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Enzymatic Removal of Sulfa Drugs from Synthetic Wastewater by Soybean Peroxidase

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By

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ABSTRACT

Sulfa drugs are a broad family of antibiotics that are widely used in the treatment of a wide range of infections, and they have been found in surface and groundwater, as well as present in sewage and effluent (treated sewage and sludge) municipal or industrial wastewater treatment plants (WWTPs). in concentrations of ng/L to >g/L. The continued presence of these so-called emerging pollutants (ECs) and their metabolites can cause adverse ecological effects, including bacterial resistance, even at very low concentrations. In this study, the first aim was to explore the feasibility of oxidation processes catalyzed by soybean peroxidase as an eco-friendly and economically advantageous alternative method for the conversion of selected sulfonamide class compounds. Soybean peroxidase (SBP) is extracted from the seed sell (husk), which is a by-product of the crushing process and is used in animal feed. In the second step, the most important operational parameters, pH, H_2O_2 concentration, and enzyme activity were optimized for two compounds that were SBP substrates. Thirdly, a redox mediator was used to improve the final conditions. Also, in the end, a time course study was conducted under optimal conditions before and after adding the redox mediator, to determine the initial first-order rate constant and half-life of each substrate. Finally, the probable oligomerization products of enzymatic treatment were characterized by mass spectrometry analysis and showed the formation of dimers and azo compounds for the two substrates.

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LIST OF ABBREVIATIONS/SYMBOLS

Abbreviations:

SMX, Sulfamethoxazole

SMR, Sulfamerazine

SBP, Soybean peroxidase

EC, Emerging contaminant

HRP, Horseradish peroxidase

MS, Mass spectrometry

ESI, Electrospray ionization

UV-Vis, Ultraviolet-Visible

WWTP, Wastewater treatment plant

MP, Micropollutant

4-AAP, 4-Amino-antipyrine

ACN, Acetonitrile

HPLC, High-performance liquid chromatography

U.S. FDA, The United States Food and Drug Administration

CGW, Coal gasification wastewater

PAHs, Polycyclic aromatic compounds

AOP, Advanced oxidation process

MBT, Mercaptobenzothiazole

MMT, Million metric tons

USDA, The United States Department of Agriculture

ARP, *Arthromyces ramosus* peroxidase

PES, Polyethersulfone

Tof, Time-of-flight

ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

NMR, Nuclear magnetic resonance

FTIR, Fourier transform infrared

IPA : Isopropyl alcohol

Symbols:

λmax, Maximum wavelength

m/z, Mass-to-charge ratio

pK_a, -log10 K_a, where K_a is acid dissociation constant

k_{cat}/K_M, Specificity constant or, catalytic efficiency, where K_M is Michaelis constant and k_{cat} is turnover number

Arg-38, Arginine-38

His-42, Histidine-42

k, First-order kinetic rate constant

$t_{½}$, Half-life

CHAPTER 1

INTRODUCTION

Various organic contaminants, including sulfa drugs, have been recognized in wastewater treatment plant influent and effluent, therefore, they are not effectively eliminated by conventional treatment methods. As a result, they have penetrated the water sources and have been detected in concentrations between ng/L and mg/L(Lam, et al., 2005). Several of these compounds have been reported to develop bacterial resistance. Therefore, developing an efficient, economical, and environmentally friendly alternative for the treatment of sulfa drugs (SAs) in aqueous system is extremely important and necessary.

1.1 Background

Access to clean water is one of the most essential human needs. With the growing population, this need is becoming increasingly important; there are other factors which are also influencing this severity. Physical-chemical-biological characteristics of water are continuously being examined with the goal of investigating its availability to satisfy and meet the needs of humans and industries.

New compounds are being introduced to the environment daily. Emerging contaminants or “ECs” are synthetic or naturally occurring chemicals or any microorganisms that are not commonly monitored in the environment but have the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects. (Rasheed et al., 2019). Professionals in the water quality industry refer to emerging contaminants as pollutants that have been identified in water bodies that can negatively impact the environment or human health and are not usually regulated(González-González et al., 2022).

Of course, due to the high importance of this issue, it is expected that laws will be enforced in this field in the not-too-distant future. At the same time, conventional treatment methods also cannot eliminate these compounds completely. Among the sources of these pollutants are agriculture, urban runoff, common household products (like soaps and disinfectants),

and pharmaceuticals, which are disposed of in sewer system that reaches the sewage treatment plants. Accordingly, water-soluble and poorly degradable pharmaceutical residues have attracted the attention of researchers. The presence of more than 200 pharmaceuticals in river waters has been reported worldwide. In addition to their individual appearance in the environment, these chemical compounds may also appear in complex mixtures that may have undesirable synergistic effects (Prasannamedha, et al. 2020).

It is an accepted belief that antibiotics are the most problematic emerging contaminants due to their ability to induce bacterial resistance. The high solubility and at the same time low degradability of many of these compounds make them easily pass through all natural and manufactured filters and lead to danger for drinking water. Antimicrobial active compounds have caused more concern due to the development of bacterial resistance among the wide range of pharmaceutical compounds that exist. Numerous reports indicate that surface water contamination with antibiotics is spreading throughout Europe, the United States, and Canada. Some of them are also found in ground water (Lam , et al., 2005). One of the most common classes of antibiotics used in the treatment of human and animal diseases is sulfa drugs (Tadesse et al., 2012). The bacteriostatic sulfonamide drugs, often called sulfa drugs, include sulfanilamide and numerous compounds closely related to it, Figure 1. Other groups of sulfonamide drugs have been developed by exploiting observations made during the clinical evaluation of sulfanilamide derivatives. Sulfonamides belong to an important class of synthetic antimicrobial drugs that are used pharmacologically to treat a broad spectrum of bacterial infections. Sulfonamide contamination is often found in groundwater, surface water, sewage, and soil. Adverse ecological effects and human health issues arise due to the cumulative properties and toxicity of sulfonamides. Among the most frequent sulfonamides found in the environment are sulfamethazine and sulfamethoxazole, with a concentration range of 10 to 231 mg/L, and 4-12 mg/ L, respectively in wastewater (Xiong et al., 2019).

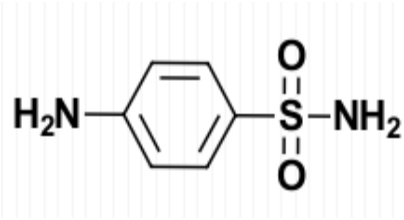


Figure 1-1 Structure of sulfonamide functional group adopted from

(PubChem, 2021)

1.2 Common wastewater treatment techniques with emphasis on sulfa drug elimination:

Given the need for reliable and improved water treatment technologies, several important factors should be addressed, such as significant population growth, the need to protect dwindling water resources, strict discharge policies, and the high cost of wastewater treatment. Generally, for wastewater treatment, common treatment methods, physical, chemical, and biological treatments can be used individually or in combination. Physical operations such as filtration, sedimentation, flotation, and skimming are purely mechanical and are used to separate solids or suspended particles. Chemical methods such as neutralization, ion exchange, and chlorination can provide specialized treatment. Biological processes, aerobic and anaerobic digestion, activated sludge, and trickling filter use microorganisms to remove pollutants from water. Even though conventional treatment technologies are well-known, they suffer from poor settling of suspended solids, high sludge production, high capital, and maintenance costs, and/or low efficacy in treating pollutants. According to traditional discharge regulations, low concentrations of biodegradable organic matter, measured in terms of biological oxygen demand (BOD), are required for effective water treatment. However, conventional methods cannot effectively treat micropollutants because of the introduction of pharmaceuticals and persistent organic pollutants into final sludge or surface water from various sources. There have been advances in treatment technologies designed to avoid the introduction of these additional pollutants to meet the therapeutic needs of the present and the future. While substitution or recycling at the source is the preferred method of pollution control, it is not always possible (Padoley et al., 2008).

As a result, solving the problem by eliminating emerging contaminants from water requires a cost-efficient and effective treatment method. It has been shown that ECs have been detected in the effluent of many WWTPs due to the inefficiency of various conventional treatment techniques, including physical, chemical, and biological (Behera et al., 2011). The purpose of the present study was, therefore, to investigate whether enzymatic treatment, with soybean peroxidase (SBP), of selected sulfa antibiotics would be an environmentally friendly and cost-effective alternative treatment method in aqueous system. Enzymatic treatment using a biological catalyst for chemical reaction combines aspects of physico-chemical and biological processes. There are several advantages to enzymatic treatment, including its ability to handle substances that are toxic to microorganisms, its ability to work over wide ranges of pH, salinity, and temperature, its relatively rapid time course, and its inability to be limited by shock loading or biomass accumulation (Al-Ansari, et al., 2011). Treatment by enzyme has some drawbacks as well, such as inactivating the enzyme, its cost, and availability (Steevensz et al., 2014a, b).

More effective and targeted treatments are necessary to remove ECs from water and wastewater, hence the development of other advanced treatment technologies are alternatives to enzymatic treatment. These technologies include membrane filtration, advanced oxidation processes, photooxidation, activated carbon adsorption, and electrooxidation. Nevertheless, these technologies suffer from low efficiency, toxic by-products, excessive costs, and/or long processing times (Hoffmann et al., 2016, Rivera-Utrilla et al., 2013). There have been several wastewater treatment methods used for treating antibiotics and other drugs. The removal of antibiotics by biological processes (as in sewage treatment plants) has been demonstrated to be ineffective. For example, it was discovered that 12 sulfonamides could not be readily biodegraded in activated sludge (Ingerslev et al., 2000).

The necessity for wastewater treatment methods in addition to conventional ones is significant due to the shortcomings of these methods. This is in addition to the need to monitor and control the entry of drugs into the environment. From this point of view, methods such as enzymatic decomposition of sulfonamide antibiotics using crude extracts of various halophytic plants, as well as the advanced technologies noted above, show promise in removing and degrading sulfonamides, there are still obstacles to their

implementation(Al-Maqdi et al., 2018). Considering the stability of the enzymatic degradation method as well as its compatibility with the environment, it is a good prospect for removing sulfonamides.

In some cases, enzymatic treatment of contaminants can lead to problems due to their resistance. In the presence of specific redox mediators, these resistant compounds were more easily degraded or transformed by enzymes. There was a significant increase in both the range of layers and the degradation efficiency of resistant compounds when these redox mediators were used. There are various redox mediators have been used for this purpose, including 1-hydroxy benzotriazole, veratryl alcohol, violuric acid, 2-methoxy-phenothiazone, 3-hydroxyanthranilic acid, etc. There are very few of these that are commonly used.1 -hydroxy benzotriazole is one of the most common redox mediators (Husain ,2007).

1.3 Objectives

The objectives of the present research are as follows:

1. To determine the feasibility of enzyme-catalyzed treatment of two selected sulfonamide antibiotics (sulfa drugs), sulfamethoxazole and sulfamerazine from synthetic wastewater; structures and chemical formulae are given in Table 1-1.

2. To optimize the catalyzed removal of the sulfonamide class of antibiotics using soybean peroxidase (SBP).

3. To identify the possible transformation products of enzymatic reaction using mass spectrometry (MS).

Preliminary studies have shown that sulfamethoxazole and sulfamerazine are substrates of SBP. These two pollutants SMX and SMR were removed with more than 88% and 76% efficiency under optimized operating conditions, respectively. The conditions were optimized, and the minimum amount of effective enzyme was achieved. By using mass spectrometry method, the possible transformed products by enzymatic reaction identified.

1.4 Scope

1. Investigate the feasibility of SBP treatment of sub-mM concentrations of sulfamethoxazole; sulfamerazine

2. Optimize the most important operational parameters, pH, hydrogen peroxide H_2O_2 concentration and SBP activity for removal of the above compounds using SBP.

3. Employ high-performance liquid chromatography (HPLC) for substrate detection to evaluate the removal efficiency of enzymatic treatment.

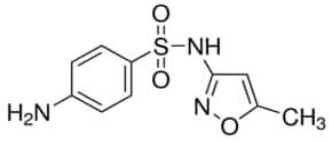
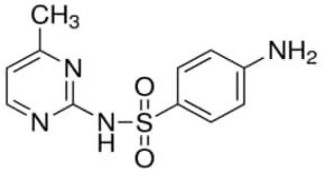
4. Study the effect of stepwise addition of H_2O_2 on the removal efficiency of the substrate.

5. Determine the initial first-order rate constants and half-lives of the substrates by monitoring the time course of substrate consumption under optimal conditions.

6. Identify the possible formation of oligomers as the result of enzymatic treatment using high resolution mass spectrometry (MS).

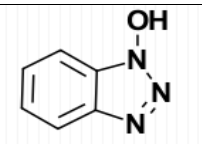
There are the name, formula, and molecular structure of the chemicals studied.

Table 1-1 Nam**e**, formula, and molecular structure of the chemicals studied (SMX , SMR)

	Chemical	Formula molecular	Molecular Weight	Structure
1	Sulfamethoxazole (SMX)	$C_{10}H_{11}N_3O_3S$	253.28	
2	Sulfamerazine (SMR) amino-N-(4-methyl-2-pyrimidinyl) benzene sulfonamide, N1-(4 methylpyrimidin-2-yl) sulfanilamide	$C_{11}H_{12}N_4O_2S$	264.30	

(Pareek, et al., 2013)

Table 1-2 Name, formula, and molecular structure of the 1-hydroxy benzotriazole as a mediator

	Chemical	Formula molecular	Molecular Weight	Structure
	1-hydroxy benzotriazole (HOBT)	$C_6H_5N_3O$	135.12 g/mol	

(Hassan, et al., 2009)

CHAPTER 2

LITRETURE REVIEW

2.1 Emerging Pollutants

The presence of ECs in water bodies has increased in recent years, and they can unbalance ecosystems and negatively affect non-target species Research has been motivated on sustainable remediation since emerging pollutants have negative impacts on the environment, are widely distributed, have high bioaccumulation rates, and are resistant to wastewater treatment plant processes. Advanced biological remediation techniques have become increasingly popular in recent years. Such technologies have exhibited numerous advantages like in-situ remediation, low costs, eco-friendliness, high public acceptance, and so on. Chemical research into ECs and improved analytical methods for detecting these chemicals have raised awareness about pharmaceuticals' existence in wastewater and aquatic ecosystems. It is possible to consider antibiotics to be pharmaceuticals of a high priority given their large production, their biological activity, and their resistance to biodegradation (Jones, et al.,2007, Kümmerer, 2009). In China, contamination with antibiotics can be seen in rivers, pools, effluents from animal farms, and sewage (Ingerslev, et al., 2000). Several wastewater treatment plants in Germany also were investigated for the treatment of hospital and pharmaceutical wastewater. This research showed that many pharmaceuticals could not be degraded during conventional biological treatment, nor could sewage sludge adsorb them (Kümmerer,et al 1997), in a system that integrated aerobic digestion with activated carbon filtration and reverse osmosis (RO). The system just reduced biochemical oxygen demand (BOD), chemical oxygen demand (COD), and total dissolved solids (TDS) in pharmaceutical wastewater. Most of the published studies, however, investigated the removal of pharmaceuticals other than antibiotics (Adams,et al., 2002). Meanwhile, it was found that more than 30 antibiotics are present in sewage, effluents and surface waters at levels ranging from ng/L to mg/L(Michael et al., 2013).

2.2 Sulfa drug compounds

The use of sulfonamide antibiotics (SNs) is widespread around the world. Clinical use of these drugs dates back to 1968. In primary care medicine, sulfonamides are mainly used to treat urinary tract infections and upper respiratory tract infections. The SA class is one of the most commonly used as antibiotics in humans, as well as in livestock and fish culture (Cháfer-Pericás, et al., 2010, Hamscher, et al., 2006). It is not uncommon for veterinary antibiotics, such as SAs, to reach wastewater treatment plants (WWTPs) only in a limited amount, but they have been detected more frequently in influents and effluents (García-Galán, et al., 2011, Göbel et al., 2007, Gros, et al., 2007) along with SAs of human use, which is predominant. Data on removal efficiencies of SAs during wastewater treatment are still insufficient, and the elimination rates reported so far have been quite low (García-Galán,et al. 2011). A number of sulfonamides are used for these purposes, such as sulfamethoxazole, sulfadiazine, sulfamerazine, sulfamethazine, sulfapyridine, and sulfathiazole. Sulfa drug compounds, having molecules containing the sulfonamide group (RSO_2NH_2NH), have been the center of drug structures as they are quite stable and well tolerated in human beings (Ashfaq, et al, 2013). Among the most frequent sulfonamides found in the environment are sulfamethazine and sulfamethoxazole, with a concentration range of 10 to 231 mg /L, and 4-12 mg/ L, respectively in wastewater (Xiong et al., 2019).

