Application of oxidative enzymes in membrane systems for the bioremediation of triazines in wastewater

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By

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DECLARATION

I declare that this thesis research and the work presented in it are my own, unaided work. It has been submitted while in candidature 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: Mahadi Lesaoana **Date:** 21 October 2024

ABSTRACT

The prevalence of herbicidal pollutants present in various environmental matrices have become a global concern. The discharge and accumulation of s-triazine agrochemicals in effluents remains a major challenge, threatening the quality of freshwater resources. These are newly identified recalcitrant contaminants of concern (CECs) with complex structures, and inadvertent exposure poses deleterious ecological risks and human health-related adverse effects. Unfortunately, they have shown resistance to conventional treatment strategies, hence their persistence in wastewater treatment plant (WWTP) effluents and water bodies. Therefore, there is an urgent need for the exploration of alternative technologies for the effective eradication of such contaminants from water samples. The bioconversion of such micropollutants using oxidative enzymes like laccase is a promising research avenue, providing a sustainable, economically and ecologically benign strategy.

The current research examined the potential of a hybrid biocatalytic membrane system to degrade common s-triazine agrochemical herbicides in aqueous solutions. Specifically, the use of Novoprime base 268 laccase coupled with hollow fibre polyethersulfone (PES) membranes was investigated for the bioremediation of atrazine (ATZ), ametryn (AMT), simazine (SMZ) , prometon (PMT) and terbuthylazine (TERB) in wastewater. In batch-mode reactions, major operating parameters (i.e. pH and temperature profiles, enzyme dosage and contact time) were varied for the laccase-assisted catalysis of s-triazine compounds. Optimised conditions provided highest removal efficiencies (> 88.9%) at pH 5.0, combined with a temperature of 25°C and 1.0 mg L^{-1} solution concentration after 24h reaction time. Through the addition of redox mediators *viz*. 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), violuric acid (VA), vanillin (VA), syringaldehyde (SRA) and acetosyringone (ASR) recalcitrant triazine degradation was enhanced by 10 to 20 % at 1.50 mM.

Subsequently, the performance of a standalone continuous flow-mode membrane system was evaluated firstly, using a bed adsorption column only operated under various conditions. The efficiencies were compared to batch-mode enzymatic experiments. The adsorption of triazines by PES was only weakly influenced by pH, and the optimum removal was attained at pH 5.0 (5.0 mg L^{-1}), 2.35 g bed mass (14.0 cm height) and 24h column operation time. The overall removal percentages were 72.6%, 75.2%, 71.4%, 67.4%, and 68.2% for ATZ, AMT, SMZ, PMT and TERB, respectively. Although the results indicated satisfactory performances by both systems, their performance is limited when used as separate units (continuous membrane vs laccase reactor). A biocatalytic membrane system was achieved by integrating laccase into the dynamic packed-bed membrane column. Relevant process control design parameters of the fixed-bed biocatalytic column were carefully evaluated and recorded an optimum of 93.2 % removal efficiency as observed at a feed flow rate 2.0 mL min^{-1}, at a bed height of 14.0 cm using an atrazine influent concentration of 5.0 mg L^{-1}. Equilibrium dynamics of the breakthrough modelling were best fitted by Thomas model. Results attained demonstrated selectivity for triazines in matrix-matched real river water samples with remarkable recyclability after six successive operational cycles. This reflects the potential workability of the integrated system for extended enzymatic reactions evaluated under robust experimental conditions. As a benchmarking exercise, cost-analysis studies showed comparable projected scalability of our configuration at 1200 m^3/d capacity at an estimated total cost of R7.036 mil.

DEDICATION

This work is specially dedicated to my beloved parents, **Martin Julius** especially my lighthouse and *guardian angel* mom, **Mataemane Christinah Lesaoana**, whose love and reassurance continue to echo in my heart. This thesis remains a testament to the dreams you nurtured and a constant reminder that those dreams are always valid. Thank you for everything.

"The Lord is not slow in keeping His promise."

(2Peter 3:9)

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RESEARCH OUTPUTS

Publications

Mahadi Lesaoana, Mbongiseni Dlamini, Dean Brady, Roger Sheldon & Heidi Richards. Integrated membrane packed enzymatic column for the removal of atrazine in wastewater: Experimental, kinetics, breakthrough curves analysis and applicability in real wastewater.

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Author's contributions: Principal author, involved in conceptualization, formal analysis, data curation, and writing of the original draft.

Conference presentations

- **Title:** Evaluation of polyethersulfone hollow fibres as potential supports for laccase in the bioremediation of atrazine (s-triazine) in aqueous solutions. Sustainable water and wastewater treatment with membrane processes, South Africa-Sweden University Forum. November 2020. **Oral presentation.**

  Authors: Mahadi Lesaoana, Dean Brady and Roger A. Sheldon & Heidi L. Richards.

- **Title:** Application of laccase enzyme for the bioremediation of persistent triazine contaminants and their effective degradation in aqueous solutions. **31st Catalysis Society of South Africa (CATSA)** international conference. November 2021. **Poster presentation**. 2nd runner up.

  Authors: Mahadi Lesaoana, Dean Brady and Roger A. Sheldon & Heidi L. Richards.

- **Title:** Performance evaluation of a packed-bed polyethersulfone biocatalytic membrane system for the remediation of s-triazine herbicides in aqueous solutions. Sustainable water and wastewater treatment with membrane processes, South Africa-Sweden University Forum. July 2022. **Oral presentation.**

  Authors: Mahadi Lesaoana, Dean Brady and Roger A. Sheldon & Heidi L. Richards.

- **Title:** Hybrid packed-bed application of a polyethersulfone-laccase column for the biocatalytic remediation of triazine herbicides in aqueous solutions. Water Institute of Southern Africa (WISA) Conference. September 2022. **Oral presentation**.

  Authors: Mahadi Lesaoana, Dean Brady and Roger A. Sheldon & Heidi L. Richards.

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ABBREVIATIONS

•OH (hydroxyl radicals)

2,2′azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)

3,5-dimethoxy-4hydroxyacetophenone and syringaldehyde (SRA)

4-hydroxy-3-methoxybenzaldehyde, acetosyringone (ASR)

Ametryn (AMT)

Atrazine (ATZ)

Chloroperoxidase (CPO)

Citrate phosphate buffer (CPB)

Contaminants of emerging concern (CECs)

Continuous stirred tank reactors (CSTRs)

Endocrine disrupting chemicals (EDCs)

Enzymatic membrane reactors (EMRs)

Food and Agriculture Organization (FAO)

Forward osmosis (FO)

High-liquid performance chromatography- mass spectrometry (HPLC-MS)

Horseradish peroxidase (HRP)

Laccase (Lacc)

Lignin peroxidase (LiP)

Manganese peroxidase (MnP)

Membrane based separation processes (MBSPs)

Micochannels (MC)

Microfiltration (MF)

Molecular weight cut-off (MWCO)

Nanofiltration (NF)

Packed bed reactors (PBRs)

Polyacetonitrile (PAN)

Polyacrylonitrile (PP)

Polyamide (PA)

Polyethersulfone (PES)

Polyetheylene (PE)

Polyvynildene (PDVF)

Promrton (PMT)

Reverse osmosis (RO)

Scanning electron microscopy (SEM)

Simazine (SMZ)

Soy bean peroxidase (SBP)

Terbuthylazine (TERB)

Ultrafiltration (UF)

Ultra-visible (UV-Vis)

Vanillin (VAN)

Violuric acid (VA)

Wastewater treatment plants (WWTPs)

CHAPTER 1 INTRODUCTION AND BACKGROUND

1.1 Background

High-quality water availability is essential for human and ecological survival. Despite nature providing numerous resources to sustain the ecosystem, water quality is severely compromised by the frequent detection of micropollutants. Thus, obtaining clean uncontaminated water has become a major global concern. The widespread occurrence and persistent detection of recalcitrant and bio-accumulative micropollutants, also known as emerging contaminants (ECs) in vital aquatic systems poses ecological risks and adversely threatens human health (Zdarta et al., 2018; Bilal, Iqbal, et al., 2019). ECs constitute of a significant portion of unregulated synthetic compounds consisting of an array of ingredients found in pharmaceutically active compounds, pesticides, personal care products, endocrine disrupting agents, textile dyes, surfactants etc (Kanaujiya et al., 2019; Jatoi et al., 2021). And since the intensification of anthropogenic and industrial activities, these compounds have been pervasively found in effluents, and subsequently discharged into other water bodies (Le Coadou et al., 2017; Rasheed et al., 2019).

Similarly, the unprecedented increase in human population has led to extensive agricultural practices intended to meet food demands and subsequently resulting to rampant contamination of waterways with xenobiotic pesticide residues *viz*. s-triazine herbicides (Jatoi et al., 2021). Owing to their persistent nature, resistance to biodegradability and incapacitated sewage treatment plants, traces of unmetabolized s-triazines have been detected in industrial run-offs, receiving surface and ground water sources (Evgenidou et al., 2015; Bilal, Iqbal, et al., 2019). Despite their presence at relatively low concentrations ranging from ng to mg L^{-1} ranges, triazine herbicides can bioaccumulate and biomagnify in food chains (Bilal and Iqbal, 2019;

Ahmed et al., 2021). Moreover, extremely low levels may still exhibit detrimental biological effects, raising ecotoxicological concerns, such as interferences of the endocrine system (Mac et al., 2016; Destro et al., 2021), neurotoxicity, gene alterations, ovary growth inhibition leading to reproductive issues, reduced fertility and higher occurrence of alteration of sex ratios in various pieces (Simonelli et al., 2007; Hayes et al., 2011; Hoskins et al., 2019; S. Wang et al., 2022; Carriquiriborde et al., 2023).

Furthermore, the heightened detection of s-triazine herbicides in waterways indicate that the current wastewater treatment plants (WWTPs) are not designed to completely eliminate these compounds and their respective metabolites (Luo et al., 2014a; Evgenidou et al., 2015; Teodosiu et al., 2018; Rasheed et al., 2019; Morsi et al., 2020). Although numerous conventional treatment technologies, based on physico-chemical approaches have shown potential in remediating triazine residues from treatment effluents, the narrow concentration range for mineralization of organic compounds from wastewaters, insufficient purification and generation of large sludge quantities, limits the application of these approaches (Bilal, Adeel, et al., 2019; Rasheed et al., 2019). Given these effects, the pursuit of novel, efficient, and greener alternative processes to tackle the persistent occurrence of agrochemicals and to ameliorate the limitations associated with the aforementioned treatment technologies has become more important.

For years, enzymes have been characterized by high chemo-, regio- and stereo selectivity and have been considered as highly efficient and environmentally benign catalysts (Alneyadi et al., 2018; Holanda et al., 2019; Rasheed et al., 2019; Zdarta et al., 2021). Enzymatic reactions proceed with greater specificity, under less disruptive conditions and can be used with compounds at low concentrations which cannot be achieved using physiochemical techniques (Al-Maqdi et al., 2017; Alneyadi et al., 2018; Barrios-Estrada et al., 2018; Rasheed et al., 2019). Oxidoreductase enzymes have shown potential in the biotransformation of various

organic pollutants and subsequently explored in wastewater remediation (Auriol et al., 2007; Bilal, Rasheed, et al., 2019; Bilal et al., 2022).

Of particular interest is laccase, a multicopper containing oxidoreductase [(EC 1.10.3.2.)] catalysing the oxidation-reduction-assisted biodegradation various substrates to less toxic derivatives by reducing molecular O_2 to H_2O (Baldrian, 2006; Mayolo-Deloisa et al., 2020). Compared to other enzymatic strains such a peroxidases, which require enzyme-destabilizing agents such as hydrogen peroxide, laccase treatments utilizes dissolved O_2 as a co-factor thus rendering them desirable for bioremediation applications (Unuofin et al., 2019, 2021; Zhuo and Fan, 2021).

Albeit the merits offered by enzymatic catalysis, the use of enzymes in their soluble/native form is limited by challenges like recovery and recyclability, limited operating stability, high costs, intolerance to unfavourable environmental conditions which may compromise stability and conformation (Husain and Husain, 2008; Al-Maqdi et al., 2017; Amin et al., 2017; Pandey et al., 2017; Zdarta et al., 2019). Thus, coupling the enzymatic systems with suitable support matrices such as polymeric membranes could be beneficial for continuous operation productivity and reusability over successive catalytic cycles (Nunes and Kunamneni, 2018; Costa et al., 2019; Ren et al., 2020). Polyethersulfone (PES) membranes have gained popularity as desirable supports in hybrid bioremediation systems (Jalali et al., 2016; Asif et al., 2018). These matrices possess exceptional mechanical properties, thermochemical and oxidative stability, robustness, and easy scalability. These qualities have led to their extensive exploration in coupled enzymatic treatments of various organic pollutants (Asif et al., 2017; Shakerian et al., 2020; Pekgenc et al., 2022; Sitanggang et al., 2022; George et al., 2023).

1.2 Problem statement

The contamination of water bodies with xenobiotic herbicidal compounds presents detrimental ecological and human health effects thus, remains an alarming worldwide

concern. Despite stringent permissible limits set by regulating bodies globally (Morsi et al., 2020), trace amounts of these persistent organic pollutants are still regularly detected in agricultural runoff, industrial effluents, and other water sources across South Africa (Rimayi et al., 2018; Tudi et al., 2021). s-triazines are highly hydrophobic, recalcitrant and are of relatively high environmental persistence in their unmetabolized form, making them unsusceptible to complete removal in traditional treatment regimes, hence their consistent prevalence in treated wastewater discharges and subsequently across surface water and sediments (Horak et al., 2021). Therefore, considerable interest continues to grow in the eradication of s-triazine herbicides, such as atrazine, ametryn, prometon, terbuthylazine and simazine (Rimayi et al., 2018; Horak et al., 2021; Triassi et al., 2022). In this case, there is an urgent need to develop 'greener', stable and highly efficient alternative processes that can improve existing technologies with the complete conversion of persistent triazines into harmless chemical species (Morsi et al., 2020). In this context, application of ligninolytic biocatalysts such as laccases have been most explored and engineered for the bioconversion of these contaminants (Jin et al., 2016; Morsi et al., 2020). Nonetheless, the use of pristine enzymes is impeded by harsh operational conditions, stability challenges, susceptibility to wash-out thus restricting reusability and recyclability (Asif et al., 2020; Morsi et al., 2020). Promisingly, the incorporation of solid support matrices like PES membranes in the biocatalytic system may afford highly stable, reusable systems with reduced inhibition improved biodegradation efficiencies of target analytes (Qu et al., 2020; Jankowska et al., 2022). To address these challenges, this thesis explores the use of laccase enzyme to fabricate an eco-friendly biocatalytic membrane system presenting facile and efficient pathway for enhanced removal of triazine herbicides in wastewater. This innovative approach aims to leverage the mechanical robustness, thermal stability, and scalability of PES membranes with the high specificity and catalytic efficiency of the enzyme, laccase.

1.3 Aims and objectives

The study was aimed at investigating the potential application of a hybrid biocatalytic PES-laccase membrane system for the bioremediation of triazine herbicides, with a focus on improving the removal efficiencies and understanding the underlying degradation mechanisms.

The specific objectives were outlined as follows:

(i) Evaluate the biocatalytic activity of the enzyme, laccase.

(ii) Investigate the catalytic efficiency of laccase in degrading each of the selected triazine herbicides under varying experimental parameters (pH, temperature, initial solution concentration, dosage and contact time).

(iii) Characterize the surface properties of PES membranes using scanning electron (SEM) microscopy.

(iv) Test the performance of a standalone PES membrane system for the rejection of triazines (atrazine, ametryn, simazine, prometon and terbuthylazine) at varying process parameters.

(v) Develop an integrated PES-laccase packed-bed biocatalytic system for removal of triazine herbicides and conduct kinetic breakthrough modelling

(vi) Assess the reusability of the biocatalytic membrane system in repeated cycles of bioremediation.

(vii) Propose and compare cost-analysis estimates for the potential scalability of the designed biocatalytic system with existing configurations.

1.4 Thesis outline

Chapter 1: Gives a general introduction background and motivation of the study. This chapter also details out the problem statement, aims and objectives of the study.

Chapter 2: Presents a concise review of literature and theoretical overview on the physicochemical characteristics, occurrence and contamination of water resources of ECs. A discussion on existing technologies used for remediation, with emphasis on merits and limitations is also given. Thereafter, a concept and justification of the presented bioremediation approaches is underscored.

Chapter 3: An outline of the description of chemicals, materials and methodologies used in the research study. Experimental set-ups, analytical instruments used and formulated statistical data analysis conducted are also included.

Chapter 4: This chapter presents the findings/ results and discussion of the study inclusive of a published paper.

Chapter 5: A summary of key findings and major outcomes of the study. These are given as general conclusions and recommendations.

CHAPTER 2 LITERATURE REVIEW

This chapter provides a comprehensive review of literature on the occurrence, environmental fate of endocrine disrupting environmental contaminants in water sources. A concise discussion on the conventional treatment approaches employed for the eradication of selected organic pollutants in wastewater with an emphasis given on biocatalytic systems.

2.1 Endocrine disrupting contaminants (EDCs)

Over the past years, there has been a growing concern over inadvertent exposure of humans and animals to endocrine disrupting agents. The adverse effects associated with long-term exposure to these pollutants have led to their classification as emerging contaminants of concern (CECs). These consist of a portion of synthetic agents which mimic the biological activity mechanism of receptor activation (Jung et al., 2022). Among the various EDCs, triazine pesticides have been listed as Tier one of the list of substances in the European Protection Agency's endocrine disrupting (ED) screening program and also included in List 2 of the Dangerous Substance Directive (76/464/EEC) by the EU (Hecker and Hollert, 2011). ED pesticides are widely disseminated in the environment, leading to intensified pollution of freshwater sources (Bilal et al., 2017; Xia et al., 2022). An illustration of the environmental distribution of pesticides is given in **Figure 2.1**.

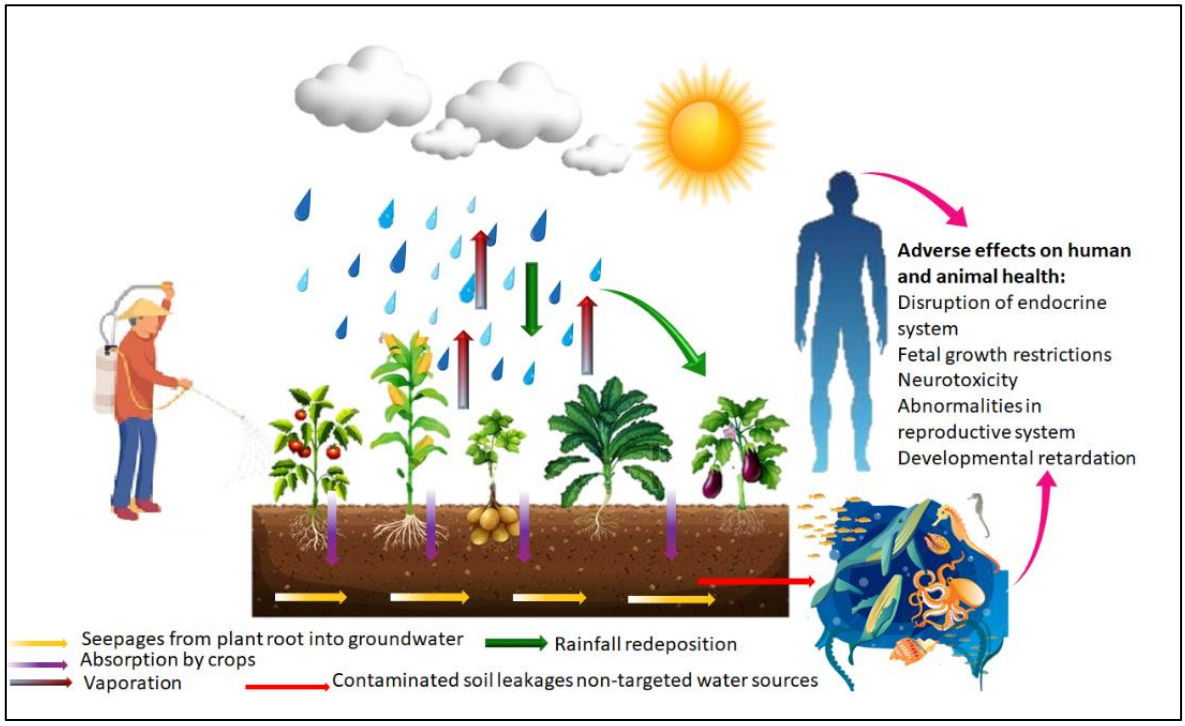


Figure 2.1: Environmental dissemination of pesticides, contamination of non-target areas and their adverse effects on human and animal life [adapted from Bilal et al., (2019)].

2.2 Agrochemical compounds: Pesticides

Pesticides form part of the widely applied class of agrochemicals and are employed in crop cultivation or post-harvest storage to manage yield and enhance production quality (Kass et al., 2020; Horak et al., 2021). These can be classified into different categories (**Figure 2.2**) based on the target pest (bactericides, fungicides, herbicides etc), formulation type, function, mode of action/entry, source of origin and usage (Bilal, Rasheed, et al., 2019; Kass et al., 2020).

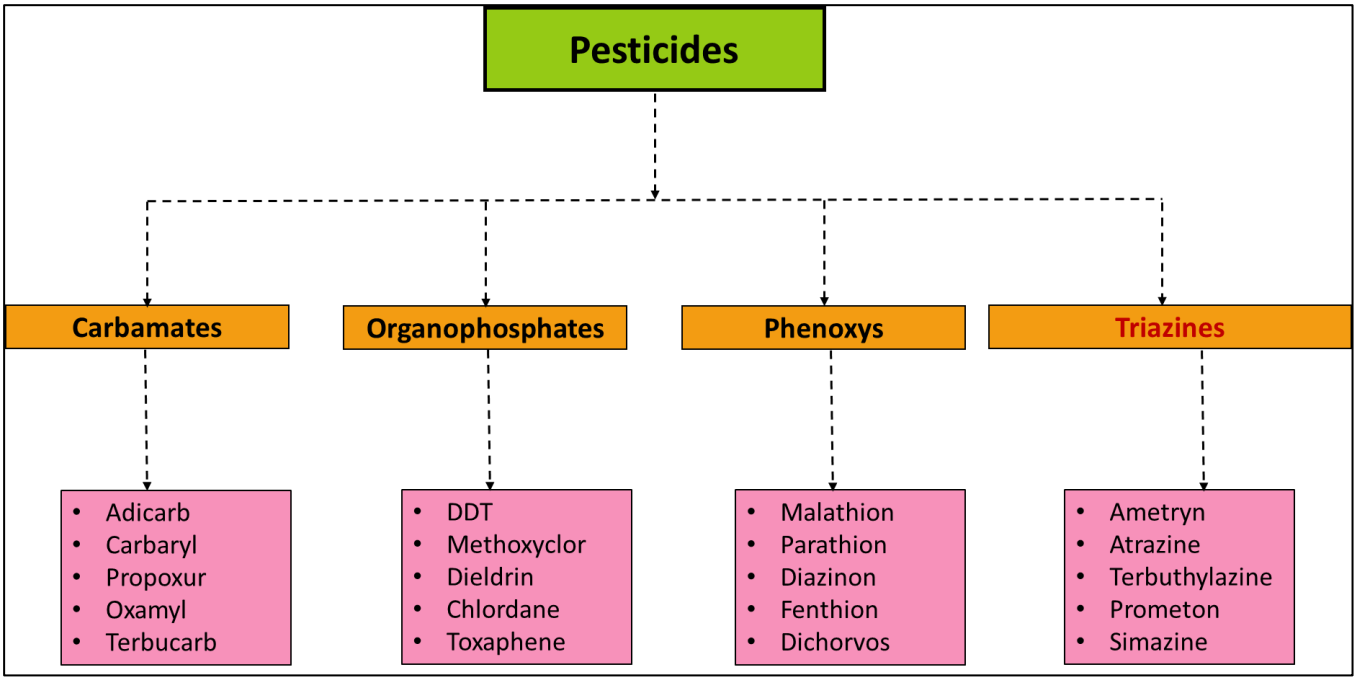


Figure 2.2: Classification of pesticides [adapted from Ore et al., (2023)].

Based on their structural backbone, pesticides are generally identified as either organic or inorganic (Bilal, Iqbal, et al., 2019; Kumar and Kumar, 2019) . Therefore, understanding their structural elucidations is not only be beneficial in appropriating the use of these compounds but also understanding their chemical behaviours, mechanisms of interactions and decomposition pathways (Ortiz-Hernández et al., 2013; Ore et al., 2023).

2.3 Triazine herbicides

2.3.1 Uses and trends

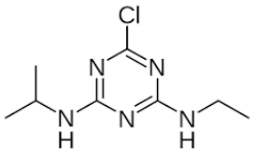
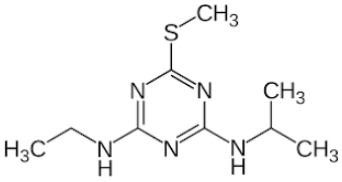
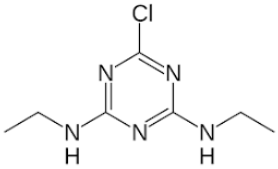
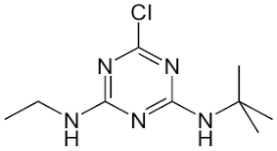
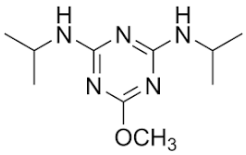
A recent report from the Food and Agriculture Organization (FAO) estimated the global consumption of pesticides at about 4.1 million tons whereby 46 % was accounted for by herbicides, 29 % by fungicides and 17 % insecticides (Balmer et al., 2019). The predominant herbicides were chloroacetanilides, amino phosphates and triazines and triazinones, which accounted for 28.4, 26.7 and 17.6 %, respectively (Fingler et al., 2017). Considering the large quantities of herbicides in current use, their environmental quantification remains a great concern.

2.4 Physico-chemical properties of triazines

Triazines are effective pre- and post-emergent weed-controlling agents and are primarily used in agricultural production. They consist of a 1,3,5- ring or symmetric-triazine (s-triazine) that contains two alkylamino substituted carbon atoms along with an alkyl, alkylmercapto or chlorine substituent on the third carbon. These include 20 different s-triazines which account for 20-25% of the world's active herbicides (Bilal, Iqbal, et al., 2019).

Atrazine is an example of a popular s-triazine herbicide (**Table 2.1**) predominantly used in combination with other triazine herbicides such as simazine, terbuthylazine and prometon (Ghosh and Philip, 2005; Dabrowski et al., 2014; Masiá et al., 2015). Due to their intensive use, trace resides of triazines have been widely detected in numerous water streams (Dabrowski et al., 2014; Rimayi et al., 2018; Horak et al., 2021; Masiá et al., 2015; Barchanska et al., 2017; Menchen et al., 2017; Mojiri et al., 2020).

