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DEVELOPMENT OF NOVEL PEROXIDASE-TiO2/ZnO HYBRID CATALYSTS FOR THE DEGRADATION OF EMERGING POLLUTANTS

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry

Under the Supervision of Dr. Iltaf Shah

November 2019

Declaration of Original Work

I, Rana Fawzi Mohamed Mohamed Morsi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Development of Novel Peroxidase-TiO_2/ZnO Hybrid Catalysts for the Degradation of Emerging Pollutants*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Iltaf Shah, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Water pollution by different organic compounds is one of the most important environmental issues that is attracting the attention of many scientists due to its direct potential bad effect on human health. These organic pollutants include pesticides, pharmaceuticals, industrial wastes and personal care products that are released in water bodies from domestic and industrial discharge. Various chemical, physical and biological approaches have been proposed to degrade these contaminants from the polluted water. In the present study, we immobilized versatile thermostable enzymes, Soybean peroxidase (SBP) and Horseradish peroxidase on a functionalized photocatalysts - TiO_2 and ZnO to create novel bio-composite catalysts (SBP-TiO_2, SBP-ZnO, HRP-TiO_2 and HRP-ZnO). These hybrid bio-catalysts appeared to have similar pH optima as 'free/un-immobilized" peroxidases, as well as similar thermal stabilities. Furthermore, we used the "combined enzyme-chemical oxidation" remediation strategy which combined a photocatalytic oxidation step with peroxidase activity to study the degradation of 21 different emerging organic pollutants, using LC-MSMS. Our results showed that immobilization of the enzymes onto solid photocatalytic supports not only allowed for the recycling of enzymes, but also created a potentially more potent hybrid catalyst as compared to free enzyme. Many emerging pollutants were degraded more efficiently using the hybrid biocatalysts rather than using the enzyme alone or the photocatalyst alone.

Keywords: Emerging pollutants, bioremediation, advanced oxidation process, enzymes, horseradish peroxidase, soybean peroxidase, photocatalysts, zinc oxide, titanium dioxide, immobilization.

Title and Abstract (in Arabic)

تطوير طريقة جديدة لمعالجة المياه الملوثة باستخدام انزيم البيروكسيداز مع ثاني أكسيد التيتانيوم وأكسيد الزنك

الملخص

يعد تلوث المياه بواسطة المركبات العضوية المختلفة أحد أهم القضايا البيئية التي تجذب انتباه العديد من العلماء بسبب تأثيرها الضار المحتمل على صحة الإنسان. تشمل هذه الملوثات العضوية المبيدات الحشرية والأدوية والنفايات الصناعية ومنتجات العناية الشخصية التي يتم إطلاقها في المسطحات المائية من التصريف المنزلي والصناعي. وقد تم اقتراح العديد من الطرق الكيميائية والفيزيائية والبيولوجية المختلفة لتحلل هذه الملوثات من المياه الملوثة. في هذه الدراسة، قمنا بتثبيط انزيمان من انزيمات البيروكسيداز على ثاني أكسيد التيتانيوم وأكسيد الزنك لإنشاء محفزات كيميائية حيوية جديده مهجنة. تبين بأن الظروف الأمثل من الرقم هيدروجيني ودرجات الحرارة اللازمة لكفاءة هذه المحفزات الحيوية الهجينة مماثله للظروف الأمثل لكفاءة انزيمات البيروكسيداز. علاوة على ذلك، استخدمنا استراتيجية معالجة "الأكسدة الانزيميه-الكيميائية المشتركة" التي جمعت بين خطوة الأكسدة عن طريق التحفيز الضوئي مع نشاط انزيما البيروكسيداز لدراسة تحلل واحد وعشرون ملوثًا عضويًا مختلفًا باستخدام تقنيه ال LC-MSMS. أظهرت نتائجنا أن تثبيط الأنزيمات على الحافز الضوئي يسمح بإعادة تدوير واستخدام الأنزيمات، وأيضًا قد خلق محفزًا هجينًا أكثر فاعلية مقارنةً بالإنزيم الحر. تم تحلل العديد من الملوثات العضوية بكفاءة أكثر باستخدام المحفزات الحيوية الهجينة بدلاً من استخدام الإنزيم وحده أو المحفز الضوئي وحده.

مفاهيم البحث الرئيسية: الملوثات العضوية، المعالجة البيولوجية، عملية الأكسدة المتقدمة، انزيمات، محفزات ضوئية، ثاني أكسيد التيتانيوم، أكسيد الزنك.

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Dedication

To my beloved parents, family and friends

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List of Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AOPs	Advance Oxidative Processes
APTES	3-Aminopropyl)triethoxysilane
CECs	Contaminants of Emerging Concern
CLEAs	Cross Linking of Enzyme Aggregates
CLECs	Cross Linking of Enzyme Crystals
CP6R	Crystal Ponceau 6R
CPO	Chloroperoxidase
CR	Congo Red
CV	Crystal Violet
DEET	N,N-Diethyl-meta-toluamide
E1	Estrone
E2	17β-Estradiol
EE2	17α-Ethinylestradiol
EPs	Emerging Pollutants
FTIR	Fourier-transform Infrared Spectroscopy
HMCs	Hollow Mesoporous Carbon Spheres
HOBT	1-Hydroxybenzotriazole
HRP	Horseradish Peroxidase
Lac	Laccase
LC	Liquid Chromatography
LiP	Lignin Peroxidase
MBT	2-Mercaptobenzothiazole
M-CLEAs	Magnetic Cross-linked Enzyme Aggregates
MCPA	2-Methyl-4-chlorophenoxyacetic acid

MDA	4,4′-Methylenedianiline
MMTD	5-Methyl-1,3,4-thiadiazole-2-thiol
MnP	Manganese Peroxidase
MPs	Micropollutants
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NSAIDs	Nonsteroidal Anti-inflammatory Drugs
PAHs	Poly Aromatic Hydrocarbons
PFAS	Polyfluoroalkyl Substances
PPCRs	Pharmaceuticals and Personal Care Products
RB5	Reactive Black 5
RBBR	Remazol Brilliant Blue R
SBP	Soybean Peroxidase
SEM	Scanning Electron Microscopy
SMX	Sulfamethoxazole
TC	Tetracycline
TCS	Triclosan
TDA	4,4′-Thiodianiline
UV	Ultraviolet
WWTPs	Waste Water Treatment Plants
XRD	X-ray Diffraction Spectroscopy

Chapter 1: Introduction

1.1 Overview

Over recent decades, the world's population is growing rapidly which resulted in numerous ecological impacts, with water being one among the foremost affected resources (Peña-Guzmán et al., 2019). The unprecedented rise in population caused higher consumer demand leading to intensified ecological pollution. All different kinds of pollution have a profound impact on human health and aquatic organisms either directly or indirectly. Human-made, and industrial and agricultural disposals play a significant role in causing pollution in wastewater. The compounds which are released in water bodies due to the different agricultural and industrial processes produced numerous types of pollutants that have modified the water cycle resulting in a worldwide problem associated with their possible effects on living organisms (Deblonde, Cossu-Leguille, & Hartemann, 2011).

1.2 Emerging pollutants

Emerging Pollutants (EPs), also known as Contaminants of Emerging Concern (CECs) or Micropollutants (MPs) (Teodosiu, Gilca, Barjoveanu, & Fiore, 2018) are a new class of organic chemicals that are found in water bodies. These emerging pollutants can be defined as man-made or manufactured synthetic chemicals or naturally occurring materials that are found in the natural environment without being monitored or regulated in most cases and have the ability to affect the health of living beings significantly (Sauvé & Desrosiers, 2014). They comprise an extensive array of various compounds and their transformation products: pharmaceuticals (e.g. nonsteroidal anti-inflammatory drugs (NSAIDs), analgesics, antibiotics, textile dyes, hormones, personal care products and pesticides (Lapworth, Baran, Stuart, & Ward,

2012). They are mainly detected in wastewater treatment plants (WWTPs), pharmaceutical production plants, hospitals, graveyards, household products, landfills, aquatic environment, industrial effluents and municipal sewage (Ahmed et al., 2017; Deegan et al., 2011). The concentration of EPs in the environment ranges from ng/L to few hundreds of μg/L (Ahmed et al., 2017; Tran, Urase, Ngo, Hu, & Ong, 2013). These concentrations are suspected to cause serious ecological threats such as interfering with the endocrine system of high organisms, reproductive impairments, physical abnormalities and congenital disorders in some species, feminization of some fish species and many others (Belhaj et al., 2015). A study in 2011 concluded that the presence of perfluorinated compounds in serum could be correlated to breast cancer risk in Greenlandic Inuit women (Bonefeld-Jorgensen et al., 2011). Additionally, it has been reported that pollutants such as perfluorooctanoate and perfluorooctane sulfonate may be linked to decreased human reproductive abilities (Vélez, Arbuckle, & Fraser, 2015). Higher concentrations have been detected for some pollutants such as ciprofloxacin and per- and polyfluoroalkyl substances (PFAS) as their concentrations reached mg/L and g/L, respectively, in the water supplies (Kelly & Brooks, 2018; Nakayama et al., 2019). Due to the ability of EPs to cause undesirable and deleterious effects to the human health and to the ecosystem, they have become the main focus of many academic research groups. For example, a 2012 study found that N,N-Diethyl-meta-toluamide (DEET) can cause an inhibition in the activity of acetylcholinesterase, which is a central nervous system enzyme, in mammals and insects (Corbel et al., 2009). Dealing with EPs requires hard work as there are many challenges a researcher can face. There is a lack of knowledge about the ecotoxicological information and a deficiency regarding the sampling and analytical techniques (Geissen et al., 2015).

Moreover, the long-term effect of EPs on living beings and environment is not available (Deblonde et al., 2011).

The term emerging pollutant covers three categories of compounds; the first category includes newly developed compounds that are introduced to the environment, the second category consist of compounds that are presented in the environment for a long time but are only being recognized newly, and the last category includes compounds that are detected since a long time but their significant impact on the environment and human health have been recognized recently such as hormones (Geissen et al., 2015). More than 1000 EPs have been identified and categorized into different classes which include pharmaceuticals, personal care products, pesticides, hormones, etc. Table 1 shows the different classes of EPs (Farré, Pérez, Kantiani, & Barceló, 2008).

Table 1: Classes of emerging compounds (Farré et al., 2008)

Compound	Example
Drugs of abuse	Amphetamine, cocaine, tetrahydrocannabinol
Flame retardants	C_{10}–C_{13} chloroalkanes, hexabromocyclododecane, polybrominated diphenyl ethers, tetrabromo bisphenol A, tris (2-chloroethyl)phosphate
Gasoline additives	Dialkyl ethers, methyl-t-butyl ether
Industrial additives and agents	Chelating agents (EDTA), aromatic sulfonates
Personal-care products Fragrances, insect repellants, soaps, antimicrobials, sun-screen agents	Benzophenone; N,N-diethyltoluamide; methylbenzylidene, nitro, polycyclic and macrocyclic musks; triclosan
Pharmaceuticals Analgesics and anti-inflammatory drugs, human and veterinary antimicrobials, antiepileptics, blood-lipid regulators and psychiatric drugs, anti-tumoral drugs, cardiovascular drugs (b-blockers) and b2-symphatomimetics, X-ray contrast agents	Acetaminophen, acetylsalicylic acid, diclofenac, diazepam, carbamazepine, benzafibrate, iopromide, iopamidol
Steroids and hormones	Diethylstilbestrol, estradiol, estriol, estrone
Surfactants and surfactant metabolites	Alkylphenol ethoxylates, alkylphenols (nonylphenol and octylphenol), alkylphenol carboxylates
New classes	Nanomaterials 1,4-dioxane swimming-pool-disinfection by-products

EPs can result from agricultural, industrial, household and hospital discharges. As mentioned previously, there are many sources for the emerging contaminants, but the major source is the WWTP effluents. WWTPs are not designed for the complete elimination and degradation of EPs and their metabolites, therefore, they can pass through WWTPs and enter our aquatic environments such as rivers and streams (Petrović et al., 2003). Figure 1 shows the different sources of EPs and their

transformation to our water supplies. Table 2 represents an interesting analysis conducted to document physiologically active concentrations of various hormones, antibiotics, and other emerging pollutants in the water bodies in several countries. As can be seen from the table, disturbingly high concentrations of various emerging pollutants are detected in various water bodies. A literature review that has been conducted by Peña-Guzmán et al. (2019) reported the concentration of different EPs that were found in the WWTPs effluents. A total of 102 EPs were found in the effluents from WWTPs. The concentrations of some of these pollutants are reported in Table 3 (Peña-Guzmán et al., 2019).

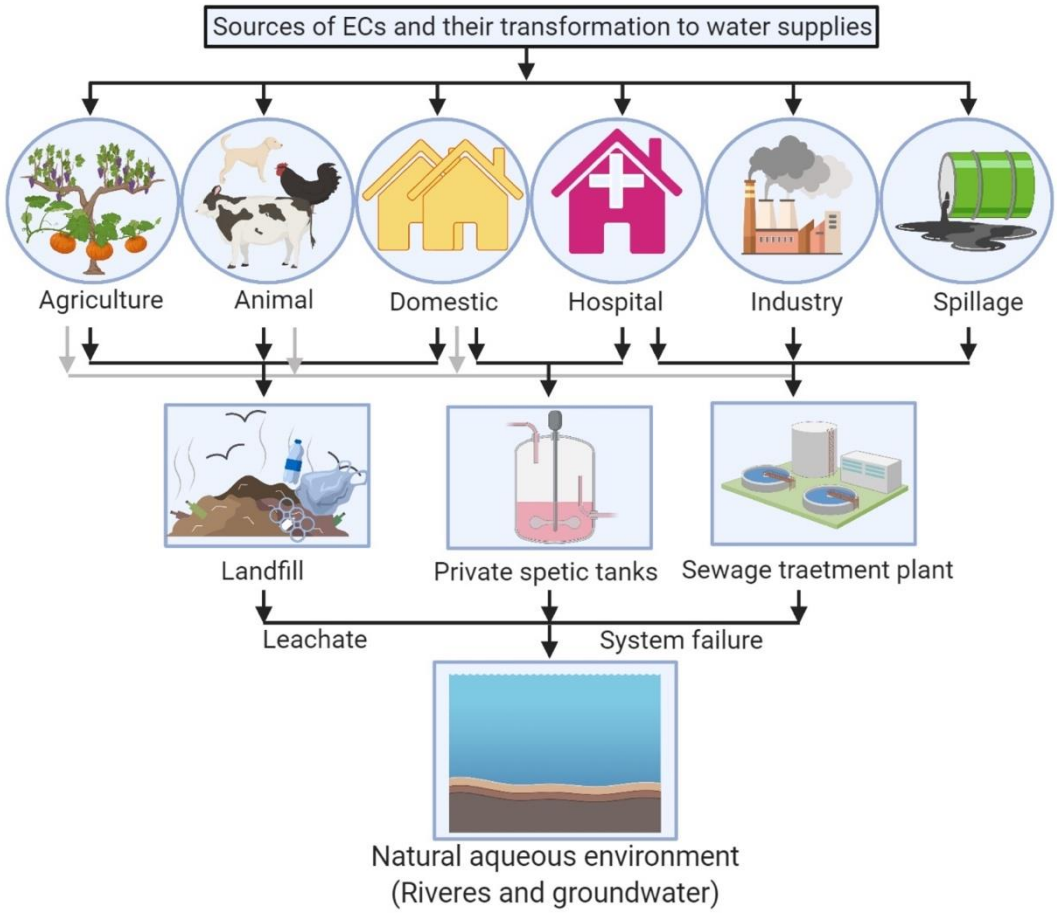


Figure 1: Different sources of emerging pollutants and their transformation to our water supplies

Table 2: Example of some emerging pollutants detected in drinking water supply

Category	Pollutant	Concentration (μg/L)	Reference
Pharmaceuticals	Caffeine	1.47	(Peña-Guzmán et al., 2019)
	Diclofenac	15	(Jux, Baginski, Arnold, Krönke, & Seng, 2002)
	Hydrochlorthiazide	1.9	(Huerta-Fontela, Galceran, & Ventura, 2011)
	Phenytoin	0.14	(Huerta-Fontela et al., 2011)
	Ibuprofen	12	(Lapworth et al., 2012)
	Atenolol	0.9	(Huerta-Fontela et al., 2011)
	Diazepam	18.5	(Peña-Guzmán et al., 2019)
	Trimethoprim	1.4	(Peña-Guzmán et al., 2019)
	Sulfamethoxazole	1.9	(Kolpin et al., 2002)
Pesticides	Fluometuron	317.6	(Papadakis et al., 2015)
	Lindane	0.034	(Huerta-Fontela et al., 2011)
	Prometryn	0.48	(Papadakis et al., 2015)
	DEET	6.5	(Lapworth et al., 2012)
Personal care products	Diethylphthalate	0.165	(Peña-Guzmán et al., 2019)
	Dibutylphthalate	0.37	(Peña-Guzmán et al., 2019)
	Triclosan	0.035	(Azzouz & Ballesteros, 2013)
Plasticizer	bis(2-ethylhexyl) phthalate	20	(Kolpin et al., 2002)
	Bisphenol A	12	(Kolpin et al., 2002)
Hormones	17β-estradiol	1.05	(Peña-Guzmán et al., 2019)
	Estriol	0.072	(Huerta-Fontela et al., 2011)
	Estrone	2.85	(Peña-Guzmán et al., 2019)

Table 3: Concentration of some of the pollutants that were found in WWTP effluents (Peña-Guzmán et al., 2019)

Category	Pollutant	Average concentration (ng/L)
Pharmaceuticals	Caffeine	3053.40
	Metronidazole	290
	Metoprolol	84.14
	Norverapamil	1.78
	Ibuprofen	244.62
	Cotinine	32.88
	Naproxen	890.35
	Tetracycline	68.95
	Trimethoprim	651.52
	Fluoxetine	5.51
	Atenolol	64.72
	Sulfamethoxazole	660.62
	Venlafaxine	24.50
Metabolites	Benzoylecgonine	487.26
	4-epitetracycline	12.30
	Paraxanthine	338.0
Endocrine disruptors	Estrone	611.45
	Progesterone	2.07
	Mestranol	135.94
Personal care products	Triclocarban	29.60
	N,N-diethyl-meta-toluamide	54.32

1.3 Removal of emerging pollutants by physiochemical methods

A wide range of approaches have been developed for the removal of these synthetic aromatic pollutants from water bodies, as well as wastewaters thus, reducing their impact on the environment. Various chemical, physical and biological methods have been used for the treatment of contaminated wastewater. Furthermore, hybrid systems in which two methods are combined and used have also been developed to enhance the removal efficiency of EPs. Figure 2 shows some of the physical, chemical and biological techniques that are used to degrade different EPs.

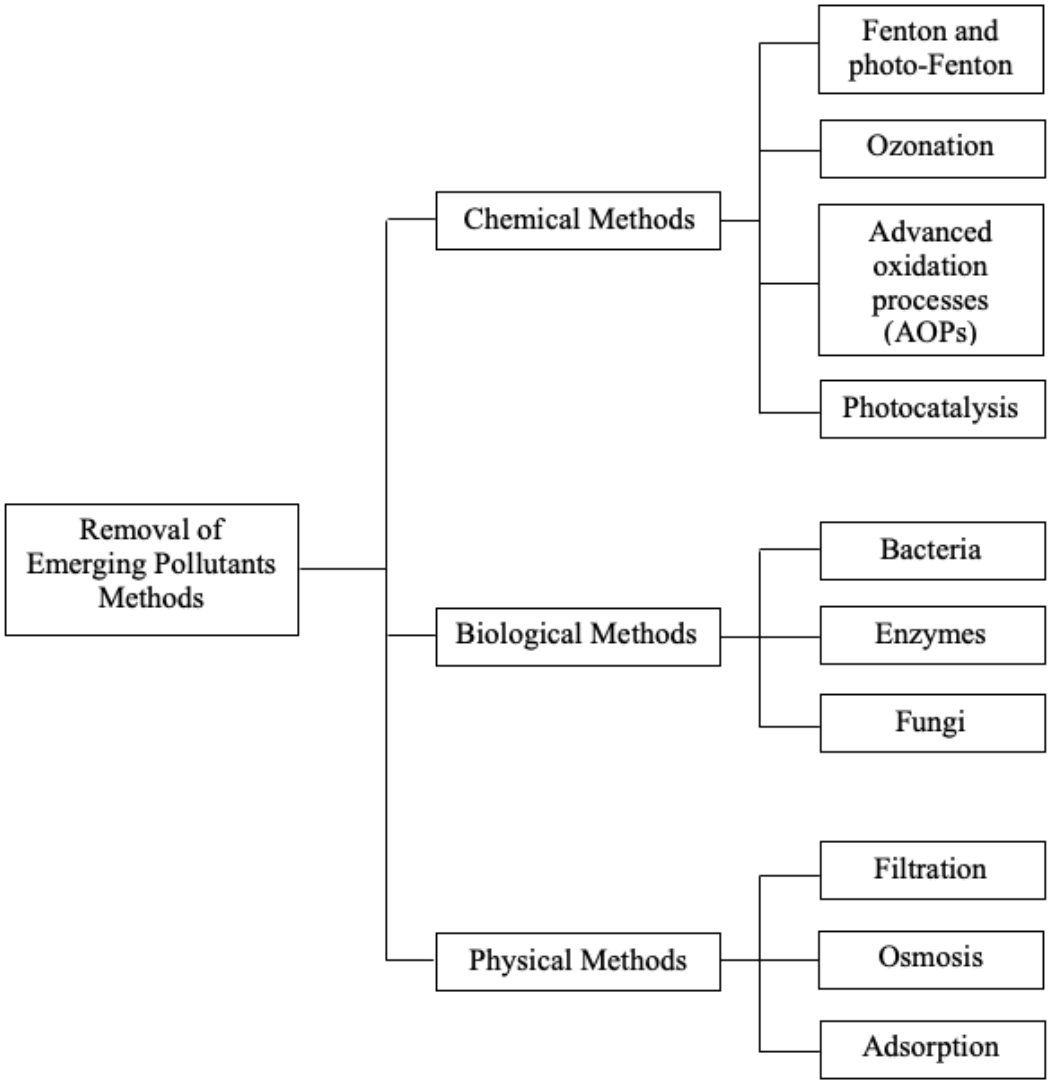


Figure 2: Various techniques used for the degradation and removal of EPs

1.3.1 Chemical methods

These include methods such as ozonation and advanced oxidation processes (AOPs). Many studies showed the ability of AOPs in degrading different types of emerging contaminants in wastewater effectively, therefore, they have become the most commonly used approaches. AOPs are defined as aqueous phase oxidation techniques that depend on the generation of highly reactive chemical species (Ahmed et al., 2017; Comninellis et al., 2008), such as hydroxyl radicals ($OH^{\bullet}$), that are used for the degradation or removal of EPs efficiently. In addition to the chemical agent (i.e. hydroxyl radical), an energy source such as ultraviolet-visible radiation, electric current, gamma-radiation, and ultrasound are needed for the reaction to occur (Ahmed et al., 2017; Deegan et al., 2011; Ikehata, Naghashkar, & El-Din, 2006). The treatment processes are initiated when the reactive free radicals are produced, the contaminants will undergo a series of oxidation reactions spontaneously that convert them into less harmful and more biodegradable products (Ikehata et al., 2006). Although this technique is efficient and useful, but it is worth mentioning that there are some challenges related to using AOPs in wastewater treatments. Sometimes it results in the production of reaction intermediates, the relatively high cost due to the continuous addition of costly chemicals and huge amounts of energy and in some cases, biologically active products that are still harmful can be produced when the parent pollutant is degraded which means no treatment has been achieved (Alneyadi, Rauf, & Ashraf, 2018; Ikehata et al., 2006; Teodosiu et al., 2018). Table 4 summarizes some of the AOPs that were used efficiently to remove various EPs.

Table 4: Summary of efficiently used AOPs for the degradation of different EPs

Type of AOPs	EPs	Removal %	Reference
UV	Estrone	90	(Sarkar, Ali, Rehmann, Nakhla, & Ray, 2014)
UV/H_2O_2	Doxycycline	100	(Bolobajev, Trapido, & Goi, 2016)
UV/Ozone	Caffeine	>95	(Souza & Féris, 2015)
	Estradiol (E2)	>99	
Ozone/H_2O_2	Naproxen	96-98	(Feng, Watts, Yeh, Esposito, & Hullebusch, 2015)
	Piroxicam	96-98	
Ozone	Ethynilestradiol (EE2)	80	(Vallejo-Rodríguez, Murillo-Tovar, Navarro-Laboulais, León-Becerril, & López-López, 2014)
	Naproxen (NPX)	80	
	Ibuprofen (IBP)	90	
	Ketoprofen	90-96	
Ozone/H_2O_2/UV	Estrone	>99	(Sarkar et al., 2014)
Photo-Fenton	Acetamiprid	70-90	(Carra et al., 2015)
Fenton process	Doxycycline	100	(Borghi, Silva, Al Arni, Converti, & Palma, 2015)

A study that was conducted by Lopez and his colleagues (2002) showed the complete degradation of 5-methyl-1,3,4-thiadiazole-2-thiol (MMTD) which is a pharmaceutical intermediate by H_2O_2/UV treatment (Lopez, Bozzi, Mascolo, Ciannarella, & Passino, 2002). Figure 3 shows the proposed degradation pathway of seven by-products that resulted from this reaction and were identified using LC-MS (Ikehata et al., 2006; Lopez et al., 2002).

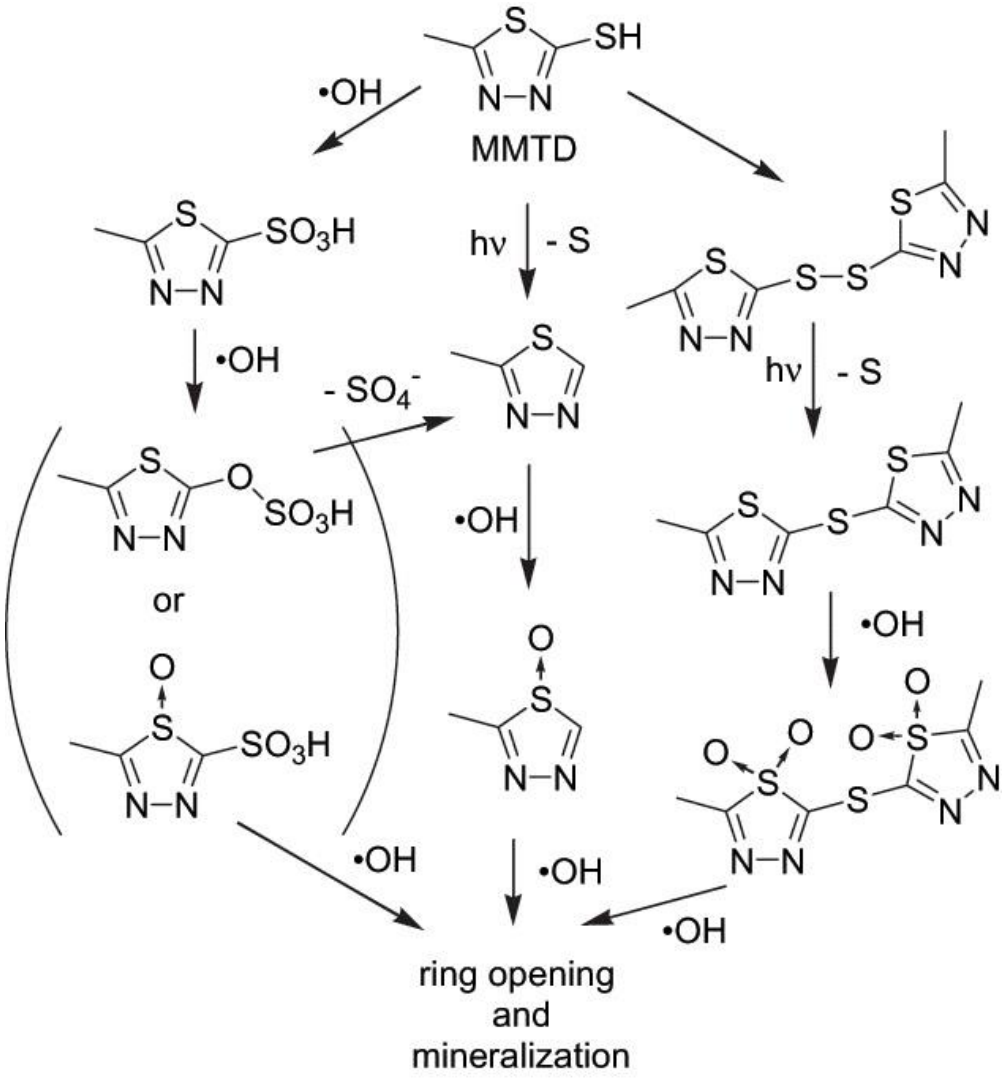


Figure 3: Proposed pathway for MMTD degradation by H_2O_2/UV process (Ikehata et al., 2006; Lopez et al., 2002)

Another study that was conducted by Calza and her colleagues (2004) demonstrated the photocatalytic degradation of buspirone, which is an anti-anxiety medicine, by TiO_2 and using xenon lamp (Calza, Pazzi, Medana, Baiocchi, & Pelizzetti, 2004). Figure 4 shows the degradation intermediates that were identified using LC-MS-MS (Calza et al., 2004; Ikehata et al., 2006).

