.3390/su12187615.

García-Morales, R., García-García, A., Orona-Navar, C., Osma, J.F., Nigam, K.D.P. & Ornelas-Soto, N. 2018. Biotransformation of emerging pollutants in groundwater by laccase from P. sanguineus CS43 immobilized onto titania nanoparticles. *Journal of Environmental Chemical Engineering*. 6(1):710–717. DOI: 10.1016/j.jece.2017.12.006.

García, M.A., Cristofoletti, R., Abrahamsson, B., Groot, D.W., Parr, A., Polli, J.E., Mehta, M., Shah, V.P., et al. 2021. Biowaiver Monograph for Immediate-Release Solid Oral Dosage Forms: Carbamazepine. *Journal of Pharmaceutical Sciences*. 110(5):1935–1947. DOI: 10.1016/j.xphs.2021.02.019.

Gascón, V., Castro-Miguel, E., Díaz-García, M., Blanco, R.M. & Sanchez-Sanchez, M. 2017. In situ and post-synthesis immobilization of enzymes on nanocrystalline MOF platforms to yield active biocatalysts. *Journal of Chemical Technology and Biotechnology*. 92(10):2583–2593. DOI: 10.1002/jctb.5274.

George, J., Rajendran, D.S., Venkataraman, S., Rathankumar, A.K., Saikia, K., Muthusamy, S., Singh, I., Singh, I., et al. 2022. Insolubilization of Tramates versicolor laccase as cross-linked enzyme aggregates for the remediation of trace organic contaminants from municipal wastewater. *Environmental Research*. 209(January):112882. DOI: 10.1016/j.envres.2022.112882.

Ghosh, S. & Sahu, M. 2022. Phthalate pollution and remediation strategies : A review. *Journal of Hazardous Materials Advances*. 6(March):100065. DOI: 10.1016/j.hazadv.2022.100065.

Grassi, M., Kaykioglu, G., Belgiorno, V. & Lofrano, G. 2012. Emerging Compounds Removal from Wastewater. *Green Chemistry for Sustainability*. 15–38. DOI: 10.1007/978-94-007-3916-1.

Griffiths, P.R. & Haseth, D.J.A. 2007. Fourier Transform Infrared Spectroscopy. 171.

Gu, J. 2021. On Enrichment Culturing and Transferring Technique. *Applied Environmental Biotechnology*. 1(6):1–5.

Guo, X.L., Zhu, Z.W. & Li, H.L. 2014. Biodegradation of sulfamethoxazole by Phanerochaete chrysosporium. *Journal of Molecular Liquids*. 198:169–172. DOI: 10.1016/j.molliq.2014.06.017.

Gupta, V.K., Ali, I., Saleh, T.A., Nayak, A. & Agarwal, S. 2012. Chemical treatment technologies for waste-water recycling—an overview. *RSC Advances*. 2(16):6380. DOI: 10.1039/c2ra20340e.

Gusmão, K.A.G., Gurgel, L.V.A., Melo, T.M.S., Carvalho, C. de F. & Gil, L.F. 2014. Adsorption studies of etherdiamine onto modified sugarcane bagasses in aqueous solution. *Journal of Environmental Management*. 133:332–342. DOI: 10.1016/j.jenvman.2013.11.040.

Habimana, P., Jiang, Y., Gao, J., Ndayambaje, J.B., Darwesh, O.M., Mwizerwa, J.P., Zheng, X. & Ma, L. 2022. Enhancing laccase stability and activity for dyes decolorization using ZIF-8@MWCNT nanocomposite. *Chinese Journal of Chemical Engineering*. 48:66–75. DOI: 10.1016/j.cjche.2021.05.044.

Hafner, B. 2007. Scanning Electron Microscopy Primer. 1–29.

Han, H., Rafiq, M.K., Zhou, T., Xu, R., Mašek, O. & Li, X. 2019. A critical review of clay-based composites with enhanced adsorption performance for metal and organic pollutants. *Journal of Hazardous Materials*. 369(October 2018):780–796. DOI: 10.1016/j.jhazmat.2019.02.003.

Hoseinzadeh, H., Hayati, B., Shahmoradi Ghaheh, F., Seifpanahi-Shabani, K. & Mahmoodi, N.M. 2021. Development of room temperature synthesized and functionalized metal-organic framework/graphene oxide composite and pollutant adsorption ability. *Materials Research Bulletin*. 142(May):111408. DOI: 10.1016/j.materresbull.2021.111408.

Hu, Y., Dai, L., Liu, D., Du, W. & Wang, Y. 2018. Progress & prospect of metal-organic frameworks (MOFs) for enzyme immobilization (enzyme/MOFs). *Renewable and Sustainable Energy Reviews*. 91(November 2017):793–801. DOI: 10.1016/j.rser.2018.04.103.

Hu, Y., Zhang, C., Jian, Z., Chen, D., Ma, Y. & Yang, W. 2022. Surface-imprinted polymer microsphere based on self-stabilized precipitation polymerization for selective removal of norfloxacin. *Applied Surface Science*. 574(June 2021):151706. DOI: 10.1016/j.apsusc.2021.151706.

Huang, S.-J., Dwivedi, K.A., Kumar, S., Wang, C.-T. & Yadav, A.K. 2022. Binder-free NiO/MnO2 coated carbon based anodes for simultaneous norfloxacin removal, wastewater treatment and power generation in dual-chamber microbial fuel cell. *Environmental Pollution*. 317(July 2022):120578. DOI: 10.1016/j.envpol.2022.120578.

Huang, S., Ras, R.H.A. & Tian, X. 2018. Antifouling membranes for oily wastewater treatment : Interplay between wetting and membrane fouling. *Current Opinion in Colloid & Interface Science*. 36:90–109. DOI: 10.1016/j.cocis.2018.02.002.

Husain, Q. 2010. Peroxidase mediated decolorization and remediation of wastewater containing industrial dyes: A review. *Reviews in Environmental Science and Biotechnology*. 9(2):117–140. DOI: 10.1007/s11157-009-9184-9.

Husin, N.A., Hashim, N.M., Yahaya, N., Miskam, M., Raoov, M. & Zain, N.N.M. 2021. Exploring magnetic particle surface embedded with imidazole-based deep eutectic solvent for diclofenac removal from pharmaceutical wastewater samples. *Journal of Molecular Liquids*. 332:115809. DOI: 10.1016/j.molliq.2021.115809.

Hwang, S.S., Choi, H.T. & Song, H.G. 2008. Biodegradation of endocrine-disrupting phthalates by Pleurotus ostreatus. *Journal of Microbiology and Biotechnology*. 18(4):767–772.

Jedynak, K., Wideł, D. & Oszczudłowski, J. 2017. Removal of selected phthalates from aqueous solution by mesoporous-ordered carbon adsorbent. *Adsorption Science and Technology*. 35(7–8):744–750. DOI: 10.1177/0263617417708675.

Ji, C., Hou, J., Wang, K., Zhang, Y. & Chen, V. 2016. Biocatalytic degradation of carbamazepine with immobilized laccase-mediator membrane hybrid reactor. *Journal of Membrane Science*. 502:11–20. DOI: 10.1016/j.memsci.2015.12.043.

Jia, Y., Chen, Y., Luo, J. & Hu, Y. 2019. Immobilization of laccase onto meso-MIL-53(Al) via physical adsorption for the catalytic conversion of triclosan. *Ecotoxicology and Environmental Safety*. 184(September):109670. DOI: 10.1016/j.ecoenv.2019.109670.

Jiang, X., An, Y., Huang, Y., Ding, W., Sun, M., Li, H. & Zheng, H. 2023. One pot synthesis of Cu–Ni–S@Ni foam for the simultaneous removal and detection of norfloxacin. *Journal of Cleaner Production*. 382(September 2022):135385. DOI: 10.1016/j.jclepro.2022.135385.

Jiménez, S., Micó, M.M., Arnaldos, M., Medina, F. & Contreras, S. 2018. State of the art of produced water treatment. *Chemosphere*. 192:186–208. DOI: 10.1016/j.chemosphere.2017.10.139.

Kaleta, J., Papciak, D. & Puszkarewicz, A. 2018. Using ion exchange process in removal of selected organic pollution from aqueous solutions. *Journal of Ecological Engineering*. 19(4):136–142. DOI: 10.12911/22998993/89662.

Kashe, S., Mehdi, S., Mohammad, N., Kashefi, S., Borghei, S.M. & Mahmoodi, N.M. 2019. Covalently immobilized laccase onto graphene oxide nanosheets : Preparation,

characterization, and biodegradation of azo dyes in colored wastewater. *Journal of Molecular Liquids*. 276:153–162. DOI: 10.1016/j.molliq.2018.11.156.

Khalid, N.R., Majid, A., Tahir, M.B., Niaz, N.A. & Khalid, S. 2017. Carbonaceous-TiO2 nanomaterials for photocatalytic degradation of pollutants: A review. *Ceramics International*. 43(17):14552–14571. DOI: 10.1016/j.ceramint.2017.08.143.

Khan, M. 2015. Zeolitic imidazolate framework materials: Recent progress in synthesis and applications. *Journal of Materials Chemistry A*. (207890):121.

Khan, N.A., Jung, B.K., Hasan, Z. & Jhung, S.H. 2015. Adsorption and removal of phthalic acid and diethyl phthalate from water with zeolitic imidazolate and metal-organic frameworks. *Journal of Hazardous Materials*. 282:194–200. DOI: 10.1016/j.jhazmat.2014.03.047.

Kim, J.J.H., Hong, S.G., Sun, H.J., Ha, S. & Kim, J.J.H. 2016. Precipitated and chemically-crosslinked laccase over polyaniline nanofiber for high performance phenol sensing. *Chemosphere*. 143:142–147. DOI: 10.1016/j.chemosphere.2015.08.011.

Knedel, T.O., Ricklefs, E., Schlüsener, C., Urlacher, V.B. & Janiak, C. 2019. Laccase Encapsulation in ZIF-8 Metal-Organic Framework Shows Stability Enhancement and Substrate Selectivity. *ChemistryOpen*. 8(11):1337–1344. DOI: 10.1002/open.201900146.

Kudlek, E. 2018. Decomposition of contaminants of emerging concern in advanced oxidation processes. *Water (Switzerland)*. 10(7). DOI: 10.3390/w10070955.

Kumar, P., Vellingiri, K., Kim, K.H., Brown, R.J.C. & Manos, M.J. 2017. Modern progress in metal-organic frameworks and their composites for diverse applications. *Microporous and Mesoporous Materials*. 253(October):251–265. DOI: 10.1016/j.micromeso.2017.07.003.

Ladole, M.R., Pokale, P.B., Patil, S.S., Belokar, P.G. & Pandit, A.B. 2020. Laccase immobilized peroxidase mimicking magnetic metal organic frameworks for industrial dye degradation. *Bioresource Technology*. 317(August):124035. DOI: 10.1016/j.biortech.2020.124035.

Laera, G., Chong, M.N., Jin, B. & Lopez, A. 2011. An integrated MBR-TiO2 photocatalysis process for the removal of Carbamazepine from simulated pharmaceutical industrial effluent. *Bioresource Technology*. 102(13):7012–7015. DOI: 10.1016/j.biortech.2011.04.056.

Lagrange, T. 2016. Basics of Transmission Electron Microscopy (TEM). 41(0):1–33.

Latif, A., Maqbool, A., Sun, K. & Si, Y. 2022. Immobilization of Trametes Versicolor laccase on Cu-alginate beads for biocatalytic degradation of bisphenol A in water: Optimized immobilization, degradation and toxicity assessment. *Journal of Environmental Chemical Engineering*. 10(1):107089. DOI: 10.1016/j.jece.2021.107089.

Lei, Z., Gao, C., Chen, L., He, Y., Ma, W. & Lin, Z. 2018. Recent advances in biomolecule immobilization based on self-assembly: Organic-inorganic hybrid nanoflowers and metal-organic frameworks as novel substrates. *Journal of Materials Chemistry B*. 6(11):1581–1594. DOI: 10.1039/c7tb03310a.

Leng, Y., Liu, F., Cai, H., Chang, F., Xiong, W., Huang, S. & Wang, J. 2023. Mechanism of norfloxacin transformation by horseradish peroxidase and various redox mediated by humic acid and microplastics. *Science of the Total Environment*. 858(September 2022):159528. DOI: 10.1016/j.scitotenv.2022.159528.

Li, H.Z., Yang, C., Qian, H.L. & Yan, X.P. 2023. Room-temperature synthesis of ionic

covalent organic frameworks for efficient removal of diclofenac sodium from aqueous solution. *Separation and Purification Technology*. 306(PB):122704. DOI: 10.1016/j.seppur.2022.122704.

Li, J., Peng, J., Zhang, Y., Ji, Y., Shi, H., Mao, L. & Gao, S. 2016. Removal of triclosan via peroxidases-mediated reactions in water: Reaction kinetics, products and detoxification. *Journal of Hazardous Materials*. 310:152–160. DOI: 10.1016/j.jhazmat.2016.02.037.

Li, X., Wu, Z., Tao, X., Li, R., Tian, D. & Liu, X. 2022. Gentle one-step co-precipitation to synthesize bimetallic CoCu-MOF immobilized laccase for boosting enzyme stability and Congo red removal. *Journal of Hazardous Materials*. 438(July):129525. DOI: 10.1016/j.jhazmat.2022.129525.

Liang, K., Coghlan, C.J., Bell, S.G., Doonan, C. & Falcaro, P. 2016. Enzyme encapsulation in zeolitic imidazolate frameworks: A comparison between controlled co-precipitation and biomimetic mineralisation. *Chemical Communications*. 52(3):473–476. DOI: 10.1039/c5cc07577g.

Liang, L., Yue, X., Dong, S., Feng, J., Sun, J., Pan, Y. & Zhou, M. 2021. New insights into the effect of adsorption on catalysis in the metal-free persulfate activation process for removing organic pollutants. *Separation and Purification Technology*. 272(April):118923. DOI: 10.1016/j.seppur.2021.118923.

Liang, S., Wu, X.L., Xiong, J., Zong, M.H. & Lou, W.Y. 2020. Metal-organic frameworks as novel matrices for efficient enzyme immobilization: An update review. *Coordination Chemistry Reviews*. 406(December):213149. DOI: 10.1016/j.ccr.2019.213149.

Liu, L.H., Yuan, T., Zhang, J.Y., Tang, G.X., Lü, H., Zhao, H.M., Li, H., Li, Y.W., et al. 2022. Diversity of endophytic bacteria in wild rice (Oryza meridionalis) and potential for promoting plant growth and degrading phthalates. *Science of the Total Environment*. 806:150310. DOI: 10.1016/j.scitotenv.2021.150310.

Liu, X., Wang, M., Zhang, S. & Pan, B. 2013. Application potential of carbon nanotubes in water treatment: A review. *Journal of Environmental Sciences (China)*. 25(7):1263–1280. DOI: 10.1016/S1001-0742(12)60161-2.

Lu, F. & Astruc, D. 2018. Nanomaterials for removal of toxic elements from water Precipitaion Adsorption Heavy metals Removal Ion Exchange Filtration Coagulation Bio-sorprtion. 356:147–164.

Lucia, L., Gemma E., Teresa, M., Gumersindo, F. & Juan L. 2013. Removal of Estrogenic Compounds from Filtered Secondary Wastewater Effluent in a Continuous Enzymatic Membrane Reactor. Identification of Biotransformation Products. *Environmental Science & Technology*. doi.org/10.1021/es304783k

Lueangjaroenkit, P., Teerapatsakul, C., Sakka, K., Sakka, M., Kimura, T., Kunitake, E. & Chitradon, L. 2019. Two Manganese Peroxidases and a Laccase of Trametes polyzona KU-RNW027 with Novel Properties for Dye and Pharmaceutical Product Degradation in Redox Mediator-Free System. *Mycobiology*. 47(2):217–229. DOI: 10.1080/12298093.2019.1589900.

Luisa, A., Guardado, P., Luisa, A. & Guardado, P. 2019. Enzymatic degradation of recalcitrant pharmaceutical micropollutants. *HAL*.

Luo, Y., Huang, X., Li, Y., Fu, Y., Wang, Z., Lu, J. & Zhang, J. 2020. CuNiN@C coupled with peroxymonosulfate as efficient catalytic system for the removal of norfloxacin by adsorption

and catalysis. *Separation and Purification Technology*. 252(March):117476. DOI: 10.1016/j.seppur.2020.117476.

Lyu, F., Zhang, Y., Zare, R.N., Ge, J. & Liu, Z. 2014. One-pot synthesis of protein-embedded metal-organic frameworks with enhanced biological activities. *Nano Letters*. 14(10):5761–5765. DOI: 10.1021/nl5026419.

Mahmoodi, N.M., Taghizadeh, M., Taghizadeh, A., Abdi, J., Hayati, B. & Shekarchi, A.A. 2019. Bio-based magnetic metal-organic framework nanocomposite: Ultrasound-assisted synthesis and pollutant (heavy metal and dye) removal from aqueous media. *Applied Surface Science*. 480(February):288–299. DOI: 10.1016/j.apsusc.2019.02.211.

Mak, T.C. & Gu, J. 2021. Degradability and Biochemical Pathways of the Endocrine-disrupting Plasticizers Phthalate Esters in Plastics by Microorganisms. *Applied Environmental Biotechnology*. 6(1):16–30.

Male, U., Srinivasan, P. & Singu, B.S. 2015. Incorporation of polyaniline nanofibres on graphene oxide by interfacial polymerization pathway for supercapacitor. *International Nano Letters*. 5(4):231–240. DOI: 10.1007/s40089-015-0160-9.

Margot, J., Maillard, J., Rossi, L., Barry, D.A. & Holliger, C. 2013. Influence of treatment conditions on the oxidation of micropollutants by Trametes versicolor laccase. *New Biotechnology*. 30(6):803–813. DOI: 10.1016/j.nbt.2013.06.004.

Martínez, F., Leo, P., Orcajo, G., Díaz-García, M., Sanchez-Sanchez, M. & Calleja, G. 2018. Sustainable Fe-BTC catalyst for efficient removal of mehylene blue by advanced fenton oxidation. *Catalysis Today*. 313(September 2017):6–11. DOI: 10.1016/j.cattod.2017.10.002.

Mbachu, A.E., Chukwura, E.I. & Mbachu, N.A. 2020. Role of Microorganisms in the Degradation of Organic Pollutants: A Review. *Energy and Environmental Engineering*. 7(1):1–11. DOI: 10.13189/eee.2020.070101.

Mehta, J., Bhardwaj, N., Bhardwaj, S.K., Kim, K.H. & Deep, A. 2016. Recent advances in enzyme immobilization techniques: Metal-organic frameworks as novel substrates. *Coordination Chemistry Reviews*. 322:30–40. DOI: 10.1016/j.ccr.2016.05.007.

Merlini, C., Barra, G.M.O., Schmitz, D.P., Ramôa, S.D.A.S., Silveira, A., Araujo, T.M. & Pegoretti, A. 2014. Polyaniline-coated coconut fibers: Structure, properties and their use as conductive additives in matrix of polyurethane derived from castor oil. *Polymer Testing*. 38:18–25. DOI: 10.1016/j.polymertesting.2014.06.005.

Meryam Sardar, R.A. 2015. Enzyme Immobilization: An Overview on Nanoparticles as Immobilization Matrix. *Biochemistry & Analytical Biochemistry*. 04(02). DOI: 10.4172/2161-1009.1000178.

Microscopy, T.E. 1996. Transmission Electron Microscopy □.

Mir, R.A. & Gulfishan, M. 2020. The Biodegradation of Organic Pollutants. *European Journal of Molecular & Clinical Medicine*. 07(10):3552–3562. Available: www.epa.gov.

Mkaddem, H., Rosales, E., Pazos, M., Ben Amor, H., Sanromán, M.A. & Meijide, J. 2022. Anti-inflammatory drug diclofenac removal by a synthesized MgAl layered double hydroxide. *Journal of Molecular Liquids*. 359:119207. DOI: 10.1016/j.molliq.2022.119207.

Moodley, B., Birungi, G. & Ndungu, P. 2016. *Detection and Quantification of Emerging Organic Pollutants in the Umgeni and Msunduzi Rivers*.

Movasaghi, Z., Rehman, S. & Rehman, I. 2017. Fourier Transform Infrared (FTIR) Spectroscopy of Biological Tissues. 4928(August). DOI: 10.1080/05704920701829043.

Nadar, S.S. & Rathod, V.K. 2018. Encapsulation of lipase within metal-organic framework (MOF) with enhanced activity intensified under ultrasound. *Enzyme and Microbial Technology*. 108(August 2017):11–20. DOI: 10.1016/j.enzmictec.2017.08.008.

Nadar, S.S. & Rathod, V.K. 2019. One pot synthesis of α-amylase metal organic framework (MOF)-sponge. *International Journal of Biological Macromolecules*. 138:1035–1043. DOI: https://doi.org/10.1016/j.ijbiomac.2019.07.099.

Naghdi, M., Taheran, M., Brar, S.K., Kermanshahi-pour, A., Verma, M. & Surampalli, R.Y. 2018a. Biotransformation of carbamazepine by laccase-mediator system: Kinetics, by-products and toxicity assessment. *Process Biochemistry*. 67(November 2017):147–154. DOI: 10.1016/j.procbio.2018.02.009.

Naghdi, M., Taheran, M., Brar, S.K., Kermanshahi-pour, A., Verma, M. & Surampalli, R.Y. 2018b. Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes. *Environmental Pollution*. 234:190–213. DOI: 10.1016/j.envpol.2017.11.060.

Navada, K.K. & Kulal, A. 2019. Enzymatic degradation of chloramphenicol by laccase from Trametes hirsuta and comparison among mediators. *International Biodeterioration and Biodegradation*. 138(December 2018):63–69. DOI: 10.1016/j.ibiod.2018.12.012.

Nguyen, L.N., Hai, F.I., Yang, S., Kang, J., Leusch, F.D.L., Roddick, F., Price, W.E. & Nghiem, L.D. 2014. Removal of pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals and pesticides by Trametes versicolor: Role of biosorption and biodegradation. *International Biodeterioration and Biodegradation*. 88:169–175. DOI: 10.1016/j.ibiod.2013.12.017.

Nguyen, T., Roddick, F.A. & Fan, L. 2012. Biofouling of Water Treatment Membranes: A Review of the Underlying Causes, Monitoring Techniques and Control Measures. 804–840. DOI: 10.3390/membranes2040804.

Oller, I., Malato, S. & Sánchez-pérez, J.A. 2011. Combination of Advanced Oxidation Processes and biological treatments for wastewater decontamination — A review. *Science of the Total Environment, The*. 409(20):4141–4166. DOI: 10.1016/j.scitotenv.2010.08.061.

Oluwole, A.O., Omotola, E.O. & Olatunji, O.S. 2020. Pharmaceuticals and personal care products in water and wastewater: a review of treatment processes and use of photocatalyst immobilized on functionalized carbon in AOP degradation. *BMC Chemistry*. 14(1):1–29. DOI: 10.1186/s13065-020-00714-1.

Ortiz, A.U., Freitas, A.P., Boutin, A., Fuchs, A.H. & Coudert, F.X. 2014. What makes zeolitic imidazolate frameworks hydrophobic or hydrophilic? the impact of geometry and functionalization on water adsorption. *Physical Chemistry Chemical Physics*. 16(21):9940–9949. DOI: 10.1039/c3cp54292k.

Othman, A.R., Hasan, H.A., Muhamad, M.H., Ismail, N. 'Izzati & Abdullah, S.R.S. 2021. Microbial degradation of microplastics by enzymatic processes: a review. *Environmental Chemistry Letters*. 19(4):3057–3073. DOI: 10.1007/s10311-021-01197-9.

Pan, S., Guo, X., Li, R., Hu, H., Yuan, J., Liu, B., Hei, S. & Zhang, Y. 2022. Activation of peroxymonosulfate via a novel UV/hydrated Fe(III) oxide coupling strategy for norfloxacin

removal: Performance and mechanism. *Separation and Purification Technology*. 300(August):121909. DOI: 10.1016/j.seppur.2022.121909.

Paredes-Laverde, M., Silva-Agredo, J. & Torres-Palma, R.A. 2018. Removal of norfloxacin in deionized, municipal water and urine using rice (Oryza sativa) and coffee (Coffea arabica) husk wastes as natural adsorbents. *Journal of Environmental Management*. 213:98–108. DOI: 10.1016/j.jenvman.2018.02.047.

Parra Guardado, A.L., Belleville, M.P., Rostro Alanis, M. de J., Parra Saldivar, R. & Sanchez-Marcano, J. 2019. Effect of redox mediators in pharmaceuticals degradation by laccase: A comparative study. *Process Biochemistry*. 78(December 2018):123–131. DOI: 10.1016/j.procbio.2018.12.032.

Prasetya, N. & Li, K. 2022. Synthesis of defective MOF-801 via an environmentally benign approach for diclofenac removal from water streams. *Separation and Purification Technology*. 301(August):122024. DOI: 10.1016/j.seppur.2022.122024.

Primožič, M., Kravanja, G., Knez, Ž., Crnjac, A. & Leitgeb, M. 2020. Immobilized laccase in the form of (magnetic) cross-linked enzyme aggregates for sustainable diclofenac (bio)degradation. *Journal of Cleaner Production*. 275. DOI: 10.1016/j.jclepro.2020.124121.

Pylypchuk, I. V., Daniel, G., Kessler, V.G. & Seisenbaeva, G.A. 2020. Removal of diclofenac, paracetamol, and carbamazepine from model aqueous solutions by magnetic sol–gel encapsulated horseradish peroxidase and lignin peroxidase composites. *Nanomaterials*. 10(2). DOI: 10.3390/nano10020282.

Rafiei, S., Tangestaninejad, S., Horcajada, P., Moghadam, M., Mirkhani, V., Mohammadpoor-Baltork, I., Kardanpour, R. & Zadehahmadi, F. 2018. Efficient biodiesel production using a lipase@ZIF-67 nanobioreactor. *Chemical Engineering Journal*. 334(October 2017):1233–1241. DOI: 10.1016/j.cej.2017.10.094.

Rahmani, S. & Mohseni, M. 2017. The role of hydrophobic properties in ion exchange removal of organic compounds from water. *Canadian Journal of Chemical Engineering*. 95(8):1449–1455. DOI: 10.1002/cjce.22823.

Rajkumar, T., Kukkar, D., Kim, K., Ryeul, J. & Deep, A. 2019. Cyclodextrin-metal–organic framework (CD-MOF): From synthesis to applications. *Journal of Industrial and Engineering Chemistry*. 72(2018):50–66. Available: https://doi.org/10.1016/j.jiec.2018.12.048.

Reddy, J., Prasanna, L., Singh, J., Choo, K., Koduru, J.R., Lingamdinne, L.P., Singh, J. & Choo, K. 2016. Effective removal of bisphenol A (BPA) from water using a goethite/activated carbon composite. *Process Safety and Environmental Protection*. 103:87–96. DOI: 10.1016/j.psep.2016.06.038.

Rodríguez-Delgado, M.M., Alemán-Nava, G.S., Rodríguez-Delgado, J.M., Dieck-Assad, G., Martínez-Chapa, S.O., Barceló, D. & Parra, R. 2015. Laccase-based biosensors for detection of phenolic compounds. *Trends in Analytical Chemistry*. 74(December):21–45. DOI: 10.1016/j.trac.2015.05.008.

Routoula, E. & Patwardhan, S. V. 2020. Degradation of Anthraquinone Dyes from Effluents: A Review Focusing on Enzymatic Dye Degradation with Industrial Potential. *Environmental Science and Technology*. 54(2):647–664. DOI: 10.1021/acs.est.9b03737.

Ruzicka, J.-Y. 2016. Particle sizing using ImageJ – MESA.

Salgaonkar, M., Nadar, S.S. & Rathod, V.K. 2019. Biomineralization of orange peel peroxidase

within metal organic frameworks (OPP-MOFs) for dye degradation. *Journal of Environmental Chemical Engineering*. 7(2):102969. DOI: 10.1016/j.jece.2019.102969.

Salimpour Abkenar, S., Malek, R.M.A. & Mazaheri, F. 2015. Dye adsorption of cotton fabric grafted with PPI dendrimers: Isotherm and kinetic studies. *Journal of Environmental Management*. 163:53–61. DOI: 10.1016/j.jenvman.2015.08.003.

Salman, S.D., Rasheed, I.M. & Ismaeel, M.M. 2023. Removal of diclofenac from aqueous solution on apricot seeds activated carbon synthesized by pyro carbonic acid microwave. *Chemical Data Collections*. 43(December 2022):100982. DOI: 10.1016/j.cdc.2022.100982.

Sargazi, G., Afzali, D. & Mostafavi, A. 2018. A novel synthesis of a new thorium (IV) metal organic framework nanostructure with well controllable procedure through ultrasound assisted reverse micelle method. *Ultrasonics Sonochemistry*. 41(June 2017):234–251. DOI: 10.1016/j.ultsonch.2017.09.046.

Sharma, V.K. & Feng, M. 2019. Water depollution using metal-organic frameworks-catalyzed advanced oxidation processes: A review. *Journal of Hazardous Materials*. 372:3–16. DOI: 10.1016/j.jhazmat.2017.09.043.

Sidy, B., Lounes, H., Carles, C., Chloé, J., Imad, E. T., Jean-Phillipe, B., Claude, Y. L, Peter, J. & Hubert, C. 2013. Synthesis and characterization of combined cross-linked laccase and tyrosinase aggregates transforming acetaminophen as a model phenolic compound in wastewaters. *Science of the Total Environment*. 487: 748-755. DOI:10.1016/j.scitotenv.2013.10.004.

Silva, A.R.M., Alexandre, J.Y.N.H., Souza, J.E.S., Neto, J.G.L., de Sousa Júnior, P.G., Rocha, M.V.P. & dos Santos, J.C.S. 2022. The Chemistry and Applications of Metal–Organic Frameworks (MOFs) as Industrial Enzyme Immobilization Systems. *Molecules*. 27(14). DOI: 10.3390/molecules27144529.

Simón-Herrero, C., Naghdi, M., Taheran, M., Kaur Brar, S., Romero, A., Valverde, J.L., Avalos Ramirez, A. & Sánchez-Silva, L. 2019. Immobilized laccase on polyimide aerogels for removal of carbamazepine. *Journal of Hazardous Materials*. 376(January):83–90. DOI: 10.1016/j.jhazmat.2019.05.032.

Sorensen, J.P.R., Lapworth, D.J., Nkhuwa, D.C.W., Stuart, M.E., Gooddy, D.C., Bell, R.A., Chirwa, M., Kabika, J., et al. 2015. Emerging contaminants in urban groundwater sources in Africa. *Water Research*. 72:51–63. DOI: 10.1016/j.watres.2014.08.002.

Spina, F., Tigini, V., Romagnolo, A. & Varese, G.C. 2018. Bioremediation of landfill leachate with fungi: Autochthonous vs. allochthonous strains. *Life*. 8(3). DOI: 10.3390/life8030027.

Stadlmair, L.F., Letzel, T., Drewes, J.E. & Graßmann, J. 2017. Mass spectrometry based in vitro assay investigations on the transformation of pharmaceutical compounds by oxidative enzymes. *Chemosphere*. 174:466–477. DOI: 10.1016/j.chemosphere.2017.01.140.

Stadlmair, L.F., Letzel, T., Drewes, J.E. & Grassmann, J. 2018. Enzymes in removal of pharmaceuticals from wastewater: A critical review of challenges, applications and screening methods for their selection. *Chemosphere*. 205:649–661. DOI: 10.1016/j.chemosphere.2018.04.142.

Stine, K.J. 2017. Enzyme Immobilization on Nanoporous Gold: A Review. *Biochemistry Insights*. 10:117862641774860. DOI: 10.1177/1178626417748607.

Strakos, C. 2021. A Brief Review on Carbamazepine – History, Pharmacological Properties

and Environmental Impact. *Insights in Chemistry and Biochemistry*. 1(4):1–4. DOI: 10.33552/icbc.2021.01.000519.

Ta, M., Wang, T., Guo, J., Wang, Y., Zhang, J., Zhao, C., Liu, S., Liu, G., et al. 2023. Enhanced norfloxacin degradation by three-dimensional (3D) electrochemical activation of peroxymonosulfate using Mn/Cu co-doped activated carbon particle electrode. *Separation and Purification Technology*. 310(December 2022):123067. DOI: 10.1016/j.seppur.2022.123067.

Taheran, M., Naghdi, M., Brar, S.K., Knystautas, E.J., Verma, M. & Surampalli, R.Y. 2017. Degradation of chlortetracycline using immobilized laccase on Polyacrylonitrile-biochar composite nano fibrous membrane. *Science of the Total Environment*. 605–606:315–321. DOI: 10.1016/j.scitotenv.2017.06.185.

Tahmasbi, H., Khoshayand, M.R., Bozorgi-Koushalshahi, M., Heidary, M., Ghazi-Khansari, M. & Faramarzi, M.A. 2016. Biocatalytic conversion and detoxification of imipramine by the laccase-mediated system. *International Biodeterioration and Biodegradation*. 108:1–8. DOI: 10.1016/j.ibiod.2015.11.029.

Tegegne, B., Chandravanshi, B.S., Zewge, F. & Chimuka, L. 2021. Solid-phase optimisation for simultaneous determination of thirteen pharmaceuticals in Ethiopian water samples with HPLC-DAD detection: an initial assessment. *Environmental Monitoring and Assessment*. 193(5):1–14. DOI: 10.1007/s10661-021-08999-y.

Terban, M.W., Banerjee, D., Ghose, S., Medasani, B., Shukla, A., Legg, B.A., Zhou, Y., Zhu, Z., et al. 2018. Early stage structural development of prototypical zeolitic imidazolate framework (ZIF) in solution. *Nanoscale*. 10(9):4291–4300. DOI: 10.1039/c7nr07949d.

Thadathil, D.A., Varghese, A., Ahamed, C.V.S., Krishnakumar, K.A., Varma, S.S., Lankalapalli, R.S. & Radhakrishnan, K.V. 2022. Enzyme based bioelectrocatalysis over laccase immobilized poly-thiophene supported carbon fiber paper for the oxidation of D-ribofuranose to D-ribonolactone. *Molecular Catalysis*. 524(March):112314. DOI: 10.1016/j.mcat.2022.112314.

Tian, Y., Jia, N., Ma, H., Liu, G., Xiao, Z., Wu, Y., Zhou, L., Lei, J., et al. 2021. 0D/3D coupling of g-C3N4 QDs/hierarchical macro-mesoporous CuO-SiO2 for high-efficiency norfloxacin removal in photo-Fenton-like processes. *Journal of Hazardous Materials*. 419(June):126359. DOI: 10.1016/j.jhazmat.2021.126359.

Tolou-Ghamari, Z., Zare, M., Habibabadi, J.M. & Najafi, M.R. 2013. A quick review of carbamazepine pharmacokinetics in epilepsy from 1953 to 2012. *Journal of Research in Medical Sciences*. 18(SPL. 1).

Tranchemontagne, D.J., Hunt, J.R. & Yaghi, O.M. 2008. Room temperature synthesis of metal-organic frameworks: MOF-5, MOF-74, MOF-177, MOF-199, and IRMOF-0. *Tetrahedron*. 64(36):8553–8557. DOI: 10.1016/j.tet.2008.06.036.

Ulu, A. 2020. Metal–organic frameworks (MOFs): a novel support platform for ASNase immobilization. *Journal of Materials Science*. 55(14):6130–6144. DOI: 10.1007/s10853-020-04452-6.

Varga, B., Somogyi, V., Meiczinger, M., Kováts, N. & Domokos, E. 2019. Enzymatic treatment and subsequent toxicity of organic micropollutants using oxidoreductases - A review. *Journal of Cleaner Production*. 221:306–322. DOI: 10.1016/j.jclepro.2019.02.135.

Vikrant, K., Giri, B.S., Raza, N., Roy, K., Kim, K.H., Rai, B.N. & Singh, R.S. 2018. Recent

advancements in bioremediation of dye: Current status and challenges. *Bioresource Technology*. 253(November 2017):355–367. DOI: 10.1016/j.biortech.2018.01.029.

Wang, H., Pei, X., Kalmutzki, M.J., Yang, J. & Yaghi, O.M. 2022. Large Cages of Zeolitic Imidazolate Frameworks. *Accounts of Chemical Research*. 55(5):707–721. DOI: 10.1021/acs.accounts.1c00740.

Wang, J., Yu, S., Feng, F. & Lu, L. 2019. Simultaneous purification and immobilization of laccase on magnetic zeolitic imidazolate frameworks: Recyclable biocatalysts with enhanced stability for dye decolorization. *Biochemical Engineering Journal*. 150(26):107285. DOI: 10.1016/j.bej.2019.107285.

Wang, Y., Li, F., Ruan, X., Song, J., Lv, L., Chai, L., Yang, Z. & Luo, L. 2017. Biodegradation of di-n-butyl phthalate by bacterial consortium LV-1 enriched from river sludge. *PLoS ONE*. 12(5):1–13. DOI: 10.1371/journal.pone.0178213.

Wang, Z., Ren, D., Yu, H., Jiang, S., Zhang, S. & Zhang, X. 2020. Study on improving the stability of adsorption-encapsulation immobilized Laccase@ZIF-67. *Biotechnology Reports*. 28:e00553. DOI: 10.1016/j.btre.2020.e00553.

Wawrzkiewicz, M. & Hubicki, Z. 2015. World ' s largest Science , Technology & Medicine Open Access book publisher Anion Exchange Resins as Effective Sorbents for Removal of Acid , Reactive , and Direct Dyes from Textile Wastewaters.

Wong, D.W.S. 2009. *Structure and action mechanism of ligninolytic enzymes*. V. 157. DOI: 10.1007/s12010-008-8279-z.

Wu, E., Li, Y., Huang, Q., Yang, Z., Wei, A. & Hu, Q. 2019. Laccase immobilization on amino-functionalized magnetic metal organic framework for phenolic compound removal. *Chemosphere*. 233:327–335. DOI: 10.1016/j.chemosphere.2019.05.150.

Wu, Y., Chen, Y. & Wei, N. 2020. Biocatalytic properties of cell surface display laccase for degradation of emerging contaminant acetaminophen in water reclamation. *Biotechnology and Bioengineering*. 117(2):342–353. DOI: 10.1002/bit.27214.

Xia, H., Li, N., Zhong, X. & Jiang, Y. 2020. Metal-Organic Frameworks: A Potential Platform for Enzyme Immobilization and Related Applications. *Frontiers in Bioengineering and Biotechnology*. 8(June):1–16. DOI: 10.3389/fbioe.2020.00695.

Xu, R., Si, Y., Li, F. & Zhang, B. 2015. Enzymatic removal of paracetamol from aqueous phase: horseradish peroxidase immobilized on nanofibrous membranes. *Environmental Science and Pollution Research*. 22(5):3838–3846. DOI: 10.1007/s11356-014-3658-1.

Yang, H.M., Liu, X., Song, X.L., Yang, T.L., Liang, Z.H. & Fan, C.M. 2015. In situ electrochemical synthesis of MOF-5 and its application in improving photocatalytic activity of BiOBr. *Transactions of Nonferrous Metals Society of China (English Edition)*. 25(12):3987–3994. DOI: 10.1016/S1003-6326(15)64047-X.

Yaohua, G., ping, X., feng, J. & keren, S. 2019. Co-immobilization of laccase and ABTS onto novel dual-functionalized cellulose beads for highly improved biodegradation of indole. *Journal of Hazardous Materials*. 365(November 2018):118–124. DOI: 10.1016/j.jhazmat.2018.10.076.

Yousefi-Ahmadipour, A., Bozorgi-Koshalshahi, M., Mogharabi, M., Amini, M., Ghazi-Khansari, M. & Faramarzi, M.A. 2016. Laccase-catalyzed treatment of ketoconazole, identification of biotransformed metabolites, determination of kinetic parameters, and

evaluation of micro-toxicity. *Journal of Molecular Catalysis B: Enzymatic*. 133:77–84. DOI: 10.1016/j.molcatb.2016.07.015.

Yuan, Y., Wang, F., Li, H., Su, S., Gao, H., Han, X. & Ren, S. 2022. Potential application of the immobilization of carbonic anhydrase based on metal organic framework supports. *Process Biochemistry*. 122:214–223. DOI: 10.1016/j.procbio.2022.10.019.

Zaki, G. & Shoeib, T. 2018. Concentrations of several phthalates contaminants in Egyptian bottled water: Effects of storage conditions and estimate of human exposure. *Science of the Total Environment*. 618:142–150. DOI: 10.1016/j.scitotenv.2017.10.337.