Table 2.1: Molecular structures and physico-chemical properties of selected commercial s-triazine herbicides.

s-triazine compound	Molecular structure	pKa	Log K_{ow}	H_2O solubility (mg L^{-1})
Atrazine (ATZ)		1.7	2.7	70
Ametryn (AMT)		3.93	2.63	185
Simazine (SMZ)		1.6	2.3	5.0
Terbuthylazine (TERB)		1.12	3.4	8.5
Prometon (PMT)		4.05	2.91	620

These compounds are generally characterized as weak organic acids with pKa values (acid dissociation constants) varying from 1.7 to 4.05 (**Table 2.1**). Among the indicated parameters, hydrophilicity or Log K_{ow} values (octanol-water partition coefficients) and water solubility are key factors indicative of the environmental fate of contaminants. These compounds have moderately low hydrophilicity with Log K_{ow} values ranging from 2.3 to 2.9, exhibiting low soil adsorption thus promoting mobility to environmental matrices through long-range environmental transport, such as soil seepages (Bernal-González and Durán-Domínguez-de-Bazúa, 2012). Low K_{ow} values also promote bioaccumulation of triazines, thus leading to their

biomagnification in marine and terrestrial food webs (Ghosh and Philip, 2005; Moliner-Martínez et al., 2015; Mojiri et al., 2020). Moreover, s-triazines are of polar nature and exhibit high water solubility contributing to their high mobility rates in wastewater treatment systems (Luo et al., 2014a).

2.4.1 Environmental fate of triazines

2.4.1.1 Non-point versus point sources

The widespread dissemination of s-triazines emanates from either non-point (diffuse) or point sources. According to Chen et al. (2019) non-point sources are defined as those derived from direct application of herbicidal compounds onto farmlands and plantation areas. As shown in **Figure 2.3** the non-point pathways include irrigation-generated surface and under-ground run-offs, and soil leaching / seepages of spray-drifts (Ghosh and Philip, 2005; Tang et al., 2012; Cosgrove et al., 2019; Mojiri et al., 2020). Subsequent to application, triazine herbicides are transported to surface and groundwater bodies through sloping farmland, which usually consists of tile drain configurations permitting rapid transportation of herbicides to various areas (Flury, 1996; Dabrowski et al., 2014; Tudi et al., 2021). Point sources comprises of improper disposal of herbicides by agrochemical retailers, surface water overspray, herbicides manufacture, handling and distribution activities, commercial applicator facilities as well as accidental spills or leakages (Tang et al., 2012; Bilal et al., 2019 a). As a result, water ways adjacent to agricultural fields are severely compromised (Brillas, 2021; Horak et al., 2021).

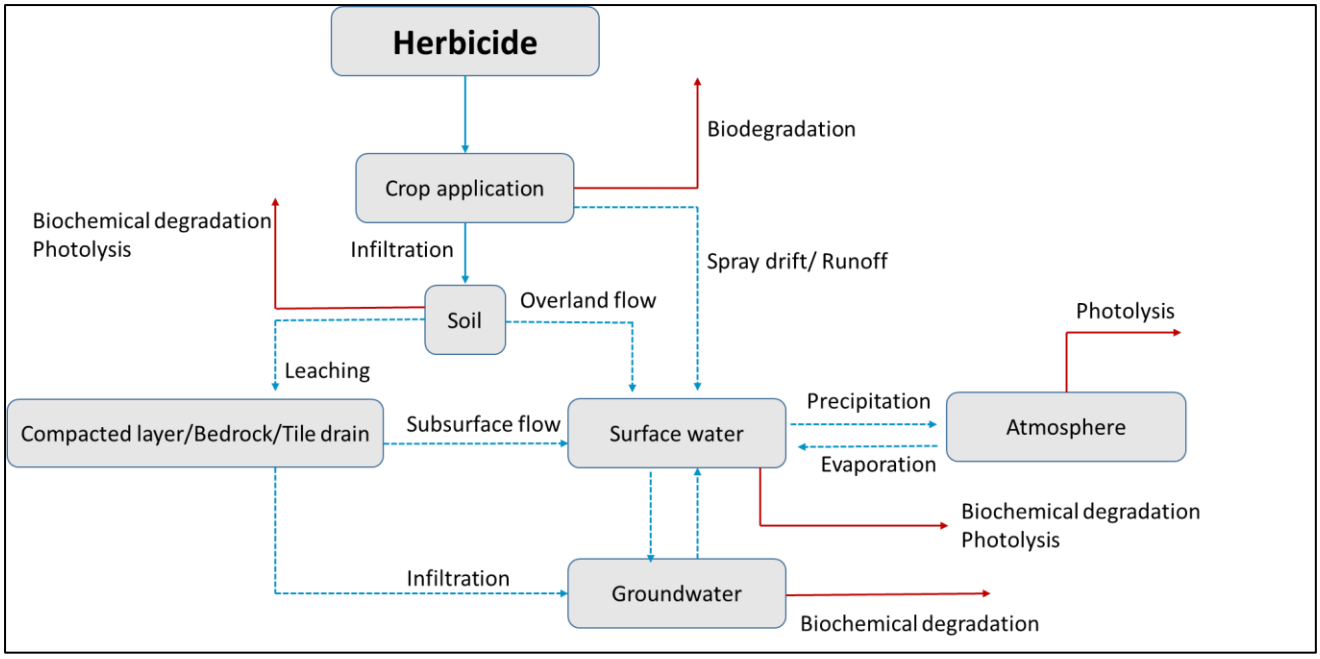


Figure 2.3: Different herbicide transport pathways in the environment. Continuous lines: direct pathways; dashed lines: indirect pathways [adapted from Tang et al. (2012); Barbosa et al. (2016)].

The constant detection of recalcitrant s-triazines in waterways suggest that conventional wastewater treatment plants (WWTPs), are inefficient in removing these compounds (Teodosiu et al., 2018; Olisah et al., 2020; Saleh et al., 2020; Horak et al., 2021). Although trace quantities of these compounds can be removed by convention treatment approaches, unmetabolized forms of s-triazines still persist in treated effluents, sewage networks (Luo et al., 2014b; Kanaujiya et al., 2019; Olisah et al., 2020). As a result, aquatic and terrestrial communities remain inadvertently threatened (Rimayi et al., 2018; Lerch and Willett, 2019; Z. Wang et al., 2022a; Pan et al., 2023).

2.4.2 Effects of triazines on the living organisms (aquatic and terrestrial)

Evidently, the consistent accumulation and occurrence of s-triazines has significantly reduced the environmental quality and adversely affect organisms and human health. Research studies have reported numerous ecotoxicological effects in the presence of triazine herbicides (Marcus and Fiumera, 2016; Rimayi et al., 2018; Jakinala et al., 2019; Jung et al., 2022). For instance,

Wang et al. (2022) reported a significant reduction of *Phaeodactylum tricornutum* biomass after exposure to a mixture of triazine compounds (atrazine, simazine, prometryn and terbutryn) at concentration ranges of 1.28 to 28.38 μg L^{-1}. Similarly, Zhao et al. (2023) observed a decrease in microbial organisms in the presence of an equitoxic triazine mixture which compromised the microbial ecosystem and the integrity of community function.

Apart from their geno – and cytotoxic effects (Khoshnood et al., 2015; Destro et al., 2021) long term exposure to these compounds induces growth and development impairments including disruption of hormone-regulated physiological processes in living organisms (MacLoughlin et al., 2018). However, data on direct cyto-toxicological effects remain inconsistent. Resultantly, the elucidation of environmental and health issues emanating from triazines remains complex. Nevertheless, monitoring and understanding their environmental fate and transport mechanisms affords comprehensive knowledge regarding their role in both marine and terrestrial ecosystems.

2.5 Remediation routes of selected triazine compounds from wastewater

The application of triazines involves direct and extensive administration over large land areas and due to the widespread contamination of water resources by these trace contaminants, various treatment methods have been extensively reviewed in the literature (Karthigadevi et al., 2021). These include traditional chemical and physical approaches (Luo et al., 2014a; Bilal, Iqbal, et al., 2019), such as those in **Table 2.2**.

Table 2.2: Removal efficiencies of target triazines using conventional approaches in wastewater treatment works.

Compound	Treatment	Removal efficiency (%)	References
Triazines (atrazine, ametryn, simazine, terbuthylazine, prometon, prometryn)	Coagulation, ozonation and chlorination	45-65	Ormad et al. (2008)
Atrazine	Coagulation-sand filter and ozonation	< 40	Chen et al. (2017)
Atrazine, Simazine	Coagulation, ozonation and ultrafiltration	< 30	Xia et al. (2022)
Atrazine, simazine, terbuthylazine	Activated sludge and trickling filter system	82	Munz et al. (2017)
Atrazine, simazine, terbuthylazine, propazine	Coagulation, flocculation, chlorination and micro filtration	< 17	Köck-Schulmeyer et al. (2019)
Atrazine, simazine, terbuthylazine, propazine	Coagulation and filtration	<50	Campo et al. (2013)
Atrazine, prometryn, propazine	Primary treatment, activated sludge with secondary clarifier	Not specified	Li et al. (2023)

However, triazines often defy treatment by WWTPs, which are essentially not designed to treat these contaminants (Evgenidou et al., 2015; Di Marcantonio et al., 2020). Hence the exploration of alternative post-treatment strategies:

- Physical techniques such as adsorption and membrane filtration. For instance, Suo et al. (2019) reported the adsorption of > 96 % of four triazines (atrazine, ametryn, simazine, simetryn) using phosphorus doped cornstraw. Although there was no report on the transformation of the analytes, the material outperformed other commercial adsorbents such as activated carbon, reduced graphene oxide and carbon nanotubes. However, sorbent bed aging and saturation of adsorption sites often requires secondary clarifiers and additional steps for long-term pollutant treatment (Grandclément et al., 2017). Navaratna et al. (2016) observed 65 % removal of ametryn in a sequential membrane reactor combined with granular activated carbon after 12 hrs in which 46 % removal was attributed to biodegradation alone. After 7.5 days of hydraulic and sludge retention time, triazine adsorption was negligible. Meanwhile biodegradation was regarded as the overall removal mechanism with 99 % removal efficiency achieved. Such integrated processes greatly enhance the elimination of micropollutants from wastewater (Besha et al., 2017).
- Microbial remediation by algae, fungi and bacteria (Alvarino et al., 2018; Pileggi et al., 2020; Rios-Miguel et al., 2023). These include the use of various microbe species such as *Pseudomonas, Bacillus*, and *Arthrobacter* in degrading various aromatics (i.e. phenols, quinones and amino acids) through catabolic pathways (Kundu et al., 2019; Wang et al., 2020). A study by Alattas et al. (2023) investigated the degradation of atrazine using different marine bacteria isolated from water and sediment samples. They showed that the marine isolates exhibited 75 %, 97 % and 87 % removal efficiencies associated with *Bacillus pacificus, Bacillus cereus* and *Bacillus paramycoides* strains, respectively. Similarly, Jakinala and co-workers (2019) found that *Bacillus*, *velezensis* degraded atrazine.

Moreover, the degradation efficiency increased from 69.4 ± 1.9 %, to 84.1 ± 4.2 % after four days of incubation. These findings indicate the flexibility of bacterial metabolic pathways, which have prompted their use for the bioremediation of atrazine-contaminated samples.

2.5.1 Membrane technology in wastewater treatment

Membrane-based separation processes (MBSPs) are broadly explored water treatment strategies that have been extensively applied in water desalination, toxic metal separation and micropollutant removal (Goh et al., 2022; Xia et al., 2022). The separation of undesirable constituents occurs through their movement from the feed solution to the membrane barrier while retaining other species on the other side. MBSPs are available in a variety of modules depending on the membrane type, produced from different materials (polymers, zeolites, ceramics etc.), exhibiting specific filtration features such as pore size, morphology, hydrophilicity or hydrophobicity, separation requirements and target pollutant (Saleh et al., 2020; Khan and Boddu, 2021; Shehata et al., 2023). As illustrated in **Figure 2.4**, the mechanism of separation of each membrane is driven by the solubility and/or diffusion of the solute through the membrane (Goh et al., 2022). The membrane-solute interaction is dependent on factors such as, molecule diffusion, solution-diffusion and the size-exclusion principle (Dharupaneedi et al., 2019; Jatoi et al., 2021). Plakas and Karabelas (2012) have elucidated the processes governing the removal of pesticides and other trace organic contaminants.

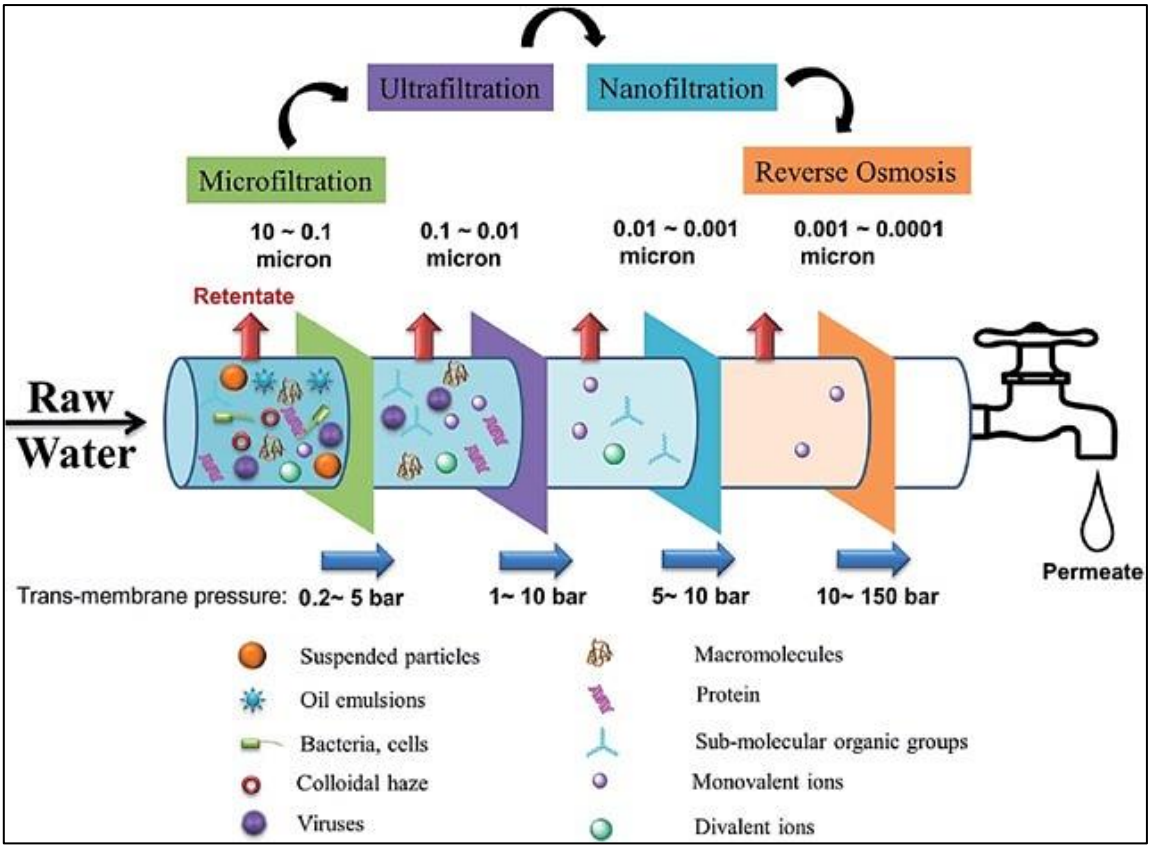


Figure 2.4: Membrane treatment characteristics [Obtained from Ahmed et al.(2021)].

Nanofiltration (NF), microfiltration (MF), reverse osmosis (RO) and ultrafiltration (UF) are types of classical pressure-driven membranes used for the remediation of micropollutants including pesticides (Khan and Boddu, 2021). Relative to other physical approaches, MBPs not only provide high quality but are also considered energy efficient, they offer flexibility in system design and ease of upscaling with reliability and durability for various water reclamation applications globally (Goh et al., 2022).

2.5.1.1 Ultrafiltration (UF)

These are membranes with pore sizes ranging between 0.01– 0.1 µm which allow the removal of colloidal particles, macromolecules, bacteria, and viruses in the range 0.01 to 0.2 µm (Dharupaneedi et al., 2019). Separation proceeds by applying a pressure differential across the membrane in which the retentate is collected on while water and dissolved solutes diffuse through the membrane. The retained residue is either continuously or periodically removed

from the system as a concentrated suspension or sludge (Plakas and Karabelas, 2012; Miao et al., 2023). The removal of atrazine and other organics were investigated by Acero et al. (2010) using single UF and MF systems and higher membrane fouling was observed for UF. Due to relatively open membranes of NF and RO, UF allows the use of low transmembrane pressure however, the retention of low molecular weight organic contaminants is low. This constitutes one of the vital drawbacks of UF for wastewater treatment (Acero et al., 2010). Acero et al. (2017) reported increased retention of atrazine from 35 to 62 % by an enhanced micellar-ultrafiltration membrane. The presence of surfactants increased the size of the low molecular weight pollutant and thus allowed enhanced atrazine removal.

2.5.1.2 Microfiltration (MF)

Microfiltration allows effluent clarification by retaining colloidal materials and suspended particles (inorganic particles, microorganisms etc.) (Titchou et al., 2021). The operation is based on a physical separation technique and comprises micropores in the range 0.1–10 μm where micrometer-sized substances such as large bacteria, suspended particles, pathogens, yeast cells and proteins are rejected (Gul et al., 2021). Similar to UF, MF is characterized by relatively larger pores which cannot remove dissolved organics. This aspect plays a crucial role in the rejection of pesticides considering that their filtration mechanism is based on size-exclusion. As a result MF and UF are generally implemented as a pre-treatment for reverse osmosis (RO) (Fonseca Couto et al., 2018). Rodriguez-mozaz et al. (2015) investigated the performance of a MF coupled with a RO plant for the rejection of pharmaceuticals and pesticides including atrazine, cyanazine, simazine and terbuthylazine. Standalone MF did not retain any of the target compounds in the WWTP effluent, however, the efficiency increased after the RO step. This was related to the larger pore sizes in MF, hence the need for an additional RO treatment. Studies on improved MF efficiency by integrated systems such as

flocculation-sedimentation (Li et al., 2023) and RO systems for triazines have been reported (Luo et al., 2015).

2.5.1.3 Nanofiltration (NF)

NF operates on a low-pressure separation with a semi-permeable membrane generally characterized by an effective pore size of $0.01 - 0.001$ μm, corresponding to molecular weight cut-off (MWCO) ranging from 100 to 1000 Da (Fane et al., 2011; Zhao et al., 2021). These features allow NF to effectively remove diverse pollutants from water (e.g. divalent ions, and organics like herbicides and pesticides) based on the Donnan effect and size exclusion mechanism (Guo et al., 2022; Behroozi et al., 2023). Compared to RO, NF processes operate at low pressure (i.e. 5-10 bar) which surmounts the osmotic pressure barrier thus sparing about 20 % of the energy (Amiri et al., 2020). Several types of NF membranes have been fabricated including dense, ceramic, electrically charged, non-porous, isotropic micro-porous, and liquid membranes based on pores and surface structure of the membrane (Wang et al., 2021; Yadav et al., 2022). Non-solvent induced phase separation technique has been widely utilized for the fabrication of most commercially available NF using a range of suitable polymers (Abdel-karim et al., 2017). Among these substrates, polysulfone (PSF)/polyether sulfone (PES), polyethylene (PE), polyvinylidene fluoride (PVDF), aromatic polyamide (PA), cellulose acetate (CA), polyacrylonitrile (PAN), and polypropylene (PE) have been explored (Brown, 2007).

2.5.1.4 Polyethersulfone membranes

Over the years, the polysulfone family consisting of tetramethyl bisphenol-A polysulfone (TM-PS), bisphenol-A polysulfone (PS) and polyethersulfone (PES) have been extensively explored in the preparation of UF and MF membranes (Xu and Alsalhy Qusay, 2004), producing thermally and mechanically stable membranes with excellent hydrolytic features. PES is considered one of the most critical polymeric materials for use as a separation tool for

drinking water purification and wastewater treatment (Tsehaye et al., 2018). A general mechanism of removal using PES is given in **Figure 2.5**.

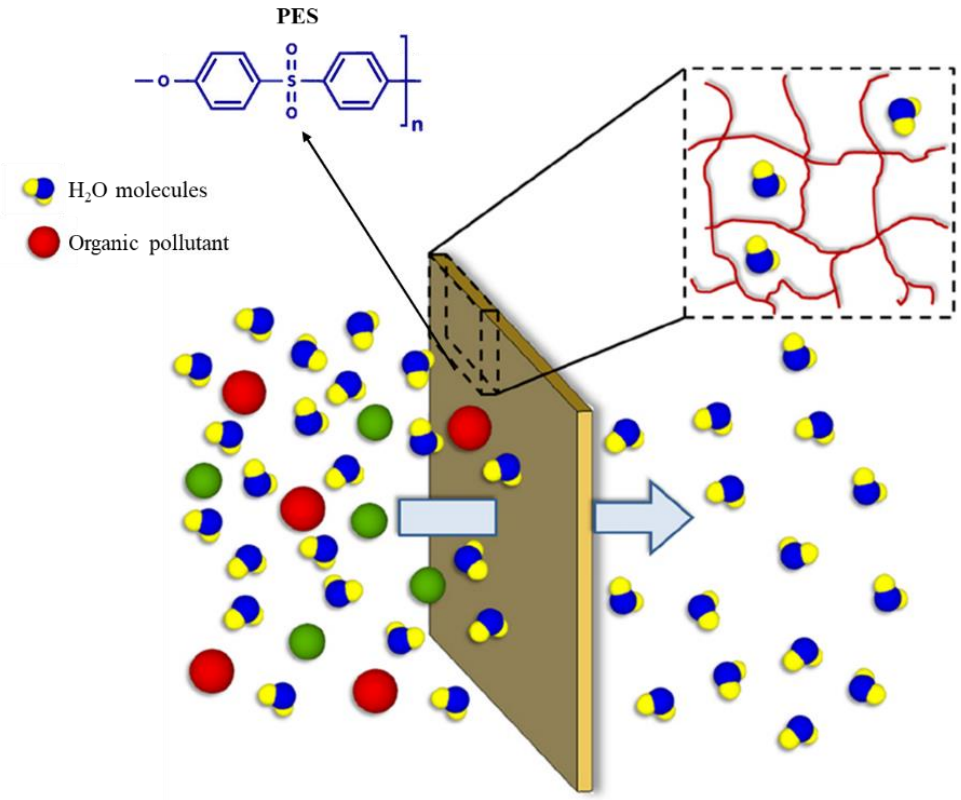


Figure 2.5: Removal mechanism of pollutants using PES membrane (Chuaicham et al., 2023).

This polymer exhibits high mechanical and thermal resistance (Ahmad et al., 2013) with outstanding oxidative, chemical (alternate sulphonyl (SO_2) and ether (-O-) linkages) and structural (presence of aromatic groups) stability (Tsehaye et al., 2018). Nevertheless, the inherent hydrophobic nature of PES makes it susceptible to fouling during operation (Liu et al., 2013; Luo et al., 2020; Ahmed et al., 2021; Gul et al., 2021). Antifouling membranes are often achieved by surface modification through various approaches (Rostam et al., 2018, Kolesnyk et al., 2019, Nasrollahi et al., 2018), and hybrid membrane processes (Yu et al., 2019) or integrated oxidative membrane reactors (Abdel-karim et al., 2017). Detailed reports on various modification approaches using different components have been reviewed (Brown, 2007; Ahmad et al., 2013).

2.5.2 Removal of triazines using advanced oxidation processes

Advanced oxidation processes (AOPs) have emerged as one of the effective treatment technologies applied for the sequestration of various organic micropollutants in wastewater through the generation and utilization of reactive radical intermediates (Glaze and Kang, 1989; Boczkaj and Fernandes, 2017). An example of the mechanism of AOPs is given in **Figure 2.6**.

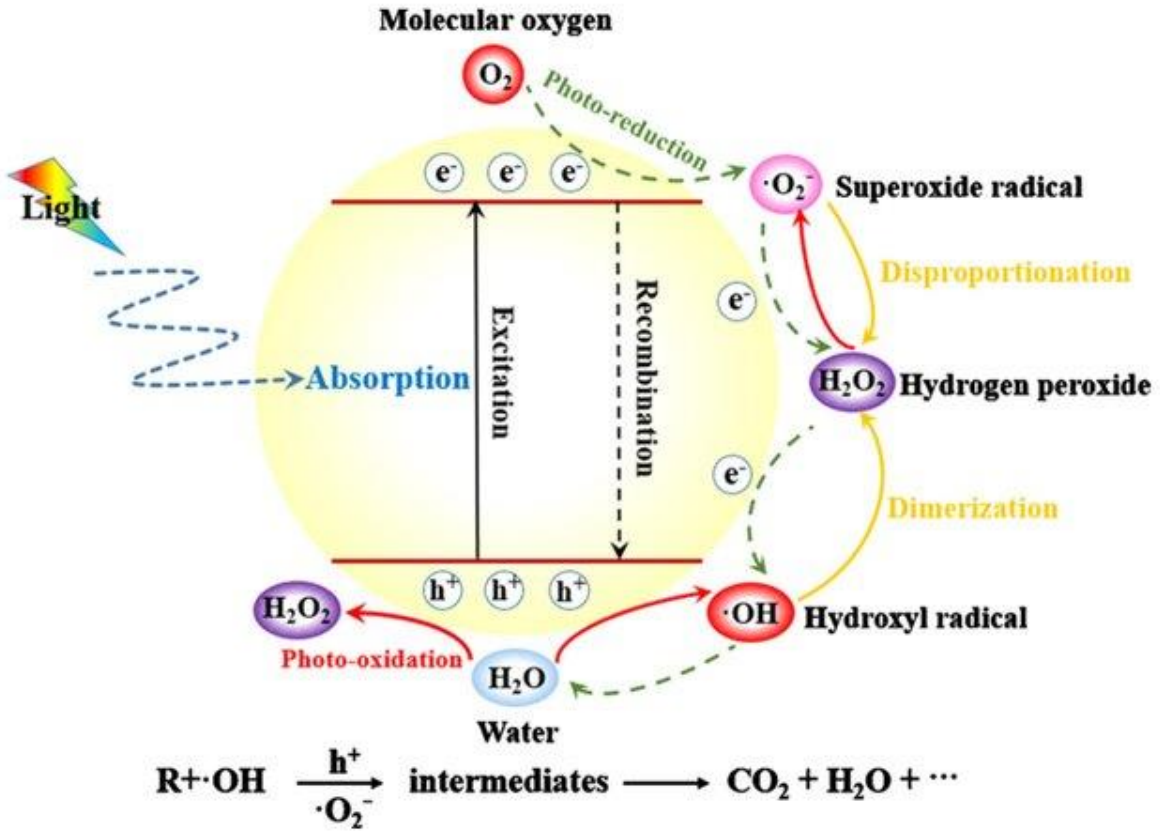


Figure 2.6: Schematic diagram representing mechanism of photocatalysis (Obtained from Chuaicham et al. (2023).