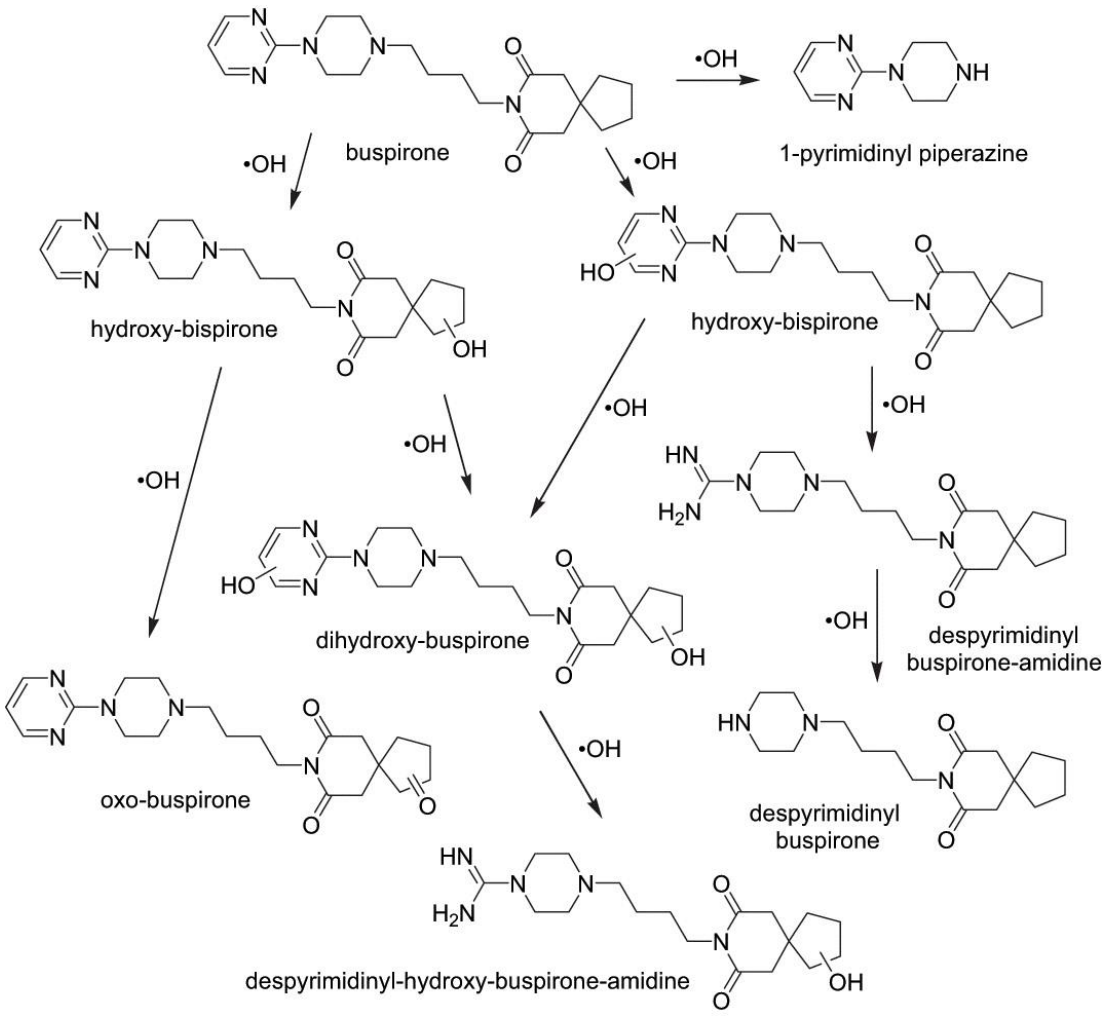


Figure 4: Degradation intermediates for buspirone treated with UV/hv (Calza et al., 2004; Ikehata et al., 2006)

Diclofenac is an anti-inflammatory drug used to treat pain and inflammatory diseases. Recently, it was conceived as a baleful ecological pollutant due to its accumulation in the food chain, and identification in drinking water and aquatic systems. It has been detected in water supplies with different concentration levels that has reached to 1.3 μg/L (Ternes et al., 2003). Many AOPs have been applied on diclofenac to evaluate their ability to degrade it effectively. For example, Vogna and her colleagues have reported the treatment of diclofenac by ozonation and H_2O_2/UV. The treatment resulted in the formation of quinoneimine intermediates that were decomposed into aniline and hydroquinone intermediates (Vogna, Marotta, Napolitano, Andreozzi, & d'Ischia, 2004). Figure 5 shows the pathway of diclofenac degradation by ozone and H_2O_2/UV (Vogna et al., 2004).

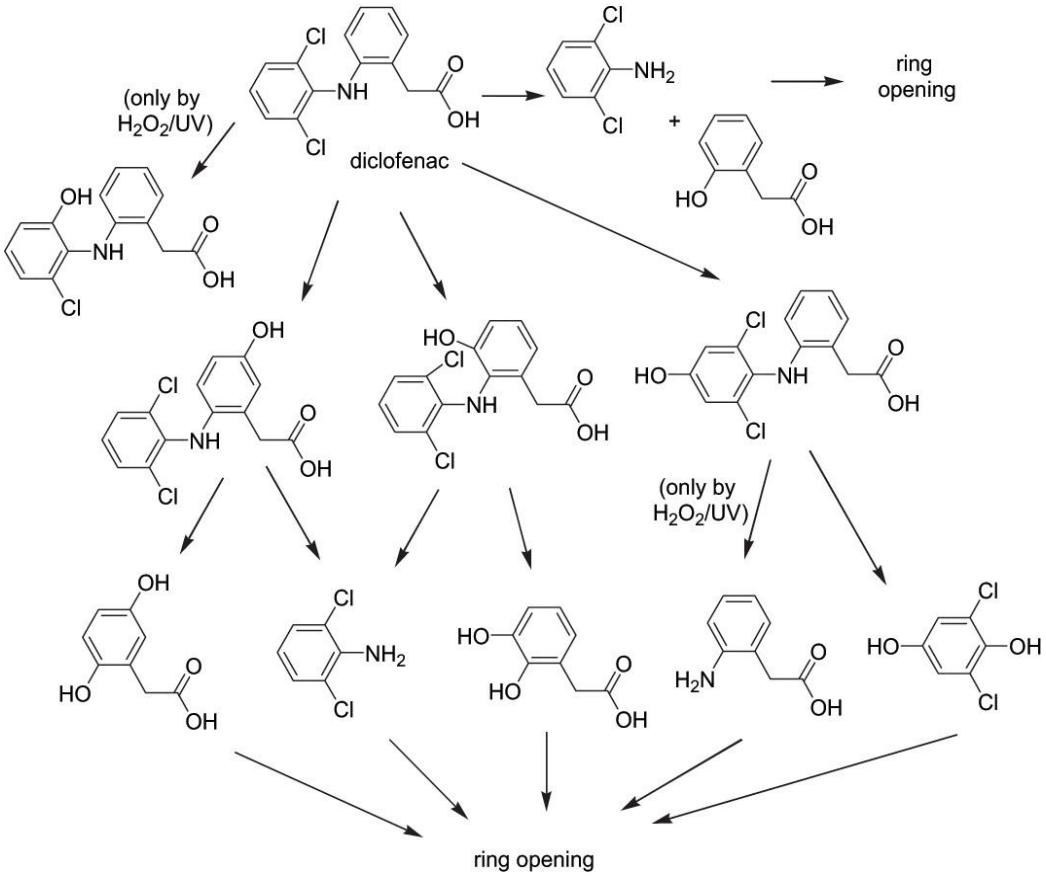


Figure 5: Breakdown of diclofenac by ozone and H_2O_2/UV (Vogna et al., 2004)

1.3.2 Physical methods

They include methods such as adsorption, filtration and osmosis. Adsorption is the most commonly physical method used for the treatment of wastewater. In adsorption, the pollutants are attracted to a solid surface. Many studies showed that different adsorbents such as activated carbon, silica gel, zeolites and metal oxides are able to eliminate organic contaminants from wastewater successfully. A study that was conducted by Ali (2012) showed that nanomaterials as adsorbents can efficiently degrade various types of pollutants (Ali, 2012). Adsorption methods have many advantages as they are inexpensive, simple to operate and adsorbents are available in adequate quantities (Teodosiu et al., 2018). However, adsorption processes generate large amount of sludge, so there is a need for an additional sludge disposal (Ali, 2012). Other physical methods such as filtration and osmosis are efficient as well, but the cost of materials is expensive compared to the other adsorption method. Table 5 shows some of the physical methods that have been used previously for the removal of different emerging pollutants.

Table 5: Summary of effectively used physical methods for the removal of different emerging pollutants

Physical method	EPs	Removal %	Reference
Adsorption on activated-carbon	Paracetamol	74	(Cabrita et al., 2010)
Adsorption on Lignin-activated	Tetracycline	76	(Huang, Wang, Shi, Huang, & Zhang, 2014)
	Ciprofloxacin	80	
Adsorption on Zeolite	Ciprofloxacin	51	(Genç & Can, 2015)
Adsorption on Graphene oxide	Tetracycline	71	(Gao et al., 2012)
Adsorption on Al_2O_3/Fe	Norfloxacin	90	(Liu, Zhang, Zhang, & Ren, 2011)
Nano-filtration	Caffeine	84	(Acero, Benitez, Teva, & Leal, 2010)
Ultrafiltration	Triclosan	98	(Melo-Guimarães, Torner-Morales, Durán-Álvarez, & Jiménez-Cisneros, 2013)
	Ibuprofen	62	
Osmosis	Acetaminophen	89	(Valladares Linares, Yangali-Quintanilla, Li, & Amy, 2011)
Reverse Osmosis	Bisphenol A	90	(Valladares Linares et al., 2011)

1.4 Biodegradation for wastewater treatment

Despite the fact that physical and chemical methods are widely used and these methods can work effectively, they have several potential limitations, such as overall high cost, inefficiency, production of high sludge, and formation of toxic side products. Hence, it is well accepted that there is a dire need to find better, novel, and more environmentally safe approaches for wastewater remediation. Biological (specifically microbial and enzyme-based) approaches for degrading various kinds of organic pollutants are promising new area of research in water treatment (Al-Maqdi, Hisaindee, Rauf, & Ashraf, 2017). Biodegradation or bioremediation has been successfully used for the removal of emerging contaminants from wastewaters. In this

process, microorganisms such as bacteria, fungi or yeasts (or enzymes from these microorganisms) are used for the removal of organic chemicals from water bodies. In biodegradation, the microorganisms utilize the pollutant as a substrate and induce enzymes, then the pollutants are enzymatically converted into smaller molecules that are usually less toxic (Ahmed et al., 2017; Tran et al., 2013). Biodegradation processes have many advantages compared to the physiochemical techniques as they are safer, less disruptive, less expensive, require lower energy employment, considered as green catalysis process and can be used with pollutants having very low concentrations which cannot be achieved using physiochemical techniques (Al-Maqdi et al., 2017; Holanda et al., 2019; Rauf & Salman Ashraf, 2012). Major drawback of biological treatments is that they require longer time and the microorganisms may not be able to survive and grow under harsh and adverse environmental conditions (Al-Maqdi et al., 2017; Rauf & Salman Ashraf, 2012).

1.4.1 Enzymatic biodegradation-towards greener oxidation route

The biological approach that uses oxidoreductase enzymes (such as peroxidases) for pollutant degradation is a relatively new and promising research area. Numerous enzyme systems have been employed for the efficient degradation of diverse organic pollutants and have shown to oxidize and degrade the pollutants into smaller intermediates. The use of enzyme-based treatments offer many advantages such as the ability to operate at high and low concentrations of pollutants, reduced amount of sludge generation, work in a catalytic manner, can be applied over a wide range of pollutants, low energy input and many others (Nicell, Al-Kassim, Bewtra, & Taylor, 1993; Unuofin, Okoh, & Nwodo, 2019). Figure 6 summarizes the advantages of using enzyme systems for the treatment of wastewater. Although enzymatic remediation has many advantages, it is important to mention that there are some

challenges such as the high cost of catalytic enzymes, inability to re-use the enzyme, possibility of having changes in the conformation of the enzyme under harsh environmental conditions (i.e. enzymes may lose their stability under harsh environmental conditions such as high temperatures or high and low pH values) and the possibility of forming hazardous soluble by-products (Al-Maqdi et al., 2017; Nicell et al., 1993; Pandey et al., 2017). However, most of these issues can be solved by immobilizing the enzyme on different solid supports.

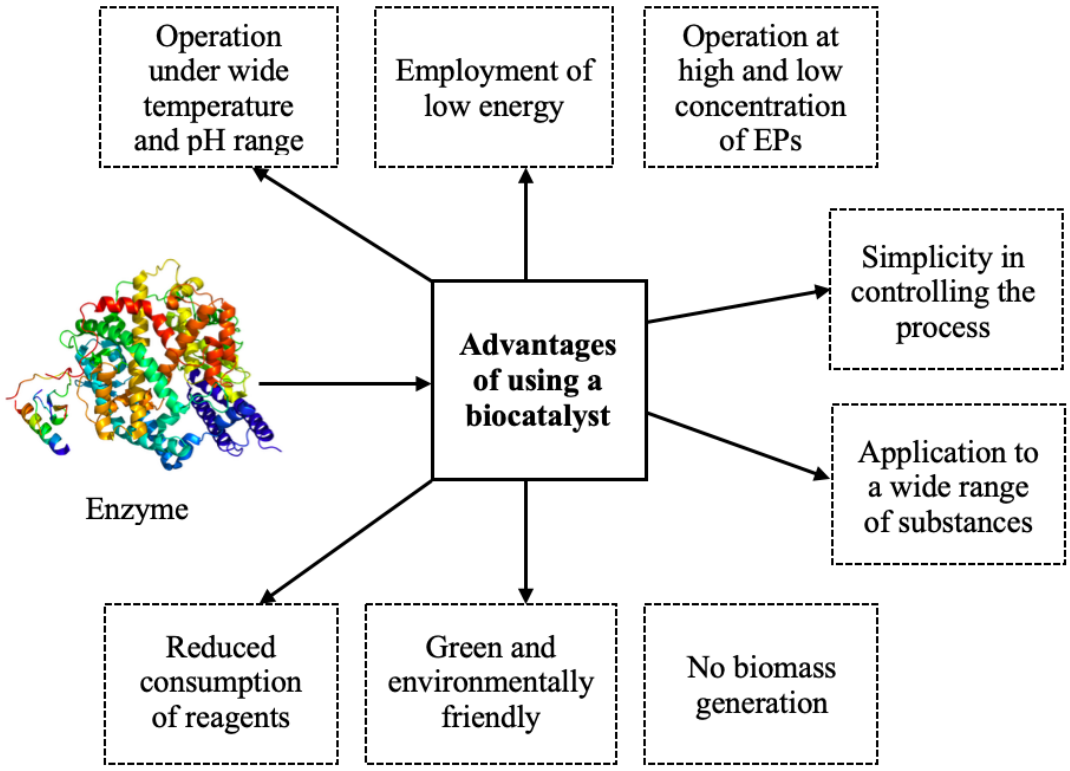


Figure 6: Advantages of using enzyme for wastewater treatment

Oxidoreductases are the most widely investigated class of enzymes for the bioremediation of wastewater. These enzymes catalyze the oxidation-reduction-assisted biodegradation of various classes of hazardous organic pollutants including cresols, phenols, chlorinated phenols, herbicides, pesticides, dioxins, synthetic textile

dyes, pharmaceuticals and personal care products (PPCRs) and many others (Bilal, Adeel, Rasheed, Zhao, & Iqbal, 2019a; Bilal, Rasheed, Iqbal, & Yan, 2018). Oxidoreductases include oxidases, peroxidases, dehydrogenases and oxygenases. Among oxidoreductases, peroxidases and laccase (oxidase enzymes) are the most commonly used enzymes for the enzymatic-remediation studies due to their high ability in degrading different organic contaminants. These enzymes form radicals that degrade the parent pollutant into smaller products that are more biodegradable and exhibit minimal toxicity (Unuofin et al., 2019).

1.4.2 Laccases- A multifaceted biocatalyst for removing emerging pollutants

Laccases (Lac) are a class of multi-copper oxidases that are mainly obtained from fungi, various plants, bacteria and insects. However, laccases from microbial origins, particularly, from wood-decaying fungi have garnered increasing attention owing to great oxidation ability to multiple compounds and a wider spectrum of substrate specificity. Laccases catalyze the single electron oxidation of hydrogen donating substrates with the concomitant reduction of molecular oxygen to water (Lloret et al., 2010; Pandey et al., 2017; Unuofin et al., 2019). Consumption of atmospheric oxygen as a source of electron acceptor is beneficial in laccase-mediated catalytic reaction than the use of hydrogen peroxide by peroxidases. Nonetheless, these enzymes necessitate the use of redox mediators i.e. acetosyringone, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1-hydroxy benzotriazole, and vanillin for abatement of numerous recalcitrant and non-phenolic environmental contaminants. In spite of this shortcoming, the use of laccases has gained pronounced interest because of their wider biotechnological applications. In the recent decade, there has been a paradigm revolution from the laccases application in conventional lignin depolymerization to oxidative removal of a wide range of emergent

contaminants, organic micro-pollutants, and xenobiotics hormones, phenolics, plasticizers, PAHs, and many others, which can significantly affect the human health and the aquatic biota (Table 6). Figure 7 illustrates the breakdown of some hospital wastewater pollutants that have been studied previously (Unuofin et al., 2019). A special thing about laccases is that they can cleave various substrates without using an external source of H_2O_2 or Mn^{2+} or any other co-factor that are required when some peroxidases such as lignin peroxidase (LiP) or manganese peroxidase (MnP) are used (Unuofin et al., 2019).

Table 6: A list of laccases used for the removal of recalcitrant emerging pollutants

Enzyme	Pollutant	Removal %	Reference
Lac	Triclosan	100	(Sun et al., 2019)
Lac	Diclofenac	98	(Mukherjee, Bhattacharya, Taylor, & Biswas, 2019)
Lac	Bisphenol A	99	(Lassouane, Aït-Amar, Amrani, & Rodriguez-Couto, 2019)
Lac	Carbamazepine	95	(Naghdi et al., 2018)
Lac	Phenanthrene	97	(Balcázar-López et al., 2016)
Lac	Benzo[α]pyrene	99	(Balcázar-López et al., 2016)
Lac	Nonylphenol	>95	(Ramírez-Cavazos et al., 2014)
Lac	Triclosan	>95	(Ramírez-Cavazos et al., 2014)
Lac	Naproxen	60	(Tong, Qingxiang, Hui, Qin, & Yi, 1997)
Lac	Ethinylestradiol	100	(Lloret et al., 2010)
Lac	Estradiol	100	(Lloret et al., 2010)

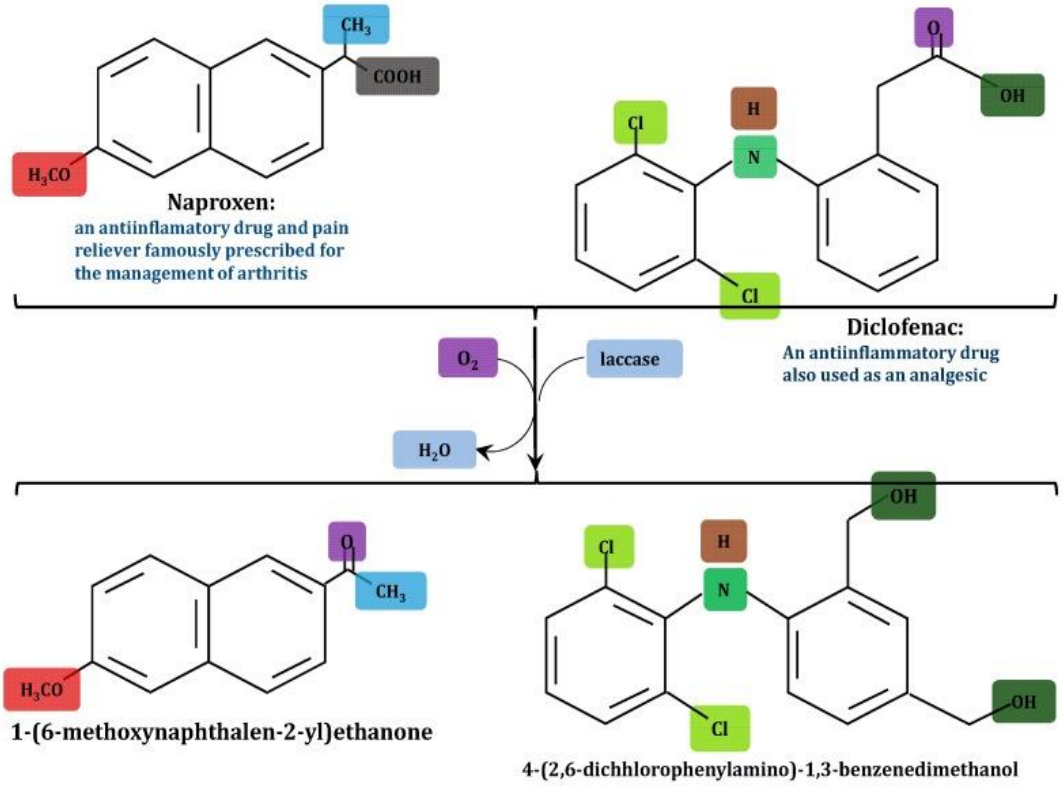


Figure 7: The oxidative cleavage of some pharmaceuticals by laccase enzyme (Unuofin et al., 2019)

Laccases have been successfully used for the degradation of different EPs and diverse classes of aromatic dyes. For example, a study that was conducted by Lloret et al. (2010) demonstrated the ability of laccase to breakdown different estrogen hormones such as estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) into products that have lower or no estrogenic activity efficiently (Lloret et al., 2010). In another study, Asghar and his team (2016) showed the ability of laccase to degrade phenolic azo dyes into smaller products that have less toxicity (Figure 8). A very recent review described the hypothetical breakdown of lindane which is an insecticide into organic acids that are greener and environmentally friendlier by-products (Unuofin et al., 2019). The hypothetical degradation pathway of lindane by laccase is shown in Figure 9 (Unuofin et al., 2019).

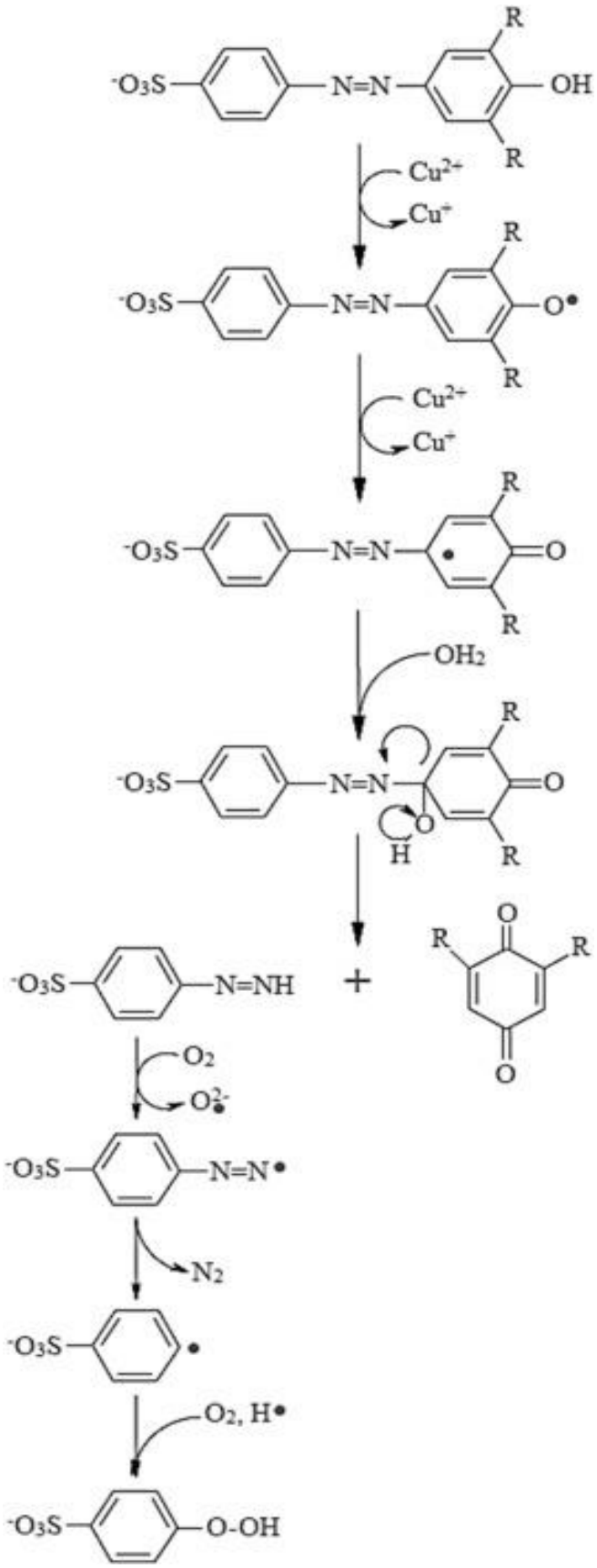


Figure 8: Mechanism of oxidative cleavage of phenolic azo dyes into less/no harmful products (Asgher, Shah, & Iqbal, 2016)

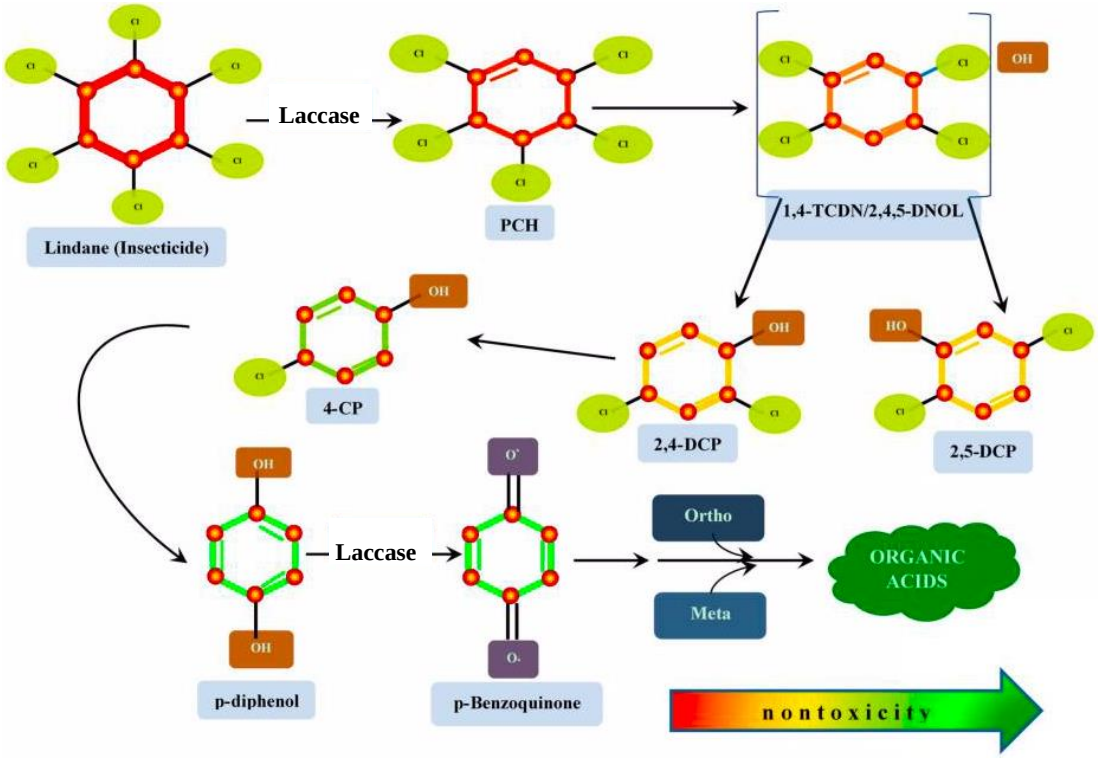


Figure 9: Pathway of hypothetical breakdown of lindane to give organic acids that are eco-friendly (Unuofin et al., 2019)

1.4.3 Peroxidases for emerging pollutants

Most peroxidases are heme-containing antioxidant proteins that are found in microbes, fungi, bacteria, animals and plants. They utilize hydrogen peroxide (H_2O_2) or organic hydroperoxides as a co-substrate to catalyze the oxidation of a broad range of organic and inorganic substrates (Battistuzzi, Bellei, Bortolotti, & Sola, 2010). These enzymes are capable to degrade pollutants efficiently due to their high specificity (Chiong, Lau, Lek, Koh, & Danquah, 2016). It is important to add the right amount of H_2O_2, as adding excess amounts of it can inactivate the enzyme. Peroxidase reactions proceed when the enzyme reacts with a hydrogen peroxide (H_2O_2) molecule. The enzyme will be oxidized to generate a cation radical, which is called Compound I and H_2O_2 will be reduced to water. Then Compound I will undergo a reduction and oxidizes an organic substrate to form Compound II and organic radical. Lastly,

Compound II oxidizes a second organic molecule to give another organic radical and the enzyme will be reduced back to its resting form (Battistuzzi et al., 2010). Figure 10 shows the generic scheme of all peroxidase enzymes reactions. The free organic radicals, which are formed during the process, are responsible for the degradation of pollutants. Many studies reported the ability of peroxidases to bio-remediate a wide range of emerging contaminants. The peroxidases that are most commonly used for wastewater treatments are soybean peroxidase (SBP), manganese peroxidase (MnP), lignin peroxidase (LiP), horseradish peroxidase (HRP), and chloroperoxidase (Alneyadi et al., 2018). Table 7 depicts a list of various peroxidases that have been used successfully for the degradation of recalcitrant emerging pollutants.

