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Enzymatic Treatment of Selected Pesticides in Aqueous System

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By

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ABSTRACT

Widely applied pesticides have been detected in water bodies, which threatens the environment and non-target life. Thus, a promising treatment method, soybean peroxidase (SBP)-catalyzed process was studied to remove two halogenated benzonitrile pesticides Bromoxynil and Ioxynil through enzymatic oxidation from synthetic wastewater. SBP can be extracted from soybean hulls, a by-product of the soybean industry. First, the experiments studied the viability of SBP-catalyzed removal on these two compounds, then the operational parameters including pH, the molar ratio between hydrogen peroxide and substrate and minimum effective enzyme concentration were optimized. The first-order rate constant and half-life of each substrate were also determined under the established optimum conditions. The results demonstrated SBP is robust enzyme to achieve more than 95% removal efficiency for both compounds. In addition, the possible oligomeric products after enzymatic treatment were characterized by mass spectrometry and both dehalogenation and hydroxylation were observed after the reaction.

DEDICATION

I would like to dedicate this thesis to my beloved parents, Baowen Zhang and Lihua Sun, who have offered me unselfish support and encouragement over many years of every academic endeavor in my life.

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LIST OF ABBREVIATIONS/SYMBOLS

Abbreviations

ASAP, Atmospheric solid analysis probe

HRP, Horseradish peroxidase

MMT, million metric tonnes

MS, Mass spectrometry

SBP, Soybean peroxidase

U. S. EPA, United States Environmental Protection Agency

UV-VIS, Ultraviolet-Visible

Symbols

m/z, Mass-to-charge ratio

mDa, millidalton

$pK_a = -\log_{10}K_a$, the negative base-10 logarithm of the acid dissociation constant (K_a) of a solution.

K_{oc}, the organic carbon normalized soil-water partition coefficient for organic compounds

k_{cat}/K_M (specificity constant or, catalytic efficiency)

ppm, parts per million

CHAPTER 1 INTRODUCTION

1.1 Background

Pesticides have significantly enhanced modern agriculture. They are applied to farmland to assist the weed control and pest infestation thereby enhancing crop production, improving food nutrition and saving labor and related costs. To an extent they are used for the same purpose in residential area (Kim et al., 2017). However, the 35 to 40 % annual increase rate of pesticides stagnates or decreases in cereal production in 47% of countries from 1961 to 2010 (Liu et al., 2015). United States Environmental Protection Agency (U.S. EPA) (2017) reports that the US annual usage of pesticide use at the producer level was over 1.1 billion pounds in 2012, nearly one-sixth of global consumption. Approximately half of the world and US pesticide use were herbicides from 2008 to 2012. In 2012, U.S. herbicide use reached 2847 million pounds, accounting for 24% of the global pesticides market.

The utilization of pesticides and herbicides not only brings benefits but also creates many challenges. Nowadays, herbicides are water-soluble, volatile, and have the risk of droplet drifting after application, which causes a low usage rate of the active ingredients (Tang et al., 2017). Only an estimated 1% of pesticides reach their targets (Berberidou et al., 2017), contributing to their dispersion to the soil, air, water, and food products. Herbicides are becoming less persistent and bioaccumulative due to the increasingly strict regulations, but some of the commonly used herbicides such as atrazine, bromoxynil, chlorpyrifos, and trifluralin are still detected in water (Gerónimo et al., 2014; Chelme-Ayala et al., 2010).

Pesticides enter water bodies from point as well as diffuse sources. Point sources originate from accidental releases at manufacturing plants or spills from spraying equipment during filling and cleaning (Holvoet et al., 2007). Diffuse contamination stems from pesticide application, which is considered the primary pathway. Farmers spray pesticides over agricultural fields, leading to water contamination through leaching, runoff and erosion, drain flow, and spray drift to large areas (Aravinna et al., 2017). Generally, pesticide residues are seasonal; their concentrations are the highest in late spring and summer; therefore, high levels are detected during this period, especially from large-scale pesticide usage in intensive farm practices (Rashid et al., 2010).

The presence of these pesticides in rivers and streams has become an increasingly important environmental issue, which poses a harmful impact on the ecosystem, flora and fauna, ultimately on human beings. Exposure to pesticides is unavoidable. People involved in pesticide industries and related fields are exposed to pesticides directly during the pesticide production, application, transportation, and utilization process. Meanwhile, indirect exposure can be through personal pesticides use, eating, drinking, or inhalation of pesticide-contaminated food, water, or air (Kim et al., 2017; Gavrilescu et al., 2005). The exposure level and corresponding pesticide hazards vary widely, which is mainly influenced by the environmental conditions of affected areas, exposure duration, pesticides' physical and chemical properties. (Anderson and Meade, 2014). Numerous health disorders have been reported even at a low level of pesticides exposure, including 300,000 annual deaths worldwide, cancer, respiratory problems, reproductive disorders, hormone disruption, memory disorders (Sabarwal et al., 2018).

Therefore, scientists and government agencies are very concerned particularly about how to remove pesticides from non-point sources. Conventional approaches at wastewater treatment plants such as physical, chemical, and biological methods are of high cost and inefficient to treat trace levels of pesticides (Fagan et al. 2016). Pesticides have been observed in wastewater treatment plant effluents in Germany, even occasionally higher than the legally permissible concentration; and the treatment efficiency varied considerably amongst different treatment facilities; occasionally, the efficiency was different from one year to the other at the same treatment plant. (Münze et al., 2017; Donald et al., 2007). However, most of pesticide-contained runoff will not flow into municipal wastewater treatment plants. Recently, constructed wetlands and chemically enhanced treatment wetlands have been studied for the treatment of agricultural runoff. These combine engineering design with natural processes and have been proposed for the potential to remove suspended solids and nutrients (Berberidou et al., 2016; Bachand et al., 2019). However, these have varying removal efficiency and usually require long retention time (Vymazal and Tereza., 2015). Some other techniques such as activated carbon adsorption, advanced oxidation process (AOP), photo-oxidation and treatment using UV/ H_2O_2 suffer from issues such as the generation of toxic by-products, longer contact time, high chemical doses and low efficiency, resulting in an economic burden in real-world practice (Ali et al., 2016; Franciscon et al., 2010).

Accordingly, overcoming the disadvantages of conventional methods and developing a rapid, economical, significantly efficient and environmentally friendly promising alternative for the treatment of trace concentrations of pesticides in aqueous system is the aim of this study. Here, two halogenated benzonitrile herbicides Bromoxynil

and Ioxynil have been selected. They are structurally analogous and have been extensively used for agricultural lands and households to limit the growth of unwanted weeds but their residues remain in water (Donald et al., 2007).

Enzymatic treatment as a bioremediation method for wastewater that has displayed its potential for treating recalcitrant contaminants and can be employed in the primary treatment or be combined with a biological unit (Steevensz et al., 2014 a). It is environmentally friendly due to low energy and chemical consumption (Watanabe et al., 2011). Peroxidases are oxidoreductases from living organisms like microorganisms or plants that catalyze the reaction rate and promote the transformation of phenolic compounds (Watanabe et al., 2011). Notably, the oxidoreductase soybean peroxidase (SBP) has shown its ability to remove halogenated phenolic pollutants in the presence of H_2O_2 (Steevensz et al., 2014 a). Compared with other conventional treatment technologies, enzymatic treatment agglomerates rather than degrades the target chemical compounds, leading to the precipitation of oligomers which then can be removed through filtration or sedimentation (Cordova-Villegas et al., 2016). Peroxidase-catalyzed removal can be a significantly robust approach for the transformation of recalcitrant target pesticides because it:

1) has high substrate specificity and excellent removal performance (Agarwal et al., 2016);
2) has the ability to catalyze under boarder range of pH and temperature (Al-Ansari et al., 2011);
3) is eco-friendly because of their biodegradability (Mugdha and Usha. 2012);

4) has shorter retention time, simple process control, and a wider applicable substrate concentration range (Mukherjee et al., 2019).

Therefore, enzymatic treatment offers valuable potential into the removal of the two selected Bromoxynil and Ioxynil.

1.2 Objectives

The objectives of this research were:

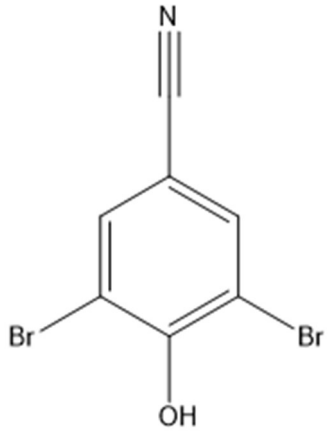
- To test the feasibility of enzymatic-catalyzed removal of two selected herbicides, Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and Ioxynil (3,5-diiodo-4-hydroxybenzonitrile), from synthetic wastewater (structures are demonstrated in Figure 1.1);
- To optimize the soybean peroxidase (SBP) catalyzed treatment for the two herbicides;
- To identify the possible polymeric products after enzymatic reaction by mass spectrometry.

1.3 Scope

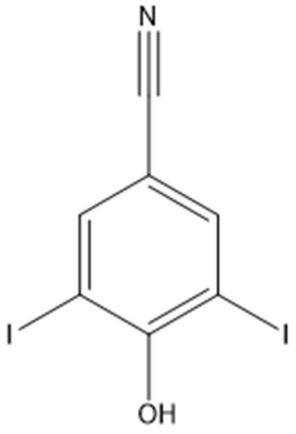
The scope of this research was to:

- Investigate the viability of SBP to convert 0.5mM Bromoxynil and 0.1mM Ioxynil in the presence of H_2O_2;
- Determine the optimal operational conditions for pH, hydrogen peroxide (H_2O_2) concentration, SBP concentration, and reaction time to accomplish $\geq$ 95 % pesticide conversion;

- Compare the removal efficiency of the selected pesticides in aqueous solutions.
- Determine the first-order rate constants and half-lives of Bromoxynil and Ioxynil from the reaction under optimal conditions;
- Characterize the formed products after reaction by using mass spectrometry (MS) with an atmospheric solids analysis probe (ASAP).



Bromoxynil
(3,5-dibromo-4-hydroxybenzonitrile)



Ioxynil
(3,5-diiodo-4-hydroxybenzonitrile)

Figure 1.1 Name and Structure of Selected Pesticides

CHAPTER 2 LITERATURE REVIEW

This chapter includes three main topics. The first part is the studies of halogenated benzonitriles, including their use, properties, concentrations in water and toxicity, in order to demonstrate the need for treatment of these compounds. The second part is related to the mechanism of soybean peroxidase, its advantages, and current applications, for the purpose of studying its potential for benzonitrile removal. The last part explains dehalogenation in the enzyme-catalyzed oxidative coupling process, which also occurs in the SBP-H_2O_2 oxidation system.

2.1 Halogenated Benzonitriles

2.1.1 Use

The two herbicides Bromoxynil and Ioxynil are both dihalogenated hydroxybenzonitriles with similar chemical structures (Pei et al., 2017). They have intensely used since the 1960s as selective contact post-emergence herbicides to control annual broad-leaved weeds (Veselá et al., 2012; Oliveira et al., 2018). Their mechanism is to hinder photosynthesis by impeding oxidative phosphorylation and the activity of mitochondria and chloroplasts (Pawlicova et al., 2006).

Bromoxynil is currently authorized in 156 commercial products in Canada, although it has been banned in Norway since 2000 (PMRA, 2019). It is generally used for control of seedling grasses of *Boraginaceae*, *compositae*, and *Polygonaceae* in a wide variety of crops (such as wheat, alfalfa, corn, sorghum), turf and non-crop land (Chen et al.,2011). Bromoxynil is also frequently combined with other herbicides like MCPA (2-methyl-4-chlorophenoxyacetic acid) at suggested doses of 0.4 – 1.1 kg/ha (Cupples et al.,

2005; Frková et al., 2014). However, a successful agricultural practice commonly requires the multiple times of applications at the suggested dosage (Pasquarelli et al., 2015). According to the U.S. Geological Survey (2019), the estimate of annual Bromoxynil usage was approximately 3 million pounds in 2016; around two-thirds was applied to wheat. Meanwhile, Bromoxynil is one of the top 10 most applied pesticides in Alberta, Canada (Munira et al.,2018). As atrazine and paraquat have been prohibited in some regions, Bromoxynil has become a preferred substitute for the two pesticides (Bettiol et al., 2016; Baxter et al., 2008).

Ioxynil is an approved herbicide in the European Union for food and feed crops, for example, cereals, onions, leeks, shallots, as well as forage grasses, lawns, and turf (Cupples et al., 2005; Lovecká et al., 2015). Forty-five tonnes of Ioxynil were traded in Denmark in 2009 (Frková et al., 2014). Ioxynil causes disruption of the thyroid system (Akiyoshi et al., 2012).

2.1.2 Chemical and Physical Properties

Some of the chemical and physical properties of two pesticides are listed in Table 2.1.

Table 2.1 Chemical and Physical Characteristics for the Two Pesticides

Compound	**Bromoxynil**	**Ioxynil**
chemical formula	$C_7H_3Br_2NO$	$C_7H_3I_2NO$
molecular mass (g/mol)	276.91	370.91
Chemical Abstracts Service (CAS) number	1689-84-5	1689-83-4
melting point (°C)	194 - 195 °C	212-213 °C
solubility in water	130 mg/L at 20°C (0.47mM)	50 mg/L at 20°C (0.13mM)
p*K*a	3.86	3.96
vapor pressure (milli-Pa)	6.3×10^{-3} at 20°C	<1 at 20°C
K_{oc} (L/kg)	87-140	155-540

Reference: Holtze et al., 2008

The *para*-position of the hydroxyl group and the relatively low soil adsorption coefficient (K_{oc}) of Bromoxynil and Ioxynil indicate both pesticides have higher possibility to partition into water and are less likely to sorb on soil (Holtze et al., 2008; Aravinna et al., 2017). The vapor pressure of Bromoxynil indicates it is easily evaporated, which can cause low utilization efficiency and potential secondary environmental contamination (Tang et al., 2017). As much as 33% of Bromoxynil volatilized into air within 48-hour in wind tunnel tests (Schweizer et al., 2000). Besides, the biologically recalcitrance of carbon-halogen bond leads to their environmental accumulation (Torres-Duarte et al., 2009).