These are radical-mediated oxidation processes used to disintegrate or completely mineralize contaminants through the non-selective attack of highly oxidizing hydroxyl species (·OH) (Boczkaj and Fernandes, 2017; Zheng et al., 2022). AOPs have demonstrated substantial pollutant mineralization and degradation efficiencies in herbicide-contaminated water (Bhat

and Gogate, 2021; Sandoval et al., 2022). **Table 2.3** summarizes the merits and drawbacks of AOPs in the decontamination of triazines.

Table 2.3: Summary of AOPs used for triazine herbicide removal.

AOP method	Mechanism	Advantages	Limitations
Fenton's reaction	H_2O_2 reacts with $FeSO_4$ which react together to form reactive oxygen species that oxidize micropollutants	Wide pollutant spectrum Absence of bromated by-products	Flocculation of reagent and pollutant produces undesirable Fe sludge Low pH requirements (< 2.5) High operational costs
H_2O_2/O_3	Generation of reactive hydroxyl radicals or the auto-decomposition cycle of extremely reactive	Generation of strong non-selective $OH^{\bullet}$ capable of degrading conjugated double bonds O_3 used in gaseous state reduces effluent volume No formation of sludge	Additional treatment steps for access H_2O_2 Less generation of hydroxyl radicals Toxic by-products by O_3 High cost
Photo-catalysis	Relies on the use of semiconducting photocatalysts, an oxidizing agent and an energy light source, to transform pesticides into carbon dioxide, water and mineral salts	Catalyst recyclability Broad wavelength Minimal energy requirements (solar radiation)	Additional multi-reactions required Slow throughput rates for aqueous systems High concentrations require addition of excess electron scavenger Presence of colour and turbidity reduces efficiency
UV/H_2O_2 UV/H_2O_2		Established technique Rapid reactions	Deterioration of lamp performance at high temperatures Highly alkaline wastewaters produce scaling on quarts sieves

2.6 Enzyme mediated degradation of target pollutants: a “greener” alternative

In the current study, the use of laccase (an oxidative enzyme) is proposed as an alternative approach for the oxidation of triazine herbicides. Biotechnological applications of ligninolytic enzymes, especially laccases, enable catalytic reactions with a wide substrate specificity under moderate conditions (temperature, pH, solvents etc.), with low energy requirements, produce less-toxic by-products and can be used with low pollutant concentration. This is usually a challenge in physicochemical approaches (Morsi et al., 2020; Al-Maqdi et al., 2017; Holanda et al., 2019). Enzymatic selectivity and specificity impede side reactions, eradicate the production of undesired by-products and also enhance the enzyme’s ability to transform complicated chemical substances (Gan et al., 2022; Sellami et al., 2022). Hence, enzymes represent promising tools as sustainable and environmentally benign alternatives for mitigating pollution of streams than conventional herbicides degradation strategies. The following section details the use of oxidative enzymes in biocatalytic remediation of triazines.

2.6.1 Oxidative fungal enzymes

These are extracellular enzymes that catalyze the oxidation-reduction assisted transformation of xenobiotics (Morsi et al., 2020). Oxidoreductases include dehydrogenases, oxidases, peroxidases, and oxygenases (Bilal et al., 2022). Amongst the various enzymes, laccases and peroxidases are the most used in enzymatic-remediation due to their high ability to degrade different organic toxins (Unuofin et al., 2021). Unlike many chemical oxidants, oxidative enzymes do not mineralize the pollutant. Instead, they rely on a free radical mechanism to degrade and detoxify the parent compound (Naghdi et al., 2018; Bilal et al., 2019b; Unuofin et al., 2019).

2.6.2 Peroxidases

The peroxidase superfamily (EC 1.11.1. X), consists of heme and non-heme containing proteins widely distributed in nature, primarily in fungi, bacteria, microbes, plants and animals

(Alneyadi et al., 2018; Gan et al., 2022). Peroxidases mediate the reduction of peroxides (H_2O_2 or organic peroxides) as co-substrates with concomitant oxidation of diverse chemical substrates (Naghdi et al., 2018; Unuofin et al., 2019). Their mode of action, depicted in **Figure 2.7** entails two-electron oxidation-reduction mechanism in which substrates are converted into radicals, which then undergo non-enzymatic reactions.

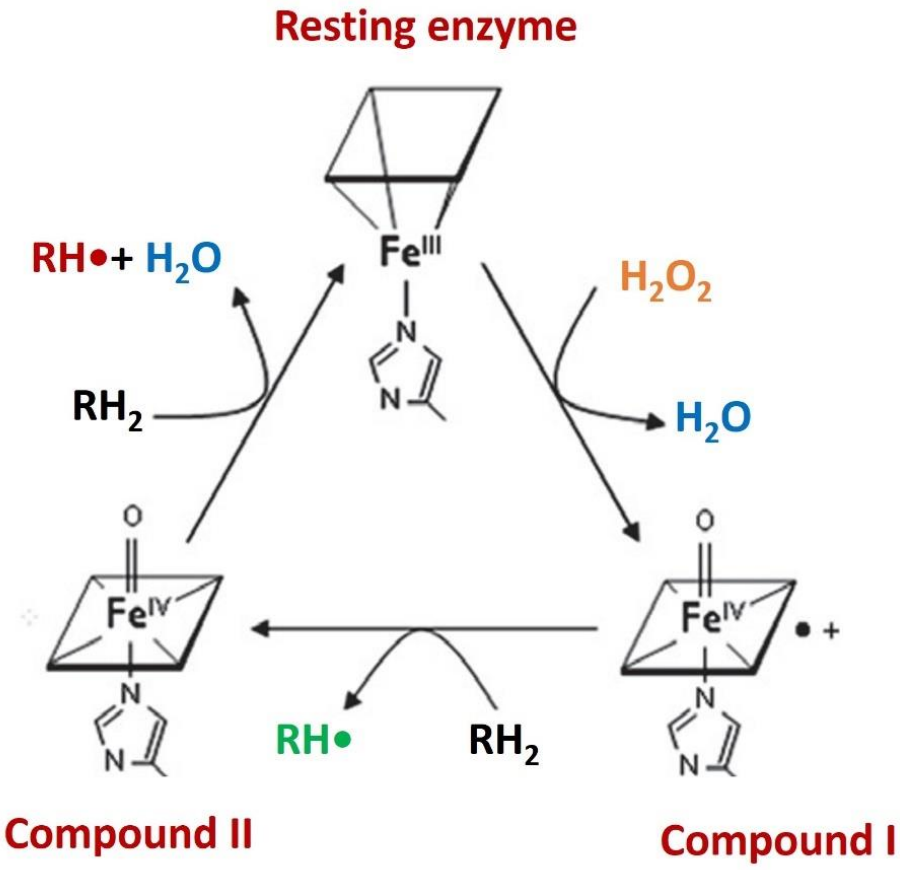


Figure 2.7: Catalytic cycle of peroxidases [adapted from Battistuzzi et al. (2010)].

Interestingly, evidence points to substrate binding and subsequent oxidation taking place in one of two ways: (i) on the protein surface site proximal to the heme channel or adjacent to the heme-Fe catalytic active site pocket. The enzymatic cycle proceeds by oxidation of the native (ferric) enzyme by H_2O_2, producing a cation radical (oxo-ferryl intermediate, Compound I) and reducing H_2O_2 to H_2O. Compound I is then reduced back through the Fe^{4+} intermediate to form Compound II and a radical with subsequent oxidation of the organic

substrate. Lastly, another oxidation of the substrate molecule involving Compound II occurs to give one more radical product permitting the reduction and restoration of the enzyme back to its resting state (Battistuzzi et al., 2010; Alneyadi et al., 2018; Sellami et al., 2022).

The free radicals generated during the catalytic process are responsible for the degradation of micropollutants (Sharma et al., 2018; Morsi et al., 2020). Examples of peroxidases commonly used for water pollution abatement include manganese peroxidase (MnP), soybean peroxidase (SBP), lignin peroxidase (LiP), horseradish peroxidase (HRP) and chloroperoxidase (CPO) (Singh and Cameotra, 2014; Unuofin et al., 2019; Bilal et al., 2022).

Notwithstanding the effectiveness of peroxidases in wastewater remediation, they possess low functional stability which contributes to the irreversible enzyme deactivation especially in the presence of excess quantities of H_2O_2 as their natural electron acceptor source (Rasheed et al., 2019; Gan et al., 2022). The use of laccases in the oxidative degradation of emerging organic pollutants is currently of much interest as it obviates the need for toxic H_2O_2 (Bilal et al., 2017, Bilal et al., 2019c; Morsi et al., 2020).

2.6.3 Laccase (Lac): a multifaceted biocatalyst for degrading triazines

Laccases (EC 1.10.3.2) are an important class of multicopper containing oxygen oxidoreductases predominantly present in a variety of organisms such as plants and bacterial species and are widely distributed in fungi (Gianfreda et al., 1998; Sugumaran and Barek, 2016). The high redox potential of microbial (fungal) lacc such as *Trametes versicolor, Cerrena unicolor* or *Trametes vilosa* has led to their wide application since they exhibit high catalytic activity, are of low price and abundant availability (Antecka et al., 2018; Bilal et al., 2019 b). Owing to their broad substrate specificity, high oxidation capacity and utilization of molecular O_2 as their natural co-substrate for the catalytic activity, laccase enzymes are regarded as suitable candidates for many environmental, biotechnological and bioremediation

applications (Pandey et al., 2017; Daccò et al., 2020; Zhuo and Fan, 2021; Bilal et al., 2022; Pathak et al., 2022).

They catalyse the one-electron oxidation reactions of organic substrates with the concurrent reduction of atmospheric O_2 to H_2O (Baldrian, 2006; Demarche et al., 2012). Laccase-assisted oxidation processes generate reactive radicals capable of oxidizing non-phenolics i.e. in the presence of suitable redox mediator(s) (Bilal, Rasheed, et al., 2019). Literature reports indicate that the presence of non-phenolic chemicals, such as most triazines may present lower catalytic efficiencies owing to: (i) their greater redox potential than laccase; and/or (ii) steric hindrance caused by electron withdrawing groups (EWGs) such as halogens and amide functional groups (Asif et al. 2018). Thus, the addition of mediators enhances the efficacy of laccase.

The presence of oxidized radical form from the redox mediator 1-hydroxybenzotriazole (HBT) permitted the degradation of high redox-potential target compounds hence improving the degradation of triclosan from 67.4 to 89.6 % during an 8 h reaction (Le et al., 2016). Interestingly, the use of molecular oxygen as electron acceptor represents a distinct advantage of lacc compared with the utilization of H_2O_2 by peroxidase-mediated catalytic reactions, thus facilitating the implementation of laccase catalysed treatments in water bioremediation (Morsi et al., 2020).

2.6.4 Catalytic mechanism and molecular structure of Laccase

Laccases are extracellular proteins containing four different cupric centres arranged in two different sites:

- Type I (T1 Cu), exhibits coordination with equatorial ligands of histidines (two) and a cysteine (one) with one Cu atom acting as the primary electron acceptor site where the catalytic oxidation of substrate takes place, T1 also imparts the blue colour to the enzyme.

- The second domain carries two histidines (His) and a H_2O, a single copper atom of Type II (T2 Cu) and type III (T3 Cu) with six His amino acid which form a tri nuclear cluster (T2/T3) responsible for the reduction of oxygen to water (Sugumaran and Barek, 2016; Falade et al., 2018; Kumar and Chandra, 2020). A depiction of the catalytic cycle of Lac is given in **Figure 2.8**.

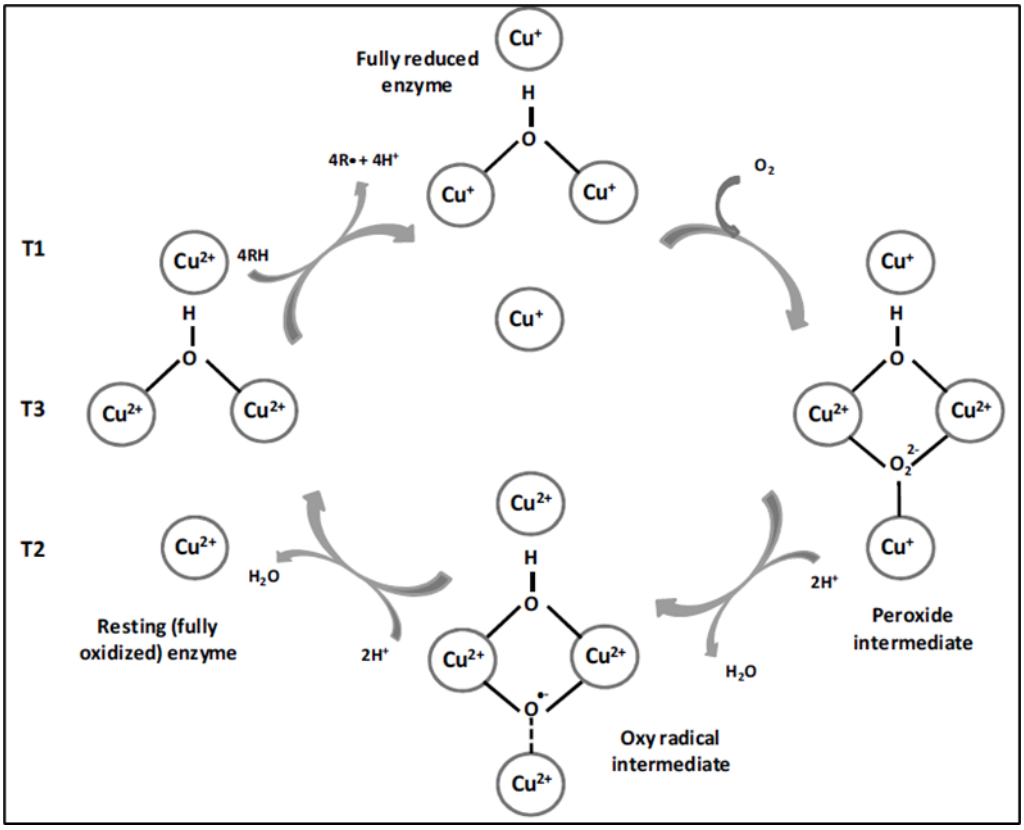


Figure 2.8: Catalytic cycle of laccase [adapted from Kulikova et al.(2011)].

The inherent advantages of laccases, renders their utilization in enzymatic based treatments to oxidatively decontaminate and eradicate diverse micropollutants from water systems (Grandclément et al., 2017). Despite their versatility, their use in native forms is strongly hampered by comparatively high costs and lack of biocatalyst reusability and stability during the remediation process (Demarche et al., 2012; Bilal et al., 2017 b). These are often mitigated by immobilization onto stable carrier matrices (Zdarta et al., 2018, 2019) or by coupling the enzymatic system to a support matrices such as membrane of appropriate MWCO to fabricate

an enzymatic membrane reactors (Nguyen et al., 2014). This approach renders benefits including improved biodegradation efficiencies, long term operational stability, enhanced enzyme retention with usage of free enzymes, avoiding mass transfer limitations, reusability, and simple product separation frequently associated with attachment to supports (Asif et al., 2018; Rasheed et al., 2019).

2.6.5 Application of biocatalytic membranes for the removal of triazines

Generally, enzyme-catalysed biotransformations have been predominantly carried out in their pristine states under batch mode, however such systems present limitations such as enzyme washout with the treated effluent during operation (Morsi et al., 2020). Moreover, batch operation involves substrate and/or product inhibition, and the recovery of products is attained by stopping the catalytic reaction before a new batch cycle proceeds again. The complex start-up and end-processes present non-productive operational time and increased labour costs (Sitanggang et al., 2022). Therefore the application of continuous catalytic transformations can be a preferred solution (Baxendale et al., 2015).

A continuous enzyme-catalysed bioconversion can be fabricated using a variety of reactor types such as packed bed reactors (PBRs), continuous stirred tank reactors (CSTRs), and microchannels (MC) (Sitanggang et al., 2022). The configuration of an enzymatic membrane systems is regarded as a novel strategy capable of expediting simultaneous catalytic conversion and product isolation and thus realizing a more cost-effect process (Rios et al., 2004; Liu et al., 2022). In addition, the selection and integration of a membrane with an appropriate molecular weight cut-off (MWCO) is critical to ensure complete retention of pollutant and dispersed enzyme molecules (i.e. heterogeneous catalysis) (Giorno and Drioli, 2000; de Cazes et al., 2014). The membrane support in the system, serves as both a separating unit and a catalytically active surface thus expanding the uses of such designs, and avoiding the need for secondary clarifiers especially in wastewater treatment (Besha et al., 2017; Asif

et al., 2018; Sitanggang et al., 2022). Various strategies for enzyme attachment to the membrane carriers were highlighted. However, compared to enzyme immobilization, the employment of packed biocatalytic systems offer advantages, such as less mass transfer restrictions, simplicity of enzyme replenishment during prolonged operation and efficient enzyme retention (Asif et al., 2018). Further advancements of biocatalytic membrane designs with various membrane designs were discussed in a comprehensive review by Luo et al. (2020). Other than presenting different applications of packed bed membrane reactors (PBMRs), they also discussed the morphological properties of membranes and major challenges encountered in the PBMR systems.

The efficacy of UF-enzymatic membrane bioreactor (EMBR) and NF-EMBR to remove a mixture of trace organic contaminants including atrazine and ametryn was compared under varying experimental conditions such as pH, temperature and reaction time. Higher triazines' removal was achieved with the NF-EMBR which further improved upon the addition of a redox-mediator (violuric acid) for the non-phenolic triazines (Asif et al., 2018). To-date, reports on the performance of NF based enzymatic membrane biocatalysts, particularly for triazines is scarce (Asif et al., 2017).

Given these effects, the next chapter provides a comprehensive overview of methods explored in developing a facile and environmentally benign PES-laccase membrane biocatalytic for the bioremediation of recalcitrant triazine herbicide in aqueous solutions as an alternative approach.

CHAPTER 3 MATERIALS AND METHODS

This chapter outlines the specific materials, instruments, and methodologies employed in the research. It details the experimental design, including the selection and preparation of samples, procedural steps followed, and the analytical techniques used for data collection and analysis. Emphasis is focused on key aspects such as the analytical approaches used, experimental parameters investigated, data acquisition, tools used for data interpretation.

3.1 Chemicals and reagents

Analytical grade standards (Pestanal grade, > 99% purity) for the selected triazine compounds: atrazine [ATZ; 6-chloro-*N*-ethyl-*N′*-(propan-2-yl)-1,3,5-triazine-2,4-diamine], ametryn [AMT; 2-(ethylamino)-4-isopropylamino-6-methyl-thio-s-triazine], prometon [PMT; 6-methoxy-1,3,5-triazine-2,4-diamine], simazine [SMZ; 2-chloro-4,6-bis(ethylamino)-s-triazine] and terbuthylazine [TBA; 2-chloro-4,6-bis(ethylamino)-s-triazine] were purchased from Merck (Johannesburg, South Africa). Formic acid and acetonitrile (ACN), a high-liquid performance chromatography (HPLC)-grade solvent were also supplied by Merck (Johannesburg, South Africa).

Citric acid ($C_6H_8O_7$), sodium dihydrogen phosphate ($NaH_2PO_4H_2O$) and disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$) of analytical grade, were purchased from Sigma-Aldrich (Johannesburg, South Africa). Redox-mediators (analytical grade, >98% purity, *viz.* 2,2′azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), violuric acid (VA), vanillin (VAN), 4-hydroxy-3-methoxybenzaldehyde, acetosyringone (ASR), 3,5-dimethoxy-4-hydroxyacetophenone and syringaldehyde (SRA), 2,2,6,6-tetramethylpiperidin-1-yl were all analytical grade chemicals, mostly from Merck (Johannesburg, South Africa). Ultrapure water obtained from Millipore, Direct-Q® 3UV water purification system (Merck, Darmstadt, Germany) was used.

3.2 Biocatalyst of interest

Laccase (Novoprime® base 268) was supplied by Novozymes (Bagsvaerd, Denmark). The enzyme was prepared by submerged fermentation of a genetically modified microorganism (units and origin not defined). Three-dimensional and general structures of the enzyme are available in literature and are shown in **Figure 3.1.** The copper ions and coordinating water molecules are depicted as purple-blue and red spheres, respectively. The internal pathway from the Type 1 Cu to the Type 2/3 trinuclear cluster is represented in yellow and an

illustration of the catalytic site and coordination sphere of the catalytic coppers is given in as a schematic diagram (Mate and Alcalde, 2017).

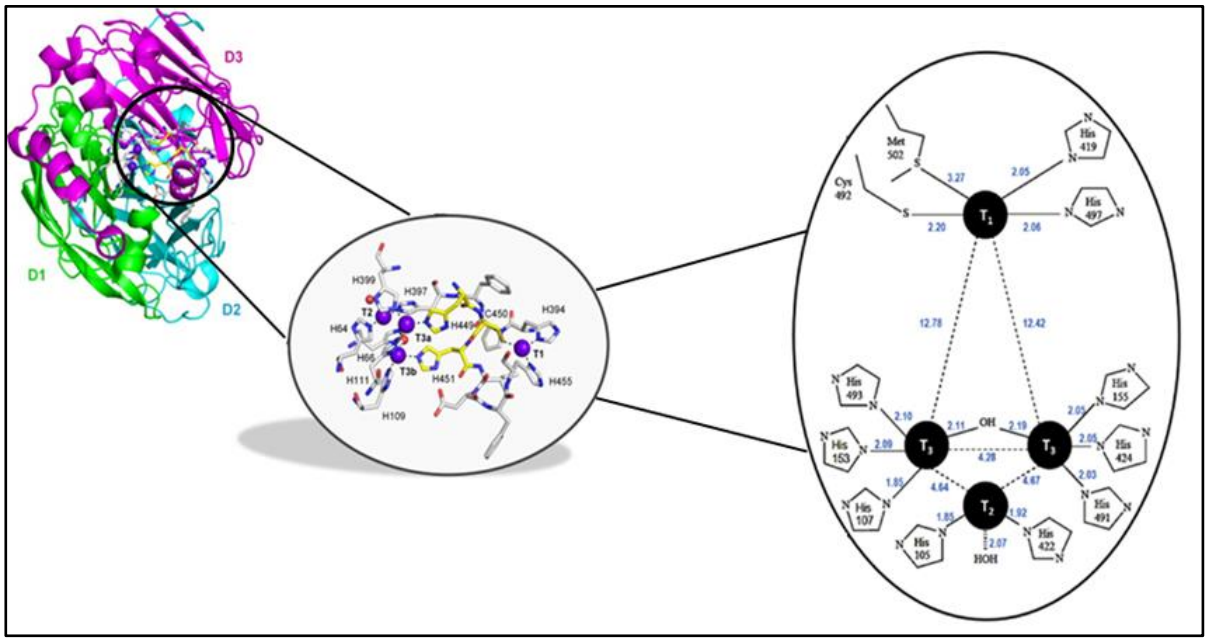


Figure 3.1: Three-dimensional, general structure and active sites of laccase enzyme. D1 (green), D2 (cyan blue) and D3 (magenta) represent the cupredoxin-like domains, respectively.

3.2.1 Characterization of the biocatalyst

Novoprime® base 268 laccase (Novozymes, Bagsvaerd, Denmark) was biochemically characterized by assaying its catalytic activity and stability over varying experimental conditions.

3.2.2 Determination of enzymatic activity

Laccase activity was measured through a colorimetric assay employing ABTS as a substrate, wherein the rate of colour development directly corresponded to enzyme activity. This was carried out as per previous method reported by (Morsi et al., 2020). The conversion of 1.0 mM ABTS to its cation radical ($ABTS^{\bullet+}$) by laccase (20 mg) was monitored at a wavelength of 420 nm wavelength in citrate-phosphate buffer (50 mM CPB, 5 mL) after stirring at pH 5 and ambient temperature (23 ± 5 °C) using an ultra-visible (UV-vis) spectrophotometer

(Agilent Technologies, Cary Series 1000, USA). Calculations were computed using the molar extinction coefficient of ABTS (ε_{420nm} = 36,000 $M^{-1}cm^{-1}$) where one unit of laccase activity was defined as the amount of enzyme that oxidized 1µmol of ABTS min^{-1}.

3.2.3 Estimation of laccase kinetic parameters

Laccase affinity for the substrate ABTS was measured by conducting kinetic experiments in 5.0 mL CPB (pH 5.0) consisting of 20 mg of enzyme (laccase) and substrate (ABTS) concentrations in the range 0.1 to 5.0 mM (Tiarsa et al., 2022). The kinetic parameters were assessed by quantifying the enzymatic reaction rate at varied concentrations (1.0 to 20 mg L^{-1}) of ATZ (a common triazine herbicide) as competitor. The rate of enzymatic reaction was described by a plot of reaction rate v/s initial substrate concentration ([V]/[S]) with the estimation of the K_m and V_{max} parameters analyzed by fitting the experimental data into the non-linear Michaelis-Menten model and by Lineweaver-Burk plots (1/[V] vs 1/[S]) (Baldrian, 2006; Lassouane et al., 2019; Mehandia et al., 2020). These were manually drawn using Origin Software version 8.0.

3.2.4 Effects of solution pH and temperature profile on laccase catalytic activity

The pH and temperature profile of laccase were determined according to published procedures (Lassouane et al., 2019). The optimum pH was studied using ABTS as a substrate following the protocol mentioned above wherein pH levels ranging from 1-10 were assayed by pe-incubating laccase in 50 mM CPB at room temperature over 24 h. Enzymatic activity was investigated at varying incubation times; for this purpose, sample aliquots of known volume were drawn and transferred to standard reaction vessels to measure the enzymatic activity with 1.0 mM ABTS and the relative activities were computed by comparing the activity obtained at each pH to the maximum achieved.

Similarly, thermal stability was determined by pre-incubating the enzyme at optimal pH and selected temperatures (20-65 °C) at increments of 10 °C for 24 h. Reaction samples were

taken periodically, and the residual laccase activity was measured at the corresponding temperature and under standard conditions previously detailed.