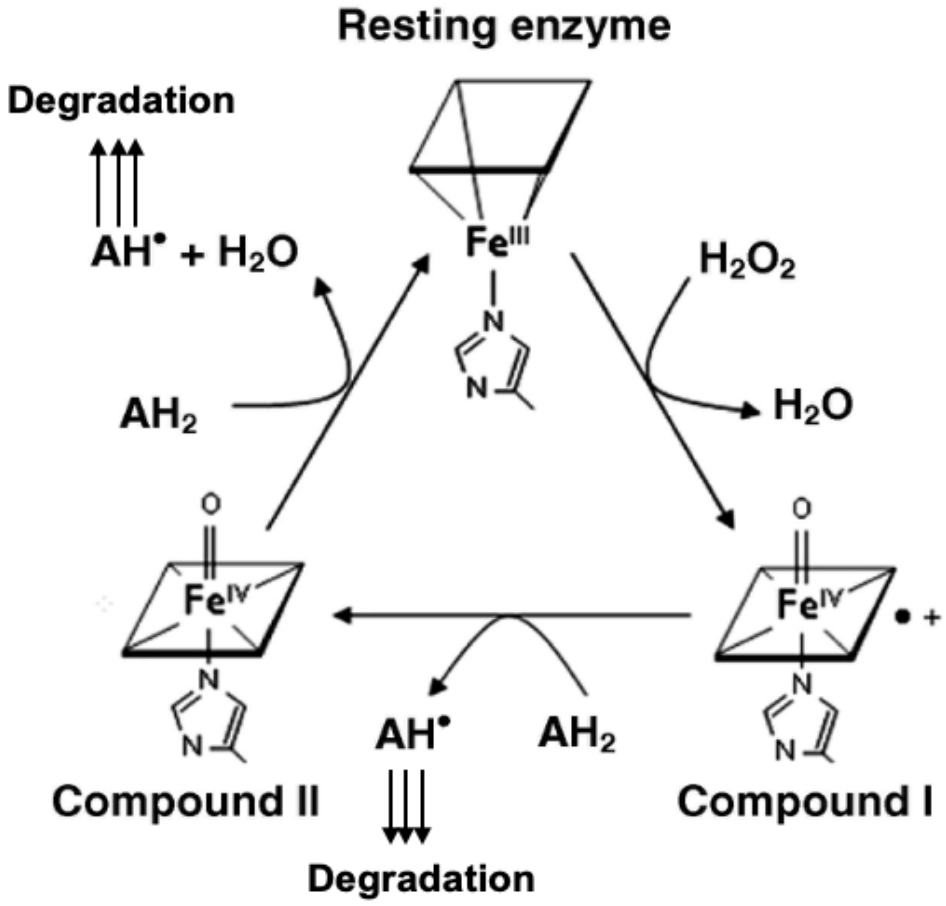


Figure 10: Reaction cycle of peroxidases

Table 7: A list of peroxidases used for the biodegradation of hazardous pollutants

Peroxidase	Pollutant	Removal %	Reference
SBP	4,4′-methylenedianiline (MDA)	>95	(Mukherjee et al., 2019)
SBP	4,4′-thiodianiline (TDA)	>95	(Mukherjee et al., 2019)
SBP	Sulforhodamine B dye	100	(Alneyadi, Shah, AbuQamar, & Ashraf, 2017)
SBP	Methyl orange	81.4	(Chiong et al., 2016)
SBP	Triclosan (TCS)	98	(J. Li et al., 2016)
HRP	Congo Red	94.35	(Bilal, Iqbal, Hu, Wang, & Zhang, 2017)
HRP	17α-ethinylestradiol (EE2)	>90	(Rathner, Petz, Tasnádi, Koller, & Ribitsch, 2017)
HRP	Phenol	99	(Tong et al., 1997)
HRP	4-chlorophenol	80	(Tong et al., 1997)
MnP	Triclosan	75	(Bilal, Asgher, Iqbal, Hu, & Zhang, 2017)
MnP	Nonylphenol	96	(Bilal, Asgher, Iqbal, Hu, & Zhang, 2017)
MnP	Drimaren Yellow X-8GN	90.2	(H. Xu, Guo, Gao, Bai, & Zhou, 2017)
MnP	Tetracycline	72.5	(Wen, Jia, & Li, 2010)
MnP	Oxytetracycline	84.3	(Wen et al., 2010)
LiP	Tetracycline	95	(Wen, Jia, & Li, 2009)
LiP	Oxytetracycline	95	(Wen et al., 2009)
LiP	Carbamazepine	15	(Zhang & Geißen, 2010)
Chloroperoxidase	Sulfamethoxazole	>80	(García-Zamora et al., 2018)
Chloroperoxidase	Naproxen	>80	(García-Zamora et al., 2018)
Chloroperoxidase	Tetracycline	>80	(García-Zamora et al., 2018)

1.4.3.1 Remediation applications of peroxidases

As mentioned earlier, peroxidases have been used in the degradation of many environmental contaminants due to their abilities in catalyzing the oxidation-reduction reactions of a broad range of organic compounds. Ali and his team (2013) reported the detailed mechanism of Crystal Ponceau 6R (CP6R), which is an azo dye, degradation using commercially available SBP enzyme (Ali et al., 2013). Their work showed that the degradation of CP6R has two different routes; symmetric azo bond cleavage and asymmetric azo bond cleavage. They found that SBP could efficiently degrade 100% of the azo dye under optimized conditions. Figure 11 shows the asymmetric azo bond cleavage route of CP6R degradation (Ali et al., 2013). Figure 12 shows the symmetric azo bond cleavage route of CP6R degradation (Ali et al., 2013).

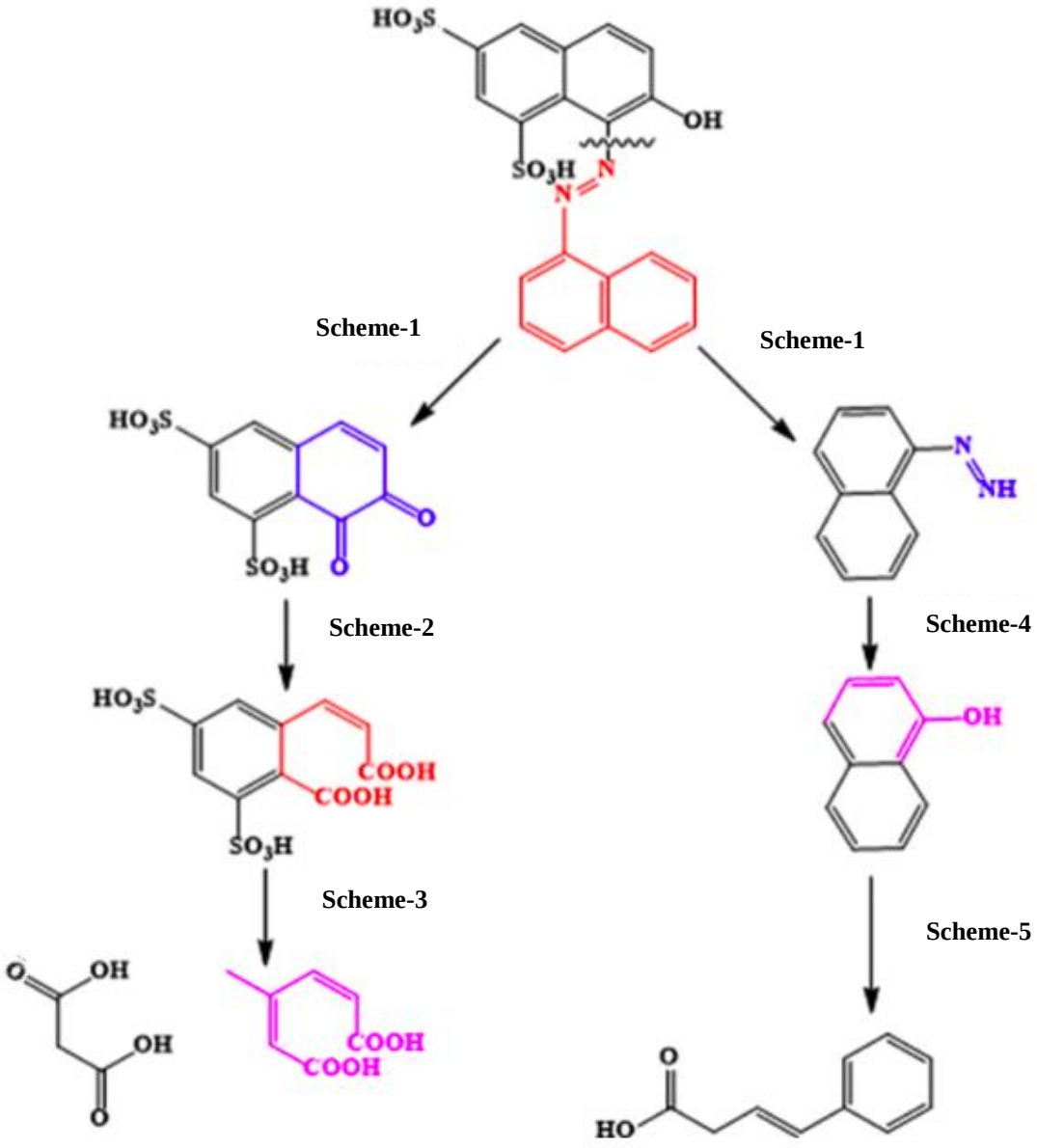


Figure 11: Asymmetric azo bond cleavage pathway for CP6R degradation (Ali et al., 2013)

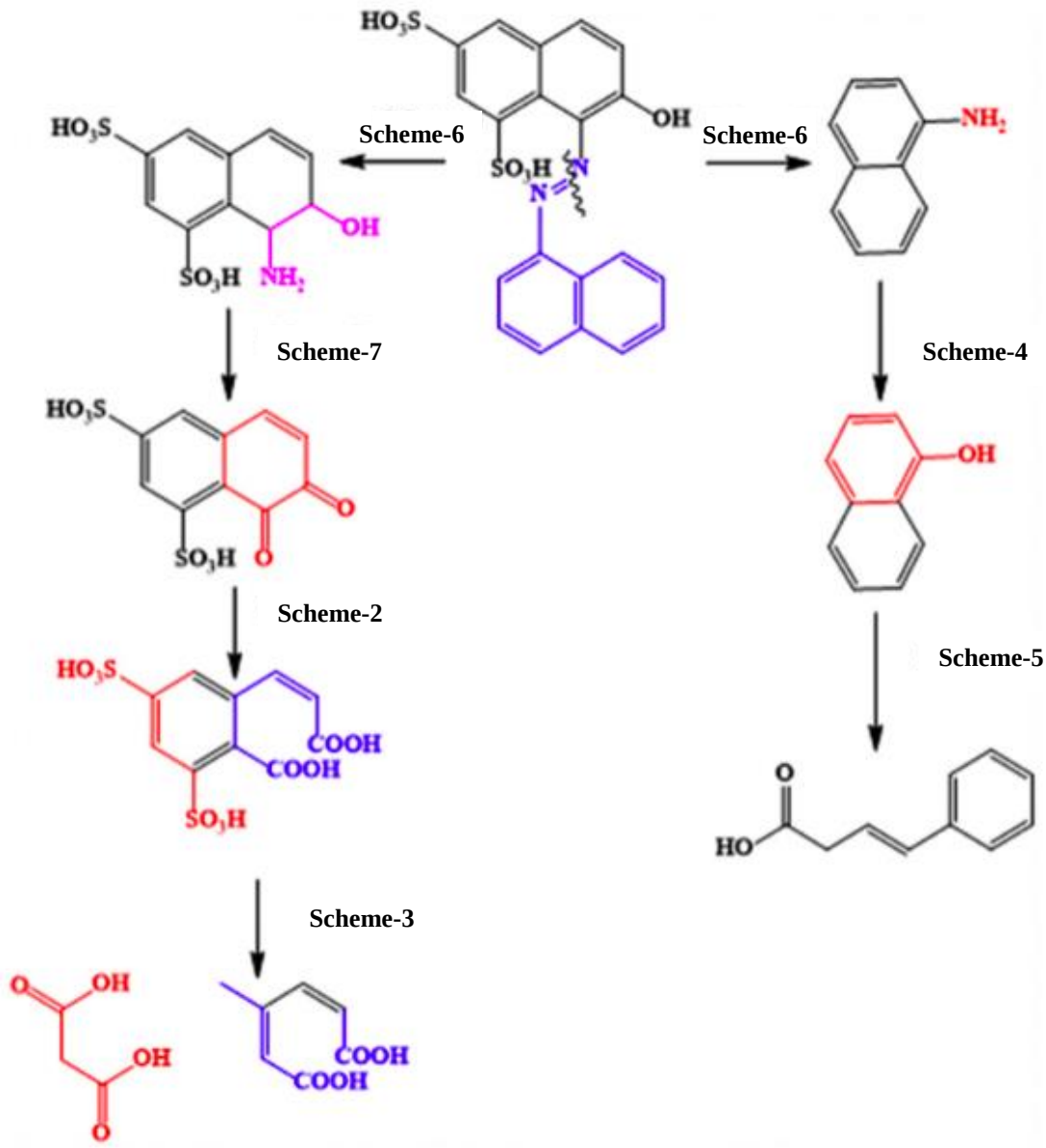


Figure 12: Symmetric azo bond cleavage pathway for CP6R degradation (Ali et al., 2013)

Sulfamethoxazole (SMX) is a drug that can be used to treat many different bacterial infections and it has been detected in our water supplies (Al-Maqdi, Hisaindee, Rauf, & Ashraf, 2018). A very recent study that was conducted by Al-Maqdi and her colleagues (2018) documented the ability of SBP in degrading SMX (Al-Maqdi et al., 2018). In order for SMX to be degraded efficiently, a redox mediator in addition to H_2O_2 should be added to the reaction. In their work, they used 1-hydroxybenzotriazole (HOBT) as a redox mediator. In the presence of H_2O_2 and HOBT, SBP successfully degraded more than 80% of SMX. The degradation scheme of SMX by SBP is presented in Figure 13 (Al-Maqdi et al., 2018).

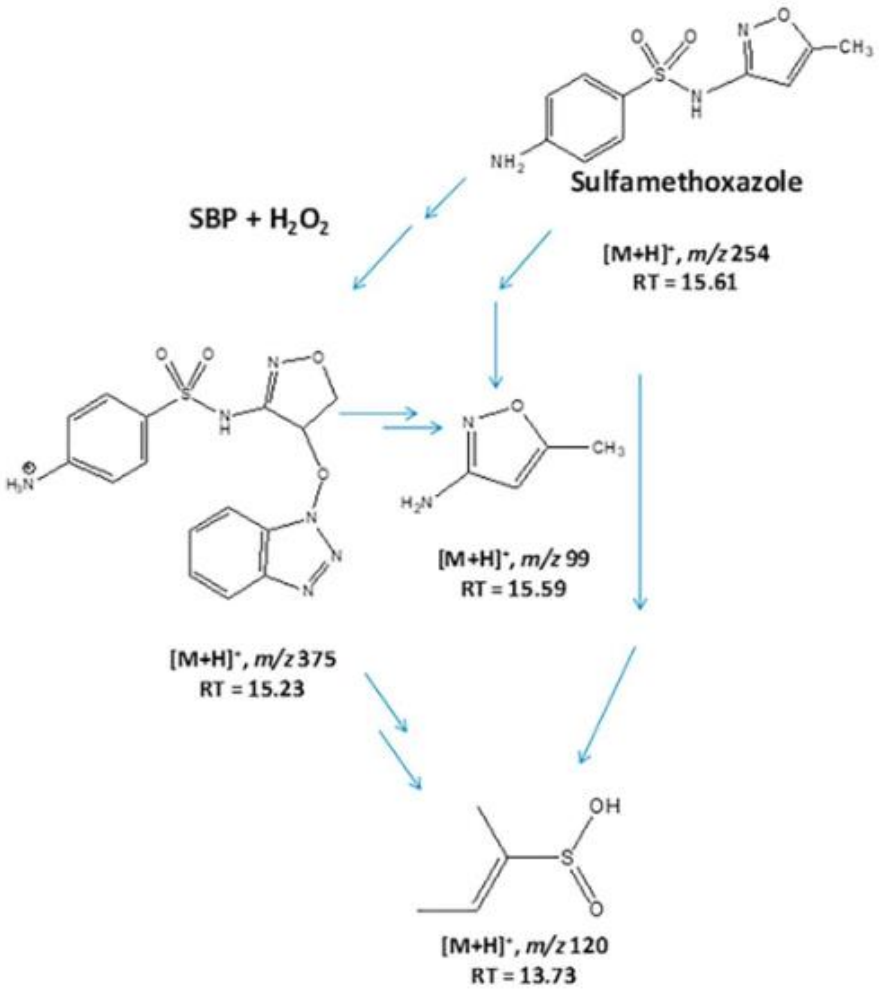


Figure 13: Degradation pathway of SMX by SBP enzyme (Al-Maqdi et al., 2018)

Another study that was published by Alneyadi and Ashraf (2016) reported the degradation of 2-mercaptobenzothiazole (MBT) which is an emerging pollutant that is extensively detected in the WWTPs and in the aquatic environment (Alneyadi & Ashraf, 2016). MBT is an organosulfur compound mainly used in manufacturing rubber items such as tiers (Li, Liu, Liang, Li, & Zhang, 2008). The work of Alneyadi and Ashraf (2016) showed that MBT can be degraded by two different peroxidases which are SBP and chloroperoxidase (CPO). SBP was able to degrade 100% of the MBT into smaller by-products effectively, while CPO degraded only 35% of MBT. Figure 14 shows the schematic pathway of MBT degradation using SBP (Alneyadi & Ashraf, 2016). Figure 15 shows the pathway of MBT degradation using CPO (Alneyadi & Ashraf, 2016).

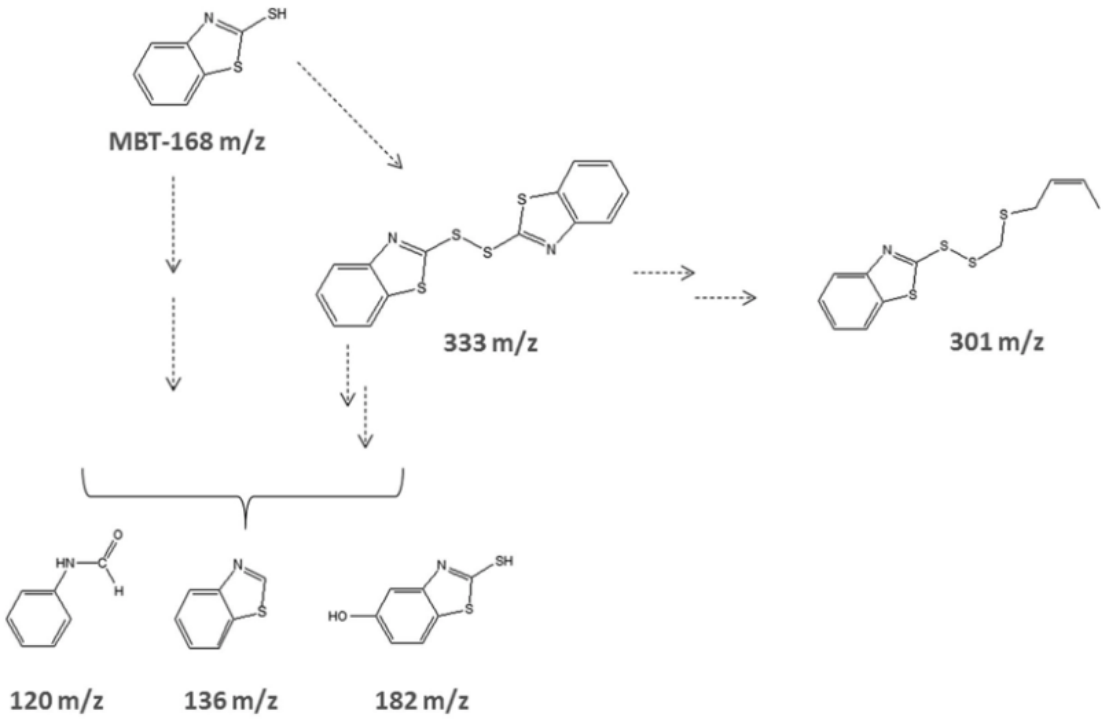


Figure 14: Degradation route of MBT by SBP (Alneyadi & Ashraf, 2016)

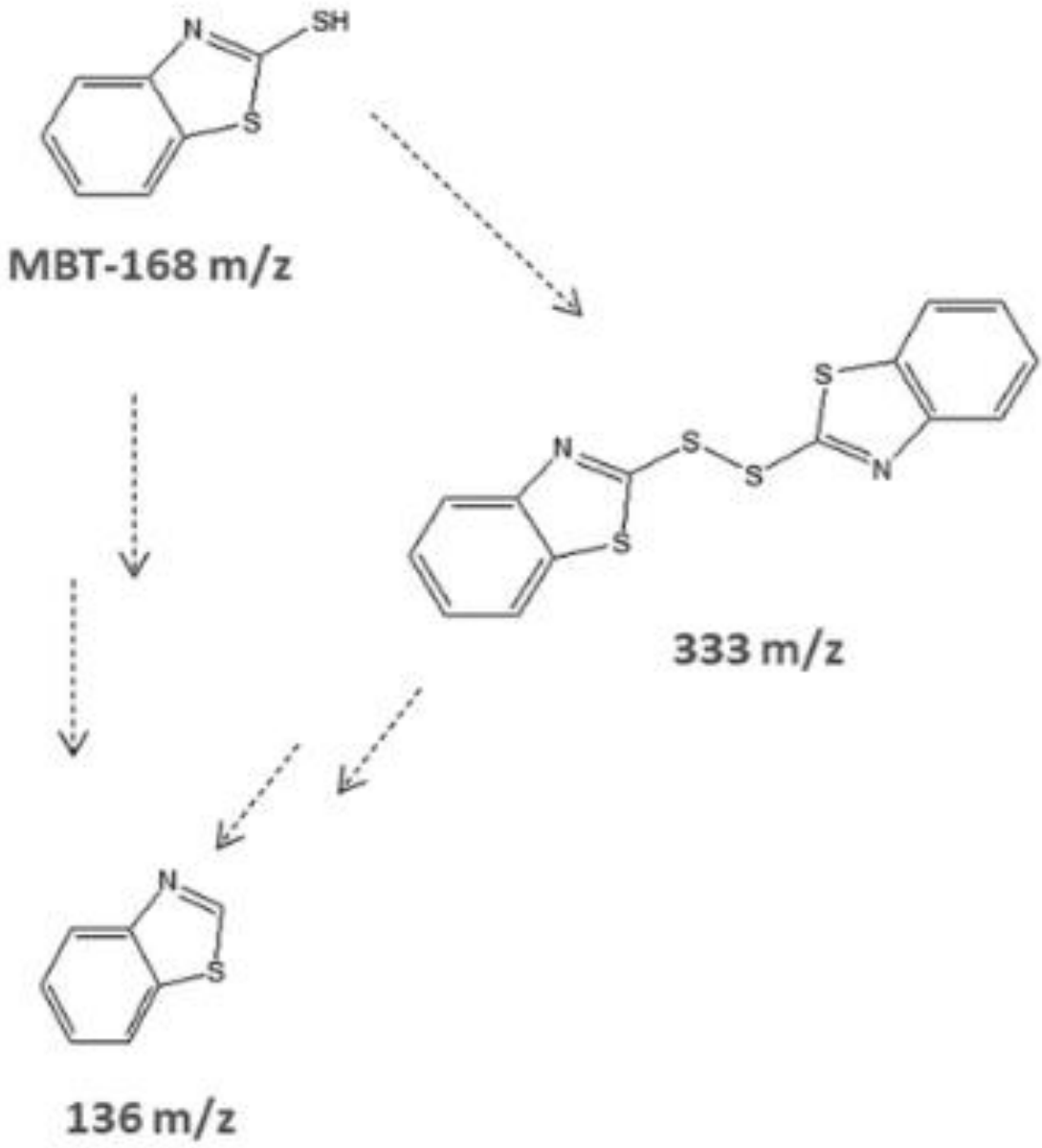


Figure 15: Degradation route of MBT by CPO (Alneyadi & Ashraf, 2016)

Methyl orange is a dye that is mainly used as pH indicator, especially in titration. Bilal and his team (2018) have reported breakdown of methyl orange dye by lignin peroxidases (LiP). The schematic route of this degradation is shown in Figure 16 (Bilal et al., 2018).

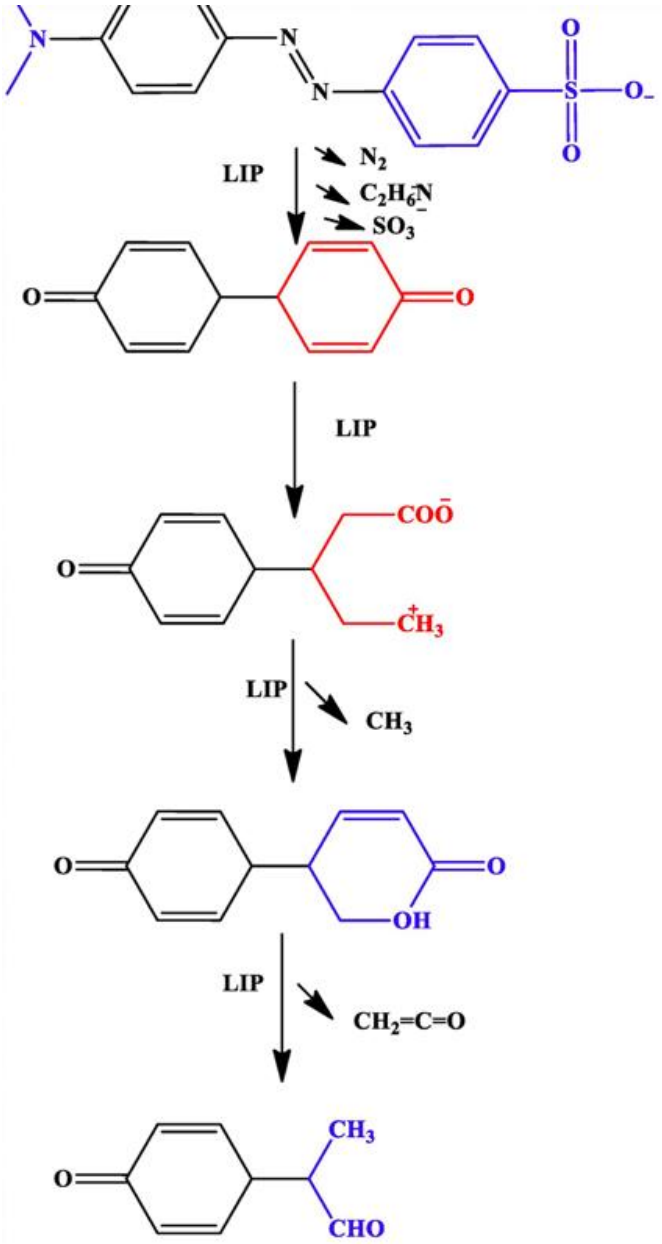


Figure 16: Degradation scheme of methyl orange dye by LiP enzyme (Bilal et al., 2018)

Lastly, the degradation of diclofenac by CPO was reported by Li and his team (2017). It is hard to eliminate diclofenac from wastewater by WWTPs which resulted in significant ecological issues. Figure 17 shows the possible degradation pathway of diclofenac by CPO (Li et al., 2017).

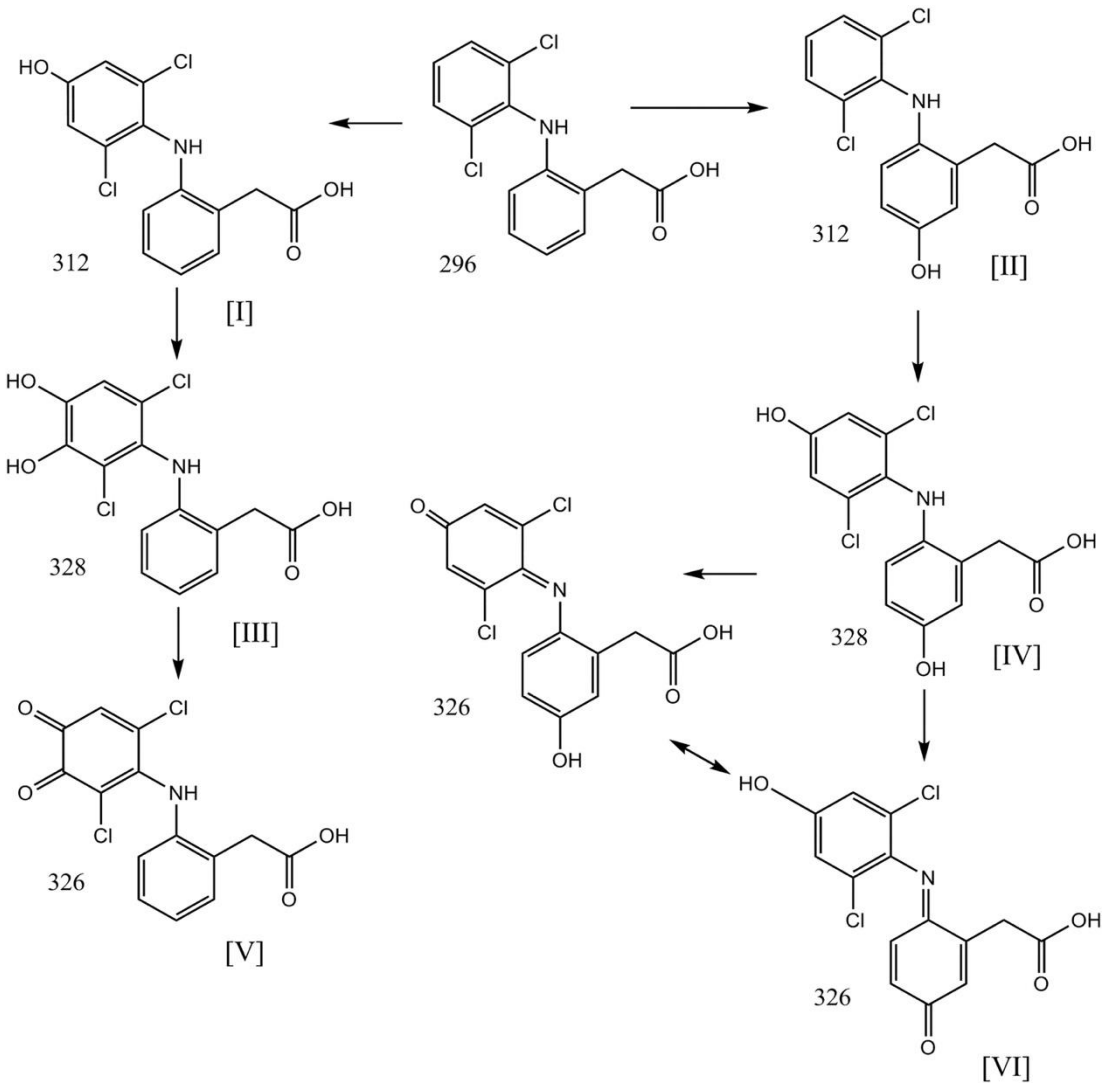


Figure 17: Proposed transformation route of diclofenac by CPO enzyme (Li et al., 2017)

1.4.4 Identification of transformation products

Enzymes cause the degradation of various environmental pollutants by different pathways resulting in the generation of various metabolic intermediates and end products during the biocatalytic reaction. In the majority of the degradation studies, scientists and researchers principally focus on the parent compounds disappearance rather than the scrutinization of transformation pathways, intermediate metabolites, and evaluation of toxicity and estrogenicity of the transformed products (Becker et al., 2016; Naghdi et al., 2018). Nevertheless, the determination of properties of transformed products and removal of toxicities have the greatest concern following the degradation of venomous contaminants in the environment.