Both compounds decompose in soil with less than 1-day half-life (Holtze et al., 2008). Another study reported the dissipation rate of Bromoxynil, which was slower in soil than in maize seedlings, with a mean half-life as 4.12 days and 1.14 days, respectively (Chen et al., 2011). The degradation of benzonitriles has been reported to be mainly through hydrolysis and microbial degradation in soil (Pasquarelli et al., 2015). The

pathways for nitrile metabolism is shown in Figure 2.1. The nitrile group is converted to the corresponding amide and further to the acid through nitrile hydratase and amidase or transform benzonitriles directly to the corresponding acid with nitrilase catalysis (Detzel et al., 2013; Oliveira et al., 2018). The photodegradation also was observed, but the rate was less than the degradation rate by soil microbes (Tang et al., 2017). The principle derived metabolites in soil are their corresponding amide 3,5-dibromo-4-hydroxybenzamide (BrAM) and 3,5-diiodo-4-hydroxybenzamide (IAM), presenting a greater solubility than their parent compounds and are more prone to leaching into water (Veselá et al., 2012).

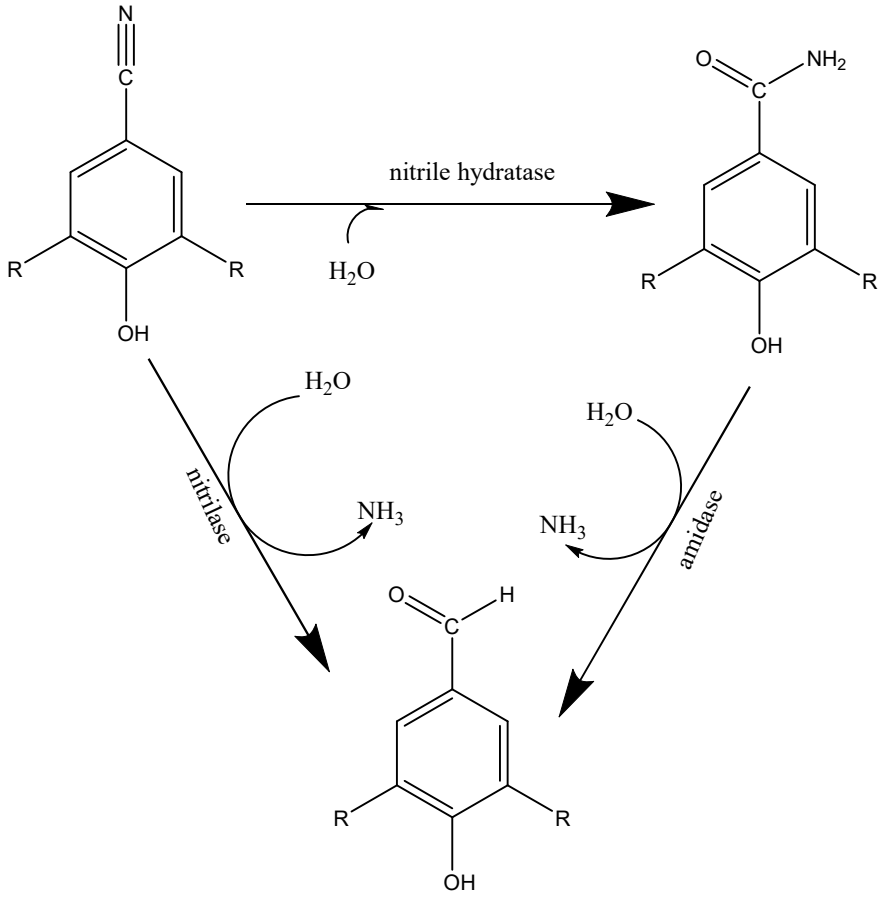


Figure 2.1 Degradation Pathway by Nitrile-degrading Enzymes

[R = Br or I; adopted from (Pei et al., 2017)]

2.1.3 Concentrations

Heavy but improper use of Bromoxynil and Ioxynil and their specific properties cause their residues to occur in receiving streams, rainfall, groundwater, and reservoirs (Semchuk et al., 2004). Bromoxynil has been found in 34% of groundwater samples in Alberta in 1991, of which 11% exceeded the Canadian drinking water guideline (Miller et al., 1995). A few years later, a study showed that Bromoxynil was present in rainfall during 1999 to 2000 in the same province with a maximum concentration of 26 µg/L (Hill et al., 2002). Donald et al. (2007) detected Bromoxynil in 54% of samples with maximum concentration at 384 ng/L in reservoirs and 227 ng/L in drinking water in Canada. The mean percentage of Bromoxynil reduction by drinking water treatment plants from 15 communities in Manitoba, Saskatchewan and Alberta was 46% (Donald et al., 2007). It was also observed that the concentration of Bromoxynil in reservoir water samples collected from same communities in July were considerably higher than samples in early spring (Donald et al., 2007). A high detection frequency (87%) of Bromoxynil in Canadian wetlands was also reported (Donald et al., 2001). Moreover, Bromoxynil were detected in 16 Canadian watersheds and rivers in 2007 (Glozier et al., 2012). A recent study identified Bromoxynil as well as the mixture of Bromoxynil, MCPA and Fluroxypyr in central Alberta with concentration at 29 ng/L and 244 ng/L, respectively (Munira et al., 2018). Not only in Canada but Bromoxynil has been found in surface waters in China as well (Zhu et al., 2008). It also has been detected in air and soil samples collected worldwide (Liaud et al., 2017; Poßberg et al., 2016). Few studies investigated or monitored Ioxynil, but its high potential of leaching into water bodies cannot be disregarded (Croll, 1991). Accordingly, both non-target aquatic lives and non-occupational residents are all under an exposure

potential to benzonitriles via polluted drinking water or dermal contact to benzonitrile-containing rainfall (Hill et al., 2002).

2.1.4 Toxicity

Toxicity studies are necessary to evaluate negative impact on human health. Toxicity information and drinking water guidelines for Bromoxynil and Ioxynil are listed in Table 2. Compared to Bromoxynil, Ioxynil has received less attention in terms of its toxicity. Both compounds presented moderate cytotoxic effects at several-fold of the allowable concentration in drinking water by European Union (0.5 µg/L) (Lovecká et al., 2015). Ioxynil is more toxic than Bromoxynil based on phytotoxicity assay results, with *L. sativum* EC50 4.99 µM and 8.70 µM, respectively (Bettiol et al., 2016). The main metabolites BrAM and IAM are more toxic than the original compounds, with a 50 % lower luminescence (EC50) value (Veselá et al., 2012).

Table 2.2 Toxicity and Drinking Water Criteria for the Two Pesticides

Toxicity Information		Bromoxynil	Ioxynil
Acute Toxicity	U.S. EPA Acute rating	Moderately Toxic	No Consensus Value
	WHO Acute Hazard	II, Moderately Hazardous	II, Moderately Hazardous
Cancer	U.S. EPA Carcinogens	C, Possible	Not Listed
Endocrine Disruption	EU list	category III	category III
Reproductive and Developmental Toxicity	CA Prop 65 Developmental Toxin	Listed	Not Listed
	U.S. TRI Developmental Toxin	Listed	Not Listed
Chemicals of Special Concern	PAN Bad Actors	Listed	Not Listed
Drinking Water Standards	maximum acceptable concentration (MAC)	5.0µg/L in Canada	Not established by the U.S. or Canada

Reference: Pesticide Action Network (PAN) Pesticide Database

The carcinogenic effect of Bromoxynil in mice and an increase of incidence rate caused by Bromoxynil exposure are reported in the recent review conducted by PMRA Canada (2019). This review also reported the strong acute toxicity of Bromoxynil to freshwater fish, LC50 as low as 29 µg/L in bluegill sunfish. A fatal case due to a Bromoxynil/MCPA co-formulation herbicide was recorded as well (Berling et al., 2015). Compared with other phenolic herbicides, Bromoxynil persists longer period in the human

body (Semchuk et al., 2004). Workers under Bromoxynil exposure more than one year had symptoms like headache, weight loss, fever, and urinary issues (Maddila et al., 2014).

Ioxynil, as an endocrine-disrupting chemical, inhibits the conversion to thyroid hormones, binding proteins, and induces tumors in animals (Lovecká et al., 2015; Leithe et al., 2010). Ioxynil presents moderate toxic effects on human cells in the liver and kidneys (Oliveira et al., 2018). Fish and amphibians are more vulnerable to Ioxynil exposure than birds and mammals (Akiyoshi et al., 2011).

2.2 Peroxidases

Peroxidases are oxidoreductase enzymes, mainly heme proteins, widely available in microbial and plant sources, as well as in animals and fungus (Jun et al., 2019). Their appealing biocatalyst properties such as board distribution, high redox potential, substrate specificity and high thermal stability have been demonstrated in catalyzing the oxidation of numerous organic and inorganic compounds under the presence of co-substrate hydrogen peroxide (H_2O_2) (Dunford and Stillman, 1976; Steevensz et al., 2013; Mukherjee et al., 2019). Treatment of phenols and halogenated phenols are one of the main biotechnological applications of peroxidases (Al-Ansari et al., 2009; Watanabe et al. 2011; Feng et al., 2013; Levin et al., 2018).

Compared to animal peroxidases, plant peroxidases have been employed extensively for wastewater bioremediation (Raven and Dunford, 2015). The peroxidase superfamily has been subcategorized into three classes based on their sequence homologies. Class I is a group of intracellular peroxidases, for example cytochrome c peroxidase and ascorbate peroxidase. Class II consists of secretory fungal peroxidases, such as manganese

and lignin Peroxidase. Class III includes secretory plant peroxidases, for instance horseradish peroxidase (HRP) and soybean peroxidase (SBP) (Jun et al., 2019; Demarche et al., 2012).

2.2.1 Peroxidase Mechanism

The mechanism of peroxidase-catalyzed pollutant removal is a modified ping-pong mechanism (Steevensz et al., 2014 a). The first step (Equation 1) is the oxidation of native peroxidase (Fe^{3+}) by hydrogen peroxide (H_2O_2), the electron acceptor, resulting in an active form, compound I (Fe^{4+}–R^{+}). Second, Compound I oxidizes a substrate molecule (AH) by one electron to become Compound II (Fe^{4+}) and generate a substrate radical (A·) (Equation 2). The substrate can be a phenol or aniline. Then Compound II oxidases another molecule of the substrate and generates the second free radical and returns to the native enzyme (Equation 3). The total reaction balance can be described in Equation 4 (Krainer and Glieder. 2015; Mashhadi et al., 2019 a).

Equation 1) Native peroxidase (Fe^{3+}) + H_2O_2 ⟶ Compound I (Fe^{4+}–R^{+}) + H_2O

Equation 2) Compound I + AH ⟶ Compound II (Fe^{4+}) + A·

Equation 3) Compound II + AH ⟶ Native peroxidase (Fe^{3+}) + A·

Equation 4) 2 AH + H_2O_2 $\xrightarrow{\text{Peroxidase}}$ 2 A· + 2 H_2O

For phenolic compounds, the substrate free radicals engage in the non-enzymatic coupling, which leads to the generation of higher oligomers through C-C or C-O coupling in the *ortho-* or *para-* positions (Steevensz et al., 2014 a). Eventually, insoluble oligomeric precipitates are formed, which can be simply removed by filtration or sedimentation (Mashhadi et al., 2019 a).

However, inactivation of peroxidase has been reported via two pathways under excess H_2O_2 and deficient level of substrate (Steevensz et al., 2014 a). First, a reversible intermediate enzyme-H_2O_2 can be produced with the generation process of Compound I, which may irreversibly further convert to inactive intermediate P-670 (absorbance peak at 670 nm) (Arano et al., 1990). The second pathway of inactivation is the formation of Compound III from the oxidation of Compound II with extra H_2O_2. Compound III can decay back to the native enzyme, but the low rate of decomposition allows accumulation of Compound III, a reversible inactive enzyme form (Krainer and Glieder. 2015). In addition, end-product inactivation by polymerized substrate which adsorbs peroxidase, leads to peroxidase inactivation also reported by Feng et al., (2013). Inactivation leads to a shorter catalytic lifetime and a higher cost of enzyme requirment for the enzymatic reaction.

Another challenge was found with HRP, one of the peroxidases has been much studied last four decades, that HRP stays active within a relatively narrow range of temperature (5 to 55 ºC) and pH (4.0 - 8.0) (Ryan et al., 2006). On the other hand, the difficulty of HRP extraction and purification brings about a limitation on its availability in large quantity at an affordable price (Al-Ansari et al., 2009). Accordingly, more scientists have diverted their attention to the more feasible soybean peroxidase (Al-Ansari et al., 2011).

2.2.2 Soybean Peroxidase

Soybean Peroxidase (SBP) is a heterogeneous glycoprotein in the group of class III plant peroxidases with molecular weight and isoelectronic point (pI) as 37 kDa and 4.1, respectively (Marchis et al., 2012; Rezvani et al., 2015; Cordova-Villegas et al., 2018).

SBP is extracted from an agricultural by-product soybean seed hulls (soybean seed coat), the major by-product of the soybean industry and utilized for animal feed (Kumar et al., 2019; Chagas et al., 2015). The extraction process of SBP starts with softening the soybean seed hulls by soaking them in water for 1-2 hours, then wash these hulls with water; thus, the extraction of SBP can be achieved and hulls can still keep the feed value (Steevensz et al., 2014 b).

Soybean is one of the principal crops in North and South America. The global soybean production has grown since 1960 from 17 million metric tons (MMT) to 363 MMT in 2018/19 (USDA, 2019). The United States is the most significant contributor of global soybean production among all countries, produced 123 MMT in 2018/19, which was approximately one-third of the total yield. According to Statistics Canada (2019), the Canadian soybean production was 7.27 MMT in 2018/19, 103% more than the production in 2009. The rise in Canadian soybean production and seeded area from 2005 to 2018 is shown in Figure 2.2. In this way, SBP can be readily available from the agricultural commodity in bulk quantities due to the cheap and abundant source, offering a great opportunity for SBP's commercial application.