3.3 Batch-mode study and parametric optimization

Method development involves optimization of various experimental parameters especially for real matrix applications. For this purpose, variations in solution pH on degradation, initial pollutant concentration, contact time and optimum amount of enzyme required for maximum degradation efficiency were all considered for optimization. The experimental conditions for the remediation of triazines are listed below.

After the reaction time has elapsed, agitation was stopped, then the enzymatic reaction was stopped by addition of 0.1 mol L^{-1} HCl solution. Following which the solutions were filtered using 0.22 µm filters. Following which, the supernatants were analyzed for residual triazines using high liquid-performance chromatography (HPLC). Instrument details and conditions employed for the chromatographic separations and/or quantification are outlined in **Section 3.4.7** and more comprehensively on the published paper in **Chapter 4.4**. All experiments were conducted in duplicates, samples were analyzed twice, and statistical tools (i.e. Microsoft Excel) were employed to compute the mean values and standard deviations.

3.4 Removal of triazines by Novoprime® base 268 laccase

3.4.1 Influence of solution pH on the biodegradation of triazines

Laccase-catalyzed removal of triazines by Novoprime® base 268 was evaluated in batch mode. The experiments were conducted in 50 mL Eppendorf tubes under continuous orbital shaking (150 rpm) at ambient temperature (23 ± 5ºC). The reaction medium (20 mL) consisted of 50 mg laccase and an initial concentration of the triazine solution (5 mg L^{-1}). To assess the solution pH effect on the degradation of triazines experiments were carried out at varying solution pH (pH 1, 3, 5, 7 and 10) in 50mM CPB in the absence of mediators. Corresponding

controls without the laccase were analyzed in parallel and demonstrated that bioremediation of the compounds only occurs in the presence of enzyme.

Removal yields (%) were calculated based on the residual concentration at each time point compared to the initial concentration

3.4.2 Effect of pollutant concentration

The effect of initial solution concentration on the biotransformation of triazines was investigated by agitating (150 rpm) the triazine solutions in the presence of laccase (50 mg) at ambient temperature (25 ± 5°C) overnight. The initial solution pH was adjusted to 4.5 and the range of pollutant concentration studied was 1, 3, 5, 10 and 20 mg L^{-1}. The solution volumes were fixed at 20 mL.

3.4.3 Effect of laccase dosage on triazines degradation

Different amounts of laccase (10, 20, 30, 40, and 50 mg) were shaken (150 rpm) in separate sample vials containing 20 mL aliquots of 5 mg L^{-1} of the triazine herbicide at 25 °C. The solution pH was kept at 4.5 in a CPB, and this was chosen based on the from the pH optimization studies, where the enzyme showed maximum degradation efficiency. The residual triazine concentrations were quantified using HPLC.

3.4.4 Effect of contact time on triazines removal

To assess the influence of varying contact time on the degradation of selected triazines by laccase, experiments were carried out at room temperature (23 ± 5°C) and the solution pH was adjusted to 4.5. The time intervals studied were 15, 60, 180, 480 and 1440 min. The solution volume was kept at 20 mL with the enzyme mass of 50 mg. After 24 hrs of agitation, analysis was conducted as described earlier.

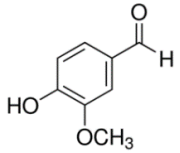
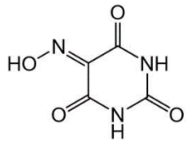
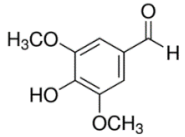
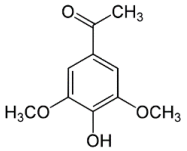
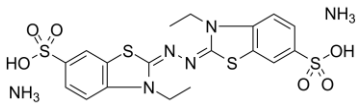
3.4.5 Screening of redox mediators for triazines removal by mediated system

A range of natural and synthetic redox mediators (analytical grade, >98% purity, Sigma Aldrich, South Africa), namely violuric acid (VA), vanillin [4-hydroxy-3-

methoxybenzaldehyde, (VAN)], acetosyringone [3,5-dimethoxy-4-hydroxyacetophenone (ASR)], syringealdehyde [2,2,6,6-tetramethylpiperidin-1-yl, (SRA)] and 2,2′azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), were compared and evaluated for their effect at aiding the degradation of the target contaminants. A detailed outline of the physicochemical properties of these mediators is given in **Table 3.1**. The oxidation mechanisms are denoted as HAT (hydrogen atom transfer) or ET (electron transfer). Experiments were analogous to those of triazine removal as a multicomponent system: ATZ, AMTR, SMZ, PMT and TERB.

The sample vials consisting of enzyme beads were filled with aliquots of the working solution (20 mL, 5 mg L^{-1}, pH 4) containing the five herbicides. Each mediator (1.0 mM) was added to the pollutant solutions separately. Control samples included simulated triazine solution in Milli-Q water and laccase without mediator (s). Reaction vials were sealed and incubated at room temperature (23 ± 5 °C) on a rotary shaker at 150 rpm for 24 hrs. After the reaction, final triazine concentrations in solution were measured using the LCMS-TOF. All tests were conducted in duplicate, and results were reported as average.

Table 3.1: Physico-chemical properties of selected redox mediators.

Redox mediator	Chemical structure	Generated radical species	Type of mediator	Oxidation mechanism	Nature of mediator
Vanillin (**VAN**)		Phenoxyl $C_6H_5O^{\bullet}$	$C_6H_4(OH)(OCH_3)$	HAT	Natural
Violuric acid (**VA**)		Aminoxyl -N-O$^{\bullet}$	N-OH	HAT	Synthetic
Syringaldehyde (**SRA**)		Phenoxyl $C_6H_5O^{\bullet}$	$C_6H_4(OH)(OCH_3)$	HAT	Natural
Acetosyringone (**ASR**)		Phenoxyl $C_6H_5O^{\bullet}$	$C_6H_4(OH)(OCH_3)$	HAT	Natural
ABTS		ABTS+$^{\bullet}$, ABTS++	ABTS	ET	Synthetic

3.4.6 Effect of varying mediator concentrations on triazines degradation

Mediators that exhibited the greatest results in triazine removal in the screening experiments were selected to evaluate the effect of varying mediator concentration on the mediated degradation of the triazine herbicides (0.05, 0.1, 0.25, 0.5, and 1.0 mM). These tests were conducted in same manner as described above.

3.4.7 Real water application

The effectiveness of the biocatalytic system in the presence of matrix components was evaluated using real water samples collected from the Crocodile River, South Africa. This river originates from Constantia Kloof in Gauteng province, flows through the Witwatersrand Mountain range, and merges with the Jukskei River to the north. It passes downstream through the Hartbeespoort area, which is popular for recreational fishing. The river samples' physicochemical properties were recorded (as described in the published paper, refer to Chapter 4.4), then filtered to remove suspended particulates and quantified for the initial concentrations of triazines using the LC-MS-TOF. Following which the samples were spiked, and the pH was adjusted to the optimal range for laccase activity.

Degradation experiments were conducted as described in previous sections and analysis was performed using a Thermo Scientific Dionex Ultimate 3000 high-performance liquid chromatography (HPLC) system (ThermoFisher Scientific, Waltham, MA, USA), equipped with an HPG-3200RS pump and a WPS-3000TRS autosampler. The HPLC was connected to a Bruker Daltonics compact quadrupole time-of-flight mass spectrometer system (Billerica, MA, USA). Data collection and processing were done using Bruker Compass Data Analysis version 4.3, with chromatographic elution carried out in positive ionization mode using formic acid (FA) in two solvent compositions: (H_2O + 0.1% FA) and (ACN + 0.1% FA).

3.4.8 Storage stability

Storage stability tests were assessed by re-incubating the used enzyme samples in CPB (0.5 mM, pH 4.5). The samples were used to determine the residual enzymatic activity measured in five-day intervals up to a period of twenty days, under standard conditions i.e., 20 mL of 5 mg L^{-1} triazines (pH 4.5) added to 50 mg laccase dosage at room temperature (i.e. 23 ± 5°C) over 24 hours.

3.4.9 Reusability/recyclability experiments

Following the degradation studies, the enzyme particles were kept at 4 °C, and in the dark for up to 20 days. During this period laccase was washed with CPB (0.05 M, pH 4.5) to remove any residual substrates or products. Thereafter, the cleaned enzyme beads were added again to a fresh reaction solution triazines degradation was repeated as per previous steps. The regeneration was carried out in six consecutive cycles.

3.5 Membrane materials

Commercial polyethersulfone (PES) hollow-fibre membranes used were kindly provided by Inge®, BASF, Greifenberg, Germany). The multibore membrane operates on an in-to-out filtration and consisting of seven capillaries per fibre with an inside diameter of 0.9 mm and pore size of 0.02 µm shown in **Figure 3.2**.

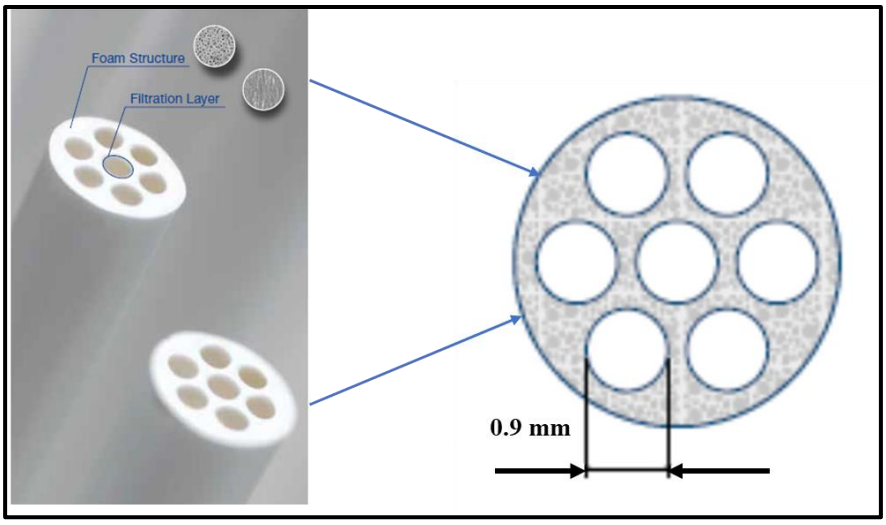


Figure 3.2: Illustration of PES multi-bore membrane material used in the study.

3.5.1 Membrane treatment experimental set-up

In this study, we employed continuous fixed-bed flow mode as a treatment technique, preceded by filtration. The experimental configuration consisted of a glass column with dimensions: internal diameter of 3 cm and a height of 50 cm. PES hollow-fiber membranes were packed into the system and supported on glass-beads following which distilled water was pumped through the column to displace the air trapped between the packing materials. After column stabilization, the contaminant effluent was continuously percolated downstream through the column in different reaction cycles and a constant flow rate using a peristatic pump (Masterflex, Vernon Hills, IL, USA). The column was operated continuously for 24 hrs and duplicate samples of the treated effluent were collected from the PES column at predefined intervals to assess the efficacy of membrane-mediated triazines removal.

The PES-packed bed system was studied by optimizing experimental parameters outlined below:

3.5.2 Continuous flow-mode study and parametric optimization of triazines removal using PES membranes

The process parameters were optimized by varying one variable at a time while maintaining all other factors constant. In the optimization experiments the process parameters were investigated by varying pH levels (at 1, 3, 5, 7, and 10), influent concentrations of triazines (ranging from 1 to 20 mg L^{-1}), and column operational time (1, 2, 4, 8 and 24 h). The membrane beds were also varied according to different masses: 0.15, 0.32, 0.65, 1.26 and 2.35 g for the chosen bed heights 10.875, 1.75, 3.5 3.5, 7.0 and 14.0, respectively. The wastewater influent flow rate was maintained constant at 2.0 mL/min throughout the study and for each of the tested parameters, 1L of the influent solution was fed into the column, varying each parameter at a time. All other operational aspects, such as sample handling and analysis, were identical to those detailed in previous batch experiments. Removal efficiency by the membrane was calculated using Eq.3 in Section 4.4.

3.5.3 Biocatalytic degradation of triazines: continuous mode packed-bed column and cost estimation study.

In order to optimize the biodegradation process parameters, the biocatalytic column was operated in continuous mode under optimum operating conditions obtained from the batch mode enzymatic and membrane studies. Relevant information on the concentration-time profiles of a fixed-bed column *viz*. sorption mechanisms, kinetic constants and adsorption capacities were predicted by fitting experimental data onto breakthrough curves model. Further details on the performance of the biocatalyst, procedures employed and cost-analysis for potential large-scale application of the system are presented in the published paper given in Chapter 4.

CHAPTER 4 RESULTS AND DISCUSSIONS

This chapter presents the findings of the research and interprets their significance with a systematic display of results using tables, graphs, and figures to provide a clear and concise overview of the data collected. Each section of results is followed by a discussion that contextualizes the findings within the broader field of study, comparing them with previous research. Two of the of the subsections in this chapter outline comprehensive analyses of separate process optimization for the enzymatic and membrane treatments while the final section is presented as a published article with referencing style detailed by the journal.

4.1 Biochemical characterization of the biocatalyst

4.1.1 Determination of enzymatic activity

Figure 4.1(a) illustrates the results for the effect of different pH levels (1.0-10) on the enzymatic activity of Novoprime Base 268 laccase. It was observed that the enzyme exhibited maximum activity at pH 5.0 corresponding to 79.7 % relative activity, indicating the optimal pH for laccase function with ABTS as the substrate. The enzymatic activity was relatively stable with sustained increments at mildly acidic pH (pH 3.0 - 5.0), however with a significant lower activity was notable activity at pH levels lower than pH 4 and higher than pH 6. This may be attributable to possible denaturation or conformational transitions in the enzyme at extremely acidic or basic conditions, reducing its catalytic efficiency (Xu, 1997; Alneyadi et al., 2018; Singh et al., 2023).

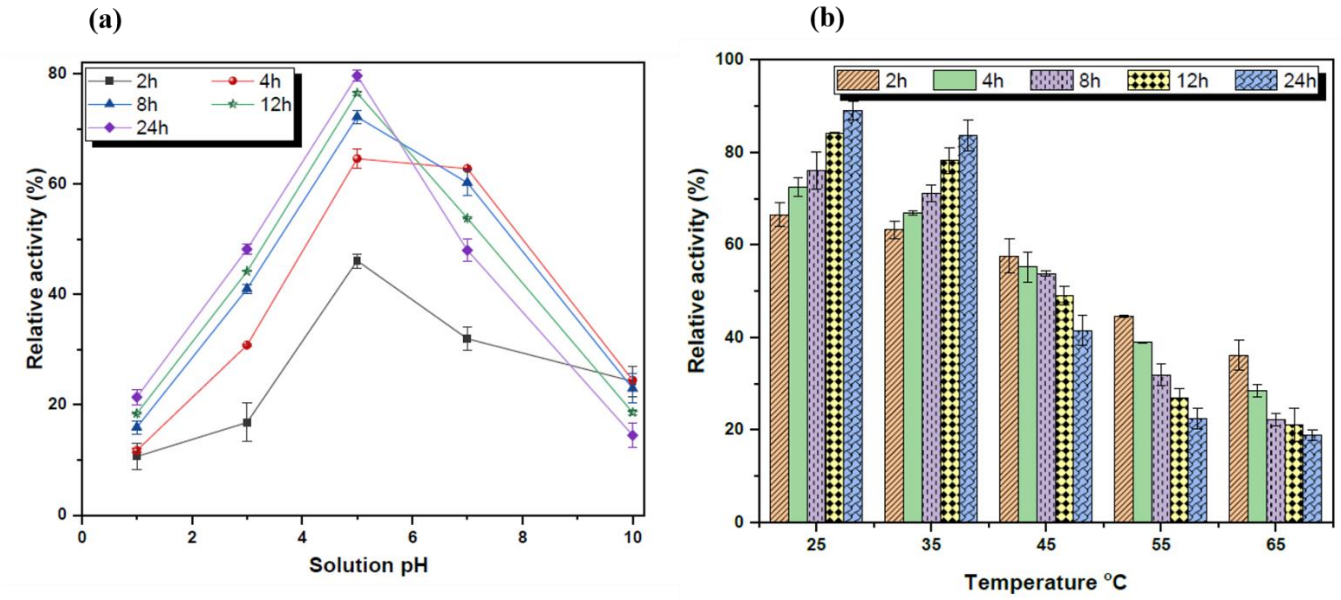


Figure 4.1: Effect of (a) solution pH and (b) temperature profile on optimum enzymatic activity of laccase.

The findings suggests that Novoprime Base 268 laccase is stable and highly active in moderate acidic conditions, which is typical for laccases. This stability is crucial for applications in environments where pH can fluctuate, such as in wastewater treatment or bioremediation.

The effect of temperature on laccase activity was assessed at different temperature profiles 25, 35, 45, 55, and 65°C at initial ABTS concentration of 0.05 mM and pH 5.0. Findings showed a general decrease in catalytic activity with an increase in temperature from 25 to 65 °C (**Figure 4.1b**). The enzyme exhibited high activity at 25 °C (88.9 %) and 24 hr incubation time, which was almost stable and maintained until 35 °C. The enzyme showed optimal activity at 25°C with relative substrate conversion of 89.4 %. Low temperatures are known to maximize the enzyme and substrate's kinetic energies, promoting effective collisions and catalytic conversions (Peterson et al., 2007; Daniel and Danson, 2013; Mehandia et al., 2020). The calculated enzyme activities notably dropped from 41.4 % to 18.8 % with a further increase in temperature from 55-65 °C. The diminished activity may be ascribed to the possible loss of the enzyme's functional conformation and denaturation arising from disruptions in the amino acid residues critical for catalysis (Othman and Wollenberger, 2020; Pekgenc et al., 2022).

4.2 Laccase-catalyzed degradation of triazines in water

4.2.1 Influence of solution pH

Enzymatic catalysis of triazines is dependent on solution pH, which affects substrate ionization, enzyme activity thus the catalytic efficiency. **Figure 4.2** shows the investigation of varying solution pH on the removal of the selected triazine compounds at pH 1, 3, 5, 7 and 10. Findings revealed general increase in degradation efficiency from pH 1-5 for all the studied triazines which slightly decreased at pH 7 and followed by a sharp decline at pH 10. Beyond neutral pH, OH^- ions binds and induces inhibitory effects on the type II Cu leading to disruptions of the internal electron transfer from T1 to T2/T3 copper active sites thus lowering the removal efficiency (Lassouane et al., 2019).

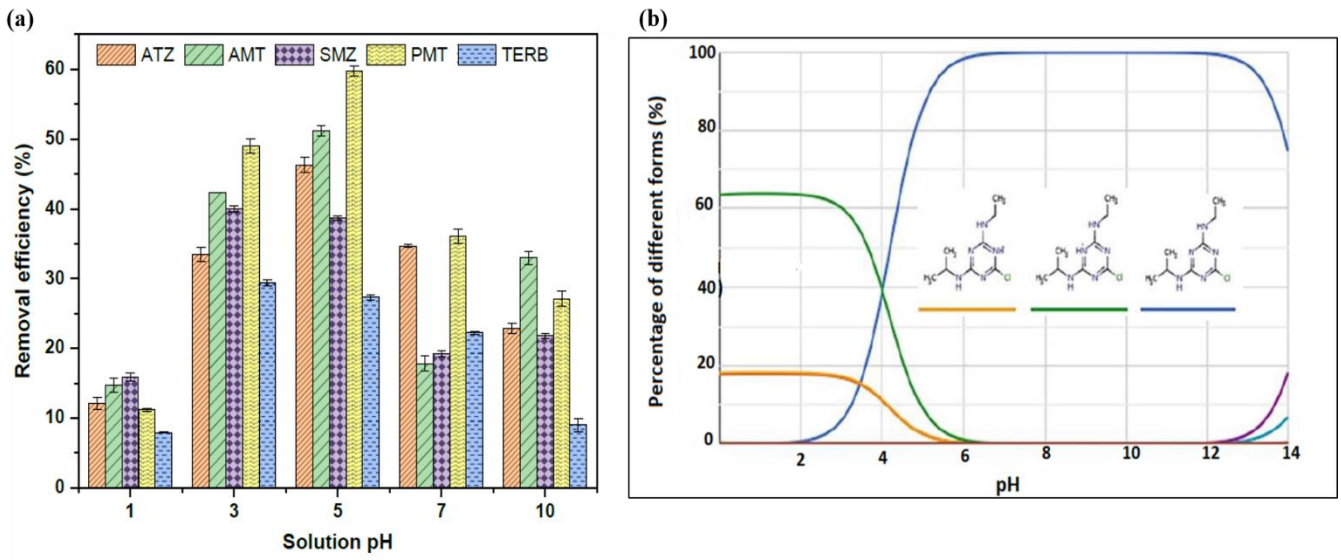


Figure 4.2: (a) Effect of solution pH on removal of triazines using laccase and (b) speciation zones diagram of predominant ATZ species with varying pH.

The general trend observed in terms of highest degradation efficiency was observed at pH 5.0 with 59.7 % and 51.1% for PMT and AMT, respectively. These triazines contain methylthio groups which are more susceptible to laccase oxidation followed by ATZ (46.3 %) and SMZ 38.6 %) with ethylamino moieties that undergo partial protonation at pH 4-6, enhancing binding with laccase active site. On the other hand, a considerably lower catalytic efficiency (27.3%) was observed for TERB affected by steric hindrance from the tert-butylamino group. It is important to note that each triazine exhibits distinct behaviour based on factors such as ionic size, functional moieties, structure and subsequent binding affinity of the compounds to the enzyme's active site under different pH activity (Baldrian, 2006; Giardina and Sannia, 2015). In addition, the enzyme proved to retain high activity at pH 5.0 which afforded optimal catalytic degradation suggesting that both the enzyme and triazines are in favourable ionic forms (Si et al., 2021). Therefore, all successive studies were investigated in CPB solution maintained at pH 5.0.

4.2.2 Effect of enzyme dosage

Figure 4.3 depicts the results for the effect of different enzyme mass was studied over a range of 10 - 50 mg at room temperature (23 ± 5°C), and a solution pH of 5.0 (20 mL, 5.0 mg L^{-1}). As shown, the catalytic conversion of triazine herbicides using Novoprime Base 268 laccase improved with higher dosages. The efficiencies increased from 24.0 – 56.3% (ATZ), 22.9 -41.6 % (SMZ) 22.7 – 59.2 % (AMT), 25.2 – 59.1 % (PMT) and 12.9 – 40.0 % (TERB) as the dosage was increased from 10 to 50 mg. With the graph showing an upward trend the maximum degradation was observed to be 56.3, 41.7, 59.2, 40.0 and 59.1 % using the highest enzyme dosage used (50 mg) for ATZ, SMZ, AMT, TERB and PMT, respectively.

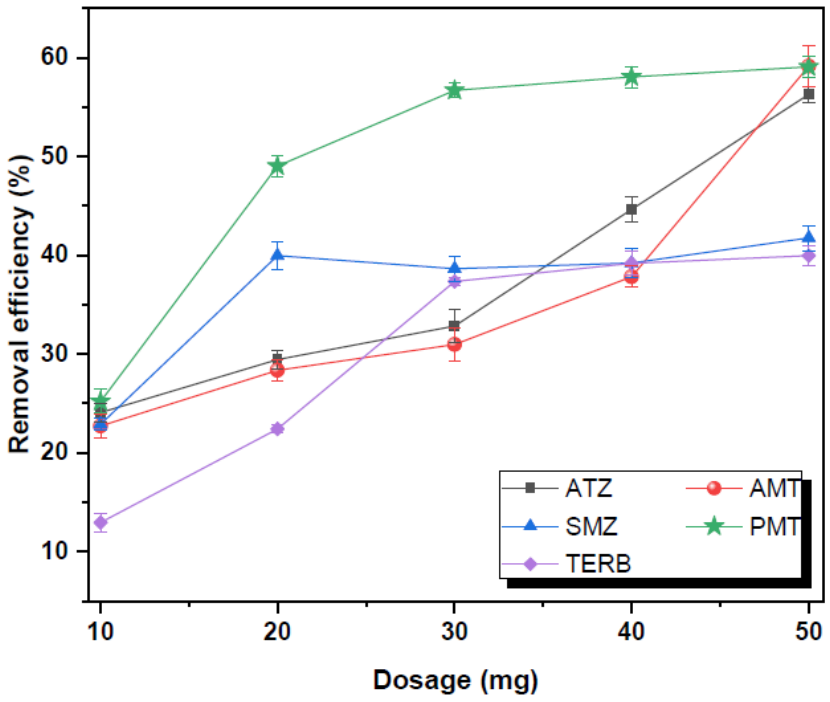


Figure 4.3: Effect of enzyme dose on the removal of triazine herbicides (5.0 mg L^{-1} solution of pH5.0, at 24hr and 150 rpm agitation).

This implies that higher dosages could potentially achieve greater degradation efficiencies, especially for compounds like TERB that have bulky moieties and may need more active sites to break down efficiently (Torres-Duarte et al., 2009; Kudanga et al., 2017; Pileggi et al., 2020).

Despite this fact, determining the optimal mass does not only afford maximum efficiencies but it's also critical in avoiding high operational costs without proportional benefits.

4.2.3 Effect of contact time

The influence of contact time on the degradation of triazine herbicides was studied over a 24h period under continuous agitation (150 rpm), at pH 5.0 and enzyme dose of 50 mg. Sample vials containing the reaction mixture were removed from the orbital shaker at predefined time intervals and the residual triazine concentrations were determined (**Figure 4.4**). Studies conducted at different contact times (30 min, 1h, 2h, 4h, 6h, 8h and 24h) revealed a trend of increasing degradation efficiency with longer contact times.

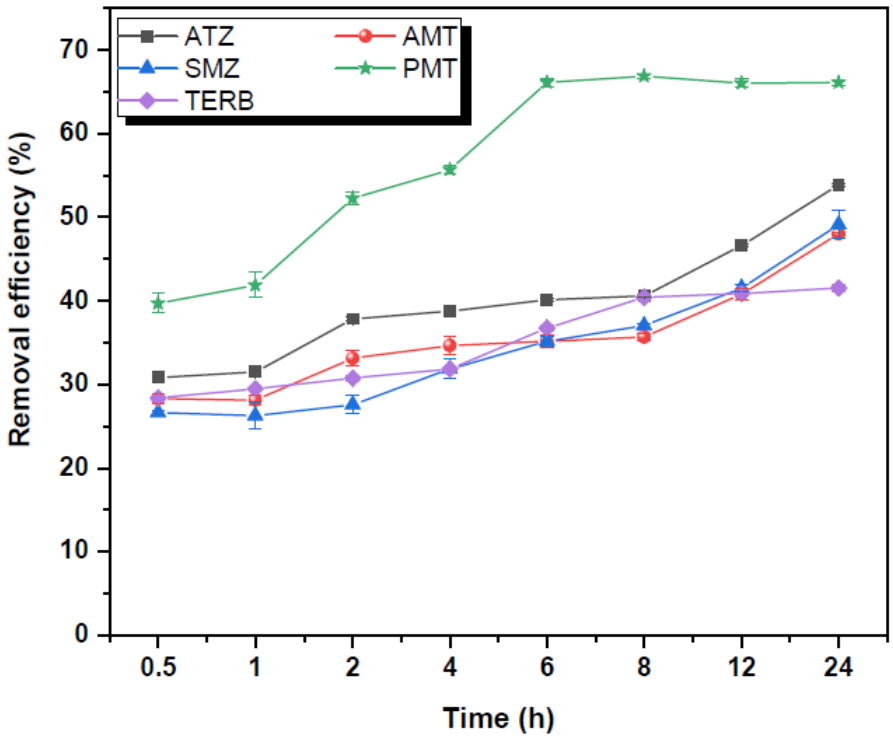


Figure 4.4: Effect of contact time on the removal of atrazine, ametryn, simazine, prometon and terbuthylazine using laccase.