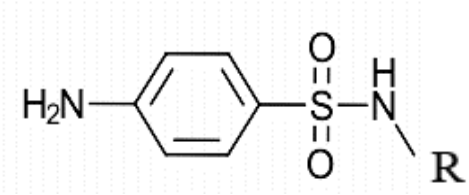
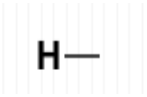


Figure 2-1 Structure of sulfonamide functional group adopted from

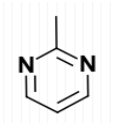
(Maren, 1967)

R =

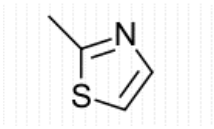
1. Hydrogen



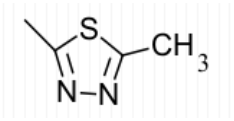
2. Sulfadiazine



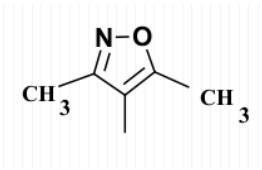
3.sulfathiazole



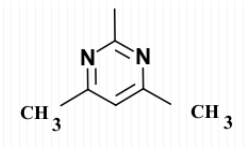
4.sulfamethizole



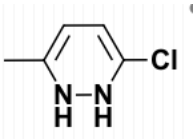
5. sulfisoxazole



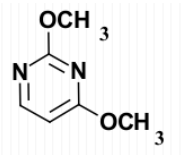
6. sulfamethazine



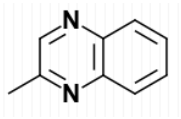
7.sulfachloropyridazine



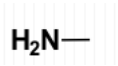
8. Sulfadimethoxine



9. sulfaquinoxaline



10. sulfanilamide



11.sulfadoxine

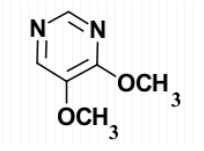


Figure 2-2 some common sulfanilamide compounds adopted from (Maren, 1967)

SAs are found in the environment over a concentration range from 1 ng/L to 1 mg/L. SAs increase the risk of endangering human life by stimulating bacteria to produce antibiotic resistance genes. Based on estimates, about 23,000 Americans die each year from antibiotic-resistant infections. In fact, in the common methods of distilled water purification, the removal of sulfonamides does not reach the expected level. For example, the highest amount of antibiotics found in water bodies in China are sulfamethazine sulfadiazine and sulfamethoxazole at maximum concentrations of 211, 17, and 63 ppb, respectively. The mean concentration of antibiotics was found to be 17.7 μg/L in Asia-Pacific, 11.3 μg/L in Africa, 0.9 μg/L in America, and 0.4 μg/L in Europe (Danner, et al, 2019). According to the United States Geological Survey reports, sulfamethoxazole was among the most prevalent antimicrobial contaminants found in groundwater with a peak concentration of (11 μg/L) (Steevensz et al., 2013). In comparison to other organic compounds, antibiotics are more likely to persist in the environment (called, pseudo-persistent pollutants). As a result, these sources continued to increase despite reactions with certain environmental processes such as photochemical degradation, biochemical degradation, and sorption (Gogoi et al., 2018). Resistance genes are created by the presence of antibiotics in the ecosystem and in turn, create resistant bacteria. These statistics show the need to develop an efficient method to remove sulfonamides is essential and a priority (Bielen et al., 2017).

Sulfa drugs, in addition to their roles as antibacterial agents, anti-infectives, inhibitors, antimicrobial agents, and drug allergens, function as environmental contaminants too. They are functionally related to sulfanilamide (SN) (Knappe, et al, 2007). Based on the 30% excretion rate of SN, it is capable of entering water reservoirs at relatively high levels (Singh, 2012). In some parts of the United States, levels of SN in wastewater treatment plants effluents have reached 3.25μg/L from its 18.3μg/L influent (Bhandari, et al , 2008). In residential, industrial, and agricultural waste effluent in Taiwan (Lin et al., 2008), average SN levels are higher than in South Korea and Canada (Choi et al., 2008, Singh, 2012). SN concentrations are high not only in the water systems of these four technologically advanced countries, but also in many other countries, and in some cases even at higher concentrations (Singh, 2012). In the pharmaceutical industry, SN is a persistent organic pollutant. A series of transformation reactions occur after consumption,

resulting in environmental oxidation, acetylation, and hydrolysis. There is evidence of transformation by-products in rivers, lakes, groundwater, sediments, and the oceans even today, although many countries have banned its use of it (Prasannamedha, 2020).

There are some methods to remove these classes of pharmaceuticals from the wastewater but some of them are not efficient and environmentally friendly enough. For example, Liu and his co-worker in 2020, used CuO-Cu_2O and light to activate persulfate (PS) for the degradation of sulfamerazine. In the CuO-Cu_2O/PS/UV system, the SMR removal efficiency increased to 100% in a half an hour. CuO-Cu_2O 's high efficiency and its outstanding catalytic stability for PS activation were attributed to the fact that CuO and the photoinduced electron of Cu_2O promoted the conversion of Cu_2^+ to Cu^+. Based on the results obtained, it appears that hydroxyl radicals and sulfate radicals play a role in the degradation of SMR. In contrast, the initial pH of the solution had a significant influence on radical concentrations and fractions (Liu et al., 2020). But one of the drawbacks of this method is the use of copper and of the reagent preparation which is not easy and affordable.

In another case study by Huang et al. 2020, experiments were conducted to evaluate the performance of ball-milled biochar in removing two sulfonamide antibiotics sulfamethoxazole (SMX) and sulfapyridine (SPY) from water and wastewater. The results showed that the adsorption of SMX with 450°C ball-milled biochar had the best removal efficiency, about (83.3%). This method is not eco-friendly enough to be widespread because of the high temperature required and high energy consumption (Huang et al., 2020). Delia et al. 2007 also succeeded in obtaining the removal of sulfamerazine at an initial concentration of up to 90 mg/L, with a removal efficiency of 97% to 100%, by using a sequential anaerobic/aerobic reactor system (Sponza et al., 2007). There are some drawbacks to this method too, including long setup time, long recovery time, need for specific nutrients, and more sensitivity to changes in environmental conditions, which makes the use of this method not widespread.

2.2 Selected sulfa drugs

Sulfonamide contains two important functional groups in the pharmaceutically relevant pH range of 4 to 9 a very weakly acidic amide moiety and one basic arylamine moiety. The arylamine nitrogen atom (-NH2) is able to gain a proton, while the amide nitrogen atom (-NH-) is able to release a proton under specific pH conditions. Thus, the first dissociation equilibrium refers to the dissociation of amine moiety (pK_1), and the second equilibrium to the dissociation of amide moiety (pK_2), Figure 1. SAs are ordinary ampholyte compounds. In ordinary ampholytes, when the difference (ΔpK_a) between acidic pKa and basic pK_b is greater than 3, only one kind of group (acid or basic) is ionized to any extent at a time. When pH is about equal to the average pKa, the neutral form is the dominant species in ampholytes. The hydrophobic nature of the neutral species is naturally greater than those of the associated ions (Baran et al., 2011).

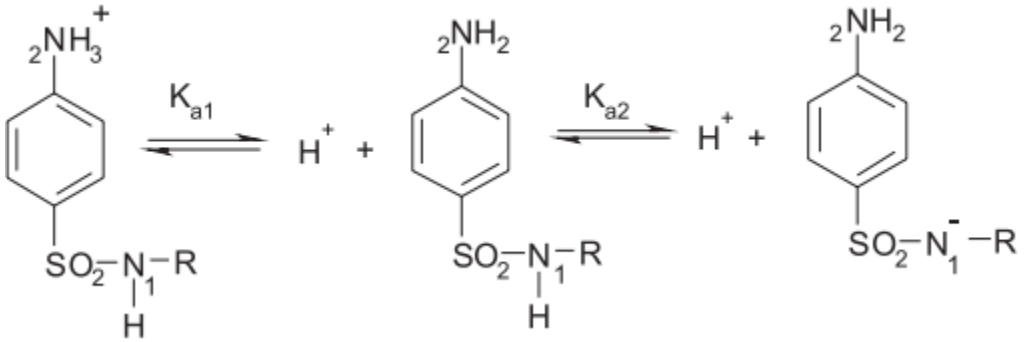


Figure 2-3 Protolytic equilibria of sulfonamide adopted from (Nurullah et al 2022)

SAs are compounds with low molecular weight and high-water solubility (Baran et al., 2011). It is well established that SAs are common anthropogenic pollutants in groundwaters, lakes, and rivers; they have been detected in major Asian (Asghar et al., 2018) and European (García-Galán et al., 2012) rivers. Hydrolysis is not able to degrade SAs or their metabolites (Białk-Bielińska et al., 2012). The concentration of SAs varies depending on the type of water reservoir and how it is exploited. Accordingly, the presence of different concentrations from ng/L to µg/L in water of these compounds or their derivatives, which are known as emerging contaminants (ECs), have potential effects on the environment and human health.

Some SAs have been studied for the possibility of SBP-catalyzed removal, while those listed below have not been studied.

Table 2-1 Chemical and Physical Characteristics for the Two Antibiotics (sulfa drugs)

Compound	sulfamethoxazole	sulfamerazine
Chemical Abstracts Service (CAS) number	723-46-6	127-79-7
Molecular Weight	253.28 g/mol	264.30 g/mol
Solubility in water	up 610 mg/L at 37°C	202 mg/L (at 20 °C)
pK_a	1.6-1.85 and 5.57-5.7	2.17-2.29 and 6.77
Melting point (°C)	168-172	236
λ max (nm)	265	275

Adopted from (PubChem, 2021)

2.2.1. Sulfamethoxazole

Sulfamethoxazole or 4-amino-N-(5-methyl-1,2-oxazole-3-yl) benzene sulfonamide (PubChem, , 2021) , exists as a yellow-white solid or sometimes crystal at standard pressure and room temperature; it is odorless, bitter(O'Neill, 2006), and soluble in water to 2.4 mM. It is UV-light sensitive (Knappe, et al, 2007).

Sulfamethoxazole is an antibacterial agent, meanwhile an environmentally harmful substance too. It is functionally related to sulfanilamide (Knappe, et al. , 2007). As well as other pharmaceutical compounds that may be present in groundwater, surface water, and wastewater, antibiotic active compounds are more concerning because of the rise in antibiotic resistance in hospitals (Lindsey, et al., 2001, David L. et al. , 2002). As a result of antibiotic consumption and its prevalence in the water cycle, resistance to SMX can develop (García-Aljaro, et al., 2014). Sulfamethoxazole, an antibiotic often combined with trimethoprim, is bacteriostatic. For humans, infections of the urinary tract are the most common reason for prescribing it. There is a variation in its concentration between countries, however, it is detected in wastewater effluent 100% of the time (Miao, et al., 2004). Due to SMX's high stability, it is proposed as an indicator of pharmaceutical entry into the environment. Various types of water can contain sulfamethoxazole, including surface waters, groundwater, drinking water, and wastewater (Watkinson et al., 2009). A

range of 8–3180 ng/L has been measured for wastewater treatment plant effluents, and 243 to 2000 ng/L has been measured for wastewater treatment plant influents (Miao, et al., 2004). Water treatment processes vary greatly in their removal rates, ranging from 7.5% to 88% (Le-Minh et al., 2012). It is also detected in surface waters due to incomplete removal in WWTPs. The measured concentrations are generally smaller, 3.6 ng/L have been measured for France (Tuc Dinh et al., 2011) and 30–85 ng/L for Germany (Hartig et al., 1999), but can rise to 4870 ng/L, as in China (Miao , et al., 2004). In the United States, Kolpin and his colleagues detected SMX in 13 of 104 water streams with a mean concentration of around 150 ng/L and a maximum concentration of 1900 ng/L. SMX was also detected in groundwater, and it was found at 12 ng/L in drinking water (Miao, et al., 2004,Schaider et al., 2014).

Approximately 85% of ingested SMX is metabolized in the human body and thus appears in urine and feces, whereas only 15% remain unchanged (David et al., 2002). Known metabolic products are N4-hydroxy-, 5-methylhydroxy-, N4-acetyl-, and N1-glucuronide-sulfamethoxazole (Vree et al., 1994). Based on its 30% excretion rate, it is able to enter water supplies in relatively large quantities (David et al., 2002). In some parts of the United States, levels of SMX in wastewater treatment plants' effluent have touched 3.25µg/L from its 18.3µg/L influent level (Vree et al., 1994). In residential, industrial, and agricultural waste effluent in Taiwan, SMX levels average 5.8 µg/L(Ye, et al., 2007), whereas in South Korea and Canada the levels in the effluent are lower at 193 ng/L and 516 ng/L, respectively (Bilal et al., 2018). SMX is not only prevalent in the water systems of these four relatively technologically advanced countries, but in many other countries as well, and in some cases, in higher concentrations. In 2022, Zdarta et al. were able to remove 40% of 10 mg/L of sulfamethoxazole using horseradish enzyme at pH 7 and at ambient temperature within 24 hours of reaction. By increasing the amount of SMX to 50 mg/L, the removal rate decreased to 15% (Zdarta J, et al., 2022).

2.2.2. Sulfamerazine

Sulfamerazine (SMR) or amino-N-(4-methyl-2-pyrimidinyl) benzene sulfonamide or N1-(4 methylpyrimidin-2-yl) sulfanilamide (Pareek, et al., 2013), a sticky, white, or creamy-

white crystalline powder at standard pressure and room temperature. Sulfamethazine is odorless and tastes slightly bitter. It is soluble in water to 0.76 mM. SMR is sensitive to light and may also be sensitive to heat (PubChem,2021, Chiong et al., 2016).

Sulfamerazine is a sulfonamide antibiotic, with pKa,1 (2.17) and pKa,2 (6.77)(Chiong et al., 2016). Sulfamerazine is a long-acting sulfanilamide antibacterial agent . It has a role as an anti-infective agent and a drug allergen. It is functionally related to sulfanilamide (PubChem, 2021). In the pharmaceutical industry, Sulfamerazine is a persistent organic pollutant. A series of transformation reactions occur after consumption, resulting in environmental oxidation, acetylation, and hydrolysis. There is evidence of its presence in rivers, lakes, groundwater, sediments, and the ocean even today, even though many countries have banned the use of sulfamerazine (Prasannamedha,et al., 2020).

Zhao Shu (2022) and his colleagues worked on degradation of sulfamerazine by horseradish peroxidase (HRP)- H_2O_2 This is an environmentally friendly and sustainable method, but efficiency is relatively low. In this study, the presence of bromide (Br^-) raised sulfamerazine (SMR) breakdown in the procedure of HRP/ H_2O_2. Due to its degradation efficiency, it is far from practical application (Wang et al., 2022).

2.3. Enzymatic treatment of water and wastewater

There are various techniques, including physical, chemical, and biological, which are widely used in advanced treatments to remove emerging pollutants, which will be briefly mentioned in this section. At the same time, there are potential limitations to these methods. In general, the formation of toxic side products and high sludge production, low removal efficiency, high operation cost and high energy consumption, long processing time, and the ability to use in low concentrations are among the problems that exist in the way of these methods (Rivera-Utrilla et al., 2013). Among the major problems of biological treatment methods, there is the possibility that microorganisms cannot grow in harsh environmental conditions and are not able to treat high concentrations of pollutants or need a long time for treatment (Lam, et al., 2005).

The use of green microalgae by Xiong et al. in 2018, was effective as a biological method to remove sulfamethazine and sulfamethoxazole, but the drawback of this method was its long time (12 days). About 62% of 0.25 mg/L of sulfamethazine and 27% of the same amount of sulfamethoxazole were removed by this mechanism (Xiong et al., 2019).

Biocatalytic processes as an alternative method to other physicochemical methods, which are also environmentally friendly, have many advantages(Almaqdi,et al., 2019, Morsi et al., 2020). These kinds of reactions are catalyzed by enzymes, especially under mild conditions. In the past few decades, research has been expanded on the ability of oxidoreductase enzymes to polymerize arylamines using H_2O_2 or O_2 as oxidants (Ćirić-Marjanović, et al., 2017). In 1984, Bollag and his colleagues were pioneers in the use of enzymes. They extracted the enzyme from the fungus *Rhizoctonia Praticola* and used it for the polymerization of 2,6-dimethoxyphenol (Bollag, et al., 1984). Klibanov and his colleagues in 1987, followed by using horseradish peroxidase for the polymerization of more than dozens of phenols and anilines in industrial wastewater, which was effective for some of these compounds with an efficiency of more than 99% (Dordick, et al., 1987). After that, extensive research has been done on the use of peroxidases in the treatment of a wide range of phenols and anilines, which are considered hazardous pollutants or have the potential to become pollutants in the future (ECs), by various regulatory agencies (Morsi et al., 2020). An enzyme can oxidize anilines and phenols to free radicals in the presence of an oxidant (hydrogen peroxide for HRP and oxygen for laccase). Oligomers can form non-enzymatic coupling. If these oligomers are soluble and have a phenolic or aniline group, they can be further oxidized by the enzyme and form higher oligomers. This process continues to form higher oligomers until the polymer reaches its solubility limit. Sedimentation and filtration methods can be used to remove possible sediments from the solution. In 2016, Xiao Zhang et al. used the enzymatic method to remove sulfamethoxazole by chloroperoxidase and activated sludge (Zhang, et al., 2016). Although this method was effective in removing this antibiotic, the disposal of the sludge produced was a limitation that reduced the effectiveness of this method (Ding et al., 2016). Wang et al. in 2022 by adding iodide ion (I^-) as mediator to improve the efficiency of HRP/ H_2O_2 significantly increased the degradation efficiency of SMR from 16% to 91%, but still, the

potential ecological risks of iodized products produced in the reaction process are one of the limitations of this method (Wang et al., 2022).

Among the advantages of biocatalytic remediation of contaminated water and wastewater, which is done using enzyme-based treatment, would be mentioned many things, such as the effectiveness of this method in a wide range of pH, salinity, and different temperatures. It also works with less input energy, without restrictions on the loading of either high or low concentrations of pollutants, ease of process control and high reaction speed, and produces less sludge (Al-Ansari, et al., 2011). There are some disadvantages of enzymatic treatment which this method may suffer. The main obstacles for its use on a large scale concern its cost and availability of the enzyme, which can be mitigated by advancement of engineering these days and novel resources and methods. Enzyme inactivation, and the possibility of forming hazardous by-products, to be discussed later, are drawbacks too (Steevensz et al., 2014a,b).

