

University of Windsor

## Scholarship at UWindor

---

Electronic Theses and Dissertations

Theses, Dissertations, and Major Papers

---

2023

# Enzymatic Removal of Diclofenac and Aceclofenac from Synthetic Wastewater by Soybean Peroxidase

Sarah Pishyar

Follow this and additional works at: <https://scholar.uwindsor.ca/etd>



Part of the [Chemistry Commons](#), and the [Environmental Engineering Commons](#)

---

### Recommended Citation

Pishyar, Sarah, "Enzymatic Removal of Diclofenac and Aceclofenac from Synthetic Wastewater by Soybean Peroxidase" (2023). *Electronic Theses and Dissertations*. 8937.

<https://scholar.uwindsor.ca/etd/8937>

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email ([scholarship@uwindsor.ca](mailto:scholarship@uwindsor.ca)) or by telephone at 519-253-3000ext. 3208.

**Enzymatic Removal of Diclofenac and Aceclofenac from Synthetic Wastewater by  
Soybean Peroxidase**

By

**Sara Pishyar**

A Thesis

Submitted to the Faculty of Graduate Studies through the Department of Civil and  
Environmental Engineering in Partial Fulfillment of the Requirements for the Degree of  
Master of Applied Science  
at the University of Windsor

Windsor, Ontario, Canada

2023

© 2023 Sara Pishyar

**Enzymatic Removal of Diclofenac and Aceclofenac from Synthetic Wastewater by  
Soybean Peroxidase**

by

**Sara Pishyar**

APPROVED BY:

---

R. Ruparathna  
Department of Civil and Environmental Engineering

---

E. Tam  
Department of Civil and Environmental Engineering

---

N. Biswas, Co-Advisor  
Department of Civil and Environmental Engineering

---

K. E. Taylor, Co-Advisor  
Department of Chemistry and Biochemistry

January 6<sup>th</sup>, 2023

## **DECLARATION OF ORIGINALITY**

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis and have included copies of such copyright clearances to my appendix.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

## ABSTRACT

Pharmaceutical medications that are a class of emerging contaminants have been detected in wastewater treatment facilities' influent and effluent, and they have reached water sources in amounts ranging from ng/L to ug/L. They threaten the environment and non-target life. Soybean peroxidase (SBP)-catalyzed process was studied to remove two non-steroidal anti-inflammatory drugs (NSAIDS) diclofenac and aceclofenac through enzymatic oxidation from synthetic wastewater. SBP can be extracted from soybean hulls, a by-product of the soybean industry. First, the experiments studied the viability of SBP-catalyzed removal on these two compounds, then the operational parameters including pH, the molar ratio between hydrogen peroxide and substrate and minimum effective enzyme concentration were optimized. The first-order rate constant and half-life of each substrate were also determined under the established optimum conditions. The results demonstrated SBP is robust enzyme to achieve more than 95% removal efficiency for both compounds. Finally, possible oligomerization products of enzymatic treatment were characterized by mass spectrometric analysis and showed formation of tetramer.

## **DEDICATION**

I would like to dedicate this thesis to my beloved parents, Azam Aslani and Aliasghar Pishyar, and my sisters Elham, Neda and Mahsa who have offered me unselfish support and encouragement over many years of every academic endeavor in my life. Also, I would like to dedicate it to who have supported me emotionally and motivated me during these years, Maryam, Mahsa, Elham, Abtin and Dilusha

## ACKNOWLEDGEMENTS

I am deeply indebted to my supervisors Dr. Keith E. Taylor and Dr. Nihar Biswas for their patience, motivation, and guidance all the time of the research and during the writing of this thesis. I have been highly lucky to join this research group to get their shared wisdom and knowledge. I also offer gratitude to Dr. R. Ruparathna and Dr. E. Tam for the honor of being my committee members and for the valuable suggestions and comments that helped me complete this thesis. I am truly grateful to Miss. Samira Narimannejad for her technical support and training. I also would like to express my gratitude to my lab colleague and my best friend Maryam Sharifzadeh for her help and support, motivated me to write thesis and thanks to all the great time we had together. Also, Thanks to University of Windsor for financial support. Finally, my profound indebtedness goes to my parents, my family members, and friends for believing in and motivating me to finish this journey.

## TABLE OF CONTENTS

DECLARATION OF ORIGINALITY .....	III
ABSTRACT.....	IV
DEDICATION.....	IV
ACKNOWLEDGEMENTS.....	VI
LIST OF TABLES.....	IX
LIST OF FIGURES.....	X
LIST OF APPENDICES.....	XI
LIST OF ABBREVIATIONS/SYMBOLS .....	XII
Chapter 1 INTRODUCTION.....	1
1.1 Background .....	1
1.2 Research gap .....	4
1.3 Research objectives .....	4
1.4 Scope .....	5
Chapter 2 LITERATURE REVIEW.....	7
2.1 Compounds.....	7
2.1.1 Diclofenac.....	7
2.1.2 Aceclofenac.....	11
2.2 Enzymatic Treatment .....	12
2.2.1 Application of Enzymes in Wastewater Treatment .....	14
2.2.2 Oxidases.....	15
2.2.3 Peroxidases .....	15
2.2.4 Soybean Peroxidase .....	18
Chapter 3 MATERIAL AND METHODS .....	22
3.1 Materials.....	22
3.1.1 Substrates and Enzymes.....	22
3.1.2 Buffer and solvents .....	22
3.1.3 Other chemicals .....	22
3.1.4 HPLC solvents and materials.....	22
3.1.5 Other materials.....	23
3.2 Analytical Equipment.....	23
3.2.1 UV-Visible spectrophotometry.....	23



3.2.2	High performance liquid chromatography (HPLC) analysis .....	23
3.2.3	Electrospray ionization (ESI) mass spectrometry .....	24
3.2.4	Other equipment.....	24
3.3	Analytical Methods .....	25
3.3.1	Enzyme stock solution preparation .....	25
3.3.2	Catalase stock solution and Buffer preparation .....	25
3.3.3	SBP activity assay.....	25
3.4	Experimental protocols .....	26
3.4.1	Enzymatic oxidation of substrates with SBP and feasibility of treatment of target aromatics.....	26
3.4.2	Optimization of important enzymatic reaction parameters.....	26
3.4.3	Preliminary identification of products .....	27
3.4.4	Sources of error.....	27
Chapter 4 RESULTS AND DISCUSSION .....		29
4.1	Parameter optimization of SBP-catalyzed treatment .....	29
4.1.1	pH Optimization.....	29
4.1.2	Enzyme optimization .....	34
4.1.3	Hydrogen peroxide optimization .....	36
4.1.4	Summary of parameter optimization .....	39
4.2	Time course of reactions .....	41
4.3	Mass spectrometry (MS) results.....	46
4.3.1	Diclofenac .....	47
4.3.2	Aceclofenac.....	48
Chapter 5 SUMMARY AND CONCLUSIONS.....		49
5.1	Summary .....	49
5.2	Limitations .....	50
5.3	Conclusions .....	50
5.4	Contributions.....	50
5.4.1	Theoretical contributions .....	50
5.4.2	Practical contributions .....	51
5.5	Future Work .....	51
REFERENCES.....		52
APPENDICES.....		64

VITA AUCTORIS .....	68
---------------------	----

### LIST OF TABLES

Table 1-1 Name, formula, and molecular structure of the chemicals studied .....	5
Table 2-1. Chemical and physical properties for Diclofenac sodium salt .....	7
Table 2-2 Chemical and physical properties for Aceclofenac .....	12
Table 2-3 Summary of efficiencies of various substrates with SBP in synthetic wastewater (Sources: Bideh et al., 2021; Kumar et al., 2019; Morsi et al., 2020) .....	20
Table 3-1 HPLC conditions for substrates run under isocratic elution.....	24
Table 4-1 Results for Standard Control Test .....	32
Table 4-2 DCF solubility in pH 3.0 buffer .....	32
Table 4-3 Optimized conditions for SBP-catalyzed removal for 0.10 mM Diclofenac and Aceclofenac.....	39
Table 4-4 Half-lives and normalized half-lives of various SBP substrates <sup>a</sup> .....	45
Table 4-5 Summary of MS results for standard and identified products of SBP-catalyzed of diclofenac <sup>a</sup> .....	48
Table 5-1 Summary of optimized conditions for SBP-catalyzed process and rate constants.....	49

## LIST OF FIGURES

Figure 2-1. Proposed Mechanism for peroxidases. (Adopted from (Dunford, 1999)) .....	17
Figure 2-2. Global Soybean Production 2021-2022 according to USDA (2022).....	19
Figure 4-1. Diclofenac pH Optimization. (0.1 mM diclofenac, 0.4 mM H <sub>2</sub> O <sub>2</sub> , 0.3 and 0.15 U/mL SBP, 3-hour reaction, room temperature) .....	31
Figure 4-2. Aceclofenac wide-range pH optimization. (0.1 mM aceclofenac 0.4 mM H <sub>2</sub> O <sub>2</sub> , 0.15 U/mL SBP, 3- hour reaction, room temperature) .....	33
Figure 4-3. Aceclofenac narrow-range pH optimization. (0.1 mM aceclofenac 0.4 mM H <sub>2</sub> O <sub>2</sub> , 0.15 U/mL SBP, 3- hour reaction, room temperature) .....	33
Figure 4-4. Diclofenac enzyme optimization. (pH 5.0 buffer, 0.1 mM diclofenac, 0.4 mM H <sub>2</sub> O <sub>2</sub> , 3-hour reaction, room temperature) .....	35
Figure 4-5. Aceclofenac Enzyme Optimization. (pH 4.0 buffer, 0.1 mM aceclofenac, 0.4 mM H <sub>2</sub> O <sub>2</sub> , 3-hour reaction, room temperature) .....	36
Figure 4-6. Diclofenac H <sub>2</sub> O <sub>2</sub> Optimization. (pH 5.0 buffer, 0.1 mM diclofenac 0.15 U/mL SBP, 3-hour reaction, room temperature) .....	37
Figure 4-7. Aceclofenac H <sub>2</sub> O <sub>2</sub> Optimization. (pH 4.0 buffer, 0.1 mM aceclofenac, 0.6 U/mL SBP, 3-hour reaction, room temperature).....	38
Figure 4-8. Aceclofenac Enzyme Optimization. (pH 4.0 buffer, 0.1 mM aceclofenac, 0.45 mM H <sub>2</sub> O <sub>2</sub> , 3-hour reaction, room temperature) .....	39
Figure 4-9. Time dependence of degradation of diclofenac in optimal conditions Batch reactor. (pH 5.0 buffer, 0.1 mM diclofenac, 0.4 mM H <sub>2</sub> O <sub>2</sub> , 0.15 U/mL SBP, 3-hour reaction, room temperature) .....	42
Figure 4-10. First-order degradation of diclofenac at the beginning of the reaction presented in Figure 4.9 .....	43
Figure 4-11. Time dependence of degradation of aceclofenac in optimal conditions Batch reactor. (pH 4.0 buffer, 0.1 mM aceclofenac, 0.45 mM H <sub>2</sub> O <sub>2</sub> , 0.6 U/mL SBP, 3-hour reaction, room temperature) .....	43
Figure 4-12. First-order degradation of aceclofenac at the beginning of the reaction presented in Figure 4.11 .....	44

## LIST OF APPENDICES

Appendix A .....	64
Appendix B .....	66

## LIST OF ABBREVIATIONS/SYMBOLS

### Abbreviations

NSAID: Non-steroidal anti-inflammatory drug

SBP: Soybean peroxidase

EC: Emerging contaminant

HRP: Horseradish peroxidase

ARP: *Arthromyces ramosus* peroxidase

TP: Turnip peroxidase

OA: Osteoarthritis

RA: rheumatoid arthritis

LBP: Low back pain

BGP: Bitter gourd peroxidase

CPO: Chloroperoxidase

MnP: Manganese peroxidase

DCF: Diclofenac

ACF: Aceclofenac

2-ABT: 2-Amonibenzothiazole

2-AT: 2-Aminothiazole

3-AQ: 3-Aminoquinoline

3-HQ: 3-Hydroxyquinoline

2-AI: 2-Aminoimidazole

2-ABI: 2-Aminobenzimidazole

3-AP: 3-Aminopyrazole

4-AAP: 4-Amino-antipyrine

HOBT: Hydroxybenzotriazole

HBT: Hydroxybenzothiazol

ASAP: Atmospheric solids analysis probe

WWTP: Wastewater treatment plant

CAN: Acetonitrile

HPLC: High-performance liquid chromatography

MS: Mass spectrometry.

UV-Vis: Ultraviolet-Visible

AOPs: Advanced oxidation processes

### **Symbols**

$\lambda_{\text{max}}$ : Maximum wavelength

$m/z$ : Mass-to-charge ratio

$\text{p}K_{\text{a}}$ ,  $-\log_{10} K_{\text{a}}$ : where  $K_{\text{a}}$  is acid dissociation constant and  $k_{\text{cat}}$  is turnover number.

$k_{\text{cat}}/K_{\text{M}}$ : Specificity constant or, catalytic efficiency, where  $K_{\text{M}}$  is Michaelis constant.

$K$ : First-order kinetic rate constant

$t_{1/2}$ : Half-life

## **CHAPTER 1**

### **INTRODUCTION**

#### **1.1 Background**

Emerging contaminants (ECs) are known as newly recognized environmental contaminants that have negative influences on the environmental and/or human health (Rasheed et al., 2019). Pharmaceutical medications are a class of emerging contaminants, and the widespread occurrence of these substances in rivers and effluents is currently of concern in water quality regulation due to the potential threats they provide to human health and the environment. Pharmaceutical medications have been found in wastewater treatment facilities' influent and effluent, and they have reached water sources in amounts ranging from ng/L to ug/L. Numerous investigations have demonstrated the acute toxicity, cytotoxicity, carcinogenicity, and mutagenicity of these compounds (Brack & Schirmer, 2003). It is critical to create an environmentally acceptable, economically viable, and efficient alternative to address this class of pollutions because these contaminants cannot be effectively eradicated by existing treatment methods.

Due to their excellent anti-inflammatory, antipyretic, and analgesic properties, non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, ibuprofen, aceclofenac, naproxen, ketoprofen, and indomethacin are among the most frequently used pharmaceuticals in both human and veterinary medicine. These properties make them effective for reducing the pain, inflammation, fever, and stiffness that are associated with rheumatic (Moore et al., 2019; Psomas, 2020). Inhibiting COX-1 and COX-2, two cyclooxygenase isoforms involved in the generation of different prostaglandins from arachidonic acid, is the main mechanism underlying their therapeutic effects ((Vane & Botting, 1998)). Most NSAIDs are reasonably priced and typically among the first drugs prescribed for aching joints, stiffness, and inflammation. In the US alone, it is estimated that more than seventy million medical prescriptions are written, and more than 30 billion dosages are utilised annually (Parolini, 2020). The European Union is the second-largest user of these medications, using between 50 and 150 g of NSAIDs per person annually, or around 24 percent of the global total (Service, 2013).

The use of NSAIDs not only has benefits, but also presents a number of challenges. Due to the fact that many NSAIDs are over-the-counter drugs, this may be one of the main reasons for their overuse and has aided in their buildup to detectable levels in the environment. In recent years, a

number of studies have documented the widespread, ongoing, and rising presence of NSAIDs in marine, surface, and ground water, as well as sewage sludge, industrial and hospital effluents, and wastewater treatment facilities (Carmona et al., 2014; Chaves et al., 2020; Eslami et al., 2015; Gumbi et al., 2017; K'oreje et al., 2016; You et al., 2015). Non-steroidal anti-inflammatory drugs are both directly and indirectly released into the environment. Surface waters are impacted by the direct receipt of urban wastewater and effluents discharged by businesses, healthcare facilities (hospitals, dental clinics, etc.), and wastewater treatment facilities. Due to inadequate elimination in wastewater treatment plants (WWTPs) and water treatment plants (WTPs), enormous amounts of NSAIDs are discharged into the aquatic environment (Jaeschke & Bajt, 2006). Most of the time, wastewater treatment facilities cannot effectively remove them using conventional sewage treatment methods (Ravi et al., 2020). Due to their slow metabolism (30–90%), non-steroidal anti-inflammatory medications specifically are excreted in the urine or faeces of both humans and animals. These chemicals, either as unmodified parent or metabolized compounds, make up to 15% of all pharmaceuticals that have been discovered in water bodies around the world (Kasprzyk-Hordern et al., 2009; Kermia et al., 2016).

These compounds are now widely recognised as a serious environmental issue having detrimental consequences on the ecology, flora, and fauna, as well as eventually on humans. NSAIDs, can negatively affect the health, behaviour, reproduction, and survival of species across multiple trophic levels in aquatic ecosystems at concentrations as low as ng to ug/L. (Kwak et al., 2018; Pandelides et al., 2021; Xiong et al., 2020). The contamination of water with NSAIDs not only endangers aquatic animals and flora but also human health (Gómez-Oliván, 2020). Moreover, the growth in antibiotic resistance in harmful strains of many bacteria may have an indirect effect on human and animal health. By design, NSAIDs can affect the biochemical and physiological functions of aquatic species throughout their whole life cycle because they are created to be effective at very low doses. Furthermore, there is indisputable evidence that NSAID residues are harmful to aquatic and scavenger species. Diclofenac, for instance, has been added to the list of products that the European Union has to monitor due to the negative effects it has even at low doses (Ebele et al., 2017).

Primary control at the source of pollutant discharge into the environment by recycling or raw material substitution is one of the most effective solutions to reduce pollution load, albeit it is not



always practical (Padoley et al., 2008) . As a result, there is a lot of interest in creating a treatment process that can effectively and affordably remove pharmaceuticals from water. According to several studies (de Jesus Gaffney et al., 2017a; Pojana et al., 2011), NSAID have been discovered in the effluent of several WWTPs. Physical, chemical, and biological treatment methods that are frequently used for the removal of pharmaceuticals are ineffective and unable to completely remove NSAIDs. More effective and targeted treatment methods are needed to eliminate NSAIDs from water and wastewater. Therefore, certain new treatment technologies have been introduced to improve the efficacy of water and wastewater treatment for the elimination of NSAIDs. Advanced oxidation processes (AOPs), adsorption on activated carbon, membrane filtering, photooxidation, and electrooxidation are some of these technologies. However, these techniques have a number of shortcomings, such as low effectiveness, the production of toxic by-products, excessive costs, and/or protracted processing periods (de Jesus Gaffney et al., 2017b; Paíga et al., 2016; Pojana et al., 2011).

As a result, one of the objectives of the present investigation was to determine if the enzymatic treatment of two NSAIDs is feasible as a cost- and environmentally friendly alternative strategy for addressing the shortcomings of conventional methods. Diclofenac and aceclofenac have been selected in this case. They are structurally similar and have been widely used as non-steroidal anti-inflammatory drugs (NSAIDs) to treat painful and inflammatory processes throughout the world, however their residues persist in water (Chaves et al., 2020; Gumbi et al., 2017; Praveena et al., 2018; Yao et al., 2018).

Enzymatic treatment combines elements of physical, chemical, and biological processes by using a biological catalyst for a chemical reaction. Peroxidase is an oxidoreductase enzyme that originates from living organisms like plants. It catalyses the transformation of pollutants into free radicals in the presence of hydrogen peroxide as an oxidant, much like AOPs, which are based on the generation of free radicals like hydroxyl ( $\bullet\text{OH}$ ) that can attack the organic molecules. Free radicals can couple non-enzymatically to create insoluble oligomers, which can subsequently be eliminated using filtration or sedimentation (Cordova Villegas et al., 2018).

Enzymatic treatment has several benefits, including the ability to work with a variety of specific chemicals, treating bio-refractory chemicals, operating over wide temperature, pH, salinity, and substrate concentration ranges, reducing sludge volume, having no shock loading effect, having

no start-up and shut-down delays, it is fast and easier to control than conventional biological processes (Caza et al., 1999; Mantha et al., 2001; Taylor et al., 1995). Enzymatic treatment can be used as the primary treatment or in conjunction with a biological unit as a bioremediation technique for wastewater that has shown its capacity to remediate resistant pollutants (Steevensz et al., 2014). As a result of its minimal energy and chemical usage, it is environmentally beneficial (Watanabe et al., 2011). Like any other form of treatment, the enzymatic one has inherent downsides, such as the potential for enzyme inactivation and issues with cost and availability (Steevensz et al., 2014, 2015).

## **1.2 Research gap**

It is evident that diclofenac and aceclofenac pose negative impacts to eco system and human through contamination. Yet, there were no efficient treatment methods for the removal of diclofenac and aceclofenac. Furthermore, there is no published literature available on polymerization products of diclofenac and aceclofenac using any peroxidase.

However, Younes et al. (2019) used laccase for diclofenac transformation using enzymatic treatment method. Similarly, chloroperoxidase was used for diclofenac treatment in synthetic wastewater by Onaizi et al. (2020). There was no literature for the removal of aceclofenac using enzymatic treatment methods.

Steevensz et al (2014), Zhang et al (2019), Mukherjee et al (2019) and Ziayee Bideh (2021) have used SBP for enzymatic treatment methods as the enzyme to treat compounds such as phenol, halogenated benzonitrile pesticides, methylenedianiline, and azo dye. The removal percentage has been identified as over 95% in all these scenarios. Even though it is distinct that SBP has a potential to achieve higher removal percentage of emerging contaminants, a clear knowledge gap in literature is identified in terms of using SBP as the enzyme for diclofenac and aceclofenac treatment.

## **1.3 Research objectives**

This study intends to examine the viability of treating two chosen compounds, diclofenac and aceclofenac, with soybean peroxidase (SBP) in synthetic wastewater (structures and chemical formulae are given in Table 1-1). The following are the sub-objectives of this research.

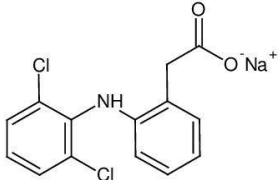
1. Optimize the catalyzed removal of the diclofenac and aceclofenac using soybean peroxidase (SBP).
2. Utilize mass spectrometry to identify potential enzymatic reaction transformation products (MS).

#### 1.4 Scope

The scope of the study included:

1. Examine the viability of employing SBP-catalysis in the presence of hydrogen peroxide to remove diclofenac and aceclofenac from water at about tenth-mM concentrations.
2. Determining the ideal reaction conditions, about pH, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) concentration, and SBP activity, for more than 95% conversion of diclofenac and aceclofenac with SBP.
3. To determine whether the enzymatic treatment was successful in eliminating the substances, use high-performance liquid chromatography (HPLC) detection techniques for the substrates.
4. By observing the time course of substrate consumption, find the initial first-order rate constants and half-lives of the substrates.
5. Utilizing high resolution mass spectrometry, determine whether enzymatic treatment could have resulted in the creation of oligomers (MS).