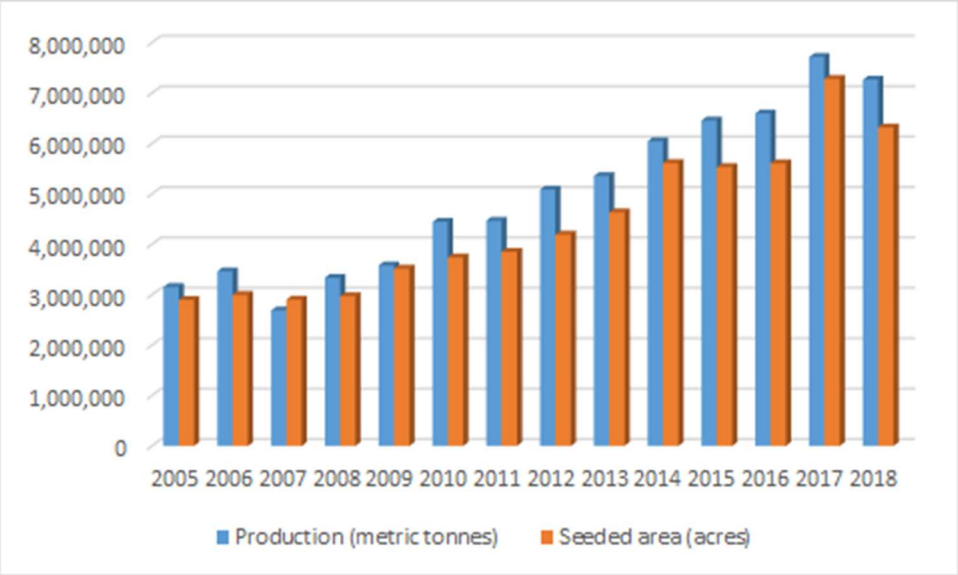


Figure 2.2 Canadian Soybean Production and Seeded Area, Statistics Canada (2019)

Besides the economic availability, SBP has exhibited other advantages, significantly enhancing its feasibility for application in treating wastewater than other peroxidases. Bódalo et al. (2008) summarized that SBP has lower susceptibility to inactivation by H_2O_2 and is more potent due to its higher k_{cat}/K_M constant than HRP. SBP based technique can be viewed as a low energy and chemically efficient process (Demarche et al., 2012). In addition, the SPB remains active from pH 2.0 to 10.0, considerably wider active range than HRP (Ryan et al., 2006). It also has better thermal stability than HRP, which allows SBP to keep functioning under harsher temperature conditions (70 ºC) without a change in the secondary structure (McEldoon and Dordick, 1996).

2.2.3 Peroxidases in Wastewater Treatment

Numerous studies have explored toxic compounds removal by using peroxidases. The first study of enzymatic treatment on phenols and anilines removal from wastewater was conducted by Klibanov et al. (1980) in the early 1980s using horseradish peroxidase

(HRP) and hydrogen peroxide. Bollag et al. (1979) studied the polymerizing ability of two halogenated phenolic compounds 2,4-dichlorophenol and 4-bromo-2-chlorophenol by laccase. Since then, many researchers have started investigations in enzyme-catalyzed treatment from aqueous solutions (Cordova-Villegas et al., 2016).

SBP has shown the catalytic ability for oxidation of a wide array of halogenated phenolic compounds (Marchis et al., 2012; Steevensz et al., 2014 a). Tolardo et al. (2019) found that SBP could effectively treat pentachlorophenol (PCP) in the presence of H_2O_2 at pH 5.0 at lab scale. Li et al. (2016) have obtained better catalytic performance of SBP than HRP. They conducted SBP and HRP removal of triclosan and achieved 98% conversion with 0.1 U/mL SBP and 0.3 U/mL HRP, respectively. SBP also can reduce the number of phenolic pharmaceuticals from synthetic wastewater (Mashhadi et al., 2019 b). Both bisphenol A (BPA) and its derivatives can be removed entirely or effectively by SBP and laccase (Watanabe et al., 2011; Modaressi et al., 2005).

In addition, SBP has shown its oxidation power in real wastewater. Steevensz et al. (2014 b) successfully converted ≥ 95% phenol from industrial wastewater (alkyd resin manufacture). Chagas et al. (2015) investigated both the free and immobilized SBP to treat caffeic acid from synthetic wastewater and wastewater from coffee processing. They discovered that both enzymes could oxidize the target compound, but two times longer reaction time was required by immobilized SBP. The enzymatic treatment using SBP for other chemicals such as azo dyes, dye-derived arylamines, quinolines also was achieved (Cordova-Villegas et al. 2018; Mukherjee et al. 2019; Mashhadi et al. 2019a).

To our knowledge, only one study was related to enzyme-catalyzed treatment for benzonitriles in water, where laccase was employed for Bromoxynil transformation.

Bromoxynil was converted while the laccase catalytic activity was 48.8 nmol min^{-1} U^{-1}, the worst performance among the tested pesticides (Torres-Duarte et al., 2009). A combined UV/H_2O_2 technique was also implemented for Bromoxynil treatment, degraded 90% of Bromoxynil from pure and natural water (Chelme-Ayala et al., 2010). It is one of the advanced oxidation processes (AOPs) that forms free hydroxyl radicals to mineralize the treated organics, which may encounter the formation of toxic intermediates (Cordova-Villegas et al., 2016). Bromoxynil can be decomposed through riboflavin photosensitized oxidation as well but with limited treatment performance (Escalada et al., 2011). The decomposition of both Bromoxynil and Ioxynil was identified in water by photohydrolysis of carbon–halogen bonds to generate monohalogenated compounds, while the degradation was incomplete (Malouki et al., 2004). The first study of SBP-catalyzed reaction for benzonitriles removal from aqueous solution could be a prospective means of overcoming these disadvantages.

2.3 Dehalogenation of Phenolic Compounds

The dehalogenation of halophenolic substrates has been discovered through oxidative coupling catalyzed by peroxidase, which may be a benefit for toxic compound removal due to chemical decomposition (Dec et al., 2003). Schultz et al. (2001) demonstrated a dehalogenation of chlorinated hydroxybiphenyls by laccase due to the presence of de-chlorinated dimers formed during the oxidative coupling process; dimers with one or two chlorine remaining were also found via gas chromatography-mass spectrometry (GC-MS) analysis. Another peroxidase, HRP, has been identified as the dehaloperoxidase for 2,4,6-trichlorophenol in the presence of H_2O_2 (Ferrari et al., 1999).

Osborne et al. (2006) employed chloroperoxidase from *Caldariomyces fumago* (CCPO) to dehalogenate the same compound under harsher conditions. The hypothetical catalytic equation has been shown in Figure 4, which shows that the mechanism can be related to electron-transfer based on the generation of a mono-dehologenated compound. Osborne et al. (2006) also reported that the dimers were the principal products rather than benzoquinone, suggesting the presence of free radical intermediate from the peroxidase oxidation process. As the similar chemical structure between 2,4,6-trichlorophenol and benzonitriles, Bromoxynil and Ioxynil may become dehalogenated via a similar catalytic pathway with the SBP- H_2O_2 oxidation system.

In terms of benzonitriles dehalogenation, Cupples et al. (2005) utilized a bacterium *D.chlororespirans* that transformed both Bromoxynil and Ioxynil under pure anaerobic culture. But no dehalogenation studies have been found using SBP as dehalogenation catalyst.

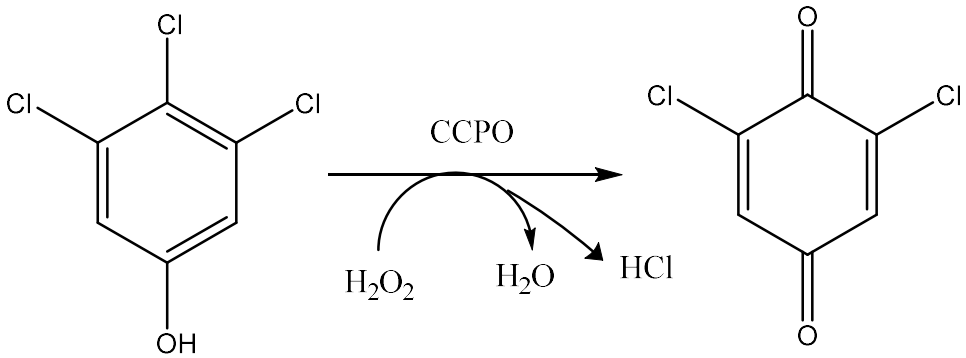


Figure 2.3 The Proposed Catalytic Dehalogenation of 2,4,6-Trichlorophenol by Chloroperoxidase Adopted from Osborne et al., 2006.

CHAPTER 3 MATERIAL AND METHODS

3.1 Materials

3.1.1 Benzonitrile Herbicides

Bromoxynil (>98% purity) was purchased from AK Scientific Inc. (Union City, CA). Ioxynil (97% purity) was obtained from Alfa Aesar (Tewksbury, MA). Both chemicals were stored at room temperature.

3.1.2 Enzymes

Crude solid SBP (activity 5 U/mg) was purchased from Organic Technologies (Coshocton, OH). Solid bovine liver catalase (activity 19,900 U/mg) was bought from Sigma Aldrich Chemical Company Inc. (Oakville, ON). Solid enzymes were both stored at − 15 °C while their aqueous solutions were stored at 4°C.

3.1.3 Reagents

4-aminoantipyrine (4-AAP) was purchased from BDH Inc. (Toronto, ON) and was kept at room temperature. Hydrogen peroxide (30% w/v) was obtained from ACP Chemicals Inc. (Montreal, QC) and was stored at 4 °C.

3.1.4 Buffer and solvents

Analytical grade monobasic and dibasic sodium phosphate, potassium chloride (KCl), hydrochloric acid (HCl), and anhydrous ethanol were purchased from ACP Chemicals Inc. Citric acid was obtained from Sigma Aldrich Chemical Company Inc.

3.1.5 Other materials

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 and Laboratory Equipment

3.2.1 UV-Visible spectrophotometry

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 determine the maximum wavelength of substrates, 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 two compounds are provided in Appendix A.

3.2.2 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 LSE^{TM} Compact Centrifuge (6×50 mL and 6×15 mL centrifuge tubes, 200 to 6000 rpm speed range) (Tewksbury, MA).

3.2.3 Atmospheric Solids Analysis Probe (ASAP) mass spectrometry

The equipment used for mass spectrometry (MS) was a high-resolution Waters Xevo G2-XS Time-of-Flight (Tof) mass spectrometer (Oakville, ON). The Atmospheric Solids Analysis Probe (ASAP) was used to analyze solid and liquid samples through atmospheric pressure ionization. The acquisition range of probe was 50 to 1500 mass-to-charge ratio (m/z). The measurement results were analyzed by the MassLynx software (version 4.1).

3.2.4 Other equipment

The study also used a vortex genie mixer (model K550-G) from Scientific Industries, Inc. (Bohemia, NY), an Accu–124 analytical balance from Fisher Scientific Company, and VWR Magnetic Stirrers (VS-C series, speed range 100-1500 rpm) from VWR International Inc.

3.3 Analytical Methods

3.3.1 SBP activity assay

The free SBP activity was determined using a colorimetric assay. The reaction of the assay was:

$$\text{4-AAP + phenol + } H_2O_2 \xrightarrow{\text{SBP}} \text{Pink chromophore } (\lambda_{max} = 510 \text{ nm})$$

SBP catalyzes the oxidative coupling of phenol and 4-AAP in the presence of H_2O_2, generating a pink chromophore (extinction coefficient $\varepsilon = 6000\ M^{-1}cm^{-1}$ relative to H_2O_2). The enzyme catalytic activity (U/mL) was the initial rate of reaction, proportional to the enzyme concentration, measured at the wavelength of maximum absorbance (510nm) of the reaction product in the solution. A unit (U) of SBP activity was defined as the amount of enzyme that converts one micromole of hydrogen peroxide per minute at pH 7.4 at room temperature. The reaction solution consisted of diluted SBP (dilution factor 30, 40, 50) and 47.5 mL of reagent. The regular regent was made of 2.4 mM 4-AAP, 0.2 mM H_2O_2, 10 mM phenol, and 0.5 mM phosphate buffer (pH 7.4) (Caza et al., 1999). First, the equipment was blanked with 950 µL of reagent and 50µL distilled water. Second, the 950 µL of reagent mixture was quickly added to 50 µL diluted SBP solution in the cuvette. Then, the increase in absorbance over 30 seconds run time and 5 seconds cycle time was monitored, and the activity was calculated through built-in instrument software using zero-order kinetics. More information is available in Appendix B.

3.3.2 Feasibility test of substrate conversion by SBP

An essential preliminary experiment of this research was to check if Bromoxynil and Ioxynil were substrates for SBP. First, the solubility test was performed for benzonitriles to determine the initial concentrations of compounds in the appropriate solvent. Bromoxynil and Ioxynil both dissolved in 30% anhydrous ethanol at concentrations of 1.0 mM and 0.2 mM, respectively as stock solutions. Second, the dissolved compounds were mixed with appropriate concentrations of SBP and H_2O_2 in

buffered solutions (pH 2.0, 4.0 and 6.0) for 3 hours, then catalase was added to stop the reaction and measure the concentration of residual compounds in filtered solutions by UV-vis. The reaction mixtures were also left overnight at room temperature for observation of precipitates formed or the possible change in color of the reaction solutions. A change in color (clear to light yellow) and a decrease in substrate concentrations with the increase of enzyme were observed; accordingly, both benzonitriles were substrates of SBP.

3.3.3 Substrate/buffer compatibility tests

The absorbance of substrates in different buffers (pH range 2.0 to 8.0) were tested by UV-vis spectrophotometer. An isosbestic point, the wavelength at which absorbance is pH-independent, was observed for both pollutants: 262.5 nm for Bromoxynil and 266 nm for Ioxynil. Therefore, one calibration curve was sufficient for each substrate. However, it was observed that there was an increase in absorbance after enzymatic treatment for Ioxynil at its isosbestic point, indicating the oligomer products may have absorbance at this wavelength. Thus, calibration curves for Ioxynil were plotted at its λmax (284 nm), instead of the isosbestic point, where there was a change in absorbance at different pHs. All calibration curves for both compounds are given in Appendix C.

3.3.4 Buffer preparation

Based on the buffer preparation manual (Gomori, 1955), different buffers with the desirable pH were prepared, including hydrochloric acid–potassium chloride buffer (pH 2.0), citrate–phosphate buffer (pH 2.6 to 5.5), and monobasic-dibasic sodium phosphate buffer (pH 6.0 to 8.0).

3.3.5 Enzyme stock solution preparation

The preparation for SBP stock solution started with mixing 1.4 g of solid enzyme with 100 mL distilled water at low speed (approximately 400 rpm) for one day; followed by centrifugation at 4000 rpm for 25 minutes. The supernatant was taken as the stock solution. Catalase stock solution was prepared by mixing 0.5 g solid catalase and 100 mL distilled water for 4 hours. Both stock solutions were kept at 4 °C.

3.4 Experimental Protocols

3.4.1 SBP-catalyzed conversion of benzonitriles

Batch reactions were employed to optimize the enzymatic removal of two substrates (at least 95%) by SBP. Optimization parameters included pH, H_2O_2 and SBP concentrations, as well as reaction time. 40 mM buffer, 0.5 mM Bromoxynil/0.1 mM Ioxynil, SBP and H_2O_2 were mixed in that order in a batch reactor (30 mL glass vials) for 3 hours on magnetic stirring plates. Catalase was then added to the mixtures at the end of the reaction to break down the residual H_2O_2, thereby stopping the reaction, followed by micro-filtering (0.2 μm) and measuring the remaining concentration of substrate by UV-vis. Three controls were used: all constituents except enzyme, all constituents except H_2O_2, only substrate in the buffered solution, respectively. The time course of reactions was conducted similarly in batch reactors under the pre-determined optimal conditions, but the substrate conversion was monitored over time. The first-order reaction rate constants and half-lives of substrates were calculated. All experiments were in triplicate, and the calculated standard deviation have been presented in figures by error bars.