Several widely used instrumental techniques for the analysis of enzyme-catalyzed degradation products include direct inlet-mass spectrometry, liquid chromatography with tandem mass spectrometry (Alneyadi & Ashraf, 2016), gas chromatography-mass spectrometry, 1H nuclear magnetic resonance (Hata, Kawai, Okamura, & Nishida, 2010). In addition, high-performance liquid chromatography diode array detection electrospray ionization mass spectrometry and liquid chromatography-electrospray time-off light mass spectrometry can also be employed to detect compounds that are not easily identified by gas chromatography (Schwarz, Aust, & Thiele-Bruhn, 2010; Stadlmair, Letzel, Drewes, & Graßmann, 2017). Very recently, transformation products are identified by a rapid and newly established laser diode thermal desorption-mass spectroscopy (Lonappan, Brar, Das, Verma, & Surampalli, 2016).

1.5 Immobilization of enzymes

Enzymes work perfectly under normal conditions, and their functionalities are highly based on their conformations. Harsh and adverse environmental conditions that

are usually experienced in effluent streams such as extreme temperatures, presence of inhibitors, very high or low pH, and high ionic strength can affect the conformation of the enzyme, thus result in its denaturation (Ambatkar & Usha, 2012). These issues can be overcome by immobilizing the enzyme on a solid support. Immobilization is the process in which the enzyme is attached to an insoluble support carrier, where it is held in a proper geometry resulting in an increased stability of the enzyme and allow its reusability (Asgher, Shahid, Kamal, & Iqbal, 2014; Bilal et al., 2019b). Immobilization process converts the enzyme from its homogenous form to a heterogenous catalyst (immobilized enzyme) to give an immobilized biocatalyst (Zdarta, Meyer, Jesionowski, & Pinelo, 2018). Immobilized enzyme can be used for the continuous bioremediation of great volumes of effluent effectively (Ambatkar & Usha, 2012).

1.5.1 Enzyme immobilization methods

The immobilization of enzymes to different supports can be achieved using three major techniques. The first method is binding to a carrier and it can be subdivided into physical binding, also known as adsorption, and chemical binding via covalent linkages (Sheldon & van Pelt, 2013). In adsorption, the enzyme is adsorbed to the outside surface of an inert support which can be a glass, matrix or alginate beads. This technique is not very efficient and the coupling between the enzyme and support is weak to keep the enzyme attached to its place. On the other hand, covalent binding involves the attachment of the enzyme to a support by covalent bonds directly or using a cross linking reagent such as glutaraldehyde which will be attached to the enzyme at one side and to the support from its other side (Figure 18). Covalent binding is stronger than adsorption and more effective. The second method is entrapment or encapsulation in which the enzyme is either trapped in a polymeric matrix network or encapsulated

within a solid carrier. The last technique is cross linking of enzyme aggregates (CLEAs) or crystals (CLECs) which are carrier-free immobilized enzymes (Asgher et al., 2014; Sheldon and van Pelt, 2013). Figure 19 shows the various methods of enzyme immobilization (Bilal et al., 2017c). The solid support that is used in immobilization should be inexpensive, ecological friendly, non-toxic and does not have a negative effect on the biodegraded solution (Bilal et al., 2019b). Figure 20 summarizes the carriers that can be used for immobilization process (Bilal et al., 2017c).

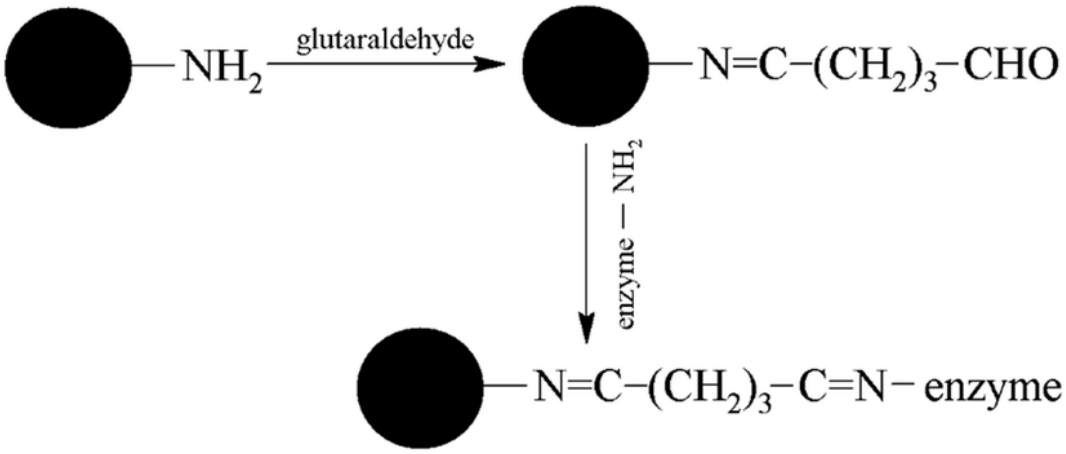


Figure 18: Immobilization of enzyme using glutaraldehyde (Kostelník, Kopel, Cegan, & Pohanka, 2017)

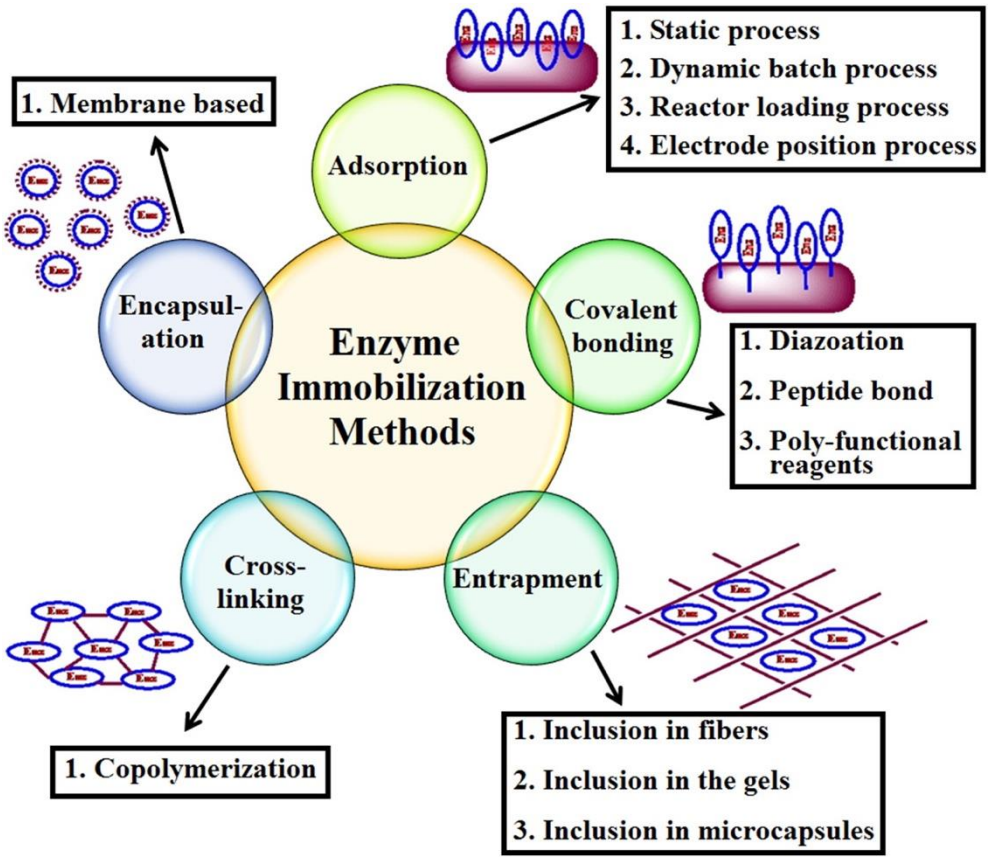


Figure 19: Methods of enzyme immobilization (Bilal et al., 2017c)

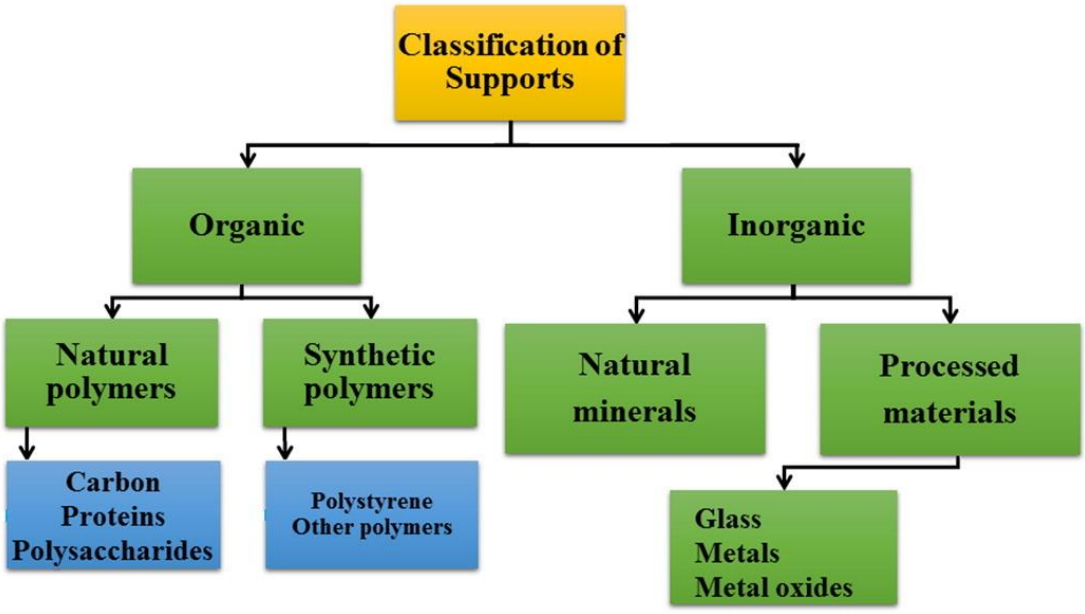


Figure 20: Classification of support materials used for immobilization (Bilal et al., 2017c)

1.5.2 Advantages offered by enzyme immobilization

Using immobilized enzymes overcome most issues that are faced when using a free enzyme. As mentioned earlier, enzymes cannot be reused after the treatment and they are considered expensive. Immobilization overcomes this issue as it allows for the repeated usability of enzymes and increases their recycling efficiencies (Bilal et al., 2019a). Moreover, immobilization increases the long-term stability of enzymes as they become more resistant to degradation and denaturation and stable against harsh temperatures, pH and pressure conditions (Bilal et al., 2019a; Bilal & Asgher, 2015). Figure 21 summarizes some of the advantages and disadvantages of using immobilized enzymes (Bilal et al., 2017c). As enzyme immobilization process offers many advantages compared to free enzymes, it can be considered as an easy and effective way to enhance the catalytic properties of enzymes.

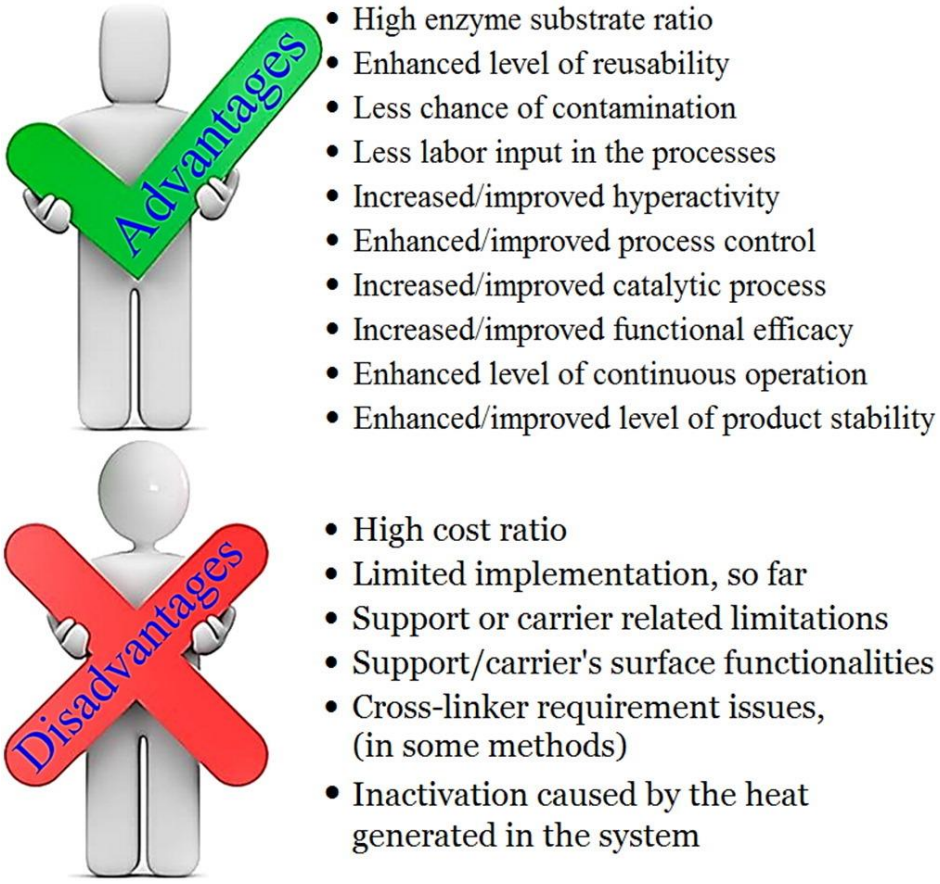


Figure 21: Advantages and disadvantages of enzyme immobilization process (Bilal et al., 2017c)

1.5.3 Applications of immobilized enzymes in degrading pollutants

Immobilized enzymes have been successfully used for the degradation of different hazardous compounds due to their stability and repeated usability. Bilal and his team (2017d) immobilized HRP onto chitosan beads (CTS-HRP) to check the ability of immobilized enzyme in degrading Remazol Brilliant Blue R (RBBR), Reactive Black 5 (RB5), Congo Red (CR) and Crystal Violet (CV) which are textile dyes (Bilal et al., 2017d). They immobilized HRP onto chitosan beads by using a simple entrapment method. The activity of immobilized HRP was tested and it showed a greater activity compared to the free enzyme and this activity was retained during six cycles of treatments. The CTS-HRP successfully degraded the dyes with removal

efficiencies as follow; RB5 (97.82%) followed by CR (94.35%), CV (87.43%) and RBBR (82.17%). Figure 22 shows the ultraviolet-visible (UV-vis) absorption spectra of the four dyes before and after the treatment by CTS-HRP (Bilal et al., 2017d).

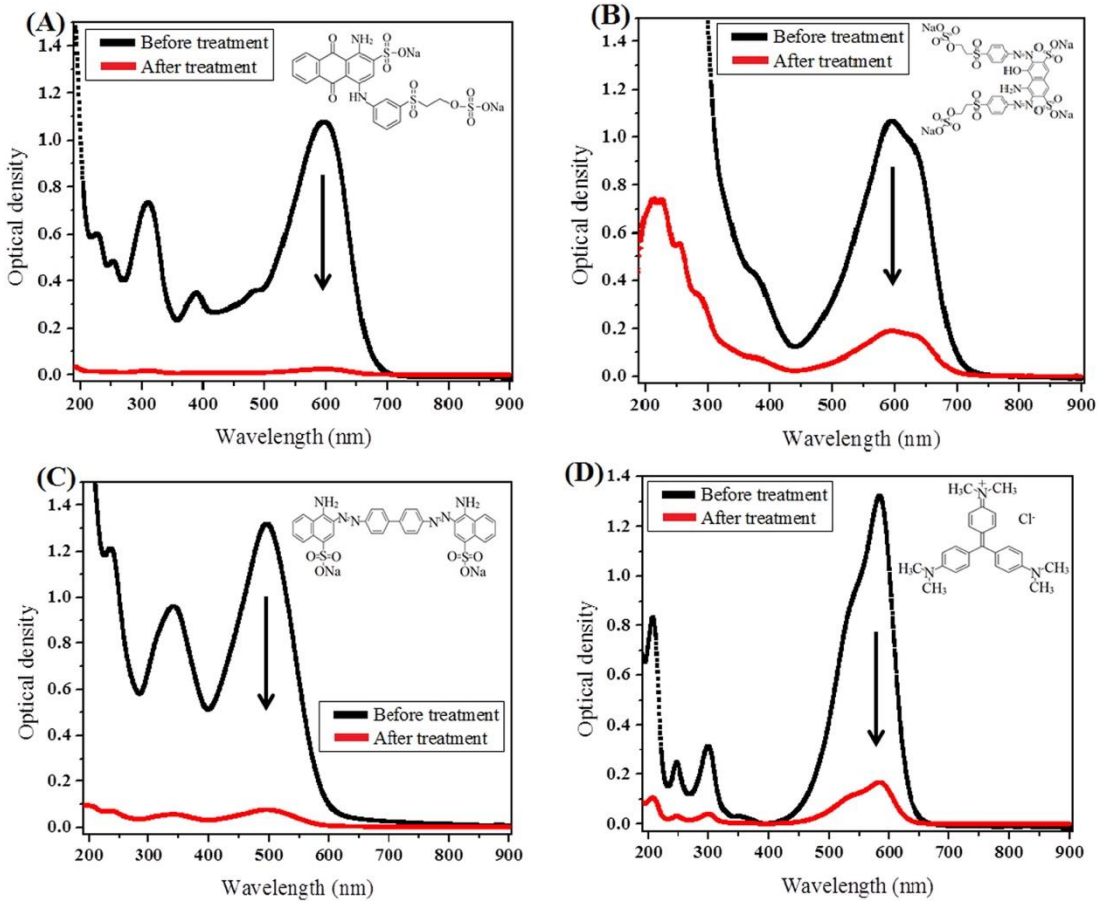


Figure 22: UV-vis spectra of the four dyes before and after CTS-HRP treatment. (A) RBBR; (B) RB5; (C) CR; and (D) CV (Bilal et al., 2017d)

Another study that was done by (Yang et al., 2017) used an immobilized laccase and tested its ability in degrading different antibiotics. The method that they used for immobilization process is cross linking of enzyme aggregates (CLEAs) as they prepared a magnetic cross-linked enzyme aggregates (M-CLEAs) for laccase and used it for the biodegradation of antibiotics. Their work showed that laccase M-CLEAs was able to degrade more than 80% of tetracycline (TC) efficiently within 12 hours. The degradation products of TC were detected using liquid chromatography coupled to time of flight/ mass spectrometry (LC-TOF MS). A three degradation products were detected which are entitled as TP 459, TP 431 and TP 396 that have elution time at 2.69, 6.01, and 6.35 minutes, respectively. Figure 23 shows the mass spectra of the three transformation products that resulted from the laccase treatment (Yang et al., 2017). Figure 24 shows the proposed mechanism of TC degradation by laccase M-CLEAs based on the identified degradation products (Yang et al., 2017).

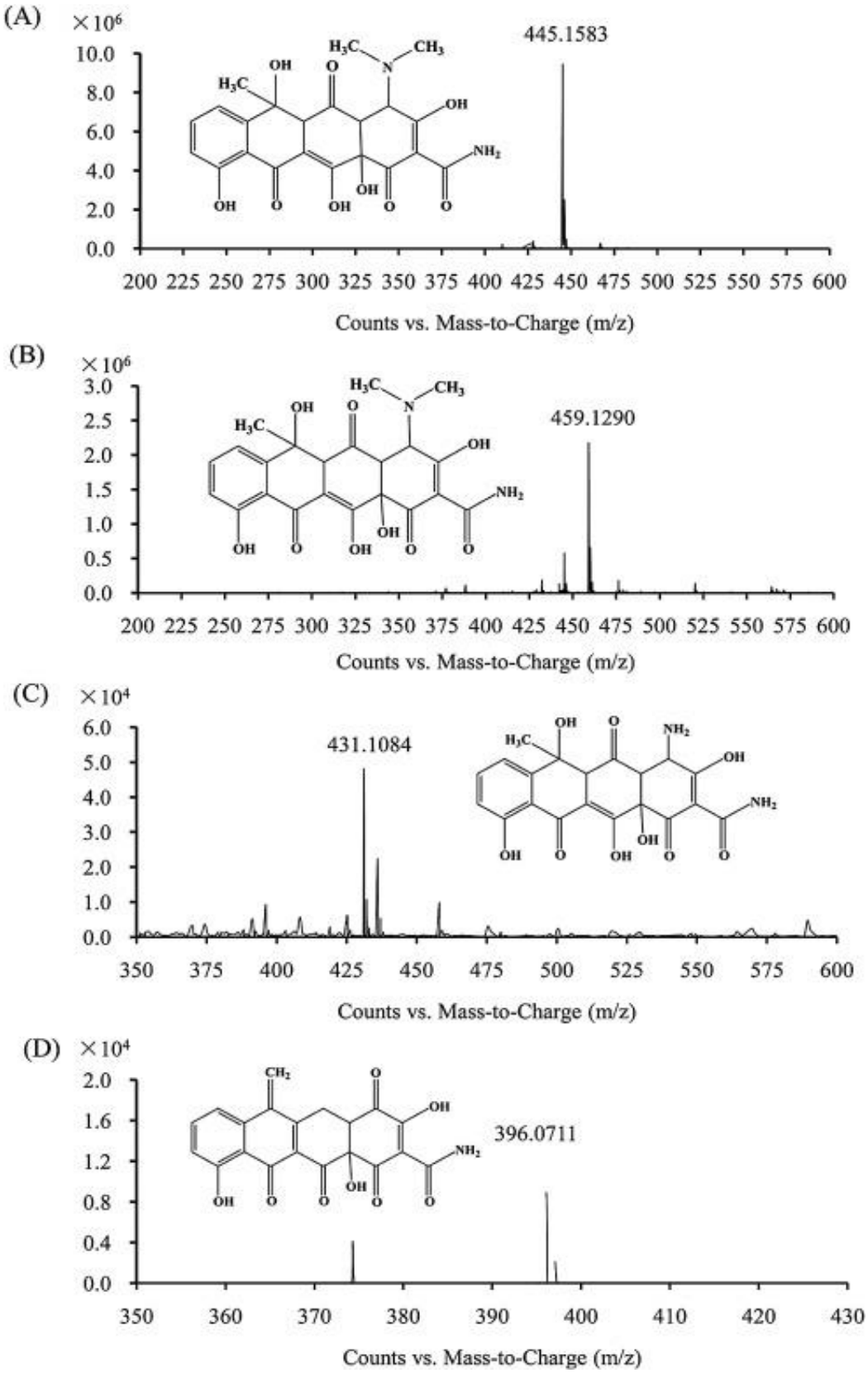


Figure 23: Mass spectra of TC degradation products after laccase treatment. (A)TC (m/z 445.16); and (B-D)TC transformation products (m/z 459.13, 431.11 and 396.07) were identified with LC-TOF MS (Yang et al., 2017)

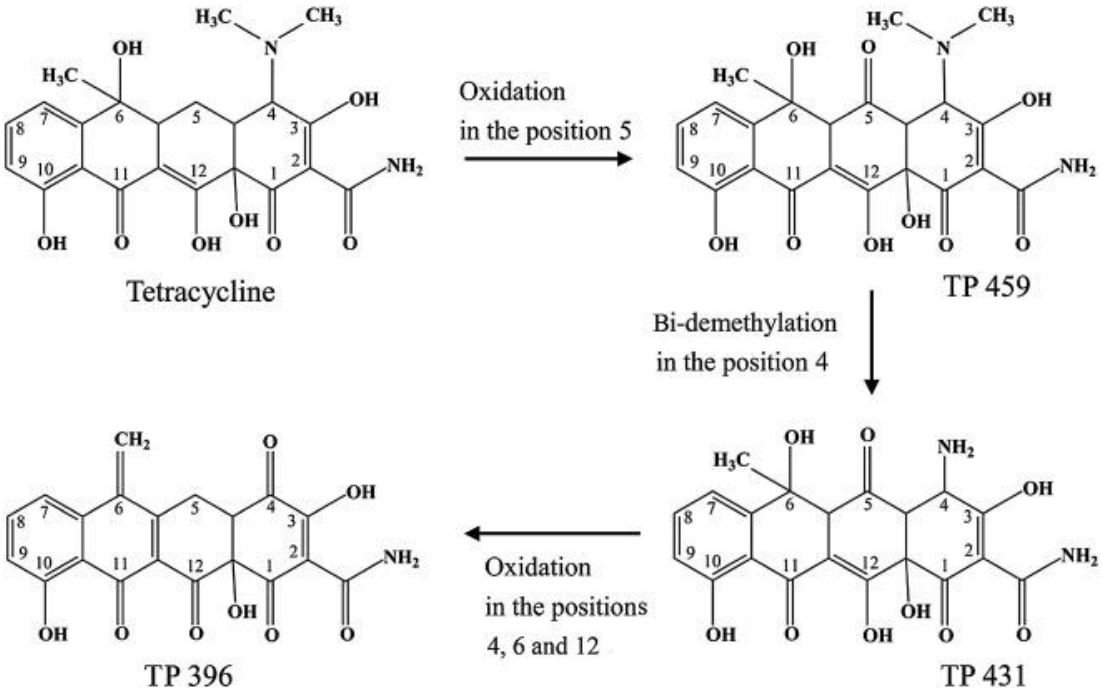


Figure 24: Proposed mechanism of TC degradation by laccase M-ClEAs treatment (Yang et al., 2017)

A very recent study which was conducted by Bilal and his colleagues (2019) reported the ability of free laccase and immobilized laccase in degrading bisphenol A which is an organic synthetic compound (Bilal, Jing, Zhao, and Iqbal, 2019b). In their work, laccase was covalently attached to chitosan beads using glutaraldehyde as a cross linking reagent. The immobilized biocatalyst showed good stability and it was able to preserve 71.24% of its activity after ten cycles of treatments. Bisphenol A was almost completely degraded (more than 99%) by immobilized laccase after 150 minutes. There are many other studies documented the ability of immobilized enzymes to degrade different contaminants efficiently. Table 8 summarizes various degradation studies of different pollutants by immobilized biocatalysts. Immobilization of biocatalysts on different supports is a promising and ecologically friendly technique for the elimination of different pollutants from wastewater.

Table 8: Removal studies of different pollutants by immobilized enzymes on different supports

Enzyme	Immobilization technique	Immobilization support	Pollutant	% Removal	Reference
LiP	Entrapment	Ca-alginate beads	Sandal-fix Red C4BLN	93	(Shaheen, Asgher, Hussain, & Bhatti, 2017)
LiP	Encapsulation	Polyacrylamide hydrogel	Bisphenol A	90	(Gassara, Brar, Verma, & Tyagi, 2013)
LiP	Adsorption	Nanoporous gold	Fuchsine	85	(Qiu et al., 2009)
LiP	Adsorption	Mesoporous 2D silica	Phenol	60	(L.-Q. Xu, Wen, & Ding, 2010)
LiP	Covalent attachment	Carbon nanotubes	Ramazol Brilliant Blue R	>50%	(Oliveira, da Luz, Kasuya, Ladeira, & Correa Junior, 2018)
HRP	Covalent attachment	Functionalized reduced graphene oxide	Phenol	100	(Besharati Vineh, Saboury, Poostchi, Rashidi, & Parivar, 2018)
HRP	Covalent attachment	Carbon nanospheres	2,4-Dichlorophenol	95	(Lu et al., 2017)
HRP	Adsorption	Kaolin	Pyroggallol	70	(Šekuljica et al., 2016)
HRP	Entrapment	Chitosan beads	Congo Red	94.35	(Bilal et al., 2017d)
MnP	Cross linking	Chitosan beads	Textile-based dye eflluents	94.5	(Bilal, Asgher, Iqbal, Hu, Wang, et al., 2017b)
MnP	Adsorption	Vulcanic nanoclay	Anthracene	65	(Acevedo et al., 2010)
SBP	Adsorption	Silica monoliths	Methyl orange	100	(Calza, Zacchigna, & Laurenti, 2016)
SBP	Covalent attachment	Glutaraldehyde-activated aminopropyl glass beads	Phenol	>95	(Gómez et al., 2006)
Lac	Covalent attachment	Hollow mesoporous carbon spheres (HMCs)	Tetracycline hydrochloride	99.2	(Shao et al., 2019)
Lac	Covalent attachment	HMCs	Ciprofloxacin hydrochloride	96.9	(Shao et al., 2019)
Lac	Cross linking	Multi-channel ceramic membrane	Bisphenol A	100	(Barrios-Estrada et al., 2018)
Lac	Covalent attachment	Functionalized TiO_2 nanoparticles	Carbamazepine	40	(Ji, Nguyen, Hou, Hai, & Chen, 2017)
Lac	Covalent attachment	Hairy polymer grafted zeolite particles	Reactive Red 120	100	(Celikbicak et al., 2014)
MnP	Adsorption	Vulcanic nanoclay	Anthracene	65	(Acevedo et al., 2010)
SBP	Adsorption	Silica monoliths	Methyl orange	100	(Calza et al., 2016)

1.6 Overall aim of this study

The overall aim of this study was to increase the applicability of two different peroxidases (SBP and HRP) by covalently immobilizing them on two different photocatalytic nanoparticles (TiO_2 and ZnO) which allows the re-usability of the two enzymes. Also, to use these immobilized peroxidases for the degradation of various emerging pollutants.