Zaluzec, N.J. 2015. Introduction to Electron Microscopy and Microanalysis.

Zdarta, J., Meyer, A.S., Jesionowski, T. & Pinelo, M. 2018a. Developments in support materials for immobilization of oxidoreductases: A comprehensive review. *Advances in Colloid and Interface Science*. 258:1–20. DOI: 10.1016/j.cis.2018.07.004.

Zdarta, J., Meyer, A.S., Jesionowski, T. & Pinelo, M. 2018b. A general overview of support materials for enzyme immobilization: Characteristics, properties, practical utility. *Catalysts*. 8(2):92. DOI: 10.3390/catal8020092.

Zeng, L.J., Huang, Y.H., Lü, H., Geng, J., Zhao, H.M., Xiang, L., Li, H., Li, Y.W., et al. 2022. Uptake pathways of phthalates (PAEs) into Chinese flowering cabbage grown in plastic greenhouses and lowering PAE accumulation by spraying PAE-degrading bacterial strain. *Science of the Total Environment*. 815:152854. DOI: 10.1016/j.scitotenv.2021.152854.

Zhang, F., Lian, M., Alhadhrami, A., Huang, M., Li, B., Mersal, G.A.M., Ibrahim, M.M. & Xu, M. 2022. Laccase immobilized on functionalized cellulose nanofiber/alginate composite hydrogel for efficient bisphenol A degradation from polluted water. *Advanced Composites and Hybrid Materials*. (0123456789). DOI: 10.1007/s42114-022-00476-5.

Zhang, S., Shao, Y., Liu, J., Aksay, I.A. & Lin, Y. 2011. Graphene À Polypyrrole Nanocomposite as a Highly Efficient and Low Cost Electrically Switched Ion Exchanger for Removing ClO 4 À from Wastewater. 3633–3637.

Zhou, H., Wang, Z., Gao, C., Sun, Q., Liu, J. & She, D. 2023. Synthesis of honeycomb lignin-based biochar and its high-efficiency adsorption of norfloxacin. *Bioresource Technology*. 369(October 2022):128402. DOI: 10.1016/j.biortech.2022.128402.

Zhu, Y., Ciston, J., Zheng, B., Miao, X., Czarnik, C., Pan, Y., Sougrat, R., Lai, Z., et al. 2017. Unravelling surface and interfacial structures of a metal-organic framework by transmission electron microscopy. *Nature Materials*. 16(5):532–536. DOI: 10.1038/nmat4852.

Zofair, S.F.F., Ahmad, S., Hashmi, M.A., Khan, S.H., Khan, M.A. & Younus, H. 2022. Catalytic roles, immobilization and management of recalcitrant environmental pollutants by laccases: Significance in sustainable green chemistry. *Journal of Environmental Management*. 309(January):114676. DOI: 10.1016/j.jenvman.2022.114676.

CHAPTER 3
MATERIALS AND METHODS

This study sought to develop a highly stable enzyme-MOF matrix referred to as Lac-ZIF. In this sense, laccase enzyme from *Aspergillus* species was immobilized onto zeolitic imidazolate framework (ZIF) for subsequent biodegradation of emerging organic pollutants. These organic contaminants consist of three pharmaceutically active compounds (PhACs): carbamazepine (CBD), diclofenac (DFC), norfloxacin (NOR), and an endocrine-disrupting chemical, dimethyl phthalate (DMP). The performance of this biocatalyst (Lac-ZIF) was compared with that of free laccase and adsorption by the pure ZIF crystals.

3.1 Synthesis of ZIF and Lac-ZIF

The synthesis procedure for ZIF particles and immobilization of laccase is presented in each of the following chapters. However, the same synthetic procedure was employed for all the applications.

3.2 Operating conditions

The capability of the synthesized Lac-ZIF biocatalytic composite to decontaminate targeted organic pollutants from aqueous solutions was investigated under various conditions. The parameters assessed were pH, biocatalyst dosage, laccase volume, initial analyte concentration, and contact time. Other factors such as temperature, storage, and organic solvent stability as well as the reusability of the material were studied.

3.3 Characterization

Characterization of the material provides an insight into the biocatalyst's morphological, physical, and chemical features. The Lac-ZIF, and ZIF particles were characterized using the following techniques: Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy, Field Emission Scanning Electron Microscopy (FE-SEM), High-Resolution Transmission Electron Microscopy (HR-TEM), Nuclear Magnetic Resonance (NMR), and X-ray Diffraction (XRD) spectroscopy.

3.3.1 FTIR

Fourier transform infrared spectroscopy is a technique used for monitoring the vibrations of functional groups that characterize the molecular structure of the material and also determine the course of chemical reactions (Griffiths & Haseth, 2007). This characterization technique was employed in the determination of functional groups present in the Lac-ZIF biocatalyst. The FTIR was principally used to confirm the successful coordination of the Zinc metal to form the ZIF crystals and subsequent immobilization of the laccase. A characteristic FTIR spectrometer includes a light source, interferometer, sample module, detector, amplifier, A/D converter, and computer (Movasaghi, Rehman & Rehman, 2017). The sample is subjected to electromagnetic radiation, which causes transitions between vibrational energy levels of the molecule's ground electronic state as shown in Figure 3. 1. Vibrations specific to the sample's functional groups cause these transitions and each functional group has a corresponding characteristic vibrational frequency.

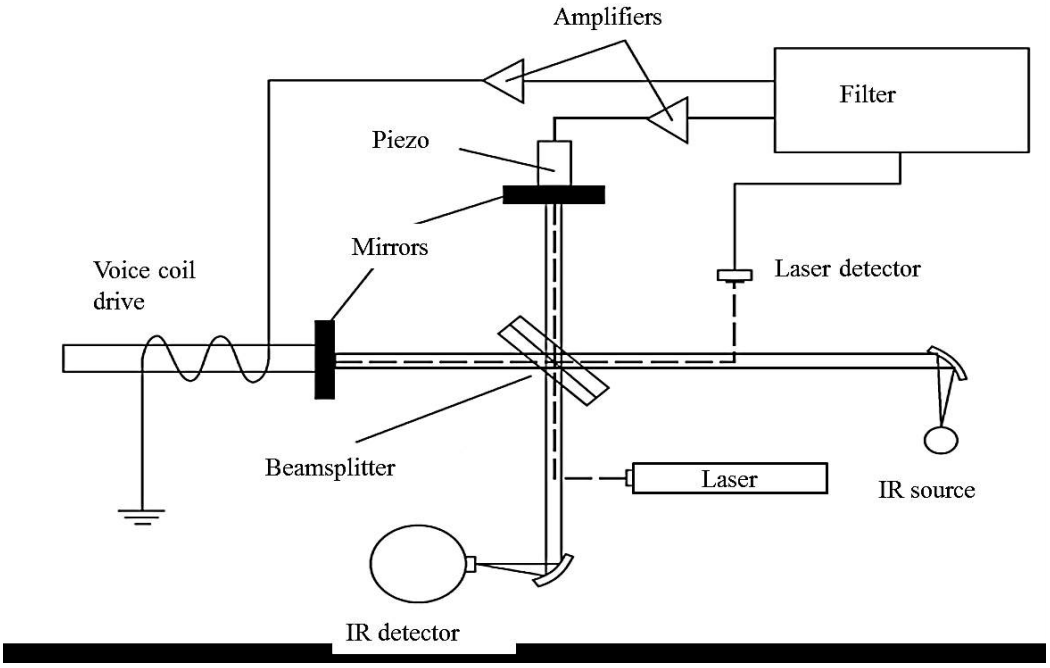


Figure 3. 1: FTIR analysis illustration (Griffiths and Chalmers, 2006)

3.3.2 FE-SEM

An FE-SEM (Figure 3. 2) can examine the structural morphology of particle surfaces with an effective resolution of about 1 nm. In this study, SEM was utilized in the analysis of the ZIF's and Lac-ZIF's morphological structure. The analysis provided an insight in the particles' surface morphology and arrangement before and after the laccase attachment. To achieve this,

the ZIF sample was irradiated with an electron beam known as an electron probe, which generates the emission of secondary electrons from the sample (Ezumi & Todokoro, 1997). This secondary radiation is of different radiation and can be analyzed to discern morphological and chemical features of the sample.

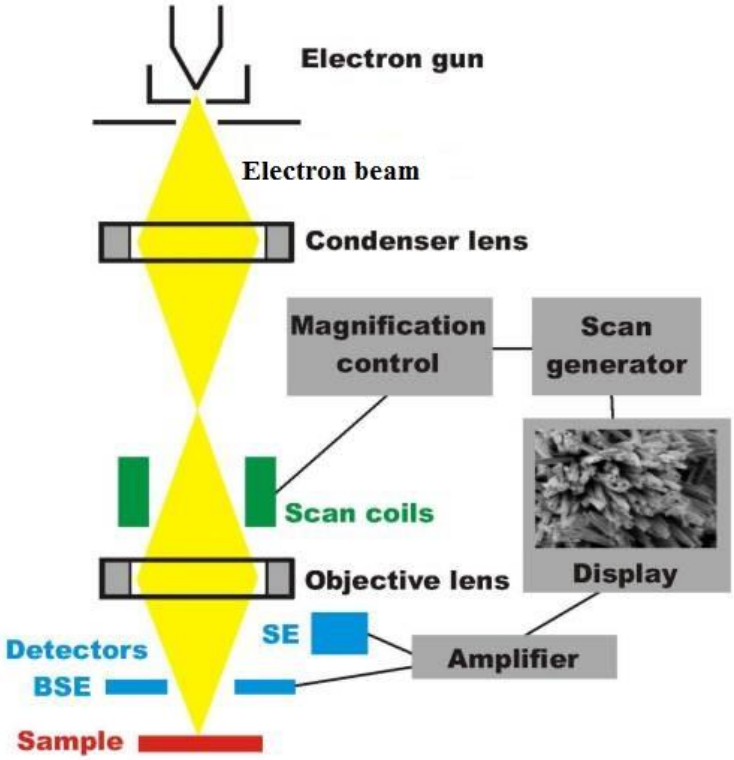


Figure 3. 2: Schematic representation of SEM (Hafner, 2007)

3.3.3 HR-TEM

Transmission electron microscopy is used for in-depth morphological analysis and it was employed in the determination of particle sizes of the prepared biocatalyst. The sizes of the pure ZIF particles were compared to that with immobilized laccase. To analyse a sample, a beam of electrons is focused through a thin specimen, and an image is created as the outcome of the electrons' interactions with the sample (Zaluzec, 2015; Lagrange, 2016). Since electrons have a short de Broglie wavelength, this technique allows for high-resolution imaging. (Microscopy, 1996; Hafner, 2007). A schematic diagram of an HR-TEM is illustrated in figure 3.3.

To analyze and discern the particle size distribution of the particles, a javascript software called ImageJ was used to extract data from the TEM images (Ruzicka, 2016).

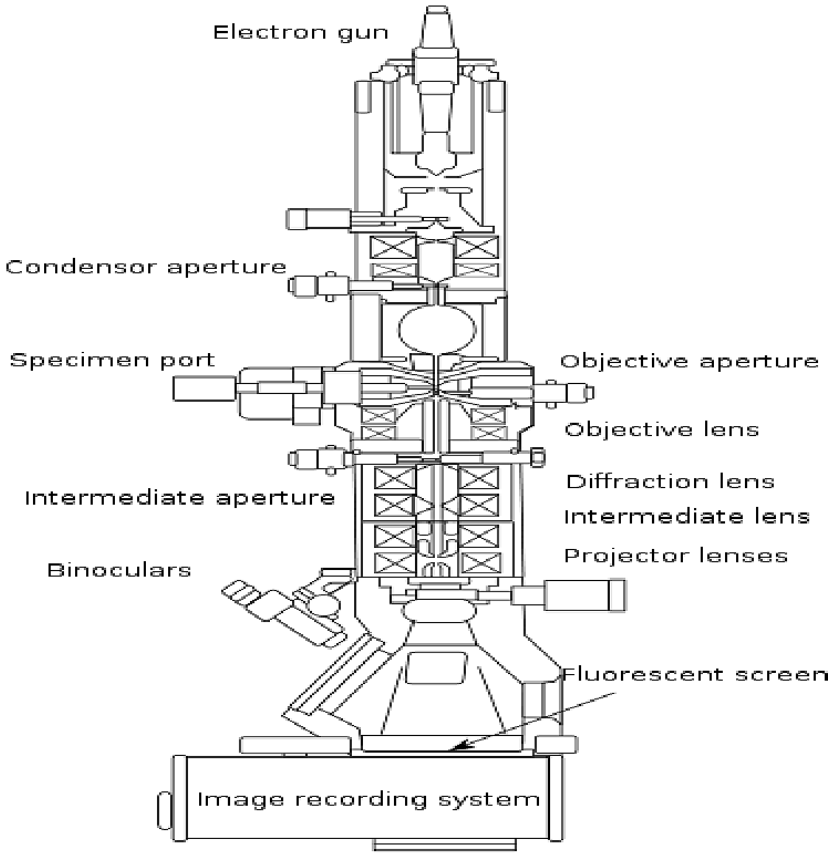


Figure 3. 3: Schematic diagram for TEM (Lagrange, 2016)

3.3.4 NMR spectroscopy

Nuclear magnetic resonance spectroscopy is a technique used to observe local magnetic fields around the nucleus of an atom. This technique was used to discern the proton and carbon shifts of the ZIF and Lac-ZIF samples, so as to ascertain the success of the laccase immobilization. The NMR analysis further provided information on the purity of the synthesized samples. To study this, the specimen is exposed to a magnetic field, and the NMR signal is generated by excitation of the nuclei sample with radio waves, subsequently detected with sensitive radio receivers. The intramolecular magnetic field surrounding an atom in a molecule changes the resonance frequency, allowing access to details of a molecule's electronic structure and distinct functional groups (Nguyen, Roddick & Fan, 2012; Naghdi et al., 2018b).

3.3.4 PXRD

In PXRD analysis, constructive interference of monochromatic X-rays and a crystalline sample exists. The X-rays are produced by a cathode ray tube that has been filtered to produce monochromatic radiation which is directed at the sample. Via Brag’s law, there’ll be an

interaction between the incident rays and the sample, which generates constructive interference. This usually occurs when the sample is subjected to X-rays with a fixed wavelength and specific incident angles. The PXRD analysis was, in this research, employed in determining the crystalline nature of the ZIF and Lac-ZIF samples. Comparisons were made to investigate the effect of attaching the laccase on the ZIF crystals.

CHAPTER 4
RESULTS AND DISCUSSION

This section presents the published articles and manuscript submitted for consideration in publication. The referencing in each manuscript is formatted according to the requirements of the journal in which they were published or submitted. The presentation format including sections, sub-sections, and referencing styles is therefore different for all three documents.

4.1 Paper 1

This paper entitled "**Zeolitic imidazolate frameworks as effective crystalline supports for *aspergillus*-based laccase immobilization for the biocatalytic degradation of carbamazepine**" has been published in Chemosphere journal.

This article evaluated the potential of laccase enzyme immobilized onto zeolitic imidazolate framework (ZIF) in the decontamination of a pharmaceutically active compound, carbamazepine.

Chemosphere, 2022, Vol. 311

https://doi.org/10.1016/j.chemosphere.2022.137142

Authors:

Mbongiseni Lungelo Dlamini – Principal author

Mahadi Lesaoana – Co-author

Izak Kotzé – Co-supervisor

Heidi Lynn Richards – Main supervisor

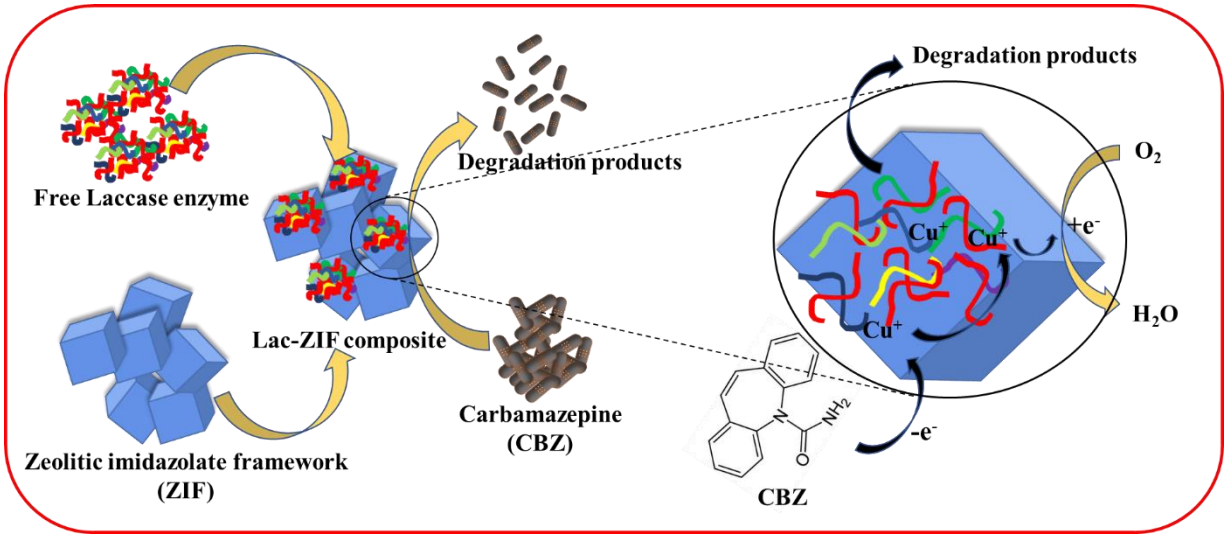
Contribution: lead author, conducted experimental work and data analysis, conceptualization, methodology, formal analysis, investigation, writing - original draft and visualization.

Zeolitic imidazolate frameworks as effective crystalline supports for *Aspergillus*-based laccase immobilization for the biocatalytic degradation of Carbamazepine

Mbongiseni Lungelo Dlamini, Mahadi Lesaoana, Izak Kotze, Heidi Lynn Richards *

Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa.

Graphical abstract



*Corresponding author: Email address: heidi.richards@wits.ac.za

Abstract

In this study, zeolitic imidazolate frameworks (ZIF) were employed as effective porous supports for laccase enzyme attachment and further explored synergistic adsorption and biocatalytic degradation of carbamazepine (CBZ) in aqueous solutions. Characterization results from FTIR and NMR analysis confirmed successful incorporation of the laccase enzyme onto ZIF particles. Further analyses from SEM and TEM revealed rhombic dodecahedral morphologies of ZIF crystals with crusts of the enzyme observed on the particles' surface. The carbamazepine degradation results showed that immobilization of the laccase improved its stability and resistance at various pH's, in comparison to the free enzyme. The immobilized laccase also exhibited relatively higher activities across the studied temperature range compared to the free form. Kinetic studies revealed a negligible decline in velocity, Vmax after immobilization, evaluated to be 0.873 and 0.692 mg L^{-1} h^{-1} for the free and immobilized laccase, respectively. The immobilized laccase demonstrated improved stabilities towards organic solvents, which qualifies the composite's application in real wastewater samples. In which case, the laccase-ZIF composite proved effective in CBZ decontamination with an efficiency of ~92%. Furthermore, the immobilized laccase exhibited appreciable storage stabilities (~70% residual activity) for up to 15 days before any significant loss in activity.

***Keywords*:** Zeolitic Imidazolate Frameworks; Carbamazepine; Biocatalyst; Laccase; Biodegradation

1. Introduction

Environmental impacts and health concerns over emerging persistent organic chemicals in domestic wastewater and effluents from wastewater treatment plants (WWTPs) have been increasing over the years. Pharmaceutically active chemicals (PhACs), which are primarily employed as prescription and non-prescription therapeutics, have been detected in wastewater treatment plants (Naghdi et al., 2018a). These compounds are designed to offer biological effects even at trace concentrations. Therefore, the discharge of such compounds and their potential metabolites into the aquatic environment may pose deleterious effects such as the enhanced evolution of resistant strains of bacteria (Zhang et al., 2009), endocrine disrupting effects (Joseph et al., 2019), and ecotoxicological impacts (Alharbi et al., 2019; Zhang et al., 2009) on the receiving biota.

Carbamazepine (CBZ), a pharmaceutical drug, principally known as an anti-epileptic or anticonvulsant drug, also prescribed for neuralgic pain relief (Gadberry and Marrero-rivera, 2018), is one of the widely detected compounds in wastewater (Alharbi et al., 2019; Naghdi et al., 2018b). Following administration, a relative portion of CBZ may be excreted in unaltered form into the environment. Due to its resistance to simple breakdown and probable adsorption onto solid materials, trace amounts of the molecule may persist after typical wastewater treatment techniques (Bahlmann et al., 2014; Rezaei Kahkha et al., 2018). Involuntary exposure to CBZ, typically through surface water runoff and the use of treated wastewater for irrigation, has been shown in studies to have detrimental effects on aquatic creatures and human health. (Schapira et al., 2020; Yan et al., 2018). These include adverse effects associated with malfunctioning of the normal physiology of aquatic creatures and potential carcinogenic effects as a result of long-term biological accumulation (Burkina et al., 2018; Ebrahimzadeh et al., 2021; Hampel et al., 2017; Nkoom et al., 2020). Consequently, several studies have been done to investigate the possibility of CBZ decontamination from wastewaters. Recently developed wastewater remediation techniques such as membrane separation, advanced oxidation processes (AOPs), and adsorption onto activated solid particles, among others, are capable of effectively removing pharmaceutically active compounds, including CBZ from wastewater. However, the formation of more harmful by-products/metabolites during AOPs (Kudlek, 2018; Oluwole et al., 2020; Yehya et al., 2020), the disposal of concentrated streams associated with membrane separation (Ji et al., 2016), and the recovery hurdles of absorbents (Al-Mashaqbeh et al., 2021; Bhattacharya et al., 2020) are all concerns that these technologies face. This, therefore, impedes the efficient application of such technologies in wastewater treatment.

Biocatalytic transformation of organic pollutants, which entails the use of living organisms or their enzymes, is a greener alternative for wastewater treatment. In comparison to other traditional technologies, this treatment approach requires less energy, operates at mild conditions, and produces fewer or no harmful by-products (Asif et al., 2017). Furthermore, the selectivity of enzymes towards substrate (Demarche et al., 2012; Li et al., 2017), aids in limiting negative side reactions where necessary (Becker et al., 2016; Phan et al., 2016). As a result, enzymes offer potential choices for selective contaminant removal from water and wastewater.

The most predominant enzymes explored in eradicating organic contaminants are oxidoreductase enzymes, such as laccases and peroxidases (Naghdi et al., 2018a), owing to their versatility, abundancy in nature, and accessibility to active sites (Varga et al., 2019). Laccases (p-diphenol: dioxygen oxidoreductases), a group of multi-copper proteins, capable of oxidizing varieties of phenolic and non-phenolic compounds, have been extensively studied over the recent years (Sharma et al., 2018). The enzyme's apparent stability, the requirement of an affordable reactant i.e. Oxygen, and lack of inhibition make it attractive and suitable for biotechnological applications (Mohammadi et al., 2018; Parra Guardado et al., 2019; Yang et al., 2017).

However, the use of enzymes in their soluble or free form tends to limit their practical application in water remediation, due to their low stabilities, low recoveries and/or re-usability potential, possible enzyme leaching during the degradation process, as well as sensitivity towards denaturing agents (Gasser and Ammann, 2014; Lassouane et al., 2019; Sheldon and van Pelt, 2013). Therefore, heterogeneous enzymatic reactions, which involve immobilization of the enzymes on/within suitable insoluble supports may alleviate such hurdles. Among the various traditional enzyme support structures such as silica-based compounds (Mohammadi et al., 2018), metal nanoparticles (Wen et al., 2019), and graphene nanosheets (Alshabib and Onaizi, 2019); metal-organic frameworks (MOFs) – a porous crystalline organic-inorganic hybrid material – have intrigued the interest of many researchers as a new type of porous material for enzymatic supports (Chen et al., 2019; Nadar and Rathod, 2018; Yang et al., 2015). The MOFs' structure and topology can be designed and modified for specific functionalities owing to their geometry richness, and connectivity of metal nodes and ligands. Furthermore, their relatively large surface area and pore volume, potentially simple pore size adjustment, and easy alteration on both metal nodes and ligands, as well as their benign synthetic conditions, qualify MOFs as effective supporting matrices for enzyme attachment (Li et al., 2018; Lian et al., 2017).

Various studies have reported the immobilization of different types of enzymes onto MOFs, however, there are limited reports on the application of the enzyme MOFs matrices to water remediation (Bilal et al., 2019; Cui et al., 2018; Gascón et al., 2018; Liu et al., 2017; Samui and Sahu, 2018). Enzyme MOFs matrices containing enzymes such as peroxidases (Ahmad et al., 2020; He et al., 2018; Salgaonkar et al., 2019), and laccases (Gascón et al., 2018; Hu et al., 2018; Knedel et al., 2019; Pang et al., 2016; Wu et al., 2019) has been explored as potentially effective biocatalysts for decontamination of organic water pollutants. However, to the best of our knowledge, there has been no report of immobilization of *Aspergillus*-based laccase on zeolitic imidazolate frameworks for the removal of pharmaceutically active chemicals. Furthermore, most of these reported works employ the utilization of redox mediators such as syringaldehyde, p-coumaric acid and 2 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) – ABTS among others, which are envisaged at enhancing the electron transfer between the enzyme and the substrate. This is principally a mechanism that entails the mediator's oxidation by the laccase thus producing stable radical species that dissipate away from the enzyme's active site to react with other substrates (Ding et al., 2016; Ji et al., 2016; Nguyen et al., 2016; Parra Guardado et al., 2019; Wang et al., 2018; Yaohua et al., 2019). However, the complexity of redox-mediator bioreactor systems, the costs associated with their use, and their potential contribution to water contamination may limit their practical application in the remediation of freshwater systems. As a result, the current study investigates the respective combined effects of adsorption and biodegradation provided by ZIF particles and laccase enzymes in the absence of redox mediators.

This study aims to effectively prepare a Laccase-Zeolitic Imidazolate Framework (Lac-ZIF) composite and evaluate its capacity and performance in synergistic adsorption and oxidoreductive biocatalytic degradation of carbamazepine (CBZ), an organic compound from synthetic and real river-water samples in mediator-free biosystems. The removal efficiency of CBZ was investigated via batch mode by optimizing experimental variables such as solution pH, initial CBZ concentration, biocatalyst dosage, temperature, and contact time, as well as the solvent and storage stability profiles of the biocatalyst. The properties of the Lac-ZIF were characterized by Fourier Transform Infrared Spectroscopy (FTIR), Powder X-Ray Diffraction (PXRD), Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM), and Nuclear Magnetic Resonance (NMR) spectroscopy. The reaction kinetic of the biocatalyst was also investigated.

2. Materials and Methods

2.1 Materials and reagents

Zinc nitrate tetrahydrate ($Zn(NO_3)_2 \cdot 4H_2O$), 2 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) – ABTS, 2-methylimidazole (2-MIm), carbamazepine (CBZ), laccase enzyme from *Aspergillus* species, disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4H_2O$), sodium acetate $C_2H_3NaO_2$, LC grade methanol (CH_3OH), acetic acid (CH_3COOH), citric acid ($C_6H_8O_7$) and sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) were purchased from Sigma Aldrich. All chemicals were of analytical grade. Distilled and ultrapure water was produced in the laboratory using Milli-Q pore system.

2.2 Preparation of ZIF and Lac-ZIF composite

For the synthesis of Lac-ZIF composite, a modified procedure by (Knedel et al., 2019) and (Shahrak et al., 2019) was employed. Typically, 2 mL of free laccase (1000 U g^{-1}) in 5 mL phosphate buffer (pH 7) was added to a solution of 2-methylimidazole (80 mmol) in 200 mL of methanol. In a separate solution, 20 mmol (5.95 g) $Zn(NO_3)_2 \cdot 4H_2O$ was dissolved in 200 mL methanol. These two solutions were stirred separately for 5 min at 150 rpm. The Zn salt solution was then poured into the 2-MIm and laccase solution. The mixture was left to stir for 2 h at room temperature. The resulting precipitate was centrifuged for 10 min at 4000 rpm and the solid residue washed two times with 50 mL of distilled water. The solid composite was then oven dried under vacuum set at 30 ℃ for 24 h. The same technique was employed in the synthesis of the pure ZIF particles, but without the laccase enzyme and phosphate buffer.

2.3 Characterization of Lac-ZIF composite

To ascertain the functional groups of the Lac-ZIF composite and pristine ZIF particles, FTIR spectra of the particles were recorded on a Perkin–Elmer, USA attenuated total reflectance Fourier Transform Infrared (ATR-FTIR) Spectrum 100 spectrometer in the range 500-4000 cm^{-1}. The spectra were processed using OPUS software with the graphs plotted using Origin software.

For PXRD measurements, A Bruker D2 XRD was employed by means of CuKα radiation with 1.5505 Å wavelength and slits at 45 kV/30 mA for 2θ values ranging from 5° to 90°. To investigate the surface morphologies of both pristine ZIF particles and Lac-ZIF composite, a Field Emission Scanning Electron Microscope (FE-SEM: ZEISS EVO 50, Oberkochen, Germany) was used. For in-depth morphologies of the synthesized particles, a High-Resolution Transmission Electron Microscope (TECNAI F20 HR-TEM) was employed, operated with a LaB6 filament at 200 kV. ^{1}H NMR spectra of pristine ZIF particles were recorded on a Bruker

400 MHz spectrometer using $CDCl_3$ as solvent. For solid-state NMR of the Lac-ZIF composite, the spectra were acquired using a Bruker spectrometer at 126 MHz. The spectra were then processed using MNova software.

2.4 Activity assay of laccase

The activity of the free laccase and immobilized laccase on ZIF particles was determined by monitoring the rate of oxidation of ABTS. One unit of laccase activity was defined as the amount of laccase required to oxidize one µmol of ABTS per minute. The absorbance of the supernatant solution was measured using UV-vis at 420 nm and the final activity of the laccase was expressed as Unit mL^{-1} of free laccase and Unit g^{-1} of Lac-ZIF composite.

To determine the pH and temperature profiles for the free and immobilized laccase activity, assays were carried out over the pH range of 3.0 to 10.0 and temperature range of 20 to 70 ℃. For the free laccase, the reaction mixture contained 1 mL of 1.5 mM ABTS solution, 100 µL laccase, and 5 mL citrate buffer (pH 3 – 6), and phosphate buffer (pH 7 – 10). For the immobilized laccase, the free laccase was replaced with 0.03 g of Lac-ZIF composite. This was reacted for 5 min. The solution pH was maintained at 7.0 for temperature profile studies.

2.4.1 Determination of kinetic parameters

The Michaelis-Menten kinetic parameters (K_m and V_{max}) were obtained by measuring the initial reaction rates with ABTS. The reactions were carried out at room temperature in phosphate buffer solutions (pH 7). The concentrations of ABTS were ranged from 0.1 to 1mM. For the evaluation of enzyme activity on carbamazepine, the ABTS was replaced with CBZ at concentrations ranging from 3 to 20 mg L^{-1}, and the reaction was carried out in the same manner. The Michaelis-Menten equation used to describe the kinetic behavior of the enzyme is given in equation (1) (Saoudi and Ghaouar, 2019):

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

Where, V (mM min^{-1}) is the reaction rate (velocity) at substrate concentration [S] (mM) with V_{max} (mM min^{-1}) and K_m (mM) being the maximum velocity and Michaelis constant, respectively. The K_m and V_{max} values are obtainable from the slope and intercept of the Lineweaver-Burk linear plot of 1/V vs 1/[S], respectively (Wu et al., 2019), (Mohammadi et al., 2018). The Lineweaver-Burk equation is presented in equation (2):

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (2)$$

2.5 CBZ degradation

The performance of ZIF particles, free laccase, and immobilized laccase on zeolitic imidazolate framework as a biocatalytic material for the removal of CBZ from aqueous media was evaluated in batch tests in distilled water and buffer solutions. In a 50 mL tube, a pre-determined amount of Lac-ZIF composite, ZIF particles, and free laccase enzyme (in separate tubes) was dispersed in 20 mL of CBZ solutions. The reaction mixture was then agitated using a rotary shaker at 160 rpm for 24 h at room temperature. The solution was subsequently filtered using a 0.45 µm nylon syringe filter prior to analysis. All the experiments were performed in triplicates, and the averages of replicates and standard deviations were determined. The removal efficiency was measured based on its initial and final aqueous phase concentrations using equation (3)

$$\%\, removal\ efficiency = \left(\frac{C_i - C_f}{C_i}\right) x100, \quad (3)$$

with C_i and C_f (mg L^{-1}) representing the initial and final analyte concentrations, respectively.

2.5.1 Stability assessment

2.5.1.1 Organic solvent stability

To investigate the solvent stability of ZIF particles, free and immobilized laccase on the removal of CBZ, 20 mL of 5 mg L^{-1} analyte solutions were used. Typically, 0.04 g of Lac-ZIF composite, 0.04 g ZIF, and 20 µL laccase volume was added (in separate vials) to CBZ solutions containing 40% of solvents such as methanol, ethanol, acetone, and acetonitrile in phosphate buffer (pH 8). The reaction mixtures were agitated on a rotary shaker at 160 rpm for 24 h.

2.5.1.2 Storage stability

For storage stability, the free and immobilized laccase, as well as the ZIF samples, were kept at room temperature in 0.05 M phosphate buffers at pH 8 for up to 40 days. During this period, the samples were used to determine the removal efficiencies for CBZ at 5-day intervals using optimized parameters, i.e 0.04 g ZIF and Lac-ZIF dosages and 20 µL free laccase added to 20 mL of 5 mg L^{-1} CBZ solutions at pH 8.

2.6 Determination of residual CBZ concentrations

Residual concentrations of the CBZ were measured with a BISCHOFF high performance liquid chromatography (HPLC) using a Fortis C18 column (250 mm x 4.6 mm, 5 µm) at room

temperature and 20 µL injection volume. An isocratic elution with an eluent composition constituting of 60% HPLC grade Acetonitrile and 40% deionized water v/v percentage at a flow rate of 0.8 mL min^{-1}, was employed. The eluted residual CBZ compounds were detected by a UV detector at 284 nm with a retention time of 5.5 min. A 100 mg L^{-1} stock solution for the CBZ compound was prepared and further diluted using distilled water to make standards in the range (0.5 – 20) mg L^{-1}, and hence, the required initial concentrations for biodegradation.

To ascertain the influence of matrix effects on CBZ degradation, real river water samples were collected and spiked with known concentrations (5 mg L^{-1}) of CBZ solutions. The composition consisted of 2:1 real water to CBZ v/v ratio. The samples were exposed to the biocatalysts for up to 24 h, filtered and analyzed using a Thermo Scientific Dionex Ultimate 3000 from ThemoFisher Scientific (Waltham, MA, USA) High Performance Liquid Chromatography (HPLC) coupled with an HPG-3200RS pump and a WPS-3000TRS autosampler. The HPLC was also linked to a Bruker Daltonics Inc. quadrupole time-of-flight compact mass spectrometer system (Billerica, MA, USA) operated in positive scan mode (range, m/z 50 – 300). The qualitative data was collected and processed using Bruker Compass DataAnalysis version 4.3 (Build 110.102.1532), while the quantitative data was collected and processed using Bruker Compass QuantAnalysis version 4.3 (Build 110.102.1532). Chromatographic separation of the analytes was achieved using the same method employed for the synthetic CBZ solutions.

3. Results and Discussion

3.1 Characterization of Lac-ZIF composite, ZIF particles, and free Laccase enzyme

The FTIR spectra of Lac-ZIF, Zeolitic Imidazolate Framework (ZIF), and 2-methylmidazole (2-MIm) are presented in fig 1 (a). The spectra for ZIF clearly indicate the disappearance of vibrational peaks corresponding to -NH bond. This vibrational peak is observed in the spectra for 2-MIm at 3200 – 2380 cm^{-1} corresponding to the -NH bond present in the 2-MIm ring. The disappearance of this peak indicates successful co-ordination of -NH with Zn^{2+} and complete deprotonation of it. This -NH vibration peak is later observed after immobilization of the

laccase enzyme confirming the attachment of the laccase onto the ZIF particles. In the Lac-ZIF spectrum, the amide I and II bands of the laccase were observed at 1660 cm^{-1} and 1460 cm^{-1}.

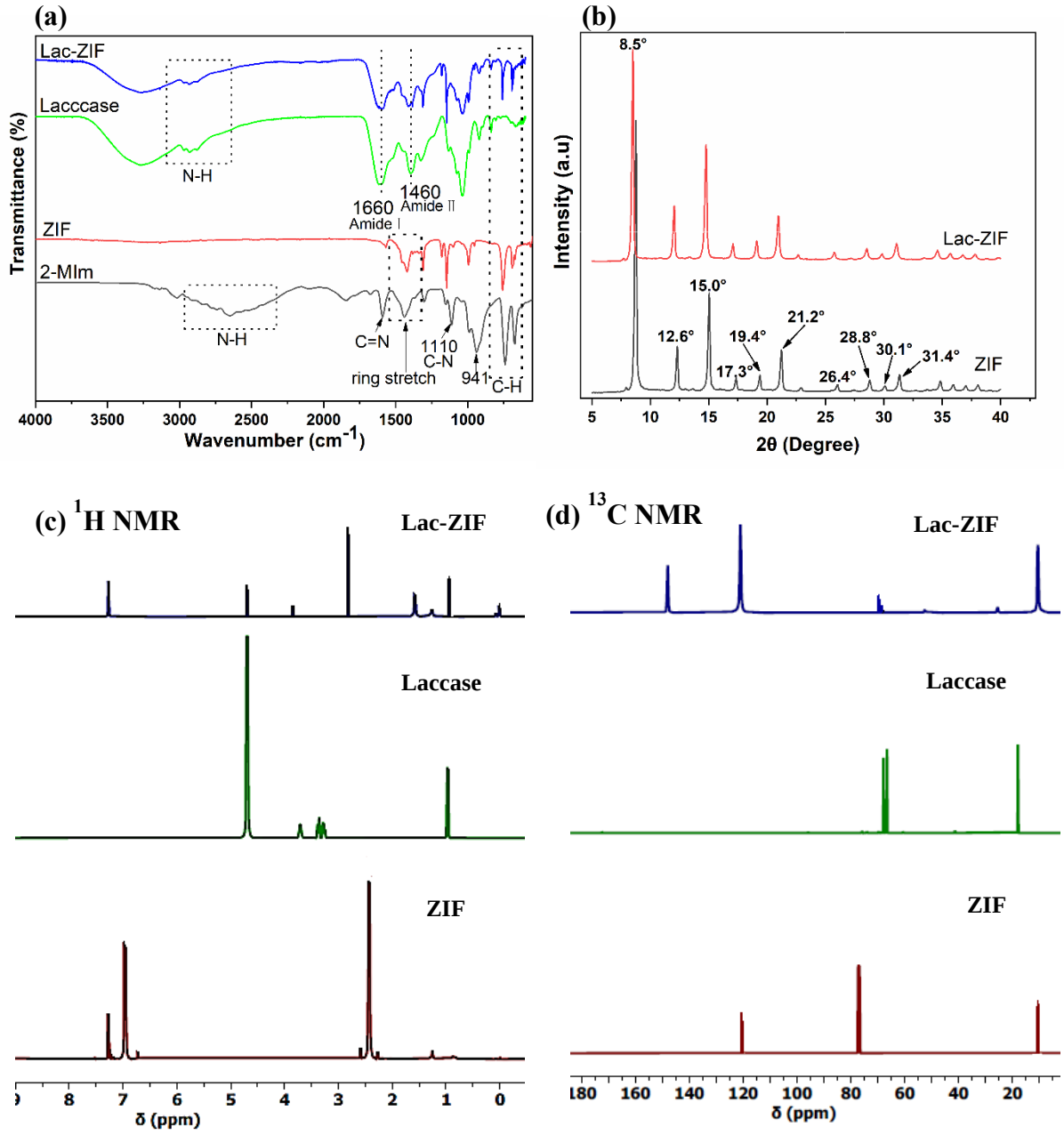


Fig. 1. (a) FTIR spectra of Lac-ZIF, pristine ZIF and 2-methylimidazole (2-MIm), (b) PXRD patterns of ZIF and Lac-ZIF particles, (c) ^{1}H NMR and (d) ^{13}C NMR spectra of Lac-ZIF, ZIF and free laccase.