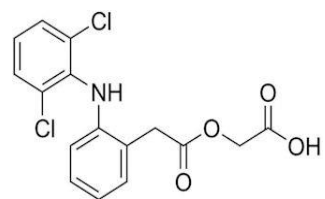
Table 1-1 Name, formula, and molecular structure of the chemicals studied

Chemical	Formula	Molecular Structure
<b>Diclofenac Sodium Salt</b>	C <sub>14</sub> H <sub>10</sub> Cl <sub>2</sub> NNaO <sub>2</sub>	

---

**Aceclofenac**

$C_{16}H_{13}Cl_2NO_4$



## CHAPTER 2 LITERATURE REVIEW

This chapter includes two key subjects. In the first section, the investigations of the non-steroidal anti-inflammatory drugs, diclofenac (DCF) and aceclofenac (ACF), including their properties, uses, concentrations in water, and toxicity, are covered to show why these compounds need to be treated. In the second section, the mechanism, benefits, and current applications of soybean peroxidase are investigated in order to assess its potential for eliminating non-steroidal anti-inflammatory medicines.

### 2.1 Compounds

#### 2.1.1 Diclofenac

Diclofenac is a NSAID that can be taken orally or applied topically. It belongs to the class of phenylacetic acid which includes secondary amino groups, phenyl acetic groups, and phenyl rings with two *ortho*-chloro groups. This class of drugs has been widely used as the mainstay for the therapeutic effects of pain, inflammation, and fever (Sayen et al., 2013). Its name “diclofenac” has been derived from its chemical name 2-(2,6-dichloroanilino) phenylacetic acid. It is sold under variety of commercial names such as Acoflam, Algosenac, Almiral, Ana-Flex, Anthraxiton, Antiflam, Arcanafenac, Arthrex, Arthrifen, Arthtotec, Diclabeta, Diclac, Dicloabac, Diclodoc, etc.(Vieno & Sillanpää, 2014). In Canada, DCF is sold with the name of Voltaren Emulgel. The physical and chemical properties of DCF, an off-white crystalline odourless powder, are listed in Table 2-1.

Table 2-1. Chemical and physical properties for Diclofenac sodium salt

<b>parameter</b>	<b>value</b>	<b>Reference</b>
Chemical Formula	$C_{14}H_{10}Cl_2NO_2$	SRC (2013)
Molecular Mass (g/mol)	381.13	SRC (2013)
Water Solubility (mg/L)	2.37	SRC (2013)
pK <sub>a</sub>	4.15	SRC (2013)
Log K <sub>ow</sub>	13.4	FELE ZILINIKCET (2007)

The log K<sub>ow</sub> (octanol-water partition coefficient) of diclofenac is around 13.4 at pH 7.4, which is used to measure the hydrophilicity/lipophilicity of a compound (Alessandretti et al., 2021). The

hydrophilicity/lipophilicity of a substance can help determine how harmful it is to living organisms or the environment, as well as its potential for bioaccumulation (Cumming & Rucker, 2017). Log  $K_{ow}$  values range from  $-3$  (extremely hydrophilic) to  $+10$  (extremely hydrophobic) (Cumming & Rucker, 2017). The US EPA considers log  $K_{ow}$  values greater than 4 to be harmful to aquatic life, while log  $K_{ow}$  for DCF is approximately 13.4 (Alessandretti et al., 2021). Therefore, substances with high log  $K_{ow}$  values prefer to be absorbed by the soil, whereas substances with low log  $K_{ow}$  values tend to remain in the water. However, depending on the binding force, these molecules may be remobilized (Karaman et al., 2012). The relative acidity or basicity of weakly ionising chemicals in aqueous solvent solutions or the miscibility between solute and solvent are assessed using the dissociation constant ( $pK_a$ ). DCF has a  $pK_a$  of 4.15, which indicates that at  $pH < 4.15$ , the predominant species is protonated (neutral), while at  $pH > 4.15$ , the majority form is deprotonated (monoanionic).  $pH$  can affect the effectiveness of some DCF removal techniques (de Rossi et al., 2020). Diclofenac has a poor water solubility and is affected by the  $pH$  of the solution due to the compound's  $pK_a$ . Therefore, at  $pH$  values below the  $pK_a$  (4.15), DCF takes on a neutral state and then has a decreased solubility, which results in DCF precipitation under these circumstances.

Since the 1970s, diclofenac has been used as an analgesic for topical administration in humans. In some nations, it has also been utilised as an all-purpose veterinary drug for domestic livestock (Van Trant et al., 2020). It can be administered orally or topically to alleviate inflammation and pain caused by diseases, and it virtually entirely undergoes biotransformation in the human body. It can be consumed for the treatment of a variety of ailments such as dysmenorrhea, severe migraine, and inflammatory syndromes, which may include arthritis, rheumatoid arthritis, polymyositis, spondylarthritis, and gout attacks (Meinicke & Danneskiold-Samsøe, 2009). Diclofenac is also crucial for treating some moderate muscular pain disorders as well as post-operative pain (which is brought on by inflammation). It is among the most popular NSAIDs worldwide and has a market share that is comparable to that of naproxen, ibuprofen, and mefenamic acid (Y. Zhang et al., 2008). For the last 35 years, diclofenac is permitted in 120 countries and is placed at 30th among the list of top 200 drugs now (Meinicke & Danneskiold-Samsøe, 2009). The precise global consumption of diclofenac is difficult to calculate because of several reasons, such as use of different trade names for DCF, use for human and veterinary purposes and that the drug is an over-the-counter drug. The current trend indicates that DCF

consumption will continue to rise in North America due to the prevalence of lifestyle disorders like arthritis and heart diseases as well as the need for medications like painkillers among an ageing population. The exact annual consumption of diclofenac in North America is not available however in the United States, DCF makes for roughly 5-6% of the whole NSAID market. In Canada, DCF accounts for 17% of NSAID use (Henry, 2013). The estimated annual consumption of DCF for the entire continent of Europe is 179.8 tonnes. (Ferrari et al., 2003). For most of the Asian and African countries, data on consumption of DCF is not available but from the frequent reports on toxicological effects observed in these countries on vultures, it is conceivable that the consumption might be enormous. Recent studies based on IMS health data (which serves 82% of the global population) from 86 countries estimated that at present on an average  $1443 \pm 58$  tons of DCF is consumed globally. However, this is only an indication on the consumption of DCF for human health related applications and does not include the consumption of DCF for veterinary uses (Acuña et al., 2015).

Diclofenac is one of the most often used non-steroidal anti-inflammatory drugs in the world. Due to its poor degradation and greater consumption rates, this medication is frequently found in surface water bodies and wastewater effluent treatment facilities in levels on the range of  $\mu\text{g/L}$  (Fatta-Kassinos et al., 2010; Zorita et al., 2009). Diclofenac has been found in concentrations of 0.5 ng/L to 177.1 ng/L in North America, 460 ng/L to 3300 ng/L in Europe, and 8.8 ng/L to 127 ng/L in Asia and Australia in wastewater effluent, whereas in surface water it was 11 ng/L to 82 ng/L in North America, 21 ng/L to 41 ng/L in Europe, and 1.1 ng/L to 6.8 ng/L in Asia and Australia. In Spain, the highest concentration detected was 810 ng/L in surface water. DCF is among the medicines that is most frequently found in the effluents of municipal wastewater treatment facilities (Verlicchi et al., 2012). Due to its poor removal after treatment, effluent concentrations rarely drop below the detection limits of few nanograms per liter when analyzed using LC-MS/MS or GC/MS. The mean values of the effluent range from 0.002 to 2.5  $\mu\text{g/L}$ , whereas the maximum concentrations range from 0.12 to 4.7  $\mu\text{g/L}$ . According to Verlicchi et al. (2012), out of 73 pharmaceuticals examined, DCF had the eighth highest average mass load in the secondary effluent of municipal wastewater treatment plants. The average DCF content in surface water is generally less than 100 ng/L. In some investigations, it has been shown to be higher, although still generally lower than 500 ng/L (Hernando et al., 2006; Hilton & Thomas, 2003; Kim et al., 2007; Lin et al., 2005; Metcalfe et al., 2003; Öllers et al., 2001; Rabiet et al., 2006). Among

NSAID pharmaceuticals, DCF was found in the highest surface water concentration at 2.20 ug/L in the Llobregat River in Spain (Acuña et al., 2015). Sim et al. (2011), indicated that in South Korea, the maximum concentration of diclofenac in hospital wastewater was about 6.88 µg/L and in pharmaceutical manufacturer's wastewater 203 µg/L, both of which were much higher than the concentrations typically found in municipal wastewater. Also, Zorita et al in 2009 demonstrated similar concentration in both hospital and municipal wastewater, around 0.2 µg/L. Municipal wastewater concentrations represent the consumption of DCF by the residents connected to the sewer system. Between and within nations, there are significant differences in consumption rates. This makes it difficult to determine typical wastewater concentrations (Carballa et al., 2005).

The presence of diclofenac in aquatic habitats in tiny amounts ranging from the ng to µg per litre can negatively impact the health, behavior, reproduction, and survival of organisms of different trophic levels such as algae, bacteria, microcrustaceans and fish (Kwak et al., 2018; Pandelides et al., 2021; Xiong et al., 2020). Diclofenac contamination in water poses concerns to human health in addition to threats to aquatic wildlife and flora (Sellami et al., 2022) Moreover, the health of human beings and animals may be indirectly impacted by the increase in antimicrobial resistance in pathogenic strains of diverse bacteria. It has been discovered that diclofenac has a more chronic toxicity than acute toxicity (Ferrari et al., 2003). The DNA damage, alterations on the oxidative stress enzymes, effects on cyto- and genotoxicity, haematological alteration, impacts on behaviour, and changes in the expression of numerous genes involved in metabolism and defence were among the main effects of diclofenac exposure that were described. Recent studies have addressed the effect of diclofenac toxicity in several species. Diclofenac's impact on ostracods and barley seedlings was examined in a recent study by Pawłowska et al., (2021). They discovered that ostracods are negatively affected by diclofenac. Diclofenac toxicity has also been demonstrated in several studies in species including rats, brown trout, and sea urchins. These effects included an increase in mortality, damage to tissues and biochemical functions, as well as gastrointestinal lesions and interference in growth rate. It is well known that DCF causes fatally destructive effects in the gastrointestinal and renal tissue of many fish. Hoeger et al., (2005), indicated that 21 days of exposure to DCF caused telangiectasia of the gills, a rise in the number of monocytes in the liver, and the usual histological abnormalities seen in the trunk kidney. Furthermore, it has been shown that following a prolonged exposure to this medicine, *rainbow trout* developed hyaline inclusions and cell necrosis in the kidney, as well as inflammatory cell foci and an increase in



basophils in the liver. DCF was the primary cause of population of three Gyps vulture species (*Gyps bengalensis*, *Gyps indicus*, *Gyps tenuirostris*) which were severely harmed, decreased by 98% in the Indian subcontinent, and were included to the IUCN's list of "critically endangered" species (Das et al., 2011). After these consecutive incidents in the first decade of 21st century, DCF received considerable international attention and was included in a watch list of compounds for widespread monitoring by the European Union as well as the priority list of the Water Framework Directive (WFD) of the European Union. According to the majority of research, aquatic animals may experience some negative consequences from ongoing exposure to DCF, even at very low doses. According to estimates from Sim et al., (2011), the no-effect concentration of DCF was 0.1 mg/L, which is extremely high when compared to concentrations seen in aquatic systems and in actual environmental conditions. Surprisingly, studies from Canada reported that DCF was a major risk even at predicted environmental concentrations (10–100 ng/L<sup>-1</sup>) (Lawrence et al., 2007). These studies in river biofilm communities showed the major impacts of DCF on community structure and function even at concentrations as low as 100 ng L<sup>-1</sup>. Additionally, In the environmental waters, DCF has been demonstrated to rapidly phototransform into several products (Svanfelt, n.d.). Not only aquatic organisms may be at risk from DCF itself but also its environmental transformation products can harm them.

#### 2.1.2 Aceclofenac

Aceclofenac is a NSAID derived from phenylacetic acid with distinct analgesic and anti-inflammatory characteristics. It is a major NSAID analogue of Diclofenac. It is a potent inhibitor of cyclooxygenase (COX), a key enzyme in the synthesis of prostaglandins and thromboxanes with selectivity for the COX-2 over COX-1 isoform. The physical and chemical properties of ACF, a white crystalline powder, are listed in Table 4.

Table 2-2 Chemical and physical properties for Aceclofenac

Parameter	Value	Reference
Chemical Formula	C <sub>16</sub> H <sub>13</sub> Cl <sub>2</sub> NO <sub>4</sub>	Celiz et al., 2009
Molecular Mass (g/mol)	354.2	Celiz et al., 2009
Water Solubility (mg/L)	0.0531	Pub Chem
pK <sub>a</sub>	2.60	Celiz et al., 2009
Log K <sub>ow</sub>	4.16	Celiz et al., 2009

Aceclofenac received its initial EU approval in 1990 and was introduced in Spain in 1992. Since then, it has received approval for usage in 69 nations throughout the world, with an estimated 171 million individuals being treated as a result. Although the authorized indications of Aceclofenac differ between nations, in general, it is used to treat inflammatory and painful conditions like osteoarthritis (OA), rheumatoid arthritis (RA), and ankylosing spondylitis as well as low back pain (LBP), odontalgia, scapulohumeral periartthritis, and extraarticular rheumatism (Iolascon et al., 2021)

To our knowledge there is no literature on investigating or monitoring aceclofenac, but its great potential of leaching into water bodies and high amount of consumption in the world cannot be ignored. Some studies suggest aceclofenac may be hazardous to both aquatic and terrestrial creatures, including mussels and Gyps vultures because of its potential to be present in the environment, considering that it has a similar structure to diclofenac, which is persistent in the environment (Celiz et al., 2009). However, data currently available are inconclusive and additional studies are required to better assess the fate and toxic effects of aceclofenac. To date, no literature has been found on the detection of aceclofenac in either wastewater or surface water.

## 2.2 Enzymatic Treatment

Treatment methods that are most frequently used for removal of emerging contaminants are physiochemical and biological methods. For the treatment of wastewater, physiochemical approaches such chemical oxidation, distillation, membrane-based separation technologies, and adsorption have been used.(Alshabib & Onaizi, 2019) . These strategies are very expensive or take a long time to complete, have low removal efficiency, and/or may even lead to further pollution and damage (Villegas et al., 2016). Biological methods have been used successfully for

removal of contaminants from wastewater. These processes are more environmentally benign and have several benefits over physicochemical methods, including lower costs, less energy usage, less disruption, and/or applicability to low pollutant concentrations (Al-Maqdi et al., 2017; Morsi et al., 2020). These techniques used plants, microorganisms, and enzymes for wastewater treatment (Alshabib & Onaizi, 2019). Organisms used in these technologies could ingest or degrade pollutants as they are being processed. However, plants and microorganisms are sensitive to some toxic pollutants from wastewater. Microorganisms may not be able to flourish in harsh environmental conditions, are incapable of treating high concentrations of pollutants and may take more time to treat some harmful chemicals from wastewaters (Ebele et al., 2017; Morsi et al., 2020), whilst enzymes could function quickly and selectively. An enzyme is a powerful biocatalyst that can degrade compounds, especially under mild conditions. (Yao et al., 2018).

Enzymes are highly specific biological catalysts which can effectively eliminate a range of target contaminants up to the satisfactory limit. They can be used for a variety of contaminants with high and low concentrations and have several benefits, including effectiveness over a wide pH, salinity, and temperature range, reduced sludge volume, short contact time, no restrictions due to shock loading or accumulation of biomass (Al-Ansari et al., 2009; Karam & Nicell, 1997; Morsi et al., 2020; Steevensz et al., 2015). Enzymes have specific active sites, ones which are able to bind with particular substrates and reduce the activation energy by this approach during enzymatic reactions. As a result, these processes have high reaction kinetics and specificity. Enzymes could also reduce the time needed for substrates transfer, which makes these processes more effective (Mishra et al., 2020; Varga et al., 2019). Enzymatic processes have better reaction kinetics than conventional chemical processes and require less water and energy (Kalia et al., 2013; Summerscales, 2021).

In contrast to bacteria and other biological processes, enzymes are not affected by competition from other lifeforms (Demarche et al., 2012). Therefore, enzymatic methods are promising for eliminating recalcitrant contaminants from wastewater, such as organic micropollutants (Feng et al., 2013). The enzymatic method has extremely low capital costs; hence, a major portion of the total cost is the cost of enzyme, which is the main drawback of enzymatic treatment process. The enzyme cost may be greatly decreased by increasing treatment effectiveness (enzyme turnovers) or by utilising a less expensive enzyme (Karam & Nicell, 1997). The enzyme's relatively limited catalytic life, caused by enzyme inactivation, is a second disadvantage of enzymatic treatment.

Peroxidases can become inactivated by suicide mechanisms in the presence of excess hydrogen peroxide and low concentration of reducing substrate (Steevensz et al., 2014). A reversible intermediate enzyme-H<sub>2</sub>O<sub>2</sub> can be produced with the generation process of Compound I, which may irreversibly further convert to inactive intermediate P-670 (absorbance peak at 670 nm) (Arnao et al., 1990). Also, peroxidases can be inactivated, when the reversible accumulation of compound III (an inactive form of enzyme) in the presence of excess H<sub>2</sub>O<sub>2</sub> through oxidation of Dimers 21 compound II; although compound III can decay back to the native form of enzyme, the rate of decomposition is very low (Valderrama et al., 2002). In addition, end-product inactivation by polymerized substrate which adsorbs peroxidase, can also leads to peroxidase inactivation (Feng et al., 2013). In addition, free radicals produced by the substrate can bind to the enzyme's active site heme (Klibanov et al., 1983). In addition, the possibility of forming hazardous by-products during the treatment process is another disadvantage of the enzymatic method, unless the products precipitate and are removed (Steevensz et al., 2009, 2014).

### 2.2.1 Application of Enzymes in Wastewater Treatment

In 1930, the first investigation into the enzymatic treatment of wastewater was put forth (Munnecke, 1976). Since then, other researchers have investigated how enzymatic treatment technology can be used to remove a wide variety of contaminants from aqueous mixtures, including phenols, anilines, synthetic colours, medicines, personal care products, and many others (Bilal et al., 2018; Mashhadi, Taylor, Jimenez, et al., 2019; Steevensz et al., 2013).

For this purpose, oxidoreductases primarily have been employed. These oxidoreductases can be classified into two groups: peroxidases and oxidases. While oxidases, such as laccase, use molecular oxygen for catalyzing the oxidation of aromatic pollutants, peroxidases, such as horseradish peroxidase (HRP), soybean peroxidase (SBP), and *Arthromyces ramosus* peroxidase (ARP), catalyse the oxidation of aromatic pollutants in the presence of hydrogen peroxide as the oxygen source. Laccases and peroxidases are the most common oxidoreductases for the degradation of organic pollutants and emerging contaminants from water and wastewater (Alneyadi et al., 2018; Bilal et al., 2018; Morsi et al., 2020). In 1979, Bollag and co-workers used laccase to polymerize 2,6-dimethoxyphenol (Bollag et al., 1979). Klibanov et al., (1983) used horseradish peroxidase to polymerize the phenols and anilines found in industrial wastewater.

### 2.2.2 Oxidases

In 1883, Yoshida first identified laccases as a component of the resin ducts of the lacquer tree *Rhus vernicifera* (Riva, 2006). They belong to a class of multi-copper oxidases that catalyze single-electron oxidation of a substrate along with reduction of oxygen to water. Amines and phenols are frequently used as substrates by these enzymes. Removal of wide range of compounds have been reported to be catalyzed by laccases (Bilal et al., 2018; Chagas et al., 2015; Morsi et al., 2020). In 2018 Naghdi et al demonstrated effective enzymatic removal of one of the most widely used medications, carbamazepine, from synthetic wastewater catalyzed by laccase in the presence of redox mediators. (Ncanana & Burton, 2007) studied the role of laccase from the white rot fungus *Trametes pubescens* in enzymatic polymerization of 8-hydroxyquinoline. Alharbi et al. (2019) reported the degradation of diclofenac, trimethoprim, carbamazepine, and sulfamethoxazole by laccase from *Trametes versicolor*. Laccase also used for removal of diphenylamine from synthetic wastewater by Saha et al (2006). Laccase from the white rot fungus *Coriolopsis polyzona* was examined by Cabana et al. (2017) for degradation of the endocrine disrupting chemicals nonylphenol and Bisphenol A and the personal care product ingredient, Triclosan. In addition, the ability of a fungal laccase to break down a mixture of 18 endocrine disruptors, pharmaceuticals, and personal care products in both a model solution and real wastewater gathered from a municipal wastewater treatment plant was tested (Spina et al., 2015). Laccases are relevant to the enzyme of focus in this thesis, a peroxidase, because they function by oxidizing the organic substrate to a free radical, as do the peroxidases.

### 2.2.3 Peroxidases

Peroxidases are oxidoreductase enzymes, mainly heme proteins, which are found in bacteria, fungi, algae, plants, and animals (Jun et al., 2019a). In comparison to animal peroxidases, plant peroxidases have been widely used in wastewater bioremediation (Raven & Dunford,2015).The peroxidases have further been sub-divided into three classes based on their sequence homologies. Intracellular peroxidases are in Class I, for example cytochrome c peroxidase and ascorbate peroxidase. Fungal peroxidases are in Class II, such as manganese 15 and lignin peroxidase and secretory plant peroxidases are in Class III that include HRP, SBP, turnip peroxidase (TP), bitter gourd peroxidase (BGP), potato pulp peroxidase and ginger peroxidase (Demarche et al., 2012; Jun et al., 2019a; Padoley et al., 2008)

Due to their capacity to catalyse the oxidation-reduction reaction of a variety of phenolic and aniline substrates, aromatic compounds, dyes, and non-phenolic aromatics in the presence of hydrogen peroxide, broad distribution, high redox potential, substrate specificity, and high thermal stability, peroxidases have been widely used in the remediation of environmental contaminants from wastewater (Jun et al., 2019b; Mashhadi, Taylor, Jimenez, et al., 2019; Steevensz et al., 2013, 2015; Villegas et al., 2016). Plant peroxidases have been employed extensively for wastewater bioremediation compared to animal peroxidases (Raven and Dunford, 2015). Enzyme-catalyzed oxidative polymerization, without any apparent degradation, transforms water-soluble hazardous organic chemicals into less water-soluble or insoluble polymers. Therefore, the enzymatic technique is the opposite of conventional biological treatment: the biological approach breaks down the target chemical, whereas the enzymatic approach builds up the target compound through oxidative polymerization (Saha et al., 2008). H<sub>2</sub>O<sub>2</sub> is used as a co-substrate by peroxidases to catalyse a variety of organic and inorganic compounds. They can effectively treat resistant pollutants in water because of their appealing biocatalytic properties, including high substrate specificity, high thermal stability, and high redox potential (Chiong et al., 2016; Mukherjee et al., 2018; Steevensz et al., 2013). Peroxidases have been employed for degradation of various emerging contaminants. Almaqdi et al., (2019) used 5 distinct peroxidases (SBP, chloroperoxidase (CPO), lactoperoxidase, manganese peroxidase (MnP) and HRP) to work on the treatment of a mixture of 21 emerging contaminants with a concentration of 2 ppm for each of them in a synthetic wastewater. The enzymatic treatment of synthetic wastewater containing two phenolic surfactant breakdown products, nonylphenol and octylphenol, two antimicrobial agents, Triclosan and sulfamethoxazole and three phenolic steroids was studied by Mashhadi, et al. (2019). Also, Mashhadi, et al (2019) investigated the enzymatic treatment of synthetic wastewater containing 0.5 or 1 mM of 3-HQ and 3-AQ. In another study, some selected azoles (imidazole, 2-AI, 2-ABI, pyrazole, 3-AP, 4-AAP, triazole, HOBT, atrazine, amitrole, trimethoprim, thiophene, benzothiophene, thiazole, benzothiazole, 2-AT, 2-ABT and 2-HBT, pyrrole, indole, indazole, furan) that are an important class of small molecules in pharmaceuticals and in pesticides and found in surface water and effluents of wastewater treatment plants, were studied by Mashhadi et al. (2019).