2.3.1. Oxidoreductase enzymes

The nonspecific nature of peroxidases makes them especially useful, i.e., for degrading many pollutants including anilines, herbicides, phenols, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls, among others (Bilal et al., 2018). There are two major types of peroxidases: heme peroxidases, which contain iron (III) in a prosthetic heme group, and non-heme peroxidases. Peroxidases can also be classified according to their origins. The comprehensive review by Pandey (Pandey, et al., 2017), shows that peroxidases are abundant in algae, bacteria, fungi, plants, and animals. They belong to the plant peroxidase superfamily, animal peroxidase superfamily, or catalase superfamily (Dunford, 1999). There are two superfamilies of heme peroxidases, the arcae superfamily and the animal superfamily. Based on their function and localization, peroxidases can be further classified as Class I, which is intracellular plant peroxidases. Class II which are extracellular fungal peroxidases, like lignin and manganese peroxidases and Class III or extracellular plant peroxidases which include soybean peroxidase (SBP) and HRP (Nurullah Şanli, et al., 2010).

Oxidoreductase enzymes (including oxidases, peroxidases, dehydrogenases, and oxygenases) catalyze the oxidation of a wide variety of pollutants, including anilines, phenols, synthetic dyes, personal care products, drugs, and many others (Zdarta J, et al., 2022, Mashhadi, et al, 2019, Steevensz, et al., 2014b).To degrade organic pollutants and emerging pollutants from water and wastewater, laccases and peroxidases are the most commonly used oxidoreductases (Zdarta J, et al., 2022, Morsi et al., 2020).

2.3.1.1. Laccases

Among the multi-copper oxidases, laccases catalyze the single-electron oxidation of a substrate along with the reduction of oxygen into the water. It has been reported that laccases can transform a wide variety of ECs enzymatically(Zdarta J, et al., 2022, Morsi et al., 2020). It has been shown that laccase is able to remove quantitatively many types of sulfonamide antibiotics, including sulfadiazine, sulfathiazole, sulfapyridine, sulfamethazine, and sulfamethoxazole (Ding et al., 2016). A study by Naghdi et al. (2018) demonstrated the efficient enzymatic removal of carbamazepine, widely used in pharmaceuticals, utilizing laccase and redox mediators to catalyze it from synthetic wastewater (Naghdi et al., 2018). According to another study, 8-hydroxyquinoline is polymerized enzymatically, by laccase from the white rot fungus *Trametes pubescent* (Goodwin, 2010). Three coumarin derivatives, 4-methyl-7,8-dihydroxy coumarin, 7,8-dihydroxy-4-phenylcoumarin, and 7,8-dihydroxy coumarin, were also oxidatively coupled using laccase (Wang et al., 2022). Also, 5 mg/L of diclofenac, trimethoprim, carbamazepine, and sulfamethoxazole was degraded by laccase from *Trametes versicolor* investigated by Alharbi (Sultan K, et al., 2019). Barrios and his team provided a comprehensive review of a number of studies of laccases used for treating endocrine disruptors (EDs) and estrogenic compounds in-vitro (Barrios-Estrada et al., 2018). An additional study evaluated the efficacy of biocatalytic elimination of 30 trace organic contaminants, including pharmaceuticals, such as carbamazepine and primidone, steroid hormones such as estriol and estrone, and personal care products were studied in an enzymatic

membrane reactor equipped with an ultrafiltration membrane (Nguyen et al., 2015). In addition, the degradation of the endocrine-disrupting chemicals nonylphenol and Bisphenol A, as well as the personal care product ingredient, Triclosan by laccase from the white rot fungus *Coriolopsis polyzoan* was studied (Garcia-Morales, et al, 2015). Additionally, Spina and his colleagues , investigated the degradation potential by a fungal laccase for some pharmaceuticals and personal care products at a wide range of concentrations from ng/L to μg/L in synthetic wastewater and real wastewater collected from an urban wastewater treatment plant (Spina et al., 2015).

2.3.1.2. Peroxidases

Due to the ability of peroxidases in wide biotechnological applications, they have been used in catalyzing the oxidation-reduction reaction of a wide range of aniline and phenolic substrates, non-phenolic aromatics, dyes, and aromatic compounds in wastewater and removing these environmental pollutants from wastewater. (Mashhadi, et al., 2019). Almaqdi studied treatment of more than 20 emerging pollutants, including non-steroidal anti-inflammatory drugs, some antibiotics, including sulfamethoxazole, norfloxacin and trimethoprim, herbicides, and fungicides, was made possible by 5 different peroxidases with or without 1-hydroxybenzotriazole as a redox mediator(Almaqdi, et al., 2019). In the past decades, studies related to HRP show that compared to soybean peroxidase (SBP), the extraction and purification of this enzyme on a large scale is not cheap (Al-Ansari, et al., 2011). Also, the temperature stability range is more limited, and the pH range is lower (from 4 to 8) for HRP compared to the range of 2 to 10 for SBP (Flock, et al., 1999).

2.3.1.3. Soybean peroxidase

Due to its appealing biological properties, SBP is receiving a lot of attention (Al-Ansari et al., 2009). SBP, in comparison to HRP, has shown to be a more potent peroxidase based on the specificity constant (k_{cat}/K_M), Michaelis-Menten parameter, less susceptibility to irreversible inactivation byH_2O_2, lower potential

cost, greater availability and higher thermal and conformational stabilities (Al-Ansari et al., 2009, Steevensz, et al., 2014b). There are a range of SAs that can be oxidized with SBP. Enzymatic treatment of sulfamethoxazole, a heterocyclic antimicrobial agent, along with other antimicrobials, phenolic steroids, and phenolic surfactants as micropollutants was also studied by using SBP(Mashhadi et al., 2019). Figure 2-3 (a) illustrates the 3-D structure of SBP; Figure 2-3 (b), shows the ferriprotoporphyrin IX (heme) prosthetic group, which is the redox center of the enzyme.

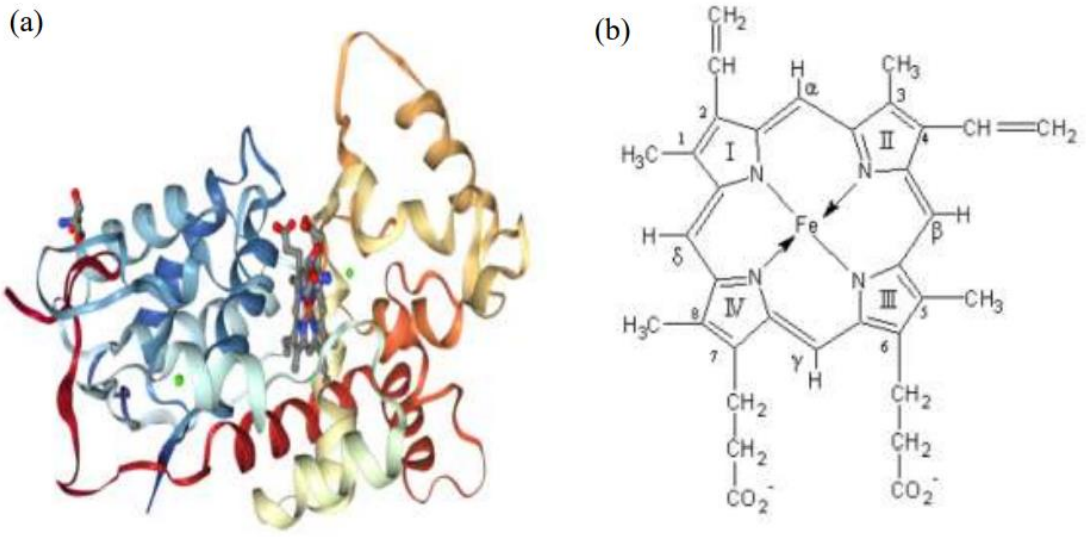


Figure 2-4 a) 3-D structure of SBP (generated from RCSB-Protein Data Base, category no. 1FHF, 2020) and (b) ferriprotoporphyrin IX (heme) prosthetic group adopted from (Al-Ansari, et al.2011)

There are studies on some substrates which were treated by soybean peroxidase, Table 2-2.

Table 2-2 Removal efficiency and optimal parameters of enzymatic treatment of previously studied of SBP

Substrate	Substrate concentration (mM)	Optimal conditions			Removal %
		pH	SBP activity (U/mL)	H_2O_2 (mM)	
2-Aminobenzimidazole	0.5	7.0	3.0	0.75	77
3-Aminopyrazole	1.0	6.0	3.0	1.50	70
Hydroxybenzotriazole	1.0	3.6	0.12	1.25	≥95
2-Aminothiazole	1.0	6.0	4.0	2.00	84
2-Aminobenzothiazole	0.5	7.0	4.5	0.75	30
2-Aminoimidazole	0.5	8	1.5	1.00	93
4-Aminoantipyrine	0.5	7.5	0.1	1.00	87
3-Hydroxyquinoline	0.5	8.6	0.1	1	≥95
3-Aminoquinoline	1.0	5.6	4.5	2	89
Indole	1.0	1.6	0.45	1.25	≥95
Pyrrole	1.0	1.6	5	1.00	85
3-hydroxycoumarin	0.5	7	0.002	0.75	95
2-aminobenzoxazole	0.1	6	3.5	0.25	45

Adopted from (Ziayee Bideh, 2020)

2.4. Peroxidase mechanism

Secretory Class III plant peroxidases include HRP, SBP, turnip peroxidase (TP), bitter gourd peroxidase (BGP), potato pulp peroxidase and ginger peroxidase (Pandey, et al., 2017, Jun et al., 2019).

A wide range of organic and non-organic chemicals are catalyzed by peroxidases using hydrogen peroxide. Through a three-step oxidation reduction cycle illustrated below, peroxidases catalyze hydrogen peroxide degradation through an irreversible ping-pong mechanism(Dunford, 1999, Pandey, et al.2017).

$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + RH \longrightarrow \text{Compound II} + R^{\circ} \quad (2)$$

$$\text{Compound II} + RH \longrightarrow \text{Peroxidase} + R^{\circ} + H_2O \quad (3)$$

$$2RH + H_2O_2 \longrightarrow 2R^{\circ} + 2H_2O \quad (4)$$

A cycle begins by reducing hydrogen peroxide to water and oxidizing native enzyme to Compound I, the active form of the enzyme with two electron-equivalents above native peroxidase. By one-electron oxidation of RH to a free radical with loss of a proton, compound I is reduced to compound II in the presence of a reducing substrate (RH). The enzyme returns to its resting state when compound II oxidizes another reducing substrate molecule into a free radical(Dunford, 1999). Dimers can be generated by coupling nonenzymatically free radicals. The dimers, if phenolic or anilino and still in solution, can undergo further peroxidase cycles to form oligomers which eventually become insoluble. Filtration or sedimentation can be used to remove the precipitated oligomers(Villeg, et al., 2016).

Figure 2-5 shows the mechanism of enzymatic reaction of SBP.

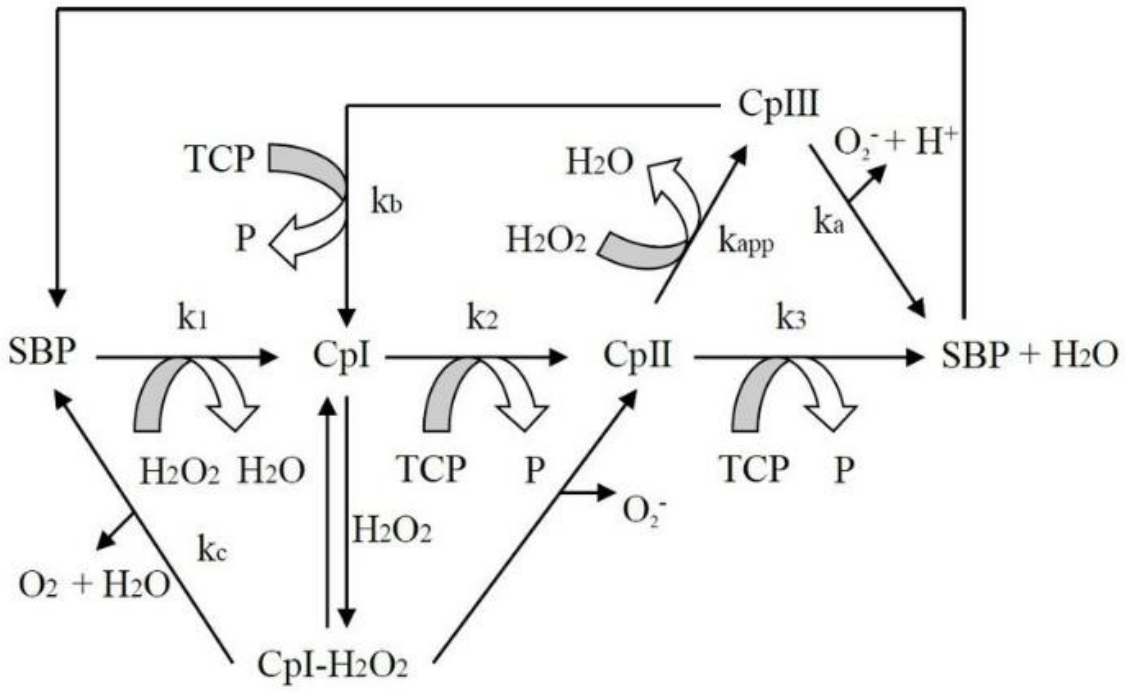


Figure 2-5 Mechanism of enzymatic reaction of SBP adopted from (Cunha, et al., 2021)

2.4.1. Peroxidase inactivation

In the presence of excess hydrogen peroxide as well as low concentration of reducing substrate, peroxidases can suffer from inactive suicidal pathways (Steevensz, et al., 2014). A reversible intermediate (compound I H_2O_2) generated in the first pathway can be further converted into a non-active intermediate P-670 (after opening the cyclic heme structure, with absorption peak at 670 nm). Secondly, compound III (a reversibly inactive form of the enzyme) accumulates in the presence of extra H_2O_2 through the oxidation of compound II. While compound III can be degraded to the enzyme's native form, it happens very slowly (Valderrama et al., 2002).A third mechanism is the apparent inactivation of peroxidases via the adsorption of the active enzyme onto oligomers of the solid end product, as demonstrated by Feng (Feng, et al., 2013). As a final point, free radicals produced from the substrate can also bind to the enzyme's active site(Klibanov et al., 1983).

2.4.2. Use of a Redox Mediator

It is well established that most peroxidases are able to oxidize a wide range of organic substrates in the presence of hydrogen peroxide. However, in some cases, peroxidases also require a small, diffusible, and easily oxidized compound known as a "redox mediator" to facilitate the oxidation-reduction reaction(Husain, et al., 2007, Van der Zee et al., 2009). Bourbonnais and his colleagues first described the use of mediators. These redox intermediates allow peroxidases to oxidize non-phenolic compounds and recalcitrant phenolics and anilines too, hope(Camarero et al., 2005). As a result, the range of substrates that can be oxidized by these enzymes is expanded by use of redox mediators. In general, the mechanism of action of redox mediators is that they are the reducing substrate for the enzyme to become the mediator radical. The mediator radical then undergoes non-enzymic radical transfer with the target compound, resulting in the target radical and the starting mediator. Low molecular weight diffusible redox mediators offer high oxidation and reduction potentials (>900 mV) to counteract recalcitrance of the target substrate.

Alneyadi and his team showed that in initial degradation experiments with Sulforhodamine B (SRB) dye neither SBP nor chloroperoxidase (CPO) was able to degrade SRB at all in the presence of H_2O_2 alone. However, when 1-hydroxy benzotriazole (HOBT), one of the most commonly used redox mediators, was added to the reaction mixture, rapid and efficient SRB dye degradation was observed. HOBT concentration was optimized for efficient SRB degradation. Higher concentrations of HOBT (beyond the optimum) caused a significant reduction in SRB degradation by both SBP and CPO. This was most likely because HOBT at higher concentrations may compete with SRB to bind to the active site of the enzymes, and hence lead to a slower degradation rate(Alneyadi et al., 2017).

Initial experiments using SBP + H_2O_2 alone showed degradation of SMX at pH 1.6, which is not desirable because of the drawbacks that exist in providing this pH in wastewater treatment plants. HOBT was chosen because it is one of the most common redox mediators used by others.

2.5. Cost and availability of soybean peroxidase

According to USDA, global soybean production and use of U.S. soybeans will change for 2022/23, with higher beginning stocks and lower production, crush, exports, and ending stocks. It is projected that soybean production will be 4.4 billion bushels, down 152 million with lower harvested area and yield. Harvested areas are down 0.6 million acres from the August prediction. The soybean yield anticipation of 50.5 bushels per acre is down 1.4 bushels from July. In both North and South America, soybeans are one of the most important oilseed products, Figure 2-3. Since 2000, Canadian soybean production and seeded areas have steadily increased, according to Agriculture and Agri-Food Canada (2022), although soybean production is expected to fall in 2023 due to a decrease in planted areas and difficult growing and harvesting conditions in Western Canada. Compared to $419/t last year and $430/t over the past five years, prices ended the crop year sharply higher at $605/t (Agriculture and Agri-Food Canada - Agriculture.Canada.Ca, n.d.) . It is estimated that soybean hulls, from which SBP can be extracted, cost $125 per ton (Mukherjee et al., 2018). Due to its widespread availability and affordability, the hulls could be a viable commercial source of SBP.