The amide I at 1660 cm^{-1} may be attributed to C=O stretching vibrations and the amide II at 1460 cm^{-1} is primarily attributed to NH bending vibration and CN stretching vibration in laccase (Cui et al., 2018; Du et al., 2017; Lian et al., 2017). The peak at 1589 cm^{-1} is due to the stretching mode of C=N while the bands in the spectral region 1500 – 600 cm^{-1} are associated with the entire vibration of the ring.

The PXRD patterns of pure ZIF and Lac-ZIF are shown in fig. 1. (b). The diffraction peaks at $2\theta = 7.27°, 12.6°, 14.9°, 16.3°, 17.8°, 21.9°, 26.4°, 27.4°, 29.4°, 30.3°$ were observed, and they are in good agreement with characteristic peaks for pure ZIF crystals in published works (Cui et al., 2018; Mehta et al., 2019). The diffraction peaks for Lac-ZIF composite crystals are similar to that of pure ZIF crystals implying that immobilization of the laccase did not significantly alter the crystalline nature of the ZIF crystals.

The ^{1}H NMR and ^{13}C NMR spectra of Zeolitic Imidazolate Framework (ZIF), free laccase and Lac-ZIF composite are shown in fig. 1. (c) and (d), respectively. From fig. 1. (c), the proton NMR spectra show two distinct signals at 2.42 ppm and 6.95 ppm for ZIF. These signals may be ascribed to the protons from CH_3- and CH- groups in the imidazolate ring, respectively and they are also observed in the spectra for Lac-ZIF. Three more peaks emerged after incorporation of the enzyme which are also distinct in the free laccase enzyme spectra. Similar results were reported by Marreiros *et al* and Karagiaridi *et al* (Karagiaridi et al., 2012; Marreiros et al., 2019). The ^{13}C NMR spectra show three signals attributed to the imidazolate ring of the ZIF molecule at 10.59 ppm, 120.99 ppm, and 147.97 ppm. The two signals at 10.59 ppm and 120.99 ppm correspond to the CH_3- and CH- groups of the 2-methylimidazole ring, respectively (Sneddon et al., 2017). The next signal obtained at 147.97 ppm may be accredited to the carbon atom between the two nitrogen atoms in the imidazole ring. A slight shift of the pure ZIF and laccase signal peaks is observed after immobilization of the enzyme implying environmental or bonding changes of the carbon atoms within the Lac-ZIF structure. Zahmakiran reported similar results in the synthesis and characterization of Iridium nanoparticles stabilized by metal-organic frameworks (Zahmakiran, 2012).

The images from FE-SEM of the as-synthesized ZIF particles and prepared Lac-ZIF composite are shown in fig. 2. (a), (b) and (c). From fig. 2. (a), it can be noted that the ZIF particles exhibited a rhombic dodecahedral morphology, typical of pure ZIF particles (Cui et al., 2018; Lian et al., 2017). Immobilization of the laccase enzyme on the ZIF particles did not alter the dodecahedral structure of the ZIF particles, but crusts of the enzyme are observed on the surface

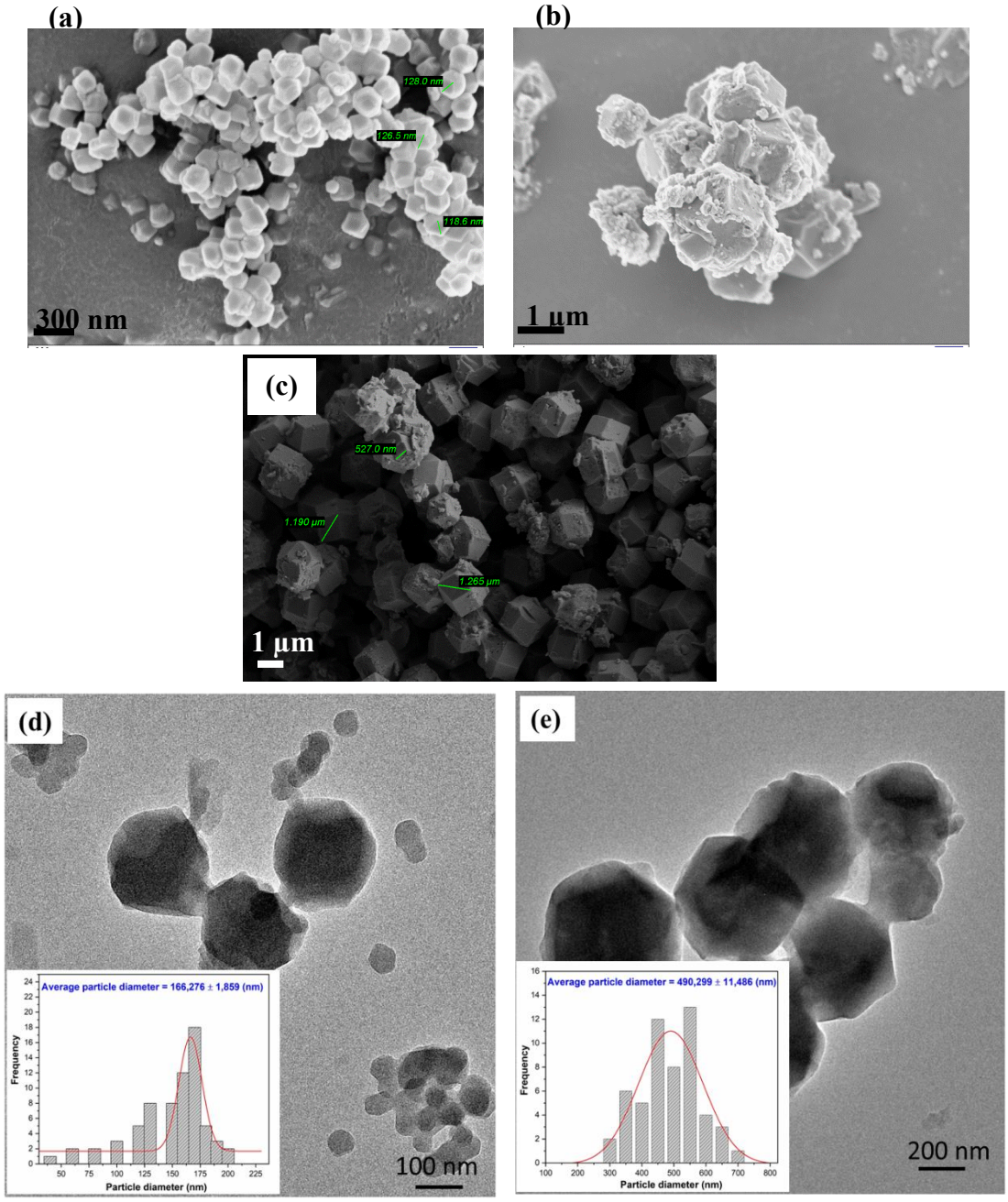


Fig. 2. SEM images of (a) ZIF particles and Lac-ZIF composite (b) and (c); TEM images of (d) ZIF particles and (e) Lac-ZIF composite.

of the ZIF particles as shown in fig. 2. (b). The Lac-ZIF SEM image further shows relative agglomeration of the particles compared to the dispersed pure ZIF particles. The assembling of nanometer-sized ZIF particles influenced by the laccase enzyme resulted in a significant change of the particles to micrometer-sized dodecahedrons. The size of the particles ranges between 118 nm – 128 nm and 520 nm – 1.265 µm for pure ZIF and Lac-ZIF particles as shown in fig. 2. (a) and (c), respectively.

These observations are in agreement with results reported elsewhere (Cui et al., 2018; Mehta et al., 2019; Wu et al., 2019).

Further confirmation of this agglomeration after immobilization of the enzyme onto the ZIF particles was revealed by transmission electron microscopy (TEM) analyses as shown in Fig. 2. (d) and (e). The TEM images show irregularly sized crystals of relatively defined decahedral morphologies of the particles with average diameters of 166.276 ± 1.859 (nm) nm and 490.299 ± 11.486 (nm) for the ZIF and Lac-ZIF, respectively. Similar morphologies were reported elsewhere (Shu et al., 2018).

In a study by P. Tan *et al.* (Tan et al., 2017), it was postulated that drying the ZIF crystals may significantly promote strong covalent bonding formation between the ZIF particles. This postulation as well as the formation of stable structures through binding of the laccase enzyme onto the ZIF particles may result in the witnessed cluster formation. Furthermore, regularly sized and smaller ZIF particles may be potentially achieved via synthesis of the crystals in an ice bath (Beh et al., 2018). However, for this study, syntheses of the ZIF and Lac-ZIF were carried out at ambient temperatures, this is because extremely low temperatures may compromise the enzyme's binding potential onto the ZIF particles due to possible reduction in molecular motions and potential collision between the enzyme and the crystals (Stwalley, 2004). Nonetheless, the crystalline nature of the ZIF after enzyme immobilization particles was preserved.

3.2 Biochemical characterization of the free and immobilized laccase

3.2.1 Enzyme Assay

Fig. 3. (a) shows the results for the effect of pH (3 – 10) on the activity of laccase. It may be observed that both free and immobilized laccase exhibited the highest activity at pH 7 corresponding to 3.9 U mL^{-1} and 7.6 U g^{-1}, respectively. For the free laccase, its activity was almost stable and maintained from pH 3 to 7 and then dropped significantly at pH values 8 – 10 (0.05 U mL^{-1}). For the immobilized laccase, the activity was relatively lower than that of free laccase at low pH (4 and 6) and considerably higher at elevated pH values, which may be attributed to possible disintegration of the ZIF structure in acidic environments (Wu et al., 2015). However, it is worth noting that immobilization of the laccase onto the ZIF particles

improved the enzyme's resistance to alkaline conditions with comparatively similar activity at acidic conditions.

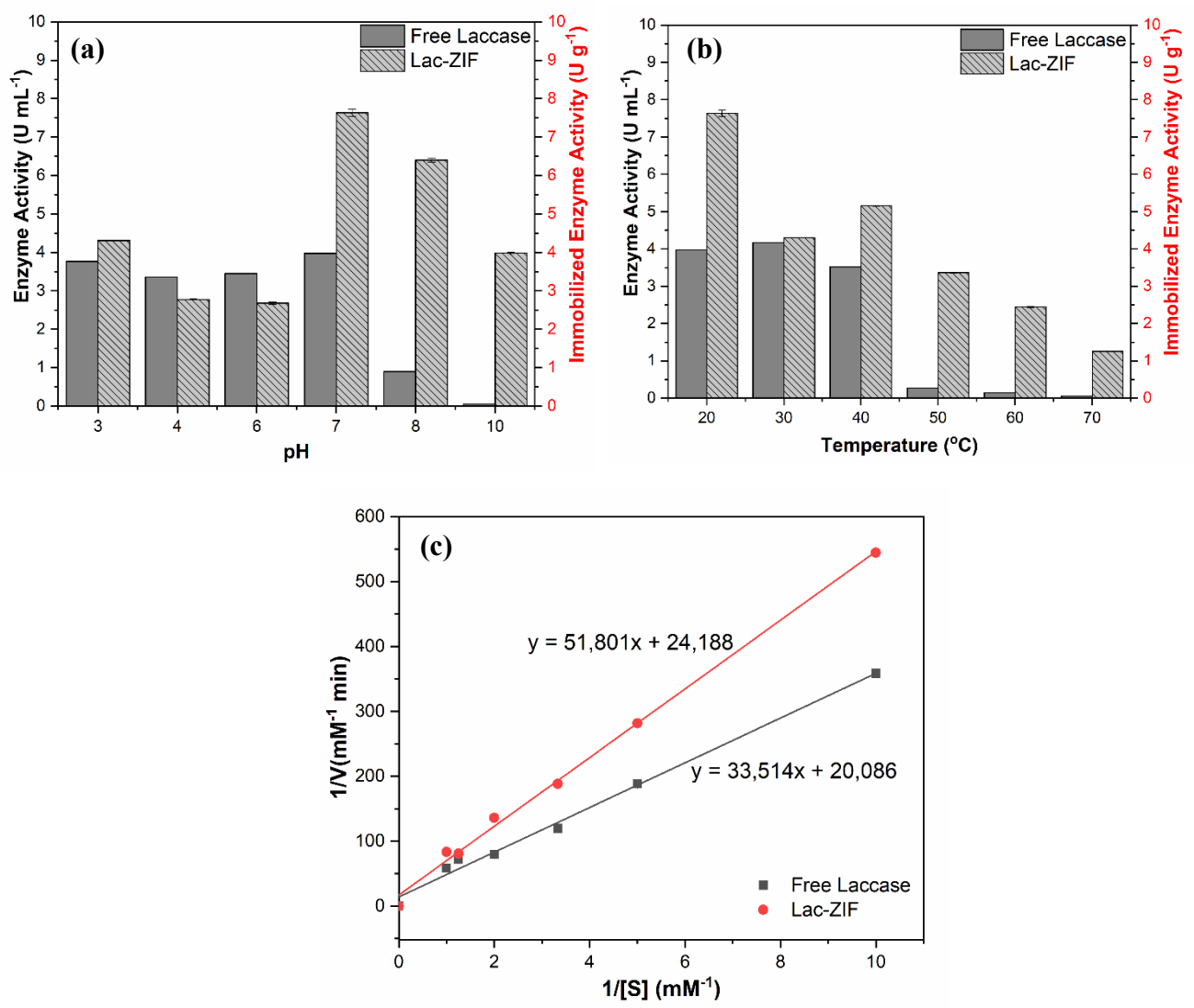


Fig. 3. Enzyme activity –Effect of (a) pH, (b) Temperature and (c) Lineweaver-Burk plots for free laccase and Lac-ZIF, for the determination of V_{max} and K_m.

The influence of temperature on laccase activity was studied over a temperature range of 20 – 70 °C and the results are presented in fig. 3. (b). The results show a general decline in enzyme activity as the temperature increased from 20 to 70 °C, with the optimum activities observed at 20 °C. The calculated enzyme activities decreased significantly from 4 to 0.1 U mL^{-1}, and 7.6 to 1.5 U g^{-1} for the free laccase and Lac-ZIF composite, respectively. These low enzyme activities for the free laccase may be ascribed to the potential denaturing of the enzyme afforded by the disintegration of the protein structure. For the immobilized laccase, however, the slow deterioration of activity may be attributed to the protection exerted by the ZIF envelope against

the temperature changes in the outside environment (Lassouane et al., 2019). Therefore, the ZIF support enhanced the thermal stability of the enzyme, hence the observed relatively high activities for Lac-ZIF across all studied temperatures.

3.2.2 Kinetic parameters of free and immobilized laccase

The laccase kinetics investigation was based on the determination of Km and Vmax for free and immobilized laccase in the presence of ABTS as a substrate at concentrations ranging from 0.1 to 1 mM. The Michaelis Menten kinetics constant (K_m) and the maximal velocity (V_{max}) were determined using a Lineweaver-Burk double reciprocal plot (Fig. 3. (c)). The calculated K_m values were 1.768 and 2.142 mM for the free and immobilized laccase, respectively. The increase in K_m after attachment of the enzyme indicates reduced affinity between the enzyme and the substrate. This phenomenon has been reported in published works (Wu et al., 2019). The K_m upsurge may be ascribed to the stable structure between the laccase and the ZIF particles, which limits the spatial freedom of the laccase molecules (Salgaonkar et al., 2019; Wu et al., 2020). This results in both restrictions for substrate (ABTS) diffusion and steric hindrance of the laccase enzyme (Farzi et al., 2019). These factors also affect the maximum velocity (V_{max}) for the enzyme. Consequently, the calculated V_{max} values were 0.0498 and 0.0413 mM min^{-1} for the free laccase and Lac-ZIF composite, respectively.

3.3 Carbamazepine decontamination from water

3.3.1 Effect of solution pH

Fig. 4 (a). depicts the results of a study that investigated the effect of solution pH on CBZ removal using ZIF, free laccase, and immobilized laccase over a pH range of 3 – 10. The results revealed an improvement in the removal efficiency when using the immobilized laccase. With a pH increase from 3 to 7, there is a general improvement in elimination efficiency. Beyond the neutral pH region, however, the free laccase enzyme activity decreased significantly. This is most likely due to the inhibition induced by the binding of the hydroxide anion (OH^-) to the T2/T3 copper of the laccase active site, which disrupts the internal electron transfer from T1 to T2/T3 copper sites (Lassouane et al., 2019). The proposed mechanism of action of the Lac-ZIF is shown in fig 4. (b). For Lac-ZIF and ZIF, the removal efficiency increases with a rise in pH from 3 to 8 and declines slightly at pH 10. The highest observed removal efficiency (11 %) was at pH 7 using free laccase, 29 % with ZIF particles, and 35 % when using Lac-ZIF at pH 8. It can also be observed that the immobilization of laccase onto zeolitic imidazolate framework particles improved the stability and resistance of the enzyme to both acidic and

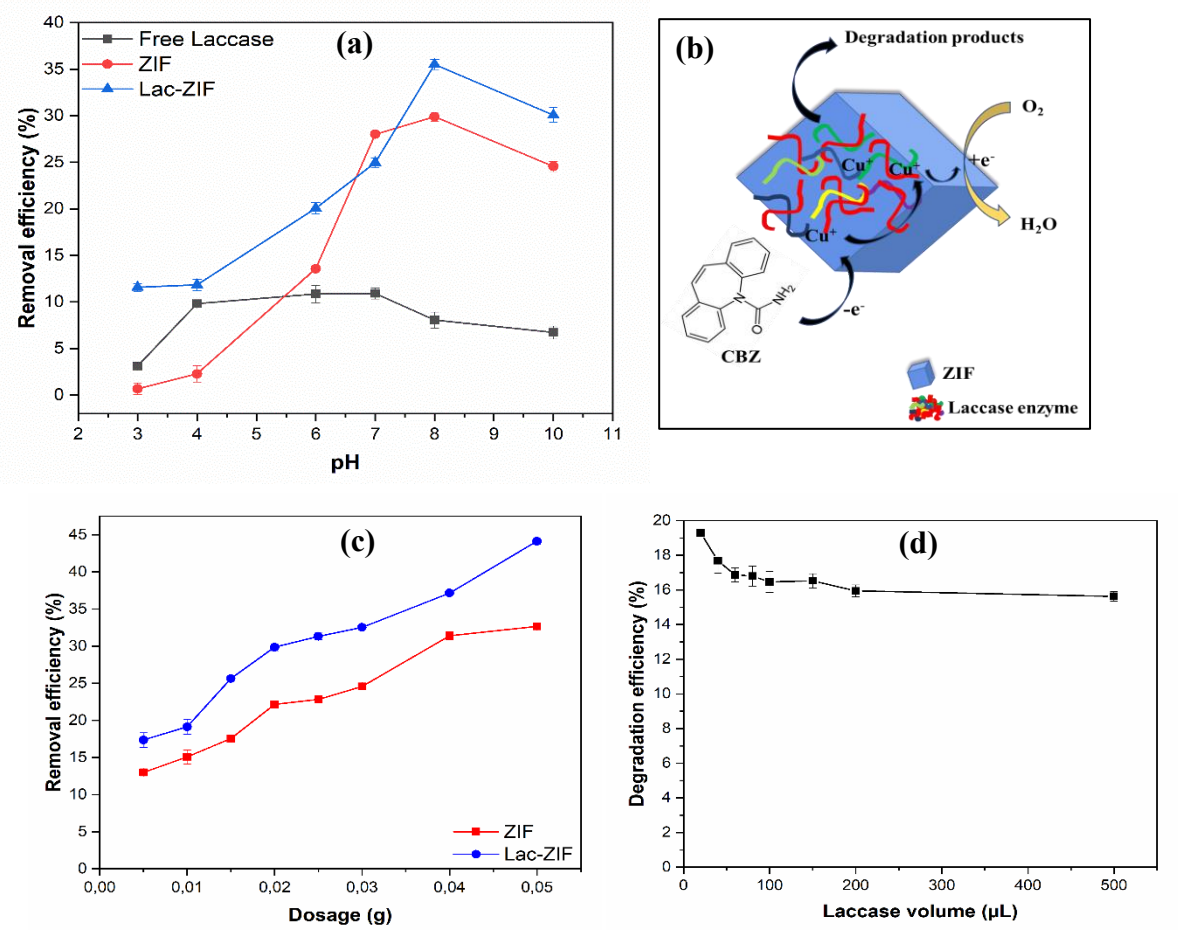


Fig. 4. (a) Effect of pH on CBZ removal using free laccase, ZIF and Lac-ZIF composite; (b) Proposed conceptual degradation mechanism; Effect of ZIF, Lac-ZIF mass (c), and free laccase volume (d) on CBZ removal

alkaline environments as a consequence of the relatively high chemical stability of the Lac-ZIF composite. Additionally, the enhanced removal of CBZ by Lac-ZIF may be attributed to synergistic biodegradation and adsorption effects of CBZ molecules offered by the composite. The low removal efficiencies observed at acidic conditions for both Lac-ZIF and ZIF particles may be attributed to possible disintegration of the ZIF structure at pH values below 5 as a result of Zinc ion leakages (Wu et al., 2015). Since high removal efficiencies were observed at pH 8, all successive studies were investigated in phosphate buffer solutions maintained at pH 8.

3.3.2 Dosage effect

To investigate the effect of ZIF, Lac-ZIF mass, and volume of free laccase on CBZ removal, varying masses of the adsorbent, composite, and volumes of free laccase were used, with maintained solution pH 8 in a phosphate buffer. The results are shown in fig. 4. (c) and (d). According to fig. 4. (c), the removal effectiveness of CBZ improves as the dosage of both the Lac-ZIF composite and the ZIF adsorbent increases. With the graph ascending, the maximum

CBZ elimination was reported to be 44% for 0.05 g Lac-ZIF and 32% for 0.05 g ZIF, indicating the possibility of even higher degradation efficiencies with higher dosages of the Lac-ZIF composite. This trend may be accredited to the fact that increasing the dose results in greater amounts of adsorbent and/or composite particles and a subsequent increase in the accessibility of sorption and degradation active sites (Pang et al., 2015). In contrast, as shown in fig. 4. (d), the CBZ breakdown efficiency utilizing free laccase enzyme declined as the enzyme volume increased. The highest degradation efficiency, 19% was achieved at 20 µL of the free laccase enzyme. Increasing the enzyme loading may potentially increase the layers around the substrate (CBZ), resulting in a decreased interaction between the enzyme and the substrate bulk, hence the observed diminishing removal efficiencies with laccase volume (Du et al., 2017). As a result, the optimal dosages for all subsequent studies were 0.05 g and 20 µL for the Lac-ZIF and free laccase, respectively.

3.3.3 Effect of initial CBZ concentration

The effect of initial CBZ concentration was investigated across a range of 3 – 20 mg L^{-1}, with the results given in fig. 5. (a). The results demonstrate a substantial increase in CBZ elimination from 3 to 5 mg L^{-1}, with a subsequent small decline at higher initial concentrations for all three materials (ZIF, Lac-ZIF, and free laccase). In all the studied concentration ranges, CBZ removal by Lac-ZIF was supreme over both ZIF and free laccase enzyme with the highest removal (55%) observed at 5 mg L^{-1}. The slight decline at high concentrations may be qualified by possible diminishing active sites for CBZ attachment and degradation on the biocatalyst and adsorbent (Nadar and Rathod, 2016; Zdarta et al., 2019). However, at low CBZ concentrations, the initial substrate concentration may not be sufficient to overcome the potential mass transfer resistance between aqueous and solid phases, as well as steric hindrances formed when free enzyme aggregates in solution, resulting in slightly lower removal efficiencies at low initial analyte concentrations (Hoseinzadeh et al., 2021). In theory, increasing the substrate concentration leads to more collisions between molecules, and the analyte's

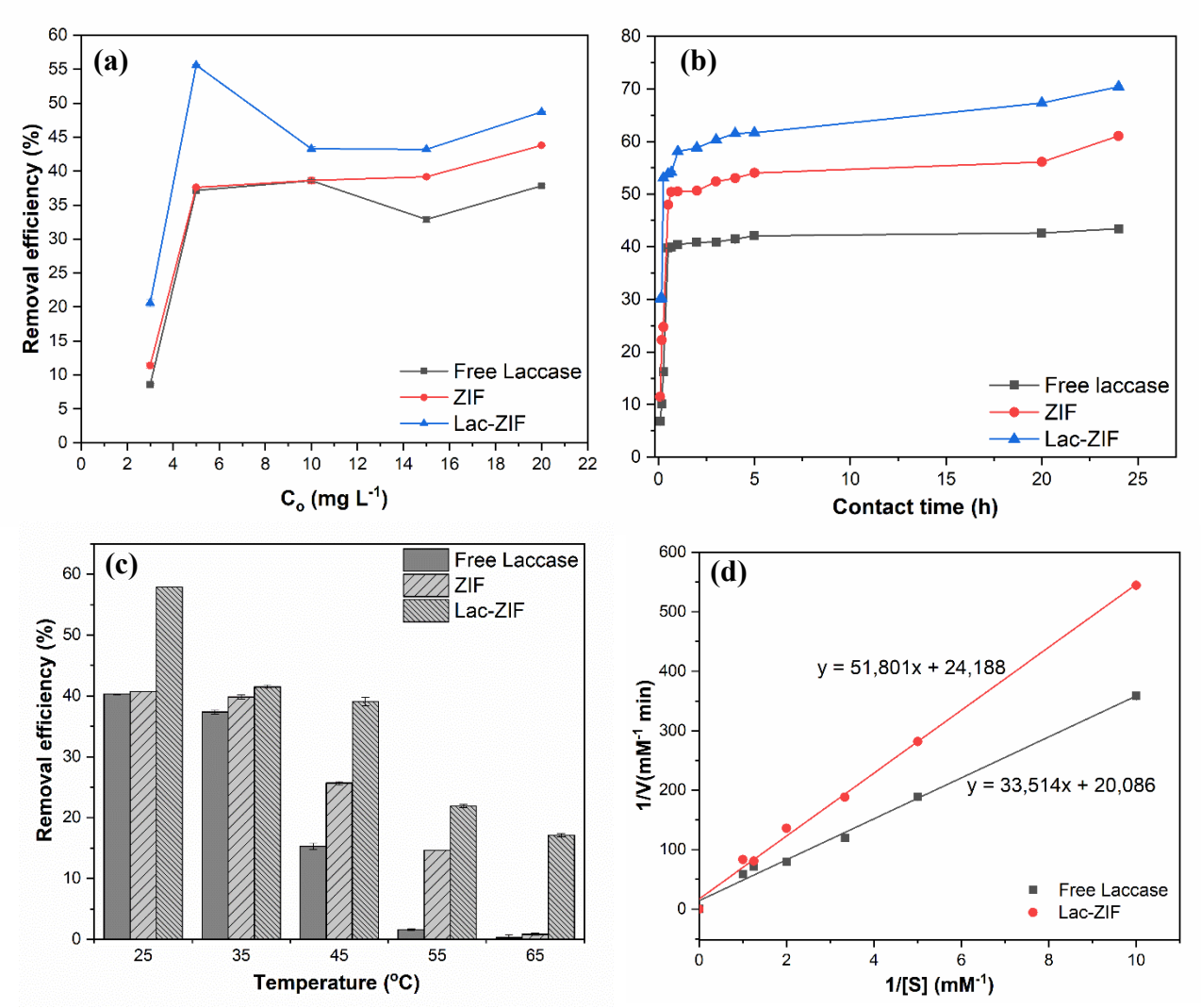


Fig. 5. Effect of (a) initial CBZ concentration, (b) contact time and (c) temperature on CBZ removal; (d) Lineweaver-Burk plots for free laccase and Lac-ZIF for the determination of V_{max} and K_m using CBZ as substrate

adsorption/degradation rate is expected to increase until equilibrium attainment (Cao et al., 2022; Ewis et al., 2022; Gao et al., 2015; Liang et al., 2021; Saravanan et al., 2021). Nevertheless, the overall results show that immobilization of the laccase enzyme considerably increased CBZ removal across the concentration range studied.

3.3.4 Effect of contact time

The effect of contact time was studied over a 24 h period under constant agitation at 160 rpm, with aliquots of the CBZ solutions drawn at predetermined time intervals. Fig. 5. (b) depicts the findings of the contact time influence on CBZ elimination. It can be observed that an increase in contact time resulted in a rise in CBZ elimination until equilibrium attainment. The removal efficiency by Lac-ZIF was highest in all investigated time intervals compared to free

laccase and ZIF particles. The CBZ removal was of quick kinetics in the first 30 minutes reaching 53% for Lac-ZIF, 47% for ZIF, and 39% for free laccase. Lac-ZIF showed a remarkable 70% removal efficiency at 24 h followed by ZIF particles (60%) with the least being free laccase at 43%. These high removal efficiencies may be due to the prolonged interaction of the substrate with the enzyme and Lac-ZIF composite.

3.3.5 Effect of temperature

The thermostability investigations of the free laccase, Lac-ZIF, and ZIF material in CBZ removal were carried out across a temperature range of 25 – 65 °C, and the results are shown in fig. 5. (c) The removal efficiency by Lac-ZIF reigns supreme over free laccase and ZIF material across all the studied temperatures. The maximum CBZ removal efficiency (57.8% for Lac-ZIF, 40.7% for ZIF, and 40.1% for free laccase) was observed at 25 ℃ with the least efficiency at 65 ℃. The enhancement in removal efficiency for the immobilized laccase may be due to the multiple linkages between the support and enzyme, through which the laccase is attached to the porous zeolitic imidazolate framework, with a subsequently stable conformation (Knedel et al., 2019; Samui and Sahu, 2018). Furthermore, the improved removal may be attributable to the synergistic adsorption and biocatalytic degradation effects offered by the ZIF particles and laccase enzyme, respectively. Conversely, the free laccase undergoes conformational alterations as the temperature increases, resulting in the efficiency diminishing at a faster rate than that of the immobilized laccase (Gascón et al., 2017). The observed decline in CBZ removal efficacy at high temperatures can be ascribed to Le Chatelier's principle of reversible reactions, whereby elevated temperatures encourage the escape of analyte molecules from the interface, hence diminishing the sorption potential of the CBZ (Pillai et al., 2019; Smitha and Thampi, 2017)

3.3.6 Kinetic parameters for the free and immobilized laccase

In general, enzymatic processes follow the Michaelis-Menten kinetic model, according to which the degradation rate is of first-order at very low substrate concentrations (Naghdi et al., 2018b). The kinetics of the laccase was based on the evaluation of K_m and V_{max} for the free and immobilized laccase deduced from Lineweaver-Burk plots as shown in fig. 5. (d), over a concentration range of 3 – 20 mg L^{-1}. The evaluated K_m values increased after immobilization which were calculated to be 28,827 ± 0.060 mg L^{-1} and 44,403 ± 0.067 mg L^{-1} for the free and immobilized laccase (Lac-ZIF), respectively, as shown in table 1. As explained in the previous section, a potential affinity decline between the enzyme and the substrate (CBZ) may be the cause for this. In this sense, the stable structure between the laccase and ZIF particles restricts

the spatial freedom of the laccase molecules (Knedel et al., 2019). The immobilization, therefore, leads to limited CBZ diffusion and steric hindrance of the laccase enzyme (Wu et al., 2019). These factors also adversely affect the maximum velocity (V_{max}) for the enzyme. Accordingly, the V_{max} values were evaluated to be 0,873 ± 0.005 mg L^{-1} h^{-1} and 0,692 ± 0.004 mg L^{-1} h^{-1} for the free laccase and Lac-ZIF, respectively, as presented in table 1.

Table 1. Kinetic parameters of free laccase and Lac-ZIF

Form	**K_m (mg L^{-1})**	**V_{max} (mg L^{-1} h^{-1})**
Free laccase	28.827 ± 0.060	0.873 ± 0.005
Lac-ZIF	44.103 ± 0.067	0.692 ± 0.004

3.3.7 Stability of biocatalyst in water-miscible organic solvents

To ascertain the feasibility of the biocatalyst for application in wastewater treatment, it is imperative to investigate its stability in water-miscible organic solvents. In this sense, the free laccase, pure ZIF, and Lac-ZIF were tried out in CBZ degradation in the presence of organic solvents including methanol, ethanol, acetone, and acetonitrile. The results are shown in fig. 6. (a) These results illustrate an improved removal efficiency (slightly over 50%) by the immobilized laccase in all studied solvents over both free and ZIF particles. Acetonitrile and acetone did not have a significant effect on the CBZ removal efficiency. Contrarily, ethanol and methanol resulted in diminished CBZ degradation efficiencies for the free laccase, which were 29% and 30%, respectively. The hydrophilic organic solvents may replace the water-shell bound to the protein surface, consequently altering the protein structure, hence the diminished removal efficiencies (Knedel et al., 2019; Wu et al., 2019). For the immobilized enzyme, the ZIF particles may protect the enzyme by forming stable structures and preventing direct contact of the enzyme with the organic solvents, thus less prone to conformational changes. This improvement of organic solvent stability would facilitate the application of immobilized laccase in wastewater treatment.

3.3.8 Storage stability

The storage stability of a biocatalyst is a critical measure of the reliability of the biocatalytic material. The free laccase, ZIF, and Lac-ZIF composite were stored at room temperature for

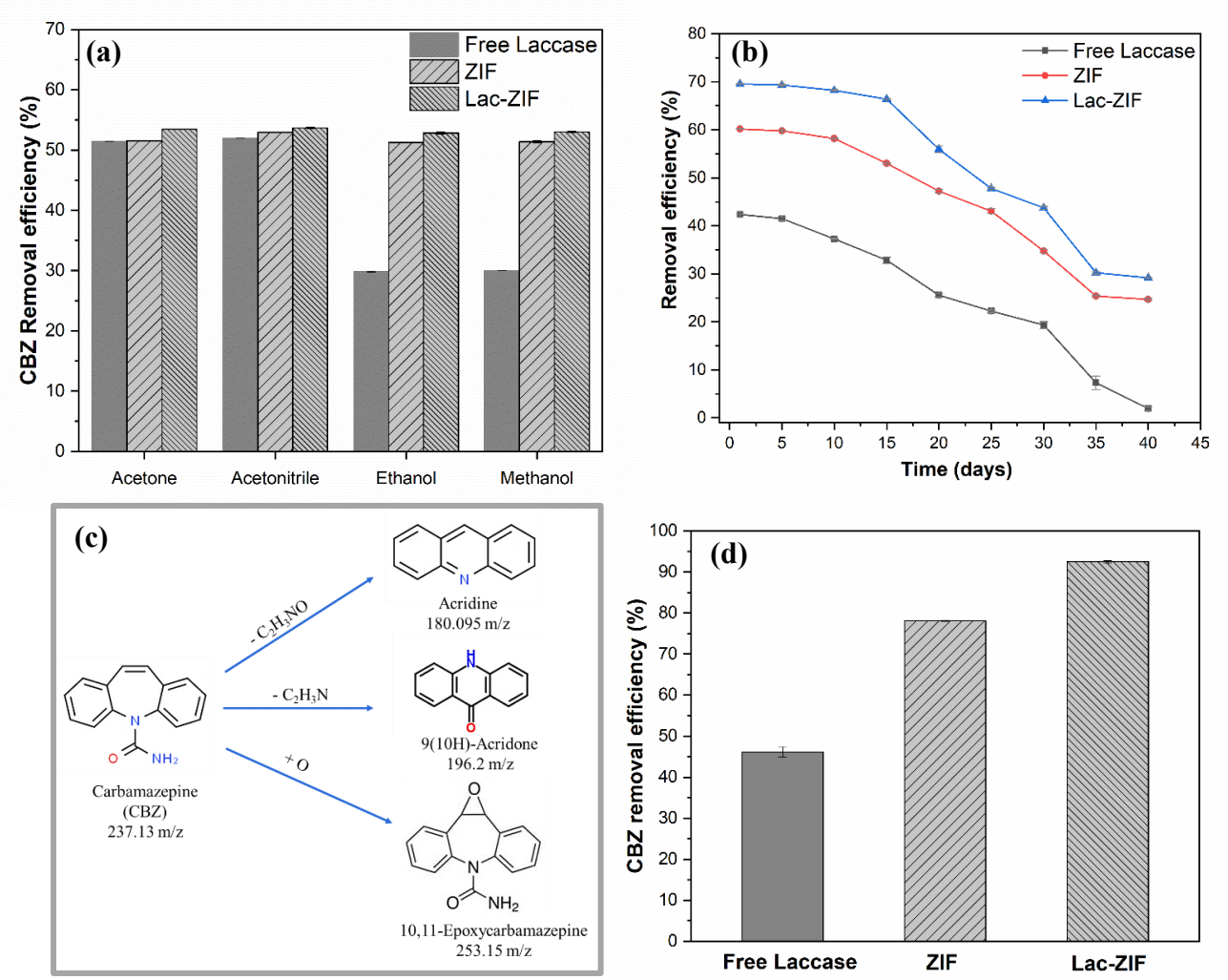


Fig. 6. Effect of (a) organic solvents on CBZ degradation, (b) storage stability studies for the biocatalytic materials, (c) CBZ biotransformation metabolites identified via LC-MS and (d) application of free laccase, ZIF and Lac-ZIF in real water remediation

storage stability experiments, and their CBZ removal efficiencies were assessed over 40 d at 5-day intervals, as shown in fig. 6. (b) The immobilized laccase (Lac-ZIF) demonstrated an improved removal efficiency compared to the degradation by free laccase and adsorption by pure ZIF crystals. It may be observed that the Lac-ZIF exhibits an almost stable activity (~70%) between 1 and 15 d, with the free laccase showing a gradual reduction (43% - 33%) for the same period. The appreciable storage stability of the Lac-ZIF may be accredited to possible shielding effects of the framework around the enzyme molecules, as well as the formation of stable structures with the enzyme (Salgaonkar et al., 2019). Therefore, the active sites and overall configuration of the laccase molecules may not undergo distortion under the storage conditions, contrary to the free laccase. The removal efficiencies by free laccase reached as little as 1.9% due to possible protein decomposition of the enzyme in the studied conditions.

3.3.9 CBZ biotransformation metabolites identification

To identify the molecular ions of CBZ transformation products, suggest empirical formulas, and deduce their chemical structures, a positive scan mode from LC-MS (range, *m/z* 50 – 300) was employed yielding a total of three CBZ metabolites. The metabolites eluted at 2.4, 2.6, and 3.1 min on the reverse-phase column with molecular ions at *m/z* 253.15, 180.095, and 196.2 in positive scan mode, respectively. Their molecular weights were thus determined to be 252.1, 179.09, and 195.2 g mol^{-1} with the corresponding formulas of ($C_{15}H_{12}N_2O_2$), ($C_{13}H_9N$), and ($C_{13}H_9NO$) and molecular structures illustrated in fig. 6. (c). The metabolite formed by simple atomic oxygen addition was identified as 10,11-epoxycarbamazepine ($C_{15}H_{12}N_2O_2$) and the two other by-products were identified as acridine ($C_{13}H_9N$) and 9(10H)-acridone ($C_{13}H_9NO$) created by the elimination of (C_2H_3NO) and (C_2H_3N) from CBZ, respectively. Similar results have been reported in previous studies on CBZ degradation (Alharbi et al., 2019; Jelic et al., 2012; Naghdi et al., 2018b). In a study by Alharbi *et al* (2019) the formation of 11-epoxycarbamazepine and acridone when CBZ is degraded by laccase from *Trametes versicolor* was reported (Alharbi et al., 2019). Other CBZ transformation products with molecular ions at *m/z* 165.5, 271.1, and 180 g mol^{-1} have been reported in literature (Alharbi and Price, 2017; Jelic et al., 2012; Ji et al., 2016; Naghdi et al., 2018b, 2017). However, these by-products were not detected under the experimental conditions used in this study, such as the absence of mediators and solely employing an enzyme to achieve CBZ degradation. Other products were a result of ABTS mediator enhancements and UV photolysis.

Since the identified biotransformation products have already been reported in previous studies, their potential toxicity was also assessed using luminescent bacteria such as *Photobacterium leiognathi* (Alharbi et al., 2019) and *Vibrio fischeri* (Jelic et al., 2012) as well as algal viability (Ji et al., 2016). According to the findings of these studies, the investigated CBZ metabolites had no significant toxicity toward the bacteria and algal cells. It should be noted, however, that the toxicity in these studies was limited to specific bacteria and algal cells. As a result, more comprehensive toxicity assessments, particularly the persistent and symbiotic ecotoxicological effects on other biologicals in real aquatic environments, are required before this process can be implemented at full scale.