The mechanism of peroxidase-catalyzed pollutant removal is a modified ping-pong mechanism (Steevensz et al., 2014). The first step (Equation 1) is the oxidation of native peroxidase (Fe<sup>3+</sup>) by

hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), the electron acceptor, resulting in an active form, compound I (Fe<sup>4+</sup>–R<sup>+</sup>). Second, Compound I oxidize a substrate molecule (AH) by one electron to become Compound II (Fe<sup>4+</sup>) and generate a substrate radical (A<sup>•</sup>) (Equation 2). The substrate can be a phenol or aniline. Then Compound II oxidizes another molecule of the substrate and generates the second free radical and returns to the native enzyme (Equation 3). The total reaction balance can be described in Equation 4 (Krainer & Glieder, 2015; Mashhadi, et al., 2019). The cycle is illustrated in Figure 2-1.

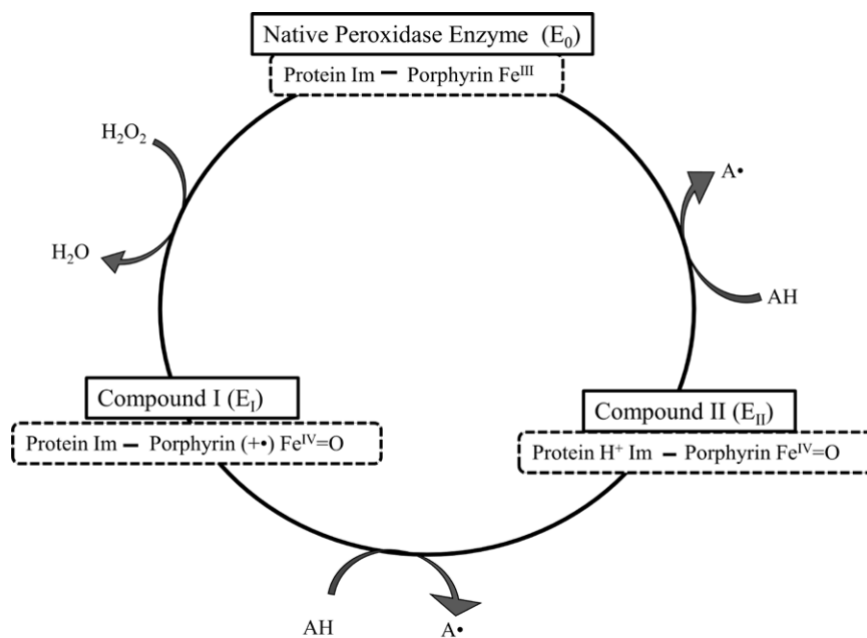
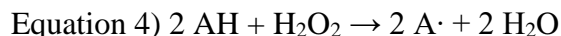
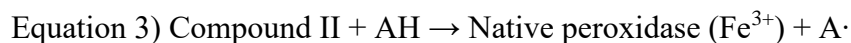
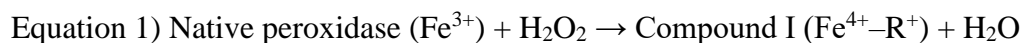


Figure 2-1. Proposed Mechanism for peroxidases. (Adopted from (Dunford, 1999))

One of the problems with using peroxidases is inactivation. There are three potential pathways for deactivating peroxidases. The first kind of inactivation is due to the free radicals that are produced during the catalytic process, irreversible inactivation, a type of suicide inhibition. Secondly, a

reversible intermediate enzyme-H<sub>2</sub>O<sub>2</sub> can be produced with the generation process of Compound I, which may be irreversibly further converted to inactive intermediate P-670 (absorbance peak at 670 nm) with excess peroxide (Arano et al., 1990). A third route to inactivation can be as a result of end-product polymers that are created during the catalytic process. When they go above their solubility limit, they may co-precipitate with adsorbed enzyme (Feng et al., 2013). The fourth inactivation, also due to excess hydrogen peroxide, is the formation of Compound III from the oxidation of Compound II with extra H<sub>2</sub>O<sub>2</sub>. Compound III can decay back to the native enzyme, but the low rate of decomposition allows accumulation of Compound III, a reversibly inactive enzyme form (Krainer & Glieder, 2015). Inactivation leads to a shorter catalytic lifetime and a higher cost of enzyme requirement for the enzymatic reaction.

#### 2.2.4 Soybean Peroxidase

Soybean seed coat peroxidase (SBP) is an anionic glycoprotein that exists as a single isozyme in the hulls and has a molecular weight and isoelectronic point (pI) of 37 kDa and 4.1, respectively (Rezvani et al., 2015; Ryan et al., 2006). Soybean peroxidase belongs to the class III secretory plant peroxidases where its classification is based on the amino acid sequence, three-dimensional structure, and biological function. SBP is an inexpensive agricultural by-product of soybean seed hulls (seed coats), the major by-product of the soybean industry and utilized for animal feed (Chagas et al., 2015; Kumar et al., 2019). It has been found in the soybean's root, leaves, and seed hulls. The extraction process of SBP starts with softening the soybean seed hulls by soaking them in water for one to two hours, the soybean seed hulls are subsequently washed with water to extract the SBP while retaining the hulls' feed value (Steevensz et al., 2014). One of the main crops in North and South America is the soybean crop. According to reports, the world will produce 385 million metric tonnes (MMT) of soybeans in 2021–2022, Figure 2-2 (USDA, 2022). Agriculture and Agri-Food Canada (2022) reports that over the previous 15 years, soybean production and seeded area have increased steadily. A report in 2021 gives the cost of soybeans as between \$395 and \$425 per tonne (Agriculture and Agri-Food Canada, 2021). SBP can be extracted from soybean hulls, which are less expensive and are predicted to cost \$125 per tonne (Mukherjee et al., 2018). Due to its inexpensive and plentiful source, SBP may be easily obtained, creating a fantastic opportunity for SBP's commercial application. In addition to being economically accessible, SBP has shown other benefits that considerably increase its viability for use in treating wastewater compared to other peroxidases. According to Bódalo et al., (1991), SBP is more potent



than HRP because it has a higher  $k_{cat}/K_M$  constant (specificity constant, a measure of enzyme efficiency) and is less susceptible to being inactivated by  $H_2O_2$ . A technique based on SBP can be thought of as being of low energy demand and chemically effective (Demarche et al., 2012). Additionally, the SPB has a greater active pH range, pH 2.0 to 10.0 (Ryan et al., 2006). It also has better thermal stability than HRP, enabling SBP to continue operating at higher temperatures (70 °C) without experiencing secondary structural changes (McEldoon & Dordick, 1996).

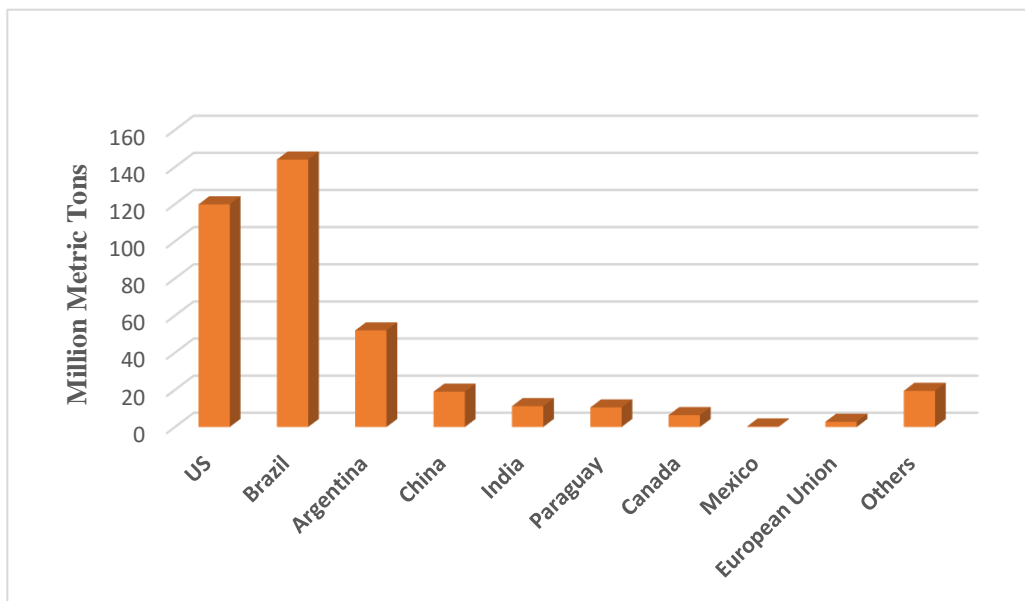


Figure 2-2. Global Soybean Production 2021-2022 according to USDA (2022)

SBP has demonstrated the ability to catalyse the oxidation of a variety of different compounds. Mashhadi et al. (2019a) assessed the viability of SBP for the treatment of nitrogen containing heterocyclic aromatics, as well as their amino- and hydroxy-derivatives. For example, at pH optima of 5.6 and 8.6, respectively, 94% of 3-amino and 3-hydroxyquinoline were removed. In the presence of 0.75 mM  $H_2O_2$  and 0.002 U/mL SBP at pH 7.0, Ziayee Bideh et al., (2021) demonstrated that SBP could effectively treat 3-hydroxyxoumarian (95%) at the lab scale. Enzymatic treatment of sulfamethoxazole, a heterocyclic antimicrobial agent, along with other antimicrobials, phenolic steroids and phenolic surfactants as micropollutants has also been investigated using SBP, where the removal efficiency of sulfamethoxazole was 80% under the optimal conditions (Mashhadi et al., 2019). Table 2-3 summarizes various substrates that were treated by using SBP (Bideh et al., 2021; Kumar et al., 2019; Morsi et al., 2020).

Table 2-3 Summary of efficiencies of various substrates with SBP in synthetic wastewater  
(Sources: Bideh et al., 2021; Kumar et al., 2019; Morsi et al., 2020)

Substrate	Substrate concentration (mM)	Optimal Conditions				Reference
		pH	Activity SBP (U/mL)	H <sub>2</sub> O <sub>2</sub> (mM)	Removal %	
Triclosan	0.01	7.0	0.1	0.01	98	Li et al. (2016)
Ioxynil	0.1	4.0	0.3	1.0	>95	Zhang (2019)
Bromoxynil	0.5	4.0	0.9	1.0	>95	Zhang (2019)
Nonylphenol	0.023	6.0	0.003	0.05	≥95	Mashhadi et al. (2019a, 2019b)
Sulfamethoxazole	0.2	1.6	4.0	0.5	80	Mashhadi et al. (2019a, 2019b)
Octylphenol	0.024	6.0	0.002	0.04	≥95	Mashhadi et al. (2019a, 2019b)
17β-Estradiol (E2)	0.039	7.0	0.12	0.08	≥95	Mashhadi et al. (2019a, 2019b)
Pyrrole	1.0	1.6	5.0	1.0	85	Mashhadi (2019)
3-Aminoquinoline	1.0	5.6	4.5	2.0	89	Mashhadi et al. (2019a, 2019b)
3-Hydroxybenzotriazole	1.0	3.6	0.12	1.25	>95	Mashhadi et al. (2019a, 2019b)
3-Aminopyrazole	1.0	6.0	3.0	1.5	70	Mashhadi (2019)
HOBt	1.0	3.6	0.12	1.25	>95	Mashhadi (2019)
4,4-methylenedianiline	0.5	6.0	0.7	0.7	>95	Mukherjee et al., (2019)
4,4-thiodianilin	0.5	6.0	0.15	0.5	>95	Mukherjee et al., (2019)
3-Hydroxycoumarin	0.5	7.0	0.002	0.75	95	Ziayee Bideh et al. (2021)
2-Aminobenzoxazole	0.1	6.0	3.5	0.25	45	Ziayee Bideh et al. (2021)

In addition, SBP has demonstrated its oxidation strength in real wastewater. More than 95% of the phenol in industrial effluent (alkyd resin manufacture) was successfully converted by Stevensz et al (2014 b). Chagas et al., (2015) looked at the treatment of caffeic acid from synthetic wastewater and wastewater from the coffee-processing industry using both free and immobilised SBP. They found that while both forms of the enzyme could oxidize the target substance, immobilised SBP required a reaction time that was twice as lengthy. Other compounds such as azo dyes, dye-derived arylamines, and quinolines also underwent enzymatic treatment utilising SBP (Mashhadi, Taylor, Biswas, et al., 2019; Mukherjee et al., 2019).

## CHAPTER 3 MATERIAL AND METHODS

### 3.1 Materials

#### 3.1.1 Substrates and Enzymes

Diclofenac and aceclofenac were purchased from Sigma Aldrich Chemical Company Inc. (Oakville, ON) and Abcam respectively with >98% purity. Both chemicals were stored at room temperature.

Crude solid SBP (activity 5 U/mg) was obtained from Organic Technologies (Coshocton, OH). Solid bovine liver catalase (activity 19,900 U/mg) was from Sigma Aldrich). Both Solid enzymes were kept at – 15 °C while their aqueous solutions were stored at 4°C.

#### 3.1.2 Buffer and solvents

Sodium phosphate, monobasic and dibasic, sodium acetate, potassium chloride, hydrochloric acid, anhydrous ethanol, iso-propanol and acetonitrile (ACN) were obtained from ACP Chemicals Inc (Montreal, QC). Citric acid was obtained from Sigma Aldrich.

#### 3.1.3 Other chemicals

Hydrogen peroxide (30% w/v) was obtained from ACP Chemicals Inc. and was stored at 4 °C. Phenol (99% pure) was obtained from Sigma Aldrich. 4-Aminoantipyrine (4-AAP) was purchased from BDH Inc. (Toronto, ON) and was kept at room temperature. All other chemicals used in this study were analytical grade and purchased either from Sigma Aldrich or BDH Inc.

#### 3.1.4 HPLC solvents and materials

HPLC grade methanol and ACN were obtained from Fisher Scientific Company (Ottawa, ON). HPLC-grade water was obtained from Waters Co. (Mississauga, ON). Gemini C18 Column, 110Å, 5 µm, 4.6 mm \*100 mm was purchased from Phenomenex (Torrance, CA).

### 3.1.5 Other materials

Syringes (10 mL), Corning plastic centrifuge tubes (50 mL), plastic disposable transfer pipets (7.5 mL) and various magnetic stirring bars were purchased from Fisher. Syringe filters (0.2  $\mu\text{m}$  pore size) were from Sarstedt (Montreal, QC). Clear glass vials (crimp top, volume 30 mL) were obtained from Sigma Aldrich. The Pipetman variable volume pipetters (20-200  $\mu\text{L}$ , 200-1000  $\mu\text{L}$ , 1.0-5.0 mL) were from Mandel Scientific (Guelph, ON). Pipette tips (100  $\mu\text{L}$ , 1000  $\mu\text{L}$ ) were purchased from VWR International Inc. (Mississauga, ON) and 5 mL pipette tips were purchased from Sarstedt.

## 3.2 Analytical Equipment

### 3.2.1 UV-Visible spectrophotometry

A UV-Visible spectrophotometer (UV-vis; Agilent (Mississauga, ON) model 8453) with  $\lambda$  range of 190 -1100 nm and 1 nm resolution controlled by a Hewlett Packard Vectra ES/12 computer was used to determine the maximum absorbance ( $\lambda_{\text{max}}$ ) of the substrates and to test SBP enzyme activity. The detected  $\lambda_{\text{max}}$  of the substrate was then used in HPLC analysis. A quartz cuvette with 1 cm path length for the measurements in UV region was purchased from Hellma (Concord, ON).

### 3.2.2 High performance liquid chromatography (HPLC) analysis

The residual substrate concentrations after enzymatic treatment were quantified using a HPLC system from Waters Co (Oakville, ON). It had a model 2489 dual wavelength absorbance detector, model 1525 binary HPLC pump and model 2707 autosampler. A C18 (5  $\mu\text{m}$ , 4.6 X 150 mm) column was used for this study. The HPLC is operated by Breeze 2.0 software. Choice and ratio of the mobile phases, detection wavelength, flow rate, operating temperature for each substrate are given in Table 3-1. The injection volume was kept constant at 10  $\mu\text{L}$  for samples. Calibration curves of the two substrates can be found in Appendix A.

Table 3-1 HPLC conditions for substrates run under isocratic elution

Substrate	Mobile phase ratio		Flow mL/min	$\lambda_{\text{max}}$ (nm)	Column temperature	Column type
	Pump A	Pump B				
Diclofenac	30% formic acid (0.1%)	70% ACN	1.0	276	40 °C	Gemini C18
Aceclofenac	30% formic acid (0.1%)	70% ACN	1.0	275	40 °C	Gemini C18

### 3.2.3 Electrospray ionization (ESI) mass spectrometry

MS analysis was carried out by Dr. Jiayi Wang, (Queen's University, Kingston, ON) using a Thermo Scientific Orbitrap Velos Pro (Easy-nLC/HESI Hybrid Ion Trap-Orbitrap Mass Spectrometer) or an Agilent AdvanceBio 6545XT LC/QTOF (1260 Infinity II LC APCI/ESI Quadrupole Time of Flight Mass Spectrometer) in high-resolution mode. Data acquisition was performed either in the positive- or negative-ion mode. The acquired mass spectra were subjected to qualitative analysis for molecular formulae targeting possibly formed oligomers and oligomer derivatives with 10 ppm difference (between measured and calculated masses) used as the cut-off for unambiguously linking a given mass to a specific chemical formula. The gas-phase ions enter the mass spectrometer and are detected based on their mass-to-charge ( $m/z$ ) ratio. In ESI, the analyte is pumped to a capillary and a high voltage is applied which makes the droplets spray from the tip of the capillary and evaporate. The evaporation process is also supported by heat and a nebulizing gas, generally nitrogen. The gas-phase ions then enter the mass spectrometer detection. The acquisition range of probe was 50 to 2000 mass-to-charge ratio ( $m/z$ ).

### 3.2.4 Other equipment

An Oakton PC 700 pH meter with range of 0.00 to 14.00 connected to a Thermo Scientific Orion pH Probe (9110DJWP, refillable/double junction/glass/semi-micro) with  $\pm 0.02$  pH accuracy was used (Vernon Hills, IL). Calibration buffers (pH 4.0, 7.0, 10.0) were obtained from BDH Inc. (Toronto, ON.). A vortex mixer model K-550-G from Scientific Industries Inc (New York, USA) was used. For mixing, various magnetic stir bars in different sizes were obtained from Cole-Parmer Canada Inc. (Montreal, QC). The magnetic stirrers from VWR international Inc. (Mississauga,

ON) with 0-1100 rpm and 100-1500 rpm was used for mixing. Centrifugation was done on a Corning LSE compact centrifuge (New York, USA).

### **3.3 Analytical Methods**

#### **3.3.1 Enzyme stock solution preparation**

The SBP stock solution was prepared by mixing 1.4 g of solid enzyme with 100 mL distilled water at low speed (approximately 400 rpm) for 24 hours. Then the solution was centrifugated at 4000 rpm for 25 minutes. The supernatant was taken as the stock solution and were kept at 4 °C.

#### **3.3.2 Catalase stock solution and Buffer preparation**

Solid bovine liver catalase (0.5 g) was dissolved in 100 mL of distilled water (99500 U/mL in this stock). The solution was magnetically stirred for approximately 3 hours before being kept at 4 °C for later use. Based on Gomori, 1955, buffer preparation manual, citrate–phosphate buffer was freshly prepared to be used in the range of pH 3.0-8.0.

#### **3.3.3 SBP activity assay**

Quantify the enzyme activity was determined using a colorimetric assay in this study (Ibrahim et al., 2001). The reaction of the assay was: 4-AAP + phenol + H<sub>2</sub>O<sub>2</sub>. A pink chromophore ( $\lambda_{\text{max}} = 510 \text{ nm}$ ; extinction coefficient,  $\epsilon = 6000 \text{ M}^{-1}\text{cm}^{-1}$  relative to H<sub>2</sub>O<sub>2</sub>) arises from SBP catalyzed oxidative coupling of phenol and 4-AAP in the presence of H<sub>2</sub>O<sub>2</sub>. The enzyme catalytic activity (U/mL) was the initial rate of reaction, proportional to the enzyme concentration, measured at the wavelength of maximum absorbance (510nm) of the reaction product in the solution by a built-in kinetic rate calculation function in the UV-Vis spectrometer. A unit (U) of SBP activity was defined as the amount of enzyme that converts one micromole of hydrogen peroxide per minute at pH 7.4 at room temperature. The reaction solution consisted of diluted SBP (dilution factor 50) and reagent solution. The regular reagent was made to be 2.4 mM 4-AAP, 0.2 mM H<sub>2</sub>O<sub>2</sub>, 10 mM phenol, and 0.05 mM phosphate buffer (pH 7.4) when a 0.95 mL aliquot was mixed with 0.05 mL of diluted SBP soln. A concentrated phenol reagent (10X phenol) was prepared in buffer containing 100 mM phenol and 0.5 M phosphate buffer (pH = 7.4) (Caza et al., 1999). First, the equipment was blanked with 950  $\mu\text{L}$  of reagent and 50  $\mu\text{L}$  distilled water. Second, the 950  $\mu\text{L}$  of reagent mixture was quickly added to 50  $\mu\text{L}$  diluted SBP solution in the cuvette. Then, the increase in absorbance over 30 seconds run time and 5 seconds cycle time was monitored, and the activity

was calculated through built-in instrument software using zero-order kinetics. More information is available in Appendix B.