3.4.2 Polymeric products identification

The mass spectrometry samples were prepared after batch reactions under determined optimum conditions. The batch reaction used a decreased buffer concentration (40 mM reduced to 5 mM) to reduce the MS interferences caused by a high concentration of buffer. After 3-hour of mixing, the reaction solutions were stopped by catalase and centrifuged at 4200 rpm for 17 minutes. All standard samples (solid and liquid), supernatant, and precipitate obtained from batch reaction mixture were analyzed by ASAP measurement in positive ionization and sensitive mode. The probe temperatures were at 250 °C for Bromoxynil and 350 °C for Ioxynil, and the temperatures for lockspray ionization source were same for both compounds at 100 °C.

3.4.3 Sources of error

Systematic errors from analytical instruments and random errors due to unexpected measurement influence the accuracy and precision of experimental results. All experiments were carried out in triplicate within 5% standard deviation range to minimize the random errors. The principal systematic error was from UV-vis since the spectroscopy-based measurement has relatively lower sensitivity and accuracy than high performance liquid chromatography (HPLC) analysis. However, the calibration of pH meter, scales, pipettes, and other equipment which were conducted each time before use, could also contribute to some systematic errors. The error caused by the influence factors of SBP activity such as room temperature and reagent age was minimized by testing enzyme activity every day before the batch reactions.

CHAPTER 4 RESULTS AND DISCUSSION

Bromoxynil and Ioxynil were studied in preliminary feasibility tests and were found to be substrates of SBP based on the reduction in absorbance at their isosbestic point or at λ_{max} (262.5 nm for Bromoxynil and 284 nm for Ioxynil), and because of a slight color change from transparent to light yellow. Accordingly, four operational parameters (pH, H_2O_2 concentration, enzyme concentration and time of reaction) were investigated to achieve ≥95% substrate removal efficiency with a minimum enzyme activity. Then reactions were operated under optimized conditions to identify possible products by mass spectrometry analysis.

4.1 SBP-catalyzed Treatment Optimization

4.1.1 pH optimization

The determination of optimum pH is necessary as it has an influence on enzyme structural stability and ionization state of amino acid side chains, therefore the catalytic activity of SBP become pH dependent (Al-Ansari et al., 2011). In addition, the optimal point relies on substrate and reaction conditions (Cordova-Villegas et al., 2018). SBP was found to have the maximum activity (>95%) in weak acidic solutions (pH 5.5 to 6.0) for guaiacol (Geng et al., 2001), and it could stay active between pH 3.0 and 9.0 for 3-substituted quinolines (Mashhadi et al., 2019). The optimal pH was the value that puts critical histidine and arginine residues of the enzyme in the best ionization state, providing the best conformational stability of the enzyme (Al-Ansari et al., 2011).

Both Bromoxynil and Ioxynil were studied across the pH range from 2.0 to 8.0 under stringent conditions with respect to enzyme concentration, in which enzyme dosage

was kept at an insufficient level so that the percentage remaining of unreacted substrates was maintained in the range of 15 - 40%; thus the optimal pH point can be clearly ascertained. The initial substrate and H_2O_2 concentrations were 0.5 mM and 0.75 mM for Bromoxynil, and 0.1 mM and 0.15 mM for Ioxynil, respectively. The batch reactors were run with two controls (one not containing enzyme and one not having H_2O_2) and a standard (no enzyme or peroxide) for 3 hours. All experiments were conducted in triplicate at room temperature (22±1 ºC).

Percentages remaining of Bromoxynil and Ioxynil as a function of pH after 3-hour reaction are shown in Figures 4.1 and 4.2. Error bars were calculated as the standard deviation, but some were not visible since they were too small and were hidden in the symbol. As shown in both figures, the influences of pH on the transformation of halogenated benzonitriles by SBP were similar, where the best performances for both substrates were at pH 4.0. Ioxynil had a slightly narrower optimum pH range (pH 4.0 - 4.5) compared to Bromoxynil (pH 3.5 - 4.5). Hence, pH 4.0 was chosen for further optimization study of Bromoxynil and Ioxynil.

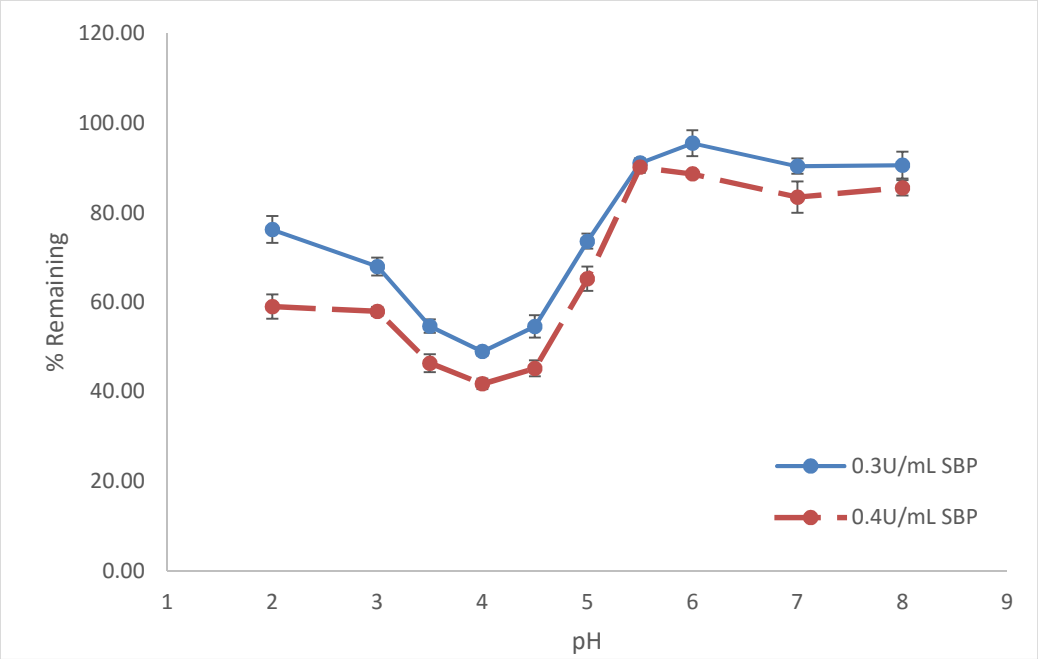


Figure 4.1 Bromoxynil pH Optimization (0.5 mM Bromoxynil, 0.75 mM H_2O_2, 0.3 and 0.4 U/mL SBP, 3-hour reaction, room temperature)

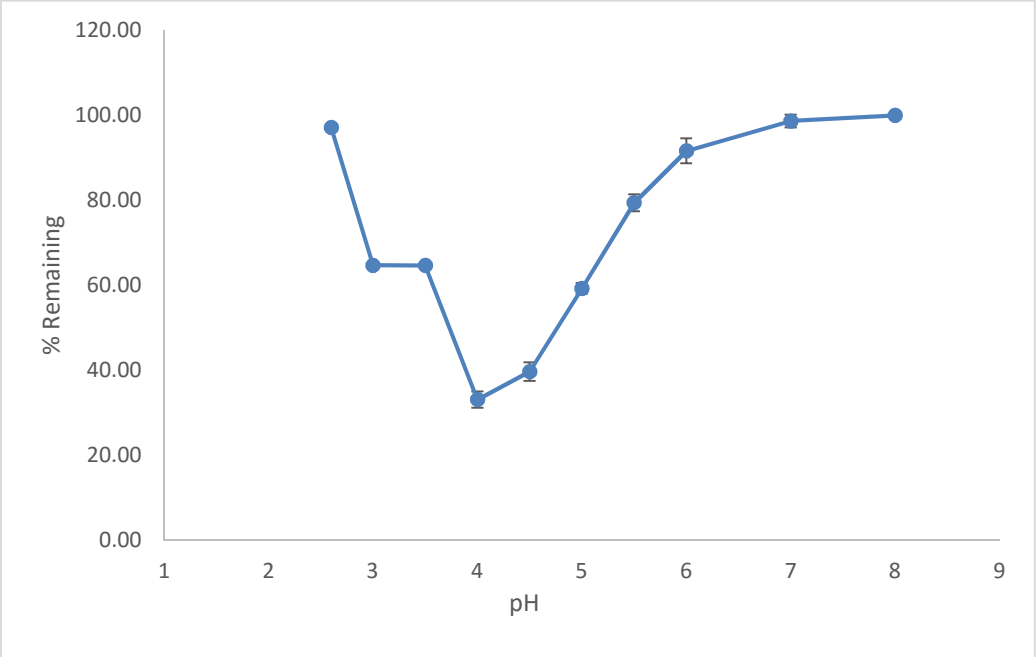


Figure 4.2 Ioxynil pH Optimization (0.1 mM Ioxynil, 0.15 mM H_2O_2, 0.1 U/mL SBP, 3-hour reaction, room temperature)

The optimum pH was very close to the pK_a of two substrates (3.86 for Bromoxynil and 3.96 for Ioxynil; Holtze et al., 2008). Similar results were found for *p*-cresidine (2-methoxy-5-methylaniline, optimal pH 4.6, pK_a 4.7) and 3-hydroxyquinoline (optimum pH range 8.0 - 8.6; pK_a 8.0) (Mukherjee et al., 2018; Mashhadi et al., 2019 a). For other phenolic compounds, such as phenol, chlorinated phenols, cresols, and Bisphenol A, the optimum pHs were between 5.0 and 8.0 with SBP as catalyst (Caza et al., 1999; Watanabe et al., 2010), lower than their corresponding pKa values (above 7.0; Criquet et al., 2015). The difference may relate to the low catalytic efficiency of the enzyme at higher pHs. In addition, no clear influence of pH on substrate conversion efficiency when pHs >6.0 are shown in either figure. This could be related to the ionization of the hydroxyl group. More than 90% of substrates were anion (OH^-) when pHs were above 6.0 and these anionic forms could not be involved in the peroxidase cycle; therefore, low transformation percentages were observed. Meanwhile, it can be seen that the conversion performance of Bromoxynil increased with increase in enzyme dosage from 0.3 to 0.4 U/mL, which supports the concept that SBP-catalyzed treatment was feasible for benzonitrile transformation.

Portion of Ioxynil precipitated out of solution at lower pHs due to the solubility issue. For this reason, the percentage remaining of standard controls from pH 2.0 to 4.0 were tested, where each standard control (only containing Ioxynil and buffer) was run in a batch reactor for 3 hours. The results in Table 4.1 illustrate a decrease in pH leaded to a decrease in solubility of Ioxynil. Specifically, at pH 2.0, only 31 ± 0.9% of the compound remained in reaction solution. These data were utilized to correct the pH optimization of Ioxynil at lower pHs, that is the absorbance of the reaction mixture after enzymatic

treatment was recalculated using the data attained from the standard control experiments. This solubility issue was not seen for Bromoxynil.

Table 4.1 Results for Standard Control Test

pHs	% Remaining of Ioxynil in Standard Controls
2.0	31 ± 0.9
2.6	40 ± 0.4
3.0	56 ± 0.8
3.5	76 ± 0.6
4.0	99 ± 0.9

4.1.2 Hydrogen peroxide optimization

Hydrogen peroxide is the co-substrate in SBP-catalyzed reactions and starts the enzymatic reaction. Less H_2O_2 limits the reaction while excess H_2O_2 could result in permanent enzyme inactivation (Al-Maqdi et al., 2018). Hence, the initial H_2O_2 concentration as one of the operational parameters was monitored to clarify its impact on the substrate degradation efficiency. Figures 4.3 and 4.4 demonstrate the removal performance under different H_2O_2 concentrations at the optimal pH 4.0. The range of H_2O_2 concentrations were from 0.3 mM to 1.0 mM for Bromoxynil and 0.03 mM to 0.3 mM for Ioxynil, respectively.

For 0.5 mM Bromoxynil, the best performance was observed at 0.4 – 0.5 mM of H_2O_2 with minimum 11.5-12.6% of substrate remaining. And for 0.1 mM Ioxynil, 0.08 – 0.1 mM H_2O_2 concentration (4.3-4.8% remaining) was its optimal range. Optimum H_2O_2-to-substrate molar ratios were 0.8-1.0 for both compounds.

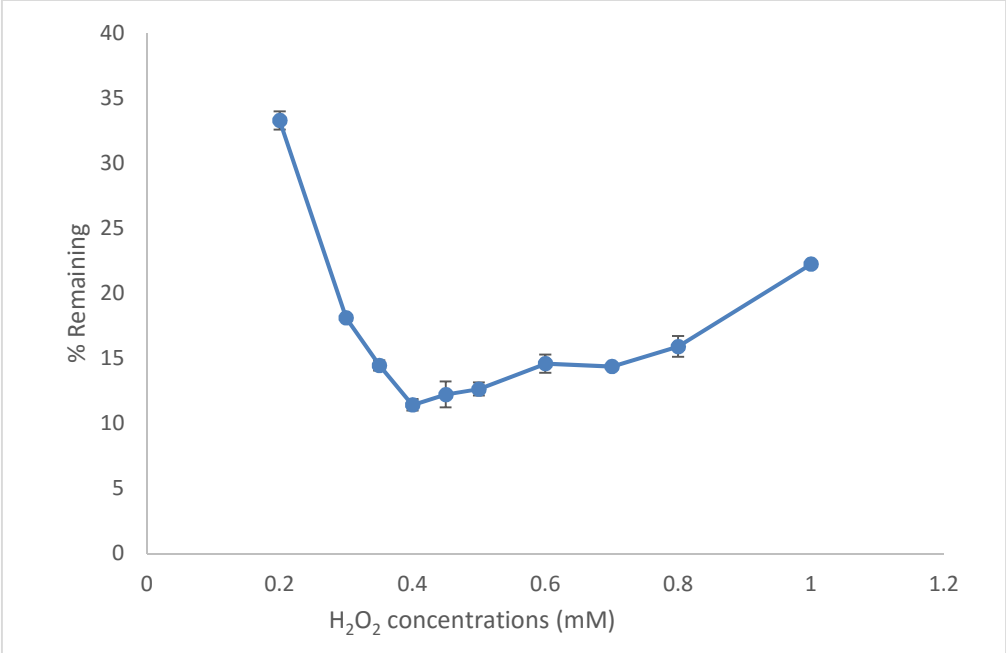


Figure 4.3 Bromoxynil H_2O_2 Optimization (pH 4.0 buffer, 0.5 mM Bromoxynil, 0.8 U/mL SBP, 3-hour reaction, room temperature)