3.3.10 Method validation for CBZ determination

A linear regression analysis of CBZ standard calibration curve was evaluated over a concentration range of 0.5 – 5 mg L^{-1} giving a good precision with low relative standard deviations (below 4%) and an R^2 value of 0.998. The detection and quantification limits (LOD and LOQ) were evaluated to be 0.690 and 2.092 mg L^{-1}, respectively. The identification of CBZ was done on the LC-qTOF/MS using retention times and 194 m/z fragmentation ion.

4. Application of the synthesized Lac-ZIF composite in real water samples

To investigate the applicability of the biocatalyst in real water remediation, real river samples were collected and spiked with known concentrations of CBZ solutions and agitated in a rotary shaker in the presence of free laccase, ZIF crystals and Lac-ZIF composite. The river water samples exhibited the following physicochemical properties: pH 7.27, conductivity: 865 µS cm^{-1}, total dissolved solids: 366 ppm, dissolved oxygen: 7.41 ppm. The decontamination efficiencies are presented in fig. 6. (d). It is worth noting that the as-synthesized Lac-ZIF proved effective in removing the CBZ from the water, reaching an efficiency of ~92%. The pristine ZIF crystals and free laccase only removed up to ~78% and ~46% of the CBZ from the samples. The increased removal efficiency by the Lac-ZIF may be attributable to the combined effects of adsorption and degradation provided by the ZIF particles and laccase enzyme, respectively. Immobilization of the enzyme, therefore, significantly improved the performance of the biocatalyst in real water CBZ decontamination.

The observed low removal efficiency by free laccase could be ascribed to possible shielding of the enzyme's active site by other organic compounds present in the water, which could be potential substrates for the laccase, as well as probable disintegration of the protein structure in toxic dissolved compounds in the water, among other factors (Knedel et al., 2019; Wu et al., 2019).

5. Conclusions

In this study, zeolitic imidazolate framework particles were successfully prepared via a facile synthetic route and used as porous support structures for laccase enzyme immobilization. Immobilization of the laccase enzyme was confirmed by characterization techniques such as FTIR, NMR, PXRD, SEM, and TEM. The as-synthesized ZIF particles displayed typical crystalline diffraction peaks with maintained crystallinity after immobilization of the laccase enzyme. Morphological analyses revealed agglomerated rhombic dodecahedrons of the ZIF nanocrystals, sparsely coated with irregular distribution of the enzyme. Immobilization of the

laccase onto the Zeolitic Imidazolate Framework demonstrated improved biocatalytic and stability properties compared to the free laccase enzyme. Kinetic investigations, on the other hand, demonstrated a minor decrease in maximum enzyme velocity (V_{max}) (from 0.873 to 0.692 mg L^{-1} h^{-1}) following immobilization, signifying a slight reduction in the biocatalytic degradation rate of the carbamazepine under the investigated concentration range. Nevertheless, the immobilized laccase displayed substantial stability towards organic solvents and a consequently remarkable performance in real river water sample remediation reaching ~92% removal efficiencies. Additionally, the Lac-ZIF composite exhibited significant storage stabilities (~70% residual activity) for up to 15 d before any notable reduction in activity was observed. As a result, Zeolitic Imidazole Frameworks could be effective laccase enzyme immobilization supports for wastewater remediation.

Acknowledgements

This work was supported by the Council for Scientific and Industrial Research (CSIR) – Department of Science and Innovation (DSI) Inter-Programme Postgraduate Bursary (IBS).

References

Ahmad, R., Shanahan, J., Rizaldo, S., Kissel, D.S., Stone, K.L., 2020. Co-immobilization of an enzyme system on a metal-organic framework to produce a more effective biocatalyst. Catalysts 10. https://doi.org/10.3390/catal10050499

Al-Mashaqbeh, O.A., Snow, D.D., Alsafadi, D.A., Alsalhi, L.Z., Bartelt-Hunt, S.L., 2021. Removal of carbamazepine onto modified zeolitic tuff in different water matrices: Batch and continuous flow experiments. Water (Switzerland) 13. https://doi.org/10.3390/w13081084

Alharbi, S.K., Nghiem, L.D., van de Merwe, J.P., Leusch, F.D.L., Asif, M.B., Hai, F.I., Price, W.E., 2019. Degradation of diclofenac, trimethoprim, carbamazepine, and sulfamethoxazole by laccase from Trametes versicolor: Transformation products and toxicity of treated effluent. Biocatal. Biotransformation. https://doi.org/10.1080/10242422.2019.1580268

Alharbi, S.K., Price, W.E., 2017. Degradation and Fate of Pharmaceutically Active Contaminants by Advanced Oxidation Processes. Curr. Pollut. Reports 3, 268–280. https://doi.org/10.1007/s40726-017-0072-6

Alshabib, M., Onaizi, S.A., 2019. A review on phenolic wastewater remediation using homogeneous and heterogeneous enzymatic processes: Current status and potential challenges. Sep. Purif. Technol. 219, 186–207. https://doi.org/10.1016/j.seppur.2019.03.028

Asif, M.B., Hai, F.I., Singh, L., Price, W.E., Nghiem, L.D., 2017. Degradation of Pharmaceuticals and Personal Care Products by White-Rot Fungi—a Critical Review. Curr. Pollut. Reports 3, 88–103. https://doi.org/10.1007/s40726-017-0049-5

Bahlmann, A., Brack, W., Schneider, R.J., Krauss, M., 2014. Carbamazepine and its metabolites in wastewater: Analytical pitfalls and occurrence in Germany and Portugal. Water Res. 57, 104–114. https://doi.org/10.1016/j.watres.2014.03.022

Becker, D., Varela Della Giustina, S., Rodriguez-Mozaz, S., Schoevaart, R., Barceló, D., de Cazes, M., Belleville, M.P., Sanchez-Marcano, J., de Gunzburg, J., Couillerot, O., Völker, J., Oehlmann, J., Wagner, M., 2016. Removal of antibiotics in wastewater by enzymatic treatment with fungal laccase – Degradation of compounds does not always eliminate toxicity. Bioresour. Technol. 219, 500–509. https://doi.org/10.1016/j.biortech.2016.08.004

Beh, J.J., Lim, J.K., Ng, E.P., Ooi, B.S., 2018. Synthesis and size control of zeolitic imidazolate framework-8 (ZIF-8): From the perspective of reaction kinetics and thermodynamics of nucleation. Mater. Chem. Phys. 216, 393–401. https://doi.org/10.1016/j.matchemphys.2018.06.022

Bhattacharya, S., Banerjee, P., Das, P., Bhowal, A., Majumder, S.K., Ghosh, P., 2020. Erratum: Removal of aqueous carbamazepine using graphene oxide nanoplatelets: Process modelling and optimization (Sustainable Environment Research (2020) 30 (17) DOI: 10.1186/s42834-020-00062-8). Sustain. Environ. Res. 30. https://doi.org/10.1186/s42834-020-00066-4

Bilal, M., Adeel, M., Rasheed, T., Iqbal, H.M.N., 2019. Multifunctional metal-organic frameworks-based biocatalytic platforms: Recent developments and future prospects. J. Mater. Res. Technol. 8, 2359–2371. https://doi.org/10.1016/j.jmrt.2018.12.001

Burkina, V., Sakalli, S., Pilipenko, N., Zlabek, V., Zamaratskaia, G., 2018. Effect of human pharmaceuticals common to aquatic environments on hepatic CYP1A and CYP3A-like activities in rainbow trout (Oncorhynchus mykiss): An in vitro study. Chemosphere 205, 380–386. https://doi.org/10.1016/j.chemosphere.2018.04.080

Cao, X., Gao, J., Yang, Y., Li, H., Zheng, X., Liu, G., Jiang, Y., 2022. Synergistic degradation of chlorophenol pollutants by a photo-enzyme integrated catalyst. J. Environ. Chem. Eng. 10, 107909. https://doi.org/10.1016/j.jece.2022.107909

Chen, Z., Wang, X., Islamoglu, T., Farha, O.K., 2019. Green Synthesis of a Functionalized Zirconium-Based Metal–Organic Framework for Water and Ethanol Adsorption. Inorganics 7, 56. https://doi.org/10.3390/inorganics7050056

Cui, J., Feng, Y., Jia, S., 2018. Silica encapsulated catalase@metal-organic framework composite: A highly stable and recyclable biocatalyst. Chem. Eng. J. 351, 506–514. https://doi.org/10.1016/j.cej.2018.06.121

Demarche, P., Junghanns, C., Nair, R.R., Agathos, S.N., 2012. Harnessing the power of enzymes for environmental stewardship. Biotechnol. Adv. 30, 933–953. https://doi.org/10.1016/j.biotechadv.2011.05.013

Ding, H., Wu, Y., Zou, B., Lou, Q., Zhang, W., Zhong, J., Lu, L., Dai, G., 2016. Simultaneous removal and degradation characteristics of sulfonamide, tetracycline, and quinolone antibiotics by laccase-mediated oxidation coupled with soil adsorption. J. Hazard. Mater. 307, 350–358. https://doi.org/10.1016/j.jhazmat.2015.12.062

Du, Y., Gao, J., Zhou, L., Ma, L., He, Y., Huang, Z., Jiang, Y., 2017. Enzyme nanocapsules armored by metal-organic frameworks: A novel approach for preparing nanobiocatalyst. Chem. Eng. J. 327, 1192–1197. https://doi.org/10.1016/j.cej.2017.07.021

Ebrahimzadeh, S., Castiglioni, S., Riva, F., Zuccato, E., Azzellino, A., 2021. Carbamazepine levels related to the demographic indicators in groundwater of densely populated area. Water (Switzerland) 13. https://doi.org/10.3390/w13182539

Ewis, D., Ba-Abbad, M.M., Benamor, A., El-Naas, M.H., 2022. Adsorption of organic water pollutants by clays and clay minerals composites: A comprehensive review. Appl. Clay Sci. 229, 106686. https://doi.org/10.1016/j.clay.2022.106686

Farzi, A., Dehnad, A., Fotouhi, A.F., 2019. Biodegradation of polyethylene terephthalate waste using Streptomyces species and kinetic modeling of the process. Biocatal. Agric. Biotechnol. 17, 25–31. https://doi.org/10.1016/j.bcab.2018.11.002

Gadberry, J.A., Marrero-rivera, P., 2018. Removal of Carbamazepine from Drinking Water.

Gao, W.W., Zhang, F.X., Zhang, G.X., Zhou, C.H., 2015. Key factors affecting the activity and stability of enzymes in ionic liquids and novel applications in biocatalysis. Biochem. Eng. J. 99, 67–84. https://doi.org/10.1016/j.bej.2015.03.005

Gascón, V., Castro-Miguel, E., Díaz-García, M., Blanco, R.M., Sanchez-Sanchez, M., 2017. In situ and post-synthesis immobilization of enzymes on nanocrystalline MOF platforms to yield active biocatalysts. J. Chem. Technol. Biotechnol. 92, 2583–2593. https://doi.org/10.1002/jctb.5274

Gascón, V., Jiménez, M.B., Blanco, R.M., Sanchez-Sanchez, M., 2018. Semi-crystalline Fe-BTC MOF material as an efficient support for enzyme immobilization. Catal. Today 304, 119–126. https://doi.org/10.1016/j.cattod.2017.10.022

Gasser, C.A., Ammann, E.M., 2014. Laccases to take on the challenge of emerging organic contaminants in wastewater. https://doi.org/10.1007/s00253-014-6177-6

Hampel, M., Blasco, J., Babbucci, M., Ferraresso, S., Bargelloni, L., Milan, M., 2017. Transcriptome analysis of the brain of the sea bream (Sparus aurata) after exposure to human pharmaceuticals at realistic environmental concentrations. Mar. Environ. Res. 129, 36–45. https://doi.org/10.1016/j.marenvres.2017.04.012

He, J., Zhang, Y., Zhang, X., Huang, Y., 2018. Highly efficient Fenton and enzyme-mimetic activities of NH2-MIL-88B(Fe) metal organic framework for methylene blue degradation. Sci. Rep. 8, 1–8. https://doi.org/10.1038/s41598-018-23557-2

Hoseinzadeh, H., Hayati, B., Shahmoradi Ghaheh, F., Seifpanahi-Shabani, K., Mahmoodi, N.M., 2021. Development of room temperature synthesized and functionalized metal-organic framework/graphene oxide composite and pollutant adsorption ability. Mater. Res. Bull. 142, 111408. https://doi.org/10.1016/j.materresbull.2021.111408

Hu, Y., Dai, L., Liu, D., Du, W., Wang, Y., 2018. Progress & prospect of metal-organic frameworks (MOFs) for enzyme immobilization (enzyme/MOFs). Renew. Sustain. Energy Rev. 91, 793–801. https://doi.org/10.1016/j.rser.2018.04.103

Jelic, A., Cruz-Morató, C., Marco-Urrea, E., Sarrà, M., Perez, S., Vicent, T., Petrović, M., Barcelo, D., 2012. Degradation of carbamazepine by Trametes versicolor in an air pulsed fluidized bed bioreactor and identification of intermediates. Water Res. 46, 955–964. https://doi.org/10.1016/j.watres.2011.11.063

Ji, C., Hou, J., Wang, K., Zhang, Y., Chen, V., 2016. Biocatalytic degradation of carbamazepine with immobilized laccase-mediator membrane hybrid reactor. J. Memb. Sci. 502, 11–20. https://doi.org/10.1016/j.memsci.2015.12.043

Joseph, L., Jun, B.M., Jang, M., Park, C.M., Muñoz-Senmache, J.C., Hernández-Maldonado, A.J., Heyden, A., Yu, M., Yoon, Y., 2019. Removal of contaminants of emerging concern by metal-organic framework nanoadsorbents: A review. Chem. Eng. J. 369, 928–946. https://doi.org/10.1016/j.cej.2019.03.173

Karagiaridi, O., Bury, W., Sarjeant, A.A., Stern, C.L., Farha, O.K., Hupp, J.T., 2012. Synthesis and characterization of isostructural cadmium zeolitic imidazolate frameworks via solvent-assisted linker exchange. Chem. Sci. 3, 3256–3260. https://doi.org/10.1039/c2sc20558k

Knedel, T.O., Ricklefs, E., Schlüsener, C., Urlacher, V.B., Janiak, C., 2019. Laccase Encapsulation in ZIF-8 Metal-Organic Framework Shows Stability Enhancement and Substrate Selectivity. ChemistryOpen 8, 1337–1344. https://doi.org/10.1002/open.201900146

Kudlek, E., 2018. Decomposition of contaminants of emerging concern in advanced oxidation processes. Water (Switzerland) 10. https://doi.org/10.3390/w10070955

Lassouane, F., Aït-amar, H., Amrani, S., Rodriguez-Couto, S., 2019. A promising laccase immobilization approach for Bisphenol A removal from aqueous solutions. Bioresour. Technol. 271, 360–367. https://doi.org/10.1016/j.biortech.2018.09.129

Li, P., Chen, Q., Wang, T.C., Vermeulen, N.A., Mehdi, B.L., Dohnalkova, A., Browning, N.D., Shen, D., Anderson, R., Gómez-Gualdrón, D.A., Cetin, F.M., Jagiello, J., Asiri, A.M., Stoddart, J.F., Farha, O.K., 2018. Hierarchically Engineered Mesoporous Metal-Organic Frameworks toward Cell-free Immobilized Enzyme Systems. Chem 4, 1022–1034. https://doi.org/10.1016/j.chempr.2018.03.001

Li, X., He, Q., Li, H., Gao, X., Hu, M., Li, S., Zhai, Q., Jiang, Y., Wang, X., 2017. Bioconversion of non-steroidal anti-inflammatory drugs diclofenac and naproxen by chloroperoxidase. Biochem. Eng. J. 120, 7–16. https://doi.org/10.1016/j.bej.2016.12.018

Lian, X., Fang, Y., Joseph, E., Wang, Q., Li, J., Banerjee, S., Lollar, C., Wang, X., Zhou, H.C., 2017. Enzyme-MOF (metal-organic framework) composites. Chem. Soc. Rev. 46, 3386–3401. https://doi.org/10.1039/c7cs00058h

Liang, L., Yue, X., Dong, S., Feng, J., Sun, J., Pan, Y., Zhou, M., 2021. New insights into the effect of adsorption on catalysis in the metal-free persulfate activation process for removing organic pollutants. Sep. Purif. Technol. 272, 118923. https://doi.org/10.1016/j.seppur.2021.118923

Liu, G., Xu, Y., Han, Y., Wu, J., Xu, J., Meng, H., Zhang, X., 2017. Immobilization of lysozyme proteins on a hierarchical zeolitic imidazolate framework (ZIF-8). Dalt. Trans. 46, 2114–2121. https://doi.org/10.1039/c6dt04582k

Marreiros, J., Van Dommelen, L., Fleury, G., de Oliveira-Silva, R., Stassin, T., Iacomi, P., Furukawa, S., Sakellariou, D., Llewellyn, P.L., Roeffaers, M., Ameloot, R., 2019. Vapor-Phase Linker Exchange of the Metal–Organic Framework ZIF-8: A Solvent-Free Approach to Post-synthetic Modification. Angew. Chemie - Int. Ed. 58, 18471–18475. https://doi.org/10.1002/anie.201912088

Mehta, J., Dhaka, S., Bhardwaj, N., Paul, A.K., Dayananda, S., Lee, S.E., Kim, K.H., Deep, A., 2019. Application of an enzyme encapsulated metal-organic framework composite for convenient sensing and degradation of methyl parathion. Sensors Actuators, B Chem. 267–274. https://doi.org/10.1016/j.snb.2019.03.116

Mohammadi, M., As'habi, M.A., Salehi, P., Yousefi, M., Nazari, M., Brask, J., 2018. Immobilization of laccase on epoxy-functionalized silica and its application in biodegradation of phenolic compounds. Int. J. Biol. Macromol. 109, 443–447. https://doi.org/10.1016/j.ijbiomac.2017.12.102

Nadar, S.S., Rathod, V.K., 2018. Magnetic-metal organic framework (magnetic-MOF): A novel platform for enzyme immobilization and nanozyme applications. Int. J. Biol. Macromol. 120, 2293–2302. https://doi.org/10.1016/j.ijbiomac.2018.08.126

Nadar, S.S., Rathod, V.K., 2016. Enzyme and Microbial Technology Magnetic macromolecular cross linked enzyme aggregates (CLEAs) of glucoamylase. Enzyme Microb. Technol. 83, 78–87. https://doi.org/10.1016/j.enzmictec.2015.10.009

Naghdi, M., Taheran, M., Brar, S.K., Kermanshahi-pour, A., Verma, M., Surampalli, R.Y., 2018a. Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes. Environ. Pollut. 234, 190–213. https://doi.org/10.1016/j.envpol.2017.11.060

Naghdi, M., Taheran, M., Brar, S.K., Kermanshahi-pour, A., Verma, M., Surampalli, R.Y., 2018b. Biotransformation of carbamazepine by laccase-mediator system: Kinetics, by-products and toxicity assessment. Process Biochem. 67, 147–154. https://doi.org/10.1016/j.procbio.2018.02.009

Naghdi, M., Taheran, M., Brar, S.K., Kermanshahi-pour, A., Verma, M., Surampalli, R.Y., 2017. Immobilized laccase on oxygen functionalized nanobiochars through mineral acids treatment for removal of carbamazepine. Sci. Total Environ. 584–585, 393–401. https://doi.org/10.1016/j.scitotenv.2017.01.021

Nguyen, L.N., van de Merwe, J.P., Hai, F.I., Leusch, F.D.L., Kang, J., Price, W.E., Roddick, F., Magram, S.F., Nghiem, L.D., 2016. Laccase-syringaldehyde-mediated degradation of trace organic contaminants in an enzymatic membrane reactor: Removal efficiency and effluent toxicity. Bioresour. Technol. 200, 477–484. https://doi.org/10.1016/j.biortech.2015.10.054

Nkoom, M., Lu, G., Liu, J., Dong, H., 2020. Biological uptake, depuration and biochemical effects of diclofenac and carbamazepine in Carassius carassius. Ecotoxicol. Environ. Saf. 205, 111106. https://doi.org/10.1016/j.ecoenv.2020.111106

Oluwole, A.O., Omotola, E.O., Olatunji, O.S., 2020. Pharmaceuticals and personal care products in water and wastewater: a review of treatment processes and use of photocatalyst immobilized on functionalized carbon in AOP degradation. BMC Chem. 14, 1–29. https://doi.org/10.1186/s13065-020-00714-1

Pang, R., Li, M., Zhang, C., 2015. Degradation of phenolic compounds by laccase immobilized on carbon nanomaterials: Diffusional limitation investigation. Talanta 131, 38–45. https://doi.org/10.1016/j.talanta.2014.07.045

Pang, S., Wu, Y., Zhang, X., Li, B., Ouyang, J., Ding, M., 2016. Immobilization of laccase via adsorption onto bimodal mesoporous Zr-MOF. Process Biochem. 51, 229–239. https://doi.org/10.1016/j.procbio.2015.11.033

Parra Guardado, A.L., Belleville, M.P., Rostro Alanis, M. de J., Parra Saldivar, R., Sanchez-Marcano, J., 2019. Effect of redox mediators in pharmaceuticals degradation by laccase: A comparative study. Process Biochem. 78, 123–131. https://doi.org/10.1016/j.procbio.2018.12.032

Phan, H. V., Hai, F.I., Zhang, R., Kang, J., Price, W.E., Nghiem, L.D., 2016. Bacterial community dynamics in an anoxic-aerobic membrane bioreactor - Impact on nutrient and trace organic contaminant removal. Int. Biodeterior. Biodegrad. 109, 61–72. https://doi.org/10.1016/j.ibiod.2016.01.002

Pillai, P., Dharaskar, S., Sasikumar, S., Khalid, M., 2019. Zeolitic imidazolate framework-8 nanoparticle: a promising adsorbent for effective fluoride removal from aqueous solution. Appl. Water Sci. 9, 1–12. https://doi.org/10.1007/s13201-019-1030-9

Rezaei Kahkha, M.R., Oveisi, A.R., Kaykhaii, M., Rezaei Kahkha, B., 2018. Determination of carbamazepine in urine and water samples using amino-functionalized metal–organic framework as sorbent. Chem. Cent. J. 12, 1–12. https://doi.org/10.1186/s13065-018-0446-x

Salgaonkar, M., Nadar, S.S., Rathod, V.K., 2019. Biomineralization of orange peel peroxidase within metal organic frameworks (OPP-MOFs) for dye degradation. J. Environ. Chem. Eng. 7. https://doi.org/10.1016/j.jece.2019.102969

Samui, A., Sahu, S.K., 2018. One-pot synthesis of microporous nanoscale metal organic frameworks conjugated with laccase as a promising biocatalyst. New J. Chem. 42, 4192–4200. https://doi.org/10.1039/c7nj03619a

Saoudi, O., Ghaouar, N., 2019. Biocatalytic characterization of free and immobilized laccase from Trametes versicolor in its activation zone. Int. J. Biol. Macromol. 128, 681–691. https://doi.org/10.1016/j.ijbiomac.2019.01.199

Saravanan, A., Kumar, P.S., Vo, D.V.N., Jeevanantham, S., Karishma, S., Yaashikaa, P.R., 2021. A review on catalytic-enzyme degradation of toxic environmental pollutants: Microbial enzymes. J. Hazard. Mater. 419, 126451. https://doi.org/10.1016/j.jhazmat.2021.126451

Schapira, M., Manor, O., Golan, N., Kalo, D., Mordehay, V., Kirshenbaum, N., Goldsmith, R., Chefetz, B., Paltiel, O., 2020. Involuntary human exposure to carbamazepine: A cross-sectional study of correlates across the lifespan and dietary spectrum. Environ. Int. 143, 105951. https://doi.org/10.1016/j.envint.2020.105951

Shahrak, M.N., Ghahramaninezhad, M., Eydifarash, M., 2019. Zeolitic imidazolate framework-8 for efficient adsorption and removal of Cr (VI) ions from aqueous solution. https://doi.org/10.1007/s11356-017-8577-5

Sharma, B., Dangi, A.K., Shukla, P., 2018. Contemporary enzyme based technologies for bioremediation: A review. J. Environ. Manage. 210, 10–22. https://doi.org/10.1016/j.jenvman.2017.12.075

Sheldon, R.A., van Pelt, S., 2013. Enzyme immobilisation in biocatalysis: Why, what and how. Chem. Soc. Rev. 42, 6223–6235. https://doi.org/10.1039/c3cs60075k

Shu, F., Lv, D., Song, X.L., Huang, B., Wang, C., Yu, Y., Zhao, S.C., 2018. Fabrication of a hyaluronic acid conjugated metal organic framework for targeted drug delivery and magnetic resonance imaging. RSC Adv. 8, 6581–6589. https://doi.org/10.1039/c7ra12969f

Smitha, K., Thampi, S.G., 2017. Experimental Investigations on Fluoride Removal from Water Using Nanoalumina-Carbon Nanotubes Blend. J. Water Resour. Prot. 09, 760–769. https://doi.org/10.4236/jwarp.2017.97050

Sneddon, S., Kahr, J., Orsi, A.F., Price, D.J., Dawson, D.M., Wright, P.A., Ashbrook, S.E., 2017. Investigation of zeolitic imidazolate frameworks using 13C and 15N solid-state NMR spectroscopy. Solid State Nucl. Magn. Reson. 87, 54–64. https://doi.org/10.1016/j.ssnmr.2017.09.001

Stwalley, W.C., 2004. Collisions and reactions of ultracold molecules. Can. J. Chem. 82, 709–712. https://doi.org/10.1139/v04-035

Tan, P.C., Ooi, B.S., Ahmad, A.L., Low, S.C., 2017. Size control and stability study of zeolitic imidazolate framework-8 to prepare mixed matrix membrane. J. Phys. Sci. 28, 215–226. https://doi.org/10.21315/jps2017.28.s1.14

Varga, B., Somogyi, V., Meiczinger, M., Kováts, N., Domokos, E., 2019. Enzymatic treatment and subsequent toxicity of organic micropollutants using oxidoreductases - A review. J. Clean. Prod. 221, 306–322. https://doi.org/10.1016/j.jclepro.2019.02.135

Wang, M., Zhao, Y., Li, J., 2018. Demethylation and other modifications of industrial softwood kraft lignin by laccase-mediators. Holzforschung 72, 357–365. https://doi.org/10.1515/hf-2017-0096

Wen, X., Zeng, Z., Du, C., Huang, D., Zeng, G., Xiao, R., Lai, C., Xu, P., Zhang, C., Wan, J., Hu, L., Yin, L., Zhou, C., Deng, R., 2019. Immobilized laccase on bentonite-derived mesoporous materials for removal of tetracycline. Chemosphere 222, 865–871. https://doi.org/10.1016/j.chemosphere.2019.02.020

Wu, C.S., Xiong, Z.H., Li, C., Zhang, J.M., 2015. Zeolitic imidazolate metal organic framework ZIF-8 with ultra-high adsorption capacity bound tetracycline in aqueous solution. RSC Adv. 5, 82127–82137. https://doi.org/10.1039/c5ra15497a

Wu, E., Li, Y., Huang, Q., Yang, Z., Wei, A., Hu, Q., 2019. Laccase immobilization on amino-functionalized magnetic metal organic framework for phenolic compound removal. Chemosphere 233, 327–335. https://doi.org/10.1016/j.chemosphere.2019.05.150

Wu, Y., Chen, Y., Wei, N., 2020. Biocatalytic properties of cell surface display laccase for degradation of emerging contaminant acetaminophen in water reclamation. Biotechnol. Bioeng. 117, 342–353. https://doi.org/10.1002/bit.27214

Yan, S., Wang, M., Liang, X., Martyniuk, C.J., Zha, J., Wang, Z., 2018. Environmentally relevant concentrations of carbamazepine induce liver histopathological changes and a gender-specific response in hepatic proteome of Chinese rare minnows (Gobiocypris rarus). Environ. Pollut. 243, 480–491. https://doi.org/10.1016/j.envpol.2018.09.009

Yang, H.M., Liu, X., Song, X.L., Yang, T.L., Liang, Z.H., Fan, C.M., 2015. In situ electrochemical synthesis of MOF-5 and its application in improving photocatalytic activity of BiOBr. Trans. Nonferrous Met. Soc. China (English Ed. 25, 3987–3994. https://doi.org/10.1016/S1003-6326(15)64047-X

Yang, J., Lin, Y., Yang, X., Ng, T.B., Ye, X., Lin, J., 2017. Degradation of tetracycline by immobilized laccase and the proposed transformation pathway. J. Hazard. Mater. 322, 525–531. https://doi.org/10.1016/j.jhazmat.2016.10.019

Yaohua, G., Ping, X., Feng, J., Keren, S., 2019. Co-immobilization of laccase and ABTS onto novel dual-functionalized cellulose beads for highly improved biodegradation of indole. J. Hazard. Mater. 365, 118–124. https://doi.org/10.1016/j.jhazmat.2018.10.076

Yehya, T., Favier, L., Audonnet, F., Fayad, N., Bahry, H., Bahrim, G.E., Vial, C., 2020. Towards a better understanding of the removal of carbamazepine by ankistrodesmus braunii: Investigation of some key parameters. Appl. Sci. 10, 1–18. https://doi.org/10.3390/app10228034

Zahmakiran, M., 2012. Iridium nanoparticles stabilized by metal organic frameworks (IrNPs@ZIF-8): Synthesis, structural properties and catalytic performance. Dalt. Trans. 41, 12690–12696. https://doi.org/10.1039/c2dt31779f

Zdarta, J., Jankowska, K., Wyszowska, M., Kijeńska-Gawrońska, E., Zgoła-Grześkowiak, A., Pinelo, M., Meyer, A.S., Moszyński, D., Jesionowski, T., 2019. Robust biodegradation of naproxen and diclofenac by laccase immobilized using electrospun nanofibers with enhanced stability and reusability. Mater. Sci. Eng. C 103. https://doi.org/10.1016/j.msec.2019.109789

Zhang, X.X., Zhang, T., Fang, H.H.P., 2009. Antibiotic resistance genes in water environment. Appl. Microbiol. Biotechnol. 82, 397–414. https://doi.org/10.1007/s00253-008-1829-z

4.2 Paper 2

This paper entitled "**An oxidoreductase enzyme, fungal laccase immobilized on zeolitic imidazolate frameworks for the biocatalytic degradation of an endocrine-disrupting chemical, dimethyl phthalate**" has been published in Journal of Environmental Chemical Engineering.

This article investigated the synergistic adsorption and biodegradation of an endocrine-disrupting chemical (EDC), dimethyl phthalate (DMP) using Lac-ZIF.

Journal of Environmental Chemical Engineering, 2023

https://doi.org/10.1016/j.jece.2023.109810

Authors:

Mbongiseni Lungelo Dlamini – Principal author

Mahadi Lesaoana – Co-author

Izak Kotzé – Co-supervisor

Heidi Lynn Richards – Main supervisor

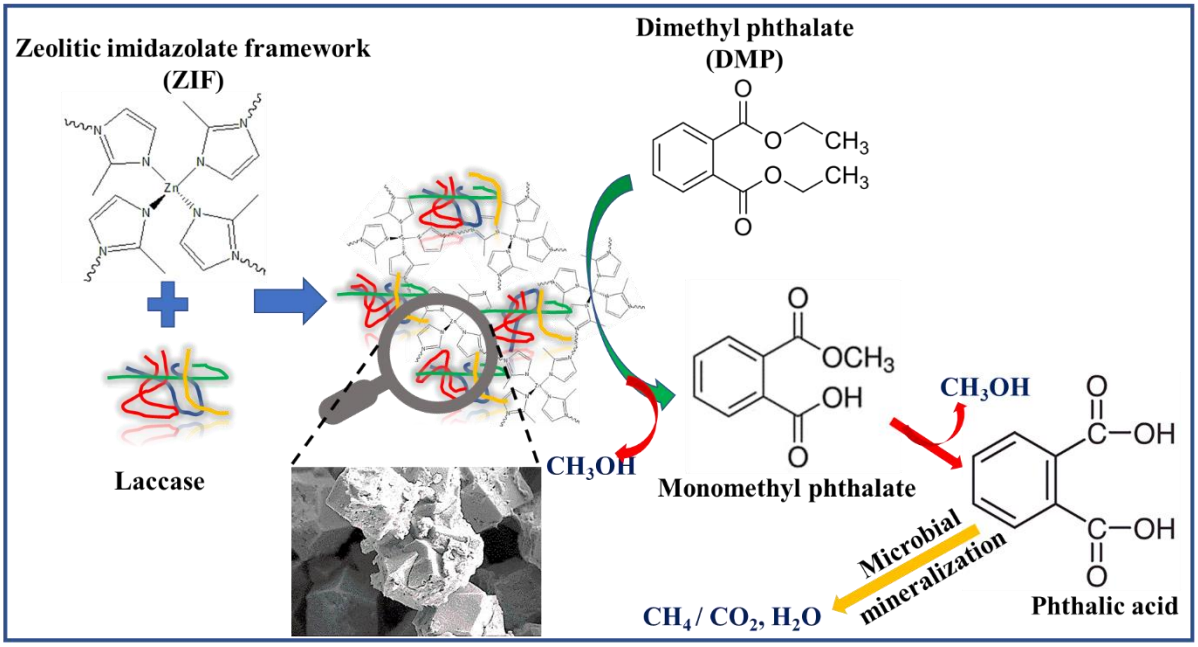
Contribution: lead author, conducted experimental work and data analysis, conceptualization, methodology, formal analysis, investigation, writing - original draft and visualization.

An oxidoreductase enzyme, fungal laccase immobilized on zeolitic imidazolate frameworks for the biocatalytic degradation of an endocrine-disrupting chemical, dimethyl phthalate

Mbongiseni Lungelo Dlamini, Mahadi Lesaoana, Izak Kotzé, Heidi Richards*

Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa.

Graphical abstract



*Corresponding author: Email address: heidi.richards@wits.ac.za

Abstract

This study investigated a facile one-pot synthesis procedure of zeolitic imidazolate frameworks (ZIF) and laccase immobilization for use in the biodegradation of an endocrine-disrupting chemical (EDC), dimethyl phthalate (DMP). The as-synthesized Lac-ZIF's characterization results revealed preserved crystallinity of the ZIF particles and lightly enzyme-coated decahedral particles. The immobilized laccase demonstrated enhanced temperature and storage stabilities for up to 65 ℃ and 20 d, respectively, for the DMP removal. While a reduced maximum velocity (V_{max}) after the laccase immobilization (0.651 to 0.238 mg L^{-1} h^{-1}) was observed, the Lac-ZIF presented a higher DMP removal efficiency (90.1%) compared to the free laccase (53.2%) within 4 h of contact time. Additionally, after 5 cycles of reuse, the Lac-ZIF retained a remarkable 95.2% efficiency and improved effectiveness in DMP decontamination from river water samples than the free laccase. Furthermore, the resulting DMP degradation metabolites were studied and revealed the production of relatively less toxic compounds after probable complete microbial mineralization. The study, therefore, demonstrates that Lac-ZIF has an admirable performance in the effective removal of phthalates from wastewater, making it a promising candidate for environmental remediation.

***Keywords*:** Phthalates; ZIF; Laccase; EDC; Biodegradation

1. Introduction

Emerging organic pollutants, such as endocrine-disrupting chemicals (EDCs), have attracted substantial interest from many researchers as significant contributors of environmental pollution [1,2]. Endocrine-disrupting chemicals are primarily exogenous agents that interfere with the synthesis, secretion, transport, and metabolism of naturally occurring hormones in the body, consequently influencing an organism's homeostasis, reproduction, and developmental processes [3–5].

Among various endocrine-disrupting chemicals, phthalates, also known as phthalate esters, have been detected in wastewater as a result of the production, use, and disposal of plastics in the environment [6,7]. Phthalates are commonly utilized as vinyl softeners in plastic synthesis, as well as a coating in food packaging, cosmetics, lubricants, and decorative clothing [8,9]. When employed as plasticizers, these phthalates are not chemically bound to the polymer of the plastics and can potentially migrate into the environment. Owing to their hepatotoxic, teratogenic, and carcinogenic properties, phthalates, and their metabolites have been shown to be potentially hazardous to humans and the environment [10].

Due to their stability, short-chain phthalates such as dimethyl phthalates (DMP) tend to persist in wastewater treatment plants, defying total elimination by traditional water treatment procedures such as coagulation, sedimentation, reverse osmosis, basic biological processes, filtration, and adsorption techniques [11–15]. Also, these technologies may present drawbacks such as the high costs associated with adsorbent regeneration, as well as sludge and by-product production with higher toxicity than the parent compound [16–20]. It is, therefore, imperative to develop an alternative environmentally friendly, economical, and effective technique to address environmental pollution.

Accordingly, biocatalytic remediation of wastewater using oxidoreductase enzymes is an environmentally benign approach for biodegrading such EDCs. In this sense, extracellular white rot fungi enzyme extracts such as peroxidases and laccase have been explored for their capacity to degrade recalcitrant pollutants including chlorophenols, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons [21–23]. These enzymes catalyze reactions under moderate conditions with high selectivity, minimal energy consumption, and toxic waste generation [24]. Peroxidases, such as horseradish peroxidases (HRP), are the most researched heme-proteins, capable of catalyzing the oxidation-reduction of a wide range of organic and inorganic chemicals, including phenolics [25]. However, the demand for H_2O_2, an

environmentally hazardous compound, for optimal peroxidase function limits their practical application in wastewater remediation [26]. As a result, researchers have investigated alternatives in this regard, and laccases provide ideal replacements for peroxidases since these enzymes only require a harmless and widely available oxidant, molecular oxygen, to initiate a reaction [27,28].

Laccases (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) are multicopper oxidases that catalyze the oxidation of a wide range of phenolic/non-phenolic chemicals and refractory environmental contaminants [29,30]. However, to enhance their application frequency and efficiency, stability, recycling, and reuse should be established. This is usually afforded by the incorporation of the biocatalyst onto/into insoluble and stable supports. The formation of linkages between the enzyme and a matrix stabilizes the peptide structure of the biocatalyst, resulting in improved enzyme stability towards strong pH, high temperatures, or organic solvents.

Recently, metal-organic frameworks (MOFs) among other support materials [31–35] have piqued the attention of several researchers as novel potential porous materials for enzyme immobilization. These are highly organized microporous crystalline hybrid materials made up of specific metal ions (or metal clusters) and organic linkers [36]. MOFs have inherent advantages such as large surface area and pore volumes, intrinsic crystalline nature, controllable topological structure [37], exceptional multifunctionality, tunable porosity, homogeneous aperture size, rich binding interaction sites for a selected reactant, and relatively high chemical/thermal/mechanical stability [38–42]. Due to the strong affinity between MOFs and enzymes, these highly porous materials have been examined as potential platforms for enzymatic attachment for organic water contaminants' degradation using oxidoreductase enzymes such as peroxidase, lipases [43], and glucoamylase [44] among others [45,46]. It may be noted that most of these works focus on synthesis and characterization of the biocatalysts with limited reports on practical application of the immobilized enzyme in real environmental water as well as comprehensive investigation of biotransformation products.

To date, various techniques for the fabrication of enzyme-MOF composites have been reported, comprising encapsulation, adsorption, and covalent bonding [47–50]. A combination of these strategies may be employed to augment the immobilization potential. In this sense, Wu et al. employed an amino-functionalized MOF (Fe_3O_4-NH_2@MIL-101(Cr)) for laccase attachment via the adsorption and covalent binding technique with a subsequent improvement in catalytic

efficiency, thermal and organic solvent stability as well as the recyclability of the biocatalyst [51]. Knedel et al. reported a facile encapsulation procedure for laccase entrapment in MOFs resulting in remarkable enzyme stabilities [52]. The enzyme encapsulation technique, however, may present drawbacks associated with a reduction in enzymatic velocity, owing to the diminished substrate access to the enzyme's active sites for binding [38,41,53,54].

De novo approaches for enzyme-MOF synthesis as outlined by Nadar et al [35] via co-precipitation and biomineralization have proven effective with a potential success in real-world applications. As a result, these viable approaches have intrigued the need to further investigate a one-pot biomineralization procedure for laccase immobilization onto ZIF, particularly for the subsequent application in real wastewater remediation and assessment of biotransformation products. Accordingly, this study reports the successful immobilization of fungal laccase enzyme via a facile adsorption and covalent binding procedure onto zeolitic imidazolate frameworks (ZIF) for the synergistic adsorption and biocatalytic degradation of dimethyl phthalate (DMP) in water. The resulting enzyme-MOF matrix referred to as Lac-ZIF was characterized by Fourier Transform Infrared Spectroscopy (FTIR), Powder X-Ray Diffraction (PXRD), Scanning Electron Microscope (SEM), and Transmission Electron Microscope (TEM). The DMP removal efficacy was studied in batch mode exploring variables such as solution pH, initial substrate concentration, biocatalyst dosage, temperature, and contact time. The storage stability profile and recyclability, as well as the reaction kinetics of the biocatalyst was also investigated.