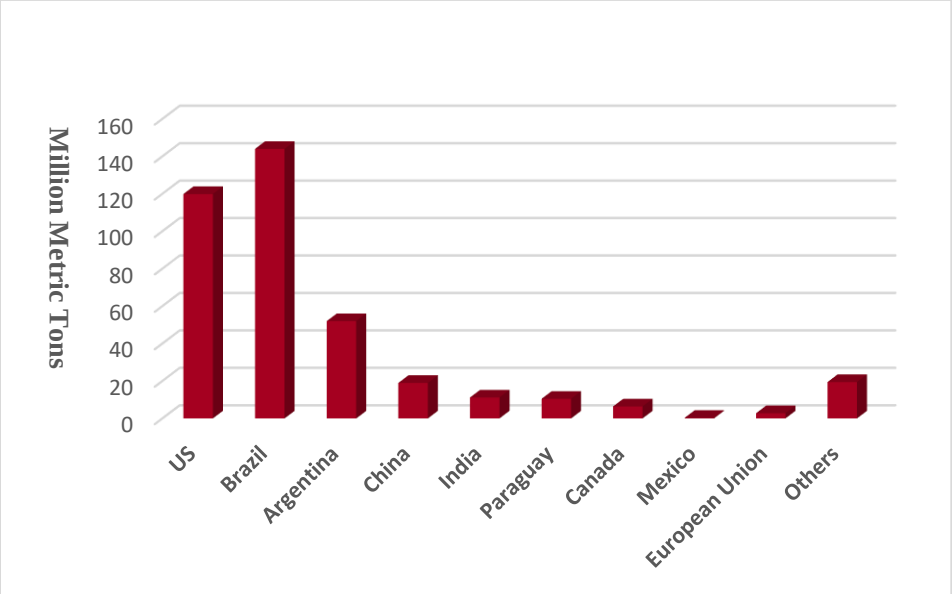


Figure 2-6 global soybean production 2021 according to USDA (2022)

2.6. Soybean peroxidase extraction

SBP extraction is a process carried out with soybean seed coats (hulls). Hulls are obtained as a by-product of crushing these seeds, which are also used for animal feed. Soaking dry soybeans in water for 1-2 hours is the start of the extraction process, so the husk/husk of the seed becomes softer and crumbly. At this stage, SBP is extracted by washing the hulls without reducing the nutritional value of them (nutritional value is in the hull fiber). Especially noteworthy, SBP activity has been reported to vary among cultivars and seed coverage(Steevensz et al., 2014).

CHAPTER 3

METHODS AND MATERIALS

3.1. Materials

3.1.1. Enzymes

Crude solid SBP (E.C. 1.11.7, Industrial Grade Lot No. 18541NX) from Organic Technologies (Coshocton, OH) was purchased. The solid bovine liver catalase was obtained through Sigma Aldrich Chemical Company Inc. (Oakville, ON). A temperature of -15 °C was used to store both enzymes. Novo ARP (Arthromyces ramosus peroxidase) was donated by Novozymes Inc. (Franklinton, NC) and 4 °C was the optimum temperature to keep Novo ARP.

3.1.2. Sulfa drug compounds

Both sulfamethoxazole (SMX) and sulfamerazine (≥99% pure) (SMR) were procured from Sigma Aldrich (Oakville, ON).1-Hydroxybenzotriazole hydrate ≥97.0% (T)(HOBT) was purchased from Sigma Aldrich (Oakville, ON). SMX and SMR were stored at room temperature and HOBT was kept at 4°C.

3.1.3. Buffers and solvents

Citric acid, sodium citrate, hydrochloric acid, potassium chloride, monobasic sodium phosphate, and dibasic sodium phosphate were provided by ACP Chemicals Inc. Tris (hydroxymethyl)-aminomethane was provided by Sigma. Buffer standards (pH 4, 7, and 10) were obtained from ACP Chemicals Inc and buffer standard pH 1.68 was from Hanna Instruments (Newmarket, ON).

3.1.4. Other chemicals

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

3.1.5. Chromatography solvents, columns, and filters

The procurement HPLC grade methanol was done from ACP Chemicals Inc. HPLC-grade water ordered and bought from Waters Co. (Mississauga, ON). HPLC-grade acetonitrile (ACN) was obtained from Fisher Scientific Company (Ottawa, ON). Gemini C18 Column, 110Å, 5 µm, 4.6 mm *100 mm was purchased from Phenomenex (Torrance, CA). Syringe filters (0.2 µm pore size) were obtained from Sarstedt (Montreal, QC). Syringes (10 mL), Corning plastic centrifuge tube (50 mL), plastic disposable transfer pipets (7.5 mL) and various magnetic stirring bars were purchased from Fisher Scientific Company (Ottawa, ON). Clear glass vials (crimp top, volume 30 mL) were obtained from Sigma Aldrich Chemical Company Inc. The Pipetman variable volume pipettes (50-200 µL, 200-1000 µL, 1-5 mL) were purchased from Mandel Scientific (Guelph, ON). Pipette tips (100 µL, 1000 µL) were purchased from VWR International Inc. (Mississauga, ON) and 5 mL pipette tips were purchased from Sarstedt.

3.2. Analytical methods

3.2.1. Buffer preparation

In accordance with Gomori , different buffers ranging from 1-10 pH were prepared(Gomori, 1955). The Hydrochloric Acid-Potassium Chloride Acid buffer was prepared to be used in the range of pH 1.0-2.2. A citrate buffer was prepared for a pH range of 3.0 - 6.0. A monobasic-dibasic sodium phosphate buffer was made to be used in the pH range of 6.0 -8.0. Tris (hydroxymethyl)- aminomethane-HCl buffer for the pH 9.0 was provided.

3.2.2. Enzyme stock solution preparation

In order to prepare SBP stock solution, 1.4 g of solid enzyme was mixed with 100 mL of distilled water at a low speed (approximately 400 rpm) for a day (24 hours). A centrifuge at 4000 rpm was used for 25 minutes to centrifuge the solution. The supernatant was taken as the stock solution and was stored at 4°C. For the preparation of catalase stock solution, 0.5 grams of solid catalase were mixed with 100 mL of distilled water for four hours. Stock solutions were stored at 4 °C too.

3.2.3. Enzyme activity assay

A colorimetric assay, reported by Ibrahim et al., was applied to determine the SBP activity. The activity of enzymes is measured in standard catalytic units (U/mL). 1.0 U is equal to the proportional to the enzyme concentration required to convert 1.0 μmol of hydrogen peroxide per minute at pH 7.4 at room temperature. The starting rate of this reaction is used for SBP activity and measured by a built-in kinetic rate calculation function in the UV-Vis spectrometer (Appendix A). The enzyme reagent assay was performed using a 10X phenol reagent prepared in a buffer containing 100 mM phenol and 0.5 M phosphate buffer (pH = 7.4). To prepare the enzyme reagent assay, 5 mL of 10X phenol, 25 mg of solid 4-AAP, and 0.2 mM H_2O_2 were made up in a 50 mL volumetric flask prior to the enzyme activity assay (Caza et al., 1999). Reagents were prepared and diluted SBP (dilution factor 50) was used as the reaction solution. To begin with, the equipment was blanked with 950 μL of reagent and 50 μL of distilled water. Following this, the 950 μL of reagent mixture was immediately added to 50 μL diluted SBP solution in the cuvette. Accordingly, the increase in absorbance over 30 seconds run time and 5 seconds cycle time was recorded, and the activity was calculated through built-in instrument software using zero-order kinetics. Additional information can be found in Appendix B. A 30-second absorbance measurement is performed by mixing the contents and monitoring the change in absorbance at 510 nm. The dilutions were designed so that their absorbances remained below 1. For the enzyme activity test performed after enzymatic treatment of substrates, if required, the sample size was increased accordingly to generate at least a 0.2 change in absorbance. This can be done by mixing 100 μL sample with 900 μL appropriately reformulated reagent or if not sensitive enough, by mixing 200 μL sample with 800 μL appropriately reformulated reagent. The final concentration of each component of the reagent was kept constant based on the sample size used. Appendix A provides more information about this assay.

In colorimetric assay, the following reaction occurred:

4-AAP + phenol H_2O_2 SBP Pink chromophore (λ_{max} = 510 nm)

SBP catalyzes the oxidative coupling of phenol and 4-AAP in the presence of H_2O_2, generating a pink chromophore (extinction coefficient ε = 6000 $M^{-1}cm^{-1}$ relative to H_2O_2).

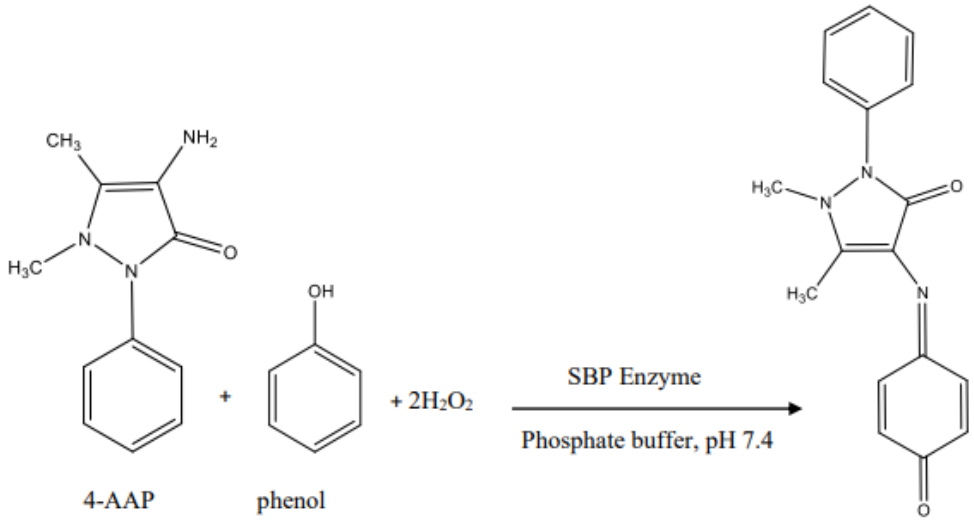


Figure 3-1Oxidative coupling of phenol and 4-AAP in the presence of H_2O_2 and SBP adopted from (Mashhadi,et al., 2019)

3.2.4. Residual enzyme activity assay

A residual enzyme activity test is conducted after the substrate has been enzymatically treated in order to determine the remaining enzyme activity. Residual enzyme activity tests work in a similar way to regular enzyme activity tests. In the case of a lower enzyme concentration, following the enzyme reaction, reagent concentration and mixing ratio were adjusted to maintain a constant concentration of each component. More information about this assay is provided in Appendix C.

3.2.5. Residual hydrogen peroxide assay

After enzymatic treatment, residual hydrogen peroxide was determined using a similar colorimetric method (Caza et al., 1999). As a reagent, phenol and Novo

ARP liquid concentrate at pH 7.4 were used. By adding the reagent to the sample, phenolic radicals form from the reaction with ARP and peroxide, couple with 4-AAP and produce a chromophore (same chromophore as in enzyme activity test) of 510 nm (maximum absorbance). The absorbance of the solution after this reaction (18 minutes) was measured and compared to a hydrogen peroxide calibration curve to determine the residual concentration of hydrogen peroxide. Detailed information regarding this assay is available in Appendix D.

3.3. Analytical equipment

3.3.1. UV-Vis spectrometry

A UV-Visible spectrophotometer (UV-Vis; Agilent (Mississauga, ON) model 8453) with λ range of 190 -1100 nm and 1 nm resolution controlled by a Hewlett Packard Vectra ES/12 computer was used to measure the maximum wavelength of compounds, to quantify the concentration of compounds by measuring the corresponding absorbance (Beer Lambert Law), and to test SBP enzyme activity. Quartz glass cuvettes (10mm optical light path, 1000 µL volume) were purchased from Hellma Canada Ltd. (Markham, ON). The spectroscopy of the two compounds is provided in Appendix A.

3.3.2. High performance liquid chromatography (HPLC) analysis

Following the guidelines laid out by the experiment performed by Ziayee (Ziayee Bideh, 2020), the curves were developed using a Waters (Oakville, ON) HPLC system with binary pumps, model 1525, dual wavelength absorbance detector, model 2489, and auto-sampler, model 2707, connected to a computer and operated by Breeze 2.0 software, in addition to quantifying residual substrate concentrations after enzymatic treatment (p.27).

Also following the previous experiment, “a gradient method with 5 µL injection volume was reproduced for SMX”(Al-Maqdi et al., 2018). The UV-detector was calibrated with reference to the predetermined λ_{max}. The mobile phase ratios, flow rate, column temperature and type of column used are given in Table 3-1. Solvent

A was 0.1% Formic acid, and Solvent B was Acetonitrile. Again, these parameters replicate the previous experiment by (Al-Maqdi et al., 2018)

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

Substrate	Mobile phase ratio		Flow mL/min	λmax nm	Column
sulfamethoxazole	Pump A	Pump B	1	265	Gemini C18
	30% formic acid (0.1%)	Acetonitrile			
sulfamerazine	30% formic acid (0.1%)	Acetonitrile	1	275	

3.3.3. Mass spectrometry

MS analysis was carried out by Dr. Jiaxi Wang, (Queen's University, Kingston, ON) using a Thermo Scientific Orbitrap Velos Pro (Easy-nLC/HESI Hybrid Ion Trap-Orbitrap Mass Spectrometer) or an Agilent AdvanceBio 6545XT LC/QTOF (1260 Infinity II LC APCI/ESI Quadrupole Time of Flight Mass Spectrometer) in high-resolution mode. Data acquisition was performed either in the positive- or negative-ion mode. The acquired mass spectra were subjected to qualitative analysis for molecular formulae targeting possibly formed oligomers and oligomer derivatives with 10 ppm difference (between measured and calculated masses) used as the cut-off for unambiguously linking a given mass to a specific chemical formula. In ESI, the analyte is pumped to a capillary and a high voltage is applied which makes the droplets spray from the tip of the capillary and evaporate. The evaporation process is also supported by heat and a nebulizing gas, generally nitrogen. The gas-phase ions then enter the mass spectrometer detection. The acquisition range of probe was 50 to 1500 mass-to-charge ratio (m/z).

3.3.4. pH meter and centrifuge

The Oakton pH/con700 benchtop meter (pH range -2.0 to 16.0, accuracy ±0.01pH) and its Thermo Orion pH probe (9110DJWP, semi-micro tip, double junction, glass body) were purchased from Oakton Instruments (Vernon Hills, IL). The calibration solutions pH 4.0, 7.0 and 10.0 were obtained from ACP Chemicals Inc., and pH 1.68 was purchased from Hanna Instruments (Laval, QC). The centrifuge was a Corning LSETM Compact Centrifuge (6×50 mL and 6×15 mL centrifuge tubes, 200 to 6000 rpm speed range (Tewksbury, MA)

3.3.5. Other instruments

Again, instrumentation for this experiment follows the design of the previous experiment by Ziayee Bideh (2020).

A LSE compact centrifuge was obtained from Corning Company (New York, USA). The centrifuge conditions are defined in the respective experimental protocols. An Oakton PC 700 pH meter with range of 0.00 to 14.00 connected to a Thermo Scientific Orion pH Probe (9110DJWP, refillable/double junction/glass/semi-micro) with ± 0.02 pH accuracy was used (Vernon Hills, IL). Various magnetic stir bars in different sizes were obtained from Cole-Parmer Canada Inc. (Montreal, QC). The magnetic stirrers from VWR international Inc. (Mississauga, ON) with 0-1100 rpm and 100-1500 rpm were used for mixing. Vortex mixer model K-550-G was purchased from Scientific Industries Inc (New York, USA) (Ziayee Bideh, 2020, p. 30).

3.4. Experimental protocols

3.4.1. Enzymatic oxidation of substrate with SBP and feasibility of treatment of target anilines

At room temperature, in covered vials (to prevent the effects of light), the enzymatic reactions were carried out in 20 mL batch reactors, in triplicate, and at room temperature, to achieve 95% removal (with the exception of pH optimization). The 20 mL reaction medium in batch reactors consisted of 40 mM buffer, 0.08 or 0.20 mM of the substrate, SBP, and hydrogen peroxide in concentrations appropriate to

the substrate. To preserve the order of addition of reaction components to start the reaction, hydrogen peroxide was added to the solution last. After mixing the solution for 3 hours using Teflon-coated magnetic stirrer bars on magnetic stirring plates, the reaction was stopped by adding 100 μL catalase stock solution (0.5 g/100 mL) to quench the reaction by immediately using up the remaining hydrogen peroxide. The sample was then microfiltered with pre-conditioned, 0.22 μm PES syringe filters, and the residual substrate concentration was analyzed by HPLC.

The substrate conversion over time was also monitored through time-course experiments using optimized parameters. A total of three batch reactors in bigger size with a volume of 200-300 mL were used, and samples (5 mL) were taken at selected time intervals, quenched with catalase, and vortexed to stop the reaction at once. Later, samples were filtered and analyzed by HPLC. As a control for each set of batch reactors, different control batch reactors were also run, formulated the same as the sample but without either hydrogen peroxide or SBP to determine the effect of enzyme or hydrogen peroxide on the substrate, respectively. Preliminary experiments were conducted to specify whether the selected sulfa drug compounds are substrates for SBP. Batch reactors with adequate enzyme and hydrogen peroxide along with sulfonamide compound and buffer were stirred for 3 hours and the reaction was then stopped by adding catalase, microfiltered, and analyzed for the residual concentration by UV-Vis and/or HPLC.

3.4.2. Optimization of important enzymatic reaction parameters

In order to achieve the removal of 95% of the target compound, experiments were conducted to define the pH and the minimum effective concentration of SBP as well as the concentration of hydrogen peroxide in the optimal state. As the first step in pH for range finding, and optimization, some preliminary experiments were conducted. It helped to find Then, the experiments were repeated with shorter pH intervals and smaller ranges. The reactions were conducted at different pHs using various buffers under stringent conditions (SBP was limited), therefore the effect of pH can be clearly monitored. 32 Later on, at optimal pH, the reactions were formulated for peroxide optimization and minimum effective concentration of SBP

to reach 95% removal. If 95% removal was not obtained, residual peroxide and residual SBP tests were conducted to determine the residual amounts of peroxide and enzyme in order to reformulate the reactions to reach 95% conversion.