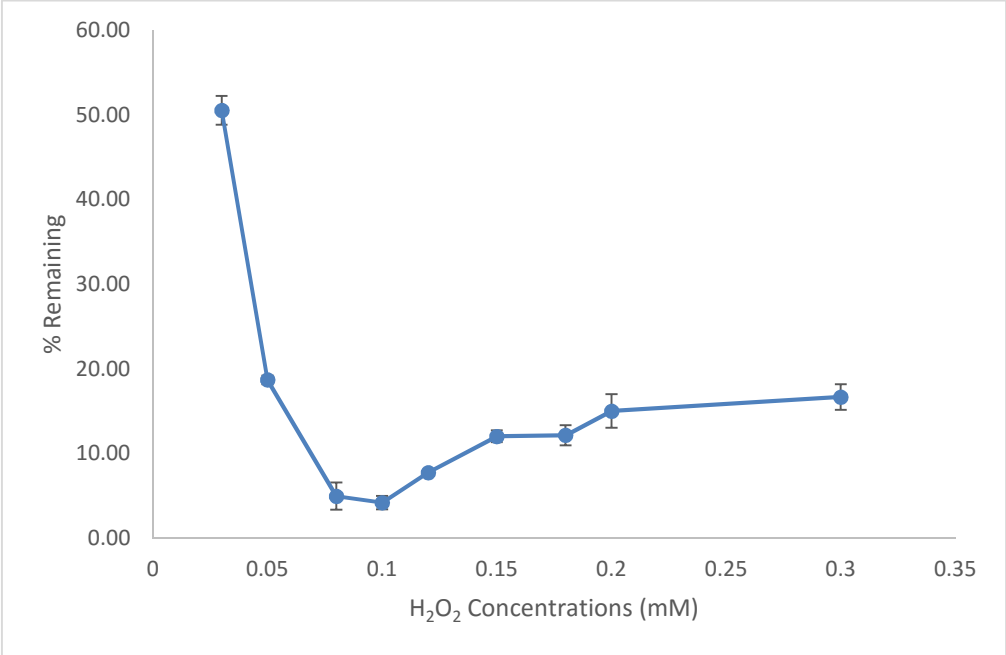


Figure 4.4 Ioxynil H_2O_2 Optimization (pH 4.0 buffer, 0.1 mM Ioxynil, 0.3 U/mL SBP, 3-hour reaction, room temperature)

According to the modified ping-pong mechanism discussed in section 2.2.1, 1 mole of H_2O_2 consumed was able to covert 2 moles of aromatic functional group (H_2O_2 to substrate ratio 1:2), whereas studies was found that the optimum ratio usually approaches 1.0 (Watanabe et al. 2010; Bódalo et al., 2008), similar to the results was obtained in this study. The ratio difference indicated additional H_2O_2 consumption. One reason could be that the soluble oligomers generated from reaction went back into the catalytic cycle (Yu et al., 1994; Raven, 2016). In that case, the optimum H_2O_2 concentration was more related to the solubility of formed products. On the other hand, the catalase activity of peroxidases and crude enzyme mixture may also result in increased peroxide consumption (Mukherjee et al., 2019). Furthermore, notable deactivations were seen in both figures at high H_2O_2 concentrations, which was possibly due to the formation of enzyme compound III (reversibly catalytically inactive) or the inactive derivative P-670 (Steevensz et al., 2014). Accordingly, to correspond with above studies (ratio as 1.0), H_2O_2 concentrations of 0.5 mM and 0.1 mM were selected for Bromoxynil and Ioxynil, respectively, for further experiments.

4.1.3 Enzyme optimization

The cost of enzyme normally restricted the practical application of enzyme treatment in wastewater treatment plant (Steevensz et al. 2009). Although SBP was a relatively low-cost enzyme, it was crucial to minimize the enzyme concentration while reaching 95% transformation efficiency of substrates. As discussed above, enzyme optimization was investigated at the above-determined optimum pHs and H_2O_2 concentrations. For 0.5 mM Bromoxynil, the range of SBP concentrations was 0.5 - 1.5

U/mL with pH 4.0 and 0.5 mM of H_2O_2 as shown in Figure 4.5. For 0.1 mM Ioxynil, 0.1 - 0.6 U/mL enzyme dosages were investigated with pH 4.0 and 0.1 mM of H_2O_2, as presented in Figure 4.6.

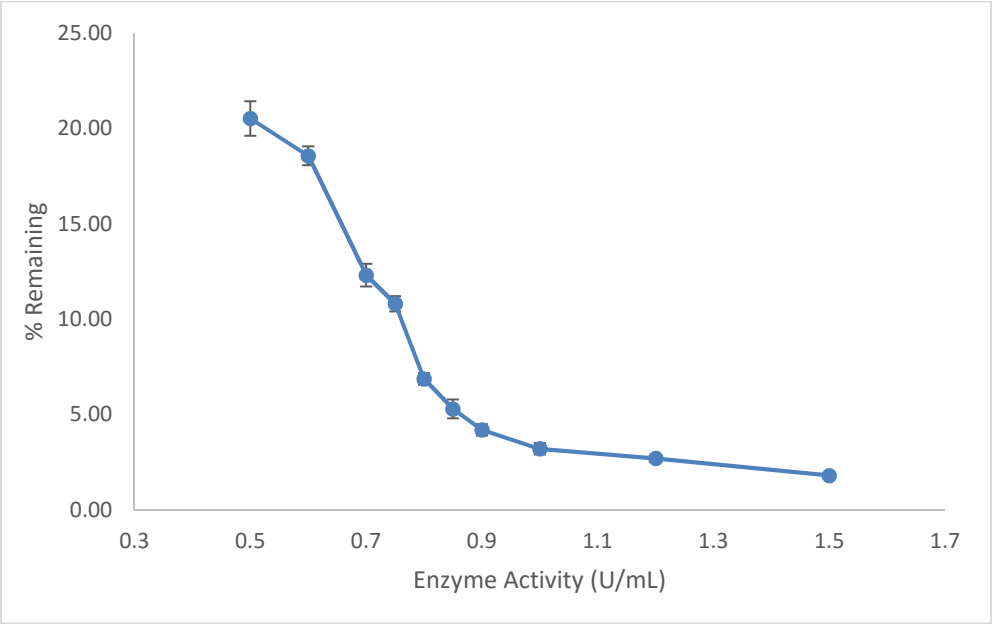


Figure 4.5 Bromoxynil Enzyme Optimization (pH 4.0 buffer, 0.5 mM Bromoxynil, 0.5 mM H_2O_2, 3-hour reaction, room temperature)

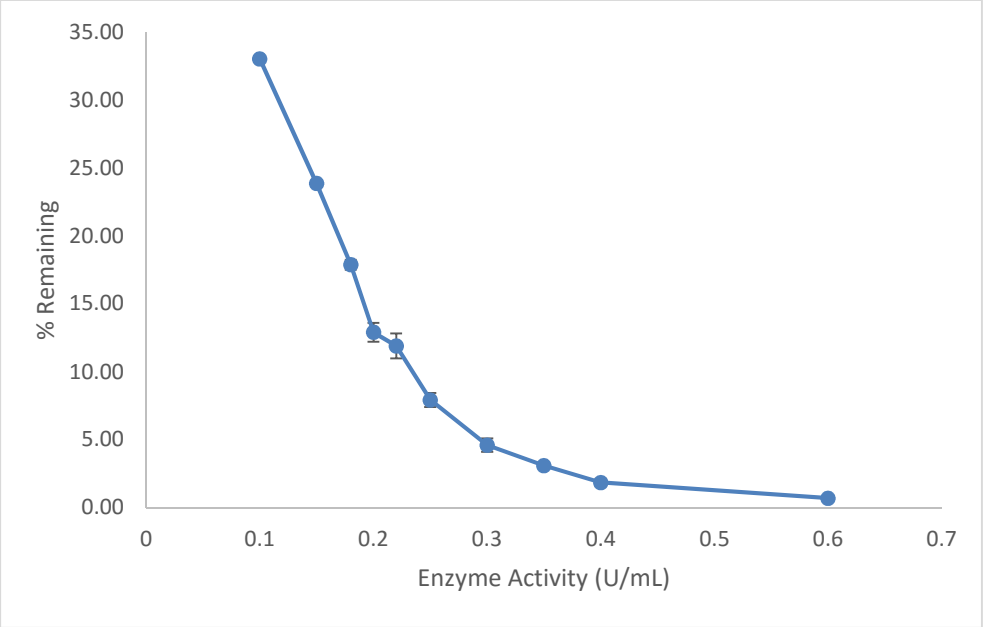


Figure 4.6 Ioxynil Enzyme Optimization (pH 4.0 buffer, 0.1 mM Ioxynil, 0.1 mM H_2O_2, 3-hour reaction, room temperature)

For Bromoxynil, the minimum enzyme concentration was at 0.9 U/mL with 4.2% substrate remaining. And for Ioxynil, the minimum SBP concentration was at 0.3 U/mL with 4.6% percent remaining. Both substrates undergone more than 95% degradation by SBP-catalyzed treatment; thus 0.9 U/mL and 0.3 U/mL were chosen as minimum effective enzyme dosages for the time course study. It also could be noted that the shape of enzyme-optimized curves for both compounds were very similar. At low levels of SBP, slight increase in enzyme concentration induced a steep increase in conversion efficiency. But no apparent changes in removal percentages were observed at higher levels of SBP, especially after the optimal point for SBP was reached. For example, 0.2 U/mL transformed 87.1% of Ioxynil, however, a doubling of the enzyme concentration only contributed an additional 11% increase in efficiency. This indicates very high removal efficiency (>95%) may not be cost-effective.

The minimum effective enzyme concentration varied with substrate. According to other studies, phenolic compounds generally required more SBP than anilines. The optimum enzyme dosages were 1.2 U/mL for 0.1 mM phenol, 0.5U/mL for 0.3 mM Bisphenol A to achieve >95% conversion (Feng et al., 2013; Watanabe et al., 2010) whereas, only 0.002 – 0.01 U/mL enzyme for 0.1 mM phenylenediamines and 0.007-0.01 U/mL enzyme for *p*-cresidine were needed for the same transformation performance (Al-Ansari et al. 2009; Mukherjee et al., 2018). The explanation can be that aniline radicals were more stable than phenoxyl radicals.

4.1.4 Time courses and substrate removal

The retention time is one of the essential parameters for engineering design due to the close association with the volume of the reactor. For this reason, time-course experiments were conducted to minimize reaction time and to reduce the cost of enzymatic treatment while achieving more than 95% degradation of pollutants. The optimum pH, H_2O_2 and enzyme concentrations established from Sections 4.1.1 to 4.1.3 were applied to 3-hour time course experiments, where 3 hours was to ensure that the reaction time is long enough. The experiments were carried out in 125 mL Erlenmeyer flask at room temperature. At appropriate time intervals (30 seconds to 1 hour), 5 mL of reaction mixture was taken then mixed with catalase and micro-filtered to analyze the residual substrate concentration. The first-order rate constants k (min^{-1}) and half-lives of Bromoxynil and Ioxynil were calculated based on Equation 5 and Equation 6.

Equation 5) $$100\% = \frac{C_0}{C} e^{-kt}$$

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

Figures 4.7 and 4.8 correspond to the removal efficiency of two pollutants with the change in time, and the red lines are the fitted first-order exponential trend-lines at the initial reaction stage (trend-line equations and R^2 are shown in the figures).

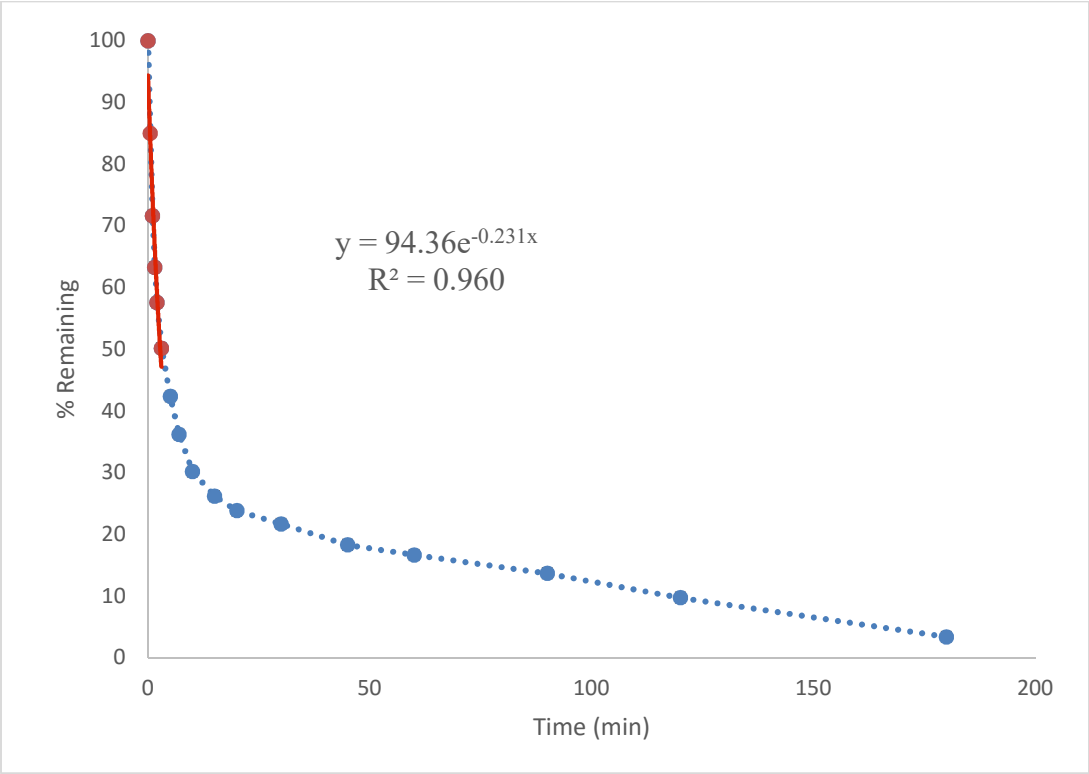


Figure 4.7 Bromoxynil Time Course Experiment (pH 4.0 buffer, 0.5 mM Bromoxynil, 0.5 mM H_2O_2, 0.90 U/mL SBP)