The main objectives of the current work are summarized below:

1. Carry out degradation experiments using two different peroxidases (SBP and HRP) in the presence and absence of redox mediator to degrade a mixture of 21 emerging pollutants.

2. Immobilize the two enzymes covalently onto two different photocatalytic supports (TiO_2 and ZnO) to produce four hybrid biocatalysts (SBP-TiO_2, SBP-ZnO, HRP-TiO_2, HRP-ZnO).

3. Characterize the immobilized enzymes to check whether the immobilization process has affected the morphology and crystallinity of the photocatalysts or the pH and thermal stabilities of the enzymes.

4. Carry out degradation experiments using the four hybrid biocatalysts to degrade a mixture of 21 emerging pollutants.

5. Carry out a comparative degradation analysis between using free peroxidases or immobilized peroxidases for the degradation of 21 emerging pollutants.

6. Test the re-usability of immobilized enzyme for emerging pollutant degradation.

Chapter 2: Materials and Methods

2.1 Materials and Reagents

Chemical standards of all emerging pollutants under study were purchased from Sigma-Aldrich (Fremont, CA, USA). Solvents used in LC-MS such as LC-MS grade water, formic acid and acetonitrile as well as hydrogen peroxide (30% w/v) were also purchased from Sigma-Aldrich (St. Louis, MO, USA). All experiments were accomplished using universal buffers (0.2 M potassium phosphate (K_2HPO_4) and 0.1 M citrate acid). Glutaraldehyde and (3-Aminopropyl)triethoxysilane (APTES) that were used in the immobilization process were purchased from Sigma-Aldrich (USA) as well. Cellulose acetate syringe filters were purchased from Medicom Distribution Fze (UAE). A ZORBAX Eclipse Plus $C1_8$ column was purchased from Agilent Technologies (Santa Clara, CA, USA). HRP with a specific activity of 279 IU/mg (1 mg/mL, 26 μM) was purchased from Sigma-Aldrich (USA). SBP with a specific activity of 2700 IU/mg (1 mg/mL, 26 μM) was purchased from Bio-Research Products (North Liberty, USA). Both photocatalysts (TiO_2 and ZnO) were purchased from Sigma-Aldrich (USA).

2.2 Immobilization process

In order to immobilize our enzymes on the photocatalytic supports, we used the procedure that was previously described by (Donadelli, García Einschlag, Laurenti, Magnacca, & Carlos, 2018). TiO_2 and ZnO particles were functionalized with APTES. To briefly describe the process, ZnO will be taken as an example. ZnO was mixed in ethanol/water (1:1) solution. The solution was left under nitrogen and sonicated for few minutes. APTES was then added to the solution and stirred for some time at 40^{o}C. The solid obtained was washed with ethanol/water solution then dried in a rotavapor. The same exact thing was done for TiO_2. Glutaraldehyde was used to link the enzyme

(SBP or HRP) to the surface of the functionalized photocatalytic support (TiO_2-APTES or ZnO-APTES). ZnO-APTES was added to a glutaraldehyde (2.5%) solution in phosphate buffer (pH=7, 0.1 M). The mixture was stirred in the dark for some time, then the products were filtered from the solution using Buchner funnel with vacuum suction. The separated producted was incubated with enzyme solution (SBP and HRP) in phosphate buffer and kept under stirring over the night. On the second day, the mixture was filtered using Buchner funnel with vacuum suction, and the solid which was obtained on the filter paper was washed few times with phosphate buffer. All solid particles were collected and preserved at 4^oC and ready to be used.

2.3 Characterization of immobilized enzymes

Immobilized enzymes were characterized using scanning electron microscopy (SEM) and X-ray diffraction spectroscopy. The morphology of the photocatalysts (TiO_2 and ZnO), functionalized photocatalysts (TiO_2-APTES and ZnO-APTES) and the four hybrid biocatalysts (SBP-TiO_2, SBP-ZnO, HRP-TiO_2 and HRP-ZnO) were investigated using a FEI SEM Quanta Inspect S50 Scanning electron microscope. Images were collected at a voltage of 25 kV and magnification of x10000. The crystalline structure of the same samples was examined using Shimadzu-6100 X-ray powder diffractometer with Cu-Kα radiation. The data were obtained from 10^o-70^o at a rate of 2^o/min.

2.4 LC-MSMS method development

When the 21 emerging pollutants were degraded with the free enzymes (SBP and HRP) and immobilized enzymes (SBP-TiO_2, SBP-ZnO, HRP-TiO_2 and HRP-ZnO), the samples were analyzed by the LCMS. All samples were filtered before their injection to the LCMS using cellulose syringe filters with a diameter of 13 mm and pore size of 0.45 μm. A ZORBAX Eclipse Plus C_{18} column was used for the analysis.

The column has a 1.8 μm particle size, an inner diameter of 2.1 mm, and a length of 50 mm. The column's temperature was maintained at 35^{o}C. The mobile phase flow rate in the column was fixed at 0.4 mL/min. The Mass Spectrometer detector was a 6420 Triple Quad detector (Agilent Technologies). Two mobile phases were used; (A) agueous solution of 0.1% formic acid, (B) 100% acetonitrile. A gradient elution was used for the method development in the LC-MS/MS analysis and it was set as follow: 2.5 min of 100% A and 0% B, then from 2.5 -15 min a 0-80% gradient of B was used, followed by 10% A and 90% B for 3 min, and lastly 95% A and 5% B for 2 min. Both positive and negative polarity modes were used in the electrospray ionization source in the LCMS system depending on the pollutant analyzed. In the LC-MS interface system, the drying N_2 gas flow was 8 L/min, and its temperature was kept at 3000^{o}C. The nebulization N_2 gas pressure was set at 45 psi, and the capillary voltage was maintained at 4000 V. The nitrogen gas was used for fragmentation in the product ion mode with different collision energies depending on the emerging pollutants.

2.5 Degradation of emerging pollutants

A mixture of 21 emerging pollutants was prepared and treated with free enzymes (SBP and HRP) and immobilized enzymes (SBP-TiO_2, SBP-ZnO, HRP-TiO_2 and HRP-ZnO) with and without a redox mediator. The degradation experiments for free enzymes were carried as follow: 0.36 μM enzyme (SBP or HRP) was added to a 2ppm mixture of 21 emerging pollutants + 0.3 mM H_2O_2 + universal buffer (pH= 5 for SBP and pH=4 for HRP). For the experiments in which redox mediator was used, 0.1 mM of 1-hydroxybenzotriazole (HOBT) was also added to the mixture. The degradation experiments for immobilized enzymes were carried exactly the same as free enzymes, but instead of using a liquid enzyme, 20 mg of each hybrid biocatalyst (SBP-TiO_2, SBP-ZnO, HRP-TiO_2 or HRP-ZnO) was added to the mixture and instead

of using 0.3 mM of H_2O_2, 0.6 mM of H_2O_2 was used. Regarding the universal buffer, all composites required pH=4 except HRP-TiO_2 which required pH=5. Mixture components were kept at room temperature to react for 30 minutes, then they were filtered and analyzed using LC-MSMS as described above.

2.6 Re-usability of immobilized enzyme

SBP-TiO_2 was used to degrade MBT for multiple times, after each reaction SBP-TiO_2 was recycled and re-used for the next reaction. The degradation experiment was carried out as follow: 20 mg SBP-TiO_2, 2 ppm MBT, 0.6 mM H_2O_2, universal buffer pH=4 and 0.1 mM HOBT. The reaction was kept at room temperature for 30 minutes, then 0.5 mL of the mixture was filtered and analyzed using LC-MSMS. The remaining was centrifuged for 5 mins. After centrifuging, the supernatant was discarded, and the pellet which contains the immobilized enzyme was kept. Components of the reaction were added again to the pellet and were kept for 30 minutes to react. Then they were analyzed using LC-MSMS and the immobilized enzyme was recycled again and tested for its ability to degrade MBT. This cycle was repeated for three more times.

Chapter 3: Results and Discussion

3.1 Bioanalytical assay development

Many different classes of emerging pollutants (EPs) have been increasingly found in the water supplies such as pharmaceuticals, hormones, pesticides, personal care products and many others. These pollutants are believed to cause serious effects to human health or ecological system; therefore, it is really important to focus on how it is possible to eliminate these pollutants from the environment. Various treatment systems have been studied previously for their abilities in degrading these pollutants successfully. In our study, we focused on the degradation of 21 different EPs by free peroxidases (SBP and HRP) and immobilized peroxidases on different photocatalytic supports (SBP-TiO_2, SBP-ZnO, HRP-TiO_2, HRP-ZnO). Table 9 shows the structures and categories of the 21 EPs that we studied. In order to quantitate the EPs and examine their degradability by free peroxidases and immobilized peroxidases, a sensitive and robust LC-MSMS method has been developed.

Table 9: Structures and categories of 21 EPs

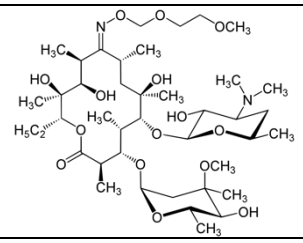
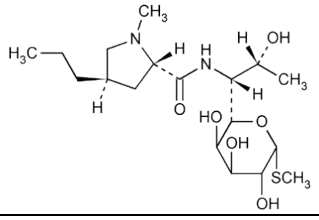
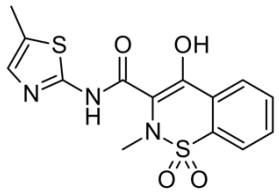
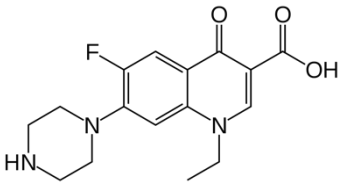
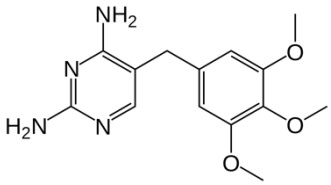
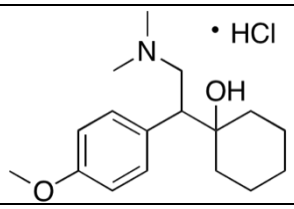
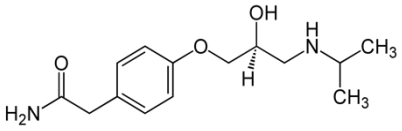
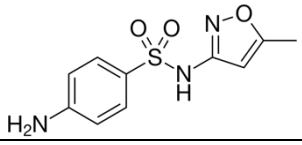
	Emerging Pollutant	Category	Structure
1	Roxithromycin	Antibiotic	
2	Lincomycin hydrochloride (Lincomycin-HCl)	Antibiotic	
3	Meloxicam	Nonsteroidal anti-inflammatory drug (NSAID)	
4	Norfloxacin	Antibiotic	
5	Trimethoprim	Antibiotic	
6	Venlafaxine hydrochloride (Venlafaxine-HCl)	Antidepressant	
7	Atenolol	Beta blocker medication	
8	Sulfamethoxazole (SMX)	Antibiotic	

Table 9: Structures and categories of 21 EPs (Continued)

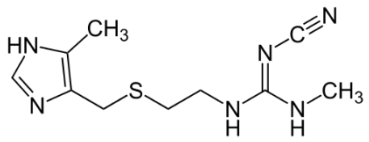
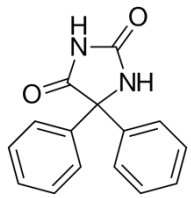
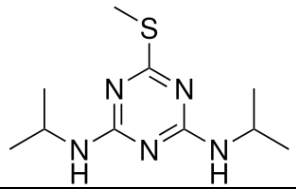
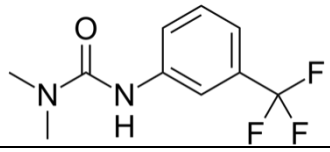
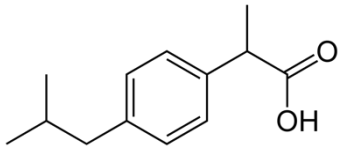
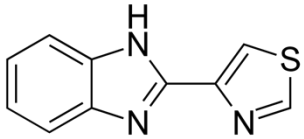
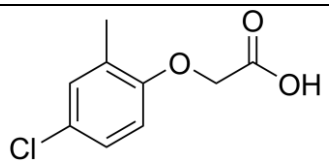
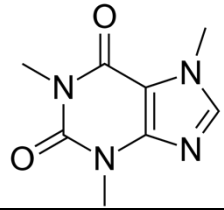
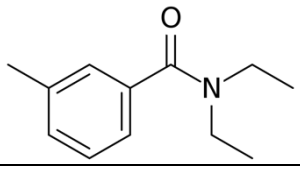
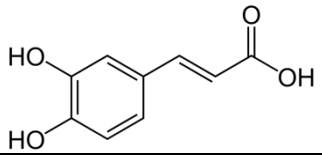
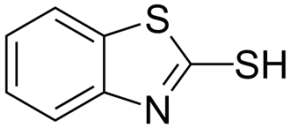
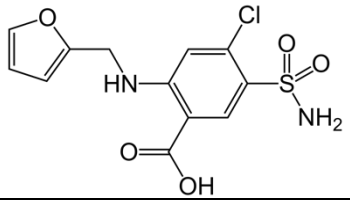
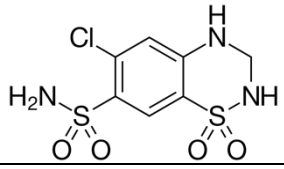
	Emerging Pollutant	Category	Structure
9	Cimetidine	Histamine H_2 receptor antagonist	
10	Phenytoin	Anti-seizure medication	
11	Prometryn	Herbicide	
12	Fluometuron	Herbicide	
13	Ibuprofen	NSAID	
14	Thiabendazole	Fungicide	
15	2-Methyl-4-chlorophenoxyacetic acid (MCPA)	Herbicide	
16	Caffeine	Stimulant	
17	DEET	Insect repellent	

Table 9: Structures and categories of 21 EPs (Continued)

	Emerging Pollutant	Category	Structure
18	Caffeic acid	Antioxidant	
19	Mercaptobenzothiazole (MBT)	Sulfur vulcanization of rubber	
20	Furosemide	Loop diuretic medication	
21	Hydrochlorothiazide	Diuretic medication	

3.1.1 LC-MS/MS method development

Many studies have reported the usage of HPLC and LC-MS based assays to quantify and detect numerous organic compounds. In our study, we developed LC-MSMS method using the Multiple Reaction Monitoring (MRM) mode that was previously described by (Alhefeiti, 2017). Taking caffeine as an example, a stock solution of caffeine was prepared and analyzed in the MS2 mode of the LC-MSMS. The MS2 scan showed a peak for caffeine in the total ion chromatogram. Then the chromatogram was extracted in order to show the expected molecular mass of the caffeine which was 195 Da. After that, the collision energy in the MS was increased gradually in order to generate product ions. When the collision energy reached 30V, the caffeine which is the precursor ion was completely broken down into a number of product ions. The product ion that had the greatest intensity (138 m/z) compared to all other product ions was used to make precursor to product ion pairs (195 → 138) which

was used to analyze the caffeine sample in the MRM mode of the LC-MSMS. The MRM mode generated a very sensitive and accurate peak for the caffeine. Figure 25 shows the overall scheme for the LC-MSMS method development for caffeine (Alhefeiti, 2017). Exactly the same thing was applied to all the other 20 emerging pollutants. Table 10 summarizes the MRM method development for all the 21 EPs by showing their MRM parameters. A mixture of the 21 EPs was prepared and analyzed by the LC-MSMS using MRM mode. Figure 26 shows the chromatogram of the mixture. Figure 27 shows the extracted chromatogram of each pollutant.

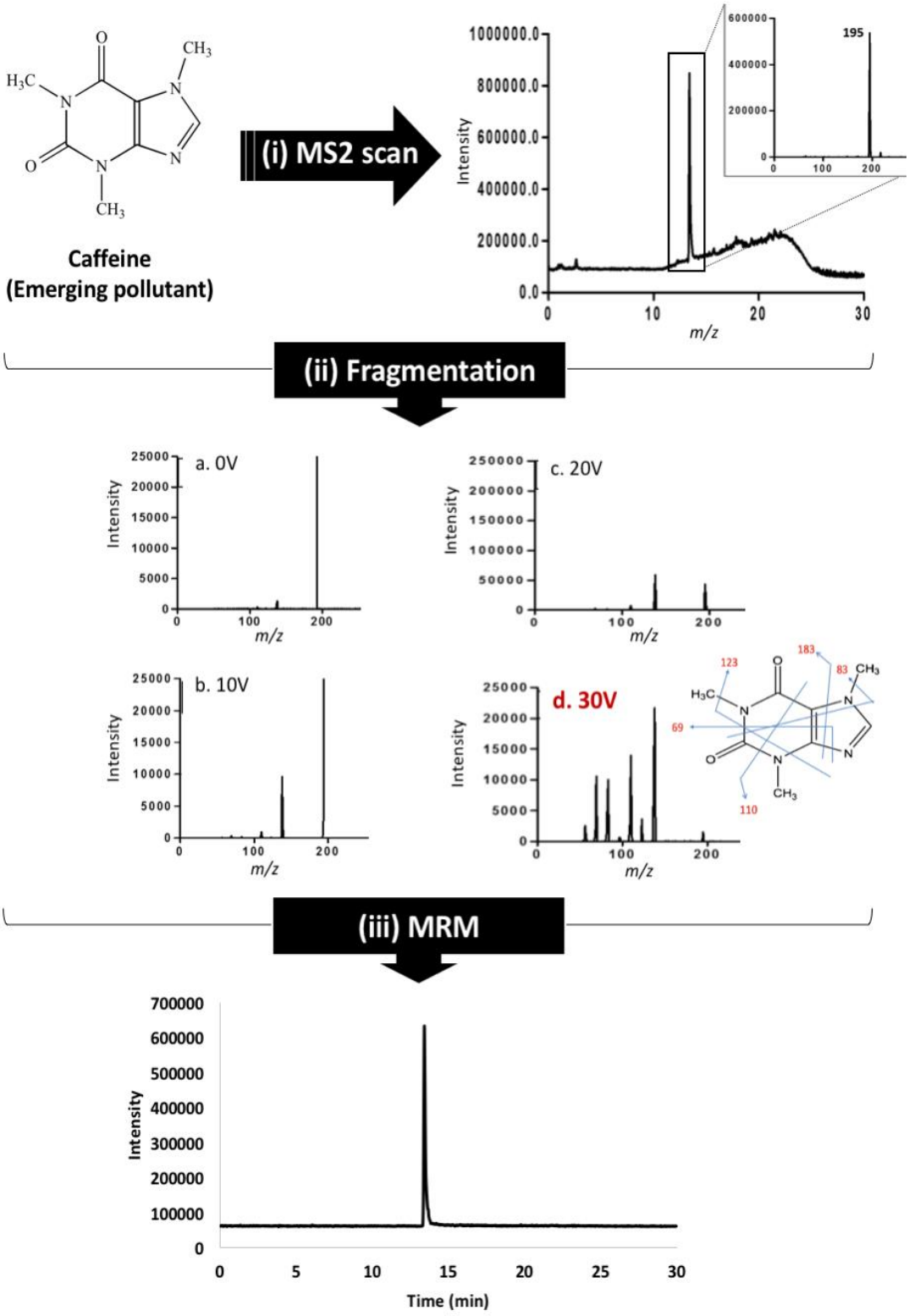


Figure 25: Schematic diagram for LC-MSMS method development of caffeine (Alhefeiti, 2017)

Table 10: Summary of MRM mode for 21 EPs

	Emerging Pollutant	Retention time (min)	Parent ion (m/z)	Daughter ion (m/z)	Polarity	Collision Energy (V)
1	Roxithromycin	11.6	837	680	Positive	20
2	Lincomycin-HCl	7.6	407	359	Positive	20
3	Meloxicam	12.8	352	115	Positive	6
4	Norfloxacin	8.2	320	302	Positive	20
5	Trimethoprim	7.9	291	230	Positive	20
6	Venlafaxine-HCl	9.4	278	260	Positive	10
7	Atenolol	7.1	267	190	Positive	20
8	SMX	9.3	254	156	Positive	20
9	Cimetidine	6.9	253	159	Positive	10
10	Phenytoin	11.1	253	182	Positive	10
11	Prometryn	11.6	242	158	Positive	30
12	Fluometuron	11.7	233	72	Positive	30
13	Ibuprofen	14.4	207	161	Positive	20
14	Thiabendazole	7.6	202	175	Positive	30
15	MCPA	12	201	125	Positive	13
16	Caffeine	7.8	195	138	Positive	30
17	DEET	11.9	192	119	Positive	30
18	Caffeic acid	7.8	181	163	Positive	20
19	MBT	10.6	168	135	Positive	30
20	Furosemide	11	329	285	Negative	15
21	Hydrochlorothiazide	6.4	167	190	Negative	20

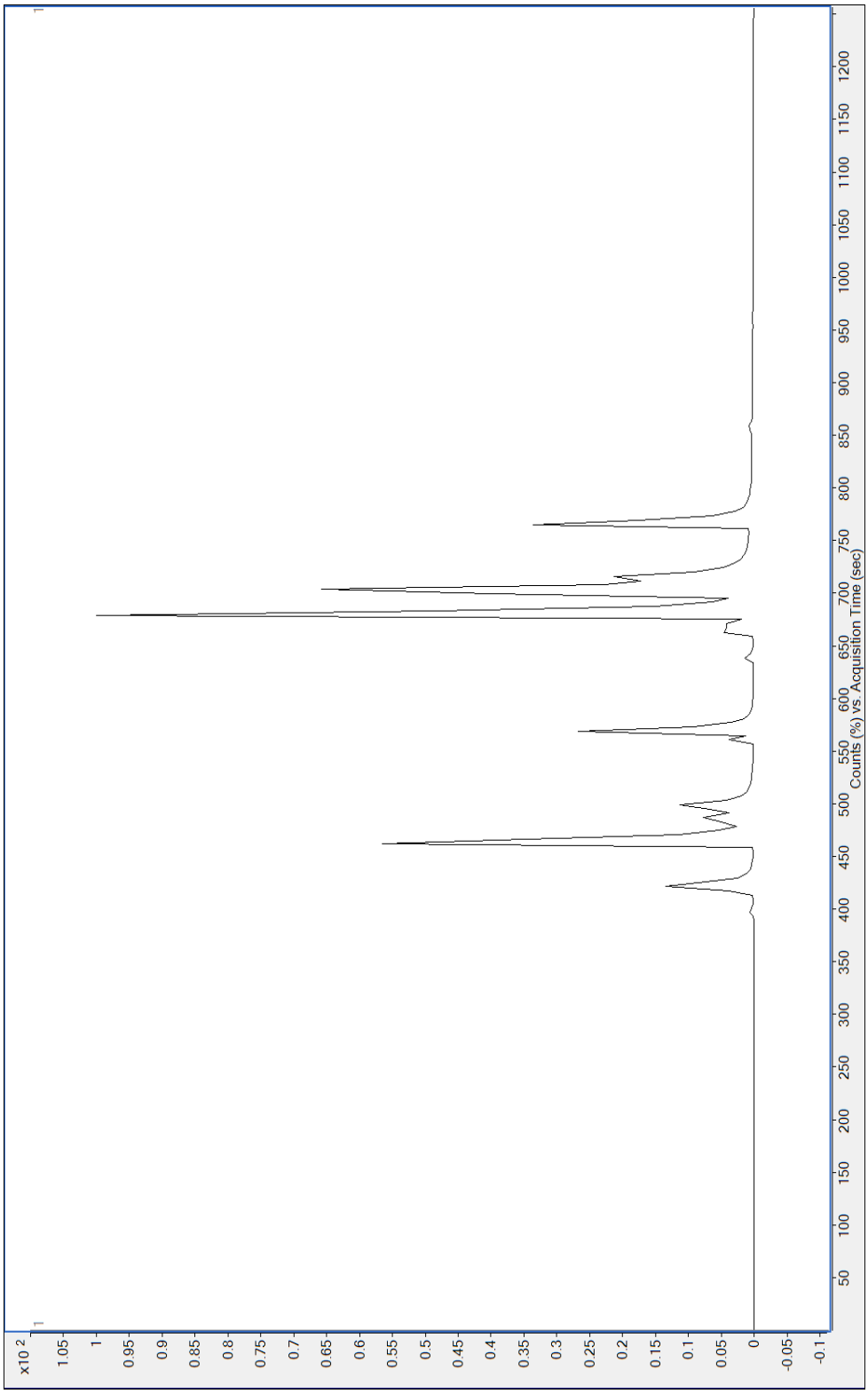


Figure 26: Chromatogram of a mixture composed of the 21 EPs

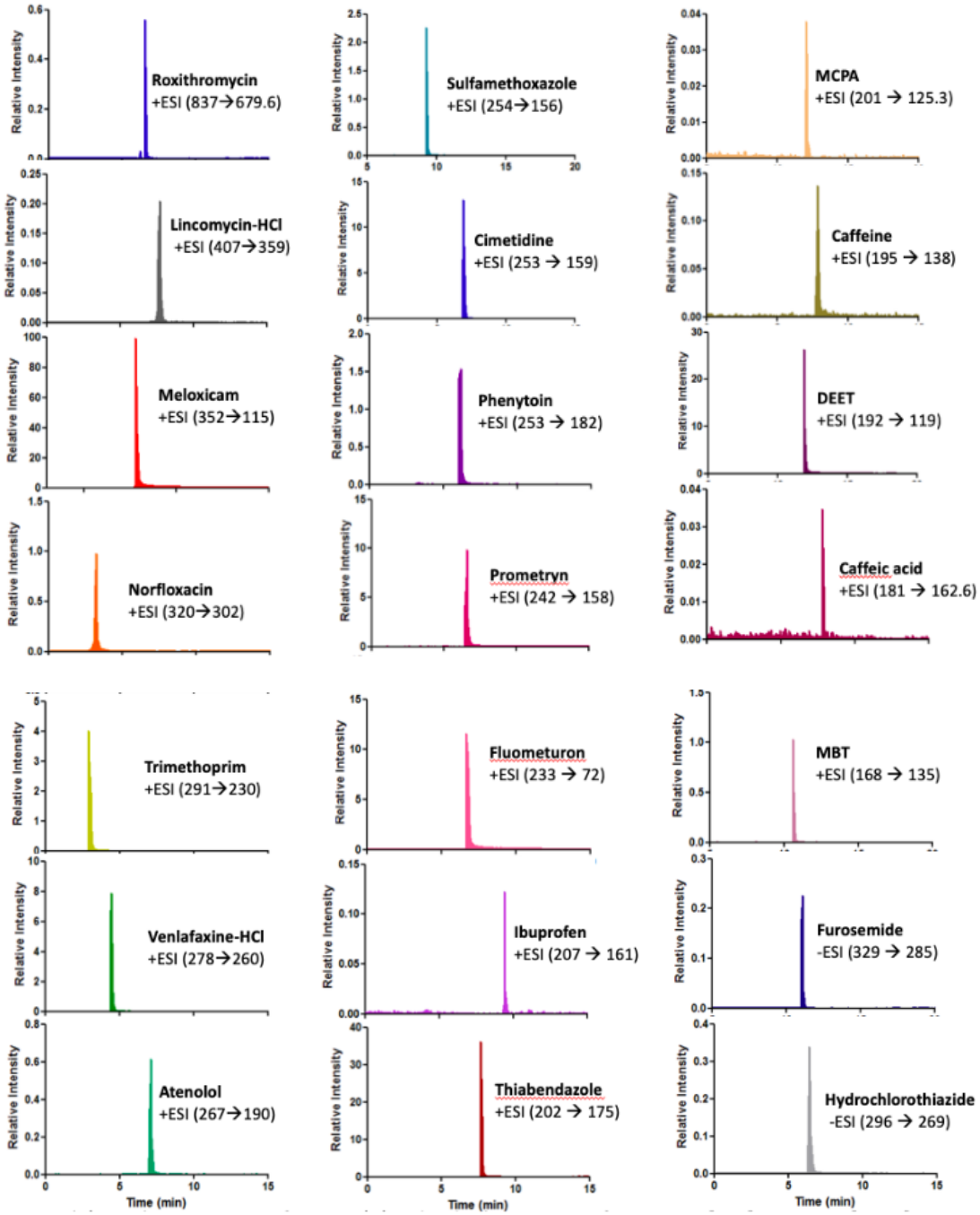


Figure 27: Extracted chromatogram for each pollutant

3.2 Degradation of 21 EPs by SBP and HRP

Among all different classes of enzymes, oxidoreductases have been the most widely used class of enzymes for the degradation of different organic compounds. Within oxidoreductases, peroxidases have shown for their successive ability in degrading various classes of organic compounds including emerging contaminants. For example, a 2019 study reported more than 95% degradation of 4,4′-methylenedianiline (MDA) by SBP enzyme (Mukherjee et al., 2019). Another study that has been conducted by (Rathner et al., 2017) showed the ability of HRP to degrade more than 90% of 17α-ethinylestradiol (EE2). Many other studies showed that different peroxidases such as LiP and MnP can efficiently eliminate EPs from wastewater supplies. In this thesis, we studied the ability of SBP and HRP in degrading the 21 EPs listed in Table 9.

A mixture of the 21 EPs was prepared and treated with SBP and HRP enzymes. Each enzyme was able to degrade some of the pollutants efficiently (greater than 80%). Other pollutants were either partially degraded (20%-80%) or not degraded (less than 20%) by the two enzymes. Figure 28 shows an extracted chromatogram for MBT; A) before the addition of SBP enzyme and B) after the addition of SBP enzyme. The area under the peak represents the amount of MBT. Figure 29 shows an extracted chromatogram for DEET; A) before SBP treatment and B) after SBP treatment. Figure 31 represents a bar graph for the percentage remaining of A) MBT and B) DEET after the enzymatic treatment with SBP. Table 11 summarizes the results that we obtained from treating the 21 EPs with SBP and HRP.