### **3.4 Experimental protocols**

#### **3.4.1 Enzymatic oxidation of substrates with SBP and feasibility of treatment of target aromatics**

A solubility test was used to determine the initial concentrations of DCF and ACF in the appropriate aqueous solvent. As stock solutions, diclofenac was dissolved in water and aceclofenac was dissolved in anhydrous ethanol (95%) at 1.0 mM. All reactions were carried out in triplicate in 30 mL glass batch reactors. The reactions were carried out at 19-25 ° C and were not temperature controlled. The enzymatic treatment of synthetic wastewater was designed to remove 95% of each substrate (except for pH optimization). In batch reactors, the 20 mL reaction medium contained 40 mM buffer, 0.10 mM substrate, SBP and hydrogen peroxide in appropriate concentrations. Hydrogen peroxide was the last chemical added to the solution to start the reaction. After mixing the solution for 3 hours by Teflon-coated magnetic stirrer bars, the reaction was stopped by adding 100 µL catalase stock solution (0.5 g/100 mL; to give 498 U/mL) to quench the reaction by immediately consuming residual hydrogen peroxide. Samples were then microfiltered with pre-conditioned, 0.22 µm PES syringe filters and analyzed by HPLC for the residual substrate in solution after treatment.

Time-course experiments were also carried out using optimized parameters to track the substrate conversion over time. Three big batch reactors (75 mL) were used, and samples (5 mL) were taken at selected time intervals, quenched with catalase and vortexed to stop the reaction immediately. Samples were later filtered and subjected to HPLC analysis. To observe the impact of the enzyme or hydrogen peroxide on the substrate, various control batch reactors with formulations similar to the sample but without SBP or without hydrogen peroxide were also run for each set of batch reactors.

#### **3.4.2 Optimization of important enzymatic reaction parameters**

The most important reaction parameters, pH, hydrogen peroxide concentration and minimum effective concentration of SBP to reach 95% removal were studied for substrates in this study. For pH optimization, initially some preliminary experiments were done to determine the approximate pH range. The studies were again repeated, but this time with shorter pH intervals and lower



ranges. The reactions were carried out at various pHs using various buffers under enzyme stringency conditions with SBP limits to clearly determine the effect of each parameter on the reaction. Each batch reactor had a pair of blanks that were formulated in the same way as the samples. One blank lacked peroxide to test the effect of enzyme on the substrate, while the other lacked enzyme to test the effect of peroxide alone on the substrate. pH optimization was followed by peroxide and minimum effective concentration optimization. The reactions were formulated for 95% removal of target compound, at optimum pH. Enzyme optimization was also repeated after optimizing the amount of peroxide to check for interaction of these two parameters.

#### 3.4.3 Preliminary identification of products

MS was used to identify potential polymerization products in reaction mixtures. A batch reactor with optimal pH, enzyme activity, and peroxide comprising 10 mM of each buffer was set up. The amount of buffer was reduced to decrease the negative effect of buffer ions on MS analysis. After three hours, the reaction mixture was quenched with catalase and the filtered and unfiltered reaction mixture in order to prevent losing possible products, and the standards were prepared to use for MS analysis.

#### 3.4.4 Sources of error

Errors is an inseparable part of any experiment and occurrence of Errors can affect reliability of results. Mainly two types of errors, namely systematic and random errors. Symmetric errors caused by analytical methods and are predictable and usually constant. This error will not be eliminated by averaging or increasing the number of experiments. Random errors are always present, can show different results for the same repeated measurement. To decrease random errors in the current study, a standard was run along with the samples in all experiments. Experiments were performed in triplicate (except time dependence), and the average was plotted on the graphs. Standard deviation of each triplicate set was calculated and shown on each graph. If the standard deviation was  $\geq 5\%$ , the data for that point was collected again. For the data points with very small deviation ( $<1\%$ ), the error bar is embedded in the icon and not visible. During enzyme activity test, the average of three runs for each dilution was taken. The activity of the enzyme was checked every day prior to the experiment to ensure correct formulation of the batch reactors. To avoid systematic errors in this study, the pipets and analytical balance were calibrated periodically. Substrate stock

solutions, reagents and buffers were prepared fresh to decrease the chance of aging, oxidation, precipitation, or any other side reactions.

## CHAPTER 4 RESULTS AND DISCUSSION

Diclofenac and aceclofenac were studied in preliminary tests of the feasibility of their SBP-catalyzed reactions with hydrogen peroxide. That they were substrates is noteworthy in that the reactive nitrogen atom is a sterically congested, secondary bis-anilino nitrogen. Three important operational parameters: pH, H<sub>2</sub>O<sub>2</sub> concentration and minimum effective enzyme activity were optimized for each substrate to reach  $\geq 95\%$  conversion efficiency. Time-course experiments based on optimal parameters were conducted to determine the time to reach 95% conversion and to find the initial first-order reaction rate constants and half-lives of substrates. Then reactions were operated under optimized conditions to identify possible products by mass spectrometry analysis.

### 4.1 Parameter optimization of SBP-catalyzed treatment

#### 4.1.1 pH Optimization

Stability is one of the most essential factors in industrial application of enzymes. The pH of the reaction medium is a crucial biocatalytic parameter that influences SBP activity and stability, and thus the effectiveness of enzymatic treatment and its industrial application. Change in the pH of the solution effects on the ionization state of specific amino acid side chains of the enzyme that are mainly responsible for catalytic function and stability of enzyme structure. Two key amino acid residues, distal histidine-42 (His42) as proton acceptor from hydrogen peroxide, and distal arginine-38 (Arg38) as the charge stabilizer (Dunford, 1999), require optimum pH that satisfies their ionization states. Also, pH affects the 3-dimensional structure of SBP and its conformational stability mainly due to interaction of amino-acid residues with heme (Al-Ansari et al., 2009). In general, the activity of enzyme can regain after being exposed to weakly acidic or basic solutions, if it is shifted back later to the optimum pH. However, if these variations in pH cause a change in overall structure of the enzyme, the change in activity may not be reversible and the enzyme cannot regain its maximum activity. Furthermore, pH of the reaction medium could influence the ionization state of the reducing substrate (diclofenac and aceclofenac here) and electron transfer rate (Parsiavash et al., 2015). Also, the catalytic activity of SBP is influenced by pH due to the presence of additional solvent-exposed heme edges in the structure of SBP (Kamal & Behere, 2003). SBP was found to have the maximum activity ( $>95\%$ ) in weak acidic solutions (pH 5.5 to 6.0) for guaiacol (Geng et al., 2001). For ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), the maximum catalytic activity of SBP was observed at pH 5.5 (Kamal & Behere,

2003) and it could stay active between pH 3.0 and 9.0 for 3-substituted quinolines (Mashhadi, et al., 2019).

In this study, optimum pH is defined as the pH which the highest removal efficiency of substrate is obtained. To determine the optimum pH, the experiments were conducted under a stringent condition with insufficient amount of SBP, leading to incomplete removal of substrate, in order to clearly distinguish pH effects. Preliminary experiments were conducted over a broad pH range. Later on, the experiments were done over a narrower range of pH. In these experiments, hydrogen peroxide concentration was generally chosen as 1.5-2.0 times the molar concentration of the substrate, based on previous experiments performed in the lab. The substrate concentration was 0.1 mM. All experiments were conducted in triplicate at room temperature ( $22\pm 1$  °C). Two control experiments were conducted for each batch reactor, the first one without H<sub>2</sub>O<sub>2</sub> and the second one without SBP. The error bars on the figures demonstrate the standard deviation; error bars are not visible for the data points with very small deviation.

The effect of pH on conversion of diclofenac after 3-hour enzymatic treatment is shown in Figure 4-1. Range finding was conducted for a broader range of pH using UV-Vis (data not shown). Later, the experiments were analyzed by HPLC with the pH range 3.0-8.0, 0.15 U/mL SBP, and 0.4 mM hydrogen peroxide.

Figure 4-1 clearly indicates the optimal pH for diclofenac enzymatic conversion is in the acidic range and the apparent optimum pH was 5.0 that showed 4% remaining with 0.15 U/mL SBP. Above this pH value, the enzymatic activity decreased drastically, causing a reduced efficiency of the catalytic degradation.

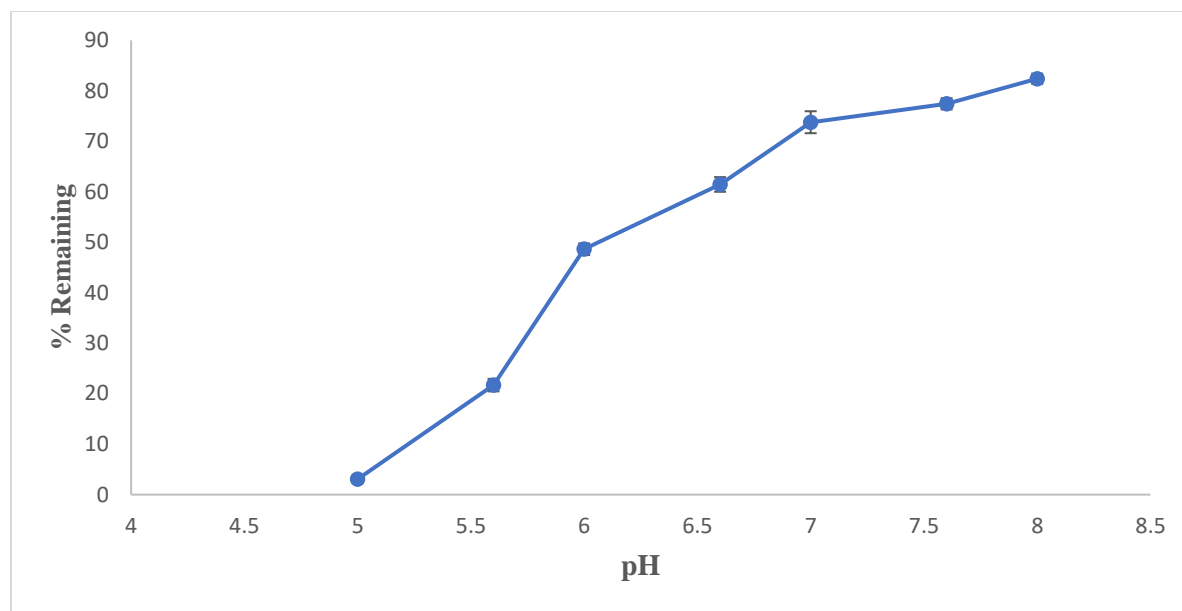


Figure 4-1. Diclofenac pH Optimization. (0.1 mM diclofenac, 0.4 mM H<sub>2</sub>O<sub>2</sub>, 0.3 and 0.15 U/mL SBP, 3-hour reaction, room temperature)

However, when analyzing the reaction of SBP on DCF in a range of pH from 3.0-8.0, DCF was found to precipitate out of solution in the no-SBP controls when pH < 5. This could be due to the pK<sub>a</sub> of DCF, 4.18 (Wang & Fang, 2008). As the pH approaches the pK<sub>a</sub> from above and then becomes is lower than, the drug is increasingly converted to the protonated (neutral) form while at pH > 4.18, the majority form is deprotonated (monoanionic), hence more soluble. Therefore, at pH values < 5, DCF becomes uncharged and precipitates (de Rossi et al., 2020; Jodeh et al., 2014). Therefore, the working pH range that was analyzed for maximal DCF removal was was restricted to pH 5.0- 7.0.

To confirm this reasoning, two experiments were conducted. First, the percentage of DCF remaining controls without SBP and peroxide from pH 3.0 to 5.0 were tested, where each control (only containing diclofenac and buffer) was run in a batch reactor for 3 hours. The results in Table 4.1 illustrate a decrease in pH lead to a decrease in solubility of diclofenac. Specifically, at pH 3.0, only 21.8 ± 0.6% of the compound remained in reaction solution. Second, the percentage DCF remaining in analogous controls but containing 0.5 mM DCF, from pH 3.0 were tested in different solvents. The results in Table 4.2 showed a decrease in the amount of compound that remained in reaction solution. This solubility issue was not seen for aceclofenac.

Table 4-1 Results for Standard Control Test

<b>pHs</b>	<b>%Remaining of diclofenac in Standard Controls</b>
3	21.8 ± 0.6
3.6	38.9 ± 0.4
4	68.5 ± 0.6
4.6	73.0 ± 0.2
5	99 ± 0.9

Table 4-2 DCF solubility in pH 3.0 buffer

<b>Solvents</b>		<b>%Remaining</b>
Ethanol	10%	5.0 ± 0.4
	20%	35.4 ± 0.7
Iso-propanol	10%	16.6 ± 0.2
	20%	39.9 ± 0.1
Acetonitrile	10%	Not soluble
	20%	Not soluble

The results of aceclofenac (0.10 mM) pH optimization are shown in Figures 4-2 and 4.3. After pH range-finding by UV-Vis (data not shown), the experiments were conducted in triplicate with HPLC detection, over a pH range of 3-8, with 40 mM buffer, 0.4 mM H<sub>2</sub>O<sub>2</sub>, and 0.15 U/mL SBP. The effect of pH on the conversion of aceclofenac (0.10 mM), is shown in Figure 4-2 and 4.3. Aceclofenac was best transformed in pH range 3.0-4.6 and the optimum pH was 4.0 which showed 30.5% remaining with 0.15 U/mL. Increasingly more remaining aceclofenac can be seen from pH 6.0 to 8.0, likely because of increasing in ionization of hydroxyl group to the anionic form of the substrate that is not able to participate in peroxidase cycle (Morales Urrea et al., 2018). The bell-shaped pH dependence shows a variation in the ionisation state of the enzyme's catalytic residues and/or the ionisation state of the substrate (Kamal & Behere, 2003).

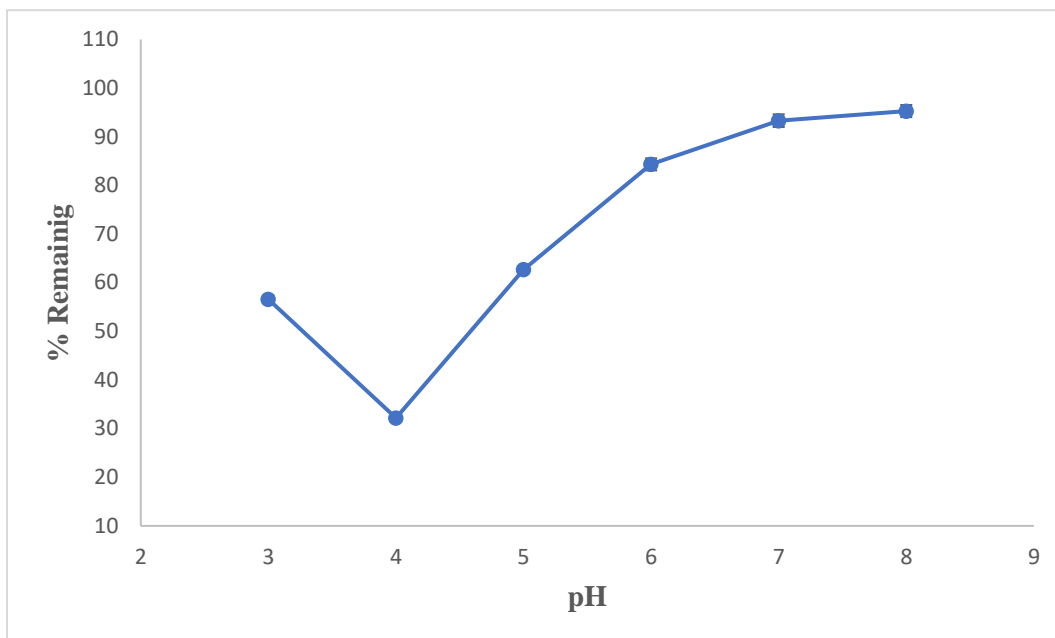


Figure 4-2. Aceclofenac wide-range pH optimization. (0.1 mM aceclofenac 0.4 mM H<sub>2</sub>O<sub>2</sub>, 0.15 U/mL SBP, 3- hour reaction, room temperature)

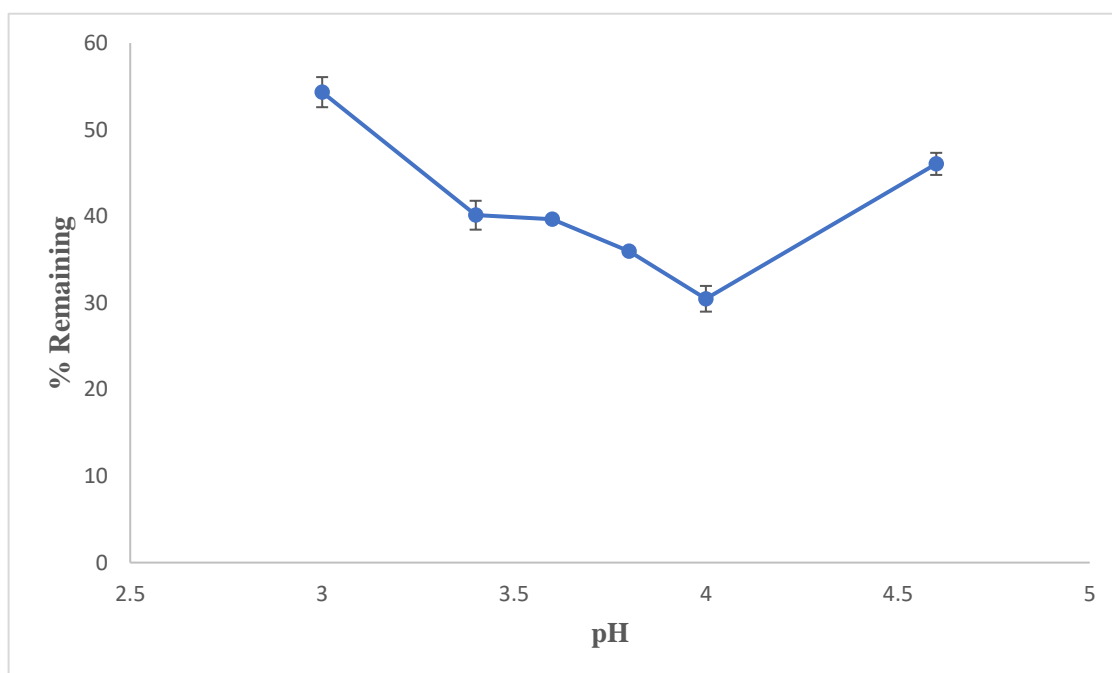


Figure 4-3. Aceclofenac narrow-range pH optimization. (0.1 mM aceclofenac 0.4 mM H<sub>2</sub>O<sub>2</sub>, 0.15 U/mL SBP, 3- hour reaction, room temperature)

#### 4.1.2 Enzyme optimization

Economic viability of a treatment method is crucial, and the cost of enzyme can be one of the barriers to application of enzymatic treatment to real wastewater (Steevensz et al., 2009). Even though SBP is a relatively low-cost enzyme, it was essential to minimize the enzyme concentration while reaching 95% transformation efficiency of substrates. So, the minimum concentration of SBP needed to reach 95% removal of organic pollutant is considered as optimum enzyme in this study. The experiments were conducted at the previously established pH optima for each substrate and the samples were analyzed by HPLC. The H<sub>2</sub>O<sub>2</sub> concentration was kept at a level where it did not become limiting during the experiments. Enzyme optimization was repeated for the substrate in which the optimum amount of hydrogen peroxide was shown to be different from what was used in SBP optimization. The graphs presented are the final optimizations of each substrate.

For 0.1 mM diclofenac, the range of SBP concentrations was 0.085-0.3 U/mL with pH 5.0 (optimal pH) and 0.4 mM of H<sub>2</sub>O<sub>2</sub> as shown in Figure 4-4. This range was chosen because preliminary enzyme optimizations by UV-vis (data not shown). As seen from the graph, increasing the enzyme activity from 0.085 to 0.15 quickly enhances the removal efficiency from ~79% to almost 96%. Further increase in enzyme activity had a very slow response in percentage of removal, hence, using 0.3 U/mL of SBP resulted in ~98% removal of substrate from the solution. So, a doubling of the enzyme concentration only contributed an additional ~2% increase in efficiency. This indicates very high removal efficiency (>96%) may not be cost-effective.



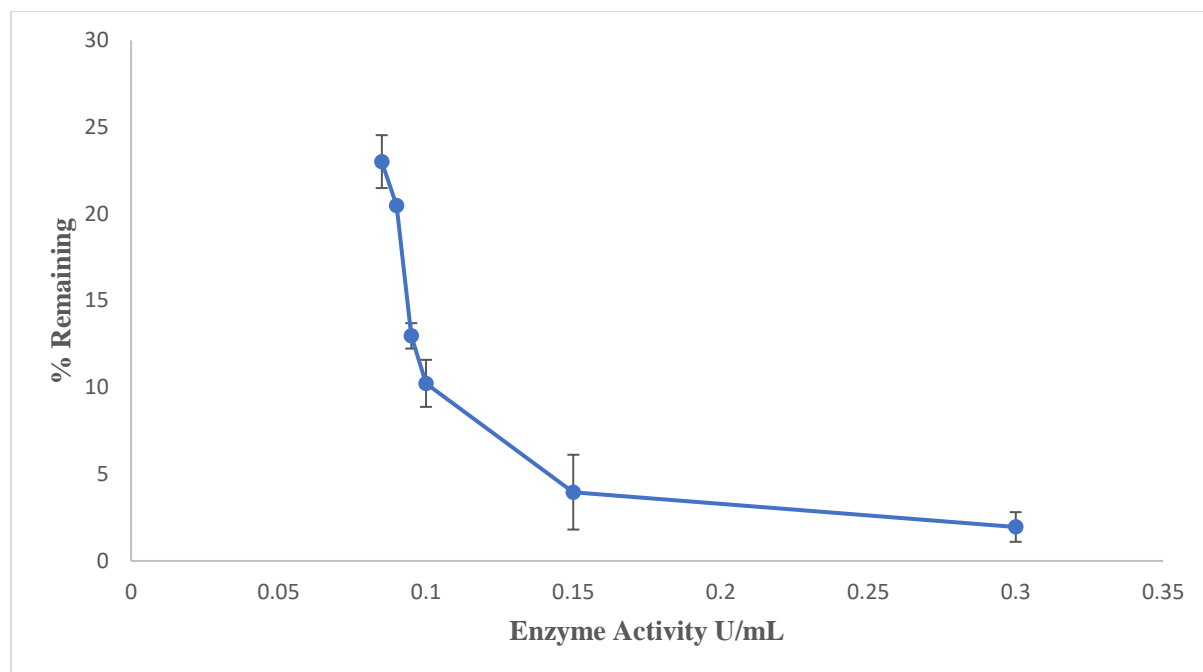


Figure 4-4. Diclofenac enzyme optimization. (pH 5.0 buffer, 0.1 mM diclofenac, 0.4 mM H<sub>2</sub>O<sub>2</sub>, 3-hour reaction, room temperature)

Enzyme optimization for aceclofenac was conducted in the range of 0.15-0.70 U/mL of SBP activity, because in the preceding experiments done for pH optimization, 0.15 U/mL enzyme activity could only contribute to ~67% conversion. Figure 4-5 is the SBP optimization for aceclofenac after pH optimization with the following conditions: 0.10 mM ACF, 40 mM citrate buffer pH 4.0 and 0.4 mM H<sub>2</sub>O<sub>2</sub>. Increasing the amount of enzyme from 0.15 to 0.6 U/mL resulted in almost ~26% improvement in removal of substrate after 3 hours (6.87% remaining). However, further increase in SBP beyond 0.6 U/mL led to a decrease in removal percentage and increase the amount of the substrate remaining to 15.8%, possibly by increased catalase activity of the enzyme. Therefore, 0.6 U/mL SBP was chosen as minimum effective enzyme activity, four times of the amount of enzyme needed for removal of the same amount of diclofenac.