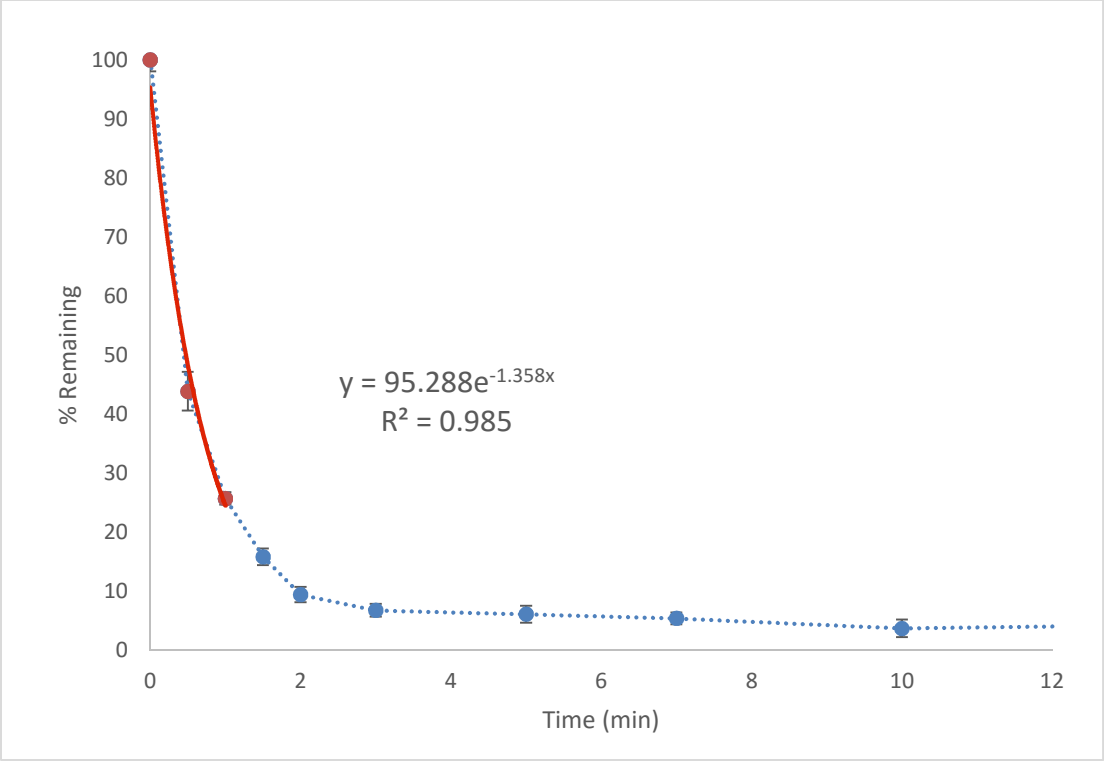


Figure 4.8 Ioxynil Time Course Experiment (pH 4.0 buffer, 0.1 mM Ioxynil, 0.1 mM H_2O_2, 0.35 U/mL SBP)

As can be seen from Figures 4.7 and 4.8, both compounds reached more than 95% degradation, 74.2% of Bromoxynil was converted within the first 15 minutes, but the degradation of the rest (21%) took 2.75 hours. Ioxynil reached 74.7% transformation at 1 minute and 96.3% degradation at 10 minutes. The slowing reaction rate is logical since the active enzyme were reduced with time, attributed to progressive enzyme inactivation (Mousa Al-Ansari et al. 2009). Similar results were found in SBP-catalyzed removal of 4-chlorophenol, 3-hydroxyquinoline, arylamines and azo dyes (Bódalo et al., 2008; Mashhadi et al., 2019a; Cordova-Villegas et al., 2018; Mukherjee et al., 2019).

Based on Equations 5 and 6 and the trend-line equations as shown in Figures 4.7 and 4.8, the rate constant for Bromoxynil and Ioxynil were 0.231±0.010 and 1.36±0.05 min^{-1}, respectively; thus the corresponding half-lives were obtained as 3.00±0.02 and

0.51±0.01 min. If normalized with respect to enzyme activity, the half-life was 3.30±0.02 min per U/mL of SBP for Bromoxynil and was 1.70±0.03 min per U/mL of SBP for Ioxynil. This indicated the SBP catalytic reaction rate of Ioxynil was two times faster than that of Bromoxynil at the beginning of the reaction.

4.2 Results for Mass Spectrometry (MS)

Different from other treatment methods, free radicals of phenolic substrates are converted to oligomers by the enzymatic reaction in the peroxidase catalytic cycle. These free radicals polymerized non-enzymatically through C-C and C-O coupling at *ortho*- and *para*- positions (O-O coupling is not stable), and, with successive cycles of enzymatic reaction, the polymerization stops when the formed oligomers precipitate out from the solution (Steevensz et al., 2014). MS analysis is beneficial for identification of reaction products and recognition of dehalogenation occurrence for the two benzonitriles. The ASAP-MS (in positive-ion mode) technique was applied to test solid standards and samples obtained after enzymatic treatment. After 3-hour enzymatic reaction, conducted under established optimum conditions, the reaction mixture of Bromoxynil was separated to supernatant and precipitates by centrifugation. Samples from these were used for MS analysis. Whereas Ioxynil samples were directly taken from the reaction mixture because the reaction products of Ioxynil were too small to be separated by centrifugation. The molecular formulae and plausible structures of reaction products were identified based on the m/z of base and discrete peaks obtained from mass spectra and were confirmed by MassLynx software to limit error within ±3 mDa and ±5 ppm. “Oxidative oligomer” were labeled for the generated reaction product. However, the determined chemical structures

were preliminary results as C-C or C-O coupling could not be distinguished by MS. The obtained structures were labeled with symbols - MH means protonated standard. Bromine and carbon have stable isotopes ^{79}Br (natural abundance 50.69%), ^{81}Br (natural abundance 49.31%) and ^{13}C (natural abundance 1.109%), symbolized as ^{79}Br-, ^{81}Br- and ^{13}C-. M_2H-2 means a protonated oxidative dimer, similar for trimer M_3H. Dehalogenation is presented by –Br or –I, and hydroxylation is denoted by +OH.

4.2.1 Bromoxynil

Since Bromoxynil contains two bromines, a total of six peaks should be found in MS analysis (three peaks for bromine isotopes and another three peaks for the corresponding ^{13}C isotope of each bromine isotopic compound). Figure 4.9 demonstrates the mass spectrum of protonated Bromoxynil standard, where the base peak with m/z 277.8644, matched with the exact mass of molecular formula $C_7H_4NO^{79}Br^{81}Br$. The other two peaks for bromine isotopes $^{79}Br_2$-MH (m/z 275.8664), and $^{81}Br_2$-MH (m/z 279.8622) were found. The m/z 276.869, 278.867 and 280.859 also matched with the exact masses of their ^{13}C isotopes ($^{79}Br_2{}^{13}C$ -MH, $^{79}Br^{81}Br\,{}^{13}C$ –MH, and $^{81}Br_2{}^{13}C$ –MH).

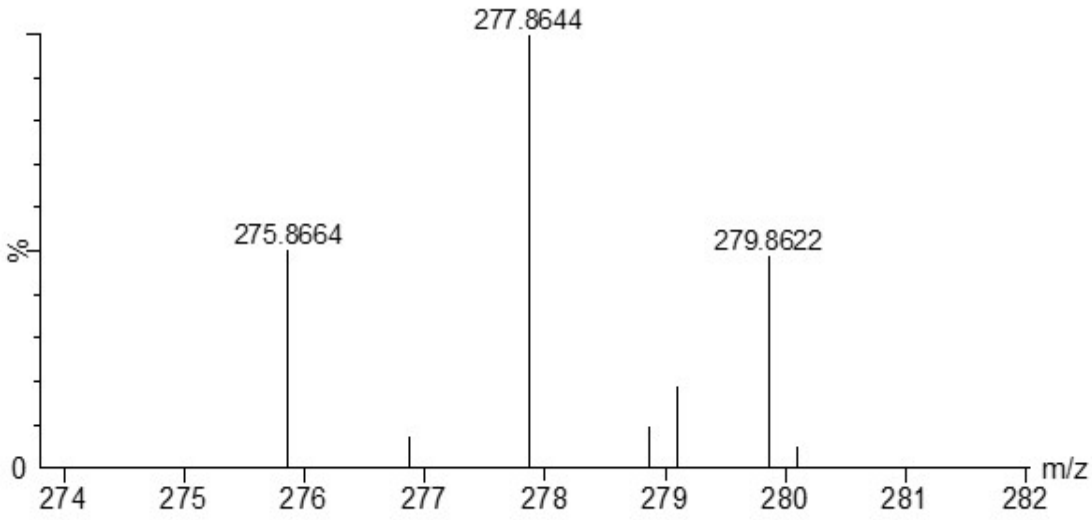


Figure 4.9 ASAP-MS (+) of protonated solid Bromoxynil standard (MH)

After 3-hour enzymatic reaction of Bromoxynil, the mass spectra of supernatant and precipitates are shown in Figure 4.10 and Figure 4.11. It can be noted that the base peaks in supernatant and precipitate (m/z = 392.8736 and 392.8703) both match with the molecular formula as $^{79}Br^{81}Br$-$C_{14}H_5N_2O_2Br_2$ (M_2H-2-2Br), proved by its isotopic masses of $^{79}Br_2$-M_2H-2-2Br (390.8714), $^{81}Br_2$-M_2H-2-2Br (394.8777), $^{79}Br_2{}^{13}C$-M_2H-2-2Br (391.8750), $^{79}Br^{81}Br^{13}C$-M_2H-2-2Br (393.8736) and $^{81}Br_2{}^{13}C$-M_2H-2-2Br (395.8712). Hence, the dimer that loses 2 bromine atoms was the dominant reaction product. In addition, the higher oligomer peaks were found in precipitate samples, which is reasonable because precipitates were generated when higher oligomers reach their solubility limits.

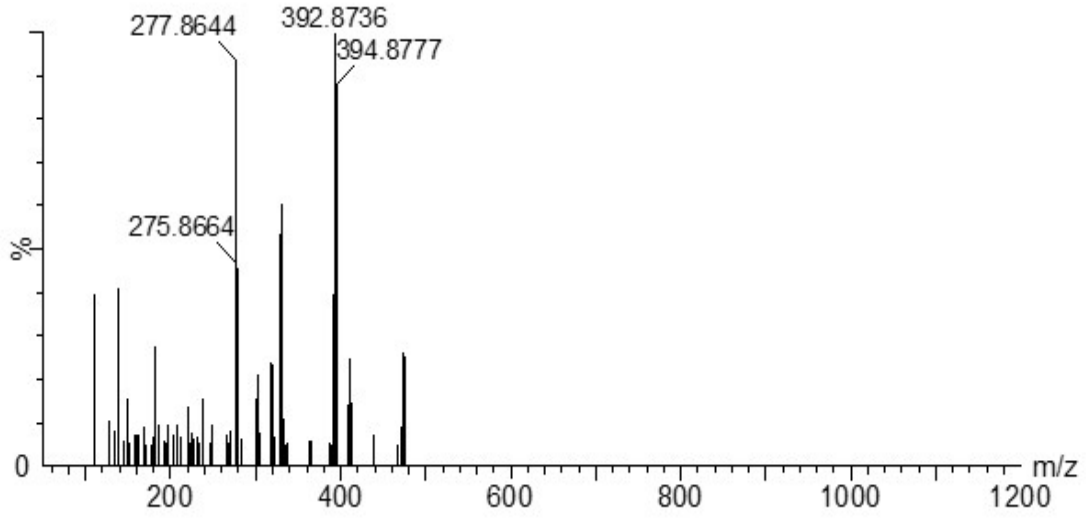


Figure 4.10 ASAP-MS (+) of Bromoxynil reaction supernatant. Full-scan mass spectrum with base peak m/z = 392.8736. Enzymatic reaction conditions: 0.5 mM Bromoxynil, 5 mM pH 4.0 buffer, 0.5 mM H_2O_2 and 0.9 mM SBP.

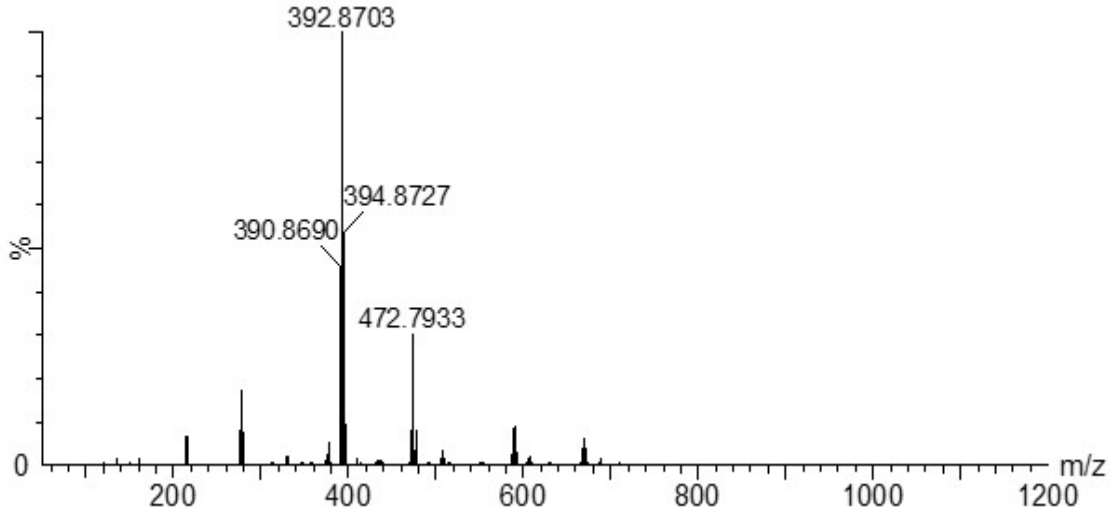


Figure 4.11 ASAP-MS (+) of Bromoxynil reaction precipitate. Full-scan mass spectrum with base peak m/z = 392.8703. Enzymatic reaction conditions: 0.5 mM Bromoxynil, 5 mM pH 4.0 buffer, 0.5 mM H_2O_2 and 0.9 mM SBP.

The reaction products included oxidative debrominated dimers and trimers as shown in Figures 4.12 to 4.15. Figures 4.12 and 4.13 illustrate the mass spectrum for each dimer, and Figures 4.14 and 4.15 demonstrate that for each trimer. In this case, there are necessarily n+1 peaks for n bromines accounting for the possible bromine isotopic

mixtures, accompanied by the corresponding ^{13}C, as discussed for Bromoxynil standard. The m/z values of all isotopes in each molecular formula were checked. It can be seen that the formation of oligomers was inevitably accompanied by the release of bromine atom(s), which is predictable. The coupling of substrate radicals only happened with *ortho*- and *para*- positions (Feng et al., 2013), but the *ortho*- positions of Bromoxynil molecule were occupied by two bromine substituents, and its *para*- position carried a cyano group. Therefore, the formation of oxidative oligomers denoted SBP contributed to the dehalogenation of *ortho* bromine(s) on Bromoxynil. Compared with the normal oxidative dimer M_2H-2, the symbol became to M_2H-1-Br or M_2H-2-2Br in this study due to the bromine loss with each H-atom loss. Similar results were found by Dec et al. (2003), the release of chlorine was observed during HRP-catalyzed transformation of 3-chlorophenol.