3.4.3. Stepwise addition of H_2O_2

For a substrate with less than 95% removal efficiency, stepwise addition of hydrogen peroxide was tested because SBP is likely to be inactivated irreversibly by addition of excess hydrogen peroxide (Valderrama et al., 2002). Optimal peroxide concentration established previously was divided into 4 equal aliquots and added in intervals, every 45 minutes, to reach the same final concentration. The reaction was stirred for 3 h in total. Half of the reaction mixture was then microfiltered for hydrogen peroxide residual test and catalase was added to the other half to stop the reaction. Later, the reaction mixture was filtered, and the removal efficiency was determined using HPLC.

3.4.4. Preliminary determination of enzymatic treatment products using mass spectrometry

For identification of plausible polymerization products of enzymatic treatment, reaction mixtures under established conditions were analyzed. The experiments were conducted in batch reactors and then, the mixtures were centrifuged for 20 minutes at 4000 rcf and the supernatant and precipitate (if any) along with the standard sample were run on the mass spectrometer with ASAP or ESI method. Since buffers produce interferences with product peaks in MS, the concentration of buffer in batch reactors was reduced to 10 mM for MS samples. MassLynx V. 4.1 software was used for data analysis.

3.5. Sources of error

There is a level of uncertainty in every analysis. The accuracy and reliability of the results are affected by inevitable systematic or random errors that can happen during the experiments. Symmetric errors are caused by analytical methods and are predictable and usually constant, whereas random errors, that are always present, can show different results for the same repeated measurement. Random errors can be minimized not avoided. In order

to minimize random errors, experiments were conducted in triplicates and standards were injected in the HPLC along with the samples. All Figures show the standard deviation of each triplicate data set as well as the average. For the data points with very small deviation (<1%), the error bars may not visible since they could be hidden by the icons. The SBP activity test using freshly prepared reagents was conducted every day prior to the experiments since the activity is affected by the temperature. Equipment such as pipets, analytical balance and pH meter were calibrated to avoid systematic errors.

CHAPTER 4

RESULTS AND DISCUSSION

An initial study was conducted on the feasibility of SBP-catalyzed transformation of two selected sulfa drugs (SAs), sulfamethoxazole and sulfamerazine. In both cases, the compounds served as substrates for SBP.

In order to reach 95% conversion efficiency, three operational parameters were optimized for each substrate: pH, H_2O_2 concentration, and minimum effective enzyme activity. Based on optimal parameters, time-course experiments were carried out to specify the time to achieve 95% conversion and to determine the initial first-order reaction rate constants and half-lives of substrates. The final step in the process was to identify possible products using mass spectrometric analysis.

4.1. Parameter optimization of SBP-catalyzed treatment

4.1.1. pH Optimization

Among the major biocatalytic parameters affecting SBP activity and stability is the pH of the reaction medium, which also affects enzymatic treatment efficiency and industrial application. Enzymes are biological catalysts. They have dynamic polymeric structures and contain ionizable side chains of amino acid residues that are responsible for both structural and catalytic functions. pH changes affect enzyme structure through ionization state of amino acid side chains participating in acid-base reactions and/or structural bridges (Al-Ansari, et al., 2011). Two key catalytic amino acid residues, distal histidine-42 (His42) as proton acceptor from hydrogen peroxide, and distal arginine-38 (Arg-38) as the charge stabilizer (Dunford, 1999), require an optimum pH that satisfies their ionization states. Additionally, the interaction of amino-acid residues with heme influences the 3-dimensional structure and conformational stability of SBP(Al-Ansari, et al., 2011). Generally, the pH-dependent activity of soybean peroxidase is reversed when exposed to weak acids and bases and the enzyme can regain its activity when shifted back to the ideal pH. But some changes in enzyme structure can be permanently irreversible, and the enzyme is unable to recover its maximum activity. Moreover, the ionization state of the reducing

substrate and the electron transfer rate could be affected by the pH of the reaction medium(Parsiavash et al., 2015). The relative activity of SBP in different pH buffers ranging from 2.0-10.0 was investigated by Geng to find that SBP was active over an extensive range of pH in the case of tetra-guaiacol formation showing its maximum activity between pH 5.5 and 6.0 (Geng et al., 2001). Due to the existence of a more solvent-exposed heme edge in the structure, the catalytic activity of SBP depends on pH as well (Kamal et al., 2003). These results gave an indication that SBP from seed hulls has significant activity over a wide pH range which enables it to oxidize a wide range of substrates. At 25 °C, SBP exhibits maximum catalytic activity for ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6 sulphonic acid)) at pH 5.(Kamal, et al., 2003). In addition, SBP remained active between pH 3.0 and 10.0 for 3-substituted quinolines(Mashhadi et al., 2019). According to this work, optimal pH range is defined as the range of pH in which substrate conversion is not less than 5% of the maximum transformation. In addition, the optimum pH value should satisfy amino acids' ionization state condition thereby providing the best conformational state for the enzyme for catalysis (Kamal, et al. , 2003)

The different substrates were studied in different buffers, pH range 1.5-10.0, in the UV-VIS to determine maximum absorbances at 265 and 275 nm for SMX and SMR, respectively, irrespective of the buffers used. To determine the optimum pH with the most removal of substrate, batch reactors were conducted under stringent conditions with an insufficient amount of SBP, resulting in incomplete substrate removal. In these initial experiments, hydrogen peroxide concentration was generally chosen as 2 times the molar concentration of the substrate, based on previous experiments performed in the lab. The substrate concentration was 0.2 mM for SMX and 0.1 mM for SMR, and experiments were run in triplicate at room temperature (22±3 °C) for 3 h. Two control experiments were carried out: one without SBP, and another without H_2O_2. The error bars on all figures illustrate the standard deviation; for the data points with exceedingly small deviations, error bars are not visible. Figure 4-1 illustrates the effects of pH on sulfamethoxazole conversion. Preliminary range finding was conducted for a wide range of pH (1.0-10.0) using UV-Vis (data not shown). Subsequent analysis by HPLC with a wide pH range 1.0-9.0 and 1.0 and 3.0 U/mL SBP, Figure 4-1, showed SMX was best transformed around pH 2.0 (33% remaining for 3.0 U/mL).

Repeating the experiment over a narrower pH range and more data points, Figure 4-2, showed the optimum pH to be 1.6 which showed 22% remaining for 3.0 U/ml SBP. Thus, acidic pH is required for SMX enzymatic conversion. For SMX, the change around the optimal pH (1.6) was found to be around 5% Thus, presumably as a result of chemical oxidation, SMX in the presence of H_2O_2, always showed 4-6% removal.

It is well known that SBP has a bell-shaped pH-dependence curve and is active from 2.0 to 9.0 (Kamal, et al. , 2003). Despite its lower catalytic activity at acidic pHs, SBP is still capable of catalyzing substrate conversion at these low pHs. It may be possible to remove contaminants effectively by adjusting the wastewater's pH to a very acidic state during such treatment, however, the cost of making such adjustments during treatment, and readjusting it afterward, may not be sensible for a given site. As a result, even a small amount of enzyme cannot effectively convert the substrate. An increase in the remaining percentage of the substrate on either side of optimum pH could be explained by the lower stability of Compound I (the active form of enzyme) in acidic solutions (Geng et al., 2001). Furthermore, the bell-shaped pH dependence indicates that the catalytic residues of the enzyme and/or substrate ionization states play a role (Kamal, et al., 2003); the lower apparent pK_a of the bell representing a group that must be in its conjugate-base form, conversely for the higher apparent pK_a.

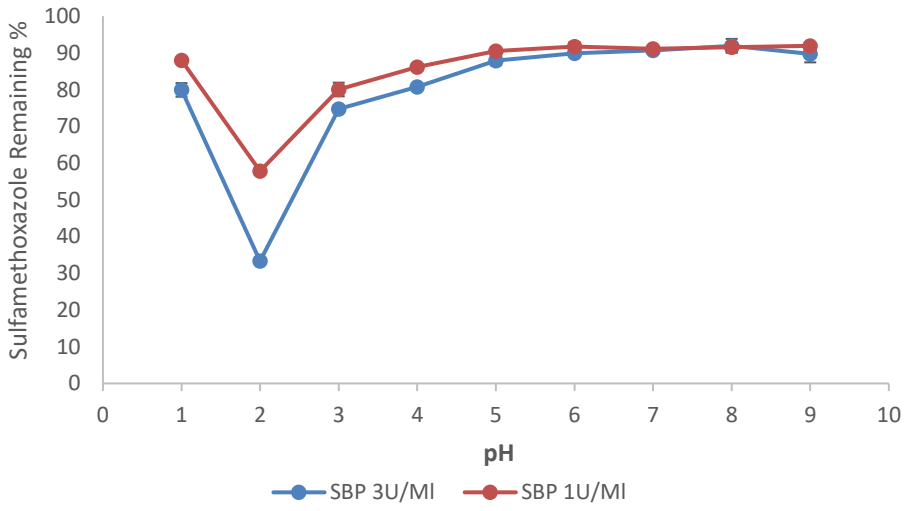


Figure 4-1-1 pH optimization for sulfamethoxazole (wide pH range)

Conditions: 0.20 mM SMX, 40 mM buffers, 3 U/mL, and 1 U/mL SBP, 0.40 mM hydrogen peroxide, 3-h reaction, analysis by HPLC.

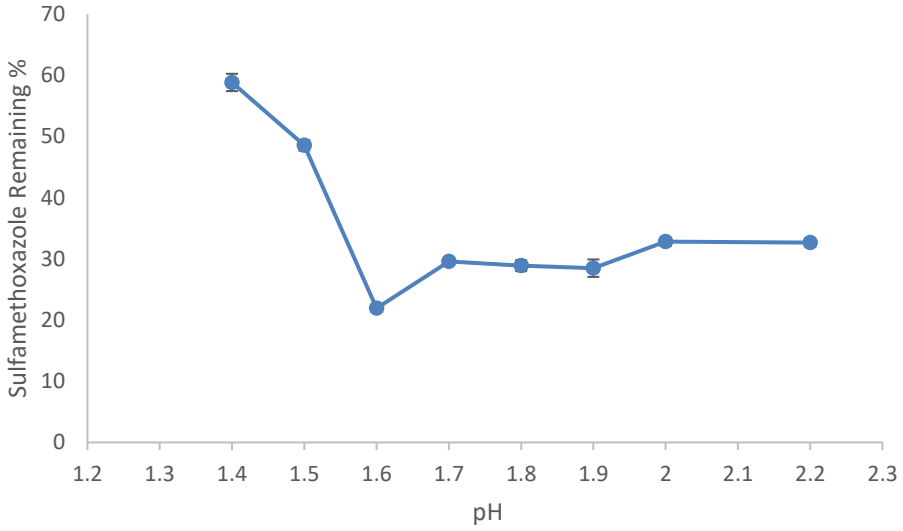


Figure 4-1-2 pH optimization for sulfamethoxazole (narrow pH range)

Conditions: 0.20 mM SMX, 40 mM buffer,3 U/mL SBP, 0.2 mM hydrogen peroxide, 3-h reaction.

Comparing the optimal pH of 1.6 to the pK_a of sulfamethoxazole (pK_a=1.6-1.85) (Knappe, 2007) suggests that the charge state of the substrate governs the lower apparent pK_a of the bell, here SMX must be in its fee-based form for reaction. Aniline and *o*-anisidine are anilines like sulfamethoxazole that have been tested with soybean peroxidase, their optimal pH was shown to be around 5, consistent with their pK_as near that pH (Al-Maqdi et al., 2018, Nurullah Şanli, et al., 2010).

Similar results were reported for 3-hydroxyquinoline (optimum pH range 8.0-8.6; pK_a 8.0)(Mashhadi et al., 2019), *p*-cresidine (2-methoxy-5- methylaniline, optimal pH 4.6, pK_a 4.7) (Mukherjee, et al.2018.) and Bromoxynil and Ioxynil (optimal pH 4; pK_a 3.86 and 3.96, respectively) (Xiong et al., 2019)

The results of pH optimization for (0.1 mM) SMR conversion, from HPLC analysis, are shown in Figure 4-2. After pH range finding by UV-Vis (data not shown) λ_{max} 275 nm, the experiments were conducted in triplicate, over a pH range of 1.5-10, with 40 mM buffer,

0.2 mM H_2O_2, and 1.0 or 2.0 U/mL SBP. Two U/mL of SBP was chosen for further experiments Acidic pH is required for SMR enzyme conversion, as shown in Figure 4-2-1, SMR showed good conversion efficiency and the optimum pH was found to be 3.6 (Figure 4-2-2) and pK_a 1 is 2.23 and pK_a 2 is 6.77.

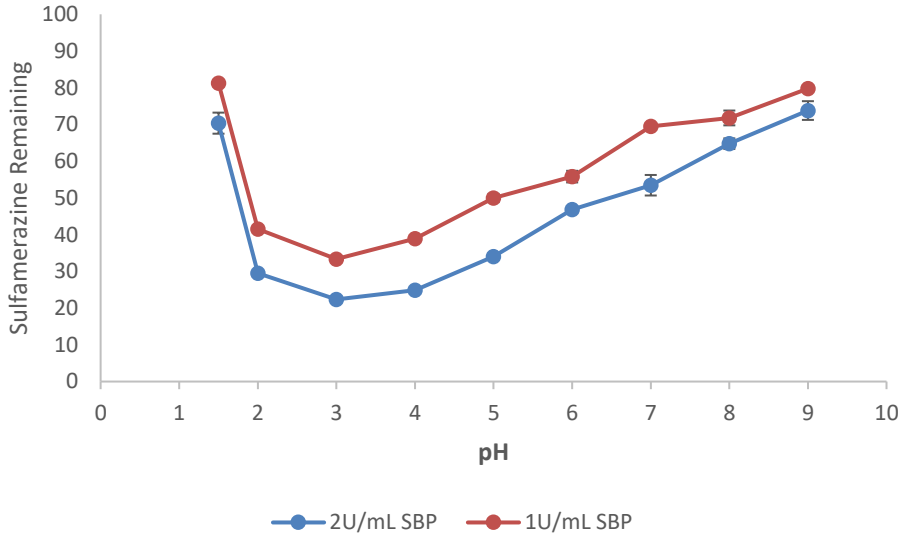


Figure 4-2-1 pH optimization for sulfamerazine (wide pH range)

Conditions: 0.10 mM SMR, 40 mM buffers ,2.0 U/mL and 1.0 U/mL SBP, 0.20 mM hydrogen peroxide, 3-h reaction.

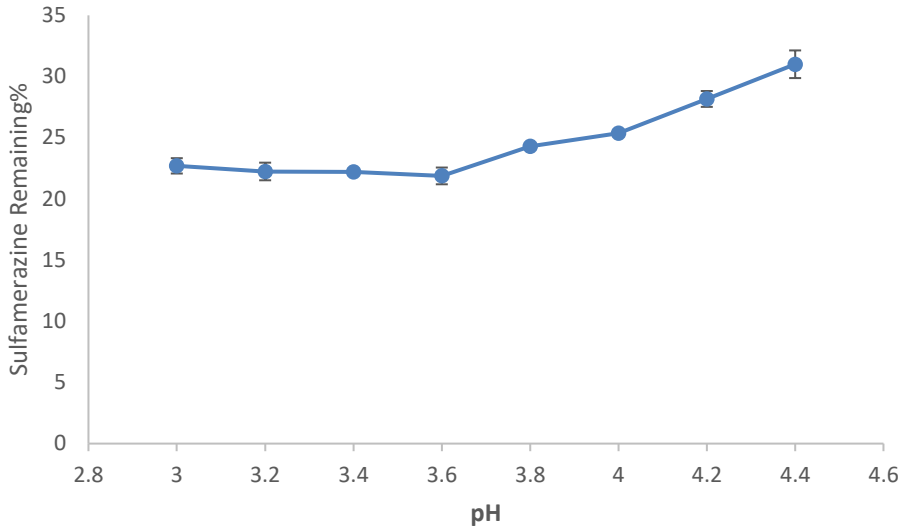


Figure 4-2-2 pH optimization for sulfamerazine (narrow pH range)

Conditions: 0.10 mM SMR, 40 mM Citrate buffer,2.0 U/mL SBP, 0.20 mM hydrogen peroxide, 3-h reaction, analysis by HPLC.

There are not many differences in substrate removal among the data for pH 3.0 to pH 3.6 in the narrow pH range, thus pH 3.6, was chosen as an optimal condition for subsequent experiments.

By observing the drastic decrease in substrate removal on either side of the optima pH range, several conclusions can be drawn from the optimal pH for the compounds studied, including that SBP can tolerate an acidic region better than a basic one, as observed by others(Al-Ansari et al., 2009, Al-Ansari, et al., 2011), and that the optimal pH depends not only on the proper ionization state of the catalytic residues, but also on the type of aromatic compound treated by the enzymatic process and its pK_a. The SBP concentration must be increased beyond the minimum effective level to achieve 95% substrate conversion for substrates with optima closer to neutrality, at which point the enzyme becomes less sensitive to pH changes. This can be of great advantage during the application of SBP in industrial wastewater treatment compared to other enzymes such as laccases that require a specific pH to be effective. The pH optima found here were used to optimize other parameters in the following experiments.

4.1.2. SBP Optimization

Cost-effectiveness is one of the most significant aspects of enzymatic treatment, but the expense of the enzyme can limit its use in real wastewater treatment(Steevensz, et al., 2009). Enzymatic treatment of target pollutants is therefore cost-effective if the minimum effective amount of SBP is appropriate for the waste in question.

The optimal amount of SBP is the minimum amount required to remove 95% of organic pollutants. An experimental design to determine the optimal SBP activity was conducted in a similar manner to optimization of the pH of the reaction. All the parameters of the reaction except for the activity of SBP were held constant at pH of 1.6 for SMX The SBP dose was set at a range of around 3U/mL, this was due to the reaction using a pH of 1.6 still not fully converting the original sulfamethoxazole, meaning the reaction may require more enzyme to fully convert the substrate. Figures 4-3 and 4-4 show the final SBP optimization for each substrate.