From the results, it is evident that the removal rate was initially high in the first 30 min reaching 39.7 (PMT), 30.8 % (ATZ), and 36.6 % (SMZ) then gradually slows as the reaction progresses to 4h. Extending the contact time from 12 h to 24 hr, continued to enhance degradation efficiency

retaining above 66.0 % (12h), for PMT which is sustained till 24h. This thus, reflecting plateaued efficiency. The highest removal was recorded at 24h for all triazines and this may be accredited to prolonged enzyme-substrate interaction, allowing binding of more triazine molecules on the active sites of the laccase thus confirming that the enzyme's catalytic function was sustained over the study period (Asif et al., 2018; Si et al., 2021).

4.2.4 Effect of solution temperature

Understanding the temperature-dependent behaviour of laccase is essential for optimization of water treatment processes. For this study, experiments were conducted across a thermal profile of 25 to 65 °C (**Figure 4.5**). The results demonstrate that the optimal bioconversion of triazines occurred at 25°C with a maximum efficiency of 69.0, 66.6, 62.6, 59.2 and 58.9 % for SMZ, ATZ, AMT, PMT and TERB, correspondingly. The removal percentage gradually decreased with ~3 % at 35°C and a further 7 % at 45°C.

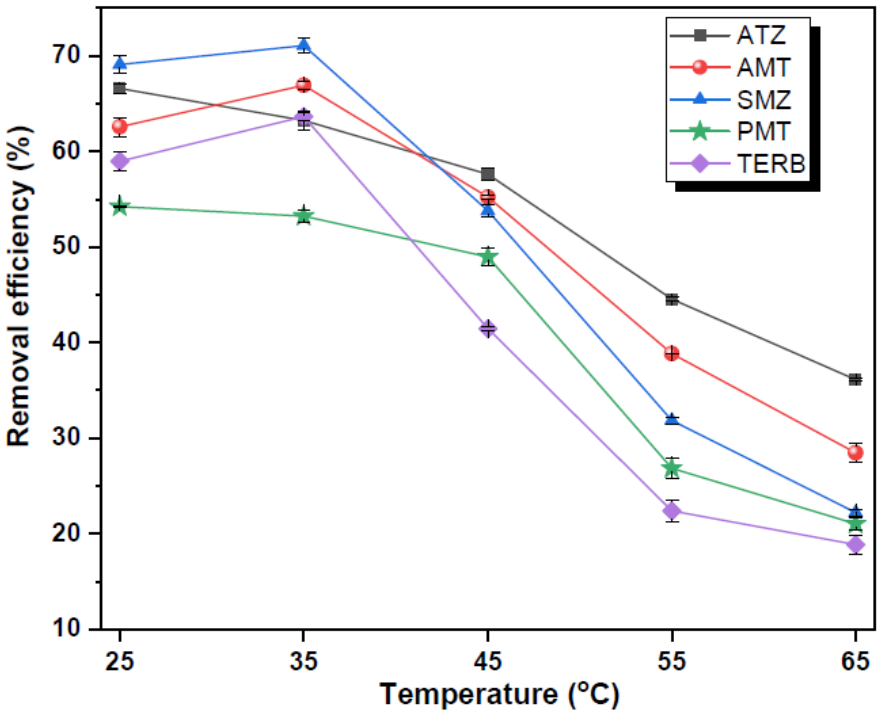


Figure 4.5: Effect of solution temperature on % degradation of triazine herbicides by laccase.

Beyond 55 °C, a sharp decline in biocatalytic efficiency until 65°C was observed following the trend TERB < PMT < SMZ < AMT < ATZ in terms of removal. This is usually ascribed to the increased rate of thermal inactivation outweighing the reaction rate, resulting in enzyme denaturation above 55°C (Peterson et al., 2007; Zeng et al. 2017).

Moreover, elevated temperatures are reported to compromise the structural stability of enzymes leading to disruptions and dysfunction of the active sites thus a marked decrease in degradation efficiency (Yousefi-Ahmadipour et al., 2016). Therefore, it may be inferred that between 25 and 45 °C, the enzyme structure remained relatively stable allowing effective functions of the active sites. This is reflected in the activity assay studies which showed a drastic reduction in specific activity at 55 and 65 °C, corroborating the observed decline in bioconversion.

4.2.5 Effect of initial solution concentration

Figure 4.6 (a) displays the results obtained for the experiments carried out to evaluate the effect of varying pollutant concentration across the range of 1 – 5.0 mg L^{-1}. In the same study, different concentrations were used in estimating the enzymatic kinetic parameters in a competitive triazines degradation through a double-reciprocal of the Lineweaver-Burk plot **Figure 4.6 (b)**. Findings revealed a substantial increase in catalytic efficiency from 1.0 to 3.0 mg L^{-1} followed by a slight decrease at higher concentration ranges (4.0- 5.0 mg L^{-1}). Theoretically, a rise in concentration increases the availability of substrate molecules, which enhances the likelihood of enzyme-substrate collisions until a plateau is attained (Zheng et al., 2022).

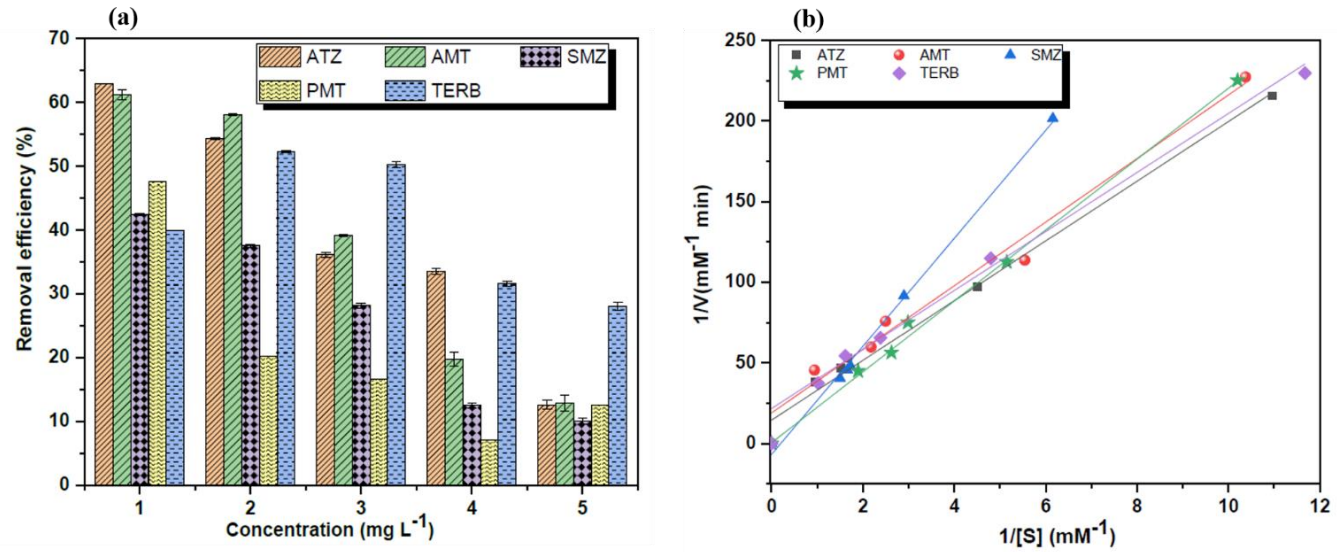


Figure 4.6: (a) Effect of solution concentration on degradation of thiazine herbicides and (b) Lineweaver-Burk plots for determination of kinetic coefficients.

Within this range, 1.0 mg L^{-1} was identified as optimal with a maximised efficiency (62.6 %) in the multicomponent thiazine system. At this concentration, the enzyme operates under conditions that balance substrate availability such that enzyme's active sites are adequately occupied, promoting effective catalysis without encountering significant substrate inhibition or mass transfer limitations.

Meanwhile at elevated substrate levels (4.0-5.0 mg L^{-1}) the efficiency reduced almost four fold for all the compounds and this may be associated with the restriction of available active sites on the enzyme promoting competition of substrate for binding, leading to substrate inhibition that further decrease the overall efficiency of the biocatalyst (Chan-Cheng et al., 2020; Si et al., 2021). This effect highlights that efficient triazine utilization at optimum level is critical for enhanced biocatalytic performance with subsequent reduction of enzyme, further lowering treatment costs.

4.2.6 Mediator screening

Substrate oxidation by laccase is generally variable and relies heavily on the presence of electron withdrawing groups (EWGs) and the difference in substrate-enzyme redox potential (Xu et al., 1996;

Asif et al., 2020). Steric shielding from EWGs hamper the electron abstraction process therefore, to fully exploit laccase's potential as a bioremediation agent, appropriate mediators are necessary. Hence this section explored laccase mediated catalysis of the selected triazines in using different redox mediators (VA, ABTS, VN, SRA, and ASR).

The experiment results in **Figure 4.7** demonstrate that regardless of the triazine compound, the presence of redox mediators improved the overall performance of the biocatalytic system. Notably, the herbicides' were most effectively degraded by laccase in the presence of VA, and this may be due to the oxidation of VA by laccase to form highly reactive aminoxyl (=N–O) radicals which induce degradation through hydrogen atom transfer (HAT) mechanism (Yang et al., 2013; Asif et al., 2017). The observation may also suggest that radical species generated by VA could potentially overcome steric hindrances and solubility factors associated with the substituents on the triazine structures.

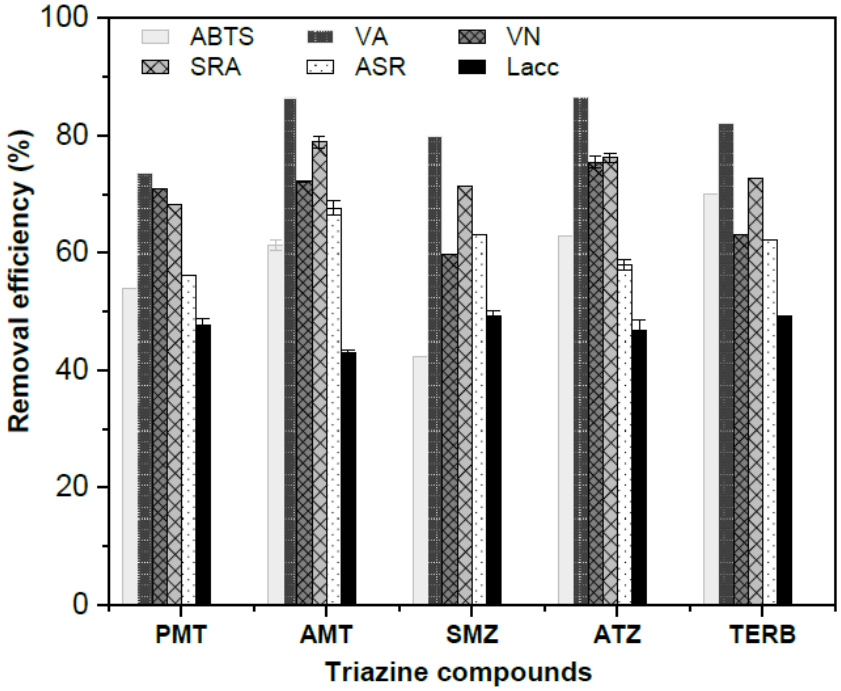


Figure 4.7: % Removal efficiency of selected triazines after treatment with laccase in the presence of separate mediators compounds at 0.5mM.

Interestingly, ABTS addition improved the herbicide degradation by a lower rate compared to VA with 40 % and 52.3 % percentage removal for simazine and prometon, respectively. These varying results indicate that optimal mediators for degrading organic contaminants are specific to each substrate. ABTS follows an electron-transfer mechanism, where an electron is obtained from the substrate instead of a hydrogen atom.

Conversely, VA utilize a hydrogen atom transfer mechanism. The relatively strong electron-withdrawing substituent in the aromatic ring of triazines, which favors hydrogen atom transfer, explains why HBT and VA are more effective mediators than ABTS for isoproturon degradation using laccase (Torres-Duarte et al., 2009; Ashe et al., 2016). Of all the tested triazines, ATZ was removed with the highest efficiency (88.9%) followed by AMT (86.3 %) violuric acid. On the other hand, triazines with EWGs (e.g. chlorine in ATZ, SMZ, and TBZ) resulted in relatively lower removal rates which may be explained by their highly stable molecular structures increasing resistance to degradation by laccase. More so, for compounds with bulky substituents like TBT, removal efficiencies are not substantial, and this is associated with inhibition and steric hinderance and induced by the tert-butylamine moiety.

Barring ABTS and VA; SRA and ASR were also able to substantially degrade the selected triazine compounds compared to a non-mediated laccase system. To avoid limitations presented by synthetic mediators, SRA was used in successive studies investigating the effect of varying mediator concentrations on the biocatalytic efficiency of the current laccase-mediated system (LMS). Moreover, though synthetic mediators (ABTS and VA) provided significant improvement in the laccase mediated system, natural (SRA, ASR and VN) mediators are derived from renewable resources presenting an environmentally benign choice also making them as sustainable options for large-scale biocatalytic treatments.

4.2.7 Effect of mediator concentrations

Figure 4.8 depicts the relationship between mediator (SRA) concentration and triazine removal. It is evident that triazine removal improved with increasing mediator concentration from 0.10 to 0.25 mM. However, the effect was insignificant with only ~ 3% improvement calculated between 0.1 - 0.25mM. Beyond this range, the increase in SRA concentration to 1.0 mM significantly increased the conversion of triazine compounds achieving over 70 % removal for AMT, ATZ, SMZ and PMT. Although lesser that the other compounds, 69.3 % of TERB, was degraded with 1.0 mM SRA after 24 h reaction time and this improved from 36.6 % in the absence of a mediator. These results are consistent with previous reports, which found limited herbicide transformation using laccase without mediators (Zeng et al., 2017).

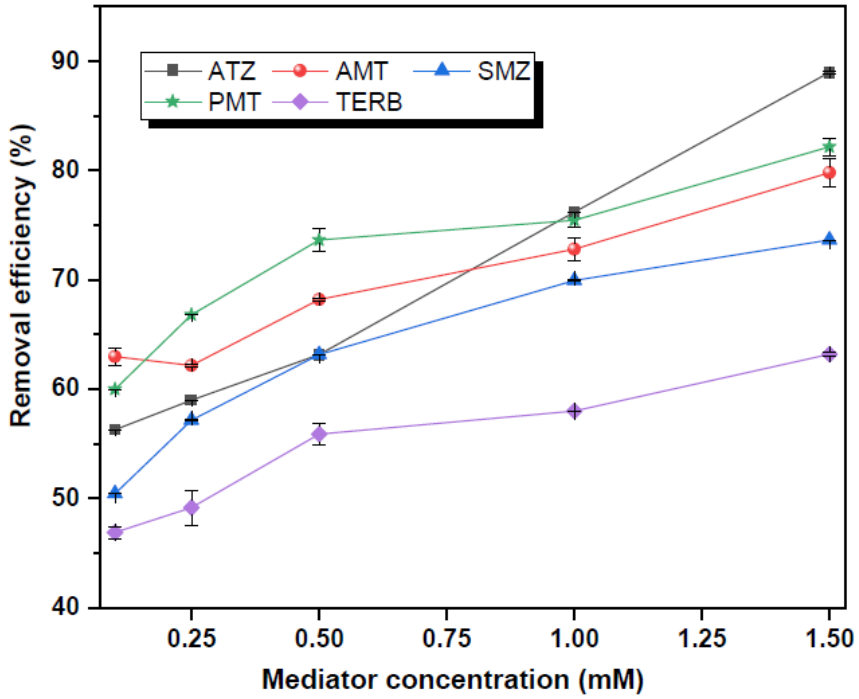


Figure 4.8: Syringaldehyde-mediated laccase degradation of triazine herbicides at different SRA concentration.

The increase in mediator concentration to 1 mM allowed the LMS to achieve the highest conversion for ATZ (82.9%) followed by PMT with 82.2 % removal, at maximum threshold concentration.

According to literature (Yang et al., 2013; Kupski et al., 2019; X. Chen et al., 2019), this threshold is influenced by factors such as the mediator-substrate type, the enzyme source and operational costs. A proposed explanation choosing 1.50 mM as the optimal point is the possible non-productive binding and/or inhibitory effects resulting from excessive mediator concentrations, thus preventing further increases in degradation efficiency (Chan-Cheng et al., 2020). This fact may also result in high operational costs from unnecessary excess mediator dosing. Overall, an improvement > 30 % in triazine degradation was achieved by Novoprime Base 268 laccase-SRA system, which could be attributed to an increase in the ORP of the reaction media in the presence of SRA. These results indicate that natural mediator SRA provided a better laccase-mediator system than the most utilized synthetic mediators (ABTS and VA).

4.3 PES membrane performance

4.3.1 Characterization of membrane materials

Scanning electron microscopy of the PES membrane is given in **Figure 4.9 (a-c)**. The image shows the overall view (a), inner (b) zoomed view and (c) outer view of the membrane. The images revealed an asymmetric structure, comprising of a porous sublayer (**Figure 4.9 a**), dense skin layer (**Figure 4.9 c**), and fully developed honeycomb-like pores at the bottom layer. These are typical structural features of pristine PES membranes. In addition, the bare PES membrane exhibited a thick skin layer and a less porous bottom layer with short, sponge-like structures.

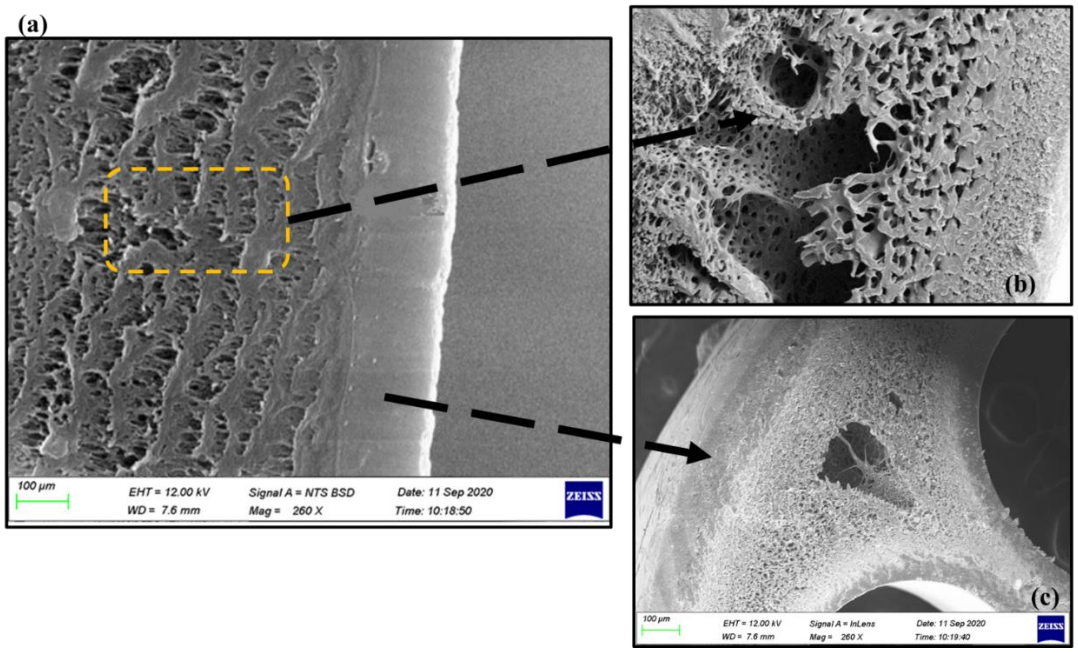


Figure 4.9: SEM image showing morphological structure of hollow-fibre PES membranes used.

4.3.2 Adsorptive removal of triazines under continuous flow-mode

4.3.2.1 Effect of solution pH

Considering that the surface properties of triazine molecules are highly influenced by solution pH, the effect was investigated across a pH range of 1.0 to 10.0 in a continuous-flow filtration process by PES membranes. Evidently, pH variation insignificantly changed the removal of triazines by PES (**Figure 4.10**). The figure illustrates that removal efficiency remained insignificantly changed from pH 1.0-3.0 retaining between 1 to 2 % increments in removal for the herbicides. It could be attributed to the predominance of protonated amine groups ($-NH_3^+$) on the triazine molecules thus, limiting PES-triazine interaction to hydrogen bonding or van der Waals forces (Romita et al.,2019).

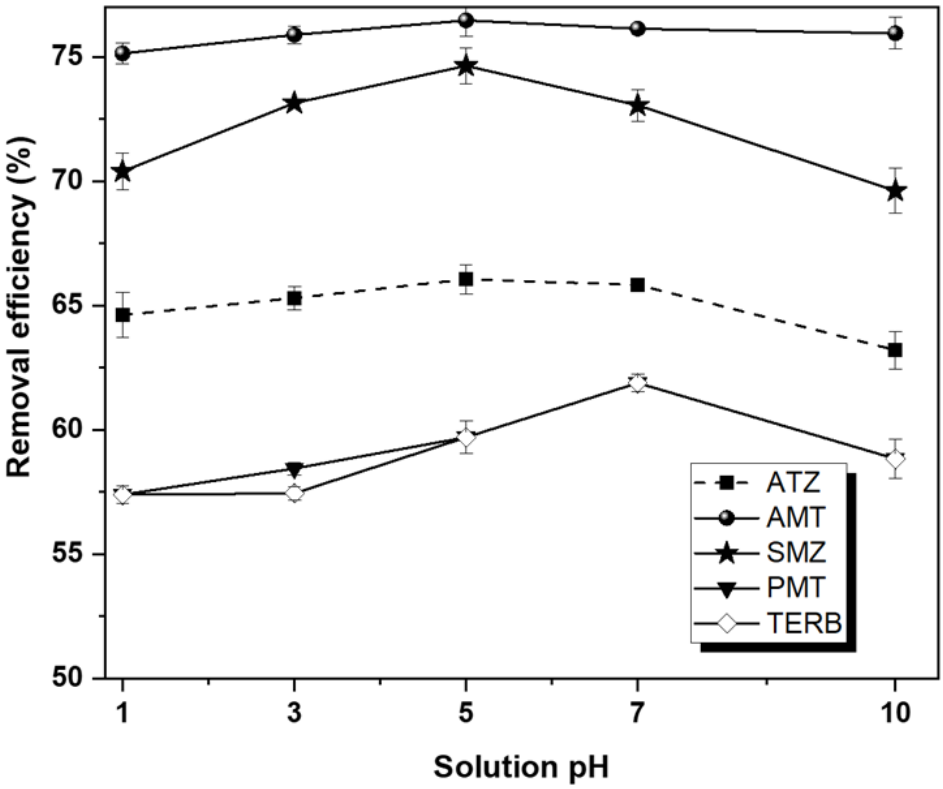


Figure 4.10: Effect of pH on % removal of triazine using PES membranes.

Triazine herbicide are generally weak bases (pka ~ 1.6 to 1.95) meaning in weakly acidic environments (pH 5.0) the triazine molecules become partially deprotonated with and exhibit polar sites, thus generating a mixture of protonated and electroneutral species; an example is given (**Figure 4.2b**) in the speciation zone diagram for ATZ chemical species at different pH values (Gong et al., 2022). This could be beneficial in facilitating more pronounced dipole-dipole moments between sulfone groups and pollutants hence a slight increase in efficiency. Similar observations have been documented (Tang et al., 2012; Hanbali et al., 2014; Gong et al., 2022).

The removal efficiency was sustained at 76.1% (AMT), 73.0% (SMZ), 65.83% (ATZ) 62.3% (PMT) and 61.8% (TERB), at neutral pH, then slightly diminished at pH 10.0. From **Table 2.1** it is indicated that triazine herbicides are hydrophobic and possess high log k_{ow} values thus their rejection

mechanism with inherently unionizable PES is predominantly governed by hydrogen bonding, dipole moments and van der Waals forces .

4.3.2.2 Variation of influent initial concentration

The performance of PES membranes at different initial solution concentrations (1.0-20 mg L^{-1}) was evaluated while keeping other parameters constant (**Figure 4.11**). Findings revealed that PES efficiently removed the triazines to between 1.0 and 5.0 mg L^{-1}, for AMT (77.1-75.3 %), ATZ (81.1-85.0 %) and SMZ (75.1-81.2 %). Contrarily, PMT (77.2-68.3 %) and TERB (65.6-59.1 %) exhibited a different trend. The case of increasing efficiency is attributed to the presence of higher membrane surface areas relative to the quantity of triazine molecules at low concentration levels which affords better efficiencies (Si et al., 2021). This means that the triazine molecules can easily access and interact with the sulfone groups on the PES membrane.