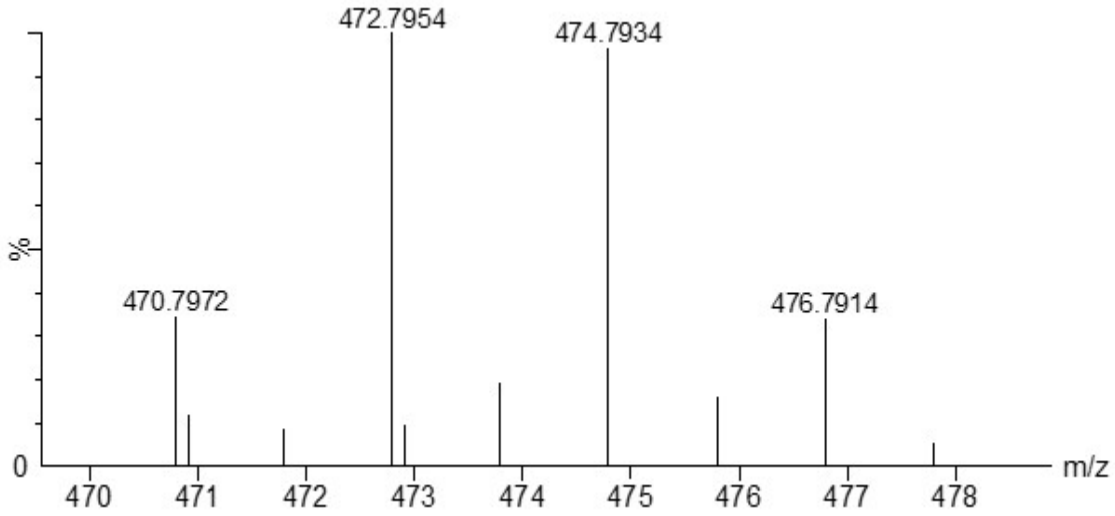


Figure 4.12 ASAP-MS (+) of Bromoxynil oxidative dimer with 1Br loss. Molecular formula for the base peak $^{79}Br_2{}^{81}Br$-$C_{14}H_6N_2O_2Br_3$ (M_2H-1-Br, m/z = 472.7954).

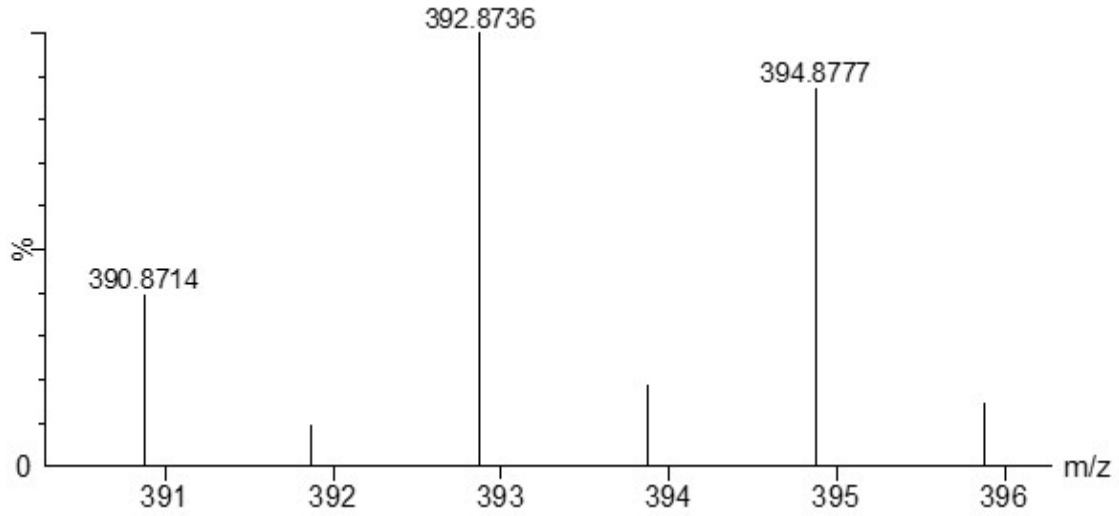


Figure 4.13 ASAP-MS (+) of Bromoxynil oxidative dimer with 2Br loss. Molecular formula for the base peak $^{79}Br^{81}Br$-$C_{14}H_5N_2O_2Br_2$ (M_2H-2-2Br, m/z = 392.8736).

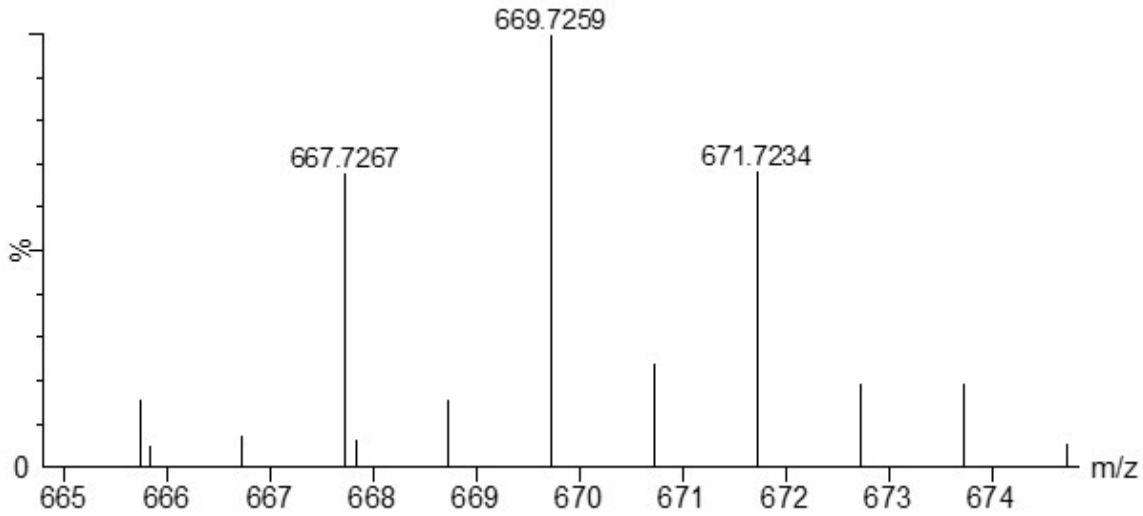


Figure 4.14 ASAP-MS (+) of Bromoxynil oxidative trimer with 2Br loss. Molecular formula for the base peak $^{79}Br_2{}^{81}Br_2$-$C_{21}H_8N_3O_3Br_4$ (M_3H-2-2Br, m/z = 669.7259).

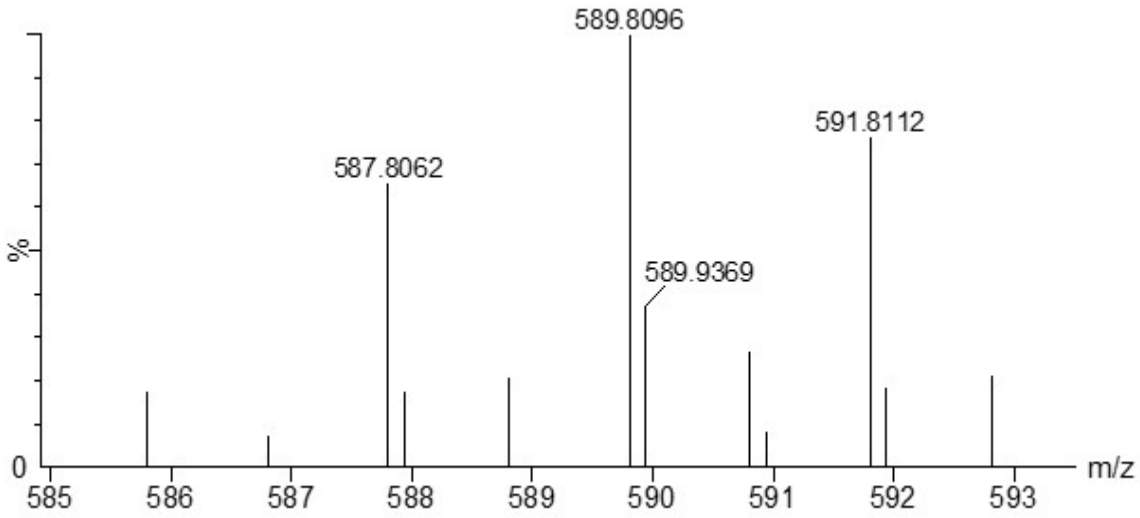


Figure 4.15 ASAP-MS (+) of Bromoxynil oxidative trimer with 3Br loss. Molecular formula for the base peak $^{79}Br_2{}^{81}Br$-$C_{21}H_7N_3O_3Br_3$ (M_3H-3-3Br, m/z = 589.8096).

Hydroxylation of Bromoxynil denotes a bromine ion being replaced by a hydroxyl group. Hydroxylated Bromoxynil residual monomer (MH-Br+OH) and oligomers (M_2H-1-2Br+OH, M_2H-2-3Br+OH and M_3H-2-3Br+OH) were recognized in the supernatant samples collected after the enzymatic reaction (Figures 4.16 to 4.19). Hydroxylated oxidative trimer with loss of 4 bromines M_3H-3-4Br+OH was also detected but it was ignored due to its fairly small peak. No hydroxylated products were found in standard samples. Therefore, we can conclude that the hydroxylation occurred in the process of enzymatic treatment. Similar findings were reported by Mashhadi et al. (2019) and Mukherjee et al. (2019), they found the hydroxylation/oxygenation in the oxidative dimers of 3-hydroxyquinoline and 4-chloro-*o*-toluidine, respectively, obtained from the supernatant samples after SBP-catalyzed reactions.

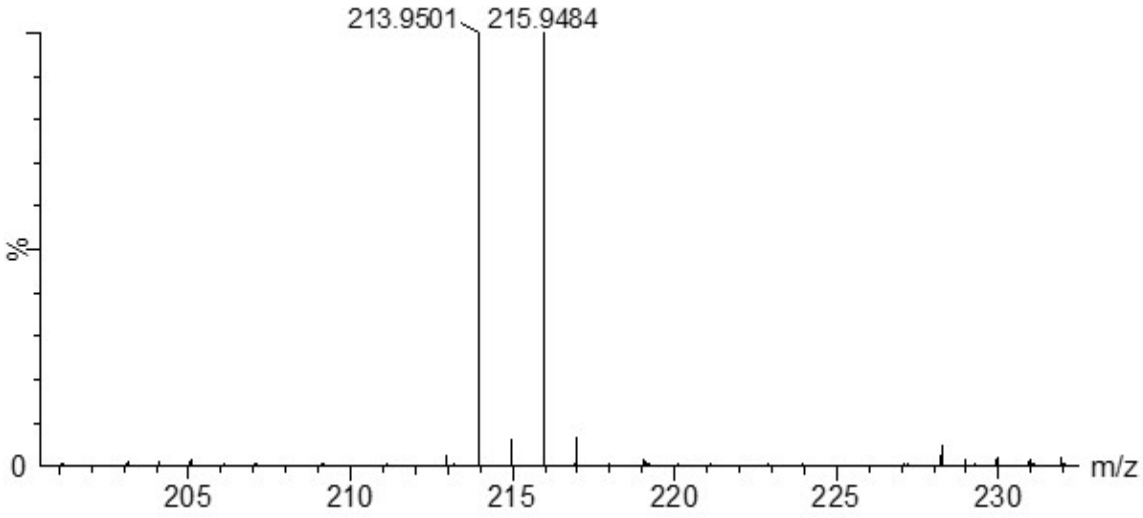


Figure 4.16 ASAP-MS (+) of Bromoxynil residual monomer with 1Br loss and hydroxylation. Molecular formula for the base peak ^{79}Br-$C_7H_5NO_2Br$ (MH-Br+OH, m/z = 213.9501).

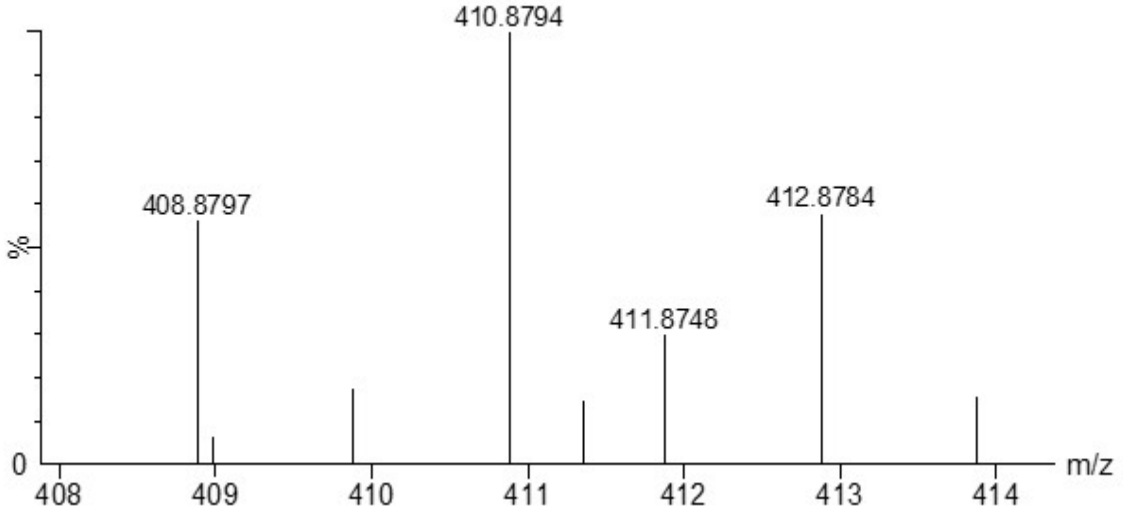


Figure 4.17 ASAP-MS (+) of Bromoxynil oxidative dimer with 2Br loss and hydroxylation. Molecular formula for the base peak $^{79}Br^{81}Br$-$C_{14}H_7N_2O_3Br_2$ (M_2H-1-2Br+OH, m/z = 410.8794).