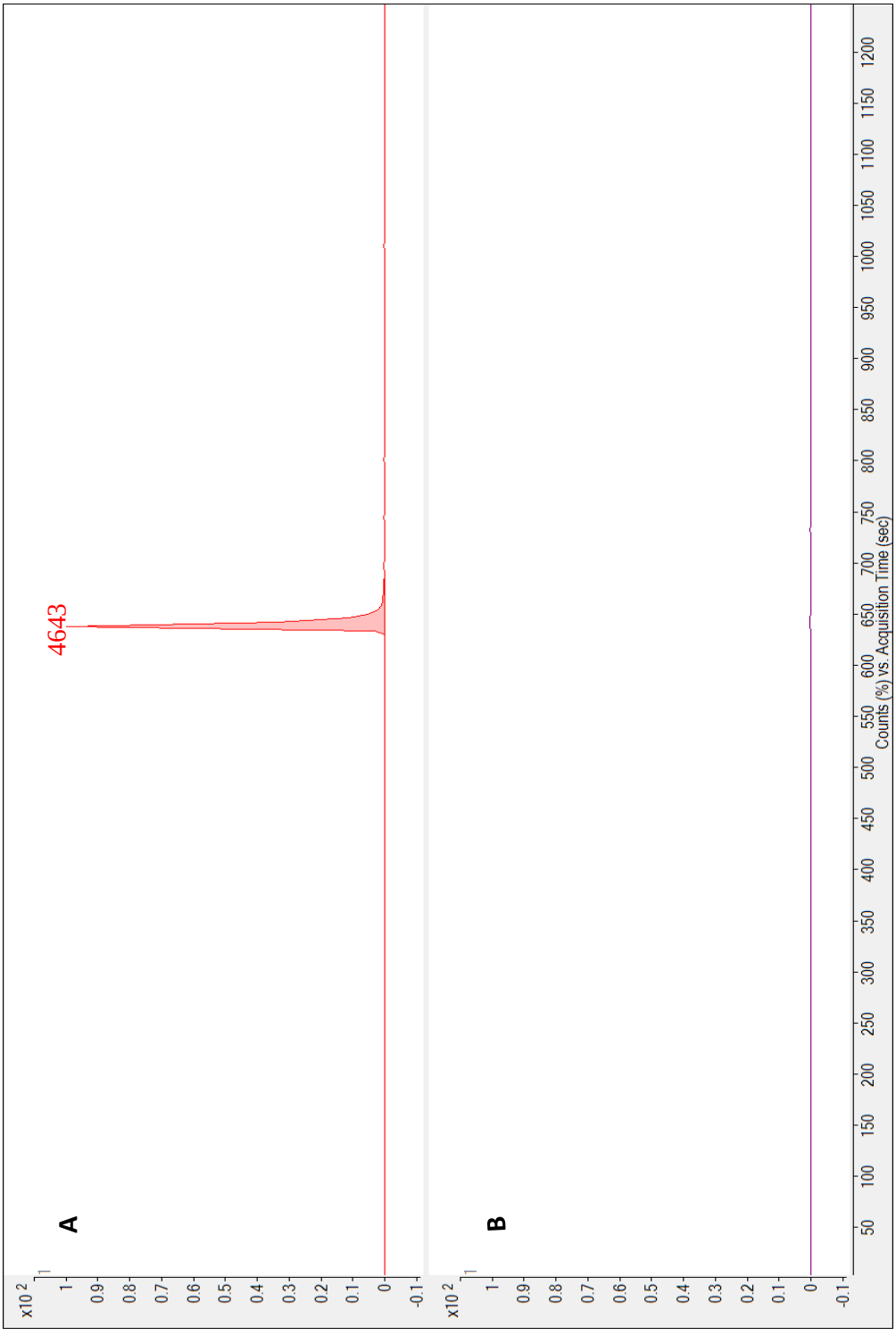


Figure 28: An extracted chromatogram of MBT. A) Before SBP treatment; B) After SBP treatment. Treatment with SBP resulted in a complete degradation of MBT

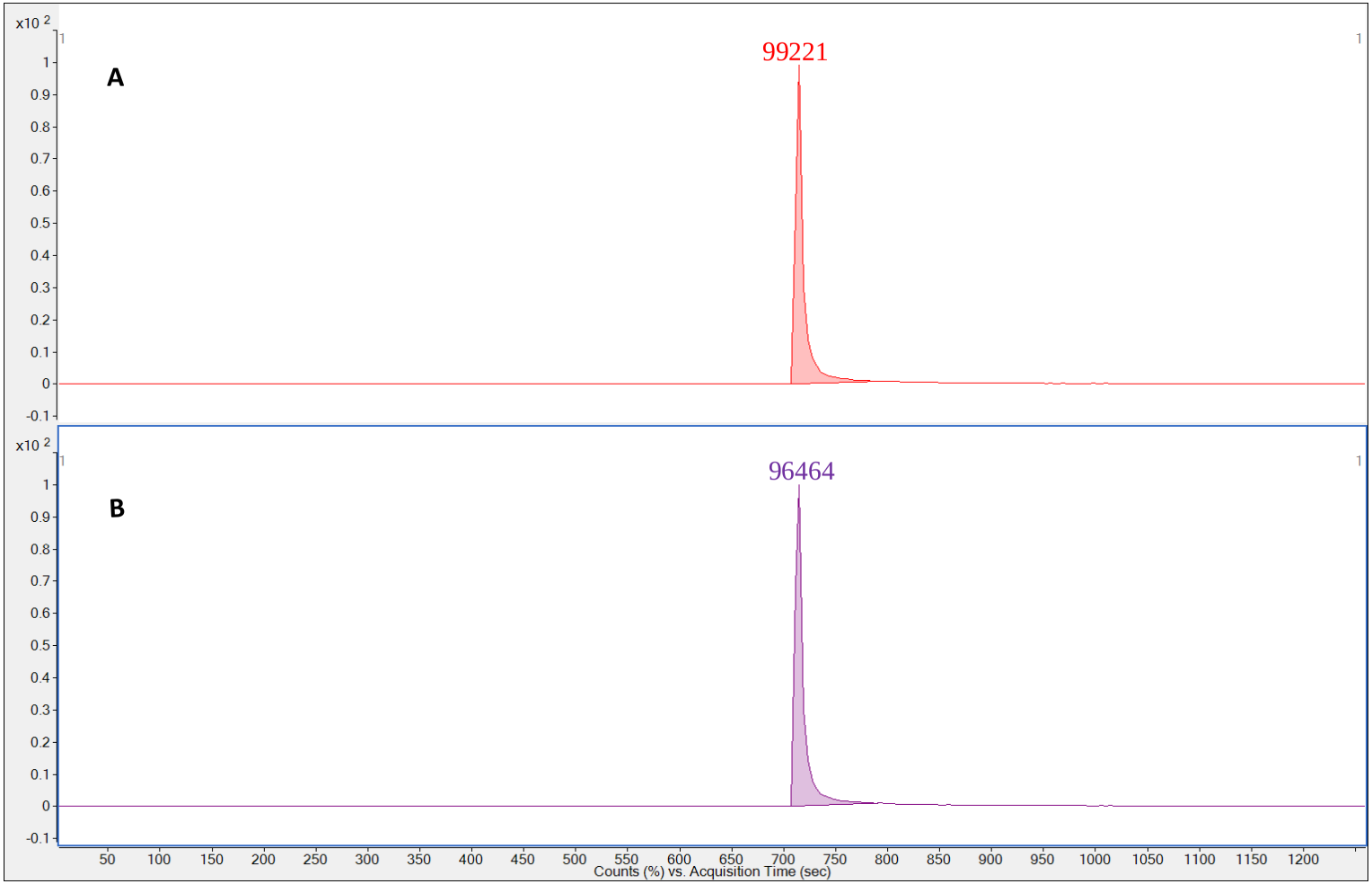


Figure 29: An extracted chromatogram of DEET. A) Before SBP treatment; B) After SBP treatment. SBP could not degrade DEET

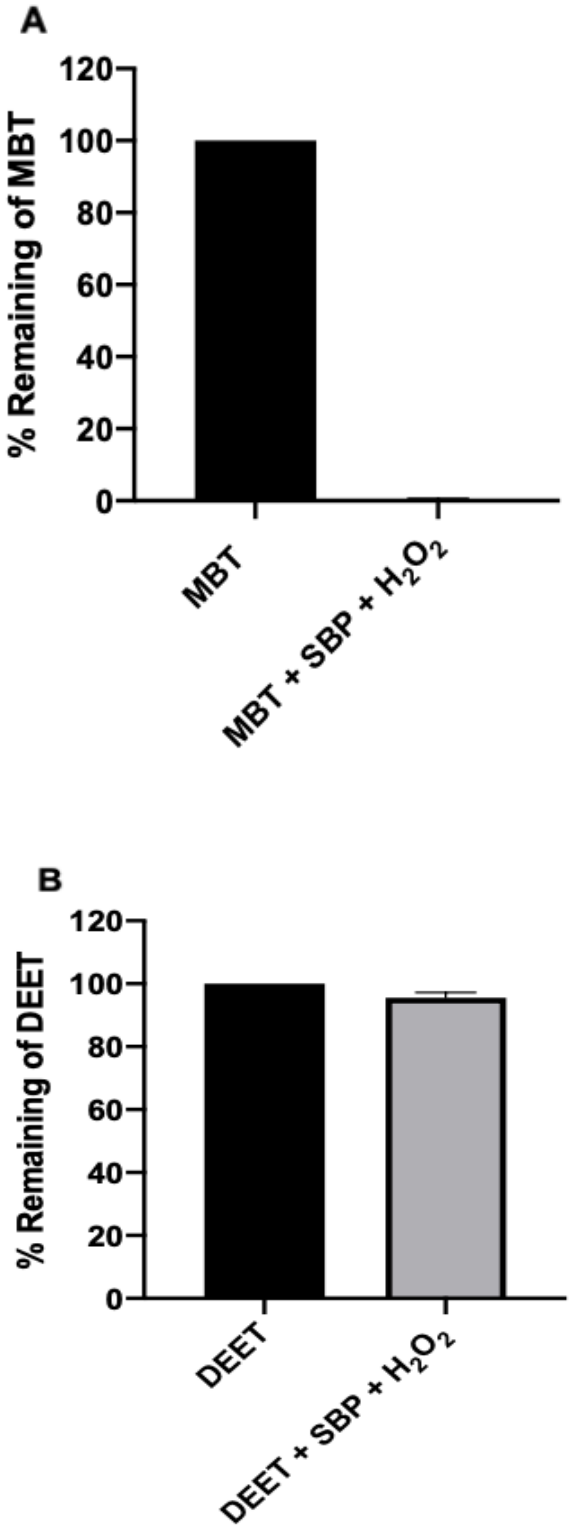


Figure 30: Percentage remaining of A) MBT; B) DEET before and after SBP treatment

Table 11: Percentage degradation of 21 EPs treated with SBP and HRP

	Emerging Pollutant	SBP		HRP	
		% Degradation	STD	% Degradation	STD
1	Roxithromycin	23.5	4.6	0.5	7.2
2	Lincomycin-HCl	2.6	7.9	0.0	0.0
3	Meloxicam	98.8	0.2	99.5	0.1
4	Norfloxacin	2.5	4.8	0.0	0.0
5	Trimethoprim	13.4	6.0	0.0	0.0
6	Venlafaxine-HCl	4.4	3.1	3.3	7.2
7	Atenolol	13.5	5.8	0.0	0.0
8	SMX	38.5	6.9	38.9	2.0
9	Cimetidine	2.1	7.8	0.3	4.8
10	Phenytoin	3.7	6.7	3.1	1.5
11	Prometryn	8.1	7.3	19.2	5.3
12	Fluometuron	1.8	4.6	0.0	0.0
13	Ibuprofen	16.6	5.3	13.2	7.9
14	Thiabendazole	0.0	0.0	3.8	3.7
15	MCPA	4.1	6.4	20	8.1
16	Caffeine	16.7	8.5	0.0	0.0
17	DEET	4.4	1.7	0.0	0.0
18	Caffeic acid	88.2	2.4	98.6	3.4
19	MBT	99.4	0.0	100.0	0.0
20	Furosemide	49.5	7.0	45.8	3.0
21	Hydrochlorothiazide	5.9	3.4	12.2	2.5

As we can see from Figure 28, the area under the peak for MBT was equal to 4659 before the addition of SBP and the peak disappeared after the addition of SBP. This means that SBP degraded MBT successfully. On the other hand, the amount of DEET before SBP treatment was equal to 99221 and after the treatment it was equal to 96464. As we can see, there is not a big difference between the two numbers which indicates that SBP cannot be used for the degradation of DEET. Figure 30 shows this more clearly, so if we consider that we have 100% of DEET before the treatment, after the treatment the amount of DEET remained is around 98% which means there is no degradation.

Based on Table 11, we can say that the effect of both enzymes (SBP and HRP) is very similar on the pollutants. Both SBP and HRP were able to efficiently degrade more than 90% of meloxicam, caffeic acid and MBT. Moreover, both enzymes degraded SMX and furosemide partially (from 38% of degradation to 50%). The only difference between them is that SBP was able to degrade 23.5% of roxithromycin but HRP did not. On the other hand, HRP was able to degrade 20% of MCPA but SBP did not.

3.2.1 Requirement of redox mediator

In some cases, oxidoreductase enzymes cannot degrade a certain organic compound due to its nature unless a low-molecular weight compound known as a redox mediator is presented in the reaction. Redox mediators are reactive diffusible chemicals that are believed to enhance the enzymatic-based reactions by increasing the reaction rate and the range of compounds that can be degraded by the oxidoreductase enzymes (Adelaja, Keshavarz, & Kyazze, 2015; Alneyadi et al., 2018). As mentioned earlier in this thesis, peroxidases produce cationic radicals that are used for the degradation of EPs. Redox mediators can produce secondary cationic radicals

which enhances the degradation of the pollutant (Husain & Husain, 2008). Examples of redox mediators that are most commonly used include 1-hydroxybenzotriazole (HOBT), veratryl alcohol, violuric acid and 2- methoxyphenothiazone. Various studies reported the ability of redox mediator in enhancing the degradation of different pollutants. For example, a study that was conducted by (Rauf & Salman Ashraf, 2012) reported the inability of SBP/H_2O_2 alone to degrade Rhodamine B dye, but when they added HOBT as a redox mediator to the reaction, the dye was almost completely degraded. It is worth mentioning that redox mediators can act as substrates for peroxidases and compete with the pollutant, therefore affecting its degradation negatively (Alneyadi & Ashraf, 2016). In our study, we tested the effect of using HOBT which is a redox mediator on the degradation of the 21 EPs.

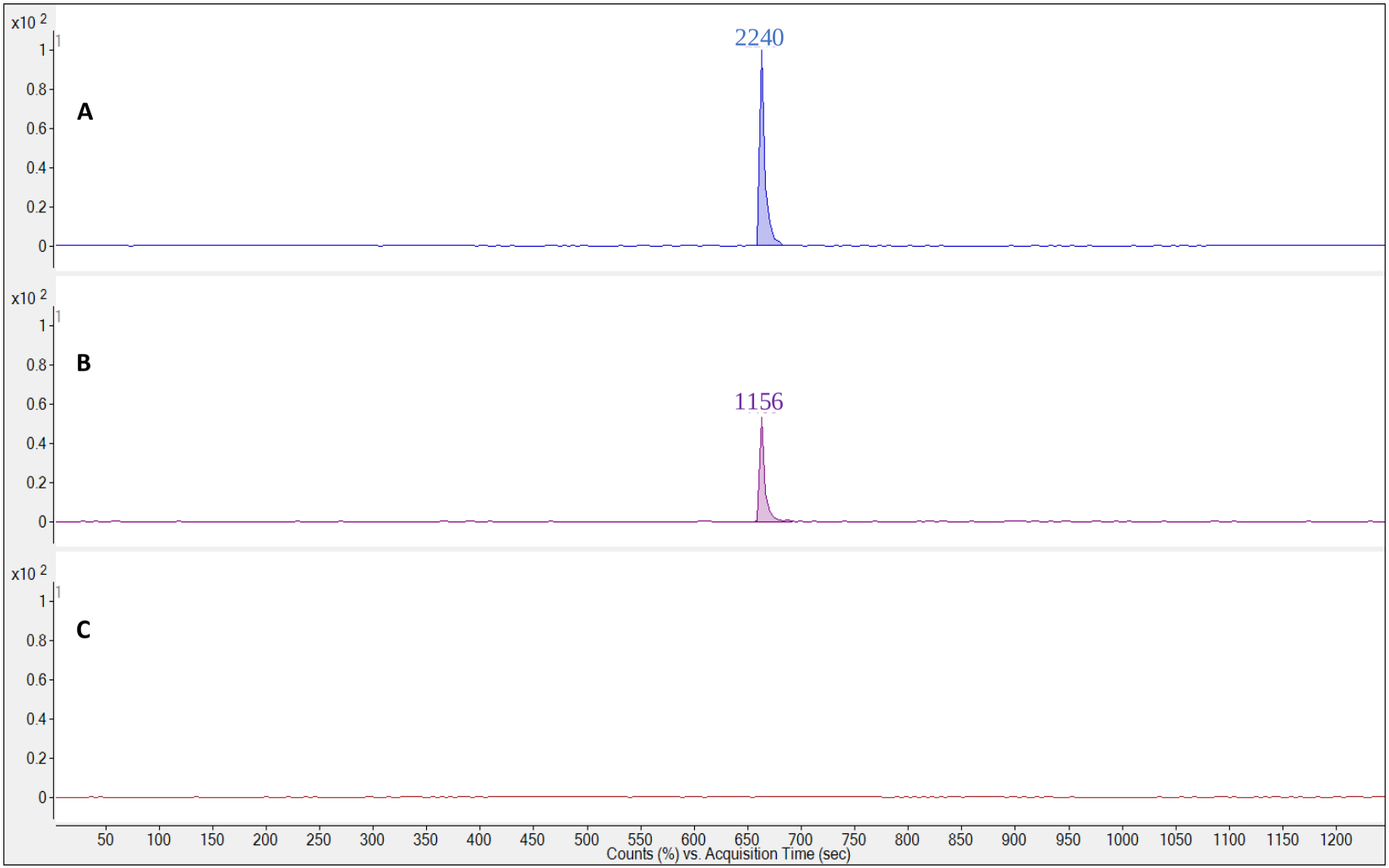


Figure 31: An extracted chromatogram of furosemide. A) Before SBP treatment; B) with SBP + H_2O_2; C) with SBP + H_2O_2 + HOBT. The presence of redox mediator resulted in a complete degradation of furosemide

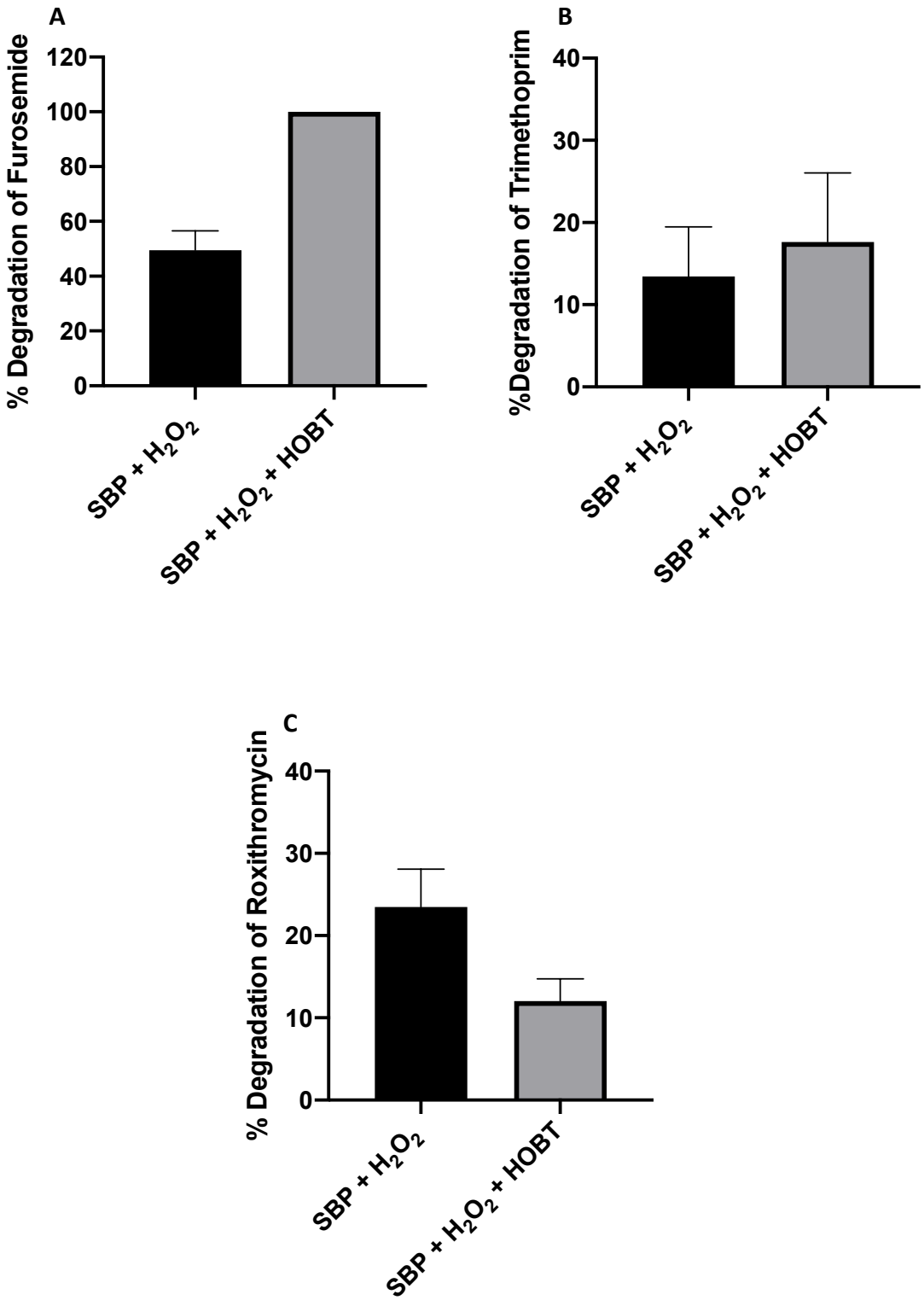


Figure 32: Percentage degradation of A) Furosemide; B) Trimethoprim; and C) Roxithromycin in the presence and absence of the redox mediator (HOBT)

The presence of a redox mediator in the reaction may affect the reaction in different ways; it can enhance the degradation of the pollutant, lowers the degradation efficiency of the pollutant, or has no effect on the reaction. Figure 31 and Figure 32A; both represent the degradation of furosemide by SBP alone and SBP with HOBT. As we can see from the figures, the presence of redox mediator enhanced the degradation of the pollutant. SBP alone was able to degrade around 50% of furosemide, but when HOBT was added to the reaction, the percentage degradation increased to reach 100%, which means the redox mediator resulted in a complete degradation of furosemide. On the other hand, if we look into the results obtained for trimethoprim (Figure 32B), we can see that the presence of HOBT had almost no effect on the reaction as % degradation of trimethoprim with and without HOBT are almost similar. Lastly, the results of roxithromycin shows the inhibition effect of HOBT (Figure 32C). When HOBT was not added to the reaction, 23.5% of roxithromycin was degraded, but when HOBT was added this percentage decreased to 12%. This means that HOBT caused an inhibition of degradation. Table 12 and Table 13 summarize all the data obtained from treating the 21 EPs with SBP and HRP with and without HOBT.

Table 12: Percentage degradation of 21 EPs treated with SBP and SBP+HOBT

	Emerging Pollutant	SBP		SBP + HOBT	
		% Degradation	STD	% Degradation	STD
1	Roxithromycin	23.5	4.6	12.0	2.7
2	Lincomycin-HCl	2.6	7.9	5.8	8.1
3	Meloxicam	98.8	0.2	99.0	0.3
4	Norfloxacin	2.5	4.8	5.5	3.6
5	Trimethoprim	13.4	6.0	17.6	8.4
6	Venlafaxine-HCl	4.4	3.1	3.8	2.4
7	Atenolol	13.5	5.8	6.9	9.5
8	SMX	38.5	6.9	99.3	0.3
9	Cimetidine	2.1	7.8	8.7	7.7
10	Phenytoin	3.7	6.7	0.0	4.2
11	Prometryn	8.1	7.3	4.3	5.6
12	Fluometuron	1.8	4.6	2.5	4.5
13	Ibuprofen	16.6	5.3	13.8	8.9
14	Thiabendazole	0.0	0.0	4.3	3.9
15	MCPA	4.1	6.4	6.1	6.3
16	Caffeine	16.7	8.5	21.8	1.3
17	DEET	4.4	1.7	5.4	1.4
18	Caffeic acid	88.2	2.4	100.0	0.0
19	MBT	99.4	0.0	98.7	0.3
20	Furosemide	49.5	7.0	100.0	0.0
21	Hydrochlorothiazide	5.9	3.4	5.5	5.3

Table 13: Percentage degradation of 21 EPs treated with HRP and HRP+HOBT

	Emerging Pollutant	HRP		HRP + HOBT	
		% Degradation	STD	% Degradation	STD
1	Roxithromycin	0.5	7.2	0.0	0.0
2	Lincomycin-HCl	0.0	0.0	36.9	8.1
3	Meloxicam	99.5	0.1	99.6	0.1
4	Norfloxacin	0.0	0.0	12.7	4.1
5	Trimethoprim	0.0	0.0	21.5	4.7
6	Venlafaxine-HCl	3.3	7.2	20.1	8.8
7	Atenolol	0.0	0.0	3.1	3.7
8	SMX	38.9	2.0	99.0	0.1
9	Cimetidine	0.3	4.8	9.0	3.3
10	Phenytoin	3.1	1.5	2.6	3.7
11	Prometryn	19.2	5.3	13.4	6.2
12	Fluometuron	0.0	0.0	17.1	4.0
13	Ibuprofen	13.2	7.9	12.1	4.0
14	Thiabendazole	3.8	3.7	13.2	2.3
15	MCPA	20	8.1	17.4	8.7
16	Caffeine	0.0	0.0	14.9	3.6
17	DEET	0.0	0.0	6.0	2.5
18	Caffeic acid	98.6	3.4	100.0	0.0
19	MBT	100.0	0.0	100.0	0.0
20	Furosemide	45.8	3.0	100.0	0.0
21	Hydrochlorothiazide	12.2	2.5	24.8	4.2

Based on Table 12, we can see that there is a dramatic increase in the percentage of degradation of SMX and furosemide when HOBT was added to the reaction. SBP alone was able to degrade 38.5% of SMX, but after the addition of a redox mediator to the reaction, the degradation was improved to reach 99.3%. In other pollutants, we can see that HOBT slightly enhanced the degradation of the pollutant such as caffeic acid. SBP alone degraded 88.2% of caffeic acid, and the presence of HOBT resulted in a small enhancement as this percentage increased to 100% which means HOBT resulted in a complete degradation of caffeic acid. On the other hand, the degradation of some pollutants was not affected by the presence of HOBT such as meloxicam, trimethoprim, and MBT. Lastly, the presence of HOBT had a negative effect on the degradation of two pollutants which are roxithromycin and atenolol as it competed with the pollutants to bind to the enzyme, thus causing an inhibition of degradation. In both pollutants, we can see that the % of degradation without HOBT is better than the % of degradation when HOBT was presented in the reaction.

Almost same things were observed in the case of using HRP instead of SBP but with slight differences. As we can see from Table 13, the presence of HOBT dramatically enhanced the degradation of SMX and furosemide. HOBT had no effect on the degradation of meloxicam, caffeic acid and MBT. Interesting results obtained for lincomycin-HCl, trimethoprim and venalafaxine-HCl as HRP alone could not degrade any of them, but the addition of HOBT resulted in some degradation with percentages ranging from 20-37%. Although these percentages are not that good, but it gives a hope in obtaining better results if we do some modifications to the reaction such as increasing the reaction time.

3.3 Development of immobilized biocatalysts

As it is well-known, one of the major drawbacks of using enzymes is their cost and lack of re-usability. Many groups have suggested different immobilization strategies in order to overcome the drawbacks of using enzymes. In our work, we covalently immobilized SBP and HRP on two different photocatalytic supports titanium dioxide (TiO_2) and zinc oxide (ZnO) to produce four hybrid bio-composites; SBP-TiO_2, SBP-ZnO, HRP-TiO_2, and HRP-ZnO. Then these composites were used to test their ability in degrading the 21 EPs that are previously mentioned. Immobilizing enzymes allows the re-usability of the enzyme, therefore, an overall lower cost. Moreover, when enzymes are used for treatment processes, enzymatic catalysis is the only reaction that lead to the degradation of the pollutants. In our case, when an enzyme is immobilized on a photocatalytic support, in addition to the enzymatic catalysis, there will be a photocatalysis and photolysis (Figure 33), so the immobilization process combines the power of a photocatalyst and peroxidase. All the three reactions will produce radicals that can degrade the pollutants into smaller intermediates. Therefore, using immobilized enzymes may result in a better degradation of contaminants.