2. Materials and Methods

2.1 Materials

2 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) – ABTS, 2-methylimidazole (2-MIm), Zinc nitrate tetrahydrate ($Zn(NO_3)_2 \cdot 4H_2O$), dimethyl phthalate (DMP), laccase enzyme (E.C. 1.10.3.2), disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$), sodium acetate $C_2H_3NaO_2$, sodium dihydrogen phosphate ($NaH_2PO_4H_2O$), Liquid Chromatography grade methanol (CH_3OH), sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), acetic acid ($CH_3CO_2H$) and citric acid ($C_6H_8O_7$) were purchased from Sigma Aldrich. All chemicals were of analytical grade. A Milli-Q pore system was used to produce distilled and ultrapure water.

2.2 Synthesis of ZIF and Lac-ZIF

Lac-ZIF composite synthesis was achieved via a facile one-pot synthesis procedure. This entails the quick mixing of 80 mmol 2-methylimidazole in 200 mL methanol and 2 mL of free

laccase (1000 U g^{-1}) in a 5 mL phosphate buffer (pH 7) solution. A 20 mmol $Zn(NO_3)_2 \cdot 4H_2O$ solution was prepared in 200 mL methanol, separately. The two solutions were left to stir for 5 min at 150 rpm. The zinc salt solution was then mixed with the 2-methylimidazole and laccase mixture and stirred for 2 h at ambient temperature. The resultant white suspension was centrifuged for 10 min at 4000 rpm and the solid residue was rinsed two times with 50 mL portions of distilled water. The solid composite was then oven-dried at 30 ℃ for 24 h. This procedure was adapted from [52] and [55] and modified. The pure ZIF crystals were synthesized using the same procedure in the absence of the laccase enzyme.

2.3 Characterization of ZIF crystals and Lac-ZIF composite

FTIR spectra recorded on a Perkin–Elmer, USA attenuated total reflectance Fourier Transform Infrared (ATR-FTIR) Spectrum 100 spectrometer in the range 500-4000 cm^{-1} were used to study the functional groups present in the Lac-ZIF composite and pure ZIF crystals. The obtained spectra were processed using OPUS software, and OriginLab software was used to plot the graphs.

PXRD measurements were done using a Bruker D2 XRD spectrometer through CuKα radiation with 1.5505 Å wavelength and slits at 45 kV/30 mA for 2θ values ranging from 5° to 90°. To examine the surface morphologies for both Lac-ZIF and pristine ZIF crystals, a Field Emission Scanning Electron Microscope (FE-SEM: ZEISS EVO 50, Oberkochen, Germany) was employed. A further in-depth morphological analysis of the particles was studied using High-Resolution Transmission Electron Microscope (TECNAI F20 HR-TEM) operated with a LaB6 filament at 200 kV.

2.4 Laccase activity assay

The rate of ABTS oxidation was used to evaluate the activity of free and immobilized laccase on ZIF particles. The amount of laccase needed to oxidize one µmol of ABTS per minute was defined as one unit of laccase activity. Typically, 100 µL laccase in 5 mL of 50 mM citrate-phosphate buffer (pH 7) was mixed with 1 mL of 1.5 mM ABTS solution for the free laccase, and these were allowed to react for 5 min to produce $ABTS^+$ at room temperature. For the immobilized laccase, the free laccase was replaced with 0.03 g of Lac-ZIF composite. The absorbance of the supernatant solution was measured using UV-vis at 420 nm (ε_{420} = 36 000 M^{-1} cm^{-1}), and the laccase's final activity was expressed as Units mL^{-1} of free laccase and Units g^{-1} of Lac-ZIF composite.

2.4.1 Determination of kinetic properties of free and immobilized laccase

The reaction kinetics of the enzyme was examined through the evaluation of Michaelis-Menten kinetic parameters (K_m and V_{max}) by measuring the initial reaction rates of the enzyme with ABTS in 50 mM citrate-phosphate buffer (pH 7). The ABTS was replaced with DMP to evaluate the laccase activity on dimethyl phthalate, and the reaction procedure was maintained. The ABTS and DMP concentrations were varied from 0.1 to 2 mM and 3 to 200 mg L^{-1}, respectively. The initial reaction rates (V) of the free laccase and Lac-ZIF were plotted against the initial substrate concentrations (S), following the Michaelis-Menten equation (1) to obtain the V_{max} and K_m parameters [28].

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

Where, V (mM min^{-1}) represents the initial reaction rate (velocity) at substrate concentration [S] (mM) with V_{max} (mM min^{-1}) and K_m (mM) indicating the maximum velocity and Michaelis constant, respectively. The K_m and V_{max} values are obtainable from the slope and intercept of the Lineweaver-Burk linear plot of 1/V vs 1/[S], respectively, [56]. The Lineweaver-Burk equation is given in equation (2) [57,58]:

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (2)$$

2.5 DMP removal

To investigate the biocatalyst's potential for DMP removal, 20 mL of DMP solutions were treated separately in 50 mL tubes with pre-determined amounts of ZIF particles, Lac-ZIF, and free laccase enzyme. The effect of experimental variables such as pH (3 - 10), material dosage (5 – 50 mg), laccase volume (20 – 500 µL), DMP solution concentration (3 – 20 mg L^{-1}), and reaction times (0.5 – 25 h) was evaluated. The reaction mixtures were placed on a thermostatic oscillator at 160 rpm for up to 25 h. Prior to analysis, the solutions were filtered through 0.45 µm nylon syringe filters.

A BISCHOFF high-performance liquid chromatography (HPLC) using a Fortis C18 column (250 mm x 4.6 mm, 5 µm) was employed for quantitative analysis of the residual DMP concentrations at room temperature. 20 µL sample solutions were injected and UV absorption was detected at 230 nm wavelength. The eluent of methanol and water (75:25 v/v) was run in isocratic mode maintaining a 1.2 mL min^{-1} flow rate. A 100 mg L^{-1} DMP stock solution was

prepared and thereafter diluted with distilled water to make standards (0.5 – 20 mg L^{-1}) and thus the required initial concentrations for each study parameter.

To determine the impact of matrix effects on DMP removal, real river water samples were collected from Crocodile River, South Africa, and spiked with known concentrations (5 mg L^{-1}) of DMP solutions. The ratio of real water to DMP v/v was 2:1. The samples were then subjected to the biocatalysts/adsorbent for up to 24 h and subsequently filtered and analyzed using a Thermo Scientific Dionex Ultimate 3000 from ThermoFisher Scientific (Waltham, MA, USA) Ultra-High-Performance Liquid Chromatography (UHPLC) coupled with an HPG-3200RS pump and a WPS-3000TRS autosampler. The UHPLC was connected to a Bruker Daltonics Inc. quadrupole time-of-flight (TOF) compact mass spectrometer system (Billerica, MA, USA). The quantitative data was collected and processed using Bruker Compass QuantAnalysis version 4.3 (Build 110.102.1532).

2.5.1 Laccase recyclability and storage stability

One of the primary goals of enzyme immobilization is to appreciate the benefits of its recyclability. Therefore, the possibility of Lac-ZIF for its repeated use in DMP decontamination was evaluated under optimized conditions for up to 8 cycles. After each cycle, the Lac-ZIF was separated by centrifugation at 4000 rpm for 5 min from the reaction medium and washed with 50 mM citrate-phosphate buffer (pH 7) and a fresh DMP solution was used. The initial removal efficiency was considered to be 100% and used as a reference value for the subsequent cycles.

To explore storage stability, the ZIF particles, free, and immobilized enzymes were separately stored in 50 mM citrate-phosphate buffers (pH 7) for up to 40 days at room temperature. On a 5-day interval, these samples were analyzed for residual DMP removal efficiencies using 40 mg ZIF and Lac-ZIF and 60 µL free laccase in 20 mL of 5 mg L^{-1} DMP analytes.

2.6 Data analysis

All experiments were carried out in triplicates, and replicate averages and standard deviations were calculated. The removal efficiencies were calculated based on the initial and final aqueous phase concentrations as shown in equation (3):

$$\%\, removal\ efficiency = \left(\frac{C_i - C_f}{C_i}\right) x100, \quad (3)$$

with C_i and C_f (mg L^{-1}) representing the initial and final DMP concentrations, respectively.

3. Results and Discussion

3.1 *Characteristic analyses of ZIF, Lac-ZIF composite, and laccase enzyme*

The successful synthesis of ZIF particles and subsequent immobilization of laccase was confirmed by FTIR analyses and the results of the spectra are shown in fig. 1. (a). A vibrational peak ascribed to the pyrrole group and the pyridinic nitrogen N-H···N bond previously detected at 3200 – 2380 cm^{-1} in the 2-methylimidazole's (2-MIm) spectrum has been eliminated in the spectra for ZIF. This suggests complete co-ordination and deprotonation of the -NH bond of the imidazole ring with Zn^{2+}. For the Lac-ZIF spectrum successful attachment of the enzyme onto the ZIF crystals was confirmed by the presence of amide I and II bands of the protein observed at 1660 and 1460 cm^{-1} respectively.

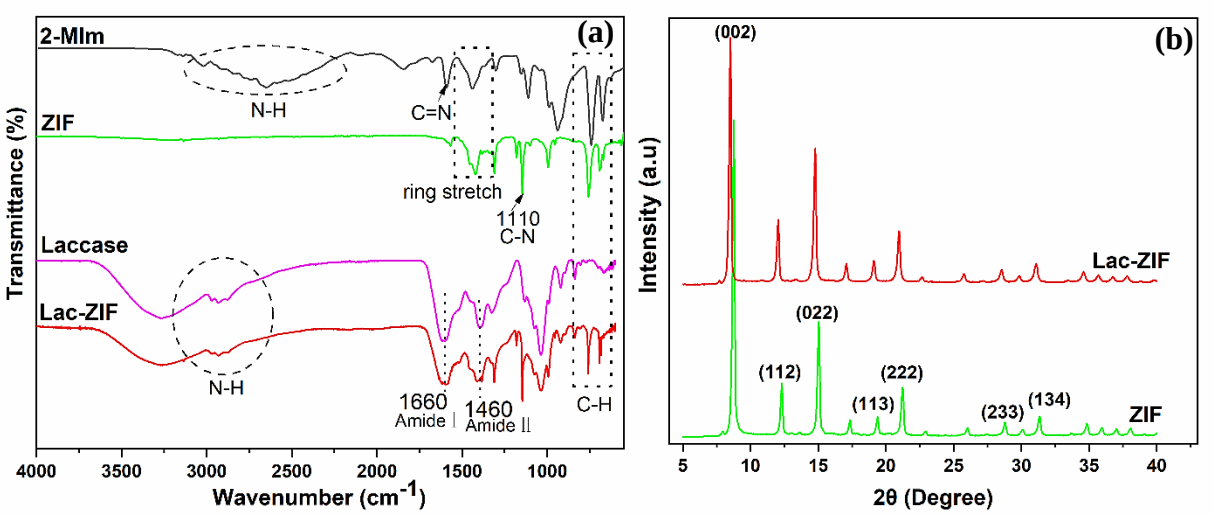


Fig. 1. (a) FTIR spectra of 2-methylimidazole (2-MIm), ZIF and Lac-ZIF composite, and (b) PXRD patterns of ZIF (JCPDS 00-062-1030) and Lac-ZIF.

The amide I band at 1660 cm^{-1} may be due to C=O stretching vibrations [49,59,60], while the amide II band at 1460 cm^{-1} may be recognized as NH and CN bending and stretching vibrations in laccase, respectively [61]. Additionally, the band at 1589 cm^{-1} is caused by the stretching mode of C=N, and the bands between 1500 and 600 cm^{-1} are related to the entire vibration of the imidazole ring [62].

The PXRD analysis results are shown in fig. 1. (b). From this figure, the pristine ZIF diffraction pattern shows peaks that are typical of pure ZIF crystals [54,60]. These were observed at 2θ = 8.5°, 12.6°, 15.0°, 19.4°, 21.2°, 31.8° [63–65] and were assigned (002), (112), (022), (113), (222), (233) and (134) planes of crystalline ZIF with a sodalite topology, respectively (JCPDS 00-062-1030) [66]. A slight shift of the (002) peak to lower 2θ values was observed after the attachment of the enzyme, indicating a slight change in the crystal phase of the ZIF particles,

and further confirming successful laccase immobilization [43]. Nonetheless, the diffraction peaks for Lac-ZIF are comparable to those of pure ZIF crystals, and this suggests that the crystallinity of the ZIF particles was not altered by the attachment of the laccase enzyme.

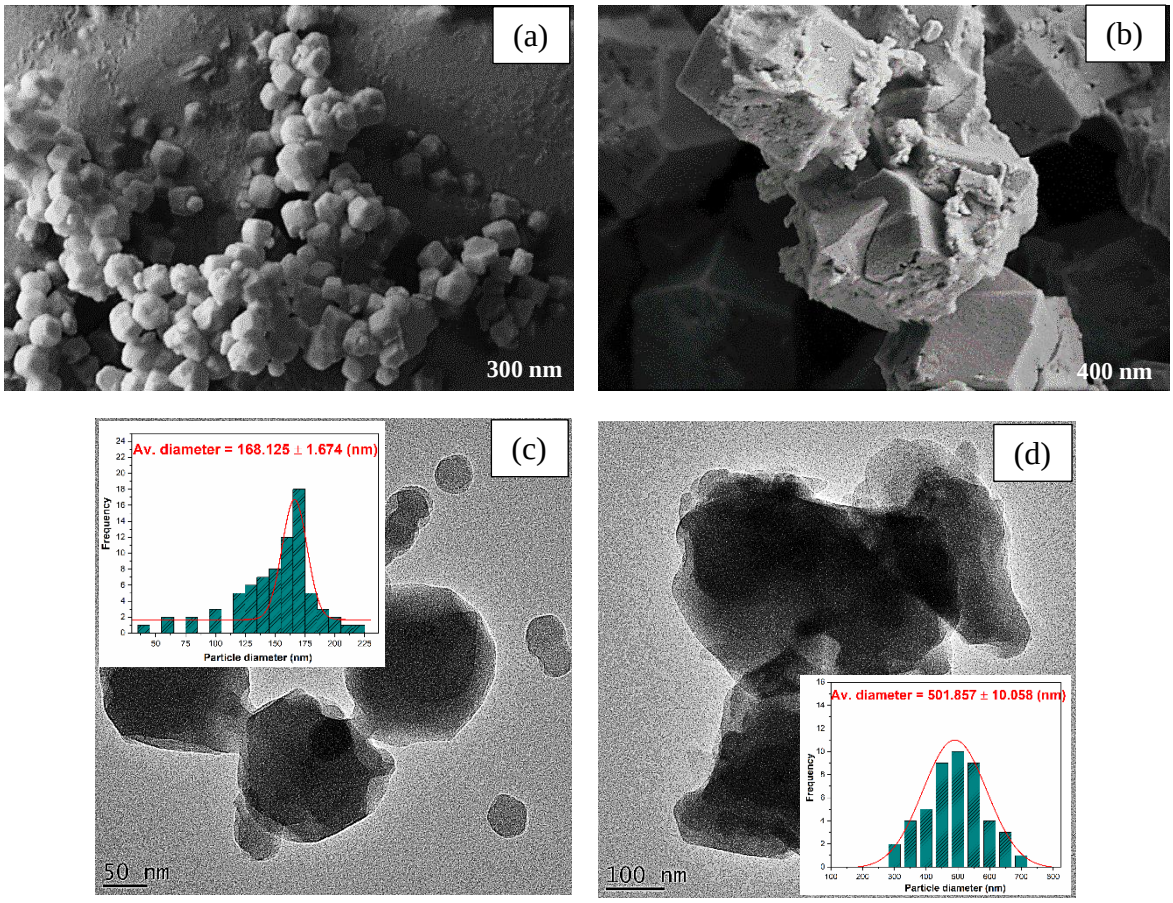


Fig. 2. FE-SEM micrographs of (a) ZIF particles and (b) Lac-ZIF composite, and TEM images of (c) ZIF and (d) Lac-ZIF.

SEM analyses of the synthesized particles yielded the micrographs shown in fig. 2. (a) and (b) (b). The particles presented the characteristic rhombic dodecahedral morphologies of pure ZIF crystals, as seen in fig. 2. (a) [49,60]. The enzyme crusts are visible on the surface of the particles following the laccase attachment (fig. 2 (b)), with the dodecahedral morphologies of the ZIF particles retained. The Lac-ZIF SEM image also demonstrates relative particle aggregation as compared to scattered pure ZIF particles, which results in increased average particle sizes from approximately 168 to 501 nm. This may be attributed to the multiple covalent linkages formed between the enzyme's protein structure and the ZIF crystals, which aid interparticle interactions and result in a lower dispersion potential of the crystals in space [67]. The agglomerated particles are apparent in the TEM micrographs (fig. 2 (c) and (d)).

Similar findings on the consequences of enzyme immobilization have been published elsewhere [51,66,68–70].

3.2 Kinetic properties of free and immobilized laccase

Fig. 3 (a) shows the Michaelis Menten plot for the free and immobilized laccase with the calculated K_m and V_{max} kinetic parameters presented in table 1. Results for the pure ZIF crystals were exempted as they proved unreactive towards ABTS exposure since the crystals did not contain any biocatalytic material. The graph indicates a general rise in velocities with increasing ABTS concentration until equilibrium is attained, which may be ascribed to more collisions between substrate and enzyme molecules at higher substrate concentrations [71]. However, it can be noted that the velocities diminished after the enzyme attachment. Accordingly, the evaluated V_{max} values dropped about two-fold, from 0.0428 ± 0.0102 to 0.0205 ± 0.0153 mM min^{-1}. The reduction in velocity after enzyme immobilization has been reported in several publications [52,69,72–74]. This observation may be attributable to restricted substrate accessibility to active sites of the immobilized laccase, which is induced by enhanced steric hindrance and diffusion resistance following enzyme immobilization [66,69], hence the observed slight increase in K_m values from 0.789 ± 0.012 to 0.998 ± 0.115 mM. This rise in K_m indicates a reduced affinity between the laccase and ABTS [57]. However, for practical application, maximum pollutant removal is assisted by the synergistic adsorption onto the porous ZIF crystals and subsequent degradation by the immobilized laccase, highlighting the significance of selecting a support material with an abundance of adsorption sites for the target analyte.

Table 1. Kinetic parameters of free laccase and Lac-ZIF using ABTS as substrate.

Sample	**K_m (mM)**	**V_{max} (mM min^{-1})**
Free Laccase	0.789 ± 0.012	0.0428 ± 0.0102
Lac-ZIF	0.998 ± 0.115	0.0205 ± 0.0153

3.3 Removal of dimethyl phthalate

3.3.1 Effect of pH

Fig. 3 (b) depicts the results for the influence of solution pH on DMP elimination. The graph demonstrates a general increase in removal efficiency as the pH draws near neutral, with the highest efficiencies obtained at pH 7. Laccase immobilization on ZIF crystals may result in combined adsorption and biodegradation effects, justifying the demonstrated superiority in removal efficiencies of Lac-ZIF (72.3%) over its counterparts which reached maximum

efficiencies of 60.8% and 25.7% for pure ZIF and free laccase, respectively. Furthermore, multipoint interactions of laccase molecules with amino groups on the MOF that result in stable conformations may have contributed to the immobilized enzyme's apparent pH stability [72]. These findings are consistent with other reports on the influence of free and immobilized laccase on activity [59,68,75]. The poor removal efficiencies at low pH levels can be attributed

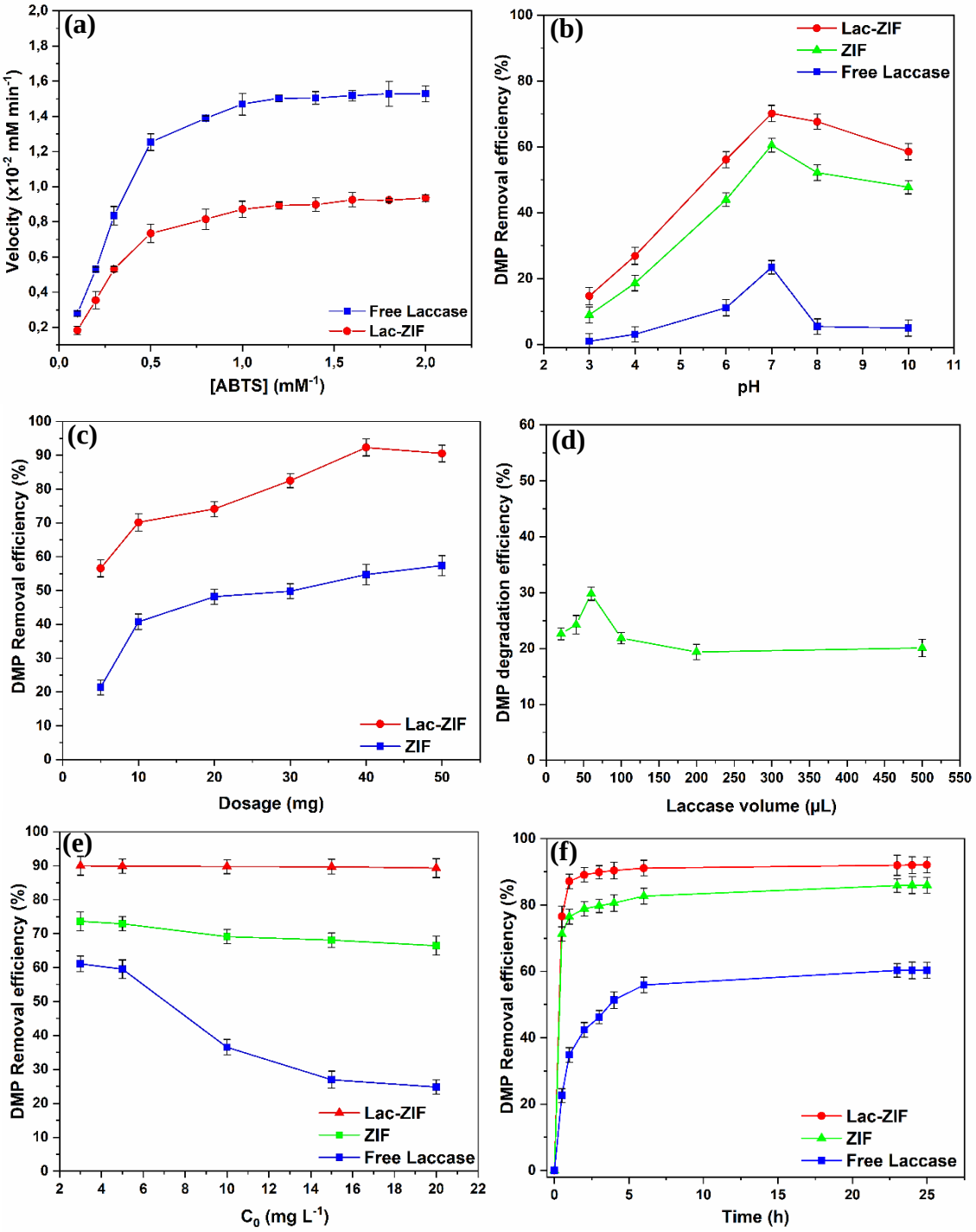


Fig. 3. (a) Michaelis Menten plot for laccase activity on ABTS, Effect of (b) pH, (c) dosage, (d) laccase volume, (e) initial solution concentration and (f) contact time on DMP removal.

to enzyme instability and disintegration of the ZIF structure, for the free and immobilized laccase, respectively [74,76]. In alkaline environments, the hydroxide anion may bind to the laccase's T2/T3 cupric site, inhibiting internal electron transport from the T1 to T2/T3 cupric sites, resulting in weaker interactions between the DMP and the free laccase, and consequently the reported low efficiencies for the free laccase. In the case of the Lac-ZIF and ZIF, the ZIF particles' stable structures enhanced the removal efficiency afforded by adsorption of the DMP molecules and subsequent biodegradation by the laccase [51,69]. The resistance of the Lac-ZIF to both acidic and alkaline media makes this biocatalyst composite more suitable for a wide range of applications, contrary to most published works where optimal activity is achieved in acidic conditions (pH < 5) [51,69,77,78]. As a result, the optimal pH for all succeeding experiments was set at 7 which mimics real environmental water.

3.3.2 Dosage effect

The results for the role of Lac-ZIF and ZIF mass, as well as free laccase volume, on DMP elimination, are shown in fig. 3 (c) and (d). The DMP removal efficiencies rise with the dose of Lac-ZIF and ZIF, reaching a maximum of 94.2% and 53.5%, respectively, at a dosage of 40 mg as shown in fig. 3 (c). This observation implies that higher doses provide more particles, resulting in greater availability of sorption and biodegradation active sites, and this is in agreement with published works [51,79–83]. Contrarily, as shown in fig. 3 (d), with increasing laccase volume, the DMP degradation efficiency improved from 23.1% to 30.2% at 50 µL, then dropped considerably with additional enzyme volume increment to 19.8%. This observation may be related to a potential reduction in interactions between the enzyme and the substrate bulk induced by multiple layers of laccase around the substrate in the presence of greater amounts of laccase molecules in the reaction media [59]. Consequently, the optimal doses for all further experiments for the Lac-ZIF and ZIF were kept at 40 mg, with the free laccase fixed at 50 µL.

3.3.3 Initial DMP concentration effect

Fig. 3 (e) illustrates the effect of initial solution concentration on DMP removal. As seen in the graph, the immobilized laccase outperformed the free laccase and ZIF particles. In this regard, the efficiency remained nearly constant over the concentrations inspected, reaching a maximum of 90.5% for the Lac-ZIF, with pristine ZIF crystal adsorption's efficiency exhibiting a slight drop from 74.2% at 3 mg L^{-1} to 68% at 20 mg L^{-1}. On the other hand, the degradation efficiency of the free laccase enzyme decreases noticeably from 63.2% at 3 mg L^{-1} to 27.8% at 20 mg L^{-1}. This observation may be ascribed to possible depleting active sites for

DMP degradation as well as inhibition of the laccase by substrate molecules, contrary to the immobilized enzyme, where removal efficiency is supplemented by adsorption of the analyte onto the ZIF particles [84]. In this sense, the degradation efficiency of the immobilized laccase would be predicted to be lower than that of the free laccase, corresponding to the reduced velocities from 0.651 ± 0.031 to 0.238 ± 0.016 mg L^{-1} h^{-1} as a result of the decreased affinity for DMP indicated by increased K_m values [52], following enzyme attachment, as shown in table 2. The evaluated Michaelis Menten constants were 18.715 ± 0.483 and 24.515 ± 0.341 mg L^{-1} for the free and immobilized laccase, respectively. Nevertheless, the ZIF particles enhanced the immobilized laccase's overall performance at elevated DMP concentrations, with subsequently higher removal efficiencies.

Table 2. Kinetic parameters of free laccase and Lac-ZIF using DMP as substrate.

Sample	**K_m (mg L^{-1})**	**V_{max} (mg L^{-1} h^{-1})**
Free Laccase	18.715 ± 0.483	0.651 ± 0.031
Lac-ZIF	24.515 ± 0.341	0.238 ± 0.016

3.3.4 Contact time effect

DMP removal at different contact times was evaluated over 25 h for an initial DMP concentration of 5 mg L^{-1} at pH 7, and the results are presented in fig. 3 (f). The efficiency of DMP elimination increased with exposure time, and equilibrium was reached within 4 h of contact time. During this period, Lac-ZIF outperformed its contenders, reaching an efficiency of 90.1%, followed by pure ZIF crystals at 80.6% and free laccase at 53.2%. The high efficiencies observed for the Lac-ZIF may be related to the mutual effects of DMP adsorption onto the ZIF backbone, followed by biodegradation provided by the immobilized laccase. The free laccase, however, undergoes quick saturation of the protein's active sites, hence the observed low removal efficiencies. After 5 h of contact time, all the investigated materials showed minimal variances in removal efficiencies, indicating complete saturation of the active sites for interactions with the DMP.

3.3.5 Temperature effect

The use of enzymes at high temperatures is well known to reduce catalytic activity. At high temperatures, the active sites of the enzymes are disrupted and altered, resulting in irreversible dysfunction [85]. It is therefore crucial to investigate the performance of the immobilized and free enzyme at different temperatures. In this regard, the DMP removal studies were conducted at temperatures ranging from 25 to 65 °C, and the results are illustrated in fig. 4 (a). Due to

multiple interconnections between the enzyme and the ZIF support, forming stable structures, the ZIF particles improved the thermal stability of the immobilized laccase, resulting in the observed superior performance in DMP decontamination after immobilization compared to the free laccase. As a result, the removal efficiency by Lac-ZIF increased slightly with temperature, from 90.1% at 25 °C to 98.4% at 65 °C, while the free laccase's efficiency declined significantly, from 57.3% to 1.2% for the same temperature range. The slight increase may be attributed to an increase in kinetic energy as temperature rises, allowing for more molecular collisions between the composite and analyte. However, for the free laccase, this effect may be insignificant as the enzyme may undergo denaturation due to protein structure deterioration with high thermal exposure, resulting in the observed diminishing efficiencies with increasing temperatures. [2]. Similar results were reported elsewhere [52,68,86]. The removal by pure ZIF crystals is primarily attributable to adsorption effects, resulting in relatively lower efficiencies (80.6% to 87.9%) compared to Lac-ZIF, which has both adsorption and biodegradation effects.

3.3.6 Recyclability and storage stability

The reusability of the immobilized enzyme is a crucial element in its cost-effectiveness and large-scale industrial application. Therefore, the performance and reusability of the immobilized laccase were investigated by assessing the DMP removal efficiency for up to 8 cycles. From fig. 4 (b), it can be noted that the Lac-ZIF can be effectively re-used for up to 5 cycles retaining 95.2% efficiency, with a drastic decline to 34.5% after 8 cycles. The significant loss of efficiency after the 5th cycle may be due to inactivation and structural denaturation of the laccase as well as potential leaching during the repeated elution process [85,87]. Nonetheless, the retained high efficiency for up to 5 cycles indicates enhanced stability of the immobilized laccase, offered by the stable linkages formed between the ZIF crystals and the protein. After 8 cycles of re-use, however, the SEM micrograph (fig. 4 (e)) shows slight distortions of the ZIF structure and loss of the laccase from the surface of the crystals, hence the significant loss in efficiency.

Another significant factor is enzyme storage, as enzymes are quite responsive to surrounding environments and can deactivate after a certain period when kept at room temperature. Immobilization of the enzyme can help to protect them from such external environments while also preserving some activity. Accordingly, the storage stabilities of the free laccase, immobilized and pure ZIF crystals were evaluated over 40 d for DMP removal at ambient temperature. As illustrated in fig. 4 (c), the immobilized laccase outperformed the free enzyme and ZIF particles in storage stability, retaining 88.6% DMP removal efficiency after 20 d of

storage, while the free laccase and ZIF demonstrated only 45.7% and 64.9% efficiencies,

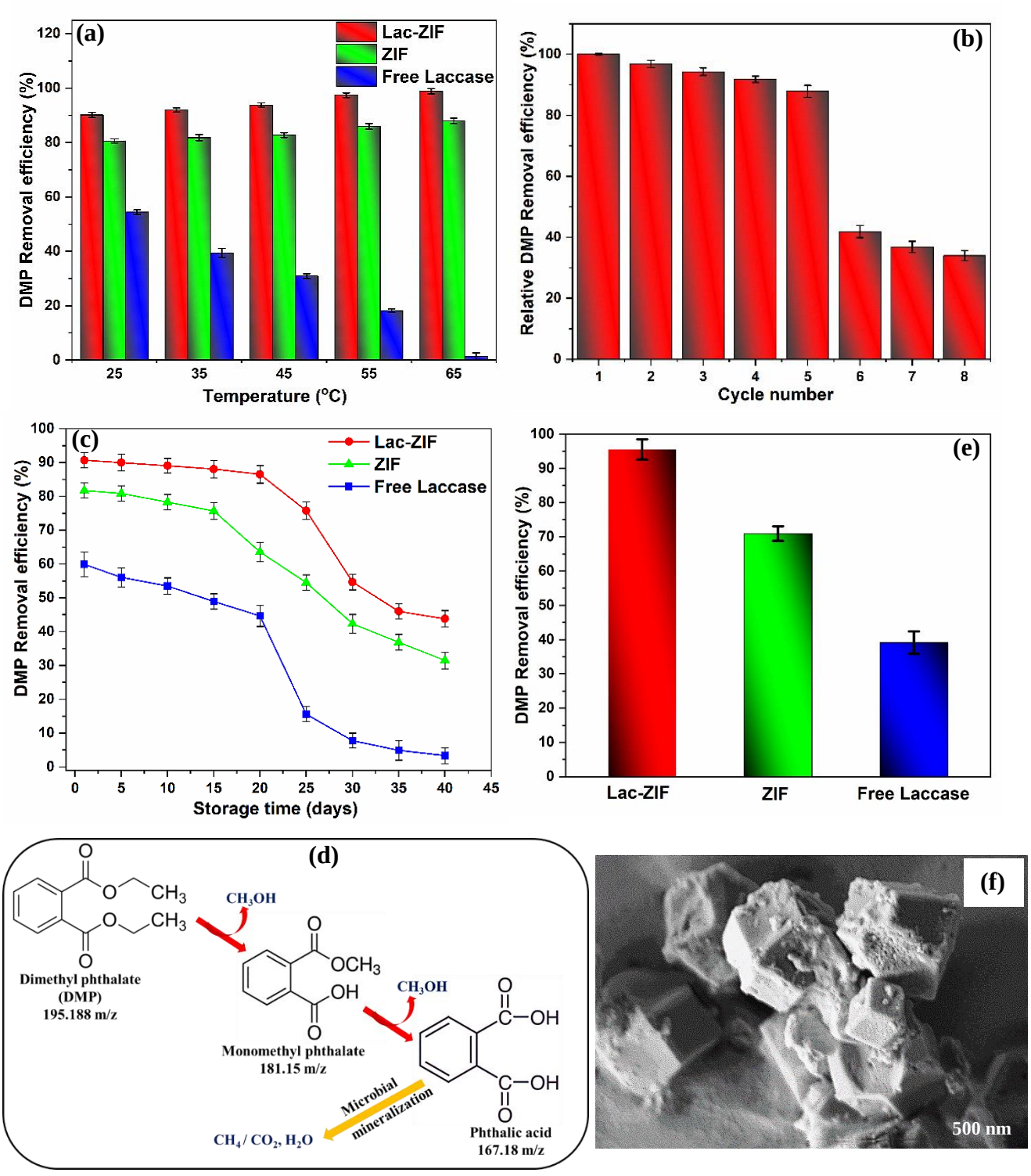


Fig. 4. (a) Effect of temperature on DMP removal, (b) Recyclability of Lac-ZIF, (c) Storage stability, (d) Probable DMP biochemical degradation pathway by laccase, (e) Application of biocatalysts in river water samples and (f) SEM image for Lac-ZIF after 8 cycles of reuse.

respectively. The attachment of the laccase to the ZIF particles protected the enzyme from structural distortion during storage, resulting in improved efficiency, as opposed to the rapid

loss of activity observed for the free laccase. [73,88]. It is worth noting that for most storage stability studies published, the storage temperature is usually maintained at 4 ℃ or lower, for example, Tocco et al. immobilized laccase onto ZIF-zni and it only retained 57 % activity after 20 d of storage at 4 ℃ [69]. Also, Pang et al used Zr-MOF as a support for laccase immobilization and it exhibited a relative 55.4% efficiency after 21 d of storage at 4 ℃ [89]. In this work, however, the biocatalyst composite proved relatively stable (88.6% efficiency after 20 d) even when kept at room temperature, making it a suitable candidate for application in real wastewater without temperature adjustments.

3.3.7 Identification of DMP biotransformation products

A positive scan mode from LC-MS (*m/z* 50 - 300) was used to identify the molecular ions of DMP degradation products, propose empirical formulas, and derive their chemical structures, and this resulted in two metabolites. On the reverse-phase column, the byproducts were eluted at retention times (t_R) of 6.1 and 6.9 min with precursor ions at *m/z* 181.15 and 167.18, respectively.

Their molecular weights were therefore ascertained to be 180.14 and 166.17 g mol^{-1}, corresponding to monomethyl phthalate ($C_9H_8O_4$) and phthalic acid ($C_8H_6O_4$), respectively. Fig. 4 (d) shows the probable DMP biochemical degradation pathway.

This suggests that the DMP is degraded via a sequential ester bond cleavage to yield the phthalate monoester with further hydrolysis resulting in the formation of phthalic acid, and these results are in good agreement with published works on phthalates and phthalate-ester degradation [90–95]. Accordingly, further mineralization of phthalic acid, typically by several microorganisms including fungi, bacteria, algae, and yeasts, can result in the formation of compounds such as CH_4, CO_2, and H_2O in the environment [95–97]. As a result, this technique could be considered a relatively environmentally friendly approach to wastewater remediation.

3.3.8 Validation of HPLC method for DMP analysis

A concentration range of 0.1 – 5 mg L^{-1} was used to compute a linear regression standard calibration curve of the DMP, resulting in good precisions with low relative standard deviations (≤ 3%) and a coefficient of determination (R^2) of 0.998. The limits of detection (LOD) and quantification (LOQ) were determined to be 0.175 ± 0.004 and 0.956 ± 0.021 mg L^{-1}, respectively. The retention times and molecular ion (195.188 *m/z*) of DMP on the LC-qTOF/MS were utilized in the identification of the compound in the solutions.

4. DMP removal from spiked river water

The potential application of the biocatalysts/adsorbent in environmental water systems was investigated using spiked river water samples collected from the Crocodile River in South Africa. The water samples exhibited the following physicochemical properties: pH 7.19, conductivity 847 µS cm^{-1}, total dissolved solids (TDS) 389 ppm, and dissolved oxygen (DO) 7.94 ppm. The Lac-ZIF outperformed the ZIF crystals and free laccase, achieving a removal efficiency of 95.4%, while the ZIF and free laccase attained efficiencies of 70.1% and 39.9%, respectively. The improved performance of the Lac-ZIF can be related to the mutual adsorption and degradation effects provided by the support material (ZIF) and the immobilized laccase. Furthermore, the stable conformation of the Lac-ZIF protected the enzyme from possible suppression by probable inhibitory molecules present in the water, in contrast to the free laccase, which may undergo structural breakdown under these environmental conditions [98]. The lower efficiencies for pristine ZIF particles may be due to competition for attachment onto the active sites of the crystals induced by other dissolved compounds in the water [99].

5. Conclusions

Laccase was immobilized on ZIF via a biomineralization approach to form Lac-ZIF. PXRD and SEM analyses revealed preserved crystallinity and dodecahedral conformations of the ZIF after laccase attachment, respectively, with an overall performance improvement compared to the free laccase. However, the Lac-ZIF exhibited reduced affinity between the laccase and DMP, resulting in a three-fold drop of V_{max} from 0.651 to 0.238 mg L^{-1} h^{-1}. Nonetheless, the Lac-ZIF demonstrated higher DMP removal efficiencies at 4 h of contact time than the free laccase. Moreover, the Lac-ZIF showed enhanced tolerance to pH variations, thermal (up to 65 °C) and storage stabilities, as well as recyclability potential. Furthermore, the DMP decontamination efficiency from river water samples was 95.4% within 24 h, which was considerably higher than that of free laccase (39.9%), indicating the potential use of this biocatalyst composite in environmental wastewater treatment.

Acknowledgements

This work was supported by the Council for Scientific and Industrial Research (CSIR) – Department of Science and Innovation (DSI) Inter-Programme Postgraduate Bursary (IBS).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] C. Barrios-Estrada, M. de Jesús Rostro-Alanis, B.D. Muñoz-Gutiérrez, H.M.N. Iqbal, S. Kannan, R. Parra-Saldívar, Emergent contaminants: Endocrine disruptors and their laccase-assisted degradation – A review, Sci. Total Environ. 612 (2018) 1516–1531. https://doi.org/10.1016/j.scitotenv.2017.09.013.

[2] F. Lassouane, H. Aït-Amar, S. Amrani, S. Rodriguez-couto, A promising laccase immobilization approach for Bisphenol A removal from aqueous solutions, Bioresour. Technol. 271 (2019) 360–367. https://doi.org/10.1016/j.biortech.2018.09.129.