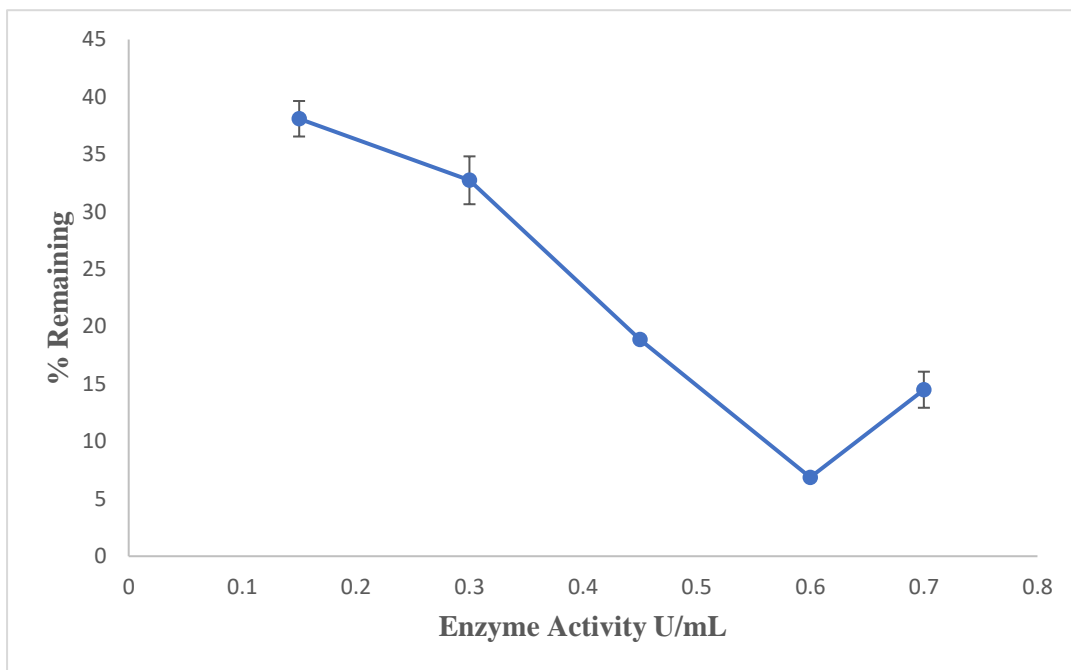


Figure 4-5. Aceclofenac Enzyme Optimization. (pH 4.0 buffer, 0.1 mM aceclofenac, 0.4 mM H<sub>2</sub>O<sub>2</sub>, 3-hour reaction, room temperature)

#### 4.1.3 Hydrogen peroxide optimization

Hydrogen peroxide is one of the important components of enzymatic treatment and it is essential for any peroxidase-based reaction. It is the co-substrate in soybean peroxidase reactions and starts the enzymatic reaction. Upon reaction of peroxide with SBP, the radical form of the enzyme (Compound I), is generated which then reacts with organic substrate. However, the presence of excess H<sub>2</sub>O<sub>2</sub> can result in enzyme inactivation through the formation of the Compound III from Compound II. On the other hand, a low concentration of H<sub>2</sub>O<sub>2</sub> might lead to insufficient conversion of the substrate. Based on the oxidation-reduction mechanism in the peroxidase cycle, for 1 mole of H<sub>2</sub>O<sub>2</sub> consumed, 2 moles of the substrate would be converted to free radicals. This ratio is subject to change depending on the treatment conditions. Catalase activity of peroxidases and the formation of higher oligomers from the first-formed dimers can increase peroxide consumption during treatment (Valderrama et al., 2002).

To determine the optimum hydrogen peroxide concentration, batch reactors were run at the previously determined optimum pH and minimum effective SBP activity (0.15 U/mL and 0.6 U/mL SBP, respectively) for each substrate for 3 hours to achieve  $\geq 95\%$  removal. Hydrogen

peroxide optimization of diclofenac and aceclofenac (HPLC analysis) were carried out. Figures 4-6 and 4-7 demonstrate the removal performance under different H<sub>2</sub>O<sub>2</sub> concentrations at the optimal pH 5.0 and 4.0 for diclofenac and aceclofenac, respectively.

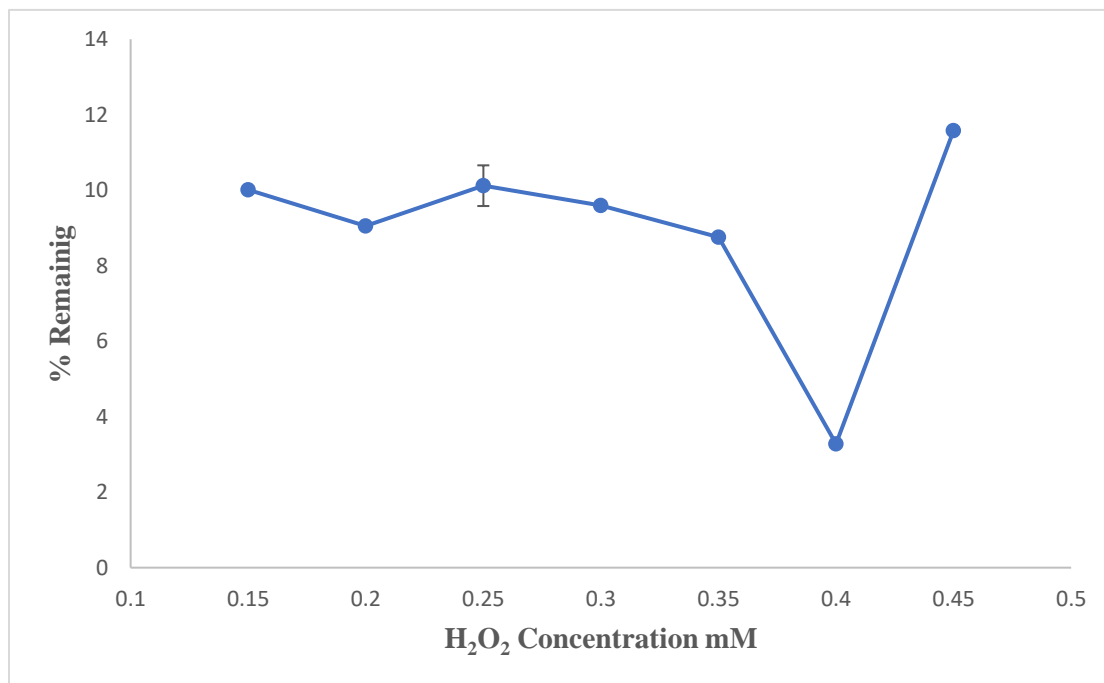


Figure 4-6. Diclofenac H<sub>2</sub>O<sub>2</sub> Optimization. (pH 5.0 buffer, 0.1 mM diclofenac 0.15 U/mL SBP, 3-hour reaction, room temperature)

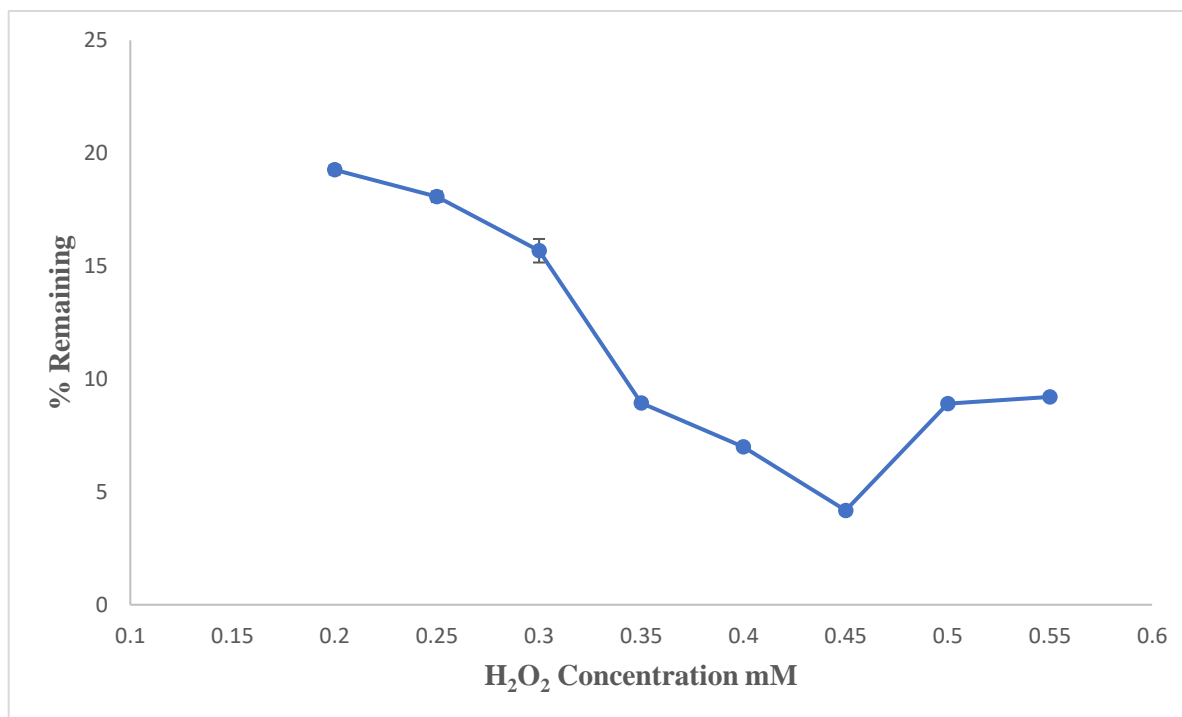


Figure 4-7. Aceclofenac H<sub>2</sub>O<sub>2</sub> Optimization. (pH 4.0 buffer, 0.1 mM aceclofenac, 0.6 U/mL SBP, 3-hour reaction, room temperature)

The range of H<sub>2</sub>O<sub>2</sub> concentrations were from 0.15 mM to 0.45 mM for diclofenac and 0.2 mM to 0.6 mM for aceclofenac. For 0.1 mM diclofenac, the best performance was observed at 0.40 mM of H<sub>2</sub>O<sub>2</sub> with minimum 3.38% of substrate remaining and for 0.1 mM aceclofenac, 0.45 mM H<sub>2</sub>O<sub>2</sub> concentration (4.18% remaining) was optimal.

The results show that increasing the concentration of H<sub>2</sub>O<sub>2</sub> over 0.4 mM for diclofenac and 0.45 mM for aceclofenac, resulted in a lower removal efficiency. This might be because of inactivation of SBP through the formation of compound III (reversibly catalytically inactive) in the presence of excess amount of H<sub>2</sub>O<sub>2</sub>, resulting in lower conversion or the inactive derivative P-670 (Steevensz et al., 2014). Enzyme optimization was repeated with optimum hydrogen peroxide for aceclofenac. Comparing the optimum point with 0.45 mM H<sub>2</sub>O<sub>2</sub> and previous amount of H<sub>2</sub>O<sub>2</sub> that used for enzyme optimization, 0.40 mM (Figure 4-8), very minor improvement is observed (a little above 3%) in the removal efficiency.

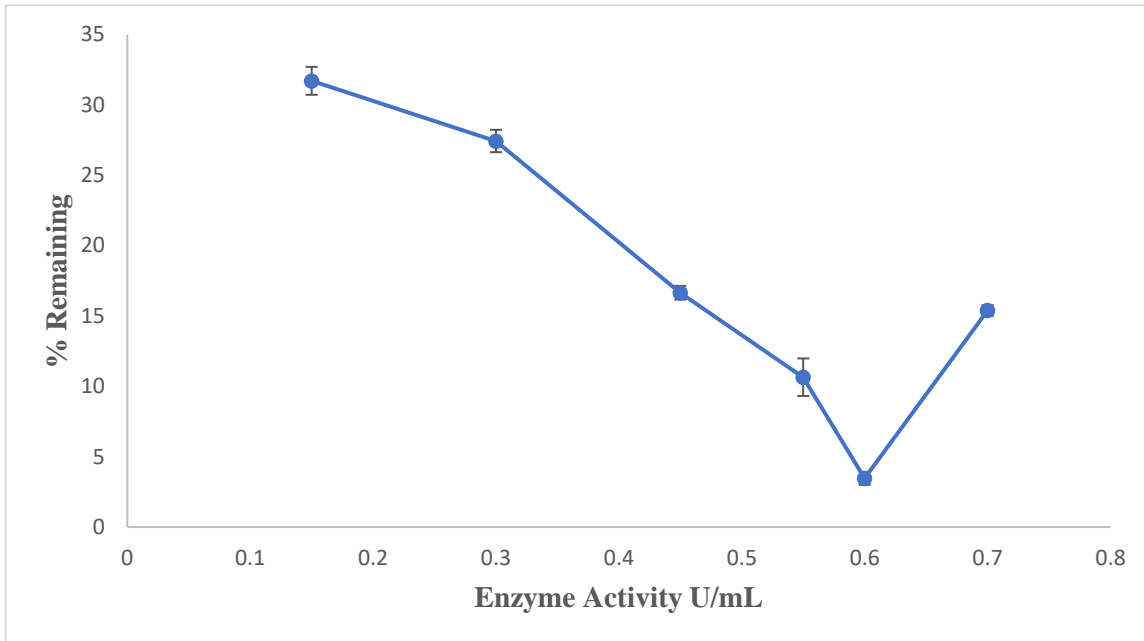


Figure 4-8. Aceclofenac Enzyme Optimization. (pH 4.0 buffer, 0.1 mM aceclofenac, 0.45 mM H<sub>2</sub>O<sub>2</sub>, 3-hour reaction, room temperature)

#### 4.1.4 Summary of parameter optimization

Table 4.3 collects the optimized parameters determined in the preceding sections and the discussion following discussion places those results in the literature context.

Table 4-3 Optimized conditions for SBP-catalyzed removal for 0.10 mM Diclofenac and Aceclofenac

Parameters	Diclofenac	Aceclofenac
[Substrate] (mM)	0.10	0.10
pH	5.0	4.0
SBP activity (U/mL)	0.15	0.60
[H <sub>2</sub> O <sub>2</sub> ] (mM)	0.40	0.45
Removal (%)	≥96.7	≥96.5

The pH optima for the compounds in this study shows that SBP can convert these anilines in a mildly acidic region, which has been observed by others (Al-Ansari et al., 2009; Altahir et al., 2016; Mukherjee et al., 2019); and that the optimum pH not only is dependent on proper ionization state of the enzyme catalytic residues but also on the type of aromatic compound being treated by enzymatic process and its pK<sub>a</sub>. Other anilino compounds such as phenylenediamines, benzidine and 4-COT showed similar trends exhibiting optima at pH 4.5-5.6, pH 5.0 and 4.4-5.0, and at pH > 7.0 there was almost no removal. (Al-Ansari et al., 2009; Altahir et al., 2016; Mukherjee et al., 2018). The same results were found for laccase-catalyzed degradation of DCF. The maximum enzyme activity was attained at pH range 4-5, acidic range, while the enzyme activity at neutral to basic pH was much lower (from 70% to 3% at pHs 7 and 12, respectively; Lloret et al., 2010).

The minimum effective SBP for DCF can be quantitatively compared to 0.1 U/mL and 0.15 required during SBP optimization of 0.1 mM of 4,4'-methylenebis(2-chlororaniline) and 4,4'-thiodianiline, respectively (Mukherjee et al., 2019).

The ratio of [H<sub>2</sub>O<sub>2</sub>]/[substrate] was found to be 4 and 4.5 for diclofenac and aceclofenac, respectively. The general experience with compounds studied in the lab group has been that the optimum ratio is in the 1.0 – 1.5 range (examples given below). From the peroxide mechanism, a ratio of 0.5 is predicted. However, this would be expected if only dimer were produced, and it precipitated quantitatively. If the dimers and higher oligomers remained soluble and underwent subsequent peroxidase cycles, more peroxide would be consumed to a limiting stoichiometry ratio of 1.0 Yu et al. (1994). Beyond a ratio of 1, additional peroxide demand could be a result of intrinsic catalase activity found in all plant peroxidases, including SBP. Catalase decomposes H<sub>2</sub>O<sub>2</sub> (Yu et al., 1994) to oxygen and water (Dunford, 2016). Another possibility for increased peroxide demand could be due to H<sub>2</sub>O<sub>2</sub> oxidation of organic matter present in the crude enzyme mixture used here (Biswas, 1999). This effect would be expected to be the greater with sluggish substrates, those requiring multi-U/mL for substantial conversion. Thus, it is especially surprising for DCF which uses low SBP activity (0.15 U/mL). Previously-studied compounds such as *o*-anisidine (optimal treatment: 1.0 mM substrate, 0.012 U/mL, 1.25 mM H<sub>2</sub>O<sub>2</sub>, Mazloun et al., 2016); *p*-cresidine (optimal treatment: 1.0 mM substrate, 0.010 U/mL, 1.25 mM H<sub>2</sub>O<sub>2</sub> (Mukherjee., 2019), 3-hydroxycoumarin (optimal treatment: 0.5 mM substrate, 0.002 U/mL, 0.75 nM H<sub>2</sub>O<sub>2</sub> , Bideh et

al., 2021), indicated the higher peroxide demand than the amount of that needed theoretically for oligomerization.

## 4.2 Time course of reactions

Reaction time is one of the essential parameters for treatment plant designing. It has close association with the reactor volume and overall cost of the plant. As a result, time-course experiments were carried out in order to minimise reaction time and to reduce the cost of enzymatic treatment while achieving more than 95% pollutant degradation. The optimal pH, H<sub>2</sub>O<sub>2</sub>, and enzyme concentrations determined in Sections 4.1.1 to 4.1.3 were applied to 3-hour time course experiments, with 3 hours ensuring that the reaction time was sufficient. The experiments were carried out in a 75 mL Erlenmeyer flask at room temperature. At appropriate time intervals (0 seconds to 2 min), 5 mL of reaction mixture was taken then mixed with catalase and micro-filtered to analyze the residual substrate concentration. The initial pseudo first-order kinetic constants *k* and half-lives of diclofenac and aceclofenac were calculated based on Equations 5 and 6. It is expected that these reactions will not remain pseudo-first order for very long because of progressive consumption of the non-monitored substrate, hydrogen peroxide. Nevertheless, in the absence of a detailed kinetic study, this rate constant will be useful in comparing reactivity of the enzyme with various substrates.

Equation 5)  $C = C_0 e^{-kt}$

Equation 6)  $t_{\frac{1}{2}} = \frac{\ln(2)}{k}$

As can be seen from Figures 4.9 and 4.11, both compounds reached more than 95% degradation, almost 86% of diclofenac was converted within the first 30 minutes, but the degradation of the rest (10.74%) took 2.75 hours; aceclofenac reached 88% transformation at 30 minutes, and it is evident that only 8.1% improvement in treatment efficiency is observed after the first 30 minutes, through to the end. The slowing reaction rate is logical since the active enzyme was reduced with time, attributed to progressive enzyme inactivation (Al-Ansari et al., 2009). Similar results were found in SBP-catalyzed removal of 4-chlorophenol, 3-hydroxyquinoline, arylamines, azo dyes and pesticides (Bódalo et al., 1991; Mashhadi, et al., 2019; Mukherjee et al., 2019; X. Zhang, 2019).

Based on Equations 5 and 6 and the trend-line equations as shown in Figures 4.10 and 4.12, the rate constant for diclofenac and aceclofenac were  $0.484 \pm 0.010$  and  $0.823 \pm 0.020$  min<sup>-1</sup>,

respectively; thus, the corresponding half-lives were obtained as  $1.43 \pm 0.01$  and  $0.84 \pm 0.05$  min respectively. If normalized with respect to enzyme activity, the half-life was  $0.22 \pm 0.02$  min per U/mL of SBP for diclofenac and was  $0.49 \pm 0.01$  min per U/mL of SBP for aceclofenac. This indicated the SBP normalized catalytic reaction rate of diclofenac was approximately two times faster than that of aceclofenac at the beginning of the reaction.

Table 4-5 summarizes normalized half-lives of various SBP substrates studied in this group and substrates of this study with respect to the optimum enzyme (Bideh, 2020). From that, it may be seen that DCF and ACF are amongst the substrates ( $t_{1/2} < 1$  min) in the table.