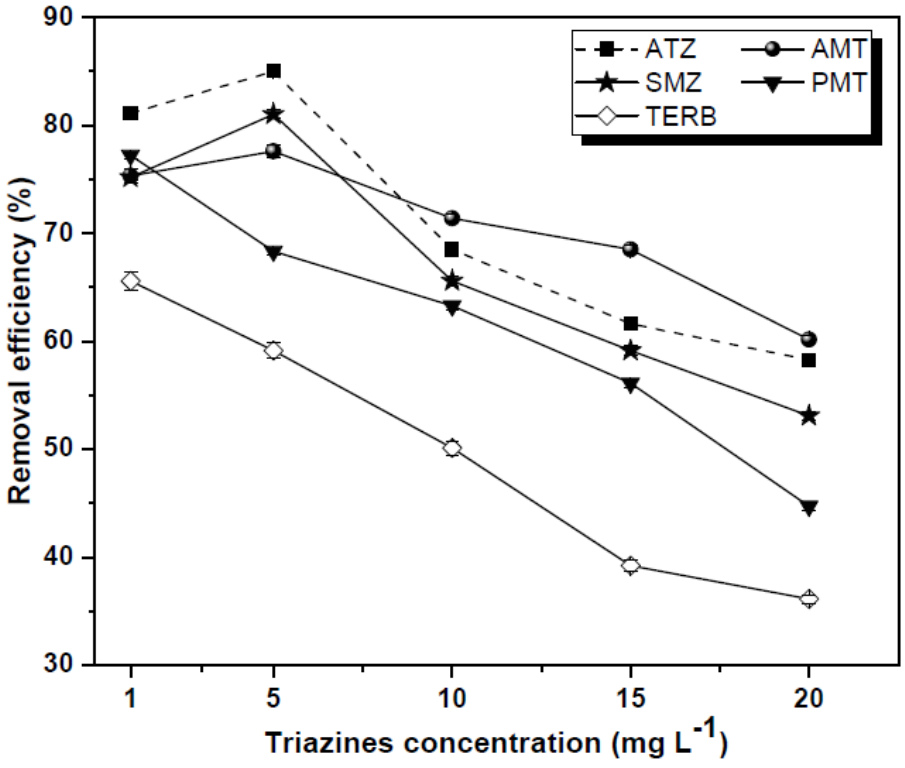


Figure 4.11: % Removal of triazines at different concentrations using PES membranes

Whereas a possible explanation for the decline may be because of the exhaustion of available PES functional sites, steric limitations and mass transfer restrictions at elevated solute concentrations, thus creating a concentration polarization effect (Mukherjee et al., 2018). Evidently, the membrane system showed better performance at 1.0 and 5 mg L^{-1}. However, since the biocatalytic experiments were optimal at 5.0 mg L^{-1}, this concentration was used in successive experiments; it was considered to be favourable for subsequent hybrid membrane biocatalytic studies.

4.3.2.3 Effect of bed mass on membrane performance

The accumulation of triazine molecules on PES generally relies on the quantity of membrane material inside the column. As depicted in **Figure 4.12**, it was seen that increasing the bed mass from 0.15 to 0.32 g gradually increased the percentage removal of the contaminants. This steadily increased four-fold from 0.65 g to 1.26 g and reaching maximum removal of 72.6% (ATZ), 75.2%(AMT), 71.4% (SMZ), 67.4% (PMT) and 68.2% (TERB) at 2.35 g, correspondingly. This phenomenon often occurs due to broadening mass transfer zone leading to a higher efficiency. In the case of low bed masses/shorter bed heights, the axial dispersion of mass transfer dominates, and tend to impede the diffusion of molecules through the membrane pores (Sivarajasekar et al., 2017). However, with a higher bed mass, the expanded mass transfer zone afforded higher percentage of triazine removal.

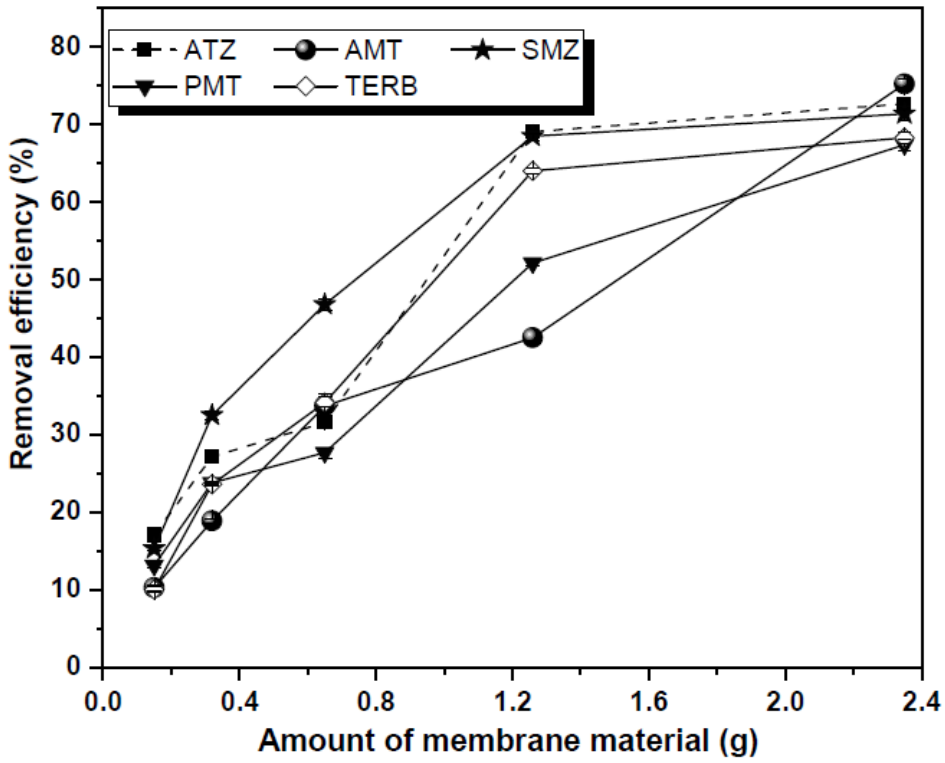


Figure 4.12: Effect of varying PES material mass on % R of triazine herbicides.

4.3.2.4 Effect of contact time

The contact time was optimized using a series of experiments carried out at 1, 2, 4, 8 and 24h time intervals while other parameters were fixed (pH 5.0, solution concentration 5 mg L^{-1} and bed mass 2.35 g). The percentage removal (%R) of ATZ, SMZ, AMT, PMT and TERB are plotted against time in **Figure 4.13**. Based on the results, the removal percentage generally increased with increasing contact time. There was rapid reaction kinetics the 1st and 4th hour of operation increasing the % R from 38.8 to 56.0 % (ATZ), 33.6 to 59.6 % for AMT, 29.7 to 53.8 (PMT), 23.5 to 59.1 % (SMZ) and 17.2 to 39.6 % (TERB) in the time interval. Then, a slower mass transfer phase is observed between 4.0 to 8h and finally, the graph continues to slightly increase at 24h. The rapid uptake at early states of operation is characteristic of enhanced surface coverage and sufficient penetration the membrane pores by the herbicides (Vitola et al., 2021). The membrane exhibited minor variations in removal

efficiency after eight hours of contact time, indicating external mass transfer limitations of the herbicide pollutants in the packed membrane column (Kundu et al., 2019).

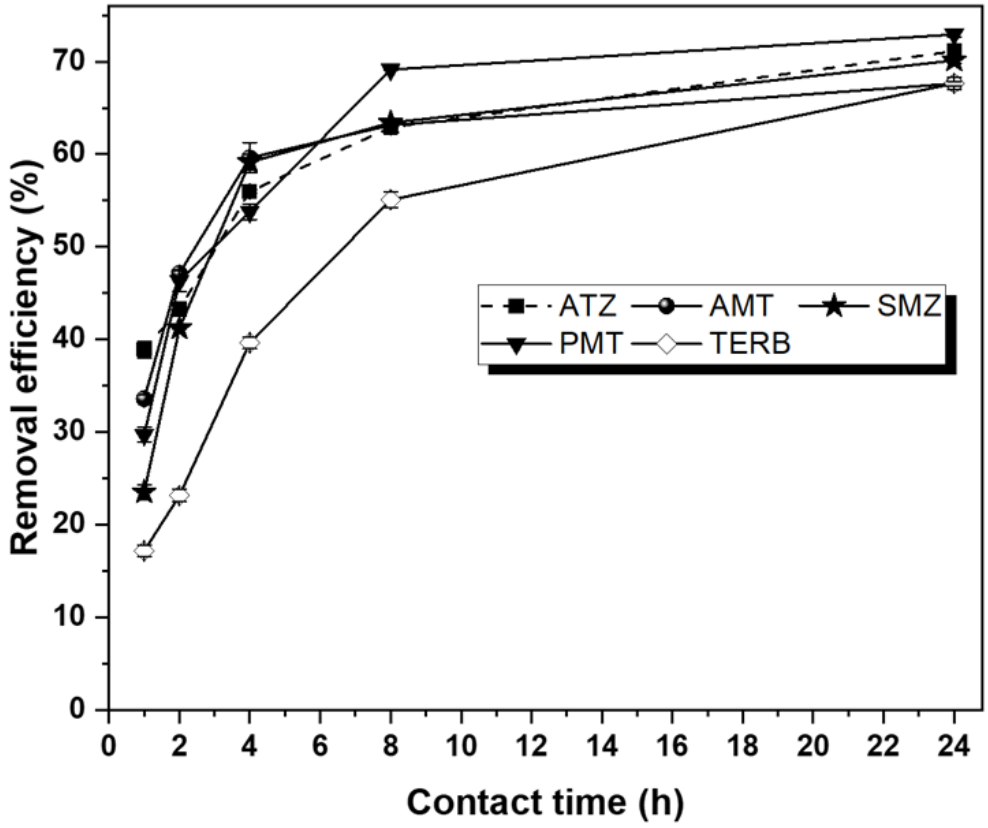


Figure 4.13: Effect of contact time on the removal of triazines using PES membranes.

The assessment of different parameters using unmodified PES membranes has shown moderate efficiency in removing triazine herbicides. Although the findings suggest that PES membranes have potential, their effectiveness is restricted when used independently. Consequently, integrated membrane biocatalytic systems, could be beneficial in developing advanced, multi-functional treatment systems.

Notably, the performance of both systems as standalone units was limited in multicomponent triazines applications, therefore the focal point was redirected by testing and implementing the biocatalytic membrane for atrazine (a prevalent s-triazine). This was done with the intention of

providing solutions in the recovery and reuse of the targeted for smallholder farming. The estimated cost of the system is given in the Appendix.

4.4 Paper

This sub-section has been published as a paper titled: **Integrated membrane packed enzymatic column for the removal of atrazine in wastewater: Experimental, kinetics, breakthrough curves analysis and applicability in real wastewater,** in the Journal of Water Process Engineering**.**

This article explored the evaluation and applicability of an integrated biocatalytic membrane system for the continuous removal of atrazine, a common triazine with an overview on real wastewater treatment and detailed cost-analysis studies.

J. Water Process Eng., 2024. 62, pp.105388, https://doi.org/10.1016/j.jwpe.2024.105388.

Integrated membrane packed enzymatic column for the removal of atrazine in wastewater: Experimental, kinetics, breakthrough curves analysis and applicability in real wastewater

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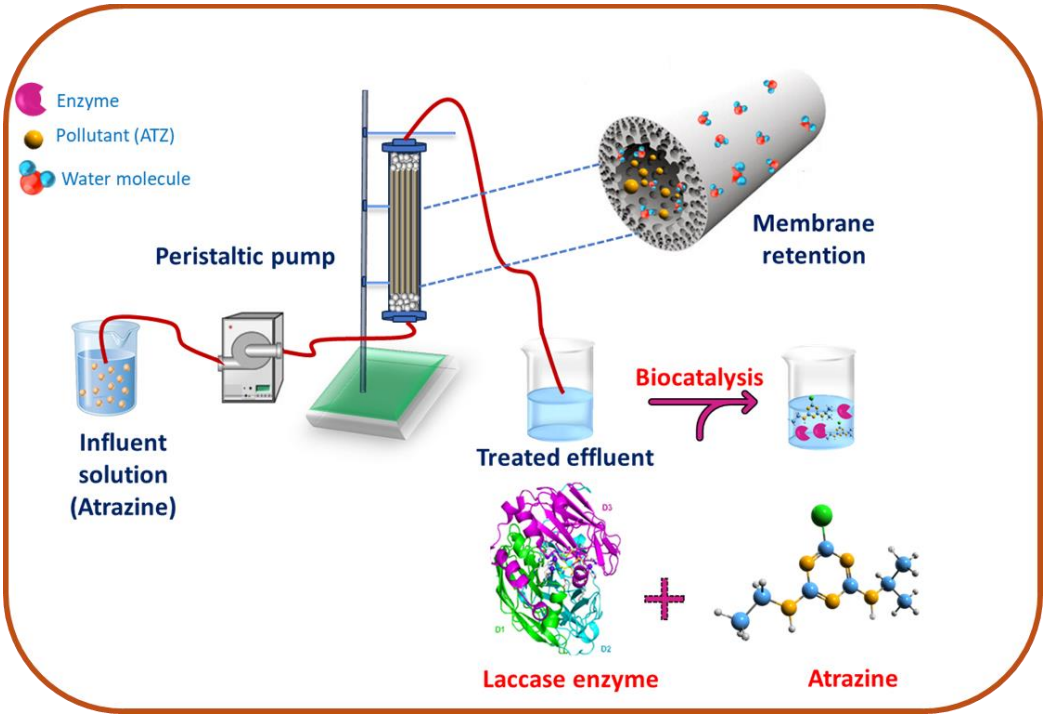
KEYWORDS*: Polyethersulfone; pesticides, wastewater, integrated processes, bioremediation*

Abstract

The current study explored the performance of a packed bed biocatalytic column equipped with polyethersulfone (PES) hollow fibre membranes and Novoprime base 268 laccase (Lac) for the biotransformation of the recalcitrant herbicide, atrazine (ATZ). Column design parameters: feed concentration (5 mg L^{-1}), flow rate (2 mL min^{-1}) and bed height (14 cm) generated optimum membrane retention (MR) in the fixed bed. A standalone MR system attained > 50 % of ATZ retention with a maximum capacity of 170.2 mg g^{-1} at bed service time of 559 min. Under optimized conditions (50 mg dosage, 5 mg L^{-1} solution concentration at 25°C) 66 % degradation efficiency was observed in the laccase-assisted conversion within 24 hrs. PES-Laccase (PES-Lac) biocatalyst improved the overall degradation efficiency to 86 %, exhibited simple regeneration and satisfactory operational reusability, retaining 46 % activity in the sixth cycle. Overall, the work demonstrated the potential of PES-Lac biocatalytic system to selectively bioremediate ATZ and provided a detailed understanding of the factors influencing its efficiency for applicability in water reclamation.

Graphical Abstract



1. Introduction

Continuous increase in agricultural productivity have intensified the environmental impacts of pesticides, is raising concerns around the world (Soledad et al., 2021; Han et al., 2023). Atrazine (ATZ) (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a selective triazine herbicide frequently used for eradicating gramineous and broadleaf weeds in essential crops (Rostami et al., 2021a). Residual amounts of ATZ permeate the environment through several non-point sources, which drive the widespread contamination of receiving water bodies, soil, and sediments (Rippy et al., 2017; C. Chen et al., 2019; Tang et al., 2023). Due to its relative stability, resistance to abiotic hydrolysis (stable at pH 5-9) and direct aqueous photolysis (Lewis et al., 2016; Alonso et al., 2018; Baran et al., 2021; Urseler et al., 2022) the ATZ's persistent environmental detection continually threatens the aquatic ecosystems and human health (He et al., 2019; Bachetti et al., 2021). Ephemeral, sub-lethal exposure of residual ATZ and its chlorinated metabolites have been

documented to disrupt key endocrine signaling pathways (Chapin et al., 1996; Wirbisky and Freeman, 2015; Cleary et al., 2019), resulting in several pathological alterations and malignancies in the reproductive system (Hayes et al., 2010; Russart and Rhen, 2016; Wirbisky and Freeman, 2017). Consequently, alternative water treatments strategies are necessitated for efficient eradication of ATZ in water sources.

Biocatalytic remediation affords an environmentally benign alternative that operates at lower energy requirements, under moderate operational conditions (Kumari et al., 2020; Morsi et al., 2020) and reduced unfavorable side reactions thus less-toxic byproducts (Zhuo and Fan, 2021). Laccase-mediated treatment for waste streams consumes ambient oxygen which greatly reduces the need for enzyme-destabilizing agents such as H_2O_2 (an oxidant required by peroxidases) [20–24]

Laccases [*p*-diphenol: dioxygen oxidoreductases (EC 1.10.3.2.)] are a class of extracellular multi-copper containing oxidoreductases catalyzing the oxidation-reduction-assisted biodegradation various substrates to less toxic derivatives (Zdarta et al., 2018; Unuofin et al., 2019; Asif et al., 2020). Lac-catalyzed degradation of ATZ promotes hydrolytic dechlorination producing hydroxyatrazine (HA) which is later converted to N-isopropylammelide or N-ethylammelide intermediates (hydrolytic deamination reactions) then finally to cyanuric acid (Rostami et al., 2021b). Another pathway proceeds through N-dealkylation of the ethyl and isopropyl moieties which generates less toxic ATZ metabolites (deethylatrazine, deethyldeisopropylatrazine and deisopropylatrazine) which are used as fuel for growth and reproduction of microorganisms [29-30].

Due to the impeded application of pristine laccases (Pekgenc et al., 2022), mitigation support onto stable carrier matrices (Zdarta et al., 2018) or coupling of enzymatic systems with polymer-based membranes (Luong N. Nguyen et al., 2014) such as Polyethersulfone (PES). PES exhibits porous,

continuously spatial structures, wide surface area, and superior thermal and chemical resilience (George et al., 2023) which have led to its integration in enzymatic membrane systems (Cen et al., 2019; Zhang et al., 2022). Extensive literature review revealed successful applications of integrated oxidoreductase systems for the remediation micropollutants including ATZ (Zdarta et al., 2018; Unuofin et al., 2019; Rostami et al., 2021a; Pekgenc et al., 2022; Triassi et al., 2022) with improved efficiencies, long term operational stability, reusability, and simple product separation (Asif et al., 2018; Bilal, Adeel, et al., 2019). However majority of the studies primarily focus on the bioconversion of pollutants in batch mode processes (Gamallo et al., 2018; Mojiri et al., 2020; Jankowska et al., 2022). For instance, three azo dyes were degraded with a reduction in the effluent's toxicity was reported in nano and ultrafiltration biosystems, enzyme deactivation and probable occlusion of active laccase sites of laccase during immobilization could not be avoided (Gamallo et al., 2018). Notwithstanding the benefits and contributions of these studies in estimating the effectiveness of such systems; findings lack accurate predictions on the dynamic nature of adsorption for the treatment of wastewater on a large scale (i.e., industrial scale) (Ronka, 2016; Patel, 2019; Saleh et al., 2020). Hence, the application of enzymatic-based separation processes in continuous systems that can be used in actual industrial settings remain scarce.

Moreover, to date, literature reports on the practical biotransformation of pesticides in real water samples remain limited. Therefore, it is essential to investigate the degradation of other emerging pollutants (i.e., triazine herbicides) at their environmentally relevant concentrations in biosystems. This will encourage more feasible developments in the comprehension of the fate of pesticides in enzymatic treatments and increase scientific understanding of their removal mechanisms thus creating an extensive database for future predictions.

Herein, our study explored a hybrid catalytically active packed-column reactor which coupled Novoprime® base 268 laccase with PES based hollow-fiber membranes for the bioremediation of atrazine in wastewater. This single unit integrated molecular retention with catalysis, two crucial components of wastewater purification.

2. Chemicals and materials

Atrazine (ATZ) was selected as a relevant pesticide pollutant based on its widespread occurrence in wastewater and various water sources. An analytical standard (Pestanal grade, > 99% purity), ATZ (6-chloro-N-ethyl-N´-(propan-2-yl)-1,3,5-triazine-2,4-diamine) was purchased from Sigma-Aldrich (Johannesburg, South Africa). Acetonitrile (ACN), (HPLC)-grade, 2,2´azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium citrate dihydrate, citric acid, sodium dihydrogen phosphate and disodium hydrogen phosphate of analytical grade were also obtained from Sigma-Aldrich (Johannesburg, South Africa). Novoprime® base 268 was supplied by Novozymes (Denmark) while commercial PES hollow-fiber membranes used were kindly provided by Inge®, BASF, Greifenberg, Germany). All solutions were prepared using ultrapure water obtained from Millipore, Direct-Q® 3UV water purification system (Merck, Germany).

3. Experimental

3.1. ATZ removal: membrane packed column studies

PES hollow fiber membranes were evaluated for the adsorption of atrazine in a fixed-bed system according to a previously reported method (Sivarajasekar et al., 2017). A cylindrical glass tube column packed with PES was fed with the influent (ATZ) at a fixed flow rate. Pre-determined volumes of effluent samples were collected consecutively at varying experimental parameters: initial analyte concentration (5 to 15 mg L^{-1}), flow rate of 2 to 6 mL min^{-1} and bed height (3.5 to 14 cm). Each parameter was varied at a time and the breakthrough curves were then constructed by plotting C_t/C_o against time.

3.2. Prediction of column performance indicators

The dynamic performance of packed-bed adsorption is influenced by experimental conditions, significantly affects mass transfer resistance and hence adsorption parameters. This is based on the permeate concentration-profile or breakthrough curves (Deokar and Mandavgane, 2015) which express the point of breakthrough from the plot of C_t/C_o over time, empty bed contact time (EBCT), the number of processed bed volumes, (BV) and the adsorbate exhaustion rate (AER) (Patel, 2019). The capacity at breakthrough (q_b) in mg g^{-1} at a given flow rate was calculated using the Eq 1. (Gupta and Garg, 2019).

$$q_b = Q.\frac{C_0}{m}\int_0^b \left(1 - \frac{C_t}{C_o}\right) dt \qquad \textbf{Eq. 1}$$

where Q represents the volumetric flow rate (mL min^{-1}), m is the PES mass (g), C_o and C_t are the concentration (mg L^{-1}) of the influent and effluent, respectively at time (t).

3.3. Breakthrough modelling

The design and industrial-scale implementation of a successful fixed-bed column is dependentant on the prediction of dynamic nature of the column. For this purpose, the well-known mathematical models i.e., Thomas, Bohart-Adams and Yoon Nelson were used to describe the lab-scale column setup. The general basis of the Thomas model involves an adsorption process which neglects internal or external diffusion restrictions (Thomas, 1944). The postulation here is that pollutant adsorption process follows the Langmuir isotherm and second-order reversible reaction kinetics without axial dispersion. Bohart-Adams model posits a surface reaction theory and suggests that equilibrium is instantaneous (Bohart and Adams, 1920; Bayat et al., 2018) while Yoon-Nelson developed a model in which it is presupposed that the probability of an adsorbate being absorbed is directly correlated with the likelihood that its concentration will exceed the breakthrough concentration on the adsorbent (Yoon and Nelson, 1984).

3.4. Enzymatic activity assay

Laccase activity was measured by observing the change in absorbance due to the oxidation of 1.0 mM ABTS recorded at 420 nm using UV-vis spectroscopy (Agilent Technologies, Cary Series 1000, USA). Known portions of laccase were agitated in a glass vials with 50 mM citrate-phosphate buffer (CPB, 5 mL, pH 5.0) followed by an addition of 1 mL of ABTS (5 mM). Enzymatic activity was computed using the molar extinction coefficient of ABTS (ε_{420} = 36,000 $M^{-1}cm^{-1}$) where one unit of laccase activity is defined as the amount of enzyme that oxidized 1µ mol of ABTS min^{-1}. This was expressed as units mg^{-1} (Asif et al., 2020).

3.4.1. Kinetic analysis of Novoprime base 268 laccase

The kinetic experiments were performed in 5.0 mL CPB (pH 5.0) consisting of laccase and ABTS concentrations in the range 0.1 to 5 mM and also at varied ATZ concentrations (1.0 to 20 mg L^{-1}). The rate of enzymatic reaction was described by estimation of the K_m and V_{max} parameters given by the Michaelis-Menten equation (Gianfreda et al., 1998), (Engasser and Horvath, 1976):

$$V = \frac{V_{max}\,[S]}{K_m + [S]} \qquad \textbf{Eq. 2}$$

where V (mM min^{-1}) and S (mM) represent the velocity of the initial reaction rate and substrate concentration, respectively. V_{max} is the maximum reaction velocity in mM min^{-1} and K_m (mM) is the Michaelis-Menten constant. V_{max} and K_{max} values were predicted according to the fitting curve of 1/[V] versus 1/[S] (Ascenzi and Amiconi, 1987; Vitola et al., 2021) following the Lineweaver-Burk plot.

3.5. Optimization of ATZ removal by laccase

Experimental parameters affecting biodegradation efficiency *viz.* initial solution pH (pH 1-7), concentration (1.0-20 mg L^{-1}), enzyme mass (10-50 mg) and reaction time (1-24 hrs) were investigated in batch mode. A high-performance liquid chromatography (BISCHOFF, Labexchange,

Deutschland) was employed for analysis using an Ascentis® C18, 25 cm x 4.6 mm, 5µm (SUPELCO®, Bellefonte, PA, USA) operated at a column temperature of 25 °C and a detection wavelength of 223 nm. A constant flow rate of 1.0 mL min^{-1} was maintained under isocratic elution with an eluent composition of acetonitrile: water (70:30, v/v). Calibration standards were prepared daily by serial dilution of the stock solution (1000 mg L^{-1}) which were kept in the dark at 4°C when not in use.

3.6. Fabrication of PES-Lac fixed-bed column

The biocatalytic remediation of ATZ was systematically determined using the PES-Lac packed bed in continuous mode. Optimum membrane retention and degradation conditions were used as described in section 3.2 and section 3.5. Removal efficiencies of ATZ by stand-alone PES system and PES-Lac treatment were determined by Eq. :

$$\% \boldsymbol{R} = \left(\frac{\boldsymbol{C_o - C_f}}{\boldsymbol{C_o}}\right) \times \mathbf{100}\% \qquad \textbf{Eq. 3}$$

where % R is the overall membrane removal and degradation efficiency (%), accordingly, C_0 and C_f are the pollutant's initial and residual concentrations (mg L^{-1}), respectively.

The applicability of the biocatalytic system in the presence of matrix components was tested using real water samples collected from the Crocodile River, South Africa. The river emerges from Constantia Kloof, in Gauteng province, and flows into the Witwatersrand Mountain range. Its confluence with the Juskei River is located to the north and the stream passes downstream Hartbeespoort area, used by residents for recreational fishing. A Thermo Scientific Dionex Ultimate 3000 high performance liquid chromatography (HPLC) system (ThermoFisher Scientific, Waltham, MA, USA) coupled to an HPG-3200RS pump and a WPS-3000TRS autosampler was utlilized for analysis. The HPLC was connected to a Bruker Daltonics Inc. compact quadrupole time-of-flight mass spectrometer system (Billerica, MA, USA). Data was collected and processed using Bruker

Compass DataAnalysis version 4.3 and chromatographic elution was conducted in a positive ionization mode using formic acid (FA) in two solvent compositions constituting of (H_2O + 0.1% FA) and (ACN +0.1% FA), respectively.