[3] G. Zaki, T. Shoeib, Concentrations of several phthalates contaminants in Egyptian bottled water: Effects of storage conditions and estimate of human exposure, Sci. Total Environ. 618 (2018) 142–150. https://doi.org/10.1016/j.scitotenv.2017.10.337.

[4] J. Wang, M. Zhang, Adsorption Characteristics and Mechanism of Bisphenol A by Magnetic Biochar, (2020).

[5] C.K. Gadupudi, L. Rice, L. Xiao, K. Kantamaneni, Endocrine Disrupting Compounds Removal Methods from Wastewater in the United Kingdom: A Review, Sci. 3 (2021) 11. https://doi.org/10.3390/sci3010011.

[6] P.N. Angnunavuri, F. Attiogbe, A. Dansie, B. Mensah, Effect of Storage on the Levels of Phthalates in High Density Polyethylene (Hdpe) Film-Packaged Drinking Water, SSRN Electron. J. 845 (2022) 157347. https://doi.org/10.2139/ssrn.4091495.

[7] D.W. Gao, Z.D. Wen, Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes, Sci. Total Environ. 541 (2016) 986–1001. https://doi.org/10.1016/j.scitotenv.2015.09.148.

[8] S. Dutta, D.K. Haggerty, D.A. Rappolee, D.M. Ruden, Phthalate Exposure and Long-Term Epigenomic Consequences: A Review, Front. Genet. 11 (2020) 1–27. https://doi.org/10.3389/fgene.2020.00405.

[9] Y. Wang, H. Qian, Phthalates and their impacts on human health, Healthc. 9 (2021) 1–9. https://doi.org/10.3390/healthcare9050603.

[10] D.W. Liang, T. Zhang, H.H.P.P. Fang, J. He, Phthalates biodegradation in the environment, Appl. Microbiol. Biotechnol. 80 (2008) 183–198. https://doi.org/10.1007/s00253-008-1548-5.

[11] S. Jiménez, M.M. Micó, M. Arnaldos, F. Medina, S. Contreras, State of the art of produced water treatment, Chemosphere. 192 (2018) 186–208. https://doi.org/10.1016/j.chemosphere.2017.10.139.

[12] E. Routoula, S. V. Patwardhan, Degradation of Anthraquinone Dyes from Effluents: A Review Focusing on Enzymatic Dye Degradation with Industrial Potential, Environ. Sci. Technol. 54 (2020) 647–664. https://doi.org/10.1021/acs.est.9b03737.

[13] H. Han, M.K. Rafiq, T. Zhou, R. Xu, O. Mašek, X. Li, A critical review of clay-based composites with enhanced adsorption performance for metal and organic pollutants, J. Hazard. Mater. 369 (2019) 780–796. https://doi.org/10.1016/j.jhazmat.2019.02.003.

[14] A.R. Othman, H.A. Hasan, M.H. Muhamad, N. 'Izzati Ismail, S.R.S. Abdullah, Microbial degradation of microplastics by enzymatic processes: a review, Environ. Chem. Lett. 19 (2021) 3057–3073. https://doi.org/10.1007/s10311-021-01197-9.

[15] N.R. Khalid, A. Majid, M.B. Tahir, N.A. Niaz, S. Khalid, Carbonaceous-TiO2 nanomaterials for photocatalytic degradation of pollutants: A review, Ceram. Int. 43 (2017) 14552–14571. https://doi.org/10.1016/j.ceramint.2017.08.143.

[16] J. Carrillo-Abad, J. Mora-Gómez, M. García-Gabaldón, E. Ortega, S. Mestre, V. Pérez-Herranz, Effect of the CuO addition on a Sb-doped SnO2 ceramic electrode applied to the removal of Norfloxacin in chloride media by electro-oxidation, Chemosphere. 249 (2020). https://doi.org/10.1016/j.chemosphere.2020.126178.

[17] Z. Shan, F. Ma, S. You, L. Shan, D. Kong, H. Guo, C. Cui, Enhanced visible light photo-Fenton catalysis by lanthanum-doping BiFeO3 for norfloxacin degradation, Environ. Res. 216 (2023) 114588. https://doi.org/10.1016/j.envres.2022.114588.

[18] H. Guo, C. Zhao, H. Xu, H. Hao, Z. Yang, N. Li, W. Xu, Enhanced H2O2 formation and norfloxacin removal by electro-Fenton process using a surface-reconstructed graphite felt cathode: New insight into synergistic mechanism of defective active sites, Environ. Res. 220 (2023). https://doi.org/10.1016/j.envres.2023.115221.

[19] Q. Sun, X. Hu, Q. He, M. Sun, L. Dai, S. Khan, H. Zheng, Polydopamine-Chitosan Modified TiO2 Nanoparticles for Temperature-Response Removal of Diclofenac Sodium Under Visible Light Irradiation, J. Taiwan Inst. Chem. Eng. 131 (2022) 104151. https://doi.org/10.1016/j.jtice.2021.11.018.

[20] Z.Z. Ariffin, M.N. Saat, N.T. Zulkifle, A review on response surface methodology optimization in microbial biotransformation, Sci. Lett. 16 (2022) 64–72. https://doi.org/10.24191/sl.v16i2.17135.

[21] M. Chen, P. Xu, G. Zeng, C. Yang, D. Huang, J. Zhang, Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs, Biotechnol. Adv. 33 (2015) 745–755. https://doi.org/10.1016/j.biotechadv.2015.05.003.

[22] F. Anasonye, E. Winquist, B. Kluczek-Turpeinen, M. Räsänen, K. Salonen, K.T. Steffen, M. Tuomela, Fungal enzyme production and biodegradation of polychlorinated dibenzo-p-dioxins and dibenzofurans in contaminated sawmill soil, Chemosphere. 110 (2014) 85–90. https://doi.org/10.1016/j.chemosphere.2014.03.079.

[23] M.B. Asif, F.I. Hai, L. Singh, W.E. Price, L.D. Nghiem, Degradation of Pharmaceuticals and Personal Care Products by White-Rot Fungi—a Critical Review, Curr. Pollut. Reports. 3 (2017) 88–103. https://doi.org/10.1007/s40726-017-0049-5.

[24] B. Varga, V. Somogyi, M. Meiczinger, N. Kováts, E. Domokos, Enzymatic treatment and subsequent toxicity of organic micropollutants using oxidoreductases - A review, J. Clean. Prod. 221 (2019) 306–322. https://doi.org/10.1016/j.jclepro.2019.02.135.

[25] C. Ely, M. de Lourdes Borba Magalhães, C. Henrique Lemos Soares, E. Skoronski, M. De Lourdes Borba Magalhães, C. Henrique Lemos Soares, E. Skoronski, Optimization of Phenol Removal from Biorefinery Effluent Using Horseradish Peroxidase, J. Environ. Eng. 143 (2017) 1–7. https://doi.org/10.1061/(asce)ee.1943-7870.0001279.

[26] M. Alshabib, S.A. Onaizi, A review on phenolic wastewater remediation using homogeneous and heterogeneous enzymatic processes: Current status and potential challenges, Sep. Purif. Technol. 219 (2019) 186–207. https://doi.org/10.1016/j.seppur.2019.03.028.

[27] P. Lueangjaroenkit, C. Teerapatsakul, K. Sakka, M. Sakka, T. Kimura, E. Kunitake, L. Chitradon, Two Manganese Peroxidases and a Laccase of Trametes polyzona KU-RNW027 with Novel Properties for Dye and Pharmaceutical Product Degradation in Redox Mediator-Free System, Mycobiology. 47 (2019) 217–229. https://doi.org/10.1080/12298093.2019.1589900.

[28] Y. Wu, Y. Chen, N. Wei, Biocatalytic properties of cell surface display laccase for degradation of emerging contaminant acetaminophen in water reclamation, Biotechnol. Bioeng. 117 (2020) 342–353. https://doi.org/10.1002/bit.27214.

[29] M. Catapane, C. Nicolucci, C. Menale, L. Mita, S. Rossi, D.G. Mita, N. Diano, Enzymatic removal of estrogenic activity of nonylphenol and octylphenol aqueous solutions by immobilized laccase from Trametes versicolor, J. Hazard. Mater. 248–249 (2013) 337–346. https://doi.org/10.1016/j.jhazmat.2013.01.031.

[30] Z.K. Bagewadi, S.I. Mulla, H.Z. Ninnekar, Purification and immobilization of laccase from Trichoderma harzianum strain HZN10 and its application in dye decolorization, J. Genet. Eng. Biotechnol. 15 (2017) 139–150. https://doi.org/10.1016/j.jgeb.2017.01.007.

[31] J.J.H. Kim, S.G. Hong, H.J. Sun, S. Ha, J.J.H. Kim, Precipitated and chemically-crosslinked laccase over polyaniline nanofiber for high performance phenol sensing, Chemosphere. 143 (2016) 142–147. https://doi.org/10.1016/j.chemosphere.2015.08.011.

[32] N. Jaiswal, V.P. Pandey, U.N. Dwivedi, Immobilization of papaya laccase in chitosan led to improved multipronged stability and dye discoloration, Int. J. Biol. Macromol. 86 (2016) 288–295. https://doi.org/10.1016/j.ijbiomac.2016.01.079.

[33] T.T. Le, K. Murugesan, C.S. Lee, C.H. Vu, Y.S. Chang, J.R. Jeon, Degradation of synthetic pollutants in real wastewater using laccase encapsulated in core-shell magnetic copper alginate beads, Bioresour. Technol. 216 (2016) 203–210. https://doi.org/10.1016/j.biortech.2016.05.077.

[34] P. Sathishkumar, S. Kamala-Kannan, M. Cho, J.S. Kim, T. Hadibarata, M.R. Salim, B.T. Oh, Laccase immobilization on cellulose nanofiber: The catalytic efficiency and recyclic application for simulated dye effluent treatment, J. Mol. Catal. B Enzym. 100 (2014) 111–120. https://doi.org/10.1016/j.molcatb.2013.12.008.

[35] S.S. Nadar, L. Vaidya, V.K. Rathod, Enzyme embedded metal organic framework (enzyme–MOF): De novo approaches for immobilization, Int. J. Biol. Macromol. 149 (2020) 861–876. https://doi.org/10.1016/j.ijbiomac.2020.01.240.

[36] S. Liang, X.L. Wu, J. Xiong, M.H. Zong, W.Y. Lou, Metal-organic frameworks as novel matrices for efficient enzyme immobilization: An update review, Coord. Chem. Rev. 406 (2020) 213149. https://doi.org/10.1016/j.ccr.2019.213149.

[37] L.B. Vaidya, S.S. Nadar, V.K. Rathod, Metal-organic frameworks (MOFs) for enzyme immobilization, Met. Fram. Biomed. Appl. (2020) 491–523. https://doi.org/10.1016/B978-0-12-816984-1.00024-X.

[38] S. Pu, X. Zhang, C. Yang, S. Naseer, X. Zhang, J. Ouyang, D. Li, J. Yang, The effects of NaCl on enzyme encapsulation by zeolitic imidazolate frameworks-8, Enzyme Microb. Technol. 122 (2019) 1–6. https://doi.org/10.1016/j.enzmictec.2018.12.003.

[39] Z. Lei, C. Gao, L. Chen, Y. He, W. Ma, Z. Lin, Recent advances in biomolecule immobilization based on self-assembly: Organic-inorganic hybrid nanoflowers and metal-organic frameworks as novel substrates, J. Mater. Chem. B. 6 (2018) 1581–1594. https://doi.org/10.1039/c7tb03310a.

[40] Y. Zhu, J. Ciston, B. Zheng, X. Miao, C. Czarnik, Y. Pan, R. Sougrat, Z. Lai, C.E. Hsiung, K. Yao, I. Pinnau, M. Pan, Y. Han, Unravelling surface and interfacial structures of a metal-organic framework by transmission electron microscopy, Nat. Mater. 16 (2017) 532–536. https://doi.org/10.1038/nmat4852.

[41] W. Liang, H. Xu, F. Carraro, N.K. Maddigan, Q. Li, S.G. Bell, D.M. Huang, A. Tarzia, M.B. Solomon, H. Amenitsch, L. Vaccari, C.J. Sumby, P. Falcaro, C.J. Doonan, Enhanced Activity of Enzymes Encapsulated in Hydrophilic Metal-Organic Frameworks, J. Am. Chem. Soc. 141 (2019) 2348–2355. https://doi.org/10.1021/jacs.8b10302.

[42] Y. Wang, P. Hou, Z. Wang, P. Kang, Zinc Imidazolate Metal–Organic Frameworks (ZIF-8) for Electrochemical Reduction of CO2 to CO, ChemPhysChem. 18 (2017) 3142–3147. https://doi.org/10.1002/cphc.201700716.

[43] N.M. Mahmoodi, J. Abdi, Metal-organic framework as a platform of the enzyme to prepare novel environmentally friendly nanobiocatalyst for degrading pollutant in water, J. Ind. Eng. Chem. 80 (2019) 606–613. https://doi.org/10.1016/j.jiec.2019.08.036.

[44] M. Salgaonkar, S.S. Nadar, V.K. Rathod, Combi-metal organic framework (Combi-MOF) of α-amylase and glucoamylase for one pot starch hydrolysis, Int. J. Biol. Macromol. 113 (2018) 464–475. https://doi.org/10.1016/j.ijbiomac.2018.02.092.

[45] M. Salgaonkar, S.S. Nadar, V.K. Rathod, Biomineralization of orange peel peroxidase within metal organic frameworks (OPP-MOFs) for dye degradation, J. Environ. Chem. Eng. 7 (2019) 102969. https://doi.org/10.1016/j.jece.2019.102969.

[46] S.S. Nadar, V.K. Rathod, Immobilization of proline activated lipase within metal organic framework (MOF), Int. J. Biol. Macromol. 152 (2020) 1108–1112. https://doi.org/10.1016/j.ijbiomac.2019.10.199.

[47] V. Gascón, E. Castro-Miguel, M. Díaz-García, R.M. Blanco, M. Sanchez-Sanchez, In situ and post-synthesis immobilization of enzymes on nanocrystalline MOF platforms to yield active biocatalysts, J. Chem. Technol. Biotechnol. 92 (2017) 2583–2593. https://doi.org/10.1002/jctb.5274.

[48] S.S. Nadar, V.K. Rathod, Magnetic-metal organic framework (magnetic-MOF): A novel platform for enzyme immobilization and nanozyme applications, Int. J. Biol. Macromol. 120 (2018) 2293–2302. https://doi.org/10.1016/j.ijbiomac.2018.08.126.

[49] X. Lian, Y. Fang, E. Joseph, Q. Wang, J. Li, S. Banerjee, C. Lollar, X. Wang, H.C. Zhou, Enzyme-MOF (metal-organic framework) composites, Chem. Soc. Rev. 46 (2017) 3386–3401. https://doi.org/10.1039/c7cs00058h.

[50] R. Ahmad, J. Shanahan, S. Rizaldo, D.S. Kissel, K.L. Stone, Co-immobilization of an

enzyme system on a metal-organic framework to produce a more effective biocatalyst, Catalysts. 10 (2020). https://doi.org/10.3390/catal10050499.

[51] E. Wu, Y. Li, Q. Huang, Z. Yang, A. Wei, Q. Hu, Laccase immobilization on amino-functionalized magnetic metal organic framework for phenolic compound removal, Chemosphere. 233 (2019) 327–335. https://doi.org/10.1016/j.chemosphere.2019.05.150.

[52] T.O. Knedel, E. Ricklefs, C. Schlüsener, V.B. Urlacher, C. Janiak, Laccase Encapsulation in ZIF-8 Metal-Organic Framework Shows Stability Enhancement and Substrate Selectivity, ChemistryOpen. 8 (2019) 1337–1344. https://doi.org/10.1002/open.201900146.

[53] S.S. Nadar, V.K. Rathod, Enzyme and Microbial Technology Encapsulation of lipase within metal-organic framework (MOF) with enhanced activity intensi fi ed under ultrasound, Enzyme Microb. Technol. 108 (2018) 11–20. https://doi.org/10.1016/j.enzmictec.2017.08.008.

[54] J. Mehta, S. Dhaka, N. Bhardwaj, A.K. Paul, S. Dayananda, S.E. Lee, K.H. Kim, A. Deep, Application of an enzyme encapsulated metal-organic framework composite for convenient sensing and degradation of methyl parathion, Sensors Actuators, B Chem. (2019) 267–274. https://doi.org/10.1016/j.snb.2019.03.116.

[55] M.N. Shahrak, M. Ghahramaninezhad, M. Eydifarash, Zeolitic imidazolate framework-8 for efficient adsorption and removal of Cr (VI) ions from aqueous solution, (2019). https://doi.org/10.1007/s11356-017-8577-5.

[56] M. Mohammadi, M.A. As'habi, P. Salehi, M. Yousefi, M. Nazari, J. Brask, Immobilization of laccase on epoxy-functionalized silica and its application in biodegradation of phenolic compounds, Int. J. Biol. Macromol. 109 (2018) 443–447. https://doi.org/10.1016/j.ijbiomac.2017.12.102.

[57] F. Zhang, M. Lian, A. Alhadhrami, M. Huang, B. Li, G.A.M. Mersal, M.M. Ibrahim, M. Xu, Laccase immobilized on functionalized cellulose nanofiber/alginate composite hydrogel for efficient bisphenol A degradation from polluted water, Adv. Compos. Hybrid Mater. (2022). https://doi.org/10.1007/s42114-022-00476-5.

[58] H.R. Wehaidy, M.A. Abdel-Naby, H.M. El-Hennawi, H.F. Youssef, Nanoporous Zeolite-X as a new carrier for laccase immobilization and its application in dyes decolorization, Biocatal. Agric. Biotechnol. 19 (2019) 101135. https://doi.org/10.1016/j.bcab.2019.101135.

[59] Y. Du, J. Gao, L. Zhou, L. Ma, Y. He, Z. Huang, Y. Jiang, Enzyme nanocapsules armored by metal-organic frameworks: A novel approach for preparing nanobiocatalyst, Chem. Eng. J. 327 (2017) 1192–1197. https://doi.org/10.1016/j.cej.2017.07.021.

[60] J. Cui, Y. Feng, S. Jia, Silica encapsulated catalase@metal-organic framework composite: A highly stable and recyclable biocatalyst, Chem. Eng. J. 351 (2018) 506–514. https://doi.org/10.1016/j.cej.2018.06.121.

[61] S. Kashefi, S.M. Borghei, N.M. Mahmoodi, Covalently immobilized laccase onto graphene oxide nanosheets: Preparation, characterization, and biodegradation of azo dyes in colored wastewater, J. Mol. Liq. 276 (2019) 153–162. https://doi.org/10.1016/j.molliq.2018.11.156.

[62] A. Ulu, Metal–organic frameworks (MOFs): a novel support platform for ASNase immobilization, J. Mater. Sci. 55 (2020) 6130–6144. https://doi.org/10.1007/s10853-020-04452-6.

[63] Y. Zhang, Y. Jia, L. Hou, Synthesis of zeolitic imidazolate framework-8 on polyester fiber for PM2.5 removal, RSC Adv. 8 (2018) 31471–31477. https://doi.org/10.1039/c8ra06414h.

[64] L. Zhou, N. Li, G. Owens, Z. Chen, Simultaneous removal of mixed contaminants, copper and norfloxacin, from aqueous solution by ZIF-8, Chem. Eng. J. 362 (2019) 628–637. https://doi.org/10.1016/j.cej.2019.01.068.

[65] N.M. Mahmoodi, S. Keshavarzi, M. Oveisi, S. Rahimi, B. Hayati, Metal-organic framework (ZIF-8)/inorganic nanofiber (Fe2O3) nanocomposite: Green synthesis and photocatalytic degradation using LED irradiation, J. Mol. Liq. 291 (2019) 2–3. https://doi.org/10.1016/j.molliq.2019.111333.

[66] J. Wang, S. Yu, F. Feng, L. Lu, Simultaneous purification and immobilization of laccase on magnetic zeolitic imidazolate frameworks: Recyclable biocatalysts with enhanced stability for dye decolorization, Biochem. Eng. J. 150 (2019) 107285. https://doi.org/10.1016/j.bej.2019.107285.

[67] A.R.M. Silva, J.Y.N.H. Alexandre, J.E.S. Souza, J.G.L. Neto, P.G. de Sousa Júnior, M.V.P. Rocha, J.C.S. dos Santos, The Chemistry and Applications of Metal–Organic Frameworks (MOFs) as Industrial Enzyme Immobilization Systems, Molecules. 27 (2022). https://doi.org/10.3390/molecules27144529.

[68] Z. Wang, D. Ren, H. Yu, S. Jiang, S. Zhang, X. Zhang, Study on improving the stability of adsorption-encapsulation immobilized Laccase@ZIF-67, Biotechnol. Reports. 28 (2020) e00553. https://doi.org/10.1016/j.btre.2020.e00553.

[69] D. Tocco, C. Carucci, D. Todde, K. Shortall, F. Otero, E. Sanjust, E. Magner, A. Salis, Enzyme immobilization on metal organic frameworks: Laccase from Aspergillus sp. is better adapted to ZIF-zni rather than Fe-BTC, Colloids Surfaces B Biointerfaces. 208 (2021) 112147. https://doi.org/10.1016/j.colsurfb.2021.112147.

[70] D. Li, Y. Cheng, H. Zuo, W. Zhang, G. Pan, Y. Fu, Q. Wei, Dual-functional biocatalytic membrane containing laccase-embedded metal-organic frameworks for detection and degradation of phenolic pollutant, J. Colloid Interface Sci. 603 (2021) 771–782. https://doi.org/10.1016/j.jcis.2021.06.155.

[71] P. Xu, H. Du, X. Peng, Y. Tang, Y. Zhou, X. Chen, J. Fei, Y. Meng, L. Yuan, Degradation of several polycyclic aromatic hydrocarbons by laccase in reverse micelle system, Sci. Total Environ. 708 (2020) 134970. https://doi.org/10.1016/j.scitotenv.2019.134970.

[72] E. Birhanlı, S.A.A. Noma, F. Boran, A. Ulu, Ö. Yeşilada, B. Ateş, Design of laccase–metal–organic framework hybrid constructs for biocatalytic removal of textile dyes, Chemosphere. 292 (2022). https://doi.org/10.1016/j.chemosphere.2021.133382.

[73] D. Wang, J. Lou, J. Yuan, J. Xu, R. Zhu, Q. Wang, X. Fan, Laccase immobilization on core-shell magnetic metal-organic framework microspheres for alkylphenol ethoxylate compound removal, J. Environ. Chem. Eng. 9 (2021) 105000. https://doi.org/10.1016/j.jece.2020.105000.

[74] X. Li, Z. Wu, X. Tao, R. Li, D. Tian, X. Liu, Gentle one-step co-precipitation to synthesize bimetallic CoCu-MOF immobilized laccase for boosting enzyme stability and Congo red removal, J. Hazard. Mater. 438 (2022) 129525. https://doi.org/10.1016/j.jhazmat.2022.129525.

[75] S.F.F. Zofair, S. Ahmad, M.A. Hashmi, S.H. Khan, M.A. Khan, H. Younus, Catalytic roles, immobilization and management of recalcitrant environmental pollutants by laccases: Significance in sustainable green chemistry, J. Environ. Manage. 309 (2022) 114676. https://doi.org/10.1016/j.jenvman.2022.114676.

[76] Y. Gao, M. Wang, K. Shah, S. Singh Kalra, L.H. Rome, S. Mahendra, Decolorization and detoxification of synthetic dye compounds by laccase immobilized in vault nanoparticles, Bioresour. Technol. 351 (2022) 127040. https://doi.org/10.1016/j.biortech.2022.127040.

[77] Z. Wang, D. Ren, Y. Cheng, X. Zhang, S. Zhang, W. Chen, Immobilization of laccase on chitosan functionalized halloysite nanotubes for degradation of Bisphenol A in aqueous solution: degradation mechanism and mineralization pathway, Heliyon. 8 (2022) e09919. https://doi.org/10.1016/j.heliyon.2022.e09919.

[78] P. Habimana, Y. Jiang, J. Gao, J.B. Ndayambaje, O.M. Darwesh, J.P. Mwizerwa, X. Zheng, L. Ma, Enhancing laccase stability and activity for dyes decolorization using ZIF-8@MWCNT nanocomposite, Chinese J. Chem. Eng. 48 (2022) 66–75. https://doi.org/10.1016/j.cjche.2021.05.044.

[79] M. Bhaumik, R.I. McCrindle, A. Maity, S. Agarwal, V.K. Gupta, Polyaniline nanofibers as highly effective re-usable adsorbent for removal of reactive black 5 from aqueous solutions, J. Colloid Interface Sci. 466 (2016) 442–451. https://doi.org/10.1016/j.jcis.2015.12.056.

[80] M. Seenuvasan, G. Vinodhini, C.G. Malar, N. Balaji, K.S. Kumar, Magnetic nanoparticles: a versatile carrier for enzymes in bio-processing sectors, IET Nanobiotechnology. 12 (2018) 535–548. https://doi.org/10.1049/IET-NBT.2017.0041.

[81] Z. Chen, J. Yao, T. Šolević Knudsen, B. Ma, B. Liu, H. Li, X. Zhu, C. Zhao, W. Pang, Y. Cao, Degradation of novel mineral flotation reagent 8-hydroxyquinoline by superparamagnetic immobilized laccase: Effect, mechanism and toxicity evaluation, Chem. Eng. J. 432 (2022). https://doi.org/10.1016/j.cej.2021.134239.

[82] D. Liu, W. Gu, W. Zhou, Y. Xu, W. He, L. Liu, L. Zhou, J. Lei, J. Zhang, Y. Liu, Magnetic Fe/carbon/sodium alginate hydrogels for efficient degradation of norfloxacin in simulated wastewater, J. Clean. Prod. 369 (2022) 133239. https://doi.org/10.1016/j.jclepro.2022.133239.

[83] J. Deng, H. Wang, H. Zhan, C. Wu, Y. Huang, B. Yang, A. Mosa, W. Ling, Catalyzed degradation of polycyclic aromatic hydrocarbons by recoverable magnetic chitosan immobilized laccase from Trametes versicolor, Chemosphere. 301 (2022) 134753. https://doi.org/10.1016/j.chemosphere.2022.134753.

[84] J. Zdarta, K. Jankowska, M. Wyszowska, E. Kijeńska-Gawrońska, A. Zgoła-Grześkowiak, M. Pinelo, A.S. Meyer, D. Moszyński, T. Jesionowski, Robust biodegradation of naproxen and diclofenac by laccase immobilized using electrospun nanofibers with enhanced stability and reusability, Mater. Sci. Eng. C. 103 (2019). https://doi.org/10.1016/j.msec.2019.109789.

[85] M.R. Ladole, P.B. Pokale, S.S. Patil, P.G. Belokar, A.B. Pandit, Laccase immobilized peroxidase mimicking magnetic metal organic frameworks for industrial dye degradation, Bioresour. Technol. 317 (2020) 124035. https://doi.org/10.1016/j.biortech.2020.124035.

[86] X. Lou, F. Zhi, X. Sun, F. Wang, X. Hou, C. Lv, Q. Hu, Construction of co-immobilized laccase and mediator based on MOFs membrane for enhancing organic pollutants removal, Chem. Eng. J. 451 (2023). https://doi.org/10.1016/j.cej.2022.138080.

[87] S. Sadeghzadeh, Z. Ghobadi Nejad, S. Ghasemi, M. Khafaji, S.M. Borghei, Removal of bisphenol A in aqueous solution using magnetic cross-linked laccase aggregates from Trametes hirsuta, Bioresour. Technol. 306 (2020) 123169. https://doi.org/10.1016/j.biortech.2020.123169.

[88] X. Qiu, S. Wang, S. Miao, H. Suo, H. Xu, Y. Hu, Co-immobilization of laccase and ABTS onto amino-functionalized ionic liquid-modified magnetic chitosan nanoparticles for pollutants removal, J. Hazard. Mater. 401 (2021). https://doi.org/10.1016/j.jhazmat.2020.123353.

[89] S. Pang, Y. Wu, X. Zhang, B. Li, J. Ouyang, M. Ding, Immobilization of laccase via adsorption onto bimodal mesoporous Zr-MOF, Process Biochem. 51 (2016) 229–239. https://doi.org/10.1016/j.procbio.2015.11.033.

[90] D.O. Jegede, G.O. Adewuyi, M.R. Gbadamosi, P.O. Oladoye, A.L. Ogunneye, O.J. Oyewola, Degradation of Diethyl Phthalate and Di (2-Ethylhexyl) Phthalate Using Chemical and Microbial Methods, Niger. J. Chem. Res. 24 (2019) 77–89.

[91] X. Zhang, L. Liu, S. Zhang, Y. Pan, J. Li, H. Pan, S. Xu, F. Luo, Biodegradation of Dimethyl Phthalate by Freshwater Unicellular Cyanobacteria, Biomed Res. Int. 2016 (2016). https://doi.org/10.1155/2016/5178697.

[92] B. Prasad, S. Suresh, Biodegradation of Dimethyl Phthalate , Diethyl Phthalate , Dibutyl Phthalate and Their Mixture by Variovorax Sp ., Int. J. Environ. Sci. Dev. 3 (2012). https://doi.org/10.7763/IJESD.2012.V3.232.

[93] J. Sun, H. Zhu, X. Yang, Y. Zheng, T. Sun, Carboxylesterase and lipase-catalyzed degradation of phthalate esters in soil and water : Congener structure selectivity and specificity, Environ. Technol. Innov. 28 (2022) 102571. https://doi.org/10.1016/j.eti.2022.102571.

[94] M. Mehrnia, M. Torun, D. Şolpan, Investigation of Degradation Mechanism for dimethyl phthalate (DMP) in aqueous solution by using analytical methods, ALKU J. Sci. (2019) 125–141. http://dergipark.gov.tr/alku/issue/41490/491251.

[95] J. Wang, W. Xu, Z. Tianhua, Degradation of dimethyl phthalate esters by a filamentous fungus Aspergillus versicolor isolated from deep-sea sediments, Bot. Mar. 60 (2017) 351–359. https://doi.org/10.1515/bot-2016-0077.

[96] T.C. Mak, J. Gu, Degradability and Biochemical Pathways of the Endocrine-disrupting Plasticizers Phthalate Esters in Plastics by Microorganisms, Appl. Environ. Biotechnol. 6 (2021) 16–30.

[97] Y.L. Feng, R. Singh, A. Chao, Y. Li, Diagnostic Fragmentation Pathways for Identification of Phthalate Metabolites in Nontargeted Analysis Studies, J. Am. Soc.

Mass Spectrom. 33 (2022) 981–995. https://doi.org/10.1021/jasms.2c00052.

[98] D. Wu, P. Lv, Q. Feng, Y. Jiang, H. Yang, M. Alfred, Q. Wei, Biomass-derived nanocellulose aerogel enable highly efficient immobilization of laccase for the degradation of organic pollutants, Bioresour. Technol. 356 (2022) 127311. https://doi.org/10.1016/j.biortech.2022.127311.

[99] K. Bjorklund, L.Y. Li, Adsorption of organic stormwater pollutants onto activated carbon from sewage sludge, J. Environ. Manage. 197 (2017) 490–497. https://doi.org/10.1016/j.jenvman.2017.04.011.

4.3 Paper 3

This manuscript entitled "**Response surface methodology mediated optimization of diclofenac and norfloxacin biodegradation using laccase immobilized on metal-organic frameworks**" is under review and communication with supervisors for submission to Separation and purification journal.

This article investigated the optimization of parameters for maximum biodegradation of diclofenac (DFC) and norfloxacin (NOR) using laccase immobilized on ZIF crystals.

Authors:

Mbongiseni Lungelo Dlamini – Principal author

Mahadi Lesaoana – Co-author

Izak Kotzé – Co-supervisor

Heidi Lynn Richards – Main supervisor

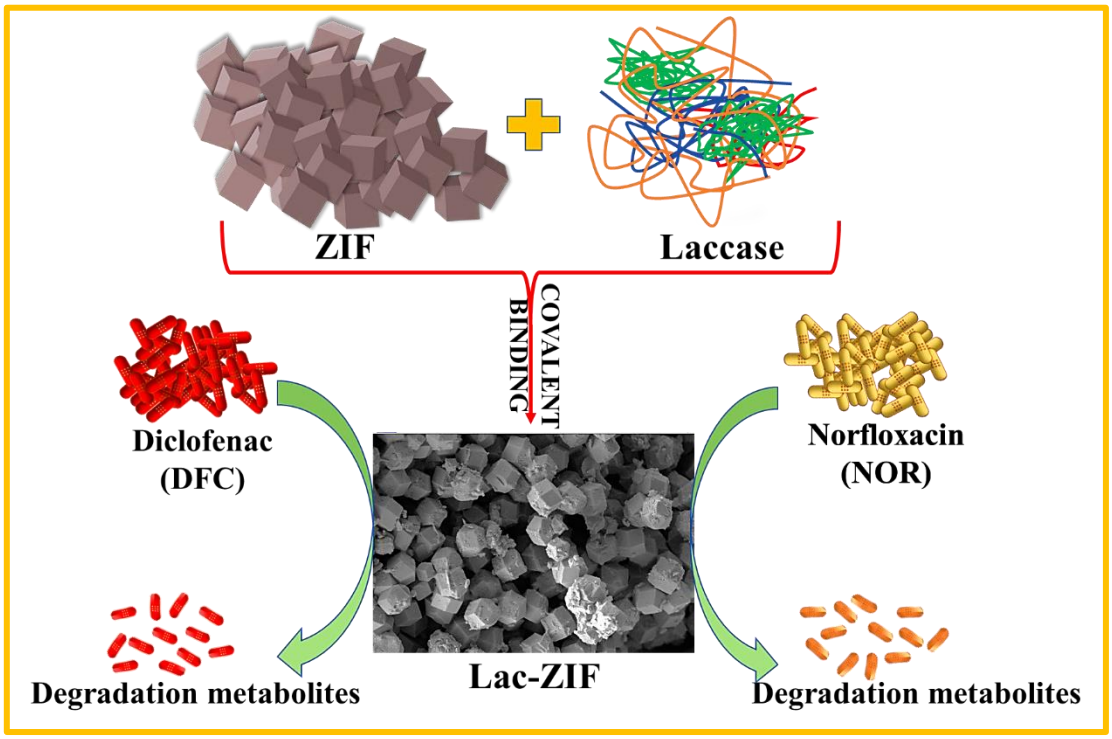
Contribution: lead author, conducted experimental work and data analysis, conceptualization, methodology, formal analysis, investigation, writing - original draft and visualization.

Response surface methodology mediated optimization of diclofenac and norfloxacin biodegradation using laccase immobilized on metal-organic frameworks

Mbongiseni Lungelo Dlamini, Mahadi Lesaoana, Izak A. Kotzé, Heidi Richards*

Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa.

Graphical Abstract



*Corresponding author: Email address: heidi.richards@wits.ac.za

Abstract

In this work, immobilized laccase (Lac-ZIF) is used as a biocatalytic composite to decontaminate pharmaceutically active compounds (PhACs): diclofenac (DFC), and norfloxacin (NOR). The central composite face (CCF) design was used, and operational parameters were optimized using a quadratic response surface methodology (RSM) regression model, resulting in pH 6.4, 10 mg Lac-ZIF dosage, 10.4 mg L^{-1} initial concentration, and 8 h contact time. The model proved viable and effective for application in this study, giving predictions of 93.9 and 95.1% removal efficiencies of DFC and NOR, which were confirmed by actual experimental results with high accuracy. The Lac-ZIF revealed enhanced thermal stability with subsequently higher deactivation energies (E_d) than the free form of laccase. Additionally, the application of the biocatalyst in the treatment of real water samples, outperformed the free laccase by approximately 1.5 folds. Furthermore, the Lac-ZIF preserved 79 and 83% residual removal efficiencies after 6 cycles of reuse for DFC and NOR elimination, respectively.

***Keywords*:** Diclofenac; Norfloxacin; RSM; Biodegradation; Laccase; Immobilization

1. Introduction

Pharmaceutically active compounds (PhACs) occurrence in the environment is a global concern, and the health risks associated with exposure of aquatic life to these compounds have led to their classification as emerging environmental contaminants (Khulu et al., 2022; Tegegne et al., 2021). PhACs are extensively utilized as prescription or non-prescription medicines, and they end up in wastewater as intact substances or metabolites via urine and fecal matter (An et al., 2018; Naghdi et al., 2018). It is concerning that wastewater treatment plants (WWTPs) are inefficient in removing these substances, owing to their recalcitrant nature, resulting in their discharge into surface water (X. Chen et al., 2021; de Carvalho Filho et al., 2022; Mkaddem et al., 2022).

Antibiotics, analgesics, and anti-inflammatory drugs are among the various synthetic and natural compounds utilized in the treatment of infectious diseases and pain relief in humans and animals, and have been detected in wastewater (Al-Kindi et al., 2021; L. Chen et al., 2021; Hu et al., 2022; Tegegne et al., 2021). Norfloxacin (NOR), an antibiotic of the fluoroquinolone class, is widely used due to its broad-spectrum antibacterial properties (L. Chen et al., 2021; Paredes-Laverde et al., 2018). Fluoroquinolones would typically undergo partial metabolism, therefore, a greater proportion of fluoroquinolones consumed may be excreted directly into the environment as parent compounds or other bioactive metabolites, resulting in enhanced antibiotic resistance in aquatic life, as well as inhibition of denitrifying bacterial growth (L. Chen et al., 2021; Domingo-Torner et al., 2023; Liu et al., 2021; Paredes-Laverde et al., 2018; Xin et al., 2022).

Another recently detected PhAC in aquatic environments at trace concentrations is the analgesic, non-steroidal anti-inflammatory drug (NSAID), diclofenac (DFC) (Mustafa and Hama Aziz, 2023). DFC's ecotoxicity may be relatively low, however, chronic exposure to environmentally relevant quantities in water significantly increases toxicity and has an adverse influence on aquatic life (Li et al., 2023; Sharma et al., 2023; B. Zhang et al., 2022).

Due to the negative effects of PhACs on the biosystem, it is essential to develop and optimize an eco-friendly, cost-effective, and efficient approach to mitigating environmental pollution. Conventional physicochemical approaches, such as photocatalytic degradation, membrane filtration, ozonation, and adsorption with modified carbonaceous materials among others, have been explored to improve the elimination efficiencies of such techniques (Choi et al., 2021; M. C. Dlamini et al., 2022; Fan et al., 2017; Peng et al., 2018; Zelaya Soulé et al., 2020). However,

major drawbacks such as the high costs associated with ozone treatment and adsorbent regeneration have been identified. Additionally, these technologies may generate large amounts of sludge and metabolites that are more toxic than the parent molecule. (Ariffin et al., 2022; Carrillo-Abad et al., 2020; Guo et al., 2023; Shan et al., 2023; Sun et al., 2022).

In the recent decade, biocatalytic degradation systems have emerged as one of the most promising advances in organic wastewater treatment, due to their eco-friendliness, enhanced efficiency, cost-effectiveness, and limited secondary pollution (Alessandretti et al., 2021; Crespí Sánchez et al., 2023; Li et al., 2023; Prasetya and Li, 2022). This involves the use of living organisms or their enzymes, and fungal oxidoreductase enzymes such as laccases and peroxidases have received the most attention due to their ability to degrade a wide range of organic pollutants with minimal energy requirements and high substrate specificity (Luisa et al., 2019; Naghdi et al., 2018; Zhang et al., 2020). However, free enzymes have shortcomings including low stability and non-repeatable application as a consequence of leaching, which are key constraints of their functional application (Habimana et al., 2022).

As a result, immobilization of the enzyme onto insoluble supports is required, making the enzymes more commercially and industrially viable (Rafiei et al., 2018). Immobilization of the enzyme improves its storage, pH, and thermal stabilities, as well as its insolubility and recyclability potential (Leynaud Kieffer Curran et al., 2022; Liang et al., 2020). Among various support materials, metal-organic frameworks (MOFs) have gained exceptional appreciation as porous hybrid materials. They are characterized by stable crystallographic morphologies, low densities, designable metal-organic ligands, and large surface area with remarkable porosity which enhances enzyme loading. Additionally, MOFs form strong interactions with the organic counterparts of organic frameworks and enzymes, preventing them from leaching out during applications (Eddaoudi et al., 2015; Liang et al., 2020; Mehta et al., 2016).