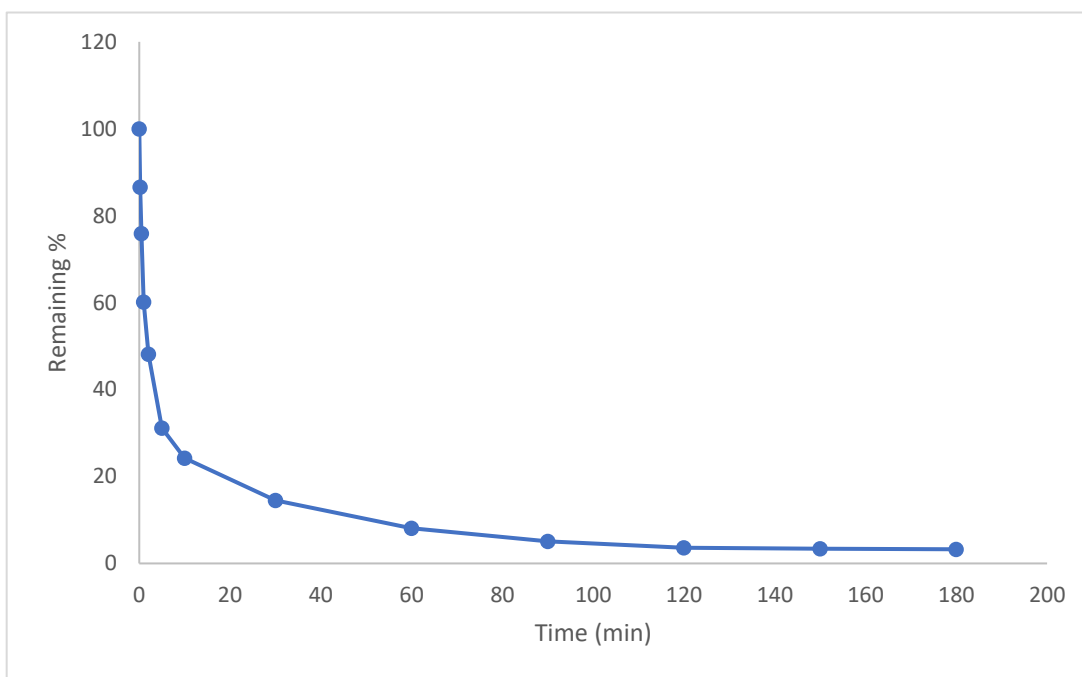


Figure 4-9. Time dependence of degradation of diclofenac in optimal conditions Batch reactor. (pH 5.0 buffer, 0.1 mM diclofenac, 0.4 mM H<sub>2</sub>O<sub>2</sub>, 0.15 U/mL SBP, 3-hour reaction, room temperature)



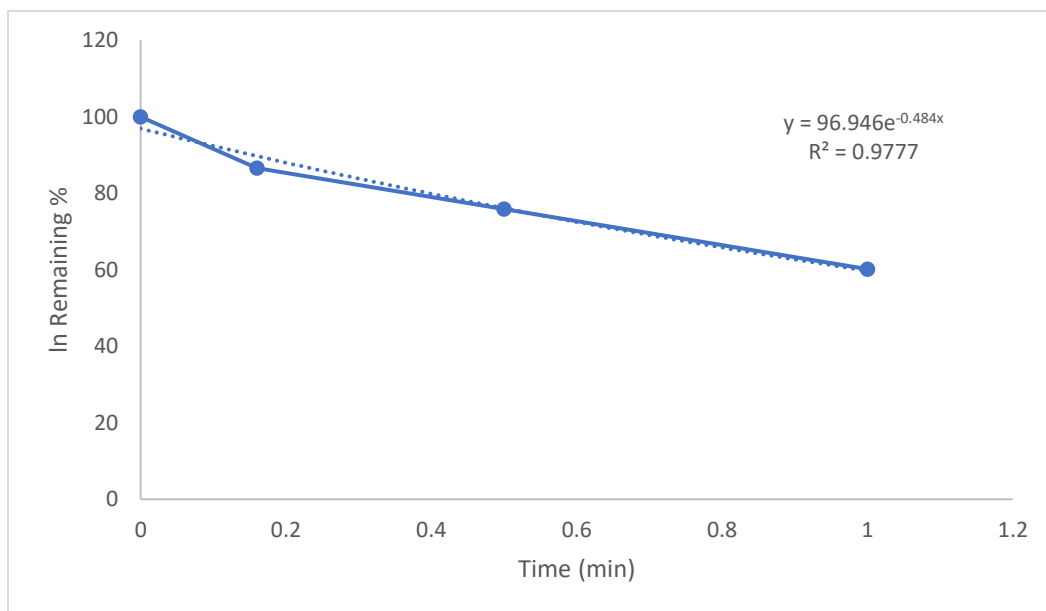


Figure 4-10. First-order degradation of diclofenac at the beginning of the reaction presented in Figure 4.9

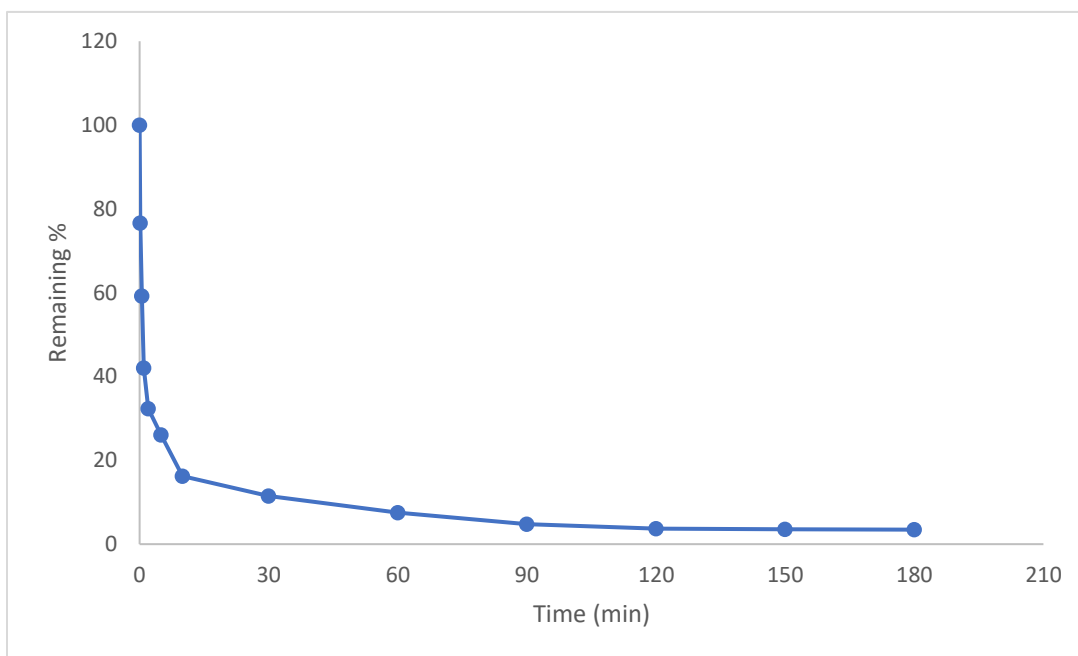


Figure 4-11. Time dependence of degradation of aceclofenac in optimal conditions Batch reactor. (pH 4.0 buffer, 0.1 mM aceclofenac, 0.45 mM H<sub>2</sub>O<sub>2</sub>, 0.60 U/mL SBP, 3-hour reaction, room temperature)

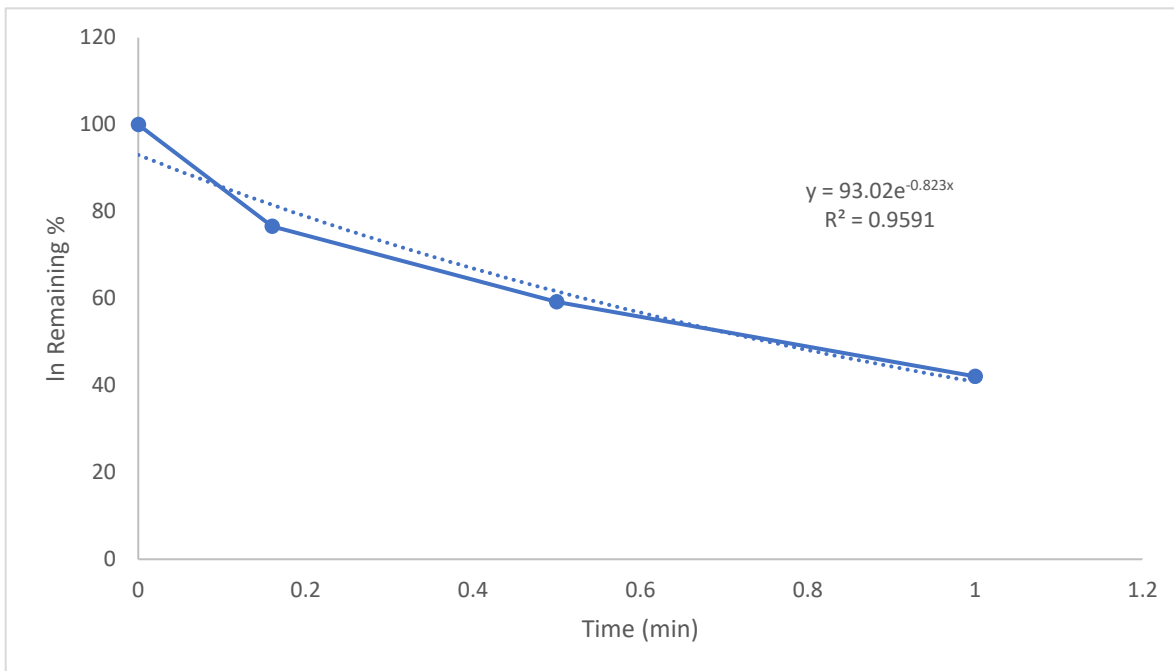


Figure 4-12. First-order degradation of aceclofenac at the beginning of the reaction presented in Figure 4.11

Table 4-4 Half-lives and normalized half-lives of various SBP substrates<sup>a</sup>

	<b>Substrate</b>	<b>Half-life (min)</b>	<b>Normalized half-life (min)</b>	
<b>Present Study</b>	Diclofenac	1.43±0.01	0.215±0.02	
	Aceclofenac	0.84±0.05	0.493±0.01	
<b>HACs (Mashhadi, 2019)</b>	Pyrrole	49.2 ± 3	246 ± 15	
	Indole	25.2 ± 1.2	11.3 ± 0.5	
	2-Aminothiazole	33.0 ± 0.6	132 ± 2	
	2-Aminobenzothiazole	720 ± 0.01	3240 ± 0.0	
	3-Hydroxyquinoline	11.9 ± 0.59	1.19 ± 0.06	
	3-Aminoquinoline	14.99 ± 0.6	67.5 ± 2.7	
	2-Aminoimidazole	5.1 ± 0.2	7.7 ± 0.3	
	2-Aminobenzimidazole	29.4 ± 0.6	88.2 ± 1.8	
	3-Aminopyrazole	36 ± 1.2	108 ± 4	
	4-Aminoantipyrine	60.6 ± 1.2	6.06 ± 0.12	
	Hydroxybenzotriazole	41.4 ± 1.8	4.97 ± 0.22	
	<b>Arylamines (Mukherjee, 2019)</b>	4,4'-Oxydianiline	12.4 ± 0.0	0.124 ± 0.0
		4-Chloro-o-toluidine	11.5 ± 0.0	0.104 ± 0.0
p-Cresidine		1.80 ± 0.02	0.072 ± 0.001	
4,4'-Methylenedianiline		0.58 ± 0.10	0.40 ± 0.07	
4,4'-Thiodianiline		0.513 ± 0.007	0.0770 ± 0.0011	
4,4'-Methylenebis (2-chlororaniline)		4.08 ± 0.02	0.408 ± 0.002	
<b>Pesticides (X. Zhang, 2019)</b>	Bromoxynil	3.00 ± 0.13	2.7 ± 0.02	
	Ioxynil	0.51 ± 0.018	0.18 ± 0.01	
<b>Azo dyes (Cordova Villegas, 2017)</b>	Acid Blue 113	8.76 ± 0.60	13.2 ± 0.9	
	Direct Black 38	2.1 ± 0.2	6.4 ± 0.6	
<b>(Ziayee Bideh, 2020)</b>	3-Hydroxycoumarin	12.4 ± 0.5	0.0257 ± 0.0010	
	2-Aminobenzoxazole	129 ± 4.44	452 ± 15	

<sup>a</sup>Aside from top two entries, this table taken from (Ziayee Bideh), MASC thesis, University of Windsor, (2020)

### 4.3 Mass spectrometry (MS) results

As discussed in Section 2.4, free radicals produced by the oxidative action of the enzyme diffuse in the peroxidase catalytic cycle into solution and couple non-enzymatically. When the oligomers precipitate out of the solution upon reaching their maximum solubility, the polymerization process would come to an end. Due to the presence of several resonance-stabilized radical structures, many coupling sites, such as C-C, C-O, O-O (O-O coupling is not stable), N-N, and N-C, with *ortho*-, *para*-orientation are expected due to the presence of different loci of unpaired electron-density in the resonance-stabilized radicals, leading to the production of various polymerization products for a substrate such as oxidative dimer, trimer, etc. (M<sub>2</sub>-2, M<sub>3</sub>-4 and so on). The mechanism or variety in type of products created during enzymatic treatment of wastewater have not been extensively studied. A thorough examination of the types of the compounds produced during enzymatic treatment helps with better comprehension of the potential toxicity of these transformation products. For instance, soybean peroxidase was quite effective at removing azo dyes from textile wastewater, but the development of soluble degradation products made it more hazardous (Silva et al., 2013). Based on the molecular weight of oligomers obtained from high-resolution MS data, unambiguous chemical formulae are assigned if the mass found and that calculated for the formula are within 10 ppm, and possible reaction product structures have been assigned based on structures of the starting compounds and the coupling 'rules' above. It should be emphasised that mass spectrometry data can only provide a rudimentary understanding of the possible products that may be generated during the treatment and cannot discriminate among isomeric structures (Mashhadi, Taylor, Biswas, et al., 2019). In this study, MS analysis was conducted using the electro-spray ionization technique (mainly in positive-ion mode, thus, protonated forms of the products were frequently detected; negative-ion mode was also available and occasionally used). Following optimal enzymatic treatment of the substrates, the filtered and unfiltered reaction mixture (to avoid losing product precipitates) and standards were analysed using high-resolution instruments at Queen's University. MS masses are mass-to-charge ratios (m/z; z values are invariably 1 as operated). The isotope abundance was considered during the analysis to support the assigned formulae. The following symbols have been used for the obtained structures: M, standard; MH, protonated standard; <sup>13</sup>C-MH, protonated natural abundance <sup>13</sup>C-isomer of standard; M<sub>2</sub>H-2, protonated oxidative dimer; M<sub>3</sub>H-4, protonated oxidative trimer, M<sub>4</sub>H-6, protonated oxidative tetramer. Free acid and dechlorination formation are denoted here by -Na and -Cl, respectively.

Thus,  $M_4H-6-Na-Cl$  denotes an oxidative tetramer as the free acid and dechlorinated. The following table provides a summary of the MS results, including peaks that were confirmed (noted with an asterisk \*) and their assignments, as well as the masses not found, but that were sought as plausibly expected.

#### 4.3.1 Diclofenac

The mass spectral analysis was conducted on DCF standard (not treated, solid) and the filtered and unfiltered solutions after enzymatic reaction under the pre-determined optimal conditions. Table 4-4-5 demonstrates that compounds were only found in their protonated forms, and DCF not being found in its natural state. The expected oxidative tetramer was found along with the protonated free acids ( $MH-Na+H$ ,  $M_4H-6-4Na+H$ ) that confirm loss of Na under reaction conditions to provide free carboxylic acid functional group (-COOH).

The protonated oxidative tetramer which is dechlorinated ( $M_4H-6-3Cl$ ) was also found. The dechlorination process was not due to MS condition. It must have occurred during the enzymatic reaction process since the standard does not show loss of Cl in the MS analysis. Loss of chlorine was reported in some previous studies as well. Wu et al. in 1993 reported dechlorination for chlorinated phenols and for their binding to humic acid during enzymatic polymerization (Wu, 1993). Also, Minard et al. (1981) reported mass spectral evidence for the loss of chlorine atoms from 2,4-dichlorophenol during incubation with a laccase from the fungus *Rhizoctonia praticola*. Therefore, it can be concluded that for DCF, the loss of Cl occurred during the oxidative coupling as the Cl on the ring was in the *ortho*-position relative to the amino functional group, which made it prone to such a dehalogenation event.

There was no evidence of dimer and trimer formation. It can be assumed that the absence of these oligomers is because they are more readily converted to the higher oligomers, i. e. tetramer, than is conversion of the monomer to more dimer during the enzymatic reaction. It should be noted that no difference was observed in the results of filtered and unfiltered samples.

Table 4-5 Summary of MS results for standard and identified products of SBP-catalyzed of diclofenac <sup>a</sup>

Compound	Standard	Symbol	Molecular formula	m/z*	Detected
Diclofenac	Standard	MH	C <sub>14</sub> H <sub>11</sub> Cl <sub>2</sub> NNaO <sub>2</sub>	318.0158	*
		M	C <sub>14</sub> H <sub>10</sub> Cl <sub>2</sub> NNaO <sub>2</sub>	317.0088	
	Identified products	MH-Na+H	C <sub>14</sub> H <sub>12</sub> Cl <sub>2</sub> NO <sub>2</sub>	296.02498	*
		M <sub>2</sub> H-2	C <sub>28</sub> H <sub>18</sub> Cl <sub>4</sub> N <sub>2</sub> Na <sub>2</sub> O <sub>4</sub>	633.009775	
		M <sub>3</sub> H-4	C <sub>42</sub> H <sub>26</sub> Cl <sub>6</sub> N <sub>3</sub> Na <sub>3</sub> O <sub>6</sub>	948.002925	
		M <sub>4</sub> H-6	C <sub>56</sub> H <sub>34</sub> Cl <sub>8</sub> N <sub>4</sub> Na <sub>4</sub> O <sub>8</sub>	1262.99608	*
		M <sub>4</sub> H-6-4Na+H	C <sub>56</sub> H <sub>35</sub> Cl <sub>8</sub> N <sub>4</sub> O <sub>8</sub>	1171.99608	*
		M <sub>4</sub> H-6-3Cl	C <sub>56</sub> H <sub>34</sub> Cl <sub>5</sub> N <sub>4</sub> O <sub>8</sub>	1158.08953	*

<sup>a</sup> with all formulae detected, the requisite <sup>13</sup>C peaks were found and occasionally the <sup>37</sup>Cl were found

#### 4.3.2 Aceclofenac

Aceclofenac falls under 2,6-dichloroanilino-group same as diclofenac. Therefore, the identical nature of both compounds with respect to the part reacting with the enzyme may produce similar results. Hence, it is safe expect similar results from mass spectrometer for both aceclofenac and diclofenac.

## CHAPTER 5 SUMMARY AND CONCLUSIONS

### 5.1 Summary

In this thesis, the feasibility of selected analgesic compounds removal from synthetic wastewater by soybean peroxidase was investigated and optimized conditions are given in Table 5-1. The findings of these preliminary investigations demonstrated that diclofenac and aceclofenac are relatively good substrates for soybean peroxidase. With respect to pH, the optima are close to the carboxyl pKa value for diclofenac (pKa: 4.15) but not for aceclofenac (pKa: 2.60). The optimum H<sub>2</sub>O<sub>2</sub>-to-substrate ratio 4 and 4.5 for diclofenac and aceclofenac respectively, that was higher than the theoretical value 0.5, indicating the higher peroxide demand due oligomer formation, or due to catalase activity of soybean peroxidase.

Based on the first-order fits for the 3-hour time-course experiments, the normalised half-lives of SBP for DCF and ACF were also computed. The first-order model accurately predicted the initial phase of the reaction, but as enzyme activity and hydrogen peroxide levels decreased, the rate of degradation slowed. The initial first-order reaction rate constants and half-lives for the two pollutants under the optimised conditions are listed in Table 5.1.

Table 5-1 Summary of optimized conditions for SBP-catalyzed process and rate constants

<b>Parameters</b>	<b>Diclofenac</b>	<b>Aceclofenac</b>
<b>[Substrate] (mM)</b>	0.1	0.1
<b>pH</b>	5.0	4.0
<b>SBP activity (U/mL)</b>	0.15	0.6
<b>[H<sub>2</sub>O<sub>2</sub>] (mM)</b>	0.4	0.45
<b>Removal (%)</b>	≥96.7	≥96.5
<b>Rate constant, k (min<sup>-1</sup> per U/mL SBP)</b>	0.484±0.02	0.823±0.01
<b>Half-life, t<sub>1/2</sub> (min per U/mL SBP)</b>	1.43±0.01	0.84±0.05

Finally, enzymatic transformation products were analyzed by mass spectrometry after 3-h SBP-catalyzed treatment under optimized conditions. For DCF, the data show evidence of only oxidative tetramer, and their further oxidized forms, which would have formed by direct radical coupling via the dimer and/or tetramer, which were not found.

## 5.2 Limitations

There are three main limitations that affect the findings of this study.

Temperature: This experiment was conducted in controlled laboratory environment. Therefore, all the experiments were done in standard room temperature. It is possible that the reaction would accelerate or decelerate, providing a variation in results if the temperature varied. Thus, the variation of temperature has been considered a limitation.

Synthetic wastewater: This study used synthetic wastewater consisting of targeted contaminants in buffered water. Therefore, the lack of use of actual wastewater whether for the targets themselves or as a matrix for spiking the targets, is considered a limitation for this study.

Reaction with other compounds: Wastewater may contain many contaminants. This could potentially cause variations of results or development of other by-products (the matrix effect). Not examining phenomenon is considered a limitation in this study.

## 5.3 Conclusions

In conclusion, crude SBP isolated from soybean seed coat was proficient in catalyzing the transformation of diclofenac and aceclofenac, as a hypothetically emerging contaminant in an ecologically friendly and sustainable manner. With SBP's availability in massive quantity from a by-product of commodity processing of the world's largest seed crop, SBP-based treatment of selected compounds in this study provides a cost-effective alternative to conventional treatment processes for removing them from wastewaters by having >95% removal efficiency. The SBP and H<sub>2</sub>O<sub>2</sub> requirements for  $\geq 95\%$  removal of these compounds were much less. The only products detected were tetramer, which provides valuable insights into the mechanism of product formation.

## 5.4 Contributions

### 5.4.1 Theoretical contributions

This study used SBP as the enzyme to fill a knowledge gap in research. Therefore, the knowledge of SBP's treatment of the target compounds contributes towards the scholarly community. Furthermore, the quantitative characterization of stoichiometry also contributes theoretically to determine the optimized parameters.



#### 5.4.2 Practical contributions

The results of this study can be directly and indirectly applied in water treatment facilities. Furthermore, the use of SBP will aid circular economy of soybean processing plants. Lastly, the optimized result of this study allows wastewater treatment plants to be cost efficient.

### **5.5 Future Work**

The findings of this study support SBP's ability to accelerate the elimination of certain non-steroidal anti-inflammatory drugs. But still more research is needed before this treatment can be used in real-world wastewater.

1. To confirm the scope, additional NSAIDs and their associated metabolites should be researched.
2. It is advised to conduct more research on polymer products' toxicity and environmental fate. This will enable the development of an appropriate technique for secure disposal and the avoidance of further contamination.
3. In real practice, a sedimentation system must be developed for the generated suspended solids.
4. Cost and benefit analyses for the combination of SBP-catalyzed treatment with wastewater treatment plants or created wetlands should be explored to determine the true cost for full-scale application.
5. In order to estimate the reactor size and operation parameters, additional investigation on the kinetic modeling of the enzyme-catalyzed reaction will be needed.