3.7. Cost analysis study

Cost estimates were developed to evaluate the economic viability of the small-scale biocatalytic system designed for domestic duty. Each process configuration was designed and sized using the previously reported data then fully costed using actual construction prices from past and recent projects. Individual cost contributions were collated to generate the overall capital expenditure (CAPEX) i.e. directs or indirect cost and operating and maintenance (OPEX) costs (fixed and variable costs) of an agricultural wastewater reclamation facility for treatment capacities of 1000 m3/day as previously detailed (Welo et al., 2023). CAPEX comprised of the equipment, instrumentation, piping, electrical and piping costs while OPEX covered the membrane and enzyme replacements, maintenance, chemicals and total energy costs (Chellam et al., 1998; Lima-ramos and Woodley, 2014; C.D, Swartz, J.G, Menge, J.A, duPlessis, G.M, 2022). The economic analysis was executed as using previous estimates described where 2, 10 and 40 % of the total equipment costs were assumed for maintenance, equipment installations and piping costs, respectively with 25 % estimated for electrical and control systems (Welo et al., 2023). Membrane suppliers were also consulted for their valuable design input.

4. Results and discussions

4.1. Effect of operating conditions on column adsorption of ATZ by PES membranes

4.1.1. Effect of initial ATZ feed concentration (C_o)

The influence of ATZ concentration (5 to 20 mg L^{-1}) on the column performance was examined at constant feed flow rate (2 mL min^{-1}) and bed height (7.0 cm). As shown in **Figure. 1**, the column BT curves became sharper as the influent concentration increased. Meanwhile, the breakthrough

time (t_b) and exhausted time (t_e) both decreased from 441 to 225 min and 730 to 550 min, respectively resulting in lower quantities of water treated.

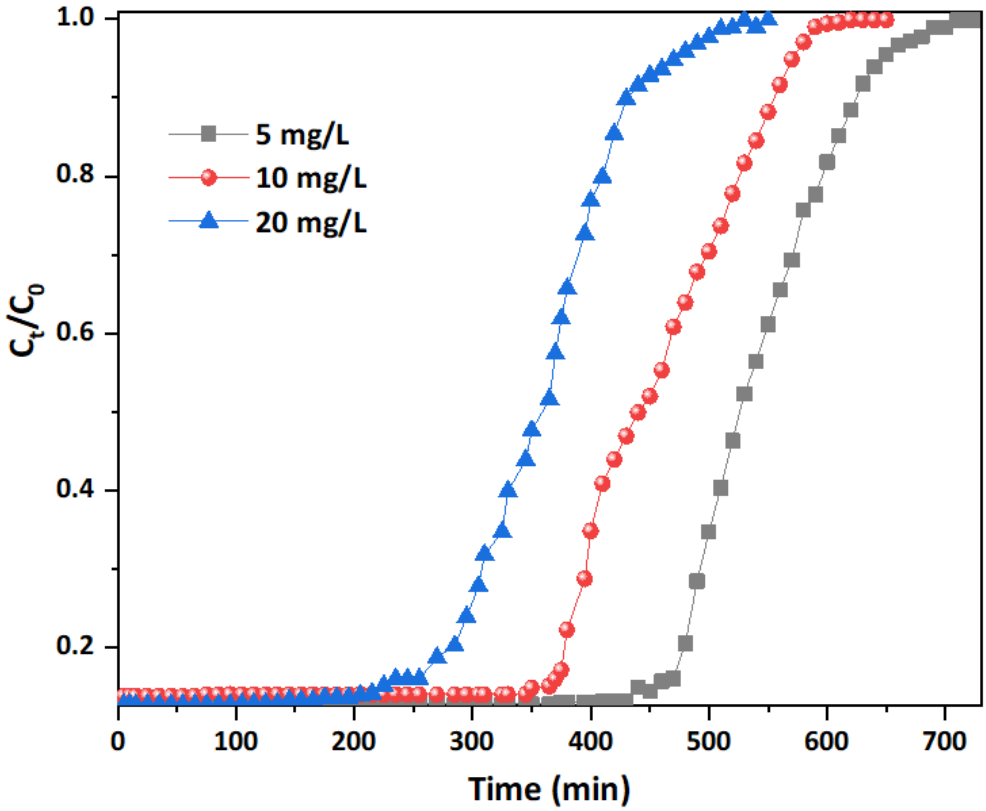


Figure. 1: Effect of influent concentration on the breakthrough curve for ATZ adsorption on PES membranes (conditions: flow rate = 2 mL min $^{-1}$, bed height = 7.0 cm).

Increasing the initial ATZ concentration prompted increased adsorption capacities (q_e) from 139.6, 129.9 and 95.68 mg g^{-1} (**Table**) corresponding to 5.0, 10 and 20 mg L^{-1}. This is attributable to accelerated concentration gradient between adsorbent and adsorbate, which provides the driving force between the solid and liquid phases and thus increases the adsorbent exhaustion rate (high ATZ loading rate onto PES binding sites) (Bilal et al., 2018). In other words, at lower influent concentrations, both the concentration gradient and the driving force between the solid and liquid phases are reduced, leading to a longer time required for PES saturation (Sivarajasekar et al., 2017). Similar trends were documented in the literature on fixed bed studies of phenol adsorption using

neem leaves (Mandal et al., 2021), diclofenac (de Franco et al., 2018) and a number of other studies (Afroze et al., 2016).

4.1.2. Effect of influent solution flow rate (Q)

Figure. 2 illustrates the effect of different feed solution flow rates (2.0, 4.0 and 6.0-mL min $^{-1}$) on the efficiency of the packed column. As the inlet flow rate rise from 2.0 to 6.0 mL min $^{-1}$, the EBCT decreased from 7.07 min to 2.34 min and the saturation time from 980 min to 405 min. This was qualified by migration of the adsorption zone leading to a gradual increase of the pollutant in the effluent until breakthrough is reached (Patel, 2019; Levio-Raiman et al., 2021).

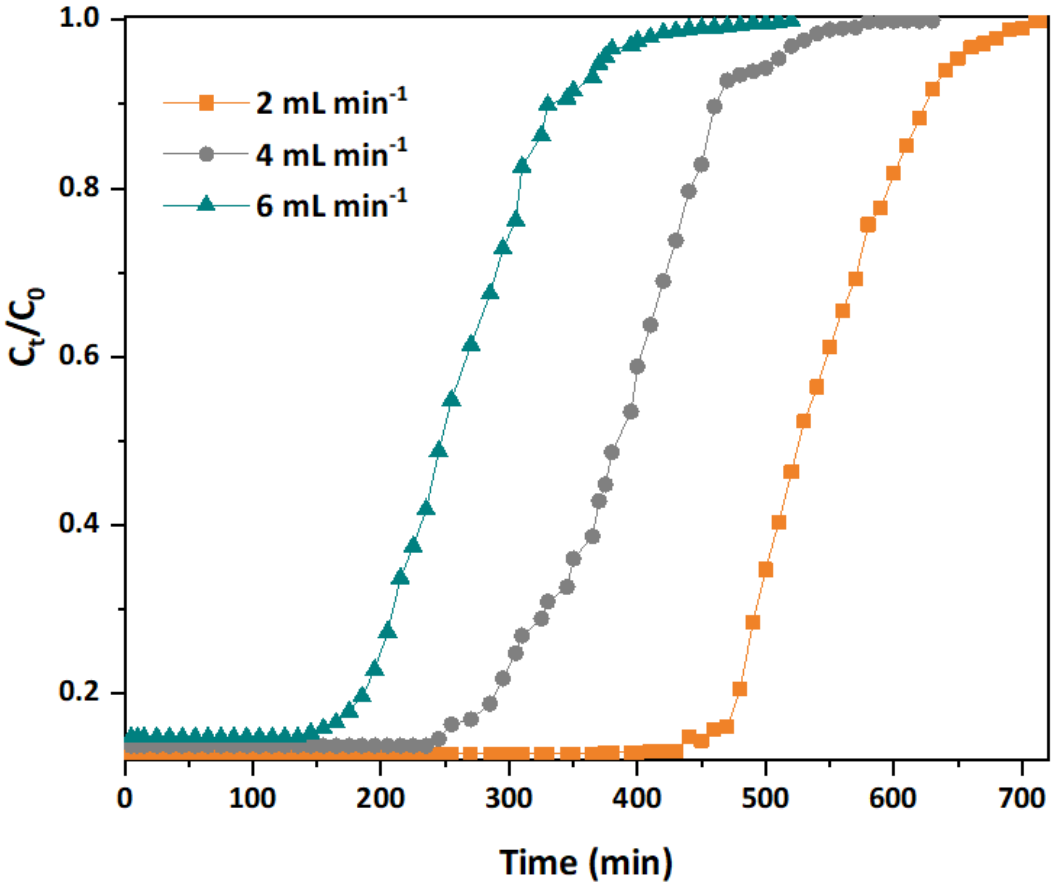


Figure. 2: Effect of inflow flow rate on the adsorption of ATZ in a packed bed column at bed height 7 cm and feed concentration of 5 mg L^{-1}.

According to Gupta and Garg (2019) (Gupta and Garg, 2019), higher volumetric flow rates tend to reduce the thickness and resistance of the external mass transfer layer while increasing the overall mass transfer coefficient and flux. Additionally, the axial dispersion and overall mass transfer coefficient rises as the inlet flow rate increases, resulting in an increase in adsorption rate and faster saturation of the fixed bed (Sivarajasekar et al., 2017). Evidently, lower feed flow rates yield higher pollutant removal. The trend agrees with other published studies on breakthrough curves at different flow rates (Deokar and Mandavgane, 2015), [70-73].

4.1.3. Effect of PES bed height (Hb)

Pollutant accumulation in a fixed bed is largely dependent on the quantity of the packing material. The column was packed with different PES bed heights (3.5 – 14 cm). For this experiment, the inlet flow rate and concentration were kept constant. **Figure. 3** demonstrates slower breakthrough time from 66.3 to 175.9 min with an increase in bed depth from 3.5 to 7.0 cm and subsequently increasing the adsorbent exhaustion time.

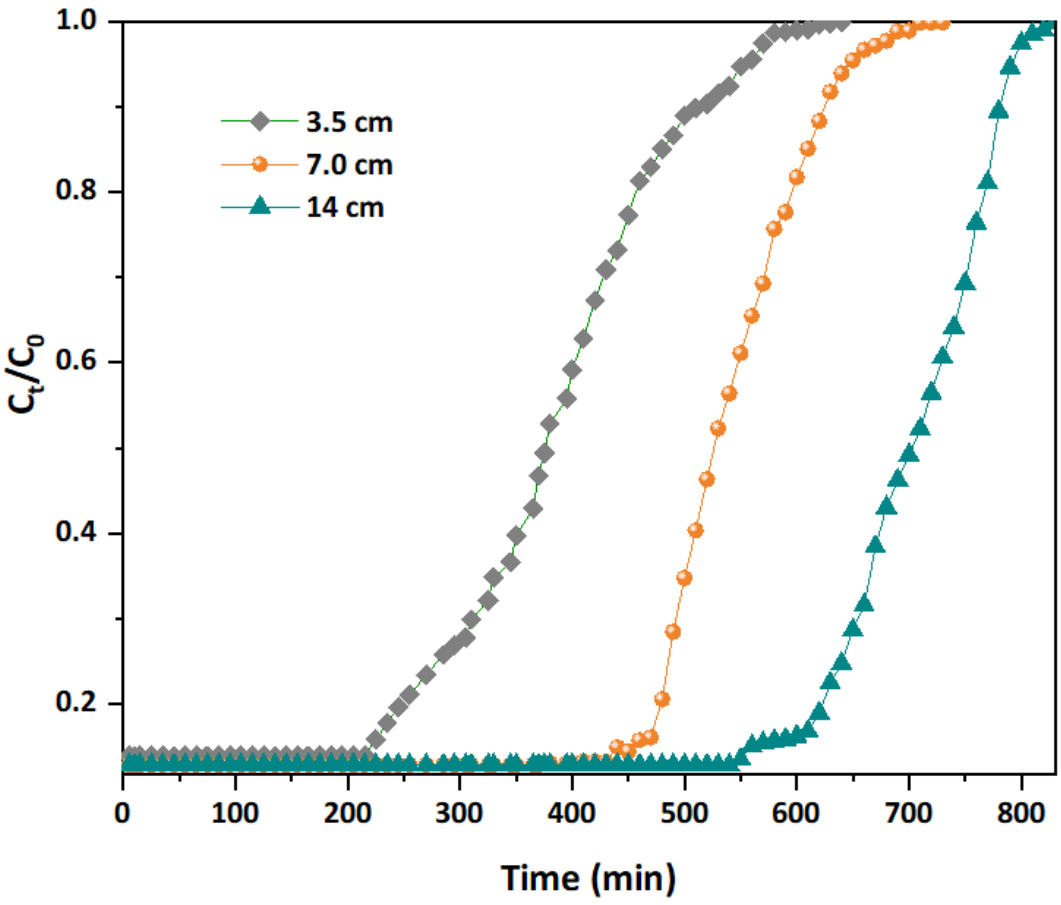


Figure. 3: Column breakthrough curves for the effect of bed mass at constant volumetric flow rate (2 mL min $^{-1}$) and feed concentration (5 mg L^{-1}).

This observation may be associated with longer pollutant residence time flowing within the column resulting decreased residual effluent concentration and thus, allowing the treatment of larger volumes of ATZ. Other researchers reported similar observations that have been ascribed to predominance of axial dispersion phenomenon of mass transfer at diminished bed heights thus, restricting the permeability of ATZ into PES membranes (Golie and Upadhyayula, 2016; Gao et al., 2019).

Table summarizes the process parameters at varying experimental conditions. The bed capacity (q_e) increased from 73.7 to 170.2 mg g^{-1} with a rise in the column bed length from 3.5 to 14 cm

resulting from increased surface area and availability of sorption sites on the PES (Golie and Upadhyayula, 2016). Song et al. (2016) also reported an extended breakthrough point (from 15 – 120 min) with an increased height of modified rice straw from 1.5 – 4.5 cm and related the behaviour to longer mass transfer zones at higher bed heights yielding larger treated volumes. Notably, the quantities of solution treated at breakthrough (V_b) also increased 452, 940 and 1118 mL corresponding to 3.5, 7.0 and 14 cm, respectively which also led to improved EBCT permitting sufficient time for the solute to diffuse into the PES (Deokar and Mandavgane, 2015). Consequently, a greater volume of the herbicide is removed. These findings suggest that the optimal performance of the bed can be attained using larger quantities of adsorbent mass.

Table 1: Estimation of breakthrough point parameters for ATZ adsorption in a fixed bed column using PES membranes as an adsorbent.

Process parameters	Service time, **t_b (min)**	Capacity, **q_e (mg g^{-1})**	Volume treated **V_b, (mL)**	AER x 10^3 **(g mL^{-1})**	ECBT **(min)**
Influent concentration (mg L^{-1})					
5.0	**470**	**139.6**	**940**	**1.33**	**7.04**
10.0	**364**	**177.3**	**728**	**1.72**	**7.04**
20.0	**271**	**203.6**	**542**	**2.31**	**7.04**
Influent solution flow rate (mL min^{-1})					
2.0	**470**	**139.6**	**940**	**1.33**	**7.04**
4.0	**254**	**129.9**	**1016**	**1.24**	**3.51**
6.0	**441**	**95.68**	**2646**	**0.47**	**2.35**
Bed height (cm)					
3.5 (0.65g)	**226**	**73.72**	**452**	**1.43**	**3.52**
7.0 (1.26 g)	**470**	**139.6**	**940**	**1.33**	**7.04**
14.0 (2.35 g)	**559**	**170.2**	**1118**	**2.99**	**14.1**

4.2. Breakthrough model analysis of ATZ on PES membranes

The performance nature of the breakthrough curves on an industrial scale was assessed using breakthrough curve kinetic plots. The experimental conditions and model parameters are summarized in **Table** .

4.2.1. Thomas model

Model constants derived from the linear graphs (**Table** indicate increasing k_{TH} values with increasing bed height, initial ATZ concentration and decreasing adsorption capacities (q_o). This observation was related to a decrease in mass transport resistance. These findings are comparable to those of other studies (Gupta and Garg, 2019), (Ranjan Rout and Mohan Jena, 2022). The maximum sorption capacity (q_o = 298.9 mg g^{-1}) was attained at 5 mg L^{-1}, feed flow of 2.0 mL min^{-1} and bed height of 14 cm.

4.2.2. Bohart-Adams

A summary of the computed Bohart Adams constants, k_{BA} and N_o values with their respective correlation coefficients is given in **Table 2**. The k_{BA} constant increased as all parameters increased. However, the R^2 for the model were relatively low (0.82 to 0.98) signifying that the Bohart-Adams model does not properly depict the equilibrium dynamics of ATZ adsorption utilizing PES membranes.

4.2.3. Yoon-Nelson

Yoon-Nelson developed a model in which it is presupposed that the probability of an adsorbate being absorbed is directly correlated with the likelihood that its concentration will exceed the breakthrough concentration on the adsorbent (Yoon and Nelson, 1984).

From the Yoon-Nelson BC modelling results in **Table 2**, R^2 values ranged between 0.83 and 0.9, and the k_{YN} constant did not uniformly follow any trend with varying parameters. Hence, this model proved to be unsatisfactory in the experimental working range. Similar trends were documented

(Mandal et al., 2021). Indeed, it is worth noting that the criterion for assessing goodness of fit for the models predominantly relies on statistical evaluation indicators such including the chi-squared value (χ^2) and root of means square error (RMSE) (Jafari et al., 2022; Hu et al., 2024).

Table 2: Column adsorption parameters under varying operational conditions in a packed-bed column.

Parameters	Thomas model				Bohart-Adams				Yoon-Nelson			
	K_{TH} x 10^{-2} (mL min^{-1} mg)	q_0 (mg g^{-1})	R^2	RMSE	K_{BA} x 10^{-3} (L mg^{-1} min^{-1})	N_0 (mg L^{-1})	R^2	RMSE	K_{YN} x 10^{-2}	τ (min)	R^2	RMSE
Influent concentration (mg L^{-1})												
5	42.4	179.5	0.991	0.004	1.48	438.6	0.819	0.019	2.35	229.6	0.99	0.031
10	24.2	198.1	0.908	0.044	3.34	814.1	0.886	0.023	2.84	207.8	0.94	0.083
20	12.2	209.9	0.923	0.072	5.18	1335.0	0.935	0.062	2.66	175.7	0.90	0.079
Flow rate (mL min^{-1})												
2	12.1	248.2	0.990	0.037	1.73	269.8	0.904	0.011	2.25	245.0	0.99	0.010
4	11.6	242.9	0.962	0.089	1.35	656.7	0.924	0.068	2.28	180.3	0.95	0.013
6	10.4	179.3	0.993	0.091	2.22	691.4	0.977	0.036	2.20	103.1	0.97	0.067
Bed height (cm)												
3.5	178.6	135.1	0.914	0.063	1.05	268.5	0.913	0.028	0.54	65.64	0.93	0.013
7.0	231.8	206.9	0.823	0.036	1.52	444.1	0.829	0.101	0.16	260.1	0.99	0.084
14	286.9	298.9	0.857	0.012	1.71	751.05	0.923	0.093	0.24	324.5	0.83	0.103

Moreover, the coefficient of k_{YN} changed as a function of bed height. The model's RMSE values were 0.010-0.103 which were relatively higher than those recorded for the other models. The results in **Table 2** also revealed lower RSME values for the Thomas model suggesting better model fitting and similarity with the experimental data (Satya et al., 2021). The viability of a PES hollow fibre membranes as an alternative sorption media for ATZ removal was compared with equilibrium adsorption capabilities of other adsorbents applied in packed-bed studies (**Table 3**). Although it was discovered that the current sorption medium was comparable to other previously documented adsorbents, there are limited research reports on the removal of ATZ in continuous fixed bed flow experiments.

Table 3: Comparison of the ATZ column equilibrium adsorption capacity of different adsorbents with PES membranes.

Adsorbent (s)	Capacity (mg g^{-1})	Reference(s)
Microwave irradiated *Aegle marmelos Correa* fruit shell	**1230**	(Sivarajasekar et al., 2017)
Poly(divinylbenzene) sorbent	**26.1**	(Ronka, 2016)
Waste activated carbon	**17.2**	(Ghosh and Philip, 2005)
Organic biomixture	**95.3**	(Levio-Raiman et al., 2021)
Ceramic ultrafiltration membrane	**NR**	(Mukherjee et al., 2018)
Hollow fibre PES membranes	**289.9**	**This study**

*NR – not reported

4.3. Enzymatic degradation

4.3.1. Substrate specificity and kinetic parameters

The biotransformation of ABTS and ATZ at varying concentration is presented by the Michaelis-Menten plot in **Figure. 4**. The kinetic parameters (K_m and $V_{max)}$ were also defined [52-53]. As illustrated, there was a general increase in the velocities with increasing substrate concentrations however, these considerably decreased when ATZ was used as an additional substrate in the ABTS solution, K_m (0.22 mM) and V_{max} (0.53 µM min^{-1}).

Table 4: Kinetic parameters of Lac with substrates: ABTS and ABTS + ATZ

	Kinetic parameters	
Substrate	K_m (mM)	V_{max} (µM min^{-1})
ABTS	**0.19**	**1.26**
ABTS + ATZ	**0.22**	**0.53**

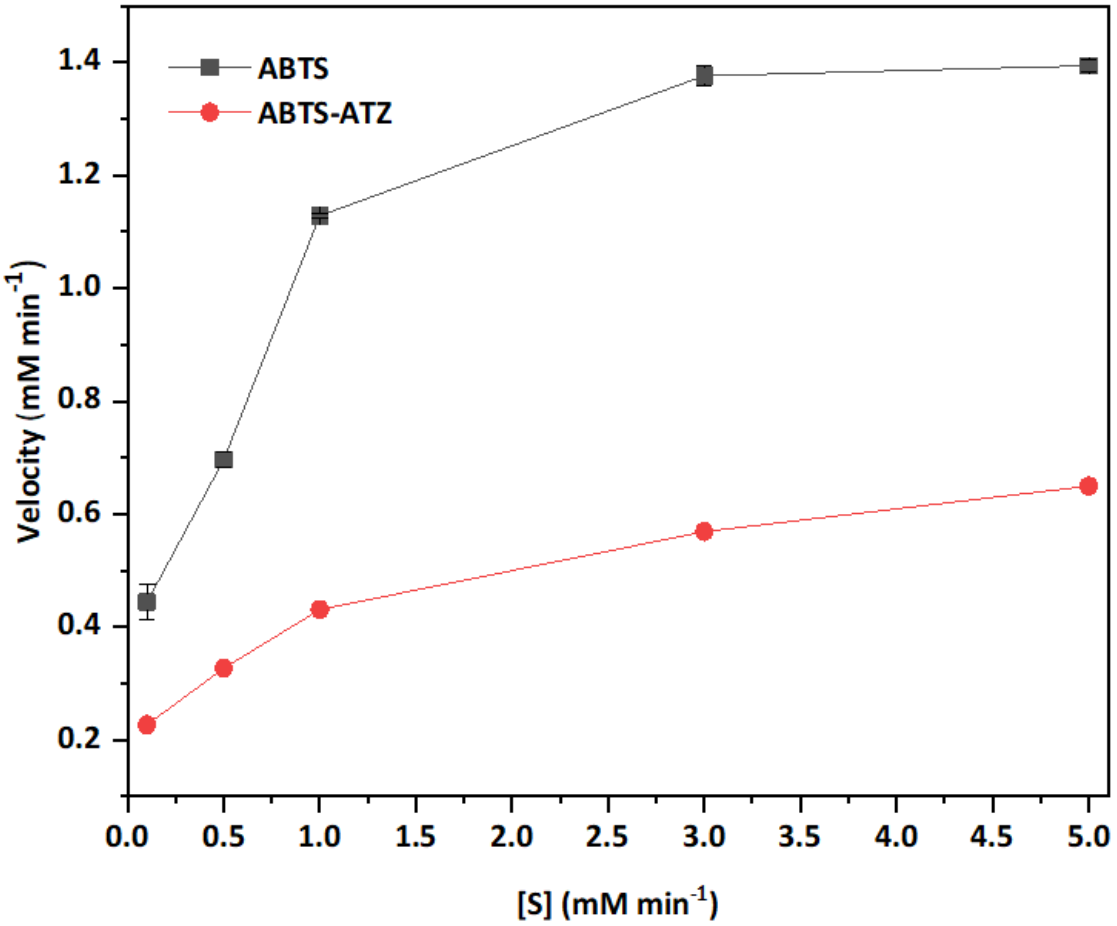


Figure. 4: Michaelis-Menten plots for Lac using ABTS and ABTS+ATZ as substrates for kinetic parameters.

The V_{max} of ATZ+ABTS decreased two folds from the V_{max} (1.26 µM min^{-1}) of ABTS alone. This observation is prevalent in the oxidation of substrates substituted EWGs (Asif et al., 2018; Kyomuhimbo and Brink, 2023). Similarly, the -NH_2 moieties and a halogen ring on the ATZ core, instigate substrate inhibition to the active catalytic sites of the enzyme (Yousefi-Ahmadipour et al., 2016; Kyomuhimbo and Brink, 2023) and thus slower oxidation of ATZ

compared to ABTS (Asif et al., 2018). An increase in K_{max} from 0.19 mM (ABTS) to 0.22mM (ABTS+ATZ) was avowed by lower affinity between ATZ and laccase however, enhanced ATZ removal was facilitated by the synergistic effect of integrating membrane retention into the enzymatic system.

4.3.2. pH and temperature profile on laccase degradation efficiency

Figure. 5 displays the temperature and pH profile on the conversion of ATZ. The enzyme showed inactivity at pH levels below optimal (pH 1-3) and a sharp reduction in activity at pH levels 7 – 10, retaining about 46 % and 35 % efficiency at pH 7 and 10, respectively. The diminished catalytic activity at non-optimum pH levels may be explained by the slower rate of substrate interaction and/or disruption of active catalytic sites (Mehandia et al., 2020) leading to irreparable denaturation (Li et al., 2022). Ideal fungal laccase activity is reportedly in the range pH 4 – 5 which tends to affect the electrostatic characteristics of the active site (Giardina and Sannia, 2015).