SMX removal efficiency didn't increase with increasing enzyme activity up to 4.5 U/mL for 0.20 mM SMX, with the highest removal efficiency 83% at 4.0 U/mL of SBP. This is comparable to the 4.5 U/mL SBP needed to remove 0.1 mM 3-aminoquinoline from synthetic wastewater (Mashhadi et al., 2019), but far more than the optimal enzyme activities needed for 95% conversion of 1.0 mM aniline and *o*-anisidine, 0.60 and 0.012 U/mL, respectively (Al-Ansari, et al., 2011).

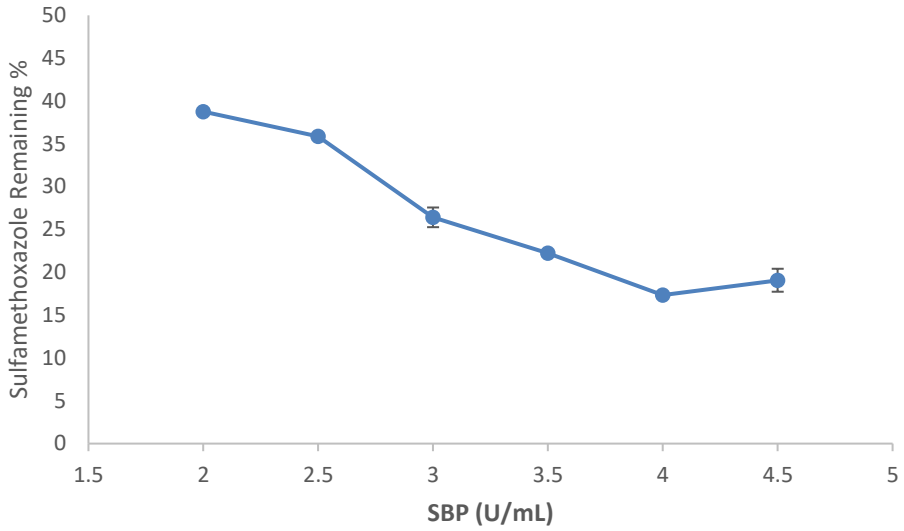


Figure 4-3 SBP optimization for sulfamethoxazole

Conditions: 0.20 mM SMX, 40 mM buffers pH 1.6, 0.40 mM hydrogen peroxide, 3-h reaction; analysis by HPLC.

For SMR, the previous pH optimization experiments(based on UV-Via data), 1.5 U/mL enzyme activity could only achieve 65% conversion. Experiments continued with the range of 1.5-4.5 U/ml enzyme activity, Figure 4-4. Increasing the amount of enzyme from 1.5 to 2.0 U/mL resulted in almost 10% improvement in the removal of the substrate after 3 hours, but further increase in SBP did not improve removal efficiency.

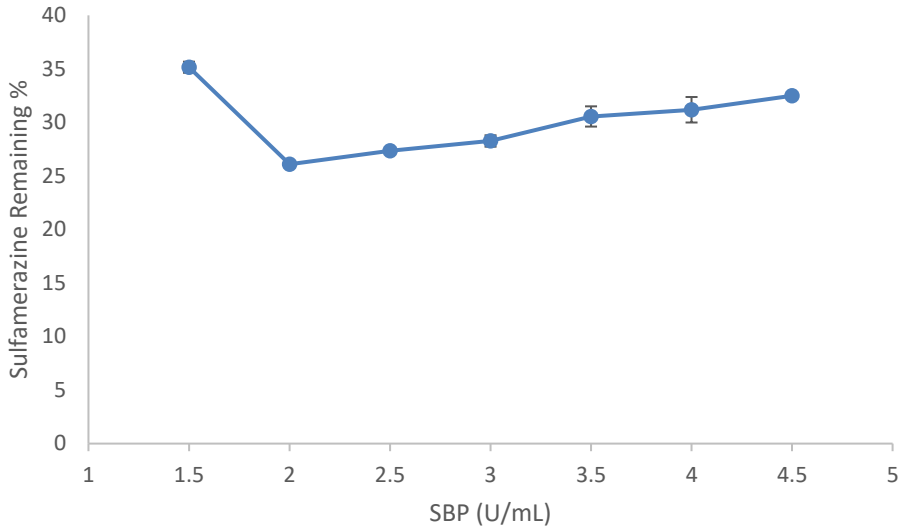


Figure 4-4 SBP optimization for sulfamerazine

Conditions: 0.100 mM SMR, 40 mM citrate buffer pH 3.6, 0.2 mM hydrogen peroxide, 3-h reaction; analysis by HPLC.

Half the SBP activity was needed for the maximum removal of sulfamerazine compared to SMX, but SMR was at half the concentration of SMX; both reactions fell substantially short of 95% conversion. This suggests that something other than SBP activity was limiting the conversions.

4.1.3. Hydrogen peroxide optimization

One of the substantial factors in enzymatic treatment is hydrogen peroxide, which begins the peroxidase reaction as a co-substrate. Based on what is explained in Section 2-4-1., peroxidase will be inactivated if H_2O_2 concentrations are in excess. Conversely, a low concentration of H_2O_2 might result in an insufficient conversion of the substrate. In the oxidation-reduction mechanism in the peroxidase cycle, 2 moles of the substrate would be converted to free radicals with the consumption of 1 mole of H_2O_2. It should be noted that this stoichiometry can change depending on the catalase activity of peroxidase and subsequent enzymatic cycles that generate higher oligomers from dimers that are first formed in peroxidase (Valderrama et al., 2002). Furthermore, the higher the SBP activity in a reaction, the higher the consumption of peroxide by catalase activity would be for example, decomposition of peroxide by endogenous catalase activity of SBP would be

expected to be far higher in a reaction with 4 U/ml than in one with 0.012 U/mL, i.e.- for a sluggish substrate than a good one.

The concentration of hydrogen peroxide for SMX (HPLC analysis) was optimized with 4.0 U/mL SBP. Figure 4-5 shows that increasing the concentration of H_2O_2 over 0.6 mM, no difference in the amount of removal observed. More H_2O_2 concentration over this resulted in lower removal efficiency. A lower conversion might be caused by the formation of compound III in the presence of excess H_2O_2, which inactivates SBP. According to the peroxidase cycle, 0.1 mM H_2O_2, which is the theoretical molar concentration of H_2O_2 required for the conversion of 0.2 mM SMX, results in low conversion efficiency, likely due to oligomer formation and catalase activity of SBP. Accordingly, 0.6 mM H_2O_2 ($[H_2O_2]/[SMX] = 3$) was considered the optimum peroxide concentration for 0.2 mM SMX, which led to 83% removal efficiency.

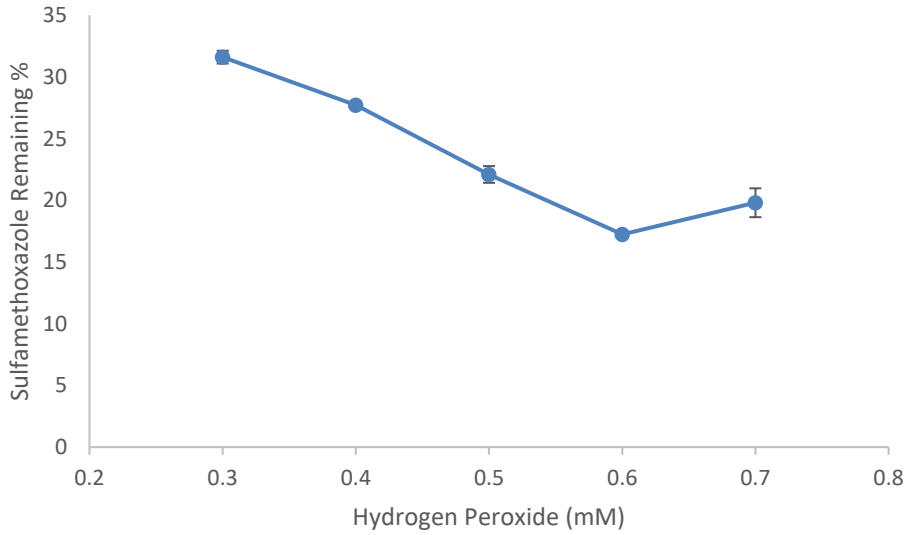


Figure 4-5 Hydrogen peroxide optimization for sulfamethoxazole**.**

Conditions: 0.20 mM SMX, 40 mM buffer pH 1.6, 4.0 U/mL SBP, 3-h reaction; analysis by HPLC.

Figure 4-6 illustrates H_2O_2 optimization for SMR (HPLC analysis). The most efficiency was identified at 0.25 mM H_2O_2 with 24% remaining. Accordingly, 0.25 mM H_2O_2 $[H_2O_2]/[SMR] = 2.5$) was considered the optimum peroxide concentration for 0.1 mM SMR, which led to almost 76% removal efficiency.

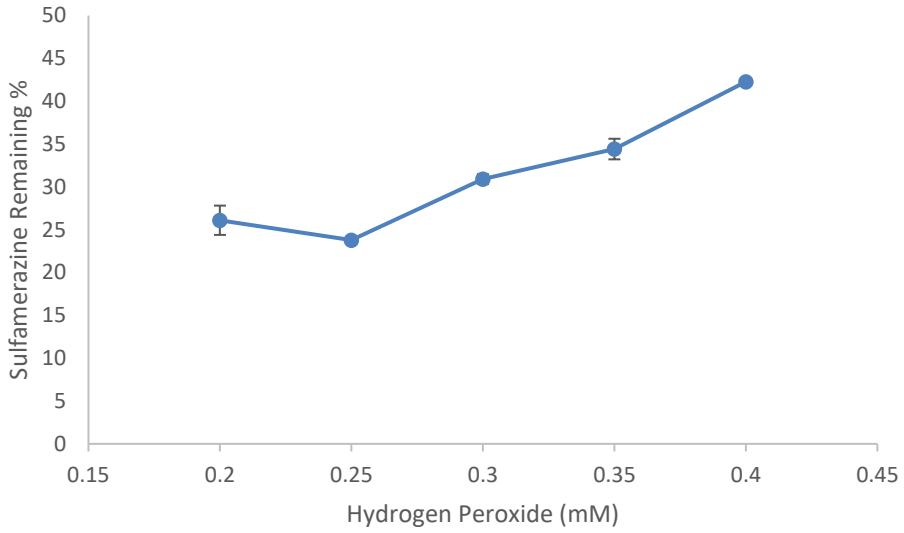


Figure 4-6 Hydrogen peroxide optimization for sulfamerazine**.**

Conditions: 0.10 mM SMR, 40 mM citrate buffer pH 3.6, 2.0 U/mL SBP, 3-h reaction; analysis by HPLC.

A series of experiments were conducted to optimize hydrogen peroxide concentration with optimum amounts of SBP for each substrate over 3 hours to achieve ≥ 95% removal as shown in Appendix C and D, residual H_2O_2 and SBP tests were conducted to increase SMX removal efficiency, as low removal efficiency may occur if enzyme and/or peroxide run out during the reaction. As a result of a 3-hour reaction under optimal conditions in Figure 4-5, the residual SBP and H_2O_2 tests for SMX were performed. The results showed the small amount of initial enzyme activity remaining and only 3.3% of the initial H_2O_2 concentration respectively.

In order to improve SMX removal efficiency, the effect of incrementally adding H_2O_2 was studied too. The optimum concentration of H_2O_2 for SMX (0.60 mM) was divided into 4 equal aliquots added to the reaction mixture every 15 minutes. Results showed 16% of the substrate remained with the stepwise addition of H_2O_2 thus only a 2% improvement in the removal efficiency over single addition.

4.2. Redox Mediator Reaction

According to section 1.2, above, one of challenges to enzymatic methods for removing emerging pollutants from industrial effluents is recalcitrance of some compounds, which might be alleviated by the presence of redox mediators. These redox mediators increased the and the degradation efficiency of resistant compounds several times. SBP is one of the enzymes for which potential of mediators has been investigated for this purpose (Husain et al. 2008). Hydroxybenzotriazole is one of these redox mediators, which has also been shown to be a good substrate, Mashhadi et al. (2019) reported removal of more than 95% of 1 mM HOBT can be achieved (with 0.12 U/mL and 1.25 mM H_2O_2 at pH 3.6; 60% conversion in less than 40 seconds). Based on the selection of HOBT for these experiments, starting conditions for the mediated reactions were chosen to be those of HOBT noted above—success with this would alleviate two problems of the results without HOBT, very low optimum pH (1.6) and very high SBP requirement (4 U/mL).

Table 4-1 Experiments after adding HOBT as mediator, optimizing parameter, and their removal efficiency for sulfamethoxazole**.**

Experiment	Substrate SMX (mM)	pH	H_2O_2 (mM)	SBP (U/mL)	HOBT (mM)	Removal %
i	0.200	1.6	0.300	0.1	0.200	≤ 16
ii	0.200	3.6	0.300	0.1	0.200	≥ 84
iii	0.200	4	0.300	0.1	0.200	≤ 69
iv	0.200	5	0.300	0.1	0.200	≤ 66
v	0.200	3.6	0.100	0.1	0.050	≤ 10
vi	0.200	3.6	0.125	0.1	0.100	≥ 50
vii	0.200	3.6	0.250	0.1	0.200	≤ 69
viii	0.200	3.6	0.375	0.1	0.300	≥ 88
ix	0.200	3.6	0.300	0.1	0	≤ 20
x	0.200	3.6	0.600	4.0	0.300	≤ 18
xi	0.200	1.6	0.600	4.0	0.300	≤54

Table 4-2 Experiments after adding HOBT as mediator, optimizing parameter, and their removal efficiency for sulfamerazine.

Experiment	Substrate SMR (mM)	pH	H_2O_2 (mM)	SBP (U/mL)	HOBT (mM)	Removal %
i	0.100	3.6	0.25	2.0	0.100	≤ 45
ii	0.100	3.6	0.25	2.0	0	≥ 78
iii	0.100	3.6	0.125	0.1	0.100	≤ 30
iv	0.100	3.6	0.125	0.1	0.150	≤ 35
v	0.100	3.6	0.125	0.1	0.200	≤ 35
vi	0.100	3.6	0.125	0.05	0.150	≥ 67
vii	0.100	3.6	0.060	0.05	0.150	≤ 55

For the initial experiment, the amount of HOBT taken to as that of substrate, and the rest of the conditions were selected as those for HOBT(Mashhadi et al., 2019), prorated for SMX concentration. Removal of 78% was found (as can be seen in table 4-1). To optimize the pH, the experiments were repeated in buffers with different pHs. As seen in Figure 4-

7, at pH 1.6, pH 4.0 and pH 5.0, the removal was less favorable, thus pH 3.6 appears to be the most suitable.

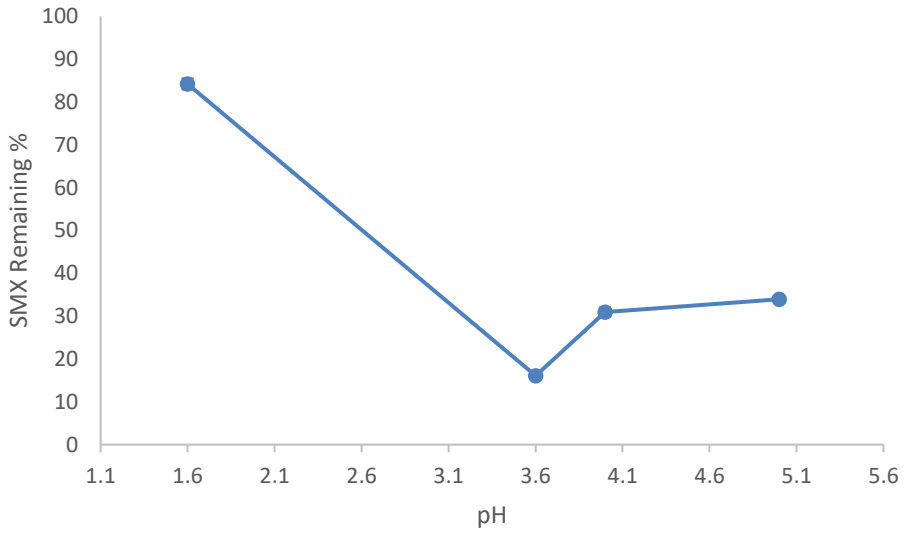


Figure 4-7 pH optimization for sulfamethoxazole in presence of HOBT.

Conditions: 0.20 mM SMX, 0.20 mM HOBT, 40 mM citrate buffer pH 3.6, 0.3 mM H_2O_2, 0.1 U/mL SBP, 3-h reaction; analysis by HPLC.

To optimize the amount of HOBT, further experiments with concentrations of HOBT and sulfamethoxazole ratios (experiments v,vi,vii,viii) in batch reactors were tested. It was found using the HOBT at 1.5 times the [substrate], equal to 0.3 mM, gave maximum removal of sulfamethoxazole

Also, to ensure the enzyme activity and H_2O_2 used in the presence of the HOBT, the amounts of these two (experiment v,vi,vii,viii) were optimized. Moreover, to ensure optimal conditions, experiments were conducted with the amount of 0.1U/mL enzyme activity and 0.3 mM H_2O_2 in the presence of the HOBT and the absence of that, at pH 3.6. Results showed that the removal of SMX without the presence of HOBT at this pH is less than 20%, which is not suitable at all (experiment ix). Following this, to compare the effects of pH and the presence of HOBT at the same time, the experiments were repeated (in experiments i and ii) . Accordingly, the results showed that at pH 1.6, these conditions are completely inappropriate, and this was only obtained less than 14% removal from the substrate. Also, a significant amount of HOBT (about 60%) remained (data not shown).