The immobilization of laccase onto MOFs have been widely explored for several applications including wastewater treatment and remediation (Knedel et al., 2019; Tocco et al., 2021; Tülek et al., 2021; Wang et al., 2021; Wu et al., 2019, 2022). However, since the biodegradation of organic pollutants is strongly affected by various factors, the empirical identification of the optimized parameters for such multifactorial investigations may not always be feasible as it necessitates evaluating several combinations of variables individually, limiting the possible detection of mutual effects between two or more experimental parameters. As a result, response surface methodology (RSM) can be an effective multivariate statistical tool for evaluating and

modeling the interactive impacts of several variables, with subsequent development of a computational model that can depict the entire process under investigation (El Telib et al., 2017; Rath et al., 2022; Shah et al., 2022). This method can easily optimize variable responses with a minimum number of experiments, thereby reducing cost and time (El Telib et al., 2017; Khulu et al., 2022).

RSM has been extensively applied in the modeling and optimization of the bioremediation potential of organic pollutants using numerous biocatalytic materials (Bankole et al., 2021; Bhattacharya and Banerjee, 2008; Chollom et al., 2022; Murugesan et al., 2007; Rath et al., 2022; Rezaei et al., 2015; Shah et al., 2022; F. Zhang et al., 2022). Rezaei et al. (2015), for example, used RSM to determine optimum conditions for acid blue 92 degradation using free laccase, and the results revealed a decolorization efficiency of 90.2%, which was about 99% of the predicted value. Also, Lou et al. (2022), used RSM to optimize the degradation of sulfonamide using a laccase–syringaldehyde mediator system. A 91.07% degradation rate was predicted and the experimental rate using the optimized parameters yielded 94.84 ± 3.98%, proving the validity and efficiency of the model. However, in most of the relevant studies, the enzymes especially laccase were used in their soluble form which limits their practical application in real water systems. Also, to the best of our knowledge, the optimization of parameters for the biodegradation of DFC and NOR using the Central Composite Face (CCF) design has not been explored. As a result, the goal of this research is to find the best parameters for maximum diclofenac and norfloxacin biodegradation in a typical bioremediation experiment using a laccase-zeolitic imidazolate framework (Lac-ZIF) composite. In this sense, a multifactorial experimental layout was developed, which was then followed by RSM modeling of the experimental data, and finally validation of the modeled prediction for enhanced removal. Additionally, the thermal deactivation and Michaelis-Menten kinetics as well as the application of the biocatalyst composite in real water samples were investigated.

2. Materials and Methods

2.1 Chemicals and reagents

Laccase enzyme (E.C. 1.10.3.2), 2-methylimidazole (2-MIm), Zinc nitrate tetrahydrate ($Zn(NO_3)_2 \cdot 4H_2O$), diclofenac sodium salt (DFC), Norfloxacin (NOR), sodium acetate $C2H3NaO_2$, sodium dihydrogen phosphate ($NaH_2PO_4H_2O$), disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$), sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), acetic acid ($CH_3CO_2H$), citric acid ($C_6H_8O_7$) and Liquid Chromatography grade methanol (CH_3OH), were obtained from

Sigma Aldrich. All chemicals were of analytical grade. Distilled and ultrapure water was obtained from a Milli-Q pore system.

2.2 Laccase immobilization

The procedure for the immobilization of laccase was adopted from our previous work (M. L. Dlamini et al., 2022). For this, a biomineralization approach was employed whereby 80 mmol of 2-MIm dissolved in 200 mL CH_3OH was rapidly mixed with 2 mL of free laccase (1000 U g^{-1}) in a 5 mL phosphate buffer (pH 7) solution. In a separate conical flask, 20 mmol $Zn(NO_3)_2 \cdot 4H_2O$ solution was also prepared in 200 mL methanol. The solutions were subjected to magnetic stirring at 150 rpm for 5 min. Thereafter, the growth of the ZIF crystals in the presence of laccase was afforded by mixing the zinc salt solution with the 2-MIm and laccase mixture, and the mixture was allowed to stir for 2 h at room temperature. This procedure yielded a white suspension which was separated from the reaction mixture via centrifugation at 4000 rpm for 10 min followed by 2 times rinsing with 50 mL portions of distilled water. The solid composite named Lac-ZIF was then oven-dried for 24 h at 30 ℃.

2.3 Characterization of ZIF crystals and Lac-ZIF composite

A Perkin–Elmer, USA attenuated total reflectance Fourier Transform Infrared (ATR-FTIR) Spectrum 100 spectrometer was used to record IR spectra in the range 500-4000 cm^{-1} to ascertain the functional groups in Lac-ZIF and its precursors. The data were processed using OPUS software and the corresponding graphs were plotted using OriginLab. For XRD measurements, a Bruker D2 XRD spectrometer was used with CuKα radiation at 1.5505 Å wavelength and slits at 45 kV/30 mA for 2θ values ranging from 5° to 90°. Morphological analyses were conducted using a Field Emission Scanning Electron Microscope (FE-SEM: ZEISS EVO 50, Oberkochen, Germany).

2.4 RSM experimental design and optimization

A Central Composite Face (CCF) design was adopted for the optimization of the decontamination of DFC and NOR using MODDE 13.1 Pro (Sartorius Stedim Biotech, Malmö, Sweden). The experiment design comprised 27 experimental runs with 3 center points. The assessed variables were pH (4, 7, 10), dosage (10, 30, 50 mg), initial concentration (4, 12, 20 mg L^{-1}), and contact time (2, 7, 12 h) with two responses being DFC and NOR removal as illustrated in table 1. Analysis of variance (ANOVA) was used to examine the effects of the four variables and their interactions on the elimination of DFC and NOR, as well as the significance of the model and regression coefficients. The polynomial equation's feature was

evaluated by determining the regression coefficient (R^2) and the P-value of the lack-of-Fit. Fischer's F-test was used to assess statistical adequacy.

Table 1. Experimental design for DFC and NOR removal with independent variables, experimental and predicted responses.

Exp No	Run Order	Independent Variables				Response Values			
		pH	Dosage (mg)	Initial Concentration (mg L^{-1})	Contact time (h)	DFC Removal Efficiency (%)		NOR Removal Efficiency (%)	
						Exp.	Pred.	Exp.	Pred.
1	13	4	10	4	2	67.9	66.5	69.9	69.9
2	26	10	10	4	2	42.8	42.6	45.1	45.1
3	3	4	50	4	2	71.3	71.5	73.4	74.0
4	7	10	50	4	2	43.3	44.8	47.4	46.8
5	21	4	10	20	2	59.9	62.8	65.3	66.1
6	10	10	10	20	2	39.6	39.0	42.3	41.4
7	15	4	50	20	2	68.7	67.8	70.9	70.3
8	25	10	50	20	2	40.6	41.1	41.9	43.1
9	19	4	10	4	12	75.8	74.4	77.7	76.6
10	9	10	10	4	12	45.5	45.9	47.8	48.2
11	20	4	50	4	12	76.9	79.4	78.7	80.8
12	11	10	50	4	12	47.3	48.0	49.2	49.9
13	16	4	10	20	12	72.1	70.8	73.5	72.9
14	1	10	10	20	12	42.7	42.3	45.8	44.4
15	6	4	50	20	12	75.8	75.8	76.4	77.0
16	23	10	50	20	12	44.9	44.4	45.9	46.1
17	24	4	30	12	7	70.6	70.1	74.8	73.1
18	8	10	30	12	7	43.8	42.5	44.7	45.2
19	17	7	10	12	7	89.7	91.8	90.1	92.9
20	22	7	50	12	7	99.4	95.4	99.9	95.8
21	27	7	30	4	7	93.4	87.5	94.7	89.8
22	18	7	30	20	7	87.1	83.9	88.4	86.1
23	4	7	30	12	2	77.9	76.0	79.8	79.5
24	5	7	30	12	12	81.5	81.6	85.3	84.4
25	14	7	30	12	7	85.4	85.7	88.7	88.0
26	2	7	30	12	7	79.4	85.7	82.6	88.0
27	12	7	30	12	7	79.5	85.7	82.9	88.0

Note: Exp. = experimental; Pred. = predicted

Prior to using RSM, preliminary experiments were conducted to compare the DFC and NOR decontamination potential of free laccase, pure ZIF crystals, and immobilized laccase (Lac-ZIF). For this, 20 mg ZIF and Lac-ZIF, 100 µL free laccase were employed in treating 20 mL of 5 mg L^{-1} DFC and NOR solutions in separate 50 mL tubes. These were agitated on a rotary shaker at 160 rpm for 24 h. The samples were then filtered using 0.45 µm nylon syringe filters

before analyzing for residual analyte concentrations. The removal efficiencies were calculated based on the initial and final aqueous phase concentrations as shown in equation (1):

$$\%\, removal = \left(\frac{C_i - C_f}{C_i}\right) x100, \quad (1)$$

where C_i and C_f (mg L^{-1}) represent the initial and final analyte concentrations, respectively.

2.5 Determination of DFC and NOR residual concentrations

Quantification of residual concentrations of DFC and NOR was evaluated using a BISCHOFF high-performance liquid chromatography (HPLC) employing a Fortis C18 column (250 mm x 4.6 mm, 5 µm) at room temperature. Typically, 10 µL sample solution injections were used with UV absorption detections at 282 (DFC) and 276 (NOR) nm wavelengths. For DFC analysis, an isocratic elution of methanol and water (80:20 v/v) with a maintained 0.9 mL min^{-1} flow rate was used. To quantify for NOR, a constant 0.8 mL min^{-1} flow rate was employed and an acetonitrile to water (70:30 v/v) eluent ratio was employed. In both cases, the water contained 0.1% formic acid. Additionally, 100 mg L^{-1} stock solutions were prepared and subsequently diluted to the required concentrations.

2.6 Thermal deactivation and Michaelis-Menten kinetics of Lac-ZIF

For thermal stability studies, the Lac-ZIF and free laccase's decontamination efficiencies were evaluated at 25, 50, and 70 ℃ in 50 mM sodium phosphate buffers (pH 6.4) using 10.4 mg L^{-1} DFC and NOR solutions. The investigations were conducted over 60 min and aliquots of samples were withdrawn at predetermined time intervals for analysis. The resultant data was used to plot the natural log of residual concentrations (lnC_t) vs time graphs. The slopes from these graphs were used to determine the thermal deactivation rate constants (k_d). From this, the time taken for the efficiency to reduce to half of its initial efficiency ($t_{1/2}$) was calculated using equation (2). Additionally, deactivation energies (E_d) were calculated using the Arrhenius equation (3) and plots of lnK vs reciprocals of temperature in Kelvin.

$$t_{1/2} = {ln2}/{K} \quad (2)$$

$$k = Ae^{\frac{-E_d}{RT}} \quad (3)$$

Where, *k* represents the rate coefficient, A is a constant, E_d (J mol^{-1}) is the deactivation energy, R is the universal gas constant (8.314 J $mol^{-1}K^{-1}$), and T is the temperature (in Kelvin).

The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) for free laccase and Lac-ZIF were separately evaluated using both DFC and NOR as substrates utilizing equation (4).

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (4)$$

Where, V (mg L^{-1} h^{-1}) is the initial reaction rate (velocity) at substrate concentration [S] (mg L^{-1}). V_{max} (mg L^{-1} h^{-1}) and K_m (mg L^{-1}) represent the maximum velocity and Michaelis constant, respectively.

2.7 Reusability of immobilized laccase

The recyclability potential of Lac-ZIF was investigated using optimum parameters for DFC and NOR elimination for up to 7 cycles. Separation of the Lac-ZIF after each cycle was afforded by centrifugation of the mixture at 4000 rpm for 5 min followed by washing with 50mM citrate-phosphate buffer (pH 7). Fresh analyte solutions were used per cycle. The calculation of residual removal efficiency was based on considering the initial decontamination efficiency as 100% and utilized as a reference value for all successive cycles.

3. Results and Discussion

3.1 Characterization of Lac-ZIF, ZIF and free laccase

The FTIR spectra for the Lac-ZIF and its precursors are presented in fig. 1. (a). The 2-methylimidazole (2-MIm) spectrum shows a significant vibrational peak between 3200 – 2380 cm^{-1} which may be ascribed to the pyrrole group and pyridinic nitrogen (N-H···N) bond. This peak disappears after complete co-ordination -NH bond of the imidazole ring with Zn^{2+}, as shown in the ZIF spectrum, indicating successful formation of ZIF particles. Subsequent attachment of the laccase enzyme to the ZIF particles is confirmed by the presence of amide 1 and II bands of the protein structure, detected at 1660 and 1460 cm^{-1} respectively.

These bands may be as a result of C=O stretching vibrations (amide I) and NH bending as well as CN stretching vibrations (amide II) in laccase (Kashefi et al., 2019). The other peaks at 1589 and peaks between 1500 – 600 cm^{-1} may be attributed to C=N stretching mode and the entire imidazole ring vibration (Ulu, 2020).

The ZIF diffraction pattern illustrated in fig. 1. (b) are representative pure ZIF crystals with peaks observed at 2θ = 8.5, 12.6, 15.0, 19.4, 21.2, 28.8, and 31.4°, (Mahmoodi et al., 2019; Zhang et al., 2018; Zhou et al., 2019) corresponding to (002), (112), (022), (113), (222), (233) and (134) planes of crystalline ZIF with a sodalite topology, respectively (JCPDS 00-062-1030) (Wang et al., 2019). It is worth noting that the crystalline nature of the ZIF particles was retained after the laccase attachment since the Lac-ZIF diffraction peaks are comparable to those of pure ZIF crystals.

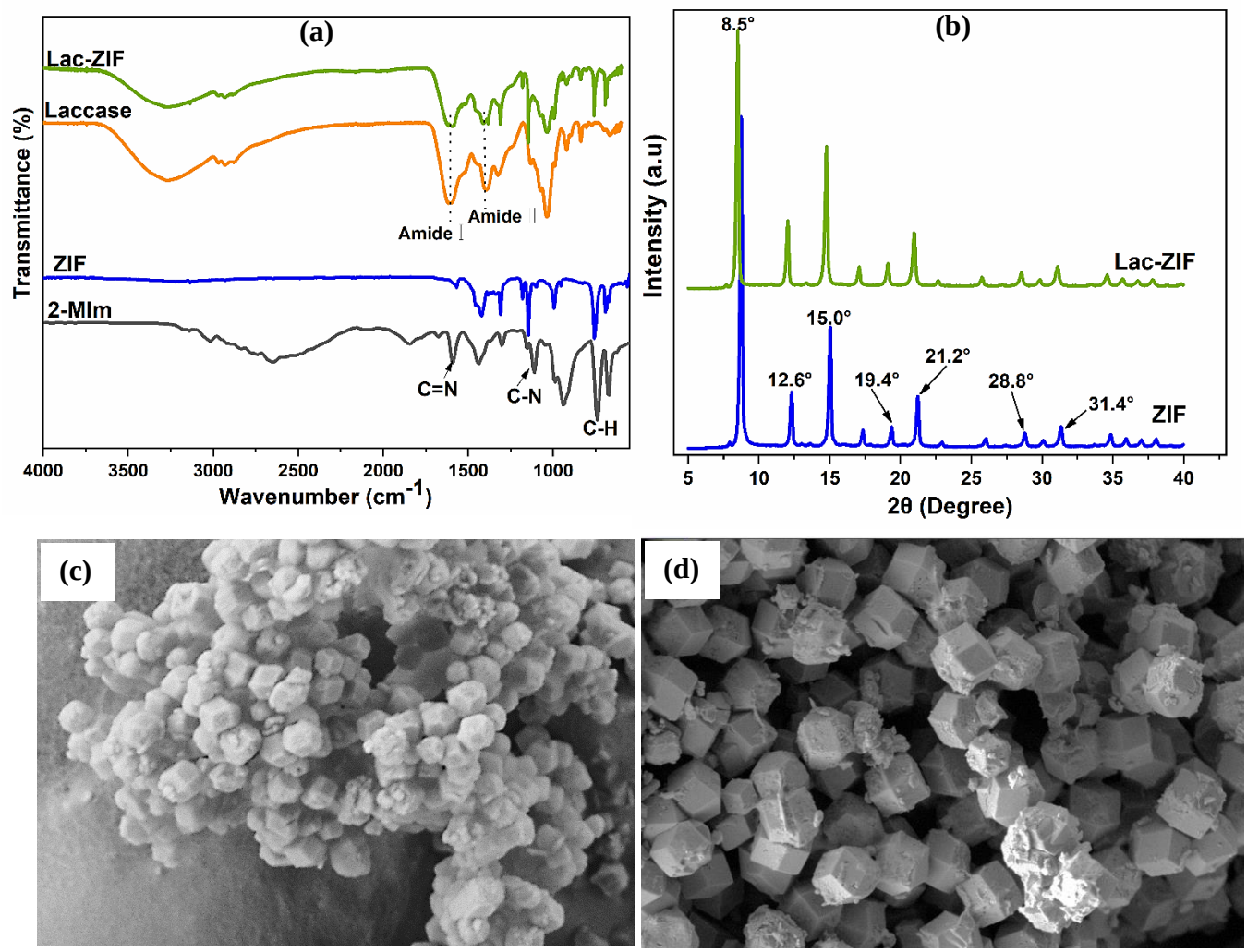


Fig. 1. (a) FTIR spectra of Lac-ZIF and its precursors, (b) PXRD patterns of ZIF (JCPDS 00-062-1030) and Lac-ZIF, and SEM micrographs of (c) ZIF and (d) Lac-ZIF ectra of Lac-ZIF and its precursors, (b) PXRD patterns of ZIF (JCPDS 00-062-1030) and Lac-ZIF, and SEM micrographs of

For surface morphological investigation, SEM analyses were conducted, and the micrographs are presented in fig. 1. (c) and (d). As shown in these micrographs, emblematic rhombic decahedral morphologies of pure ZIF particles were observed (Cui et al., 2018; Lian et al., 2017). Following immobilization of the enzyme, sparsely distributed small crusts of the laccase are noted on the surface of the ZIF particles with maintained decahedral morphologies.

3.2 DFC and NOR removal studies

For preliminary studies, the removal efficiencies of DFC and NOR using free laccase, ZIF crystals, and Lac-ZIF, decontamination studies were conducted at various pH (6.1 and 6.5 for DFC and NOR respectively) as shown in fig. 2. Interestingly, the Lac-ZIF exhibited supreme efficiency (73% for DFC and 79% for NOR removal) followed by adsorption offered by ZIF crystals (47% for DFC and 54% for NOR removal) with the least removal observed when using free laccase (31% for DFC and 42% for NOR removal). The enhanced decontamination efficiency illustrated by Lac-ZIF may be attributed to mutual adsorption and degradation effects offered by the ZIF crystals and laccase enzyme immobilized onto the ZIF particles (M. L. Dlamini et al., 2022; Liang et al., 2019). Therefore, the Lac-ZIF was used for all succeeding experiments utilizing RSM to optimize the parameters for maximum DFC and NOR decontamination.

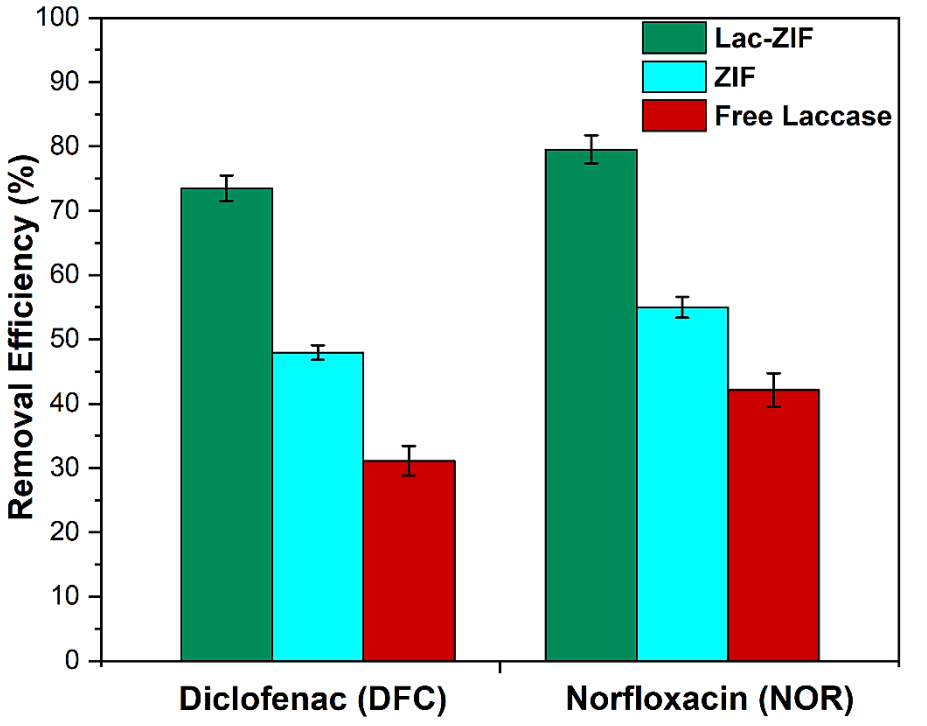


Fig. 2. Performance of Lac-ZIF, ZIF and Free Laccase on the removal of DFC and NOR

3.3 Model fitting and coefficient plots

The experimental results for the removal efficiencies of the PhACs are shown in table 1, showing maximum efficiencies of 99.4% and 99.9% for DFC and NOR, respectively.

Table 2. Analysis of variance (ANOVA) and statistical results for the removal of DFC and NOR as responses

DFC Removal	***DF***	***SS***	***MS (variance)***	***F***	***p***	***SD***
Total	27	129443	4794.18			
Constant	1	120374	120374			
Total corrected	26	9069.22	348.816			18.6766
Regression	9	8894.55	988.283	96.185	**<0.0001**	31.437
Residual	17	174.672	10.2748			3.20544
Lack of Fit (Model error)	15	151.065	10.071	0.85323	**0.663**	3.17349
Pure error	2	23.6067	11.8033			3.4356
R^2	**0.981**					
Adjusted R^2	**0.971**					
Q^2	**0.964**					
RSD	**3.205**					

NOR Removal	**DF**	**SS**	**MS (variance)**	***F***	***p***	**SD**
Total	27	137451	5090.78			
Constant	1	128561	128561			
Total corrected	26	8890.25	341.933			18.4914
Regression	9	8764.88	973.875	132.055	**<0.0001**	31.207
Residual	17	125.371	7.37475			2.71565
Lack of Fit (Model error)	15	101.724	6.78161	0.573579	**0.791**	2.60415
Pure error	2	23.6466	11.8233			3.43851
R^2	**0.986**					
Adjusted R^2	**0.978**					
Q^2	**0.972**					
RSD	**2.716**					

Note: $p < 0.05$ indicates significance; DF = degrees of freedom; MS = mean squares; SS = sum of squares; SD = standard deviation; RSD = Relative standard deviation

This indicates the ability of Lac-ZIF to eliminate the PhACs under the investigated variables. A quadratic model with individual linear effects and linear combinations of these effects was used for the experimental data analysis. Table 2 shows the statistical significance of the parameters determined by analysis of variance (ANOVA). The results show that the regression model was statistically significant at a 5% significance level (i.e $p < 0.0001$) and the R^2 and adjusted R^2 values were above 0.96 for both DFC and NOR analyses. The model's lack of fit shows insignificance ($p > 0.6$), with a significantly high future prediction precision, reliability,

and reproducibility as demonstrated by the Q^2 values that are greater than 0.96 and relative standard deviations of 3.205 and 2.716 for the two responses (DFC and NOR removal %) (Shah et al., 2022).

All the investigated variables (pH, dosage, initial concentration, and contact time) had a significant impact ($p < 0.05$) on the removal of the PhACs. In this regard, fig. 3. (a) shows that the linear factors (pH and concentration) have a negative influence on both DFC and NOR

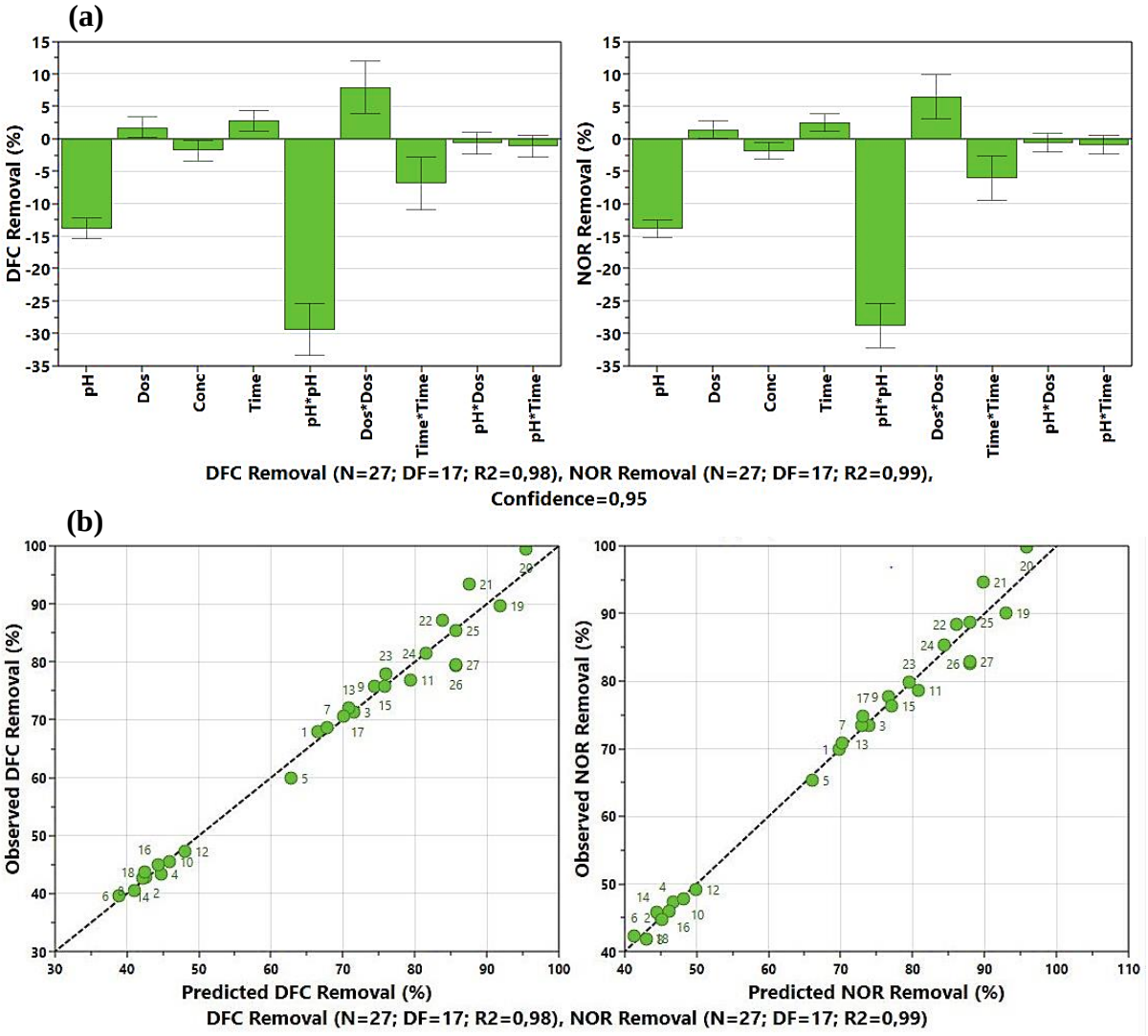


Fig. 3. (a) Coefficient plots of the investigated factors and their influence on the responses and (b) plots of observed vs predicted DFC and NOR removal efficiencies.

elimination, implying that these factors have an inverse proportionality with the studied responses. Positive effects, on the other hand, were observed for the linear variables (dosage and contact time), indicating a direct relationship with both DFC and NOR removal efficiency (Balan et al., 2012; Bankole et al., 2021). Similar results were reported by Ely et al. (Ely et al., 2017) on the bioconversion of phenol using horseradish peroxidase. Furthermore, the quadratic

regression terms (pH*pH and time*time) had a significantly strong negative impact on contaminant removal, whereas the dos*dos quadratic coefficient had a significantly strong positive impact. The model's validity was further confirmed by prediction vs. observed removal efficiency plots, as shown in fig. 3. (b), which demonstrated significantly high precision. As a result, the model was deemed valid and appropriate for optimizing experimental variables for DFC and NOR removal.

3.4 Response surface plots

The model results were also transformed into 3D response surface plots to visualize the effect of the investigated variables on the DFC and NOR removal as shown in fig. 4. Accordingly, two variables were plotted while the other two were held constant at model-optimized values. The results for the effect of pH and dosage are illustrated in fig. 4. (a) and (b) for DFC and NOR removal, respectively. The results show a significant increase in the removal efficiency of both analytes with a decrease in pH and increasing dosages, reaching a maximum efficiency of 98 and 95% at pH 6 (dosage 49.3 mg) for DFC and NOR, respectively. A further reduction in pH resulted in diminished removal efficiencies at maintained dosages. Beyond neutral pH, the DFC and NOR removal declined significantly, attaining 15% at high dosages. High hydroxide anion concentrations in basic environments may inhibit the analyte's binding potential onto the laccase's active site (T2/T3 cupric site), disrupting electron transfer from the T1 cupric site to T2/T3 (Lassouane et al., 2019; Sadeghzadeh et al., 2020), resulting in the observed decreased efficiencies beyond neutral regions. This observation supports the results of the regression coefficients analysis, which show that pH has a significant inverse correlation with analyte elimination. On the contrary, increasing the Lac-ZIF’s dosage resulted in minor improvements in efficiencies further confirming the results from the regression coefficient plots, where the dosage only has a weak positive influence on the decontamination of the analytes.

Fig. 4. (c) and (d) depict the mutual effects of pH and initial analyte concentration on DFC and NOR removal where maximum efficiency (95% for DFC and 93% for NOR) was favoured by low concentrations and near neutral pH (6.4). A rise in initial analyte concentrations may result

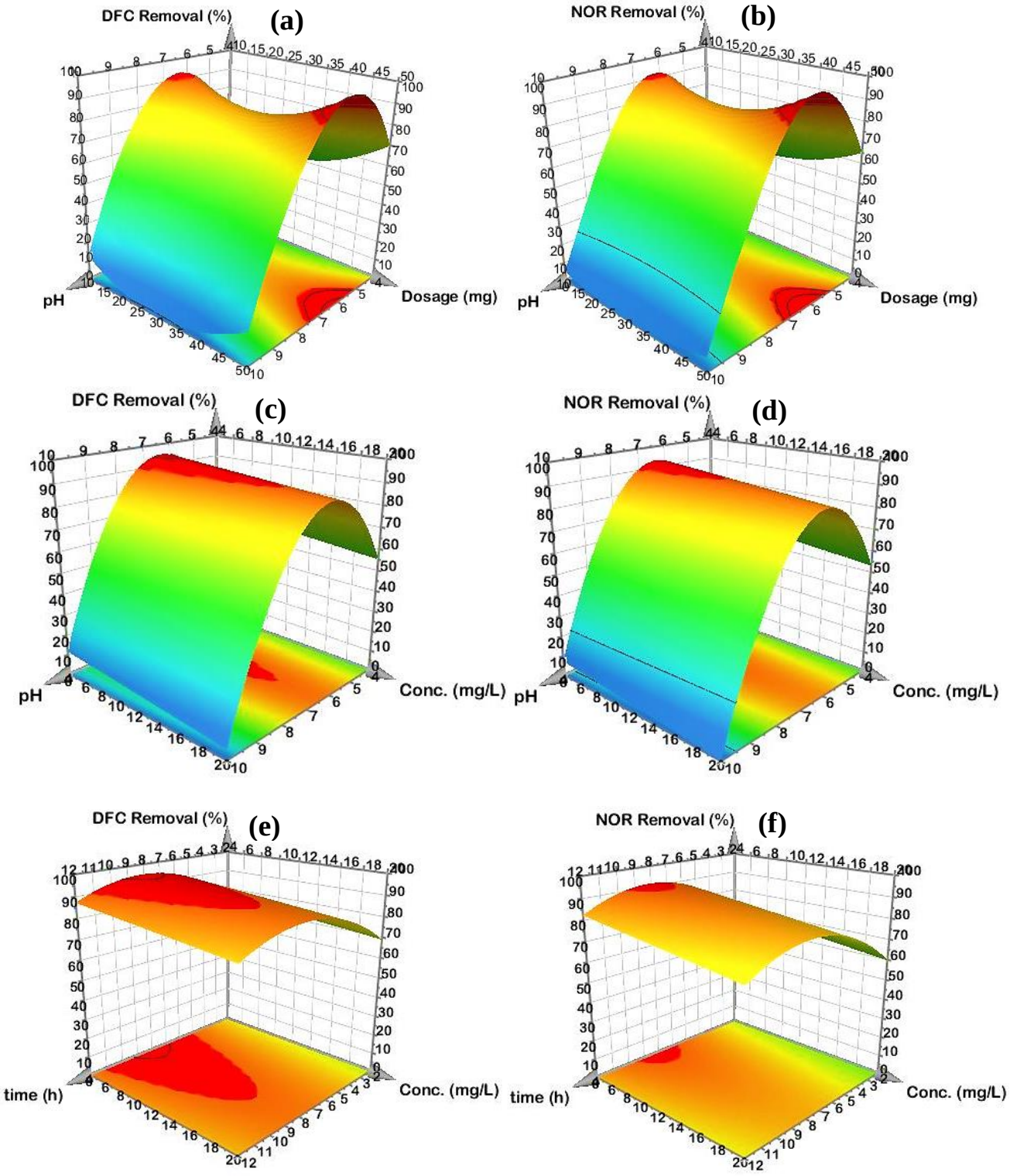


Fig. 4. 3D Response surface plots for (a) & (b) pH vs dosage, (c) & (d) pH vs concentration, and (e) & (f) time vs concentration.

in congested active sites of the Lac-ZIF, which inhibits the molecules' binding potential, hence the slight decline of efficiencies at elevated analyte concentrations (Ewis et al., 2022).

The results of the combined effects of contact time and initial concentration are presented in fig. 4. (e) and (f). The figures show significant improvements in removal efficiencies with contact time until equilibrium attainment at 8 h (94% for DFC and 91% for NOR) and minimal concentration (4 mg L^{-1}). Beyond 8 h, the Lac-ZIF particles may be saturated leading to reduced collisions between the analyte molecules and the biocatalyst's active sites (X. Cao et al., 2022), hence the slight reduction in removal efficiencies for both DFC and NOR.

3.5 Method validation

3.5.1 Optimization and model validation

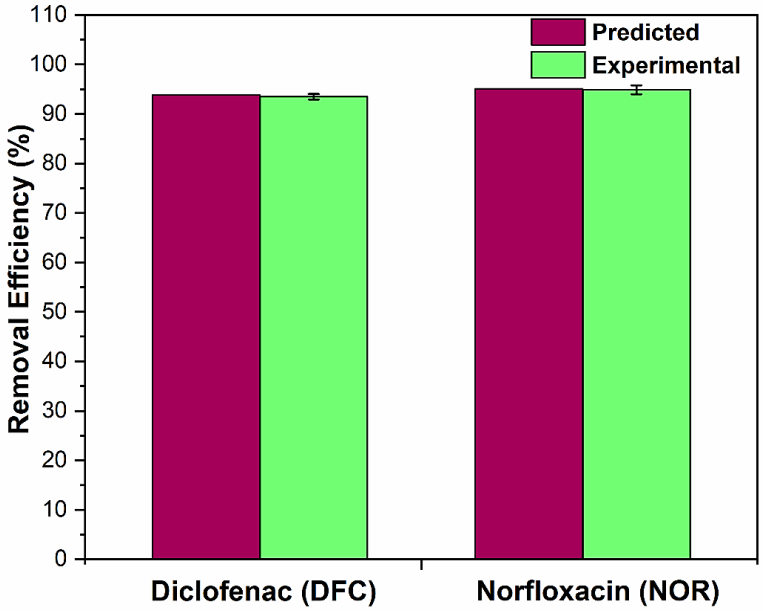


Fig. 5. A comparison of predicted and experimental results for the removal of DFC and NOR at pH 6.4, 10 mg Lac-ZIF dosage, 10.4 mg L^{-1} initial concentration and 8 h contact time.

Experiment validation is a crucial step in confirming the adequacy of the statistical model, and this was accomplished by running experiments under the model-optimized conditions: pH 6.4, 10.0 mg Lac-ZIF dosage, 10.4 mg L^{-1} initial DFC and NOR concentrations, and 8 h contact time. The experiments were carried out in triplicates and the average removal efficiencies were reported. As shown in fig. 5., the experimental values (93.5% for DFC and 94.9% for NOR) were found to be very close to the predicted removal efficiencies, 93.9 and 95.1% for DFC and NOR, respectively. Therefore, the results verified the validity of the applied model for the removal of DFC and NOR from aqueous solutions using Lac-ZIF.

3.5.2 HPLC method validation

For the validation of the analytical HPLC method, linear regression standard calibration curves were computed using 0.1 to 10 mg L^{-1} concentration ranges of DFC and NOR. The curves yielded good precisions with low relative standard deviations (RSD < 3%) and acceptable coefficient of determinations (R^2) of 0.999 and 0.998 for DFC and NOR, respectively. The detection (LOD) and quantification (LOQ) limits were evaluated to be 0.164 ± 0.003 (DFC), 0.147 ± 0.005 (NOR) and 1.02 ± 0.024 (DFC), 1.14 ± 0.013 mg L^{-1} (NOR), respectively. To identify the compounds in solution, retention times were employed and confirmed with their molecular ions (297 m/z for DFC and 320 m/z for NOR) on an LC-qTOF/MS.

3.6 Thermal deactivation kinetics studies

One of the critical parameters to consider in biocatalyst composite application is thermal stability, which is determined by thermal deactivation kinetics. The improved thermal stability would most likely broaden the application of the biocatalyst in a variety of fields. DFC (plot not shown) and NOR removal studies were performed at 25, 50, and 70 °C for 60 minutes using the model-optimized parameters (pH 6.4, 10 mg Lac-ZIF dosage, 10.4 mg L^{-1} initial concentration) to investigate the free enzyme and Lac-ZIF's thermal stability, and the results are shown in fig. 6. (a) and (b), respectively. The first-order kinetics plots were used to calculate the thermal deactivation rate constant (k_d) and half-life ($t_{1/2}$) of the biocatalysts as illustrated in table 3. The results show a general increase in k_d with temperature for both free and immobilized laccase, with a corresponding decline in $t_{1/2}$, which indicate diminishing activities of the laccase at elevated temperatures. However, the deactivation rate constants for Lac-ZIF were significantly lower than that of free laccase at respective temperatures, hence the slightly higher half-lives of the Lac-ZIF when compared to the enzyme's native form. The enhancement in thermal stability of the Lac-ZIF may be due to the localization of the laccase enzyme onto and within the ZIF framework forming stable conformations. Similar results were reported by Nadar and Rathod, and Vaidya et al (Nadar and Rathod, 2018; Vaidya et al., 2020) using lipase immobilized on ZIF-8.

Furthermore, the deactivation energy (E_d) for the free and immobilized laccase was evaluated through Arrhenius plots (fig. 6. (c)) The calculated energy required to distort the structure of free laccase and Lac-ZIF were found to be 32.1 and 47.59 kJ mol^{-1} (NOR), and 29.9 and 46.93 kJ mol^{-1} (DFC), respectively. This observation further confirms the significant improvement in thermal stability of the immobilized laccase, as a result of shielding effects provided by the ZIF crystals (Nadar et al., 2020; Salgaonkar et al., 2018).

Table 3. Thermal deactivation kinetic parameters: Thermal deactivation rate constant (k_d), half-life ($t_{1/2}$), and deactivation energy (E_d) of Lac-ZIF

Parameter	k_d (min^{-1})			$t_{1/2}$ (min)			E_d (kJ mol^{-1})
Temperature (°C)	25	50	70	25	50	70	25-70
Norfloxacin (NOR)							
Free laccase	0.0261	0.0292	0.0341	26.6	23.7	20.3	32.1
Lac-ZIF	0.0186	0.0218	0.0239	37.3	31.8	29.0	47.59
Diclofenac (DFC)							
Free laccase	0.0270	0.0309	0.0355	25.7	22.4	19.5	29.9
Lac-ZIF	0.0192	0.0225	0.0240	36.1	30.8	27.4	46.93

NB: All k_d values have SD < 0.0012, $t_{1/2}$ values have SD < 1.3, E_d have SD values < 1.7.