## REFERENCES

- Acuña, V., Ginebreda, A., Mor, J. R., Petrovic, M., Sabater, S., Sumpter, J., & Barceló, D. (2015). Balancing the health benefits and environmental risks of pharmaceuticals: Diclofenac as an example. *Environment International*, 85, 327–333. <https://doi.org/10.1016/J.ENVINT.2015.09.023>
- Al-Ansari, M. M., Steevensz, A., Al-Aasm, N., Taylor, K. E., Bewtra, J. K., & Biswas, N. (2009). Soybean peroxidase-catalyzed removal of phenylenediamines and benzenediols from water. *Enzyme and Microbial Technology*, 45(4), 253–260. <https://doi.org/10.1016/J.ENZMICTEC.2009.07.004>
- Alessandretti, I., Rigueto, C. V. T., Nazari, M. T., Rosseto, M., & Dettmer, A. (2021). Removal of diclofenac from wastewater: A comprehensive review of detection, characteristics and tertiary treatment techniques. *Journal of Environmental Chemical Engineering*, 9(6), 106743. <https://doi.org/10.1016/J.JECE.2021.106743>
- Al-Maqdi, K. A., Hisaindee, S. M., Rauf, M. A., & Ashraf, S. S. (2017). Comparative Degradation of a Thiazole Pollutant by an Advanced Oxidation Process and an Enzymatic Approach. *Biomolecules 2017, Vol. 7, Page 64*, 7(3), 64. <https://doi.org/10.3390/BIOM7030064>
- Almaqdi, K. A., Morsi, R., Alhayuti, B., Alharthi, F., & Ashraf, S. S. (2019). LC-MSMS based screening of emerging pollutant degradation by different peroxidases. *BMC Biotechnology*, 19(1), 83. <https://doi.org/10.1186/S12896-019-0574-Y>
- Alneyadi, A. H., Rauf, M. A., & Ashraf, S. S. (2018). Oxidoreductases for the remediation of organic pollutants in water – a critical review. *https://Doi.Org/10.1080/07388551.2017.1423275*, 38(7), 971–988. <https://doi.org/10.1080/07388551.2017.1423275>
- Alshabib, M., & Onaizi, S. A. (2019). A review on phenolic wastewater remediation using homogeneous and heterogeneous enzymatic processes: Current status and potential challenges. *Separation and Purification Technology*, 219, 186–207. <https://doi.org/10.1016/J.SEPPUR.2019.03.028>
- Altahir, B. M., Feng, W., Jasim, H. H., Taylor, K. E., Biswas, N., Bewtra, J. K., & Jassim, S. A. (2016). Soybean peroxidase-catalysed removal of benzidines from water. *Journal of Environmental Engineering and Science*, 10(4), 73–80. <https://doi.org/10.1680/JENES.15.00018/ASSET/IMAGES/SMALL/JENES10-0073-F6.GIF>
- Arnao, M. B., Acosta, M., del Rio, J. A., Varón, R., & García-Cánovas, F. (1990). A kinetic study on the suicide inactivation of peroxidase by hydrogen peroxide. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1041(1), 43–47. [https://doi.org/10.1016/0167-4838\(90\)90120-5](https://doi.org/10.1016/0167-4838(90)90120-5)

- ben Younes, S., ben Khedher, S., Zhang, Y., Geissen, S. U., & Sayadi, S. (2019). Laccase from *Scytalidium thermophilum*: Production Improvement, Catalytic Behavior and Detoxifying Ability of Diclofenac. *Catalysis Letters*, *149*(7), 1833–1844.  
<https://doi.org/10.1007/S10562-019-02771-1/FIGURES/4>
- Bideh, N. Z. (2020). Elimination of Selected Heteroaromatics from Wastewater Using Soybean Peroxidase. *Electronic Theses and Dissertations*. <https://scholar.uwindsor.ca/etd/8411>
- Bideh, N. Z., Mashhadi, N., Taylor, K. E., & Biswas, N. (2021). Elimination of selected heterocyclic aromatic emerging contaminants from water using soybean peroxidase. *Environmental Science and Pollution Research*, *28*(28), 37570–37579.  
<https://doi.org/10.1007/S11356-021-13403-W/FIGURES/6>
- Bilal, M., Rasheed, T., Iqbal, H. M. N., & Yan, Y. (2018). Peroxidases-assisted removal of environmentally related hazardous pollutants with reference to the reaction mechanisms of industrial dyes. *Science of The Total Environment*, *644*, 1–13.  
<https://doi.org/10.1016/J.SCITOTENV.2018.06.274>
- Biswas, Kaushik. (1999). Removal of cresols from synthetic wastewater using crude soybean peroxidase. *Electronic Theses and Dissertations*. <https://scholar.uwindsor.ca/etd/3316>
- Bódalo, A., Gómez, E., Gómez, J. L., Bastida, J., Máximo, M. F., & Díaz, F. (1991). A comparison of different methods of  $\beta$ -galactosidase immobilization. *Process Biochemistry*, *26*(6), 349–353. [https://doi.org/10.1016/0032-9592\(91\)85025-J](https://doi.org/10.1016/0032-9592(91)85025-J)
- Bollag, J. M., Liu, S. Y., & Minard, R. D. (1979). Asymmetric diphenol formation by a fungal laccase. *Applied and Environmental Microbiology*, *38*(1), 90–92.  
<https://doi.org/10.1128/AEM.38.1.90-92.1979>
- Brack, W., & Schirmer, K. (2003). Effect-directed identification of oxygen and sulfur heterocycles as major polycyclic aromatic cytochrome P450A-inducers in a contaminated sediment. *Environmental Science and Technology*, *37*(14), 3062–3070.  
<https://doi.org/10.1021/ES020248J/ASSET/IMAGES/LARGE/ES020248JF00008.JPEG>
- Carballa, M., Omil, F., & Lema, J. M. (2005). Removal of cosmetic ingredients and pharmaceuticals in sewage primary treatment. *Water Research*, *39*(19), 4790–4796.  
<https://doi.org/10.1016/J.WATRES.2005.09.018>
- Carmona, E., Andreu, V., & Picó, Y. (2014). Occurrence of acidic pharmaceuticals and personal care products in Turia River Basin: from waste to drinking water. *The Science of the Total Environment*, *484*(1), 53–63. <https://doi.org/10.1016/J.SCITOTENV.2014.02.085>
- Caza, N., Bewtra, J. K., Biswas, N., & Taylor, K. E. (1999). Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. *Water Research*, *33*(13), 3012–3018.  
[https://doi.org/10.1016/S0043-1354\(98\)00525-9](https://doi.org/10.1016/S0043-1354(98)00525-9)
- Celiz, M. D., Pérez, S., Barceló, D., & Aga, D. S. (2009). Trace Analysis of Polar Pharmaceuticals in Wastewater by LC-MS-MS: Comparison of Membrane Bioreactor and

- Activated Sludge Systems. *Journal of Chromatographic Science*, 47(1), 19–25.  
<https://doi.org/10.1093/CHROMSCI/47.1.19>
- Chagas, P. M. B., Torres, J. A., Silva, M. C., & Corrêa, A. D. (2015). Immobilized soybean hull peroxidase for the oxidation of phenolic compounds in coffee processing wastewater. *International Journal of Biological Macromolecules*, 81, 568–575.  
<https://doi.org/10.1016/J.IJBIOMAC.2015.08.061>
- Chaves, M. de J. S., Barbosa, S. C., Malinowski, M. de M., Volpato, D., Castro, Í. B., Franco, T. C. R. dos S., & Primel, E. G. (2020). Pharmaceuticals and personal care products in a Brazilian wetland of international importance: Occurrence and environmental risk assessment. *Science of The Total Environment*, 734, 139374.  
<https://doi.org/10.1016/J.SCITOTENV.2020.139374>
- Chiong, T., Lau, S. Y., Lek, Z. H., Koh, B. Y., & Danquah, M. K. (2016). Enzymatic treatment of methyl orange dye in synthetic wastewater by plant-based peroxidase enzymes. *Journal of Environmental Chemical Engineering*, 4(2), 2500–2509.  
<https://doi.org/10.1016/J.JECE.2016.04.030>
- Cordova Villegas, L. G., Mazloun, S., Taylor, K. E., & Biswas, N. (2018). Soybean Peroxidase-Catalyzed Treatment of Azo Dyes with or without Fe<sup>o</sup> Pretreatment. *Water Environment Research : A Research Publication of the Water Environment Federation*, 90(8), 675–684.  
<https://doi.org/10.2175/106143017X15131012153149>
- Cumming, H., & Rücker, C. (2017). Octanol-Water Partition Coefficient Measurement by a Simple <sup>1</sup>H NMR Method. *ACS Omega*, 2(9), 6244–6249.  
[https://doi.org/10.1021/ACSOMEGA.7B01102/ASSET/IMAGES/AO-2017-011026\\_M009.GIF](https://doi.org/10.1021/ACSOMEGA.7B01102/ASSET/IMAGES/AO-2017-011026_M009.GIF)
- Das, D., Cuthbert, R. J., Jakati, R. D., & Prakash, V. (2011). Diclofenac is toxic to the Himalayan Vulture *Gyps himalayensis*. *Bird Conservation International*, 21(1), 72–75.  
<https://doi.org/10.1017/S0959270910000171>
- de Jesus Gaffney, V., Cardoso, V. V., Cardoso, E., Teixeira, A. P., Martins, J., Benoliel, M. J., & Almeida, C. M. M. (2017a). Occurrence and behaviour of pharmaceutical compounds in a Portuguese wastewater treatment plant: Removal efficiency through conventional treatment processes. *Environmental Science and Pollution Research*, 24(17), 14717–14734.  
<https://doi.org/10.1007/S11356-017-9012-7/FIGURES/7>
- de Jesus Gaffney, V., Cardoso, V. V., Cardoso, E., Teixeira, A. P., Martins, J., Benoliel, M. J., & Almeida, C. M. M. (2017b). Occurrence and behaviour of pharmaceutical compounds in a Portuguese wastewater treatment plant: Removal efficiency through conventional treatment processes. *Environmental Science and Pollution Research*, 24(17), 14717–14734.  
<https://doi.org/10.1007/S11356-017-9012-7/FIGURES/7>
- de Rossi, A., Riguetto, C. V. T., Dettmer, A., Colla, L. M., & Piccin, J. S. (2020). Synthesis, characterization, and application of *Saccharomyces cerevisiae*/alginate composites beads for

- adsorption of heavy metals. *Journal of Environmental Chemical Engineering*, 8(4), 104009.  
<https://doi.org/10.1016/J.JECE.2020.104009>
- Demarche, P., Junghanns, C., Nair, R. R., & Agathos, S. N. (2012). Harnessing the power of enzymes for environmental stewardship. *Biotechnology Advances*, 30(5), 933–953.  
<https://doi.org/10.1016/J.BIOTECHADV.2011.05.013>
- Dunford, H. Brian. (1999). *Heme peroxidases*. 507.  
[https://books.google.com/books/about/Heme\\_Peroxidases.html?id=HnNFAQAAIAAJ](https://books.google.com/books/about/Heme_Peroxidases.html?id=HnNFAQAAIAAJ)
- Ebele, A. J., Abou-Elwafa Abdallah, M., & Harrad, S. (2017). Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. *Emerging Contaminants*, 3(1), 1–16. <https://doi.org/10.1016/J.EMCON.2016.12.004>
- Eslami, A., Amini, M. M., Yazdanbakhsh, A. R., Rastkari, N., Mohseni-Bandpei, A., Nasser, S., Piroti, E., & Asadi, A. (2015). Occurrence of non-steroidal anti-inflammatory drugs in Tehran source water, municipal and hospital wastewaters, and their ecotoxicological risk assessment. *Environmental Monitoring and Assessment*, 187(12), 1–15.  
<https://doi.org/10.1007/S10661-015-4952-1/TABLES/6>
- Fatta-Kassinos, D., Hapeshi, E., Achilleos, A., Meric, S., Gros, M., Petrovic, M., & Barcelo, D. (2010). Existence of Pharmaceutical Compounds in Tertiary Treated Urban Wastewater that is Utilized for Reuse Applications. *Water Resources Management* 25:4, 25(4), 1183–1193. <https://doi.org/10.1007/S11269-010-9646-4>
- Feng, W., Taylor, K. E., Biswas, N., & Bewtra, J. K. (2013). Soybean peroxidase trapped in product precipitate during phenol polymerization retains activity and may be recycled. *Journal of Chemical Technology & Biotechnology*, 88(8), 1429–1435.  
<https://doi.org/10.1002/JCTB.4075>
- Ferrari, B., Paxéus, N., Giudice, R. lo, Pollio, A., & Garric, J. (2003). Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. *Ecotoxicology and Environmental Safety*, 55(3), 359–370.  
[https://doi.org/10.1016/S0147-6513\(02\)00082-9](https://doi.org/10.1016/S0147-6513(02)00082-9)
- Geng, Z., Rao, K. J., Bassi, A. S., Gijzen, M., & Krishnamoorthy, N. (2001). Investigation of biocatalytic properties of soybean seed hull peroxidase. *Catalysis Today*, 64(3–4), 233–238.  
[https://doi.org/10.1016/S0920-5861\(00\)00527-7](https://doi.org/10.1016/S0920-5861(00)00527-7)
- Gómez-Oliván, L. M. (Ed.). (2020). *Non-Steroidal Anti-Inflammatory Drugs in Water*. 96.  
<https://doi.org/10.1007/978-3-030-56294-6>
- Gumbi, B. P., Moodley, B., Birungi, G., & Ndungu, P. G. (2017). Detection and quantification of acidic drug residues in South African surface water using gas chromatography-mass spectrometry. *Chemosphere*, 168, 1042–1050.  
<https://doi.org/10.1016/J.CHEMOSPHERE.2016.10.105>

- Heme Peroxidases* by Emma Raven, Brian Dunford - Books on Google Play. (n.d.). Retrieved December 20, 2022, from [https://play.google.com/store/books/details/Heme\\_Peroxidases?id=8mooDwAAQBAJ&gl=US&pli=1](https://play.google.com/store/books/details/Heme_Peroxidases?id=8mooDwAAQBAJ&gl=US&pli=1)
- Hernando, M. D., Heath, E., Petrovic, M., & Barceló, D. (2006). Trace-level determination of pharmaceutical residues by LC-MS/MS in natural and treated waters. A pilot-survey study. *Analytical and Bioanalytical Chemistry*, 385(6), 985–991. <https://doi.org/10.1007/S00216-006-0394-5/TABLES/3>
- Hilton, M. J., & Thomas, K. v. (2003). Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography–electrospray tandem mass spectrometry. *Journal of Chromatography A*, 1015(1–2), 129–141. [https://doi.org/10.1016/S0021-9673\(03\)01213-5](https://doi.org/10.1016/S0021-9673(03)01213-5)
- Hoeger, B., Köllner, B., Dietrich, D. R., & Hitzfeld, B. (2005). Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta* f. *fario*). *Aquatic Toxicology*, 75(1), 53–64. <https://doi.org/10.1016/J.AQUATOX.2005.07.006>
- Iolascon, G., Giménez, S., & Mogyorósi, D. (2021). A Review of Aceclofenac: Analgesic and Anti-Inflammatory Effects on Musculoskeletal Disorders. *Journal of Pain Research*, 14, 3651–3663. <https://doi.org/10.2147/JPR.S326101>
- Jaeschke, H., & Bajt, M. L. (2006). Intracellular Signaling Mechanisms of Acetaminophen-Induced Liver Cell Death. *Toxicological Sciences*, 89(1), 31–41. <https://doi.org/10.1093/TOXSCI/KFI336>
- Jodeh, S., Basalat, N., Obaid, A., Bouknana, D., Hammouti, B., Hadda, T., Jodeh, W., & Warad, I. (2014). *Adsorption of some organic phenolic compounds using activated carbon from cypress products*.
- Jun, L. Y., Yon, L. S., Mubarak, N. M., Bing, C. H., Pan, S., Danquah, M. K., Abdullah, E. C., & Khalid, M. (2019a). An overview of immobilized enzyme technologies for dye and phenolic removal from wastewater. *Journal of Environmental Chemical Engineering*, 7(2), 102961. <https://doi.org/10.1016/J.JECE.2019.102961>
- Jun, L. Y., Yon, L. S., Mubarak, N. M., Bing, C. H., Pan, S., Danquah, M. K., Abdullah, E. C., & Khalid, M. (2019b). An overview of immobilized enzyme technologies for dye and phenolic removal from wastewater. *Journal of Environmental Chemical Engineering*, 7(2), 102961. <https://doi.org/10.1016/J.JECE.2019.102961>
- Kalia, S., Thakur, K., Celli, A., Kiechel, M. A., & Schauer, C. L. (2013). Surface modification of plant fibers using environment friendly methods for their application in polymer composites, textile industry and antimicrobial activities: A review. *Journal of Environmental Chemical Engineering*, 1(3), 97–112. <https://doi.org/10.1016/J.JECE.2013.04.009>

- Kamal, J. K. A., & Behere, D. v. (2003). Activity, stability and conformational flexibility of seed coat soybean peroxidase. *Journal of Inorganic Biochemistry*, 94(3), 236–242.  
[https://doi.org/10.1016/S0162-0134\(03\)00004-7](https://doi.org/10.1016/S0162-0134(03)00004-7)
- Karam, J., & Nicell, J. A. (1997). Review Potential Applications of Enzymes in Waste Treatment. *J. Chem. T Ech. Biotechnol*, 69, 141–153. [https://doi.org/10.1002/\(SICI\)1097-4660\(199706\)69:2](https://doi.org/10.1002/(SICI)1097-4660(199706)69:2)
- Karaman, R., Khamis, M., Quried, M., Halabieh, R., Makharzeh, I., Manassra, A., Abbadi, J., Qtait, A., Bufo, S. A., Nasser, A., & Nir, S. (2012). Removal of diclofenac potassium from wastewater using clay-micelle complex.  
<Http://Dx.Doi.Org/10.1080/09593330.2011.619582>, 33(11), 1279–1287.  
<https://doi.org/10.1080/09593330.2011.619582>
- Kasprzyk-Hordern, B., Dinsdale, R. M., & Guwy, A. J. (2009). Illicit drugs and pharmaceuticals in the environment – Forensic applications of environmental data. Part 1: Estimation of the usage of drugs in local communities. *Environmental Pollution*, 157(6), 1773–1777.  
<https://doi.org/10.1016/J.ENVPOL.2009.03.017>
- Kermia, A. E. B., Fouial-Djebbar, D., & Trari, M. (2016). Occurrence, fate and removal efficiencies of pharmaceuticals in wastewater treatment plants (WWTPs) discharging in the coastal environment of Algiers. *Comptes Rendus Chimie*, 19(8), 963–970.  
<https://doi.org/10.1016/J.CRCL.2016.05.005>
- Kim, S. D., Cho, J., Kim, I. S., Vanderford, B. J., & Snyder, S. A. (2007). Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Research*, 41(5), 1013–1021.  
<https://doi.org/10.1016/J.WATRES.2006.06.034>
- Klibanov, A. M., Tu, T. M., & Scott, K. P. (1983). Peroxidase-Catalyzed Removal of Phenols from Coal-Conversion Waste Waters. *Science*, 221(4607), 259–261.  
<https://doi.org/10.1126/SCIENCE.221.4607.259-A>
- K'oreje, K. O., Vergeynst, L., Ombaka, D., de Wispelaere, P., Okoth, M., van Langenhove, H., & Demeestere, K. (2016). Occurrence patterns of pharmaceutical residues in wastewater, surface water and groundwater of Nairobi and Kisumu city, Kenya. *Chemosphere*, 149, 238–244. <https://doi.org/10.1016/J.CHEMOSPHERE.2016.01.095>
- Krainer, F. W., & Glieder, A. (2015). An updated view on horseradish peroxidases: recombinant production and biotechnological applications. *Applied Microbiology and Biotechnology*, 99(4), 1611–1625. <https://doi.org/10.1007/S00253-014-6346-7/TABLES/2>
- Kumar, M., Dahuja, A., Sachdev, A., Kaur, C., Varghese, E., Saha, S., & Sairam, K. V. S. S. (2019). Evaluation of enzyme and microwave-assisted conditions on extraction of anthocyanins and total phenolics from black soybean (*Glycine max L.*) seed coat. *International Journal of Biological Macromolecules*, 135, 1070–1081.  
<https://doi.org/10.1016/J.IJBIOMAC.2019.06.034>

- Kwak, K., Ji, K., Kho, Y., Kim, P., Lee, J., Ryu, J., & Choi, K. (2018). Chronic toxicity and endocrine disruption of naproxen in freshwater waterfleas and fish, and steroidogenic alteration using H295R cell assay. *Chemosphere*, *204*, 156–162. <https://doi.org/10.1016/J.CHEMOSPHERE.2018.04.035>
- Lin, W. C., Chen, H. C., & Ding, W. H. (2005). Determination of pharmaceutical residues in waters by solid-phase extraction and large-volume on-line derivatization with gas chromatography–mass spectrometry. *Journal of Chromatography A*, *1065*(2), 279–285. <https://doi.org/10.1016/J.CHROMA.2004.12.081>
- Mantha, R., Taylor, K. E., Biswas, N., & Bewtra, J. K. (2001). A continuous system for Fe0 reduction of nitrobenzene in synthetic wastewater. *Environmental Science and Technology*, *35*(15), 3231–3236. <https://doi.org/10.1021/ES0014943/ASSET/IMAGES/LARGE/ES0014943F00006.JPEG>
- Mashhadi, N., Taylor, K. E., Biswas, N., Meister, P., & Gauld, J. W. (2019). Oligomerization of 3-substituted quinolines by catalytic activity of soybean peroxidase as a wastewater treatment. Product formation and computational studies. *Chemical Engineering Journal*, *364*, 340–348. <https://doi.org/10.1016/J.CEJ.2019.01.184>
- Mashhadi, N., Taylor, K. E., Jimenez, N., Varghese, S. T., Levi, Y., Lonergan, C., Lebeau, E., Lamé, M., Lard, E., & Biswas, N. (2019). Removal of Selected Pharmaceuticals and Personal Care Products from Wastewater using Soybean Peroxidase. *Environmental Management*, *63*(3), 408–415. <https://doi.org/10.1007/S00267-018-01132-9/TABLES/1>
- McEldoon, J. P., & Dordick, J. S. (1996). Unusual Thermal Stability of Soybean Peroxidase. *Biotechnology Progress*, *12*(4), 555–558. <https://doi.org/10.1021/BP960010X>
- Meinicke, J., & Danneskiold-Samsøe, B. (2009). Diclofenac Sodium (Voltaren®) and Ibuprofen in Rheumatoid Arthritis A Randomized Double-blind Study. <Http://Dx.Doi.Org/10.3109/03009748009101380>, *9*(S35), 1–8. <https://doi.org/10.3109/03009748009101380>
- Metcalf, C. D., Miao, X. S., Koenig, B. G., & Struger, J. (2003). Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environmental Toxicology and Chemistry*, *22*(12), 2881–2889. <https://doi.org/10.1897/02-627>
- Mishra, B., Varjani, S., Agrawal, D. C., Mandal, S. K., Ngo, H. H., Taherzadeh, M. J., Chang, J. S., You, S., & Guo, W. (2020). Engineering biocatalytic material for the remediation of pollutants: A comprehensive review. *Environmental Technology & Innovation*, *20*, 101063. <https://doi.org/10.1016/J.ETI.2020.101063>
- Moore, N., Duong, M., Gulmez, S. E., Blin, P., & Droz, C. (2019). Pharmacoepidemiology of non-steroidal anti-inflammatory drugs. *Therapies*, *74*(2), 271–277. <https://doi.org/10.1016/J.THERAP.2018.11.002>