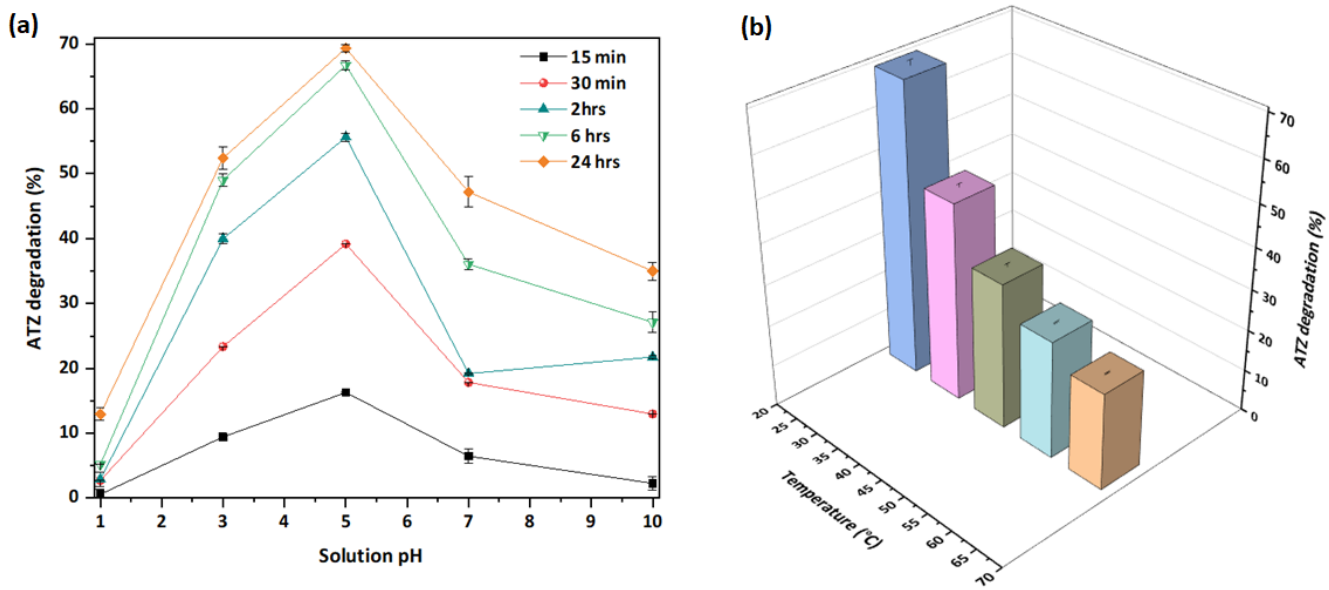


Figure. 5: (a) Influence of solution pH (b) and temperature on degradation of ATZ by laccase.

In essence, alkaline pH levels facilitate the binding of OH^- onto the copper ion at the active site, inhibiting the internal electron transfer pathway thereby decreasing laccase activity

(Baldrian, 2006; Yousefi-Ahmadipour et al., 2016). Margot et al. (2015) described a laccase-catalyzed mediated detoxification sulfamethoxazole and isoproturon at pH range 5 – 6. Additionally, the enzyme showed substantially increasing activity from 15 min to 6hrs of the reaction time maintaining approximately 55 % activity at the optimum pH. Following which the kinetics increased to 66 then 69 % from 8rs to 24hrs, respectively. These high removal efficiencies may be due to the prolonged interactions of the substrate with the enzyme at longer reaction times subsequently, 24hrs was used as the optimum time in successive batch enzymatic studies. From Figure 5(b) the thermostability profile illustrated a decrease in efficiency from 25 to 70°C, with maxima attained at 25 °C. A further increase in temperature (45-65 °C) led to diminishing ATZ degradation. This trend may be ascribed to permanent denaturation from thermal-induced conformational changes arising from irreparable unfolding of the protein structure as a result of enhanced chemical bond vibrations and linkage cleavage hence lower efficiencies (Yousefi-Ahmadipour et al., 2016; Salami et al., 2018).

4.3.3. Enzyme mass and stability tests

The quantity of enzyme required for the biocatalytic system's optimum performance was investigated at varying parameters. As shown in **Figure 6**(a) the enzymatic efficiency improved from 25.6 % to 61.4 % with increasing lac mass from 10 mg to 40 mg, respectively. Higher doses increase the availability of catalytic active sites thus reducing enzyme-substrate bulk-interactions, driven by multiple surrounding layers thus creating steric hindrance and restrained dispersion of substrate (Kyomuhimbo and Brink, 2023). These findings are consistent with previous reports (Fortes et al., 2017; Chen et al., 2021). The optimum dosage for ATZ degradation was 50 mg, which exhibits highest maximum activity (63.1 %) with a 1.7 % increment from the 40 mg enzyme mass.

The durability of Lac during storage was tested by submerging the enzyme in CPB buffer (pH 5) at 4 °C for 30 days. From **Figure 6 (b**) the enzyme retained its relative activity until day 15

(56.2 %) with a slight decrease from 67.6 %, to 66.4 % then 62.1 % and from day 1to 5 to day 10, respectively.

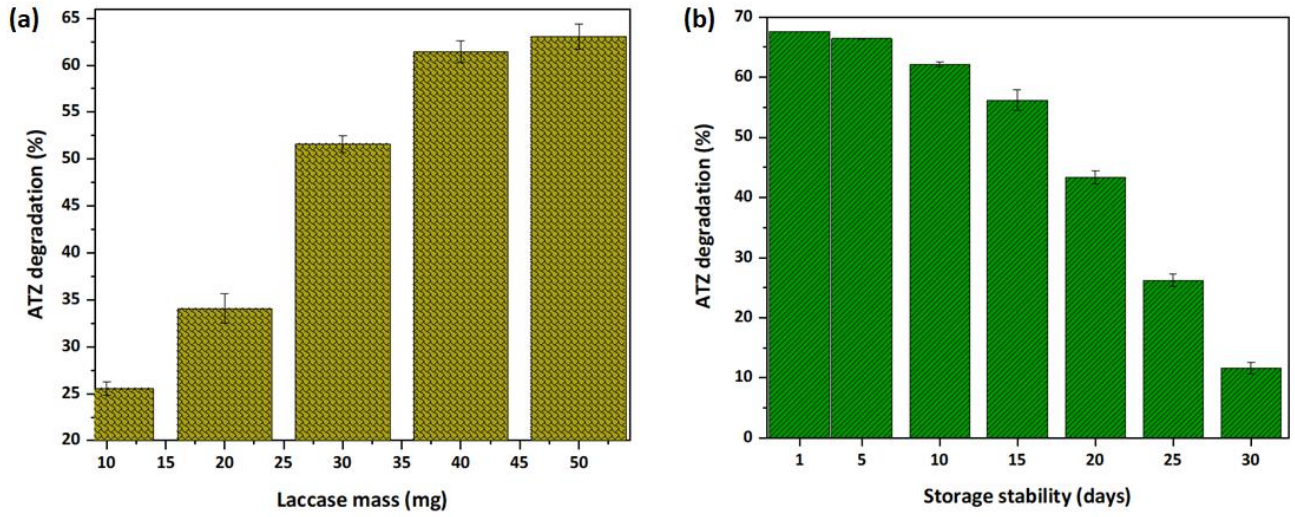


Figure 6: (a) Effect of enzyme load on ATZ degradation and (b) storage stability tests over 30 days.

The initial activity drastically dropped by close to three folds on day 25 (69 to 25 %), corresponding to degradation efficiency of 26.3 % which further decreased to 11.6 % on the last day of preservation. This observation is related to the stability in the enzyme's three-dimensional structure and its relative resistance to conformational changes under severe reaction conditions (Zdarta et al., 2019; Kyomuhimbo and Brink, 2023). The use of carrier bound enzymes leads to the dilution of catalytic activity and, hence, reduced volumetric and space-time yields and lower catalyst efficiency [89]. Therefore, the Novoprime base 258 laccase demonstrated operational stability with efficiencies that are comparable to other immobilized laccases.

4.4. Fabrication of PES-Lac biocatalytic column and operational reusability

The fabricated PES-laccase based column was applied for the bioremediation of ATZ using the previously optimized conditions. The effluent solution from the membrane packed-bed reactor subsequent to column exhaustion from 800 to 1190 min (corresponding to 10 min and

24 hrs degradation time) was used as the initial and final period for batch-mode enzymatic degradation, respectively. The overall performance of the biocatalytic system was then assessed by measuring ATZ concentration in samples collected at 10, 30, 60, 120, 240, 480, 960 and 1440 min corresponding to 880, 830, 890, 950, 1010, 1070, 1130 and 1190 min. **Figure 7b** displays the combined retention bioconversion degree of ATZ in the packed bed column as a function of time. The enzymatic degradation increased from 37.3 to 69.2 % while the membrane efficiency decreased when the reaction time was increased from 800 min (55.9 %) to 1190 min (12.2 %). This was ascribed to membrane exhaustion (**Fig. 3**).

Coupling membrane retention with enzymatic degradation improved the overall removal of ATZ. The biocatalytic system attained 93.2 % removal efficiency with 37.3 and 55.9 % enzymatic degradation and membrane retention at 800 min operational time, respectively. After 24 hrs of operation, 81.3 % degradation was observed with notable a plateau in enzyme efficiency (69.1 %) and diminished membrane performance (12.2 %).

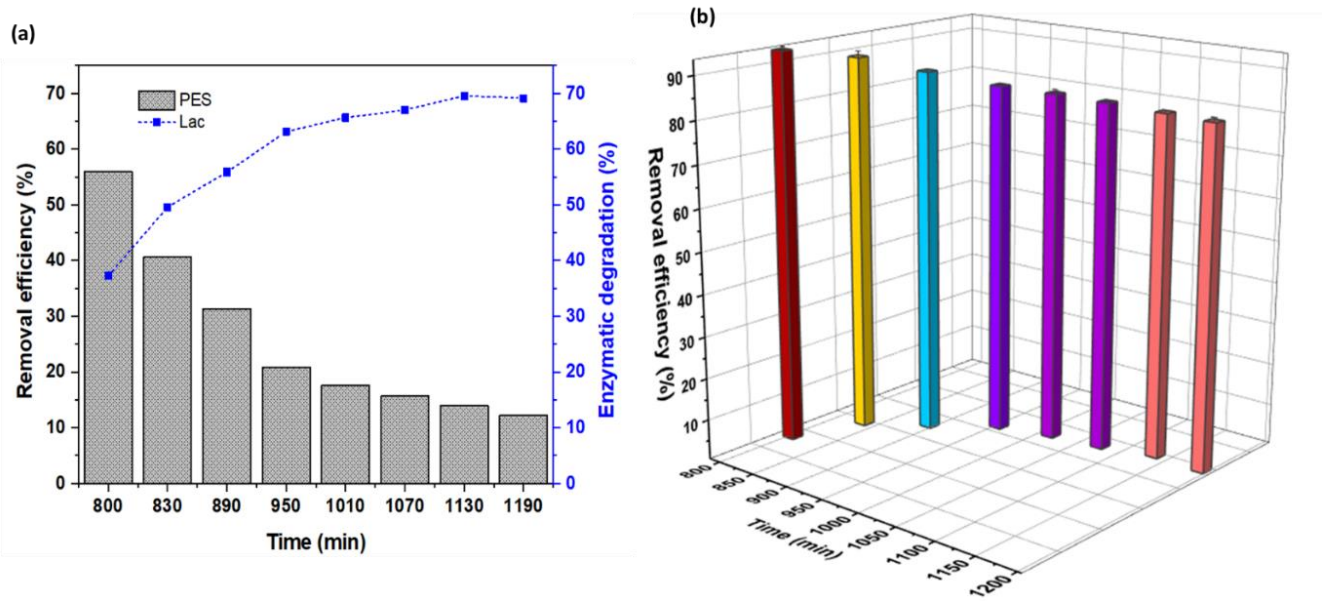


Figure 7: (a) Enzymatic degradation and retention of ATZ (b) Overall removal efficiency of ATZ in the membrane packed enzymatic system.

The effectiveness of a hybrid PES-Lac fixed bed column to effectively eradicate ATZ was highlighted with considerable improvement compared to other previously reported studies

where less than 5% removal was attained in an UF-EMBR (Luong N. Nguyen et al., 2014). Despite, laccase's ability to oxidize non-phenolic substances including those with EWGs, it is hampered by kinetic restrictions which are predominant in many enzyme-catalyzed reactions (Luong N Nguyen et al., 2014; Ashe, Luong N Nguyen, et al., 2016). Nonetheless, this study demonstrated that ATZ conversion significantly improved following retention by PES membrane in the packed-bed column.

In practical terms, the enzyme's operational stability plays a vital role in establishing a continuous treatment process. The operational durability of the biocatalytic system was assessed over six successive cycles of the degradation of ATZ (**Fig. 8**). The reusability tests showed that the biocatalytic column's catalytic efficiency was maintained at 76 % after two cycles and at 50 % at cycle 5.

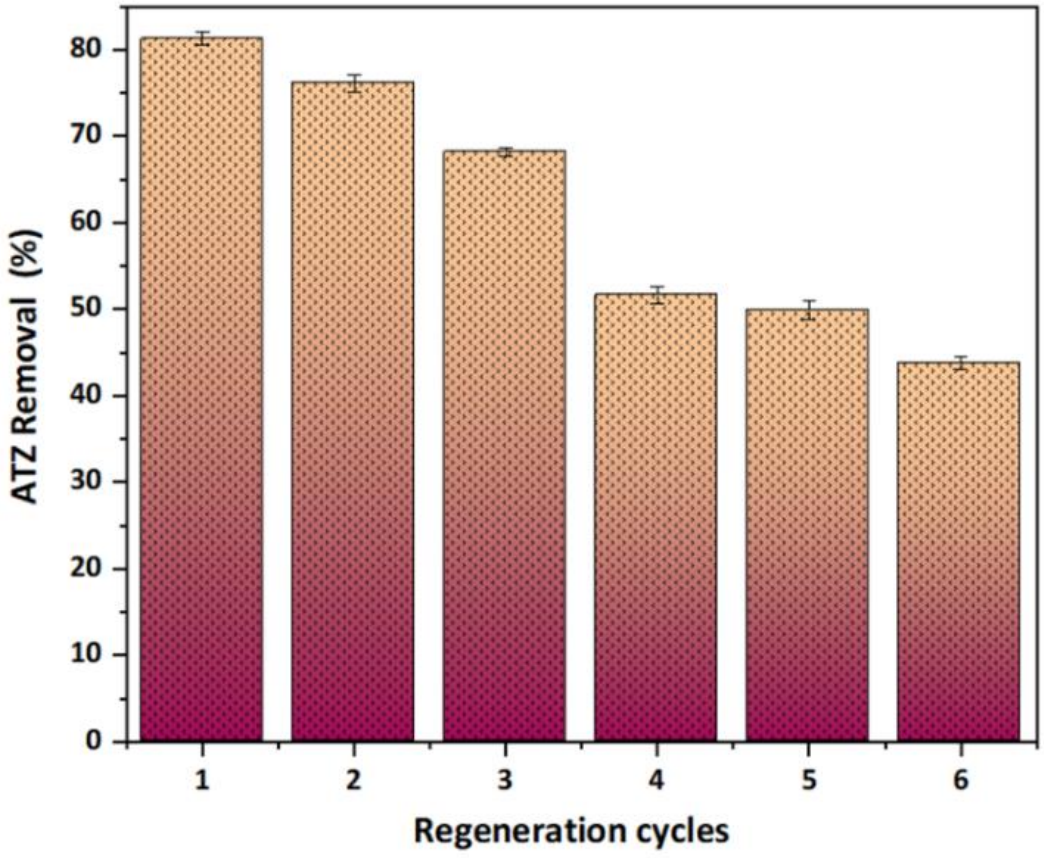


Figure 8: Reusability tests for PES-Laccase reactor in triplicates.

This favorable recyclability may be ascribed capability of the membrane support to preserve the enzyme's catalytic conformation under harsh conditions (Tiarsa et al., 2022; Yang et al., 2023). This observation suggests that Novoprime base 268 laccases may be suitable for applications in water reclamation.

4.5. Application of biocatalytic system in real water matrices

The applicability of the biocatalytic packed bed in real water samples was conducted using a water sample collected at a local river stream in Johannesburg. At the time of sampling, *in-situ* measurements of physicochemical properties of the sample included pH (7.2), conductivity (9 µS cm^{-1}), total dissolved solids (389 mg L^{-1}), temperature (27°C) and dissolved oxygen of 8 mg L^{-1}. The sample was spiked with ATZ then fed into the packed-bed column under optimized conditions. The overall conversion degree was found to be 82 %, which outperformed the efficiency of the standalone membrane reactor at 52 %. The improved efficiency could be related to the synergistic effect offered by enzymatic degradation. In this sense the availability of PES as a support may have afforded retention of other competing substrates and thus allowing better accessibility of the catalytic active sites by the target analyte.

4.6. Economic assessments

The cost parameters of the proposed full-scale PBEMBR are summarized in **Table 5**. The system design was based on a treatment capacity of 1200 m^3/d. As shown in **Table 5**, the estimated total capital cost for full-scale PBEMBR was R7.036 mil at R2.38 and R1.31 mil of CAPEX and OPEX, respectively. Compared to the other studies, the system was established at a much lower cost and the latter is because the tool projected for development and testing of a pilot plant before upscaling. In addition, the listed literature reports (C.D, Swartz, J.G, Menge, J.A, duPlessis, G.M, 2022; Welo et al., 2023) were based on fully functional facilities operated for treatment of larger quantities of water (ML/d) (Gao et al., 2021). Moreover, the cost analysis method used in this study was developed specifically for this research topic and was confined

to the research scope. The costing tool established is not generic and more comprehensive considerations should be considered for engineering decisions.

Table 5: Total cost comparison of membrane-based water reuse plant systems in South Africa.

				Total Costing			
Plant	**Process configuration**	**Year of costing**	**Capacity**	**Total Capital Cost (Rm)**	**CAPEX Rm/ML/d**	**O&M Cost (R/kL)**	**Source**
Stellenbosch DPR modelling	Reverse osmosis (RO)	2019	10 ML/d	346	34.6	Not specified	(C.D, Swartz, J.G, Menge, J.A, duPlessis, G.M, 2022)
	RO	2019	20 ML/d	504.21	25.21		(C.D, Swartz, J.G, Menge, J.A, duPlessis, G.M, 2022)
Beaufort West	Sedimentation-media UF-RO-UVAOP		2 ML/d	23.7	19.23	38.5	(C.D, Swartz, J.G, Menge, J.A, duPlessis, G.M, 2022)
Kubota FS	Membrane bioreactor	2020	50 ML/d	208.3	64.53	143.79	(Smith and Town, 2020)
Paarl WRP feasibility study	UF-RO UV/H_2O_2	2019	10 ML/d	102.8	10.2	9.73	(C.D, Swartz, J.G, Menge, J.A, duPlessis, G.M, 2022)
Zeeweed feasibility study	MBR	2020	50 ML/d	107.8	54.18	53.71	(Smith and Town, 2020)
*USA Comparative costing of MBRs	MBRs	2021	1000 ML/d		*0.84–1.71	*0.62–1.21	(Gao et al., 2021)
Packed-bed enzymatic membrane system	UF-biocatalysis	2024	1200 m^3/d	7.036	2.28	1.31	This study

* Currency in USD

5. Conclusions

The removal of atrazine, a chloro-s-triazine herbicide was investigated in an integrated biocatalytic system (membrane retention + biodegradation). The overall eradication of the ATZ in the biocatalytic packed-bed enzymatic system varied from 93.2 to 81.3 %, while the standalone membrane retention studies achieved 55.9 % pollutant removal after 24 hrs reaction time. Breakthrough modelling error statistics (R^2 and RMSE) at optimum conditions revealed that the equilibrium dynamics of ATZ adsorption utilizing PES membranes followed the Thomas Model. Notably, the incorporation of biocatalyst (Novoprime base 268 laccase) improved ATZ conversion compared to separate membrane system and unsupported enzymatic tests. In addition, the column performance showed higher stability and suitable biocatalyst regeneration, retaining activity after six successive operational cycles. The capability of the fabricated PES-Lac enzymatic column to selectively remove ATZ present in a complex real matrix (river water) was demonstrated and, hence, the potential of a PES packed bed laccase column for enzymatic conversion of chloro-s-triazines in water treatment. In addition, economic estimates provided the comparable projections of developing a pilot scale of the system for treatment of 1200 m^3/d with an estimated total cost of R7.036 m.

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CHAPTER 5 GENERAL CONCLUSIONS AND RECOMMENDATIONS

5.1 General conclusions

In this work, a biocatalytic membrane system was developed and tested as a possible alternative for the bioremediation of an equitoxic triazine herbicides mixture i.e. atrazine, simazine, ametryn, prometon and terbuthylazine. The primary objective was to explore the efficacy of applying both systems separately and as an integrated configuration to provide a facile, green approach for remediation of contaminated water.

The utlilization of Novoprime base 268 laccase as a biocatalyst afforded significant capacity to bioconvert recalcitrant triazine compounds, especially after optimization. The biocatalyst promoted degradation of these compounds highlighting the potentialities of application as a facile and alternative in bioremediation processes. Additionally, impeded by inhibition from the structural substituents of the triazine molecules. As a result, a laccase mediated system with all the different redox mediators tested afforded quicker and continuous degradation rates.

The hollow-fibre membranes were used in their pristine state and demonstrated moderate ability to eliminate the herbicides from water. While the PES was effective to some degree, the performance was limited by factors such as hydrophobicity, surface characteristics and restricted interactions which inhibits the complete removal of structurally related compounds.

The fabrication of a biocatalytic system by coupling PES membranes and laccase resulted in a synergistic effect improving the overall remediation of triazine herbicides. This hybrid approach exploited the mechanical properties of PES and the biocatalytic capabilities of laccase, resulting in a more effective bioremediation system for contaminated water samples.

The biocatalytic membrane system mimicked a real wastewater set-up operated in a continuous flow-mode thus, demonstrated a vast potential from a cost-benefit analysis standpoint and could offer comparable operational benefits to other existing configurations.

5.2 Future recommendations

The research study's aims and set objectives were achieved, future considerations can be projected by considering:

- Further studies on evaluating and utilizing various strategies to modify and/or functionalize the surface characteristics of the PES membranes for improved adsorptive performance.
- The membrane system could be applied in a multicomponent system with other prevalently detected organic contaminants to assess the selectivity and affinity of the enzyme in a mixed matrix environment.
- Perform comparative toxicity tests on the parent and generated metabolites to further elucidate their fate in the environment.
- Design and conduct pilot-testing experiments to evaluate the feasibility of the biocatalytic system in real-life scenarios. This will assist with insights into operational challenges and scalability issues.

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APPENDIX

The costing information for the reactors used in establishing the cost function was taken from past wastewater projects between 2018-2021 and estimates kindly provided by BASF, who were the consulting supplier on the projects. These prices were then escalated to 2023 at 5 % pa. The capital cost estimation software, version 2 (Turton et al., 2018) was also used wherein the total cost of the plant was expressed as a liner sum of the amortized capital (CAPEX) and operating costs (OPEX) as given below:

Total cost (TC) = αCAPEX + OPEX

where α represents the depreciation rate of the facility estimated to be 4-12% of the weighted average (Gao et al., 2021).

Table 1: Cost estimation for 1200 $m^3.day^{-1}$ PES-Lacc plant (Turton et al., 2018).

Description	Units	Values	Sources
Membrane and potting system	m^3 $year^{-1}$	388 800	Supplier estimate
Quantity of membrane modules and constructing cost	R $module^{-1}$	500	Supplier estimate
Pump	R m^{-3} h	160	
Electricity	R kW h^{-1}	2.07	Eskom through The National Energy Regulator of South Africa
Maintenance		2% of total equipment cost	Siagian et al., 2023
Equipment installation		40 % of total equipment cost	Siagian et al., 2023
Electrical and control		25 % of total equipment cost	Siagian et al., 2023
Piping		10 % of total equipment cost	Siagian et al., 2023

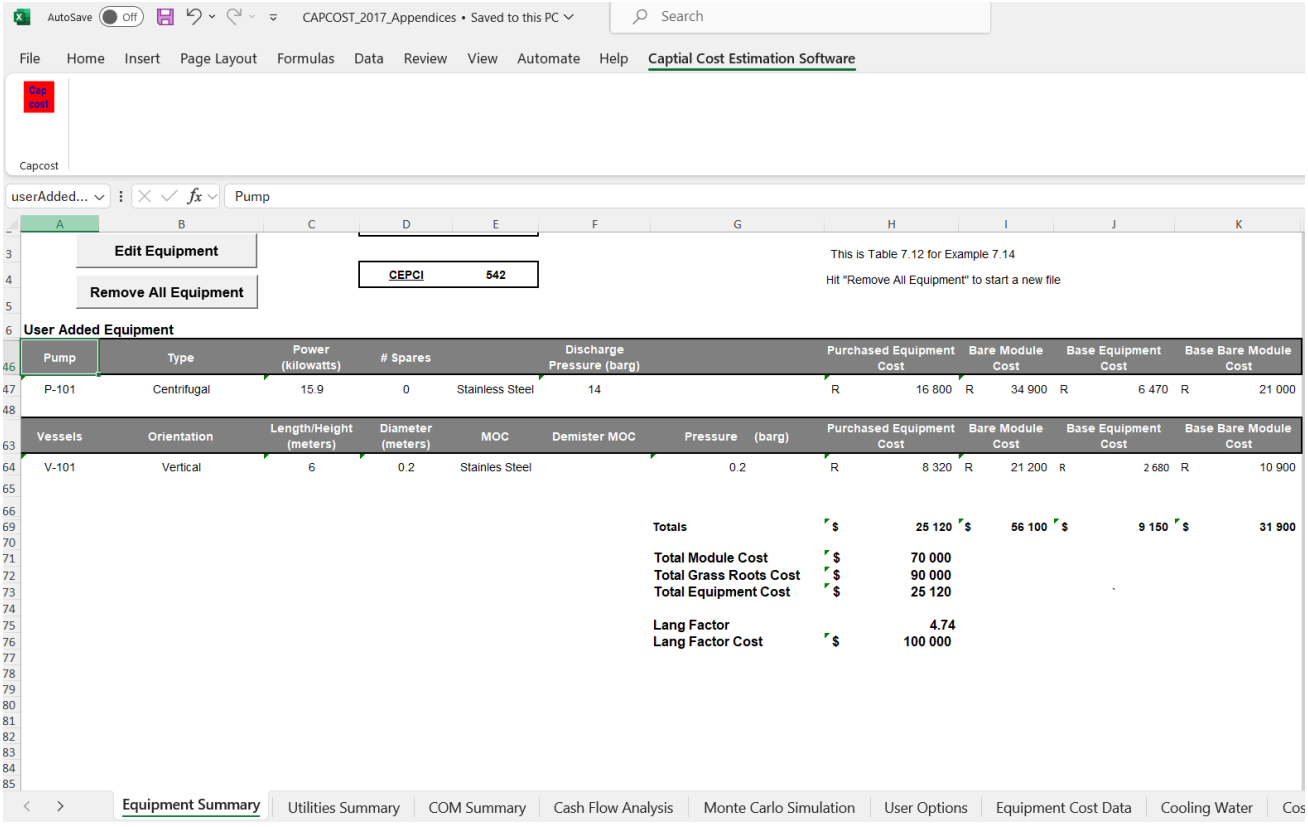

Figure 1: Capital cost estimation software for cost-analysis calculations.