3.7 Michaelis–Menten kinetic parameters

The maximum reaction velocity (V_{max}) and Michaelis constant (K_m) of free laccase and Lac-ZIF were determined by evaluating the initial reaction rates of the biocatalysts with varying concentrations of NOR and DFC substrates, and the results are summarized in table 4. It can be observed that the K_m values for Lac-ZIF were slightly higher (42.008 ± 0.019 mg L^{-1} for NOR and 43.019 ± 0.091 mg L^{-1} for DFC) than those of free laccase (39.161 ± 0.041 mg L^{-1} for NOR and 40.087 ± 0.027 for DFC). Low K_m values are favourable in enzymatic reactions as they signify high affinities between the enzyme and the substrate. In this case, however, the K_m values increased after enzyme immobilization indicating a slight decline in affinity between the immobilized enzyme and the analytes, adversely affecting the V_{max} values which showed velocity reductions following the attachment. Similar works have reported comparable observations (Ameri et al., 2021; M. Cao et al., 2022; Zofair et al., 2022).

Table 4. Kinetic parameters of free laccase and Lac-ZIF

Form	K_m (mg L^{-1})	V_{max} (mg L^{-1} h^{-1})
Norfloxacin (NOR)		
Free laccase	39.161 ± 0.041	0.924 ± 0.003
Lac-ZIF	42.008 ± 0.019	0.898 ± 0.001
Diclofenac (DFC)		
Free laccase	40.087 ± 0.027	0.895 ± 0.002
Lac-ZIF	43.019 ± 0.091	0.809 ± 0.008

3.8 Reusability studies

The prime objective of immobilization is to facilitate the recovery and recyclability of biocatalysts, which is an important aspect of economic practicability. So, the reuse efficiency of Lac-ZIF was assessed for up to 7 cycles, and the results are presented in fig. 6. (d). The results reveal retained removal efficiencies of 79 and 83% for DFC and NOR on the 6th cycle, respectively. On the 7th cycle, however, a significant loss in efficiency (54% for DFC and 58% for NOR) was observed which may be ascribed to possible deactivation or mechanical impairment of the laccase over the reuse cycles, as well as probable loss of the biocatalyst during the centrifugation and washing procedure (Nadar et al., 2020; Vaidya et al., 2020). Nonetheless, the reusability results emphasized the high chemical and conformational stability of the Lac-ZIF composite.

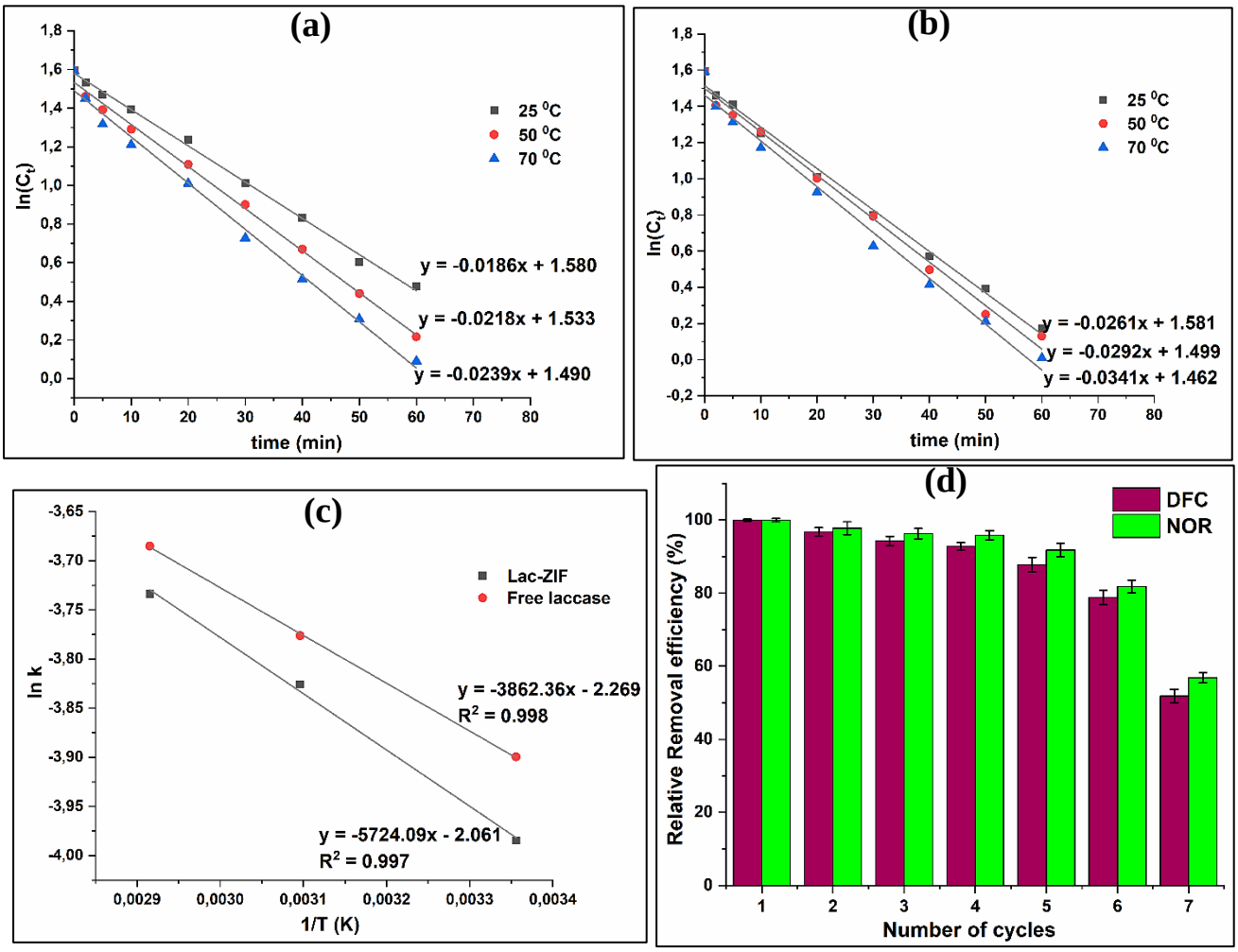


Fig. 6. Thermal deactivation kinetic studies of (a) Lac-ZIF and (b) free laccase using NOR as model substrate. (c) Arrhenius plot for inactivation of Lac-ZIF and free laccase, and (d) reusability studies of Lac-ZIF for DFC and NOR removal.

4. Treatment of DFC and NOR in real water samples

Using optimized parameters, the Lac-ZIF biocatalyst composite was used in the elimination of DFC and NOR from spiked real river water samples and the results are presented in table 5. The measured physicochemical properties of the samples are as follows: conductivity = 851 µS cm^{-1}, pH 6.89, total dissolved solids (TDS) = 374 ppm, and dissolved oxygen (DO) = 7.49 ppm. From table 5., the immobilized laccase gave a better performance than the native form, achieving removal efficiencies of 88.9% for DFC and 91.6% for NOR elimination. This observation may be due to the enhanced activity of the biocatalyst composite as a result of mutualistic biodegradation and adsorption effects provided by the Lac-ZIF as well as shielding effects of the ZIF framework around the laccase molecules protecting it from inhibition by external dissolved substances (Iqbal et al., 2022; Zhang et al., 2017).

Table 5. Application of biocatalyst in real river water samples

	Removal efficiency (%)	
Analyte	**DFC**	**NOR**
Free Laccase	51.3%	55.2%
Lac-ZIF	88.9%	91.6%

For the free laccase, however, the enzyme may be subject to activity inhibition and denaturation by dissolved organic and inorganic ions in the water, resulting in competition for the enzyme's active site and destruction of the protein structure (L. Chen et al., 2021; Jiang et al., 2023; Pan et al., 2022; Rehman et al., 2023), respectively, hence the reported reduced performance of the free laccase, 51.3 and 55.2% for DFC and NOR removal, respectively.

5. Conclusions

Immobilized laccase (Lac-ZIF) was used as a biocatalytic composite for the decontamination of diclofenac (DFC) and norfloxacin (NOR). Response surface methodology (RSM) via a central composite face (CCF) experimental design was successfully applied to determine the optimal operating parameters for maximum DFC and NOR removal, which were found to be pH 6.4, 10 mg Lac-ZIF dosage, 10.4 mg L^{-1} initial concentration and 8 h contact time. Using these process conditions, the expected removal efficiencies were 93.9 and 95.1% and the actual efficiencies were 93.5 and 94.9% for DFC and NOR removal, respectively, following experimental verification. The RSM optimization model was verified as reasonable and effective for this investigation. The immobilized laccase exhibited enhanced thermal stabilities with relatively lower thermal deactivation rate constants (k_d) and consequently higher deactivation energies (E_d) compared to the free laccase. Furthermore, the Lac-ZIF composite outperformed the free laccase in real water treatment and proved to be reusable for up to 6

cycles maintaining residual efficiencies of 79 and 83% for DFC and NOR removal, respectively.

Acknowledgements

This work was funded by the Council for Scientific and Industrial Research (CSIR) – Department of Science and Innovation (DSI) Inter-Programme Postgraduate Bursary (IBS).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Al-Kindi, G.Y., Al Ani, F.H., Al-Bidri, N.K., Alhaidri, H.A., 2021. Diclofenac removal from wastewater by activated carbon. IOP Conf. Ser. Earth Environ. Sci. 779. https://doi.org/10.1088/1755-1315/779/1/012091

Alessandretti, I., Rigueto, C.V.T., Nazari, M.T., Rosseto, M., Dettmer, A., 2021. Removal of diclofenac from wastewater: A comprehensive review of detection, characteristics and tertiary treatment techniques. J. Environ. Chem. Eng. 9, 106743. https://doi.org/10.1016/j.jece.2021.106743

Ameri, A., Taghizadeh, T., Talebian-Kiakalaieh, A., Forootanfar, H., Mojtabavi, S., Jahandar, H., Tarighi, S., Faramarzi, M.A., 2021. Bio-removal of phenol by the immobilized laccase on the fabricated parent and hierarchical NaY and ZSM-5 zeolites. J. Taiwan Inst. Chem. Eng. 120, 300–312. https://doi.org/10.1016/j.jtice.2021.03.016

An, H.J., Bhadra, B.N., Khan, N.A., Jhung, S.H., 2018. Adsorptive removal of wide range of pharmaceutical and personal care products from water by using metal azolate framework-6-derived porous carbon. Chem. Eng. J. 343, 447–454. https://doi.org/10.1016/j.cej.2018.03.025

Ariffin, Z.Z., Saat, M.N., Zulkifle, N.T., 2022. A review on response surface methodology optimization in microbial biotransformation. Sci. Lett. 16, 64–72. https://doi.org/10.24191/sl.v16i2.17135

Balan, K., Sathishkumar, P., Palvannan, T., 2012. Decolorization of malachite green by laccase: Optimization by response surface methodology. J. Taiwan Inst. Chem. Eng. 43, 776–782. https://doi.org/10.1016/j.jtice.2012.04.005

Bankole, P.O., Semple, K.T., Jeon, B.H., Govindwar, S.P., 2021. Impact of redox-mediators in the degradation of olsalazine by marine-derived fungus, Aspergillus aculeatus strain bpo2: Response surface methodology, laccase stability and kinetics. Ecotoxicol. Environ. Saf. 208, 111742. https://doi.org/10.1016/j.ecoenv.2020.111742

Bhattacharya, S.S., Banerjee, R., 2008. Laccase mediated biodegradation of 2,4-dichlorophenol using response surface methodology. Chemosphere 73, 81–85. https://doi.org/10.1016/j.chemosphere.2008.05.005

Cao, M., Yu, J., Zhang, X., Lin, Y., Huang, H., 2022. Laccase - functionalized magnetic

framework composite enabled chlorophenols degradation , a potential remediation for fungicides residues in leather. J. Leather Sci. Eng. https://doi.org/10.1186/s42825-022-00094-3

Cao, X., Gao, J., Yang, Y., Li, H., Zheng, X., Liu, G., Jiang, Y., 2022. Synergistic degradation of chlorophenol pollutants by a photo-enzyme integrated catalyst. J. Environ. Chem. Eng. 10, 107909. https://doi.org/10.1016/j.jece.2022.107909

Carrillo-Abad, J., Mora-Gómez, J., García-Gabaldón, M., Ortega, E., Mestre, S., Pérez-Herranz, V., 2020. Effect of the CuO addition on a Sb-doped SnO2 ceramic electrode applied to the removal of Norfloxacin in chloride media by electro-oxidation. Chemosphere 249. https://doi.org/10.1016/j.chemosphere.2020.126178

Chen, L., Huang, F., Zhang, C., Zhang, J., Liu, F., Guan, X., 2021. Effects of norfloxacin on nitrate reduction and dynamic denitrifying enzymes activities in groundwater. Environ. Pollut. 273, 116492. https://doi.org/10.1016/j.envpol.2021.116492

Chen, X., Zhuan, R., Wang, J., 2021. Assessment of degradation characteristic and mineralization efficiency of norfloxacin by ionizing radiation combined with Fenton-like oxidation. J. Hazard. Mater. 404, 124172. https://doi.org/10.1016/j.jhazmat.2020.124172

Choi, H.J., Ahn, Y.H., Koh, D.Y., 2021. Enantioselective mixed matrix membranes for chiral resolution. Membranes (Basel). 11. https://doi.org/10.3390/membranes11040279

Chollom, M.N., Bakare, B.F., Rathilal, S., Tetteh, E.K., 2022. Evaluating the Biodegradation of Veterinary Antibiotics Using Kinetics Model and Response Surface Methodology. Molecules 27. https://doi.org/10.3390/molecules27175402

Crespí Sánchez, N., Turnes Palomino, G., Palomino Cabello, C., 2023. Sulfonic-functionalized MIL-100-Fe MOF for the removal of diclofenac from water. Microporous Mesoporous Mater. 348. https://doi.org/10.1016/j.micromeso.2022.112398

Cui, J., Feng, Y., Jia, S., 2018. Silica encapsulated catalase@metal-organic framework composite: A highly stable and recyclable biocatalyst. Chem. Eng. J. 351, 506–514. https://doi.org/10.1016/j.cej.2018.06.121

de Carvalho Filho, J.A.A., da Cruz, H.M., Fernandes, B.S., Motteran, F., de Paiva, A.L.R., Pereira Cabral, J.J. da S., 2022. Efficiency of the bank filtration technique for diclofenac removal: A review. Environ. Pollut. 300. https://doi.org/10.1016/j.envpol.2022.118916

Dlamini, M.C., Dlamini, M.L., Mente, P., Tlhaole, B., Erasmus, R., Maubane-Nkadimeng, M.S., Moma, J.A., 2022. Photocatalytic abatement of phenol on amorphous TiO2-BiOBr-bentonite heterostructures under visible light irradiation. J. Ind. Eng. Chem. 111, 419–436. https://doi.org/10.1016/j.jiec.2022.04.023

Dlamini, M.L., Lesaoana, M., Kotze, I., Richards, H.L., 2022. Zeolitic imidazolate frameworks as effective crystalline supports for aspergillus-based laccase immobilization for the biocatalytic degradation of carbamazepine. Chemosphere 311, 137142. https://doi.org/10.1016/j.chemosphere.2022.137142

Domingo-Torner, C., García-Gabaldón, M., Martí-calatayud, M.C., Mestre, S., Pérez-Herranz, V., 2023. Norfloxacin mineralization under light exposure using Sb–SnO2 ceramic anodes coated with BiFeO3 photocatalyst. Chemosphere 313, 137518. https://doi.org/10.1016/j.chemosphere.2022.137518

Eddaoudi, M., Sava, D.F., Eubank, J.F., Adil, K., Guillerm, V., 2015. Zeolite-like metal-organic frameworks (ZMOFs): Design, synthesis, and properties. Chem. Soc. Rev. 44, 228–249. https://doi.org/10.1039/c4cs00230j

El Telib, A.E., El-Naas, M.H., Acio, J.A., 2017. Biodegradation of BTEX: Optimization through Response Surface Methodology. Am. J. Eng. Appl. Sci. 10, 20–31. https://doi.org/10.3844/ajeassp.2017.20.31

Ely, C., Lourdes Borba Magalhães, M. de, Henrique Lemos Soares, C., Skoronski, E., De Lourdes Borba Magalhães, M., Henrique Lemos Soares, C., Skoronski, E., 2017. Optimization of Phenol Removal from Biorefinery Effluent Using Horseradish Peroxidase. J. Environ. Eng. 143, 1–7. https://doi.org/10.1061/(asce)ee.1943-7870.0001279

Ewis, D., Ba-Abbad, M.M., Benamor, A., El-Naas, M.H., 2022. Adsorption of organic water pollutants by clays and clay minerals composites: A comprehensive review. Appl. Clay Sci. 229, 106686. https://doi.org/10.1016/j.clay.2022.106686

Fan, J., Luo, J., Wan, Y., 2017. Aquatic micro-pollutants removal with a biocatalytic membrane prepared by metal chelating affinity membrane chromatography. Chem. Eng. J. 327, 1011–1020. https://doi.org/10.1016/j.cej.2017.06.172

Guo, H., Zhao, C., Xu, H., Hao, H., Yang, Z., Li, N., Xu, W., 2023. Enhanced H2O2 formation and norfloxacin removal by electro-Fenton process using a surface-reconstructed graphite felt cathode: New insight into synergistic mechanism of defective active sites. Environ. Res. 220. https://doi.org/10.1016/j.envres.2023.115221

Habimana, P., Jiang, Y., Gao, J., Ndayambaje, J.B., Darwesh, O.M., Mwizerwa, J.P., Zheng, X., Ma, L., 2022. Enhancing laccase stability and activity for dyes decolorization using ZIF-8@MWCNT nanocomposite. Chinese J. Chem. Eng. 48, 66–75. https://doi.org/10.1016/j.cjche.2021.05.044

Hu, Y., Zhang, C., Jian, Z., Chen, D., Ma, Y., Yang, W., 2022. Surface-imprinted polymer microsphere based on self-stabilized precipitation polymerization for selective removal of norfloxacin. Appl. Surf. Sci. 574, 151706. https://doi.org/10.1016/j.apsusc.2021.151706

Iqbal, J., Shah, N.S., Sayed, M., Rauf, S., Haq Khan, Z.U., Niazi, N.K., Polychronopoulou, K., Howari, F., Rehman, F., 2022. Efficient removal of norfloxacin using nano zerovalent cerium composite biochar-catalyzed peroxydisulfate. J. Clean. Prod. 377, 134405. https://doi.org/10.1016/j.jclepro.2022.134405

Jiang, X., Wang, D., Wu, W., Li, F., 2023. The different toxicological effects and removal efficiencies of norfloxacin and sulfadiazine in culturing Arthrospira (Spirulina) platensis. Ecotoxicol. Environ. Saf. 250, 114468. https://doi.org/10.1016/j.ecoenv.2022.114468

Kashefi, S., Borghei, S.M., Mahmoodi, N.M., 2019. Covalently immobilized laccase onto graphene oxide nanosheets: Preparation, characterization, and biodegradation of azo dyes in colored wastewater. J. Mol. Liq. 276, 153–162. https://doi.org/10.1016/j.molliq.2018.11.156

Khulu, S., Ncube, S., Nuapia, Y., Madikizela, L.M., Tutu, H., Richards, H., Ndungu, K., Mavhunga, E., Chimuka, L., 2022. Multivariate optimization of a two-way technique for extraction of pharmaceuticals in surface water using a combination of membrane

assisted solvent extraction and a molecularly imprinted polymer. Chemosphere 286, 131973. https://doi.org/10.1016/j.chemosphere.2021.131973

Knedel, T.O., Ricklefs, E., Schlüsener, C., Urlacher, V.B., Janiak, C., 2019. Laccase Encapsulation in ZIF-8 Metal-Organic Framework Shows Stability Enhancement and Substrate Selectivity. ChemistryOpen 8, 1337–1344. https://doi.org/10.1002/open.201900146

Lassouane, F., Aït-Amar, H., Amrani, S., Rodriguez-couto, S., 2019. A promising laccase immobilization approach for Bisphenol A removal from aqueous solutions. Bioresour. Technol. 271, 360–367. https://doi.org/10.1016/j.biortech.2018.09.129

Leynaud Kieffer Curran, L.M.C., Pham, L.T.M., Sale, K.L., Simmons, B.A., 2022. Review of advances in the development of laccases for the valorization of lignin to enable the production of lignocellulosic biofuels and bioproducts. Biotechnol. Adv. 54, 107809. https://doi.org/10.1016/j.biotechadv.2021.107809

Li, H.Z., Yang, C., Qian, H.L., Yan, X.P., 2023. Room-temperature synthesis of ionic covalent organic frameworks for efficient removal of diclofenac sodium from aqueous solution. Sep. Purif. Technol. 306, 122704. https://doi.org/10.1016/j.seppur.2022.122704

Lian, X., Fang, Y., Joseph, E., Wang, Q., Li, J., Banerjee, S., Lollar, C., Wang, X., Zhou, H.C., 2017. Enzyme-MOF (metal-organic framework) composites. Chem. Soc. Rev. 46, 3386–3401. https://doi.org/10.1039/c7cs00058h

Liang, S., Wu, X.L., Xiong, J., Zong, M.H., Lou, W.Y., 2020. Metal-organic frameworks as novel matrices for efficient enzyme immobilization: An update review. Coord. Chem. Rev. 406, 213149. https://doi.org/10.1016/j.ccr.2019.213149

Liang, W., Xu, H., Carraro, F., Maddigan, N.K., Li, Q., Bell, S.G., Huang, D.M., Tarzia, A., Solomon, M.B., Amenitsch, H., Vaccari, L., Sumby, C.J., Falcaro, P., Doonan, C.J., 2019. Enhanced Activity of Enzymes Encapsulated in Hydrophilic Metal-Organic Frameworks. J. Am. Chem. Soc. 141, 2348–2355. https://doi.org/10.1021/jacs.8b10302

Liu, Z., Chen, G., Hu, F., Lu, X., 2021. Retraction notice to “Preparation of molecularly imprinted metal-organic frameworks for the selective removal of norfloxacin by an in-situ self-assembly template strategy” [Chemosphere 262 (2021) 127832] (Chemosphere (2021) 262, (S0045653520320270), (10.1. Chemosphere 262, 128260. https://doi.org/10.1016/j.chemosphere.2020.128260

Lou, Q., Wu, Y., Ding, H., Zhang, B., Zhang, W., Zhang, Y., Han, L., Liu, M., He, T., Zhong, J., 2022. Degradation of sulfonamides in aquaculture wastewater by laccase–syringaldehyde mediator system: Response surface optimization, degradation kinetics, and degradation pathway. J. Hazard. Mater. 432, 128647. https://doi.org/10.1016/j.jhazmat.2022.128647

Luisa, G., 2019. Enzymatic degradation of recalcitrant pharmaceutical micropollutants. [Doctoral thesis, Université De Montpellier] Hal theses: tel-02286745. https://theses.hal.science/tel-02286745

Mahmoodi, N.M., Keshavarzi, S., Oveisi, M., Rahimi, S., Hayati, B., 2019. Metal-organic framework (ZIF-8)/inorganic nanofiber (Fe2O3) nanocomposite: Green synthesis and photocatalytic degradation using LED irradiation. J. Mol. Liq. 291, 2–3. https://doi.org/10.1016/j.molliq.2019.111333

Mehta, J., Bhardwaj, N., Bhardwaj, S.K., Kim, K.H., Deep, A., 2016. Recent advances in enzyme immobilization techniques: Metal-organic frameworks as novel substrates. Coord. Chem. Rev. 322, 30–40. https://doi.org/10.1016/j.ccr.2016.05.007

Mkaddem, H., Rosales, E., Pazos, M., Ben Amor, H., Sanromán, M.A., Meijide, J., 2022. Anti-inflammatory drug diclofenac removal by a synthesized MgAl layered double hydroxide. J. Mol. Liq. 359, 119207. https://doi.org/10.1016/j.molliq.2022.119207

Murugesan, K., Dhamija, A., Nam, I.H., Kim, Y.M., Chang, Y.S., 2007. Decolourization of reactive black 5 by laccase: Optimization by response surface methodology. Dye. Pigment. 75, 176–184. https://doi.org/10.1016/j.dyepig.2006.04.020

Mustafa, F.S., Hama Aziz, K.H., 2023. Heterogeneous catalytic activation of persulfate for the removal of rhodamine B and diclofenac pollutants from water using iron-impregnated biochar derived from the waste of black seed pomace. Process Saf. Environ. Prot. 170, 436–448. https://doi.org/10.1016/j.psep.2022.12.030

Nadar, S.S., Rathod, V.K., 2018. Encapsulation of lipase within metal-organic framework (MOF) with enhanced activity intensified under ultrasound. Enzyme Microb. Technol. 108, 11–20. https://doi.org/10.1016/j.enzmictec.2017.08.008

Nadar, S.S., Vaidya, L., Rathod, V.K., 2020. Enzyme embedded metal organic framework (enzyme–MOF): De novo approaches for immobilization. Int. J. Biol. Macromol. 149, 861–876. https://doi.org/10.1016/j.ijbiomac.2020.01.240

Naghdi, M., Taheran, M., Brar, S.K., Kermanshahi-pour, A., Verma, M., Surampalli, R.Y., 2018. Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes. Environ. Pollut. 234, 190–213. https://doi.org/10.1016/j.envpol.2017.11.060

Pan, S., Guo, X., Li, R., Hu, H., Yuan, J., Liu, B., Hei, S., Zhang, Y., 2022. Activation of peroxymonosulfate via a novel UV/hydrated Fe(III) oxide coupling strategy for norfloxacin removal: Performance and mechanism. Sep. Purif. Technol. 300, 121909. https://doi.org/10.1016/j.seppur.2022.121909

Paredes-Laverde, M., Silva-Agredo, J., Torres-Palma, R.A., 2018. Removal of norfloxacin in deionized, municipal water and urine using rice (Oryza sativa) and coffee (Coffea arabica) husk wastes as natural adsorbents. J. Environ. Manage. 213, 98–108. https://doi.org/10.1016/j.jenvman.2018.02.047

Peng, X., Hu, F., Zhang, T., Qiu, F., Dai, H., 2018. Amine-functionalized magnetic bamboo-based activated carbon adsorptive removal of ciprofloxacin and norfloxacin: A batch and fixed-bed column study. Bioresour. Technol. 249, 924–934. https://doi.org/10.1016/j.biortech.2017.10.095

Prasetya, N., Li, K., 2022. Synthesis of defective MOF-801 via an environmentally benign approach for diclofenac removal from water streams. Sep. Purif. Technol. 301, 122024. https://doi.org/10.1016/j.seppur.2022.122024

Rafiei, S., Tangestaninejad, S., Horcajada, P., Moghadam, M., Mirkhani, V., Mohammadpoor-Baltork, I., Kardanpour, R., Zadehahmadi, F., 2018. Efficient biodiesel production using a lipase@ZIF-67 nanobioreactor. Chem. Eng. J. 334, 1233–1241. https://doi.org/10.1016/j.cej.2017.10.094

Rath, S., Paul, M., Behera, H.K., Thatoi, H., 2022. Response surface methodology mediated

optimization of Lignin peroxidase from Bacillus mycoides isolated from Simlipal Biosphere Reserve, Odisha, India. J. Genet. Eng. Biotechnol. 20. https://doi.org/10.1186/s43141-021-00284-2

Rehman, F., Parveen, N., Iqbal, J., Sayed, M., Shah, N.S., Ansar, S., Khan, J.A., Shah, A., Jamil, F., Boczkaj, G., 2023. Potential degradation of norfloxacin using UV-C/Fe2+/peroxides-based oxidative pathways. J. Photochem. Photobiol. A Chem. 435, 114305. https://doi.org/10.1016/j.jphotochem.2022.114305

Rezaei, S., Tahmasbi, H., Mogharabi, M., Ameri, A., Forootanfar, H., Khoshayand, M.R., Faramarzi, M.A., 2015. Laccase-catalyzed decolorization and detoxification of Acid Blue 92: Statistical optimization, microtoxicity, kinetics, and energetics. J. Environ. Heal. Sci. Eng. 13, 1–9. https://doi.org/10.1186/s40201-015-0183-1

Sadeghzadeh, S., Ghobadi Nejad, Z., Ghasemi, S., Khafaji, M., Borghei, S.M., 2020. Removal of bisphenol A in aqueous solution using magnetic cross-linked laccase aggregates from Trametes hirsuta. Bioresour. Technol. 306, 123169. https://doi.org/10.1016/j.biortech.2020.123169

Salgaonkar, M., Nadar, S.S., Rathod, V.K., 2018. Combi-metal organic framework (Combi-MOF) of α-amylase and glucoamylase for one pot starch hydrolysis. Int. J. Biol. Macromol. 113, 464–475. https://doi.org/10.1016/j.ijbiomac.2018.02.092

Shah, S.W.A., Rehman, M.U., Arslan, M., Abbasi, S.A., Hayat, A., Anwar, S., Iqbal, S., Afzal, M., 2022. Response Surface Methodology for Optimization of Operational Parameters to Remove Ciprofloxacin from Contaminated Water in the Presence of a Bacterial Consortium. ACS Omega 7, 27450–27457. https://doi.org/10.1021/acsomega.2c02448

Shan, Z., Ma, F., You, S., Shan, L., Kong, D., Guo, H., Cui, C., 2023. Enhanced visible light photo-Fenton catalysis by lanthanum-doping BiFeO3 for norfloxacin degradation. Environ. Res. 216, 114588. https://doi.org/10.1016/j.envres.2022.114588

Sharma, J., Mariam, I., Suresh Kareya, M., Pavan Jutur, P., Joshi, M., Harish, Bhatnagar, A., Chaurasia, A.K., Nigam, S., 2023. Metabolomic response of microalgae towards diclofenac sodium during its removal from water and concomitant recovery of pigments and lipids. Bioresour. Technol. 371, 128617. https://doi.org/10.1016/j.biortech.2023.128617

Sun, Q., Hu, X., He, Q., Sun, M., Dai, L., Khan, S., Zheng, H., 2022. Polydopamine-Chitosan Modified TiO2 Nanoparticles for Temperature-Response Removal of Diclofenac Sodium Under Visible Light Irradiation. J. Taiwan Inst. Chem. Eng. 131, 104151. https://doi.org/10.1016/j.jtice.2021.11.018

Tegegne, B., Chandravanshi, B.S., Zewge, F., Chimuka, L., 2021. Solid-phase optimisation for simultaneous determination of thirteen pharmaceuticals in Ethiopian water samples with HPLC-DAD detection: an initial assessment. Environ. Monit. Assess. 193, 1–14. https://doi.org/10.1007/s10661-021-08999-y

Tocco, D., Carucci, C., Todde, D., Shortall, K., Otero, F., Sanjust, E., Magner, E., Salis, A., 2021. Enzyme immobilization on metal organic frameworks: Laccase from Aspergillus sp. is better adapted to ZIF-zni rather than Fe-BTC. Colloids Surfaces B Biointerfaces 208, 112147. https://doi.org/10.1016/j.colsurfb.2021.112147

Tülek, A., Yıldırım, D., Aydın, D., Binay, B., 2021. Highly-stable Madurella mycetomatis

laccase immobilized in silica-coated ZIF-8 nanocomposites for environmentally friendly cotton bleaching process. Colloids Surfaces B Biointerfaces 202. https://doi.org/10.1016/j.colsurfb.2021.111672

Ulu, A., 2020. Metal–organic frameworks (MOFs): a novel support platform for ASNase immobilization. J. Mater. Sci. 55, 6130–6144. https://doi.org/10.1007/s10853-020-04452-6

Vaidya, L.B., Nadar, S.S., Rathod, V.K., 2020. Entrapment of surfactant modified lipase within zeolitic imidazolate framework (ZIF)-8. Int. J. Biol. Macromol. 146, 678–686. https://doi.org/10.1016/j.ijbiomac.2019.12.164

Wang, D., Lou, J., Yuan, J., Xu, J., Zhu, R., Wang, Q., Fan, X., 2021. Laccase immobilization on core-shell magnetic metal-organic framework microspheres for alkylphenol ethoxylate compound removal. J. Environ. Chem. Eng. 9, 105000. https://doi.org/10.1016/j.jece.2020.105000

Wang, J., Yu, S., Feng, F., Lu, L., 2019. Simultaneous purification and immobilization of laccase on magnetic zeolitic imidazolate frameworks: Recyclable biocatalysts with enhanced stability for dye decolorization. Biochem. Eng. J. 150, 107285. https://doi.org/10.1016/j.bej.2019.107285

Wu, E., Li, Y., Huang, Q., Yang, Z., Wei, A., Hu, Q., 2019. Laccase immobilization on amino-functionalized magnetic metal organic framework for phenolic compound removal. Chemosphere 233, 327–335. https://doi.org/10.1016/j.chemosphere.2019.05.150

Wu, J., Han, J., Mao, Y., Wang, L., Wang, Yi, Li, Y., Wang, Yun, 2022. Bionic mineralization growth of UIO-66 with bovine serum for facile synthesis of Zr-MOF with adjustable mesopores and its application in enzyme immobilization. Sep. Purif. Technol. 297, 121505. https://doi.org/10.1016/j.seppur.2022.121505

Xin, C., Zhu, S., Liao, J., Hou, M., Li, Q., Yu, X., Li, S., 2022. Rational design of S-scheme AgI/ZrTiO4-x heterojunctions for remarkably boosted norfloxacin degradation. Chemosphere 308, 136279. https://doi.org/10.1016/j.chemosphere.2022.136279

Zelaya Soulé, M.E., Flores, F.M., Torres Sánchez, R.M., Fernández, M.A., 2020. Norfloxacin adsorption on montmorillonite and carbon/montmorillonite hybrids: pH effects on the adsorption mechanism, and column assays. J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 56, 113–122. https://doi.org/10.1080/10934529.2020.1842042

Zhang, B., Mei, M., Li, K., Liu, J., Wang, T., Chen, S., Li, J., 2022. One-pot synthesis of MnFe2O4 functionalized magnetic biochar by the sol-gel pyrolysis method for diclofenac sodium removal. J. Clean. Prod. 381, 135210. https://doi.org/10.1016/j.jclepro.2022.135210

Zhang, C., You, S., Zhang, J., Qi, W., Su, R., He, Z., 2020. An effective in-situ method for laccase immobilization: Excellent activity, effective antibiotic removal rate and low potential ecological risk for degradation products. Bioresour. Technol. 308, 123271. https://doi.org/10.1016/j.biortech.2020.123271

Zhang, F., Lian, M., Alhadhrami, A., Huang, M., Li, B., Mersal, G.A.M., Ibrahim, M.M., Xu, M., 2022. Laccase immobilized on functionalized cellulose nanofiber/alginate composite hydrogel for efficient bisphenol A degradation from polluted water. Adv. Compos.

Hybrid Mater. https://doi.org/10.1007/s42114-022-00476-5

Zhang, W., Gao, H., He, J., Yang, P., Wang, D., Ma, T., Xia, H., Xu, X., 2017. Removal of norfloxacin using coupled synthesized nanoscale zero-valent iron (nZVI) with H2O2 system: Optimization of operating conditions and degradation pathway. Sep. Purif. Technol. 172, 158–167. https://doi.org/10.1016/j.seppur.2016.08.008

Zhang, Y., Jia, Y., Hou, L., 2018. Synthesis of zeolitic imidazolate framework-8 on polyester fiber for PM2.5 removal. RSC Adv. 8, 31471–31477. https://doi.org/10.1039/c8ra06414h

Zhou, L., Li, N., Owens, G., Chen, Z., 2019. Simultaneous removal of mixed contaminants, copper and norfloxacin, from aqueous solution by ZIF-8. Chem. Eng. J. 362, 628–637. https://doi.org/10.1016/j.cej.2019.01.068

Zofair, S.F.F., Ahmad, S., Hashmi, M.A., Khan, S.H., Khan, M.A., Younus, H., 2022. Catalytic roles, immobilization and management of recalcitrant environmental pollutants by laccases: Significance in sustainable green chemistry. J. Environ. Manage. 309, 114676. https://doi.org/10.1016/j.jenvman.2022.114676

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 General conclusions

The increasing detection of persistent organic pollutants in wastewater treatment effluents sparked the need to investigate potential remediation techniques to effectively remove such contaminants from water. This research sought to evaluate the biodegradation of three model pharmaceutical micropollutants (carbamazepine (CBZ), diclofenac (DFC), and norfloxacin (NOR)) and a plasticizer (dimethyl phthalate (DMP)) using laccase-based treatment systems. For this purpose, the enzymatic treatment employing free laccase systems and the use of immobilized laccase onto zeolitic imidazolate frameworks (ZIF) was used.

The immobilization of commercially available laccase (*Aspergillus* species) was afforded by a facile single-pot biomineralization procedure, whereby the coordination and formation of ZIF crystals proceeded in the presence of the laccase enzyme. Successful attachment of the laccase was confirmed by FTIR and NMR analyses which showed vibrational and bending peaks corresponding to the protein structure of the enzyme, with the NMR spectra exhibiting shifts attributable to the imidazole ring of the ZIF particles. PXRD analysis revealed that immobilization of the laccase had no significant effect on the crystalline nature of the ZIF particles. Moreover, SEM and TEM analyses of the enzyme-MOF (Lac-ZIF) revealed the typical dodecahedral morphologies of ZIF particles with laccase crusts visible on the surface of the crystals. Additionally, particle agglomeration was observed following laccase attachment as a result of multiple linkages between the enzyme and ZIF particles with other ZIF particles nearby. As a result, the laccase kinetics analysis of the immobilized laccase using 2 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) revealed a decrease in the enzyme's affinity for the substrate, as evidenced by an increase in Michaelis constant (K_m), affecting the maximum velocity (V_{max}), which showed a slight drop from 0.873 to 0.692 and 0.651 to 0.238 mg L^{-1} h^{-1} for CBZ and DMP removal, respectively. Additionally, the immobilized laccase exhibited enhanced thermal stabilities with relatively lower thermal deactivation rate constants (k_d) and consequently higher deactivation energies (E_d) (>46.93 kJ mol^{-1}) compared to the free laccase.

For decontamination studies, batch reaction modes using the free laccase, pure ZIF particles, and Lac-ZIF was employed for all the investigated pollutants. Mutualistic adsorption and

biodegradation of these compounds offered by the Lac-ZIF resulted in enhanced and supreme performances of the enzyme-MOF over the free laccase and pristine ZIF particles. The ZIF particles improved the immobilized laccase's resistance to harsh environmental conditions such as extreme pH, high temperature, and potentially inhibitory organic solvents. The optimized pH values were 8 and 7 for CBZ and DMP removal, respectively. Additionally, the immobilized laccase was easily recovered and reused for at least 5 cycles before any significant loss in activity or relative efficiency. Also, the Lac-ZIF exhibited remarkable storage stabilities retaining high pollutant removal efficiencies of up to 20 and 15 d for DMP and CBZ removal, respectively.

For DFC and NOR removal, the experimental plan was designed using a central composite face (CCF) design, and the optimization of operational variables was achieved via a quadratic response surface methodology (RSM) regression model, resulting in pH 6.4, 10 mg Lac-ZIF dosage, 10.4 mg L^{-1} initial concentration, and 8 h contact time. This model proved viable and effective for use in this study, predicting removal efficiencies of 93.9 and 95.1% for DFC and NOR, respectively, which were validated with great accuracy by corresponding experimental results. The actual removal efficiencies were 93.5 and 94.1% for DFC and NOR, respectively.

Finally, the Lac-ZIF was used to decontaminate the investigated compounds from spiked real river water samples, achieving elimination efficiencies of 92.0, 95.4, 88.9 and 91.6 % for CBZ, DMP, DFC, and NOR compounds, respectively. Also, the Lac-ZIF proved stable and effective in the degradation studies, indicating the promising applicability of the biocatalyst in wastewater bioremediation.

5.2 Recommendations

The research's aim and objectives were successfully achieved. However, the following recommendations may be considered to improve this study and for future beneficial considerations:

- The synthesized Lac-ZIF could be packed in a fixed-bed column allowing a continuous flow of contaminated water to fully understand and ascertain the biocatalyst’s potential in a set-up that mimics a real wastewater treatment plant. This would serve as a pre-requisite for up-scaling the biocatalytic reactor.

- Although redox mediators may increase the toxicity of biotransformation products, they are well known for increasing enzyme activity in the system. Therefore, it would be interesting to combine the immobilization approach with redox mediator additions, thereby exploring their efficacy in improving the catalytic activity of Lac-ZIF and further investigating the toxicity of the formed metabolites.

- Due to time constraints, the toxicity of the studied biotransformation products was not investigated, therefore, a study of the toxicological effects of these by-products would be highly beneficial in the contribution of new knowledge.

- The application of the Lac-ZIF biocatalyst in the treatment of multiple (mixed matrix) potential substrates for laccase may provide a better insight into the biocatalyst's selectivity and affinity in water systems.