- Morales Urrea, D. A., Haure, P. M., García Einschlag, F. S., & Contreras, E. M. (2018). Horseradish peroxidase-mediated decolourization of Orange II: modelling hydrogen peroxide utilization efficiency at different pH values. *Environmental Science and Pollution Research*, 25(20), 19989–20002. <https://doi.org/10.1007/S11356-018-2134-8>
- Morsi, R., Bilal, M., Iqbal, H. M. N., & Ashraf, S. S. (2020). Laccases and peroxidases: The smart, greener and futuristic biocatalytic tools to mitigate recalcitrant emerging pollutants. *Science of The Total Environment*, 714, 136572. <https://doi.org/10.1016/J.SCITOTENV.2020.136572>
- Mukherjee, D., Bhattacharya, S., Taylor, K. E., & Biswas, N. (2019). Enzymatic treatment for removal of hazardous aqueous arylamines, 4,4'-methylenedianiline and 4,4'-thiodianiline. *Chemosphere*, 235, 365–372. <https://doi.org/10.1016/J.CHEMOSPHERE.2019.06.182>
- Mukherjee, D., Taylor, K. E., & Biswas, N. (2018). Soybean Peroxidase-Induced Treatment of Dye-Derived Arylamines in Water. *Water, Air, and Soil Pollution*, 229(8), 1–13. <https://doi.org/10.1007/S11270-018-3936-5/TABLES/1>
- Munnecke, D. M. (1976). Enzymatic hydrolysis of organophosphate insecticides, a possible pesticide disposal method. *Applied and Environmental Microbiology*, 32(1), 7–13. <https://doi.org/10.1128/AEM.32.1.7-13.1976>
- Ncanana, S., & Burton, S. (2007). Oxidation of 8-hydroxyquinoline catalyzed by laccase from *Trametes pubescens* yields an antioxidant aromatic polymer. *Journal of Molecular Catalysis B: Enzymatic*, 44(2), 66–71. <https://doi.org/10.1016/J.MOLCATB.2006.09.005>
- Öllers, S., Singer, H. P., Fässler, P., & Müller, S. R. (2001). Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/l level in surface and waste water. *Journal of Chromatography A*, 911(2), 225–234. [https://doi.org/10.1016/S0021-9673\(01\)00514-3](https://doi.org/10.1016/S0021-9673(01)00514-3)
- Padoley, K. v., Mudliar, S. N., & Pandey, R. A. (2008). Heterocyclic nitrogenous pollutants in the environment and their treatment options – An overview. *Bioresource Technology*, 99(10), 4029–4043. <https://doi.org/10.1016/J.BIORTECH.2007.01.047>
- Paíga, P., Santos, L. H. M. L. M., Ramos, S., Jorge, S., Silva, J. G., & Delerue-Matos, C. (2016). Presence of pharmaceuticals in the Lis river (Portugal): Sources, fate and seasonal variation. *Science of The Total Environment*, 573, 164–177. <https://doi.org/10.1016/J.SCITOTENV.2016.08.089>
- Pandelides, Z., Ussery, E. J., Overturf, M. D., Guchardi, J., & Holdway, D. A. (2021). Inhibition of swim bladder inflation in Japanese medaka (*Oryzias latipes*) embryos following exposure to select pharmaceuticals alone and in combination. *Aquatic Toxicology*, 234, 105796. <https://doi.org/10.1016/J.AQUATOX.2021.105796>
- Parolini, M. (2020). Toxicity of the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) acetylsalicylic acid, paracetamol, diclofenac, ibuprofen and naproxen towards freshwater

- invertebrates: A review. *Science of The Total Environment*, 740, 140043.  
<https://doi.org/10.1016/J.SCITOTENV.2020.140043>
- Parsiavash, L., Saboora, A., Zahra, S., & Nejad, M. (2015). Investigating on the Stability of Peroxidase Extracted from Soybean (*Glycine max* var. Williams) and Effects of Na<sup>+</sup> and K<sup>+</sup> Ions on its Activity. *Journal of Cell and Molecular Research*, 7(2), 94–101.  
<https://doi.org/10.22067/JCMR.V7I2.43716>
- Pawłowska, B., Telesiński, A., & Biczak, R. (2021). Effect of diclofenac and naproxen and their mixture on spring barley seedlings and *Heterocypris incongruens*. *Environmental Toxicology and Pharmacology*, 88, 103746. <https://doi.org/10.1016/J.ETAP.2021.103746>
- Pojana, G., Fantinati, A., & Marcomini, A. (2011). Occurrence of environmentally relevant pharmaceuticals in Italian drinking water treatment plants.  
<Http://Dx.Doi.Org/10.1080/03067310903531504>, 91(6), 537–552.  
<https://doi.org/10.1080/03067310903531504>
- Praveena, S. M., Shaifuddin, S. N. M., Sukiman, S., Nasir, F. A. M., Hanafi, Z., Kamarudin, N., Ismail, T. H. T., & Aris, A. Z. (2018). Pharmaceuticals residues in selected tropical surface water bodies from Selangor (Malaysia): Occurrence and potential risk assessments. *Science of The Total Environment*, 642, 230–240.  
<https://doi.org/10.1016/J.SCITOTENV.2018.06.058>
- Psomas, G. (2020). Copper(II) and zinc(II) coordination compounds of non-steroidal anti-inflammatory drugs: Structural features and antioxidant activity. *Coordination Chemistry Reviews*, 412, 213259. <https://doi.org/10.1016/J.CCR.2020.213259>
- Rabiet, M., Togola, A., Brissaud, F., Seidel, J. L., Budzinski, H., & Elbaz-Poulichet, F. (2006). Consequences of treated water recycling as regards pharmaceuticals and drugs in surface and ground waters of a medium-sized mediterranean catchment. *Environmental Science and Technology*, 40(17), 5282–5288.  
<https://doi.org/10.1021/ES060528P/ASSET/IMAGES/LARGE/ES060528PF00004.JPEG>
- Rasheed, T., Bilal, M., Nabeel, F., Adeel, M., & Iqbal, H. M. N. (2019). Environmentally-related contaminants of high concern: Potential sources and analytical modalities for detection, quantification, and treatment. *Environment International*, 122, 52–66.  
<https://doi.org/10.1016/J.ENVINT.2018.11.038>
- Rezvani, F., Azargoshasb, H., Jamialahmadi, O., Hashemi-Najafabadi, S., Mousavi, S. M., & Shojaosadati, S. A. (2015). Experimental study and CFD simulation of phenol removal by immobilization of soybean seed coat in a packed-bed bioreactor. *Biochemical Engineering Journal*, 101, 32–43. <https://doi.org/10.1016/J.BEJ.2015.04.019>
- Riva, S. (2006). Laccases: blue enzymes for green chemistry. *Trends in Biotechnology*, 24(5), 219–226. <https://doi.org/10.1016/J.TIBTECH.2006.03.006>

- Ryan, B. J., Carolan, N., & Ó'Fágáin, C. (2006). Horseradish and soybean peroxidases: comparable tools for alternative niches? *Trends in Biotechnology*, 24(8), 355–363. <https://doi.org/10.1016/J.TIBTECH.2006.06.007>
- Saha, B., Taylor, K. E., Bewtra, J. K., & Biswas, N. (2008). Laccase-Catalyzed Removal of Diphenylamine from Synthetic Wastewater. *Water Environment Research*, 80(11), 2118–2124. <https://doi.org/10.2175/106143008X304712>
- Sayen, S., Carlier, A., Tarpin, M., & Guillon, E. (2013). A novel copper(II) mononuclear complex with the non-steroidal anti-inflammatory drug diclofenac: Structural characterization and biological activity. *Journal of Inorganic Biochemistry*, 120, 39–43. <https://doi.org/10.1016/J.JINORGBIO.2012.12.002>
- Sellami, K., Couvert, A., Nasrallah, N., Maachi, R., Abouseoud, M., & Amrane, A. (2022). Peroxidase enzymes as green catalysts for bioremediation and biotechnological applications: A review. *Science of The Total Environment*, 806, 150500. <https://doi.org/10.1016/J.SCITOTENV.2021.150500>
- Sim, W. J., Lee, J. W., Lee, E. S., Shin, S. K., Hwang, S. R., & Oh, J. E. (2011). Occurrence and distribution of pharmaceuticals in wastewater from households, livestock farms, hospitals and pharmaceutical manufactures. *Chemosphere*, 82(2), 179–186. <https://doi.org/10.1016/J.CHEMOSPHERE.2010.10.026>
- Steevensz, A., Al-Ansari, M. M., Taylor, K. E., Bewtra, J. K., & Biswas, N. (2009). Comparison of soybean peroxidase with laccase in the removal of phenol from synthetic and refinery wastewater samples. *Journal of Chemical Technology & Biotechnology*, 84(5), 761–769. <https://doi.org/10.1002/JCTB.2109>
- Steevensz, A., Madur, S., Al-Ansari, M. M., Taylor, K. E., Bewtra, J. K., & Biswas, N. (2013). A simple lab-scale extraction of soybean hull peroxidase shows wide variation among cultivars. *Industrial Crops and Products*, 48, 13–18. <https://doi.org/10.1016/J.INDCROP.2013.03.030>
- Steevensz, A., Madur, S., Feng, W., Taylor, K. E., Bewtra, J. K., & Biswas, N. (2014). Crude soybean hull peroxidase treatment of phenol in synthetic and real wastewater: Enzyme economy enhanced by Triton X-100. *Enzyme and Microbial Technology*, 55, 65–71. <https://doi.org/10.1016/J.ENZMICTEC.2013.12.005>
- Steevensz, A., Villegas, L. G. C., Feng, W., Taylor, K. E., Bewtra, J. K., & Biswas, N. (2015). Soybean peroxidase for industrial wastewater treatment: a mini review. <https://doi.org/10.1680/J.EES.13.00013>, 9(3), 181–186. <https://doi.org/10.1680/J.EES.13.00013>
- Summerscales, J. (2021). A review of bast fibres and their composites: Part 4 ~ organisms and enzyme processes. *Composites Part A: Applied Science and Manufacturing*, 140, 106149. <https://doi.org/10.1016/J.COMPOSITESA.2020.106149>

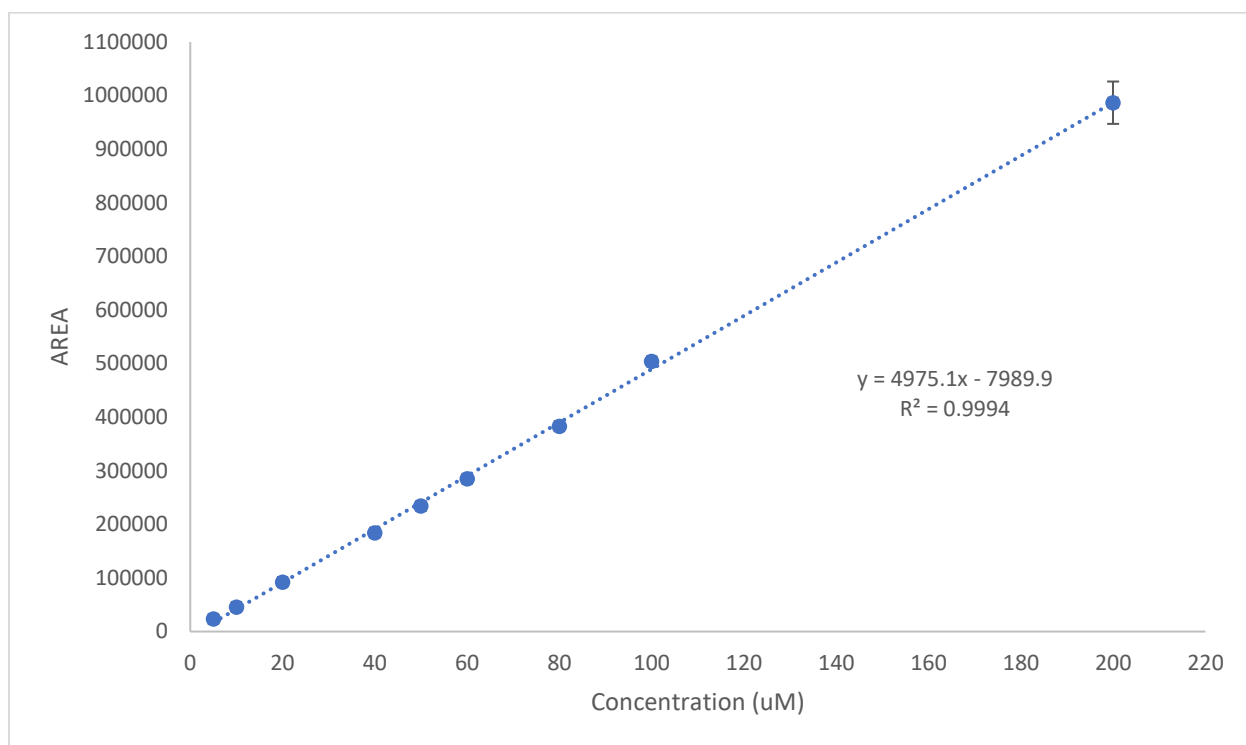
- Svanfelt, J. (n.d.). *Occurrence and photochemical fate of selected pharmaceutical active ingredients in the aquatic environment Occurrence and photochemical fate of selected pharmaceutical active ingredients in the aquatic environment*.
- Taylor, K. E., Al-Kassim, L., Bewtra, J. K., Biswas, N., & Taylor, J. (1995). Enzyme-Based Wastewater Treatment: Removal of Phenols by Oxidative Enzymes. *Environmental Biotechnology*, 524–532. [https://doi.org/10.1007/978-94-017-1435-8\\_46](https://doi.org/10.1007/978-94-017-1435-8_46)
- Valderrama, B., Ayala, M., & Vazquez-Duhalt, R. (2002). Suicide Inactivation of Peroxidases and the Challenge of Engineering More Robust Enzymes. *Chemistry & Biology*, 9(5), 555–565. [https://doi.org/10.1016/S1074-5521\(02\)00149-7](https://doi.org/10.1016/S1074-5521(02)00149-7)
- Vane, J. R., & Botting, R. M. (1998). Anti-inflammatory drugs and their mechanism of action. *Inflammation Research* 1998 47:2, 47(2), 78–87. <https://doi.org/10.1007/S000110050284>
- Varga, B., Somogyi, V., Meiczinger, M., Kováts, N., & Domokos, E. (2019). Enzymatic treatment and subsequent toxicity of organic micropollutants using oxidoreductases - A review. *Journal of Cleaner Production*, 221, 306–322. <https://doi.org/10.1016/J.JCLEPRO.2019.02.135>
- Verlicchi, P., al Aukidy, M., & Zambello, E. (2012). Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment--a review. *The Science of the Total Environment*, 429, 123–155. <https://doi.org/10.1016/J.SCITOTENV.2012.04.028>
- Vieno, N., & Sillanpää, M. (2014). Fate of diclofenac in municipal wastewater treatment plant — A review. *Environment International*, 69, 28–39. <https://doi.org/10.1016/J.ENVINT.2014.03.021>
- Villegas, L. G. C., Mashhadi, N., Chen, M., Mukherjee, D., Taylor, K. E., & Biswas, N. (2016). A Short Review of Techniques for Phenol Removal from Wastewater. *Current Pollution Reports* 2016 2:3, 2(3), 157–167. <https://doi.org/10.1007/S40726-016-0035-3>
- Wang, M., & Fang, L. (2008). *Percutaneous absorption of diclofenac acid and its salts from emulgel*.
- Wu, Yimin. (1993). Removal of phenol derivatives from aqueous solution by horseradish peroxidase in the presence of additives. *Electronic Theses and Dissertations*. <https://scholar.uwindsor.ca/etd/3318>
- Xiong, Q., Liu, Y. S., Hu, L. X., Shi, Z. Q., & Ying, G. G. (2020). Levofloxacin and sulfamethoxazole induced alterations of biomolecules in *Pseudokirchneriella subcapitata*. *Chemosphere*, 253, 126722. <https://doi.org/10.1016/J.CHEMOSPHERE.2020.126722>
- Yao, W., Ur Rehman, S. W., Wang, H., Yang, H., Yu, G., & Wang, Y. (2018). Pilot-scale evaluation of micropollutant abatements by conventional ozonation, UV/O<sub>3</sub>, and an electroperoxone process. *Water Research*, 138, 106–117. <https://doi.org/10.1016/J.WATRES.2018.03.044>

- You, L., Nguyen, V. T., Pal, A., Chen, H., He, Y., Reinhard, M., & Gin, K. Y. H. (2015). Investigation of pharmaceuticals, personal care products and endocrine disrupting chemicals in a tropical urban catchment and the influence of environmental factors. *Science of The Total Environment*, 536, 955–963. <https://doi.org/10.1016/J.SCITOTENV.2015.06.041>
- Yu, J., Taylor, K. E., Zou, H., Biswas, N., & Bewtra, J. K. (1994). Phenol Conversion and Dimeric Intermediates in Horseradish Peroxidase-Catalyzed Phenol Removal from Water. *Environmental Science and Technology*, 28(12), 2154–2160. <https://doi.org/10.1021/ES00061A025>
- Zhang, X. (2019). Enzymatic Treatment of Selected Pesticides in Aqueous System. *Electronic Theses and Dissertations*. <https://scholar.uwindsor.ca/etd/7857>
- Zhang, Y., Geißen, S. U., & Gal, C. (2008). Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere*, 73(8), 1151–1161. <https://doi.org/10.1016/J.CHEMOSPHERE.2008.07.086>
- Zorita, S., Mårtensson, L., & Mathiasson, L. (2009). Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. *Science of The Total Environment*, 407(8), 2760–2770. <https://doi.org/10.1016/J.SCITOTENV.2008.12.030>

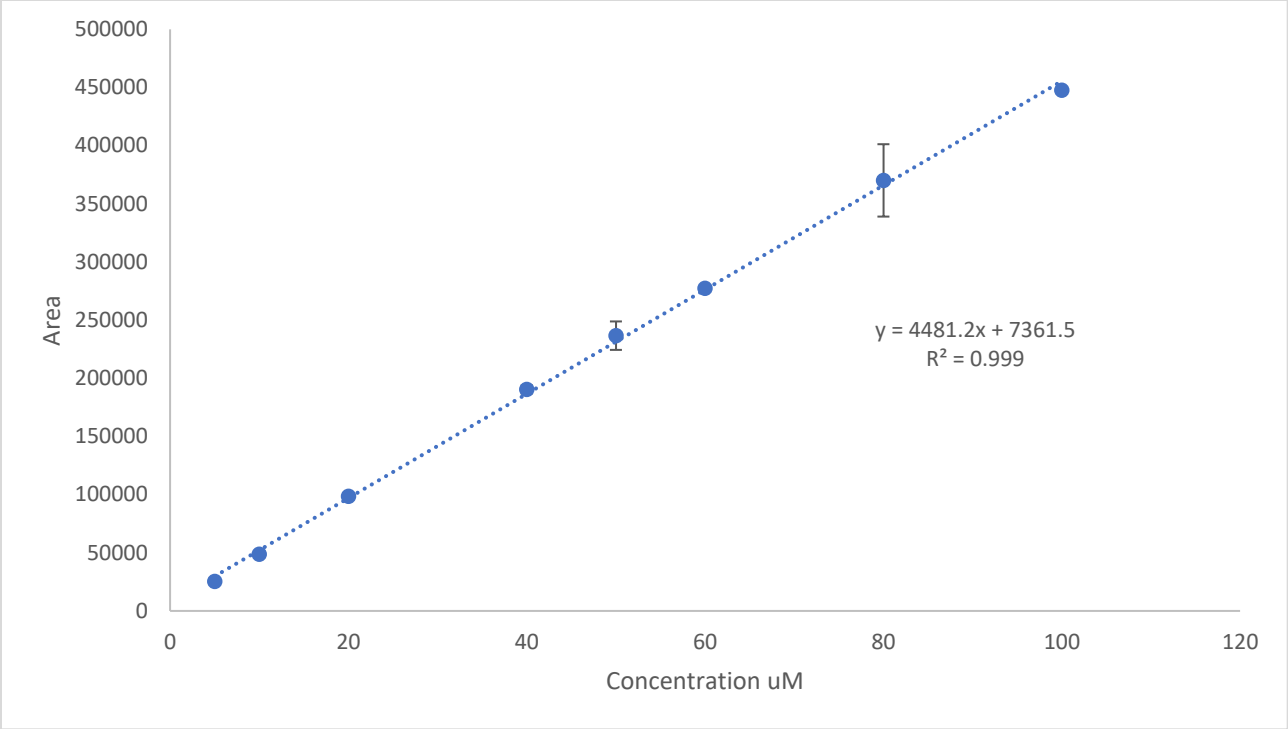
## APPENDICES

### Appendix A. HPLC Calibration curves

The standard curves for the substrates were created using the average value of triplicate experiments from the HPLC analyses. For diclofenac and aceclofenac, different concentrations of the substrate were made up. Error bars are based on calculated standard deviations.



**Figure A-1. Diclofenac calibration curve at 276**



**Figure A-1. Aceclofenac calibration curve at 275**

## Appendix B. SBP activity assay

A colorimetric assay was conducted prior to each experiment to measure the activity of SBP. A built-in kinetic rate calculation mechanism in the UV-Vis spectrometer was used to determine the initial rate of formation of a pink chromophore at 510 nm. The UV-Vis spectrometer was configured for zero-order reaction rate, kinetic mode, 30 second run time, and 5 second cycle time.

### Reagents:

1. 0.025 g of 4-aminoantipyrine (4-AAP)
2. 5 mL of 10X concentrated phenol solution (100 mM phenol in 0.5 M monobasic/dibasic sodium phosphate pH = 7.4)
3. 100  $\mu$ L of freshly prepared 100 mM H<sub>2</sub>O<sub>2</sub>
4. The contents were made up to 47.5 mL with distilled water

### Procedure in 1 mL cuvette:

1. Blank: The spectrometer was blanked with 950  $\mu$ L of freshly prepared assay reagent mixed with 50  $\mu$ L distilled water.
2. Sample: 950  $\mu$ L reagent was mixed quickly with 50  $\mu$ L of diluted SBP solution, and the rate of color formation at 510 nm was monitored.
- 3) Quickly place cuvette into UV-vis and lock the vessel
- 4) Record the activity value of SBP, calculated by software.

### Multiplication Value Calculation SBP Activity

**SBP activity in cuvette (U/mL) = initial rate (AU S) $\times$ ( 60 S 1 Min) $\times$ (dilution in the cuvette) / 6 mM<sup>-1</sup>  $\times$ cm<sup>-1</sup> = initial rate  $\times$  200**



where 200 was put in the software setting as multiplication value under UV-vis kinetics mode. 30 s run time, 5 s cycle time and zero-order were also inputted before.

## VITA AUCTORIS

**NAME:** Sara Pishyar

**PLACE OF BIRTH:** Fars, Iran

**YEAR OF BIRTH:** 1990

**EDUCATION:** Farzanegan High School, Fars, Iran, 2008  
University of Shiraz, B.Sc., Shiraz, Iran, 2013  
University of Tehran, M.Sc., Tehran, Iran, 2014