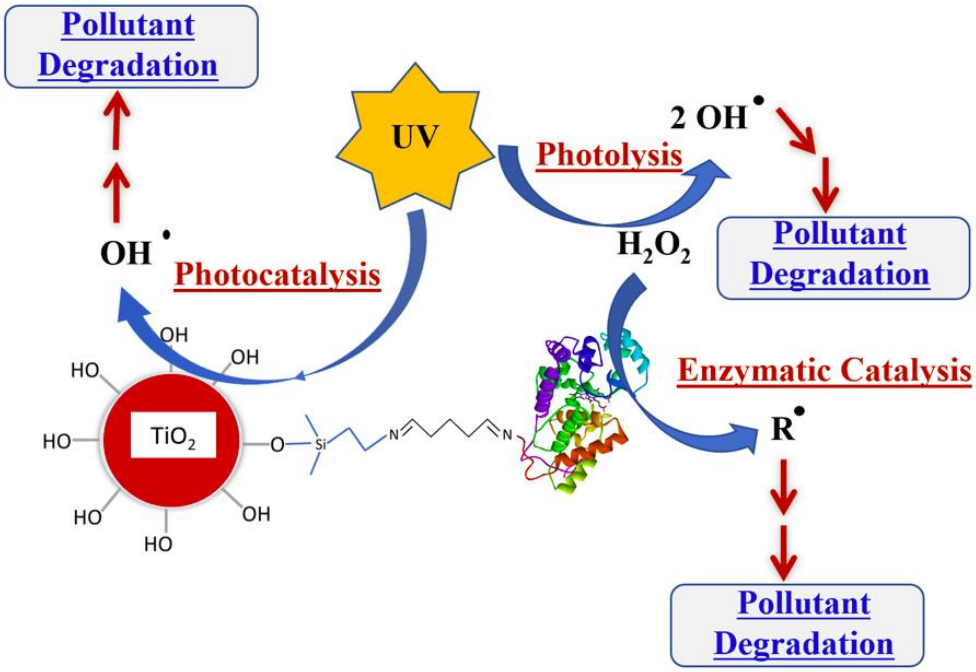


Figure 33: A schematic representation of a hybrid biocomposite

3.3.1 Preparing a hybrid biocatalyst

The main idea is functionalizing the surface of the photocatalysts, then adding a linker that links the functionalized photocatalyst at one end and the enzyme on the other end (Figure 34). At the beginning, we functionalized our photocatalysts (TiO_2 and ZnO) with (3-Aminopropyl)triethoxysilane (APTES). The functionalized photocatalysts had an amino group on their ends. Then we added a glutaraldehyde as a spacer that linked to the functionalized TiO_2 for example at one end. Then the enzyme was added to the reaction where it was linked to the other end of the glutaraldehyde from its amino group. Figure 35 shows the process of immobilization that has been done by Donadelli and his colleagues (2018), but in their case, they used iron oxide as the support (Donadelli et al., 2018). We followed their methodology to immobilize our enzymes on the photocatalysts. The detailed mechanism is explained within the materials and methods chapter.

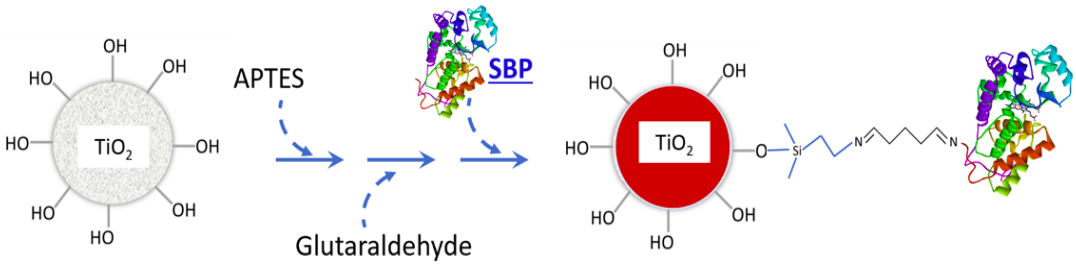


Figure 34: Preparing a hybrid biocomposite

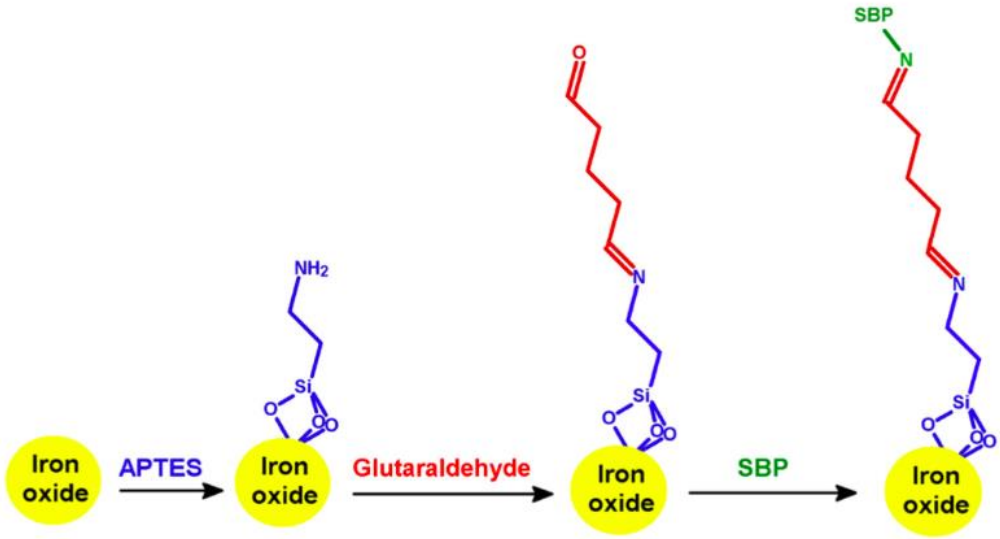


Figure 35: Synthesis scheme for the immobilization of SBP on iron oxide (Donadelli et al., 2018)

3.3.2 Characterization of the hybrid biocatalyst

3.3.2.1 Thermal and pH stability of the hybrid biocatalyst

In order to check the stability of our hybrid biocatalysts (SBP-TiO_2, SBP-ZnO, HRP-TiO_2, and HRP-ZnO), we tested their activities on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate over a range of different pH (from pH=2 to pH=9) and temperatures (from 30^{o}C to 90^{o}C). Then we compared their pH profiles and temperature profiles to the pH and temperature profiles of the free enzymes. As we can see from Figure 36 which shows the pH profile of immobilized SBP on TiO_2 and pH profile of free SBP, the pH optima for free SBP is 5 and the immobilization process caused a slight shift in the pH optima to 4. However, immobilized SBP appears to be active over a wider pH range compared to free SBP. Figure 37 shows the temperature profiles of free SBP and immobilized SBP on TiO_2. Both free and immobilized SBP appear to have almost similar thermal stabilities (Free SBP is slightly more active compared to immobilized SBP).

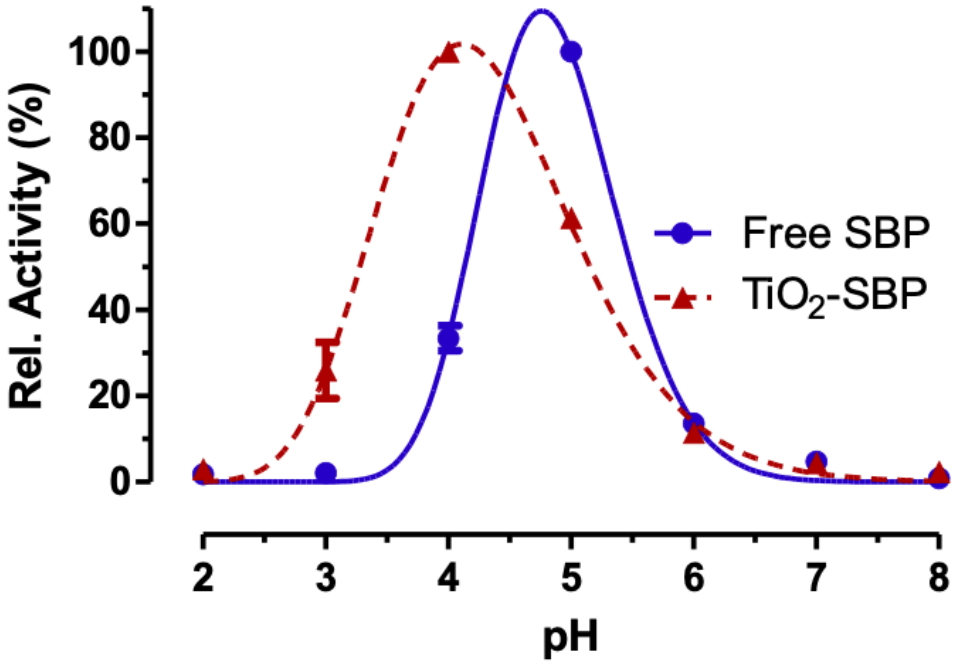


Figure 37: pH profiles of free SBP vs. immobilized SBP on TiO_2. Both show pH stability

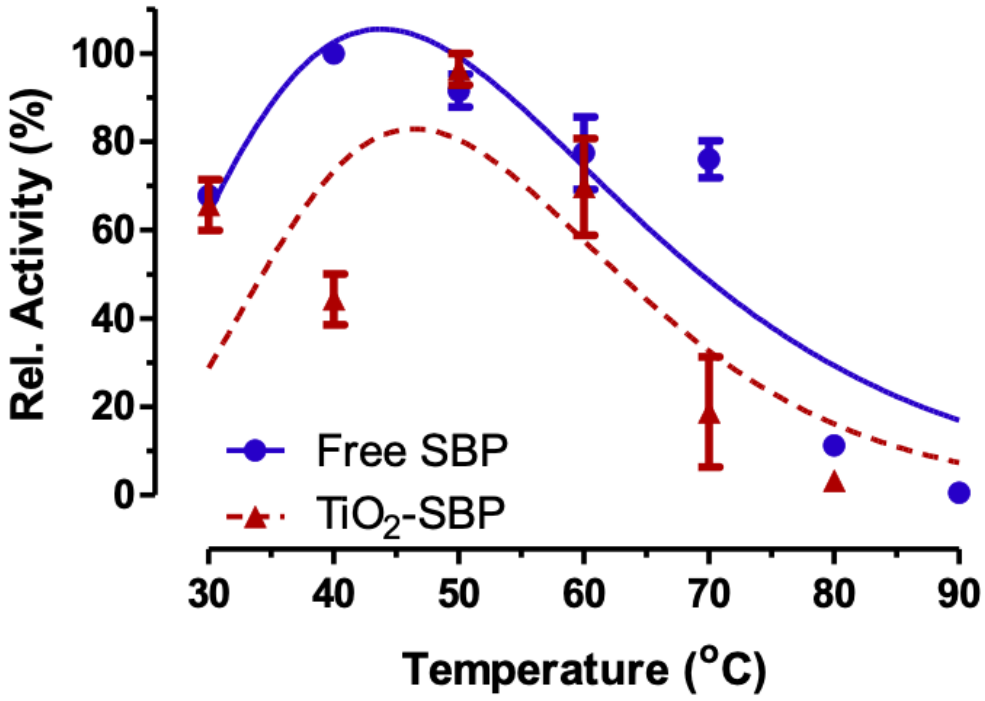


Figure 36: Temperature profiles of free SBP vs. immobilized SBP on TiO_2. Both show thermal stability

3.3.2.2 Scanning electron microscope

Scanning electron microscope (SEM) had been conducted for all the four hybrid biocatalysts, in addition to pure TiO_2 and ZnO, as well as the functionalized TiO_2 and ZnO with 3-Aminopropyltriethoxysilane (APTES) at x10000 magnification. SEM images were used to check whether the functionalization of the photocatalysts or the immobilization process caused any change in the morphology of the particles.

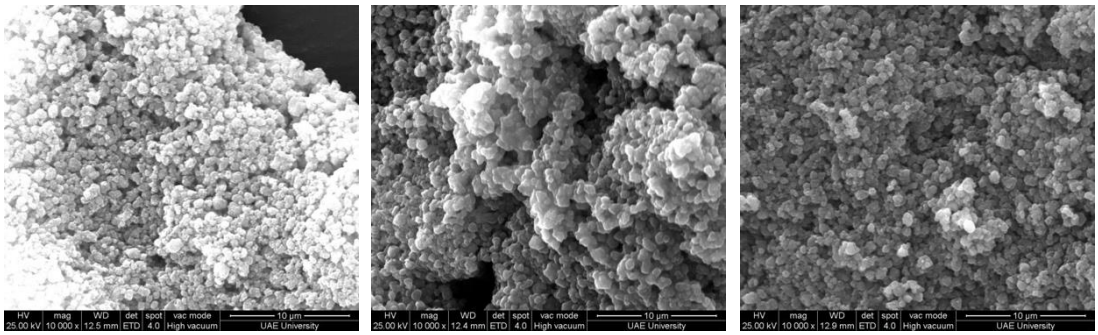


Figure 38: SEM images for A) TiO_2; B) TiO_2-APTES; and C) TiO_2-SBP at x10000 magnification

Figure 38 shows the SEM images for SBP, SBP-APTES and SBP-TiO_2 at x10000 magnification. As we can see from the figure, the particles have agglomerated sphere like morphology and all particles are uniform in their size. Both functionalization and immobilization did not affect the morphology of the particles. Same results were obtained for all the others.

3.3.2.3 X-ray diffraction

X-ray diffraction (XRD) had been done for pure TiO_2 and ZnO, functionalized TiO_2 and ZnO (TiO_2-APTES and ZnO-APTES) and for the four hybrid biocatalysts (SBP-TiO_2, SBP-ZnO, HRP-TiO_2 and HRP ZnO) in order to see if the functionalization and immobilization processes caused any changes in crystallinity of the photocatalysts. Figure 39 displays the XRD graphs for TiO_2, TiO_2-APTES, SBP-TiO_2 and HRP-TiO_2. Based on the patterns observed for pure TiO_2, the anatase TiO_2 phase is presented. As we can see from the graphs, the peaks for functionalized TiO_2 with APTES coincide with those of the pure TiO_2. Also, the peaks for SBP-TiO_2 and HRP-TiO_2 matches those of the pure TiO_2. This indicates that both the functionalization of TiO_2 with APTES and the immobilization of enzymes onto the TiO_2 did not change its crystal structure. The same results were obtained for ZnO, ZnO-APTES, TiO_2-ZnO and HRP-ZnO.

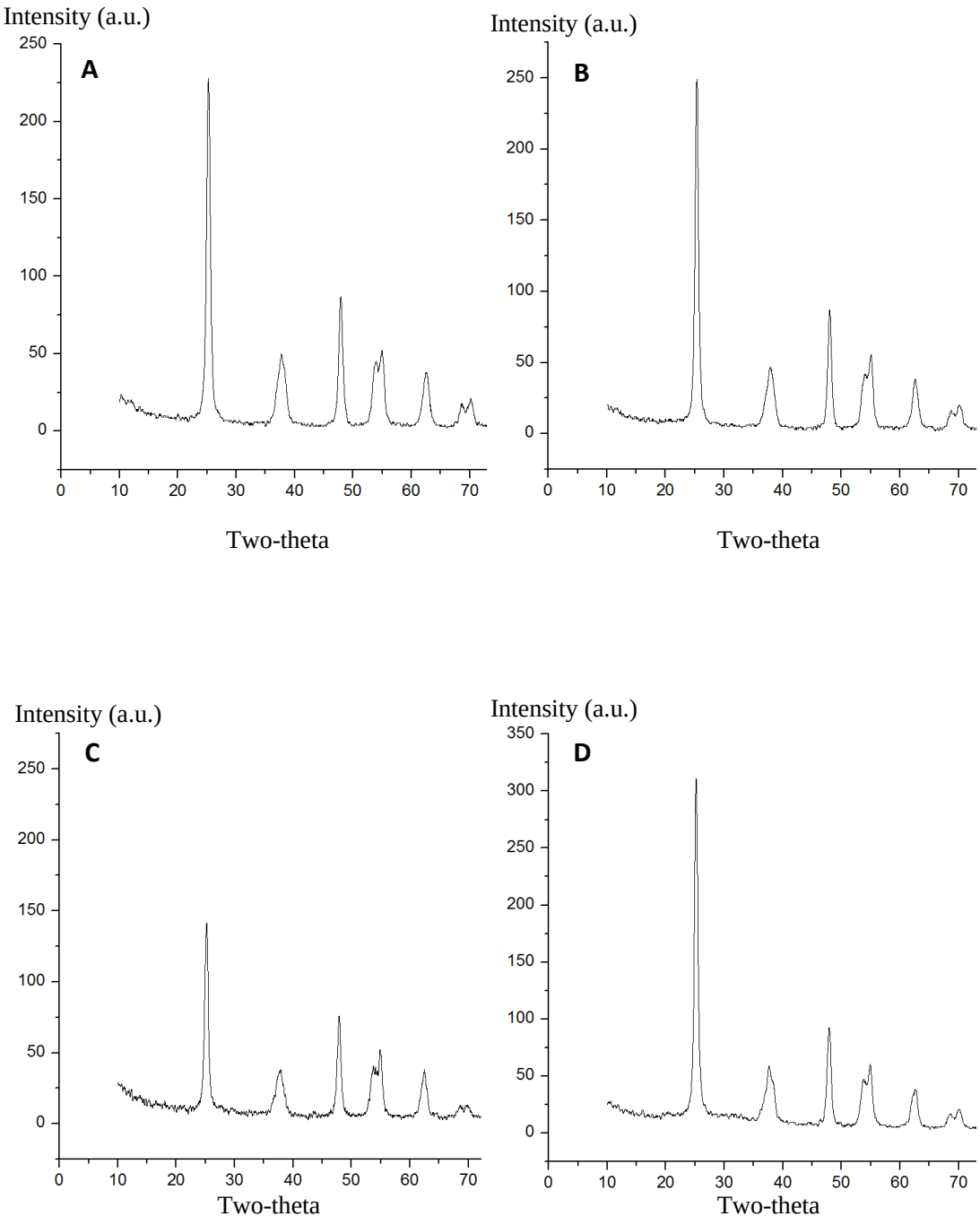


Figure 39: XRD graphs for A) pure TiO_2; B) TiO_2-APTES; C) TiO_2-SBP; and D) TiO_2-HRP

3.4 Degradation of 21 EPs by immobilized enzymes

The degradation of 21 EPs that were mentioned previously were tested using immobilized SBP on TiO_2 and ZnO and immobilized HRP on TiO_2 and ZnO with and without the redox mediator HOBT. The following tables summarize the data obtained when a mixture of 21 EPs was treated with the four hybrid biocatalysts. Any percentage of degradation of any of the pollutants that ranges from 20% to 50% was highlighted with a grey color, any percentage of degradation ranging from 50% to 80% was highlighted with a green color, and any percentage of degradation ranging from 80% and above was highlighted with a yellow color.

Table 14: Percentage degradation of 21 EPs treated with SBP-TiO_2 and SBP-TiO_2+HOBT

	Emerging Pollutant	SBP-TiO_2		SBP-TiO_2 + HOBT	
		% Degradation	STD	% Degradation	STD
1	Roxithromycin	24.4	7.3	17.7	3.4
2	Lincomycin-HCl	2.8	1.4	15.1	4.9
3	Meloxicam	26.8	4.4	99.8	0.1
4	Norfloxacin	45.1	1.3	0.0	0.0
5	Trimethoprim	3.9	3.8	7.0	3.5
6	Venlafaxine-HCl	6.2	2.5	6.5	5.5
7	Atenolol	0.0	0.0	0.2	4.2
8	SMX	9.7	1.5	37.9	3.2
9	Cimetidine	24.4	2.0	19.3	2.5
10	Phenytoin	3.2	1.9	7.4	2.5
11	Prometryn	9.4	5.0	9.2	5.4
12	Fluometuron	5.7	8.9	3.0	9.6
13	Ibuprofen	32.2	8.0	39.5	5.3
14	Thiabendazole	0.2	2.8	4.5	3.2
15	MCPA	3.1	3.8	11.5	7.1
16	Caffeine	60.2	4.1	57.2	4.6
17	DEET	3.7	2.8	17.6	2.8
18	Caffeic acid	100.0	0.0	100.0	0.0
19	MBT	100.0	0.0	100.0	0.0
20	Furosemide	15.7	5.5	100.0	0.0
21	Hydrochlorothiazide	0.0	0.0	14.3	7.5

Table 15: Percentage degradation of 21 EPs treated with SBP-ZnO and SBP-ZnO+HOBT

	Emerging Pollutant	SBP-ZnO		SBP-ZnO + HOBT	
		% Degradation	STD	% Degradation	STD
1	Roxithromycin	48.6	4.9	37.4	8.6
2	Lincomycin-HCl	5.6	3.6	14.9	3.5
3	Meloxicam	11.4	6.9	100.0	0.0
4	Norfloxacin	5.5	3.0	5.5	1.7
5	Trimethoprim	13.8	9.4	17.8	1.2
6	Venlafaxine-HCl	7.3	5.7	11.0	4.5
7	Atenolol	0.0	0.0	9.5	4.0
8	SMX	12.5	2.4	31.9	4.1
9	Cimetidine	4.7	1.0	11.3	4.4
10	Phenytoin	13.4	3.3	10.3	2.8
11	Prometryn	5.6	1.9	4.0	3.9
12	Fluometuron	2.8	6.8	2.2	7.1
13	Ibuprofen	13.1	6.4	22.2	8.8
14	Thiabendazole	4.8	1.1	6.5	1.4
15	MCPA	18.7	7.8	1.6	9.8
16	Caffeine	0.2	9.6	1.5	7.4
17	DEET	3.0	5.5	14.4	2.2
18	Caffeic acid	54.4	3.8	100.0	0.0
19	MBT	100.0	0.0	100.0	0.0
20	Furosemide	2.6	7.1	100.0	0.0
21	Hydrochlorothiazide	19.0	1.5	25.8	3.7

Table 14 shows the results obtained when SBP-TiO_2 was used for the treatment of 21 EPs. By looking into the result of using SBP-TiO_2 alone (without redox mediator), we can see that it degraded caffeic acid and MBT completely in only 30 minutes. Moreover, it was able to efficiently degrade 60.2% of caffeine. Furthermore, it was able to degrade roxithromycin, meloxicam, norfloxacin, cimetidine and ibuprofen partially with percentage degradation ranging from 24% to 45%. The addition of HOBT enhanced the degradation of some pollutants. For example, it resulted in a complete degradation of furosemide which was not degraded by the hybrid biocatalyst alone. Also, it had a wonderful effect on meloxicam, as only 26.8% of meloxicam was degraded by SBP-TiO_2, but after the addition of HOBT this percentage increased to 99.8%. The degradation of both SMX and ibuprofen was improved as well when the redox mediator was added to the reaction. On the other hand, the degradation of some pollutants was decreased when HOBT was added to the reaction such as roxithromycin, cimetidine and caffeine, which means that the redox mediator inhibited their degradation.

Table 15 shows the results obtained when SBP-ZnO was used for the degradation of 21 EPs. SBP-ZnO alone was able to completely degrade MBT. Also, it degraded 48.6% of roxithromycin and 54.4% of caffeic acid. Addition of HOBT enhanced the degradation of caffeic acid which resulted in a 100% degradation of it. Although SBP-ZnO alone was not able to degrade any of furosemide and meloxicam, the addition of HOBT resulted in their complete degradation. In addition to that, atenolol, ibuprofen and hydrochororthiazide had better degradation percentages due to the addition of HOBT. However, an inhibition of degradation of roxithromycin had occurred due to the addition of the redox mediator. Some pollutants such as phenytoin and norfloxacin were not affected at all by the addition of HOBT.

Table 16: Percentage degradation of 21 EPs treated with HRP-TiO_2 and HRP-TiO_2+HOBT

	Emerging Pollutant	HRP-TiO_2		HRP-TiO_2 + HOBT	
		% Degradation	STD	% Degradation	STD
1	Roxithromycin	43.8	9.9	4.6	3.8
2	Lincomycin-HCl	9.5	3.5	4.4	4.7
3	Meloxicam	15.1	9.8	0.0	0.0
4	Norfloxacin	7.7	5.7	11.7	5.9
5	Trimethoprim	5.2	3.4	8.7	2.2
6	Venlafaxine-HCl	6.5	2.3	12.7	5.2
7	Atenolol	1.6	8.7	14.6	9.6
8	SMX	10.4	4.3	12.2	1.2
9	Cimetidine	60.1	3.8	66.7	3.4
10	Phenytoin	9.0	4.4	9.8	6.8
11	Prometryn	14.7	5.4	9.2	0.2
12	Fluometuron	0.0	0.0	1.9	5.0
13	Ibuprofen	0.0	0.0	16.7	4.9
14	Thiabendazole	4.4	3.2	8.1	2.5
15	MCPA	3.6	4.7	15.9	3.9
16	Caffeine	24.1	7.2	8.5	9.7
17	DEET	8.8	2.7	17.8	1.8
18	Caffeic acid	81.7	1.4	0.0	0.0
19	MBT	46.2	0.9	55.1	6.7
20	Furosemide	16.2	3.1	16.8	3.9
21	Hydrochlorothiazide	0.0	0.0	8.7	4.4

Table 17: Percentage degradation of 21 EPs treated with HRP-ZnO and HRP-ZnO+HOBT

	Emerging Pollutant	HRP-ZnO		HRP-ZnO+ HOBT	
		% Degradation	STD	% Degradation	STD
1	Roxithromycin	58.2	7.8	55.4	4.4
2	Lincomycin-HCl	5.8	7.4	9.0	6.6
3	Meloxicam	5.6	7.7	13.7	2.5
4	Norfloxacin	10.9	5.4	13.3	5.1
5	Trimethoprim	3.3	2.4	3.2	4.4
6	Venlafaxine-HCl	3.8	5.5	5.2	2.2
7	Atenolol	6.1	5.2	7.2	3.3
8	SMX	7.7	1.0	8.8	2.5
9	Cimetidine	5.3	4.2	10.3	0.6
10	Phenytoin	0.0	4.3	6.0	3.4
11	Prometryn	9.8	2.2	11.7	3.3
12	Fluometuron	2.6	8.1	10.7	7.1
13	Ibuprofen	11.6	8.1	18.0	4.9
14	Thiabendazole	4.3	4.4	3.9	2.7
15	MCPA	0.0	0.0	2.8	5.3
16	Caffeine	32.7	6.2	30.6	5.9
17	DEET	3.6	0.9	14.1	1.7
18	Caffeic acid	30.2	5.5	100.0	0.0
19	MBT	86.0	2.5	90.0	3.3
20	Furosemide	9.1	1.6	8.4	7.9
21	Hydrochlorothiazide	2.2	3.0	21.4	7.1

By looking into the results obtained when HRP-TiO_2 was used for the degradation of 21 EPs (Table 16), we can see that HRP-TiO_2 alone successfully degraded 60.1% of cimetidine and 81.7% of caffeic acid. Moderate degradation percentages were obtained for roxithromycin, caffeine and MBT. The addition of HOBT inhibited the degradation of some pollutants. For example, the degradation of caffeic acid was completely inhibited due to the addition of HOBT. Moreover, the addition of HOBT decreased the percentage of degradation of roxithromycin from 43.8% to 4.6%. The degradation of prometryn and caffeine were also inhibited by HOBT. On the other hand, HOBT enhanced the degradation of cimetidine as only 60.1% of cimetidine was degraded without HOBT and it increased to 66.7% with HOBT. Furthermore, a better degradation of MBT was seen due to the addition of HOBT (from 46.2% to 55.1%). The degradation of many pollutants was not affected by HOBT such as furosemide, phenytoin and norfloxacin.

Table 17 reports the results obtained from treating 21 EPs with HRP-ZnO. Using HRP-ZnO alone degraded MBT effectively with 86% of degradation. Also, it was able to degrade 58.2% of roxithromycin, 32.7% of caffeine and 30.2% of caffeic acid. When HOBT was added to the reaction, a complete degradation of caffeic acid was reported. Moreover, HOBT enhanced the degradation of MBT from 86% to 90%. Also, it resulted in 24.1% degradation of hydrochlorothiazide which was not degraded by the composite alone. Inhibition of degradation due to the addition of HOBT had been seen for roxithromycin and caffeine.

3.4.1 Effect of concentration and time on the degradation

In all our previous experiments, 2 ppm of pollutants were used in the reaction, but in real water samples, different concentrations will be encountered, therefore, we tested the ability of the hybrid biocatalysts in degrading higher concentration of pollutants. We chose one pollutant that was degraded by different composites efficiently and increased its concentration, then we tested the ability of the same amount used previously of those composites in degrading it. As Meloxicam was degraded efficiently by SBP-TiO_2 and SBP-ZnO, we tested the ability of these two hybrid biocatalysts in degrading higher concentration of meloxicam. The concentration of meloxicam was increased to 40 ppm and the ability of SBP-TiO_2 and SBP-ZnO to degrade it was tested. After 60 minutes, 36.7% of meloxicam remained while 63.3% of meloxicam was degraded by SBP-TiO_2. On the other hand, using SBP-ZnO degraded 57% of meloxicam as only 43% of meloxicam remained after the reaction. We wanted to get better degradation; therefore, we doubled the reaction time. After 120 minutes, SBP-TiO_2 degraded 98.4% of meloxicam (1.6% remaining), but SBP-ZnO degraded 79.4% of meloxicam (20.6% remaining). Doubling the reaction time resulted in almost complete degradation of 40 ppm meloxicam using SBP-TiO_2. In order to get better results for SBP-ZnO, we increased the reaction time to 180 minutes, after that time, only 6.3% of meloxicam remained, which means 93.7% of meloxicam was degraded. The results obtained suggested that low percentages of degradation of different pollutants can be improved by longer incubation time. Results obtained for SBP-TiO_2 are shown in Figure 40. Results obtained for SBP-ZnO are shown in Figure 41.