For the last point, experiments with the optimal conditions obtained in the first step for sulfamethoxazole, 4.0 U/mL enzyme activity, and 0.6 mM H_2O_2, in the presence of HOBT were repeated at pH 1.6 and pH 3.6 (experiments x and xi). As expected, the removal of sulfamethoxazole decreased sharply and reached less than 18% at pH 3.6 and less than 54% at pH 1.6.

Finally, the optimal conditions for maximum removal of 0.200 mM sulfamethoxazole (approximately 88%) were considered as follows: 0.1 U/mL enzyme activity, 0.375 mM H_2O_2, 0.300 mM HOBT, in buffer pH 3.6. In addition to improving the appropriate pH for the reaction from 1.6 to 3.6, these conditions have also caused a significant decline (40-fold) in the enzyme activity required. It has also brought down the amount of H_2O_2 required by half.

For the other selected sulfonamide, sulfamerazine, all these steps were repeated in the same order and as expected, a similar behavior of sulfamerazine was observed. The appropriate pH was chosen as pH 3.6 and in fact no change was made in this regard. But in the optimal state in the presence of HOBT for sulfamerazine, the substrate removal conditions were improved by reducing the enzyme activity from 2.0 U/mL to 0.05 U/mL based on experiment vi. The required amount of H_2O_2 was also reduced by half. Also, the optimal HOBT amount to obtain the maximum removal for 0.100mM sulfamerazine was 1.5-fold of the substrate concentration, 0.150 mM. The noteworthy point at this stage is the reduction of the removal rate from 76% to about 67% (almost less than 10 percent).

4.3. Time course of reactions

Considering the effect of the reaction time on the necessary reactor volume and, hence, the final cost of a treatment plant, reaction time course is one of the most important parameters. Arbitrarily, the selected time for the previous experiments was 3 hours. Therefore, it would be very useful to determine the minimum required time for the conversion of more than 95% of pollutants. Thus, substrate transformation was investigated for 3 hours under optimal conditions. Aliquots taken at various time intervals were quenched with catalase, micro-filtered and analyzed by HPLC to measure the residual substrate concentration at

each time, Figures 4-8 and 4-9. The initial first-order rate constants, k, and half-lives of SMX and SMR were derived from fitting the initial data in these two figures to the exponential decay function, equation 5, from which the rate constant was derived and from it, the half-life could be calculated according to equation 6

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

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

As expected, the reaction does not stay first-order for the entire period, since the rate constant is changing due to the enzyme activity loss and H_2O_2 consumption (loss of pseudo-first-order behavior). As a result, the initial stages of the reaction have been used for reaction rate-constant determination and half-life calculation. In Figure, 4-8, sulfamethoxazole reached approximately 72% conversion the first 10 minutes, but the degradation of the rest (28%) took 2 hours and 50 minutes, whereas sulfamerazine, Figure 4.9, reached 53% transformation at 10 minutes, and treatment efficiency improved by only 15% in the ensuing 170 minutes. Clearly, there is a slowing reaction rate likely due to the enzyme inactivation by reactive radicals and/or end-product adsorption of active enzyme (Al-Ansari et al., 2009)(Feng, et al., 2013). Similar results were found in the SBP-catalyzed removal of 4-chlorophenol, 3-hydroxyquinoline, arylamines, azo dyes, and pesticides(Mashhadi et al., 2019, Villegas, et al., 2016, Mukherjee et al., 2018, Zhang, et al., 2016). Based on Equations 5 and 6, the rate constants (*k*) for sulfamethoxazole and sulfamerazine were 0.862 ±0.003 and 0.570±0.008 min^{-1}, respectively; therefore, the corresponding half-lives were obtained as 0.804 and 1.2157 min. If normalized with respect to enzyme activity, the half-life was 0.0862 min per U/mL for SBP for sulfamethoxazole and 0.06075 min per U/mL of SBP for sulfamerazine. These values are compared with many others determined in this lab (Table 4.1), aside from present entries, due to (Kaur, 2020) and(Ziayee Bideh, 2020). This indicates that the SBP normalized catalytic reaction rates of sulfamerazine, and sulfamethoxazole are close at the beginning of the reaction and amongst the fastest (shortest half-lives) in Table 4-3 and in Table 4-4.

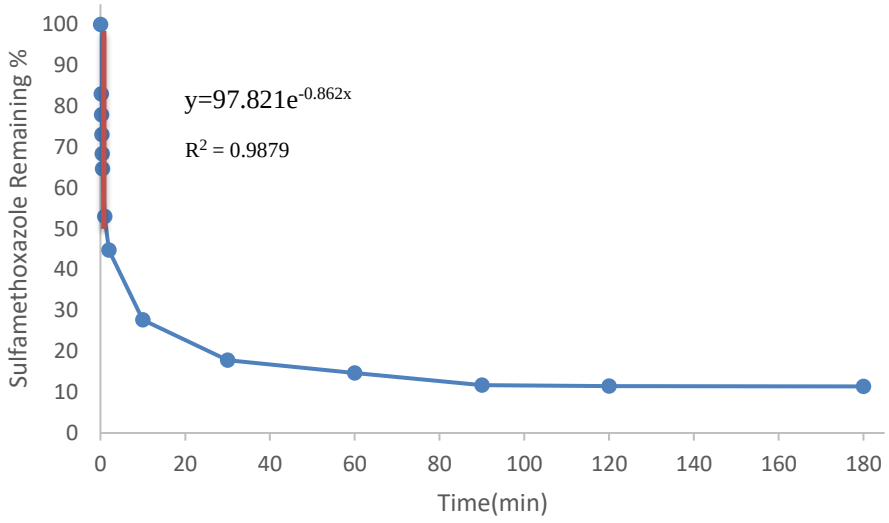


Figure 4-8-1 Time dependence of degradation of sulfamethoxazole in optimal conditions Batch reactor.

(40 mM citrate buffer pH 3.6, 0.2 mM SMX, 0.3 mM HOBT, 0.3 mM H_2O_2, 0.1 U/mL SBP, 3-hour reaction, room temperature), analysis by HPLC.

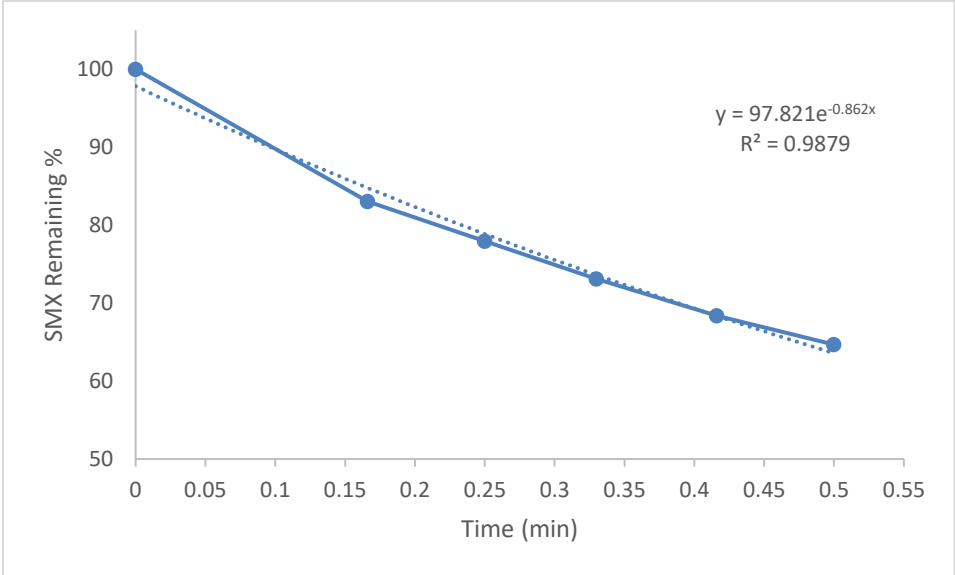


Figure 4-8-2 First-order degradation of sulfamethoxazole at the beginning of the reaction presented in Figure 4-7-1

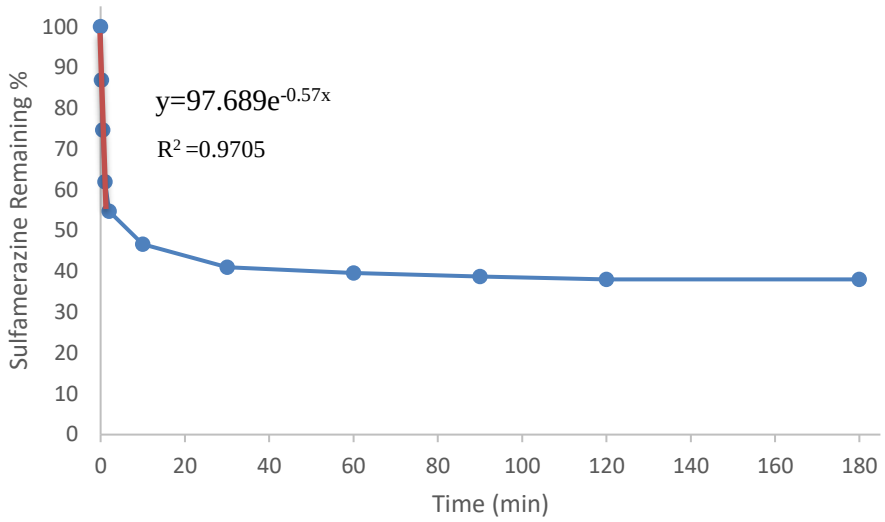


Figure 4-9-1 Time dependence of degradation of sulfamerazine in optimal conditions Batch reactor

(40 mM citrate buffer pH 3.6, 0.1 mM SMR, 0.15 mM HOBT, 0.125 mM H_2O_2, 0.05 U/mL SBP, 3-hour reaction, room temperature), analysis by HPLC.

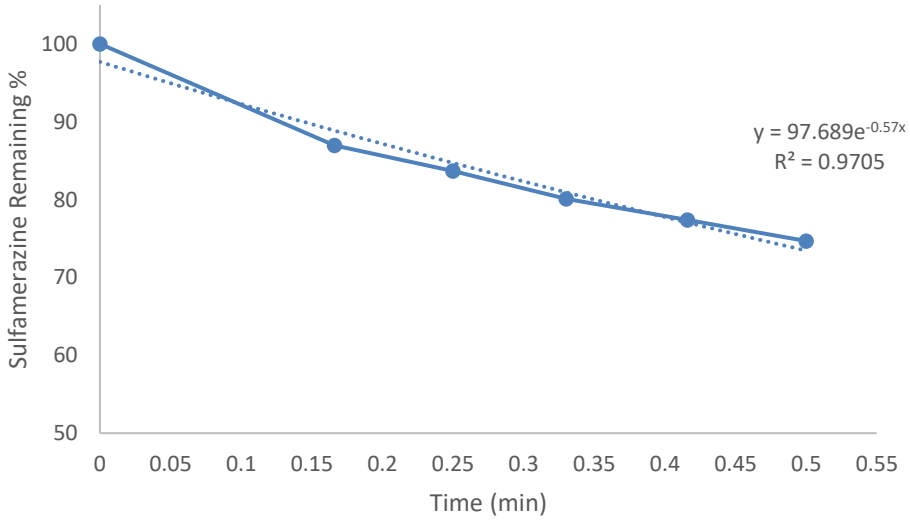


Figure 4-9-2 First-order degradation of sulfamerazine at the beginning of the reaction presented in Figure 4-8-1

Table 4-3 Half-lives, and normalized half-lives of various SBP substrates

	Substrates	Half-life (min)	Normalized Half-life (min)
This work	sulfamethoxazole	0.804 ±0.003	0.0804 ±0.0003
	sulfamerazine	1.216±0.008	0.0608±0.008
HACs (Mashhadi, 2019)	2-Aminothiazole	33.0 ± 0.6	132 ± 2
	2-Aminobenzothiazole	720 ± 0.0	3240 ± 0.0
	3-Hydroxyquinoline	11.9 ± 0.6	1.19 ± 0.06
	3-Aminoquinoline	15.0 ± 0.6	67.5 ± 2.7
	2-Aminoimidazole	5.1 ± 0.2	7.7 ± 0.3
	2-Aminobenzimidazole	29.4 ± 0.6	82 ± 2
	3-Aminopyrazole	36 ± 1	108 ± 4
	4-Aminoantipyrine	61 ± 1	6.1 ± 0.1
	Hydroxybenzotriazole	42 ± 2	5.0 ± 0.2
	Indole	26 ± 1	11.3 ± 0.5
	Pyrrole	49 ± 3	246 ± 15
Arylamines (Mukherjee, 2019)	4-Chloro-o-toluidine	11.5 ± 0.0	0.104 ± 0.0
	4,4'-Oxydianiline	1.80 ± 0.02	0.072 ± 0.001
	4,4'-Methylenedianiline	0.58 ± 0.10	0.40 ± 0.07
	4,4'-Thiodianiline	0.513 ± 0.007	0.0770 ± 0.0011
	4,4'-Methylenebis(2-chlororaniline)	4.08 ± 0.02	0.408 ± 0.002
	p-Cresidine	12.4 ± 0.0	0.124 ± 0.0
Pesticides (X. Zhang, 2019)	Bromoxyni	3.00 ± 0.13	2.70 ± 0.02
	Ioxynil	0.51 ± 0.02	0.18 ± 0.01
Azo dyes (Cordova Villegas, 2017)	Acid Blue 113	8.8 ± 0.6	13.2 ± 0.9
	Direct Black 38	2.1 ± 0.2	6.4 ± 0.6
Heteroaromatics (Ziayee Bideh, 2020)	3-hydroxycoumarin	12.4 ±0.5	0.0257 ± 0.001
	2-Aminobenzoxazole	129 ± 4	452 ±15
Dyes (Kaur2020)	*p*-Anisidine	5.46±0.84	0.0097±0.0011
	Methyl Orange	7±2	0.048±0.01

This table taken from (Ziayee Bideh), MASc thesis, University of Windsor, (2020).

Table 4-3, demonstrate half-lives and normalized half-lives of various SBP substrates studied in this group with respect to the optimum enzyme concentration.

4.4. Mass spectrometry (MS) results

Free radicals generated in the peroxidase catalytic cycle couple non-enzymatically as discussed in Section 2-4. Various coupling positions such as C-C, C-O, O-O (O-O coupling is not stable), N-N and N-C are expected due to the presence of different resonance-stabilized radical structures, leading to the formation of several isomeric coupling products. The peroxidase cycle continues until the oligomers grow to reach their solubility limit and precipitate (Villegas, et al., 2016). MS analysis helps to identify the nature of generated products which can lead to a better understanding of plausible toxicity of these compounds. Based on the molecular weight of oligomers obtained from MS data,

possible reaction product structures have been assigned. Notably, MS analysis can only offer preliminary insights into the possible products and cannot discriminate among isomeric structures. MS analysis was conducted using the electro-spray ionization technique (in positive-ion mode; thus, protonated forms of the products were frequently detected). After enzymatic treatment of the substrates under optimal conditions, the unfiltered reaction mixture (in order to prevent losing possible solid products) and the standards were analyzed in ESI mode at high resolution. Based on the molecular weight of generated oligomers (mass to charge ratio (m/z)) derived from mass spectrometry data and possible structures and formulae have been assigned. During the analysis, the isotope abundance was considered for corroboration of the assigned formulae. The following symbols have been used for the obtained structures: M, standard; MH, protonated standard; ^{13}C-MH, protonated natural abundance ^{13}C-isomer of standard, M_2H-2, protonated oxidative dimer; M_2H-4, protonated oxidized oxidative dimer, M_3H-4, protonated oxidative trimer, M_3H-6, protonated oxidized oxidative trimer. To the best of our knowledge, there is no published literature available on polymerization products of SMX and SMR using any peroxidase.

4.4.1 Sulfamethoxazole:

A sample of authentic SMX was run, although its presence in the final reaction mixture served as an internal standard. Table 4-3 shows that all identified compounds were present in their protonated forms although, surprisingly, SMX itself was also detected in its native form. The expected oxidative dimer of SMX was found, along with a peak at two mass units smaller, assigned to the azo-dimer. The latter would have arisen from a pair of nitrogen-centred radicals that coupled, to form a hydrazine, that underwent easy oxidation under the reaction conditions to the azo compound, which would be a dead-end with respect to further oligomerization. This conversion has been observed in the lab with many arylamines by previous workers (Malik Altahir, et al., 2020, Mashhadi et al., 2019, Mukherjee, 2019). The protonated homo-dimer and azo-coupled dimer, M_2H-2 and M_2H-4, were confirmed by the ^{13}C isotope signature peaks, though the heights of isotope peaks were relatively high. This might be due to the overlapping of products, and as a result peaks were not well-separated. There was no evidence of higher oligomer formation. This is not surprising in view of finding the azo dimer, but the absence of higher oligomers suggests that the initial radical coupling is only through the N-N route.

Table 4-5 Summary of mass spectrometry results for standard and identified reaction products of sulfamethoxazole and sulfamerazine after SBP-catalyzed process

Compound		Symbols	Molecular formula	m/z[a]	Detected
sulfamethoxazole	Standard	MH	$C_{10}H_{12}N_3O_3S$	254.045181	*
		M	$C_{10}H_{11}N_3O_3S$	253.037356	*
	Identified Products	M_2H-2	$C_{20}H_{20}N_6O_6S_2$	505.066887	*
		M_2H-4	$C_{20}H_{18}N_6O_6S_2$	503.051225	*
		M_3H-4	$C_{30}H_{29}N_9O_9S_3$	756.077205	
		M_4H-6	$C_{40}H_{38}N_{12}O_{12}S_4$	1007.1103	

[a]with all formulae detected, the requisite ^{13}C peaks were found and occasionally the ^{34}S were found

[b]Samples were sent to Queen's University but time constraints there precluded their running and the data being sent back. That data, when received, will be included in a paper written for the work of this thesis.