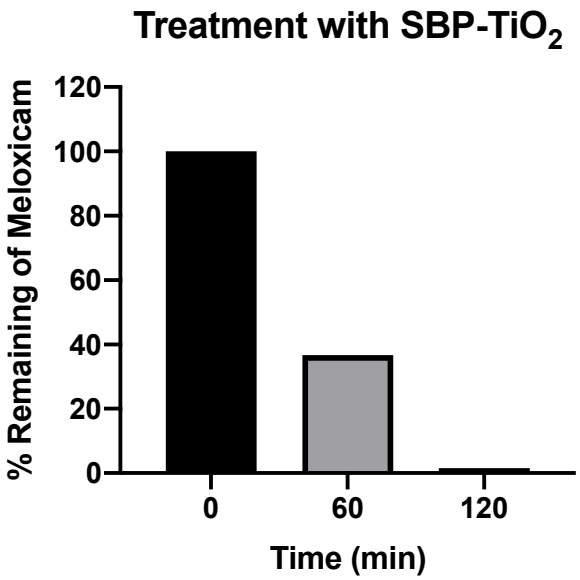


Figure 40: Percentage remaining of 40 ppm meloxicam with time using SBP-TiO_2 treatment

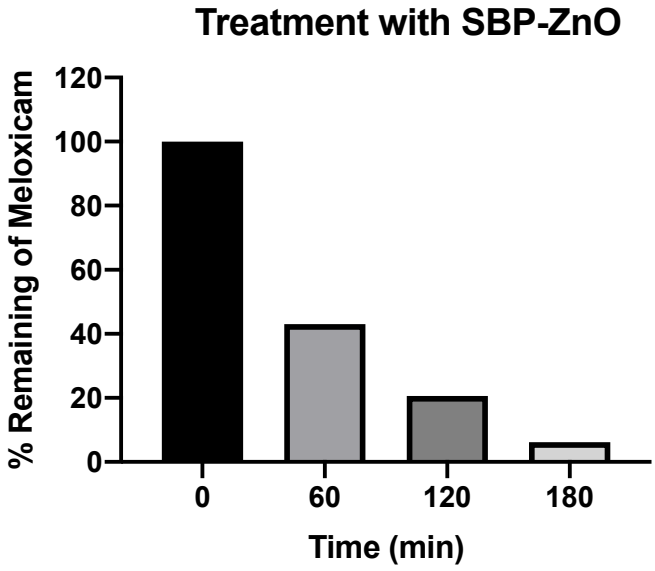


Figure 41: Percentage remaining of 40 ppm meloxicam with time using SBP-ZnO treatment

3.5 Comparison between free enzyme Vs. immobilized enzyme

This section focuses on the ability of immobilized enzymes compared to the un-immobilized enzymes in degrading the 21 emerging pollutants. Having better results with immobilized enzymes will give a hope for future applications in using

immobilized enzymes for the treatment of wastewater from emerging contaminants. The immobilization process allows the re-usability of enzymes; therefore, it is more cost-effective technique to be used for the treatment of wastewater. Table 18 summarizes all the data obtained when a mixture of 21 EPs was treated with free SBP and immobilized SBP composites (SBP-TiO_2 and SBP-ZnO). Table 19 shows all the results obtained when the 21 EPs were degraded using free HRP and immobilized HRP composites (HRP-TiO_2 and HRP-ZnO). To discuss the results obtained, we focused only on the pollutants that were degraded more than 20% either with the free enzymes or immobilized enzymes. Any percentage of degradation of any of the pollutants that was 20% or above was highlighted with a yellow color. In addition to that, for each degraded pollutant, the highest percentage of degradation was colored with a red color.

Table 18: Percentage of degradation of 21 EPs treated with free and immobilized SBP

	Emerging pollutant	SBP		SBP-TiO_2		SBP-ZnO	
		No HOBT	With HOBT	No HOBT	With HOBT	No HOBT	With HOBT
1	Roxithromycin	**23.5**	12.0	**24.4**	17.7	**48.6**	**37.4**
2	Lincomycin-HCl	2.6	5.8	2.8	15.1	5.6	14.9
3	Meloxicam	**98.8**	**99.0**	26.8	**99.8**	11.4	**100.0**
4	Norfloxacin	2.5	5.5	**45.1**	0.0	5.5	5.5
5	Trimethoprim	13.4	17.6	3.9	7.0	13.8	17.8
6	Venlafaxine-HCl	4.4	3.8	6.2	6.5	7.3	11.0
7	Atenolol	13.5	6.9	0.0	0.2	0.0	9.5
8	SMX	**38.5**	**99.3**	9.7	**37.9**	12.5	**31.9**
9	Cimetidine	2.1	8.7	**24.4**	19.3	4.7	11.3
10	Phenytoin	3.7	0.0	3.2	7.4	13.4	10.3

Table 19: Percentage of degradation of 21 EPs treated with free and immobilized SBP (Continued)

	Emerging pollutant	SBP		SBP-TiO_2		SBP-ZnO	
		With HOBT	No HOBT	With HOBT	With HOBT	No HOBT	With HOBT
11	Prometryn	8.1	4.3	9.4	9.2	5.6	4.0
12	Fluometuron	1.8	2.5	5.7	3.0	2.8	2.2
13	Ibuprofen	16.6	13.8	**32.2**	**39.5**	13.1	**22.2**
14	Thiabendazole	0.0	4.3	0.2	4.5	4.8	6.5
15	MCPA	4.1	6.1	3.1	11.5	18.7	1.6
16	Caffeine	16.7	**21.8**	**60.2**	**57.2**	0.2	1.5
17	DEET	4.4	5.4	3.7	17.6	3.0	14.4
18	Caffeic acid	**88.2**	**100.0**	**100.0**	**100.0**	**54.4**	**100.0**
19	MBT	**99.4**	**98.7**	**100.0**	**100.0**	**100.0**	**100.0**
20	Furosemide	**49.5**	**100.0**	15.7	**100.0**	2.6	**100.0**
21	Hydrochlorothiazide	5.9	5.5	0.0	14.3	19.0	**25.8**

To discuss the results of SBP and SBP hybrid biocatalysts, we focused only on the pollutants that were degraded more than 20% either with the free enzyme or immobilized enzyme. As we can see from Table 18, out of 21 EPs, only 11 EPs had percentages of degradation that are either equal to 20% or higher. The majority of these degraded pollutants were degraded with the immobilized SBP enzyme better than the free SBP enzyme. The following pollutants had better degradation percentages when immobilized SBP (either SBP-TiO_2 or SBP-ZnO) was used for their degradation; roxithromycin, meloxicam, norfloxacin, cimetidine, ibuprofen, caffeine, MBT and hydrochlorothiazide. In some of them, there was a big difference between immobilized

and un-immobilized SBP such as caffeine; free SBP degraded 21.8% of caffeine but SBP-TiO2 degraded 60.2% of caffeine which is 3 times better degradation. In others, there was not a big difference between immobilized and free SBP such as meloxicam; free SBP degraded 99% of meloxicam but both SBP-TiO_2 and SBP-ZnO degraded 100% of meloxicam. Some pollutants were only degraded by immobilized SBP, and free SBP was not able to degrade them such as norfloxacin, cimetidine, ibuprofen and hydrochlorothiazide. Some pollutants had similar degradation percentages whether an immobilized SBP or un-immobilized SBP were used such as caffeic acid and furosemide. Only SMX was better degraded by free SBP compared to immobilized SBP; 99.3% of SMX was degraded by free SBP while 32% and 38% of SMX was degraded using SBP-ZnO and SBP-TiO_2, respectively. Overall, we can say that using immobilized SBP is much better and more efficient in the degradation of emerging pollutants than using SBP alone.

Table 20: Degradation of 21 EPs treated with free and immobilized HRP

	Emerging pollutant	HRP		HRP-TiO_2		HRP-ZnO	
		No HOBT	With HOBT	No HOBT	With HOBT	No HOBT	With HOBT
1	Roxithromycin	0.5	0.0	**43.8**	4.6	**58.2**	**55.4**
2	Lincomycin-HCl	0.0	**36.9**	9.5	4.4	5.8	9.0
3	Meloxicam	**99.5**	**99.6**	15.1	0.0	5.6	13.7
4	Norfloxacin	0.0	12.7	7.7	11.7	10.9	13.3
5	Trimethoprim	0.0	**21.5**	5.2	8.7	3.3	3.2
6	Venlafaxine-HCl	3.3	**20.1**	6.5	12.7	3.8	5.2
7	Atenolol	0.0	3.1	1.6	14.6	6.1	7.2
8	SMX	**38.9**	**99.0**	10.4	12.2	7.7	8.8

Table 21: Degradation of 21 EPs treated with free and immobilized HRP (Continued)

	Emerging pollutant	HRP		HRP-TiO_2		HRP-ZnO	
		No HOBT	With HOBT	No HOBT		No HOBT	With HOBT
9	Cimetidine	0.3	9.0	**60.1**	**66.7**	5.3	10.3
10	Phenytoin	3.1	2.6	9.0	9.8	0.0	6.0
11	Prometryn	19.2	13.4	14.7	9.2	9.8	11.7
12	Fluometuron	0.0	17.1	0.0	1.9	2.6	10.7
13	Ibuprofen	13.2	12.1	0.0	16.7	11.6	18.0
14	Thiabendazole	3.8	13.2	4.4	8.1	4.3	3.9
15	MCPA	**20**	17.4	3.6	15.9	0.0	2.8
16	Caffeine	0.0	14.9	**24.1**	8.5	**32.7**	**30.6**
17	DEET	0.0	6.0	8.8	17.8	3.6	14.1
18	Caffeic acid	**98.6**	**100.0**	**81.7**	0.0	**30.2**	**100.0**
19	MBT	**100.0**	**100.0**	**46.2**	**55.1**	**86.0**	**90.0**
20	Furosemide	**45.8**	**100.0**	16.2	16.8	9.1	8.4
21	Hydrochlorothiazide	12.2	**24.8**	0.0	8.7	2.2	**21.4**

As we can see from Table 19, only 13 EPs from the 21 had percentages of degradation that are either equal to 20% or higher. Free HRP seems better in degrading the majority of these 13 pollutants compared to the immobilized HRP. From those 13 degraded pollutants, 9 had better degradation percentages when free HRP was used rather than HRP-TiO_2 or HRP-ZnO. The pollutants that were degraded better by HRP are lincomycin-HCl, meloxicam, trimethoprim, venlafaxine-HCl, SMX, MCPA, MBT, furosemide, and hydrochlorothiazide. Some of them had small degradation percentages in the range of 20%-40%, while others had high percentage of degradation

in the range of 90%-100%. Many of them were only degraded by HRP but not by immobilized HRP like lincomycin-HCl, trimethoprim, venlafaxine-HCl, SMX and MCPA. However, 3 pollutants were degraded more efficiently when either HRP-TiO_2 or HRP-ZnO were used instead of free HRP which are roxithromycin, cimetidine and caffeine. Caffeic acid was completely degraded (100%) when either free HRP or HRP-ZnO were used.

3.6 Degradation of emerging pollutants by photocatalytic oxidation

The degradation of some emerging pollutants by pure photocatalysts and hybrid biocatalysts were tested under UV light. Roxithromycin and DEET were treated with TiO_2 + UV, SBP-TiO_2 + UV, and HRP-TiO_2 + UV for 30 minutes. The results obtained are shown in Figure 42. Moreover, both pollutants were treated with ZnO + UV, SBP-ZnO + UV, and HRP-ZnO + UV. Figure 43 shows the results obtained for the degradation of roxithromycin and DEET by ZnO and ZnO composites.

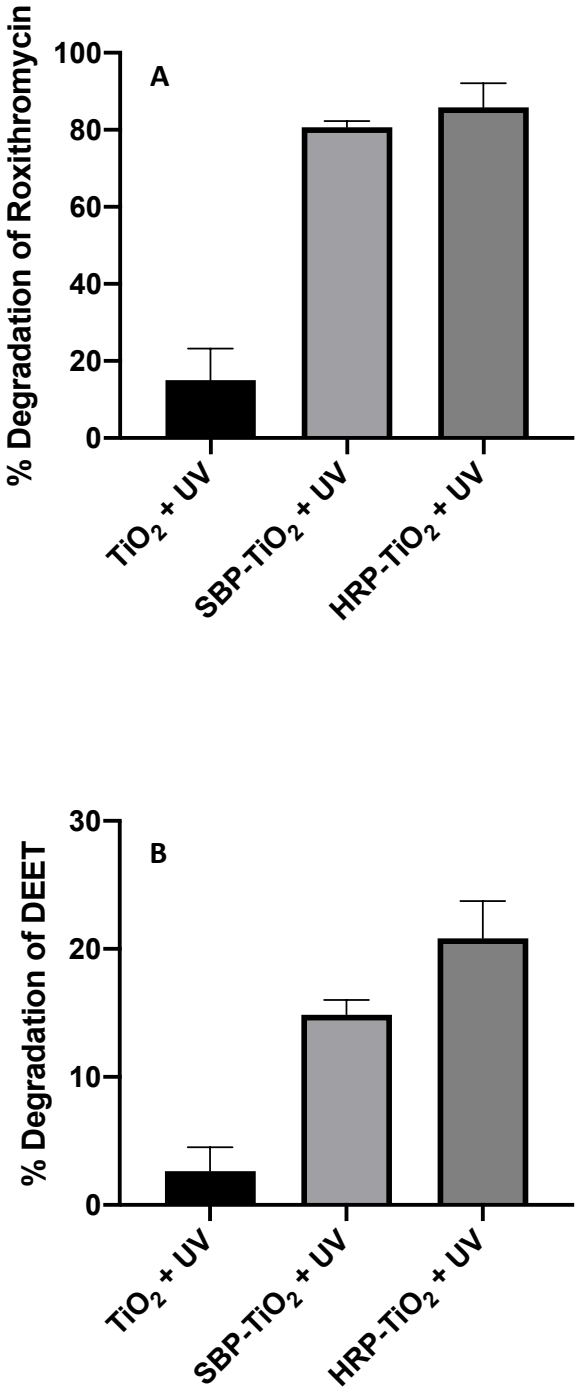


Figure 42: Degradation of A) Roxithromycin; B) DEET; by pure TiO_2, SBP-TiO_2 and HRP-TiO_2 with UV light

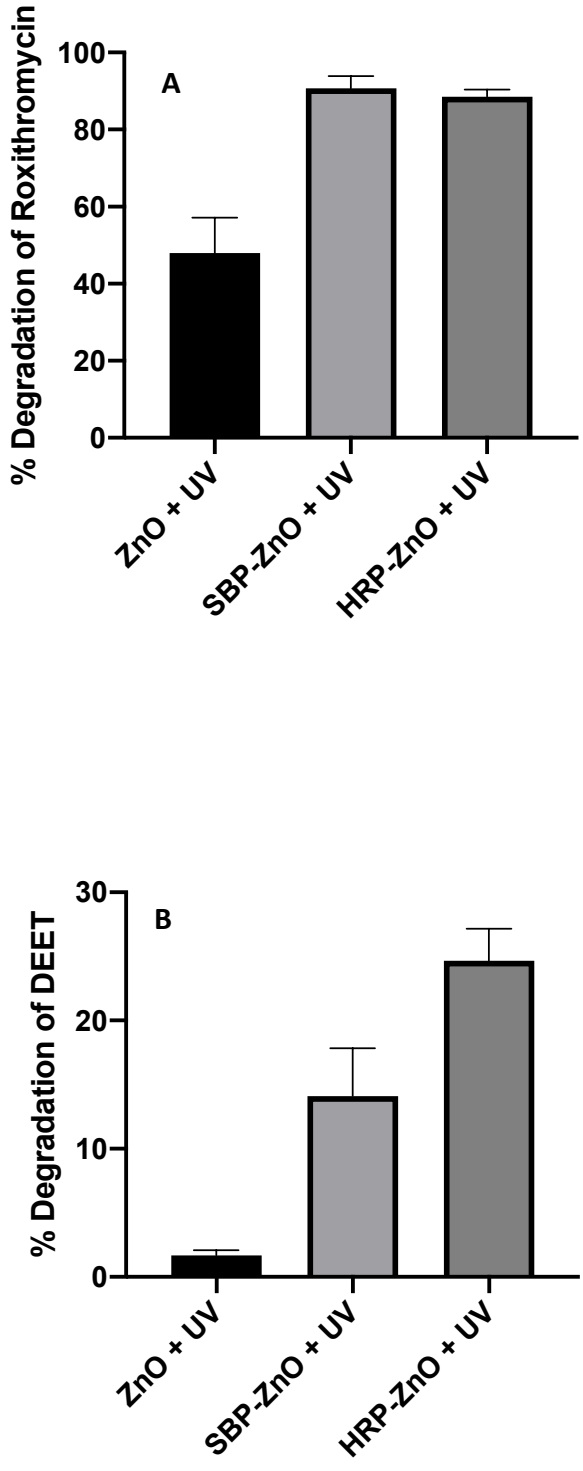


Figure 43: Degradation of A) Roxithromycin; B) DEET; by pure ZnO, SBP-ZnO and HRP-ZnO with UV light

As we can see from Figure 42, the hybrid biocatalysts (SBP-TiO_2 and HRP-TiO_2) are better catalysts compared to the photocatalyst (TiO_2). By looking into the results obtained for roxithromycin, we can see that pure TiO_2 was able to degrade 15.1% of it. While, SBP-TiO_2 and HRP-TiO_2 were able to degrade 80.3% and 85.9% of it, respectively. Only 2.9% of DEET was degraded by TiO_2. However, SBP-TiO_2 and HRP-TiO_2 enhanced the degradation of DEET as they were able to degrade 14.9% and 20.8% of it, respectively.

ZnO, SBP-ZnO and HRP-ZnO showed very similar results. Pure ZnO degraded 47.9% of roxithromycin. This percentage of degradation increased to 90.7% and 88.5% using SBP-ZnO and HRP-ZnO, respectively. Less than 2% of DEET was degraded when it was treated with ZnO. However, SBP-ZnO and HRP-ZnO were able to degrade 14.1% and 24.7% of DEET, respectively. All reactions were carried out under UV light for 30 minutes and without the addition of H_2O_2.

3.7 Hybrid biocatalysts compared to free enzymes and photocatalysts

From our previous results, we selected two pollutants that were not degraded by free enzymes (SBP or HRP), then we tested the ability of pure photocatalysts, or hybrid biocatalysts exposed to UV light in degrading them. The two pollutants that were selected are trimethoprim and DEET. Figure 45 shows the results obtained for trimethoprim. Figure 46 shows the results obtained for DEET.

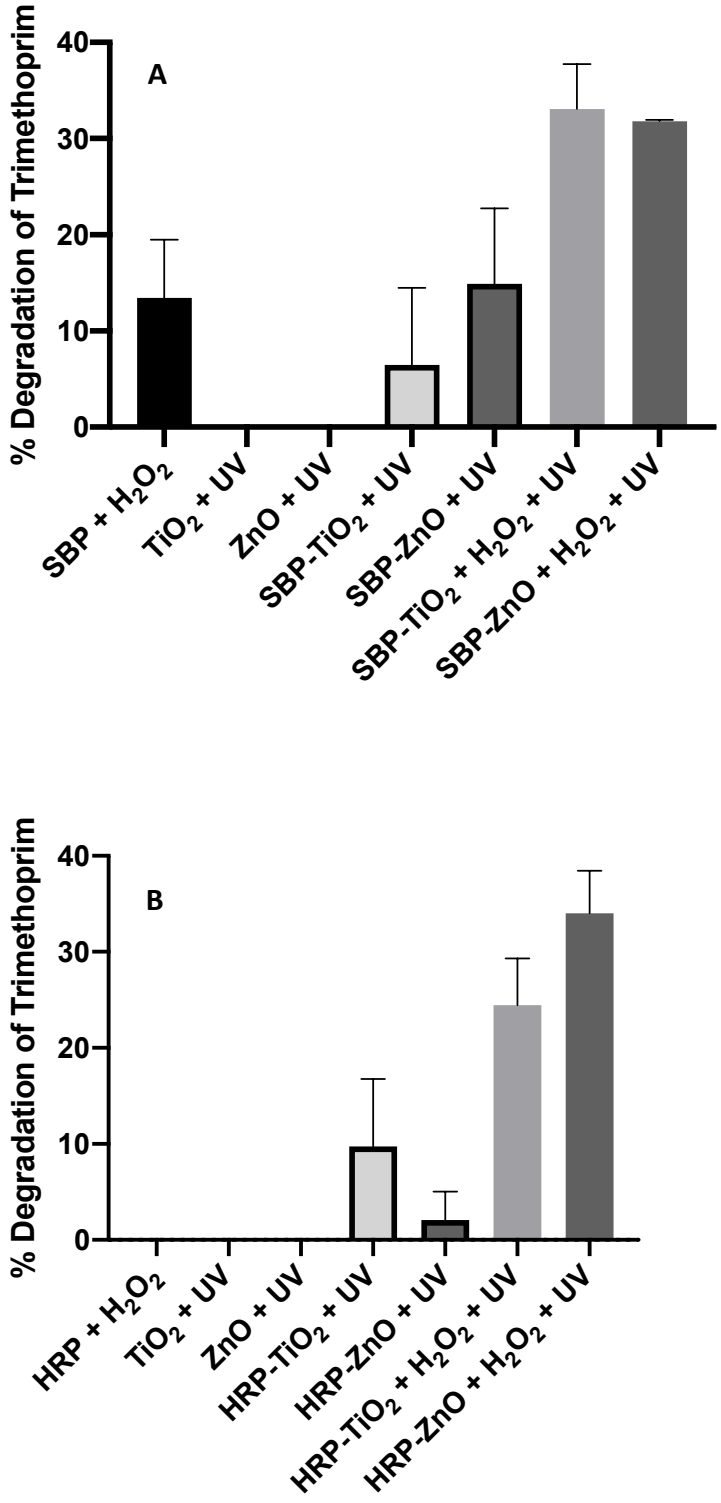


Figure 44: Degradation of Trimethoprim. A) SBP and its composites; B) HRP and its composites

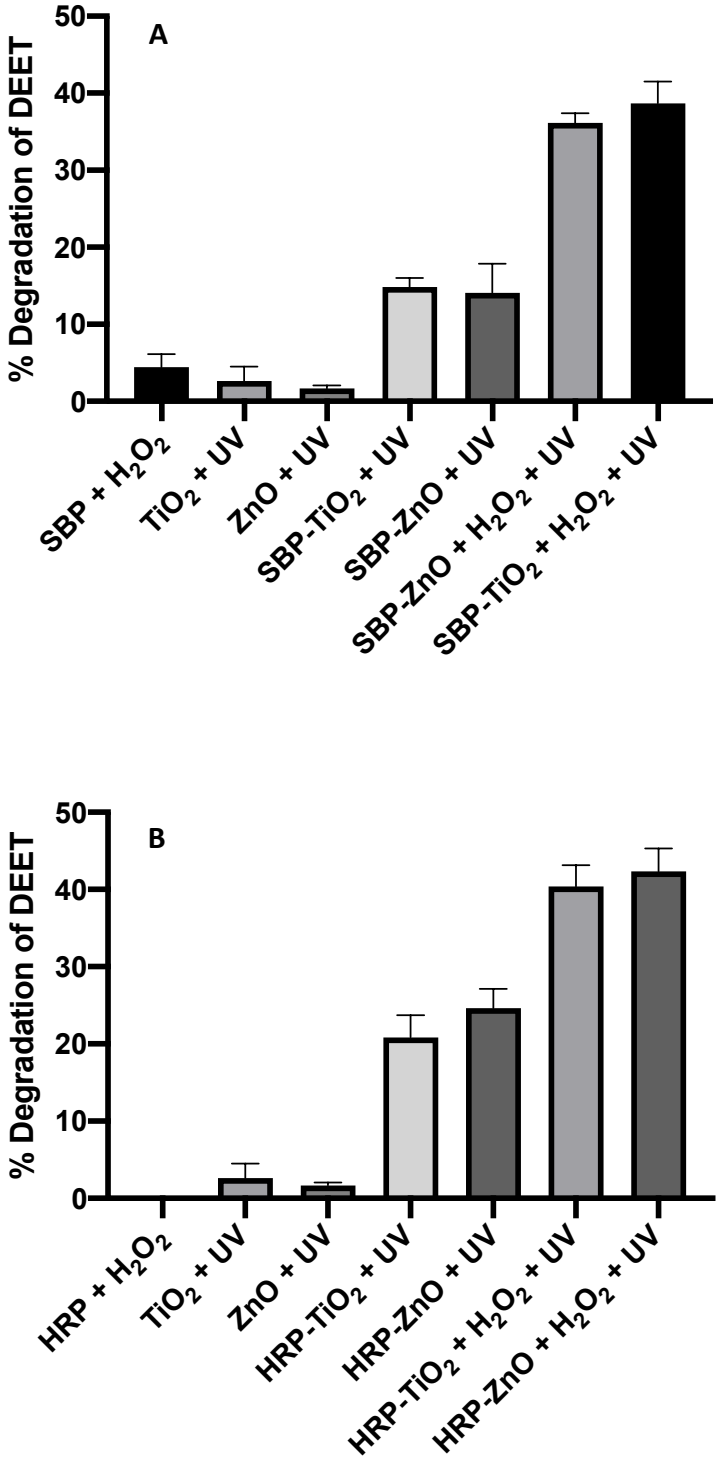


Figure 45: Degradation of DEET. A) SBP and its composites; B) HRP and its composites

As we can see from Figure 44, less than 20% of trimethoprim was degraded by SBP. Both photocatalysts (TiO_2 and ZnO) did not degrade any of trimethoprim. There was some degradation of trimethoprim (less than 20%) when SBP-TiO_2 and SBP-HRP exposed to UV light were used for its degradation. Interestingly, treating trimethoprim with SBP-TiO_2 and SBP-ZnO with the addition of H_2O_2 and exposure to UV light resulted in around 33% and 31% of degradation, respectively. By looking into the results obtained for HRP and its composites, we can see that free HRP, ZnO and TiO_2 did not degrade any amount of trimethoprim. Less than 20% of trimethoprim was degraded by HRP-TiO_2 and HRP-ZnO when exposed to UV light. Approximately, 29% and 35% of trimethoprim was degraded by HRP-TiO_2 + H_2O_2 + UV and HRP-ZnO + H_2O_2 + UV, respectively.

Similar results were obtained for DEET. Free SBP and pure photocatalysts did not degrade DEET. There was some degradation of DEET using SBP-TiO_2 and SBP-ZnO with UV (around 20% degradation). The degradation of DEET was enhanced to reach around 40% using SBP-TiO_2 and SBP-ZnO with H_2O_2 and UV light. Also, free HRP did not degrade DEET. HRP-TiO_2 and HRP-ZnO with UV degraded around 25% of DEET. The percentage of degradation of DEET increased to 45% when it was treated with HRP-TiO_2 and HRP-ZnO + H_2O_2 + UV light.

The results obtained indicates that using immobilized peroxidase enzyme onto photocatalytic support gives better degradation compared to using free enzymes alone or pure photocatalysts alone. This can be due to the combined enzyme-chemical oxidation in which our reaction includes photocatalytic oxidation step with peroxidase activity.

3.8 Re-usability of immobilized enzyme

One of the main reasons to immobilize enzymes onto different supports is to recycle the enzyme after each reaction and re-use it for multiple times. Based on our previous experiments, MBT was completely degraded by SBP-TiO_2 + H_2O_2 + HOBT. The ability of recycled SBP-TiO_2 in degrading MBT was tested for few times to check whether the recycled enzyme can still be used or no. To demonstrate the reusability of the hybrid biocatalyst, four consecutive degradation cycles of MBT were performed. In each cycle, the same amount of substrate (MBT), buffer, redox mediator and H_2O_2 was added and reaction components were kept reacting for 30 minutes at room temperature. After four times of repeated use test, the degradation efficiency of MBT was up to 95%. This means that the immobilized enzyme was still active after four cycles of degradation and it can be further used for additional degradation cycles. Figure 46 shows the results obtained for four consecutive degradation cycles of MBT.

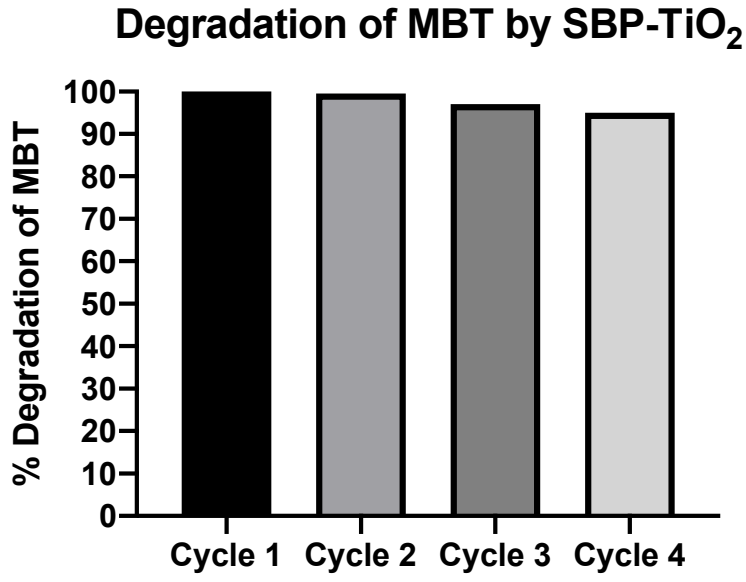


Figure 46: Four consecutive degradation cycles of MBT by SBP-TiO_2

Chapter 4: Conclusion

In summary, this study is one of the very few studies that combined two different remediation methods (chemical and biological) to degrade 21 different hazardous contaminants that belong to different classes of emerging pollutants. Moreover, this study presented a comparison between using free enzymes and immobilized enzymes for the treatment of these pollutants. SBP and HRP were successfully immobilized covalently onto two different photocatalytic supports (TiO_2 and ZnO) to produce four hybrid biocatalysts that showed pH and thermal stabilities.

SBP/HRP + H_2O_2 were used for the degradation of 21 EPs in the presence and absence of redox mediator (HOBT). Both enzymes showed very similar effect on the 21 EPs. In some pollutants, the presence of HOBT enhanced the degradation of the pollutants. While in other pollutants, HOBT inhibited their degradation.

Interestingly, immobilized SBP onto TiO_2 and ZnO showed better degradation compared to using free SBP. Roxithromycin, meloxicam, norfloxacin, cimetidine, ibuprofen, caffeine, MBT and hydrochlorothiazide were degraded better by SBP-TiO_2 or SBP-ZnO rather than free SBP. On the other hand, free HRP seemed to be better in degrading the 21 EPs compared to HRP-TiO_2 and HRP-ZnO. The degradation of the pollutants can be enhanced with increased incubation time.

The lack of reusability of enzymes is one of the major issues of using free enzymes. The immobilization of enzymes permitted the recyclability of enzymes without losing its activity. Overall, using immobilized peroxidase enzymes resulted in better degradation in most of the pollutants compared to free enzymes and allowed for the re-usability of enzymes.

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