4.4.2 Hydroxybenzotriazole

As a pure compound and as a component in the reaction mixtures, HOBT was found in unprotonated and protonated forms, Table 4-4.

In the mediated reactions both homo- and hetero-dimers were found. In addition, the azo-dimers were present as for the reactions without HOBT in the preceding table. There was no evidence of higher oligomer formation. No higher oligomers of the substrate were observed in the solution under the analysis conditions, again, not surprising with the occurrence of dead-end azo-dimers.

4.4.3 Sulfamerazine

Unfortunately, due to the lack of access to the on-campus mass spectrometer system and the sending samples to Queen's University to perform this test, the results of the mass spectrometry test for the second compound, sulfamerazine, did not reach us in time. But considering that the second substrate is also from the sulfa drug class compounds and during the testing process, behaved similarly to the first substrate, sulfamethoxazole, we can expect similar results for it.

Table 4-6 Summary of mass spectrometry results for standard and identified reaction products of sulfamethoxazole, sulfamerazine and hydroxybenzotriazole after mediated SBP-catalyzed process.

Compound		Symbols	Molecular formula	m/z[a]	Detected
Sulfamethoxazole + HOBT	Standard	M(HOBT)	$C_6H_5N_3O$	135.038335	*
		MH(HOBT)	$C_6H_6N_3O$	136.031616	*
	Identified Products	M (SMX+ HOBT)	$C_{16}H_{16}N_6O_4S$	388.075691	*
		MH-2	$C_{16}H_{17}N_6O_4S$	387.067866	*
		M_2H-2	$C_{20}H_{20}N_6O_6S_2$	505.066887	*
		M_2H-4	$C_{20}H_{18}N_6O_6S_2$	503.051225	*
		M_3H-4	$C_{30}H_{29}N_9O_9S_3$	755.06938	
		M_4H-6	$C_{40}H_{38}N_{12}O_{12}S_4$	1006.10247	

[a]with all formulae detected, the requisite ^{13}C peaks were found and occasionally the ^{34}S were found.

[b]Samples were sent to Queen's University but time constraints there precluded their running and the data being sent back. That data, when received, will be included in a paper written for the work of this thesis.

CHAPTER 5

SUMMARY AND CONCLUSIONS

In this thesis, the feasibility of selected sulfa drug compound removal by soybean peroxidase was studied. The results obtained from preliminary studies illustrated sulfamethoxazole and sulfamerazine are substrates for SBP, then the second objective: operational conditions were optimized for >95% removal of these two pollutants, Tables 5.1 and 5.2. The optimum pH for both compounds was in the acidic range, with maximum removal efficiency at pH 1.6 and 3.6 for SMX and SMR, respectively, which are very close to their pK_a value. The optimum H_2O_2-to-substrate ratio of 2.0 was higher than the theoretical value of 0.5, indicating the higher peroxide demand due to oligomer formation, or due to the catalase activity of soybean peroxidase. By adding HOBT as a mediator, the optimize parameters improved in some cases. . The optimum pH for SMX, changed to pH 3.6 , with maximum removal efficiency around 88% .Hydrogen peroxide and SBP were optimized again. In mediated reactions with adding 0.3 mM HOBT to 0.2 mM SMX, 0.375 mM H_2O_2 and 0.1 U/mL SBP were needed. Also, with adding 0.150 mM HOBT to 0.1 mM SMR at pH 3.6, 0. 125 mM H_2O_2 and 0.05 U/mL SBP were required.

Table 5-1 Summary of optimized conditions for SBP-catalyzed process and rate constants and half-lives for two compounds (without mediator)

	Optimized Conditions	
Parameters	sulfamethoxazole	sulfamerazine
Initial concentration (mM)	0.200 mM	0.100 mM
pH	1.6	3.6
H_2O_2-to-substrate ratio	0.60	0.25
SBP concentration (U/mL)	4.0 U/mL	2.5U/mL

Table 5-2 Summary of optimized conditions for mediated SBP-catalyzed process for two compounds (mediated with HOBT)

	Optimized Conditions	
Parameters	sulfamethoxazole	sulfamerazine
Initial concentration (mM)	0.200 mM	0.100 mM
Initial concentration of HOBT (mM)	0.300 mM	0.150 mM
pH	3.6	3.6
H_2O_2-to-substrate	0.375	0. 125
SBP concentration (U/mL)	0.1	0.05

The normalized half-lives of SBP for sulfamethoxazole and sulfamerazine were also calculated based on the first-order fits for the 3-hour time-course experiments. The first-order model fitted the initial stage of the reaction, then the degradation rate slowed down due to depletion of both enzyme activity and hydrogen peroxide. Under the optimized conditions, the initial first-order reaction rate constants, and half-lives for the two pollutants are summarized in Table 5-3.

Table 5-3 Summary of Rate constant, half-life and normalized half-life of two compounds under optimized conditions for mediated SBP-catalyzed process (mediated with HOBT**)**

	sulfamethoxazole	sulfamerazine
Rate constant, k (min^{-1} per U/mL SBP)	0.862	0.570
Half-life, $t_{1/2}$ (min per U/mL SBP)	0.804 ±0.003	1.216±0.008
Normalized Half-life (min)	0.0804 ±0.0003	0.0608±0.008

The possible reaction products were identified using ESI-MS in positive-ion mode. The results are summarized in chapter 4. For sulfamethoxazole, oxidative homo-dimer and hetero dimers and azo-compound were present after SBP-catalyzed treatment under optimized conditions. For sulfamerazine, only oxidative dimer and its further oxidized form, presumably the azo-compound were found, which suggests only N-N radical coupling occurred leading to the dead-end dimer.

In conclusion, this study provides an alternative wastewater treatment method for removing sulfa drugs. SBP extracted from an agricultural by-product is not only cost effective but a robust enzyme, having >88% and >78% removal efficiency on sulfamethoxazole and sulfamerazine respectively. Reaction mediation has benefits for reactions of both compounds.

CHAPTER 6

FUTURE WORK

Reinforcing the findings of the previous study in our lab, the present study also confirms that SBP holds potential for use as an agent to remove sulfa drugs from wastewater. Additionally, in keeping with the recommendations from the previous study, five specific suggestions for the real application of SBP-based treatment are as follows.

1. Other sulfa drugs, sulfanilamide, and their corresponding metabolites should be investigated to establish the scope.

2. lab studies on synthetic wastewater should be extended to real wastewater to determine the matrix effect. The current parameters may need to be re-optimized or adjusted.

3. Further study of polymeric products is recommended, such as environmental outcomes and toxicity. In this way, to avoid further contamination, proper safe disposal methods can be developed.

4. Also needed for real practice is a sedimentation system for the suspended solids generated.

5. Further study and optimization of the mediator reaction with other mediators would be useful.

6. It would be useful to carry out a cost-benefit analysis investigating the integration of SBP-catalyzed treatment with existing wastewater treatment plants or constructed wetlands. This analysis will be needed before full-scale application to clearly comprehend the real associated costs (Mukherjee, 2019).

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APPENDICES

Appendix A.

Preparation of SBP and catalase stock solutions

SBP stock solution

Distilled water (100 mL) was added to 1.4 g of solid powder of crude SBP. The mixture was stirred overnight using magnetic stirrers. This mixture was then centrifuged at 4000 rpm for 20-25 minutes and the supernatant was separated and stored at 4 °C for future use.

Catalase stock solution

Solid bovine liver catalase (0.5 g) was dissolved in 100 mL of distilled water (99500 U/mL in this stock). The solution was stirred for around 3 hours using a magnetic stirrer and stirrer plate and then stored at 4 °C for future use.

Appendix B.

SBP activity assay

To measure the activity of soybean peroxidase, a colorimetric assay was used prior to each set of experiments. The initial rate of formation of a pink chromophore at 510 nm was measured by a built-in kinetic rate calculation function in the UV-Vis spectrometer. UV-Vis spectrometer was set on kinetic mode, 30 second run time with 5 second cycle time and zero-order reaction rate.

Reagents to prepare 50 mL solution:

1. 0.025 g of 4-aminoantipyrine (4-AAP)

2. 5 mL of 10X concentrated phenol solution (100 mM phenol in 0.5 M monobasic/dibasic sodium phosphate pH = 7.4)

3. 100 μL of freshly prepared 100 mM H_2O_2

4. The contents were made up to 47.5 mL with distilled water

Test procedure in 1 mL cuvette:

1. Blank: The spectrometer was blanked with 950 µL of freshly prepared assay reagent mixed with 50 µL distilled water.

2. Sample: 950 µL reagent was mixed quickly with 50 µL of diluted SBP solution, and the rate of color formation at 510 nm was monitored.

Data calculations:

SBP activity in cuvette (U/mL) = initial rate (AU S)×(60 S 1 Min)×(dilution in the cuvette) /6 mM−1 ×cm−1 = initial rate × 200

- Initial rate = ΔA510 /Δt = ΔA510/ S

- $\Delta C = A\,\varepsilon l$, where, Ɛ = 6.0 mM -1 cm-1 and path length (l) = 1.0 cm

- Reaction takes place in l.0 mL cuvette so: mM min × 1.0 mL = 1.0 µmol min = 1.0 U

- Activity of SBP sample = SBP activity in cuvette (U/mL) SBP × dilution factor

Appendix C.

Residual SBP activity assay

To measure the remaining activity of SBP in the reaction mixture after the enzymatic treatment, residual SBP activity assay was used. The principles of residual SBP Reagent: Phenol: 10.52 mM 4-AAP: 2.59 mM Peroxide: 0.21 mM 0.95 mL reagent 0.05 mL SBP Phenol: 10 mM 4-AAP: 2.4 mM Peroxide: 0.2 mM 81 activity are the same as regular SBP activity assay. However, for low SBP activity after reaction, concentrated reagent may be needed. For instance, to prepare 2 - fold more concentrated reagent, the same amount of 10 X phenol, 4-AAP and hydrogen peroxide mentioned in Appendix B are added and made up to 25 mL with distilled water. In order to keep the final concentration of each component in the cuvette constant, the ratio of reagent to enzyme was reformulated:

For 2X concentrated reagent:

0.5 mL of freshly prepared reagent is mixed with 0.5 mL SBP sample, so SPB activity in cuvette (U/mL) =

Initial rate (AU S)×(60 S 1 Min)×(dilution in the cuvette)/ 6 mM−1 ×cm−1 = initial rate × 20

For 4X concentrated reagent*:*

0.25 mL of freshly prepared reagent is mixed with 0.75 mL SBP sample, so

SPB activity in cuvette (U/mL) = initial rate (AU S)×(60 S 1 Min)×(dilution in the cuvette) /6 mM−1 ×cm−1 = initial rate × 13.3

Appendix D.

Residual hydrogen peroxide assay

To determine the residual hydrogen peroxide after the enzymatic treatment, a colorimetric assay was conducted. A reagent consisting of phenol, 4-AAP and Novo ARP enzyme was added to the sample. Through the reaction of ARP with peroxide, phenolic radicals were formed, coupled with 4-AAP and produced a chromophore with λmax = 510 nm. The absorbance was checked with UV-Vis spectrophotometer after 18 minutes and concentration of residual hydrogen peroxide was measured using the predominated calibration curve. If the enzymatic treatment of the substrate generated color in the mixture, color correction was needed for this colorimetric assay. The spectrophotometer was blanked with water by adding 200 μL of water to 800 μL of the filtered sample, mixed, and the absorbance was then measured at 510 nm. Later, the measured absorbance was subtracted from the absorbance monitored in colorimetric assay and was plugged into the calibration curve to find the corresponding concentration of residual hydrogen peroxide. Reagents to prepare 20 mL solution: 1. 10 mL of 10X concentrated phenol solution (100 mM phenol in 0.5 M monobasic/dibasic sodium phosphate pH = 7.4) 2. 0.051 grams of 4-aminoantipyrine (4-AAP) 3. 250 μL of Novo ARP enzyme (concentrate) 4. The contents were made up to 20 mL with distilled water Test procedure: 1. Blank: The spectrometer was blanked with 200 μL of prepared reagent mixed with 800 μL distilled water. 2. Sample:

800 µL of filtered sample from the batch reactors was mixed with 200 µL of prepared reagent and the absorbance was measured at 510 nm after 18 min color development period. 3. Calibration curve: various dilutions of hydrogen peroxide (0.1 to 1.0 mM) were made from 100 mM freshly prepared stock solution; 800 µL of each diluted hydrogen peroxide sample was added to 200 µL of above-mentioned reagent and the absorbance was monitored at 510 nm after 18 min. The calibration curve for hydrogen peroxide is shown in figure D-1 and all data points are the average of triplicate values.

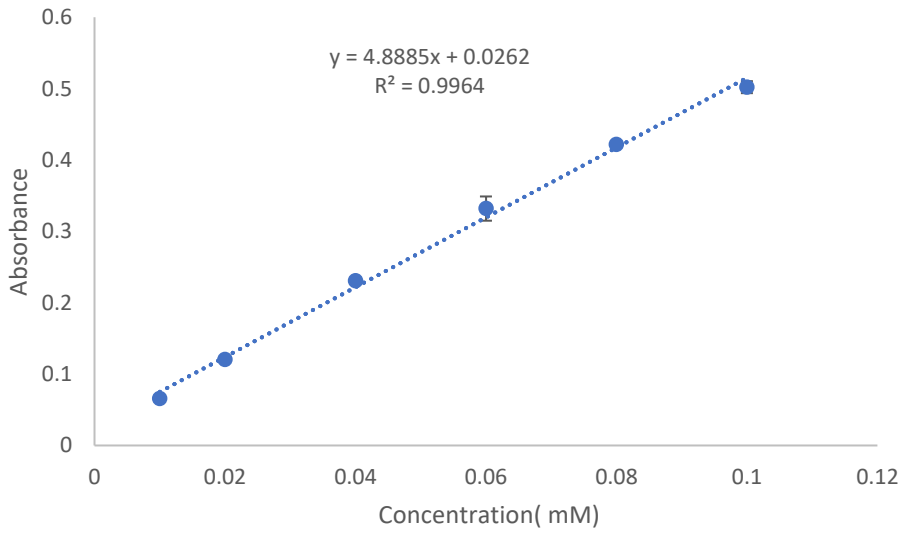

Figure D-1 Hydrogen peroxide calibration curve

Appendix E.

HPLC Calibration curves

The standard curves for the substrates were created using results from the HPLC analyses. For SMX, different concentrations of the substrate were made up in water. The calibration curve was created. The pH range finding using UV-Vis was performed previously (data not shown here).

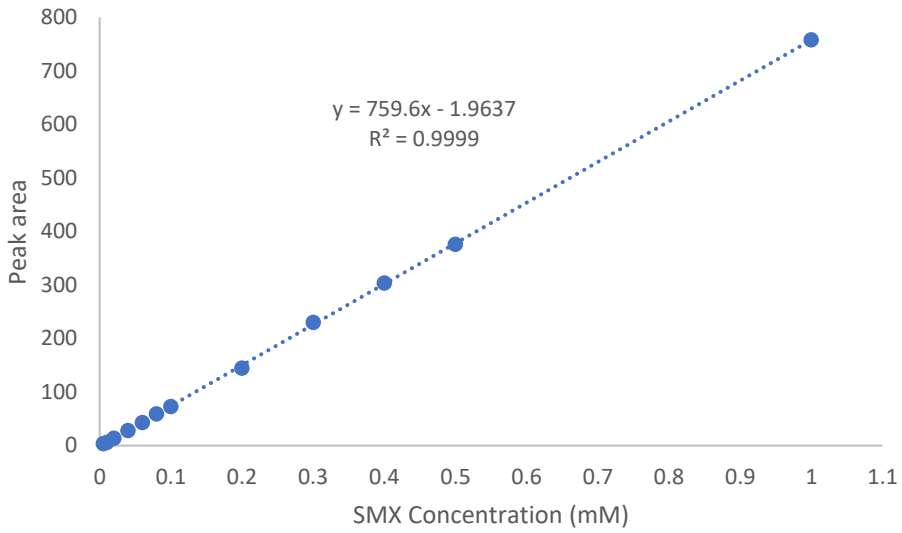

Figure E-1, SMX standard curve in water in 265 nm

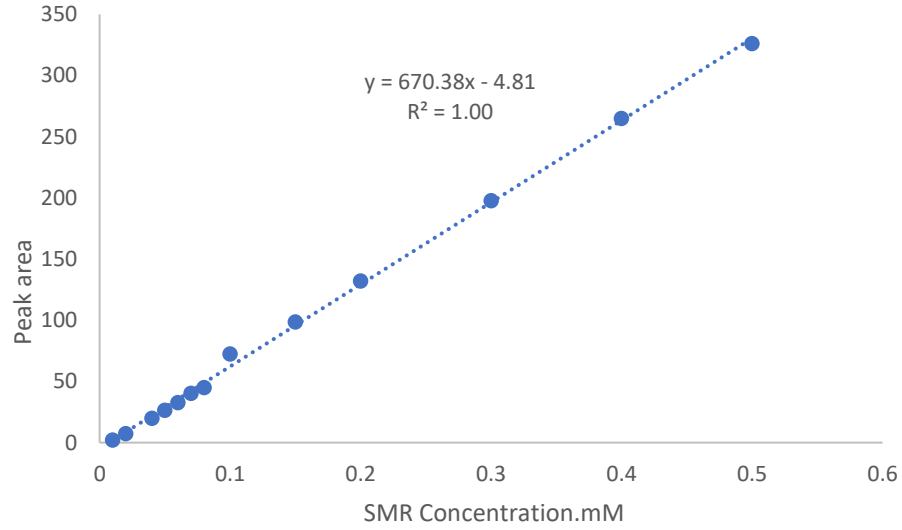

Figure E-2, SMR standard curve in water at 275 nm

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