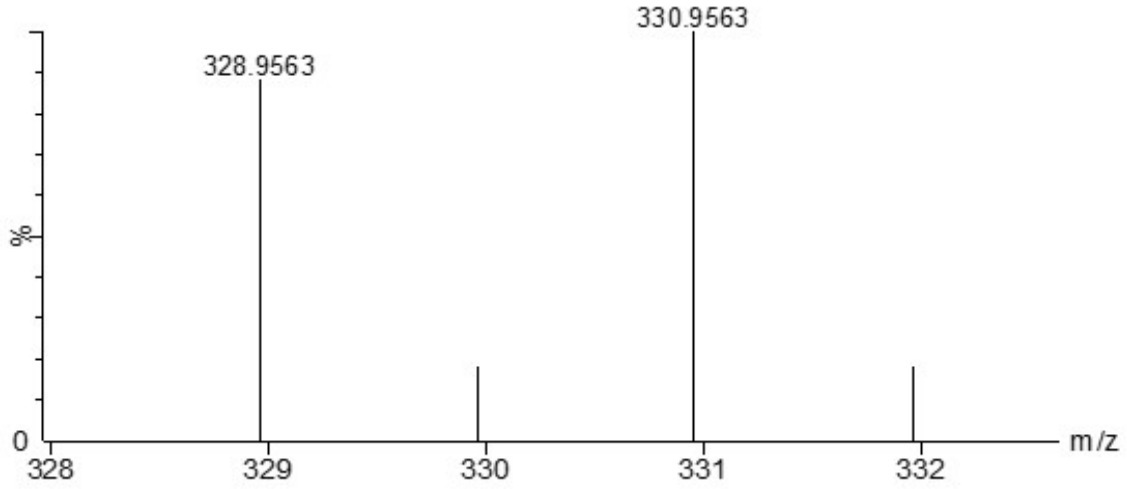


Figure 4.18 ASAP-MS (+) of Bromoxynil oxidative dimer with 3Br loss and hydroxylation. Molecular formula for the base peak ^{81}Br-$C_{14}H_6N_2O_3Br$ (M_2H-2-3Br+OH, m/z = 330.9563).

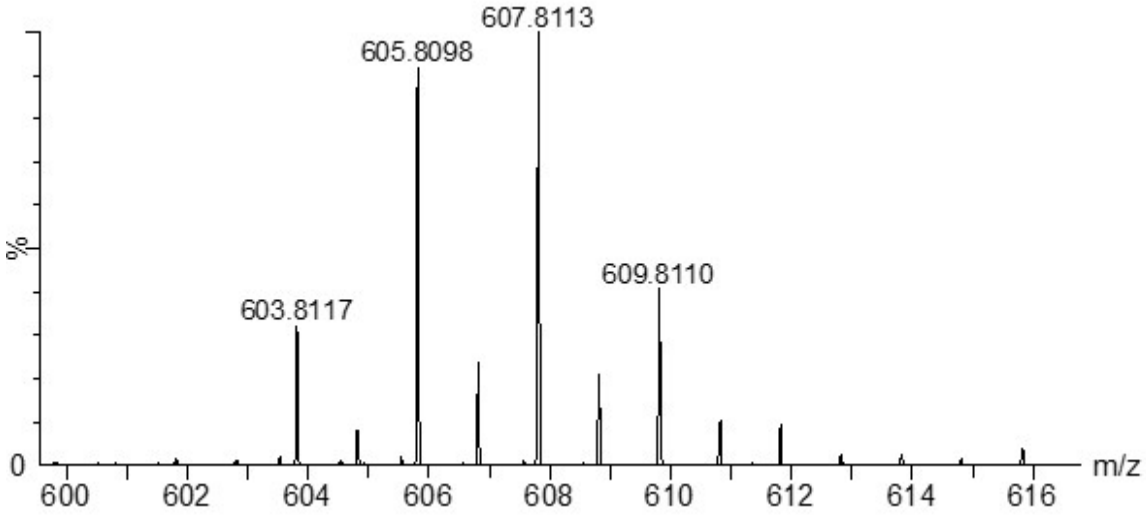


Figure 4.19 ASAP-MS (+) of Bromoxynil oxidative trimer with 3Br loss and hydroxylation. Molecular formula for the base peak $^{79}Br^{81}Br_2$-$C_{21}H_9N_3O_4Br_3$ (M_3H-2-3Br+OH, m/z = 607.8113).

4.2.2 Ioxynil

The full-scan mass spectrum of protonated Ioxynil standard (MH) shows that m/z value of the base peak was 371.8387 in Figure 4.20, corresponding to the exact mass of

molecular formula $C_7H_4NOI_2$ (^{13}C-MH, m/z = 372.8423). Since iodine has only one stable isotope (^{127}I), Ioxynil only showed ^{13}C-isotope, as seen in Figure 4.21. The loss of one iodine ion was noticed in Ioxynil standard, Figure 4.22, (MH-I, m/z = 245.9422; ^{13}C-MH-I, m/z = 247.9465), which did not happen in Bromoxynil standard. The explanation can be bromine atom is a stronger base than iodine atom (Shi et al., 2012), causing the latter to be a better leaving group.

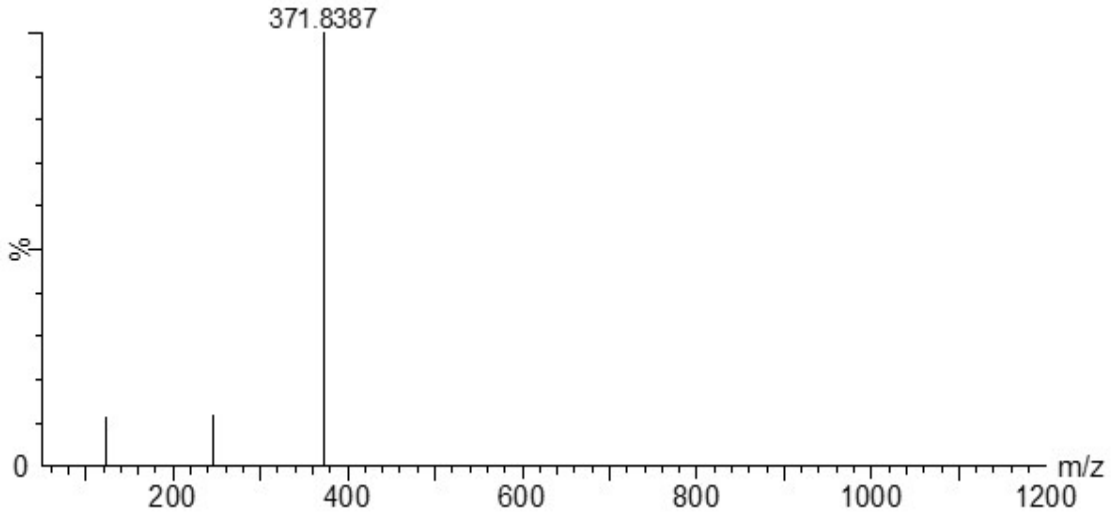


Figure 4.20 ASAP-MS (+) of solid Ioxynil standard, full-scan mass spectrum with base peak m/z = 371.8387.

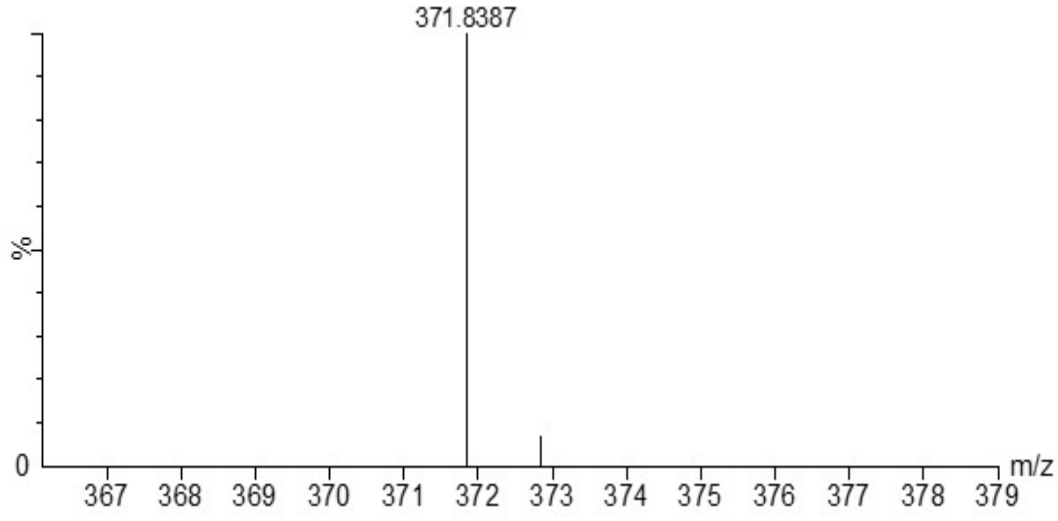


Figure 4.21 ASAP-MS (+) of solid Ioxynil standard (MH), with molecular formula $C_7H_4NOI_2$ (m/z = 371.8387, ^{13}C- isotope m/z = 372.8426).

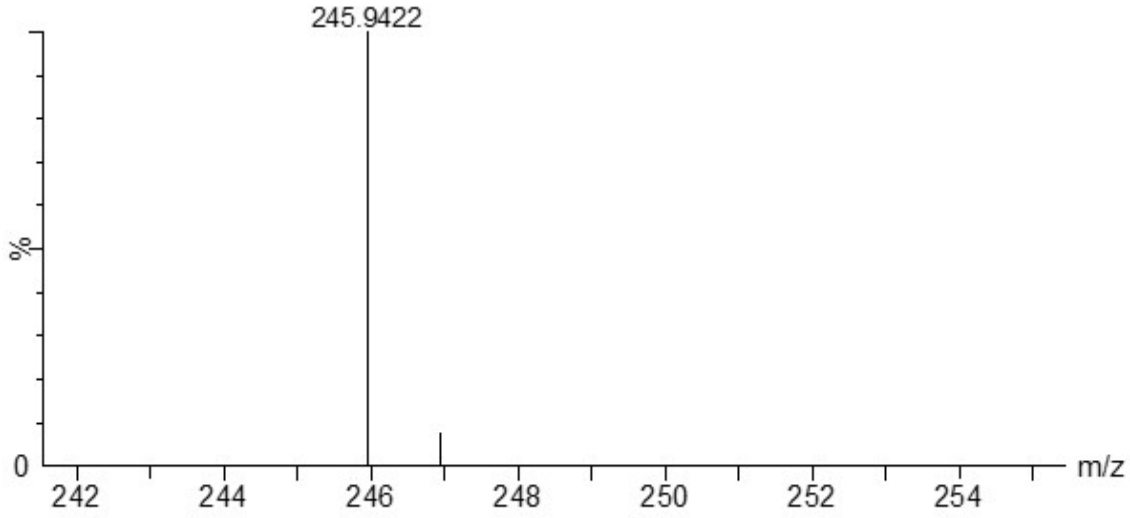


Figure 4.22 ASAP-MS (+) of solid Ioxynil standard with 1I loss (MH-I), with molecular formula C_7H_5NOI (m/z = 245.9422; ^{13}C- isotope m/z = 246.9458).

Figure 4.23 shows the full-scan mass spectrum of the reaction mixture of Ioxynil because the precipitates generated from 3-hour enzymatic reaction could not be separated by centrifugation. The base peak was found at m/z value of 488.8597, associated with molecular formula as $C_{14}H_7N_2O_2I_2$ (M_2H-2I, ^{13}C-isotope m/z = 489.8637). The reaction products with high peaks and plausible structures were two oxidative deiodinated dimers

(M_2H-2I; M_2H -3I), see Figure 4.24 and Figure 4.25. The loss of iodine atoms(s) was also identified in the dimers formed, as with the discussion in Section 4.2.1, the formation of oligomeric products in SBP-catalyzed process on Bromoxynil must be accompanied by dehalogenation. In this case, with iodine loss, the symbol for oxidative polymerization was M_2H–2I rather than M_2H-2 or M_2H-1-I. No loss of hydrogen was observed with loss of iodine radical, which may be because I was more prone to form I_2 rather than HI. The dimer M_2H-2I was considered generated by two molecules of Ioxynil standard (MH) with 2 iodine release, while another dimer M_2H-3I may be formed by one molecule of standard (MH) and one molecule of deiodinated standard (MH-I), with 2 iodine release as well.

Previous studies indicated that chloride release from phenolic compounds occured during oxidative coupling reactions, where oxidants could be phenoloxidases (SBP, HRP, laccase, tyrosinase) or metal oxides (birnessite) (Dec et al., 2003; Schultz et al., 2001; Hammel and Tardone, 1988). The debromination of Bromoxynil could be achieved by permanganate oxidation and by the soil microbe *Desulfitobacterium chlororespirans* (Zhao et al., 2016; Cupples et al., 2005), and deiodination of Ioxynil also occurred with the same bacterial culture (Cupples et al., 2005). Limited studies existed for dehalogenation of brominated and iodinated compounds. To our knowledge, this is the first study on debromination and deiodination by SBP.

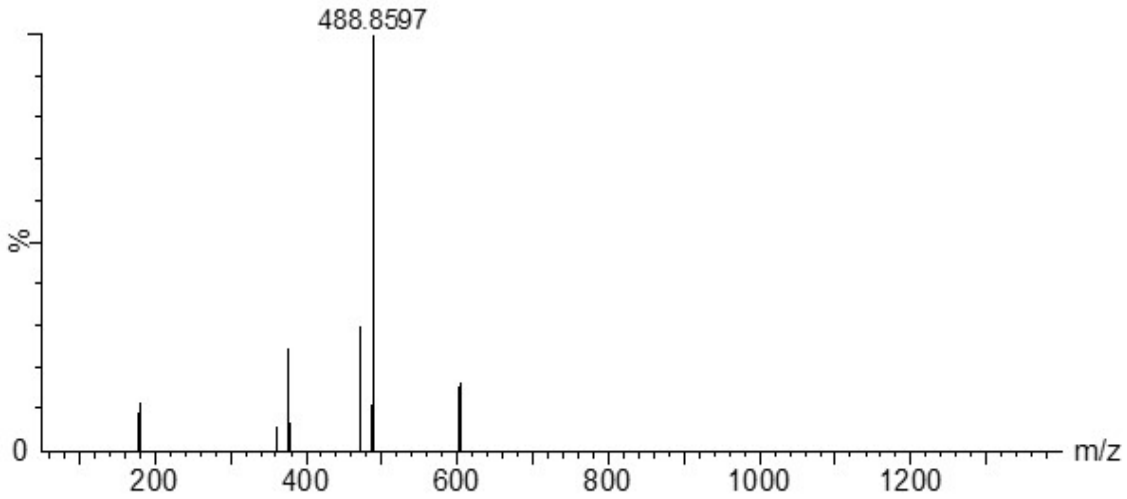


Figure 4.23 ASAP-MS (+) of Ioxynil reaction mixture. Full-scan mass spectrum with base peak m/z = 488.8597. Enzymatic reaction condition: 0.1 mM Ioxynil, 5 mM pH 4.0 buffer, 0.1 mM H_2O_2 and 0.3 mM SBP.

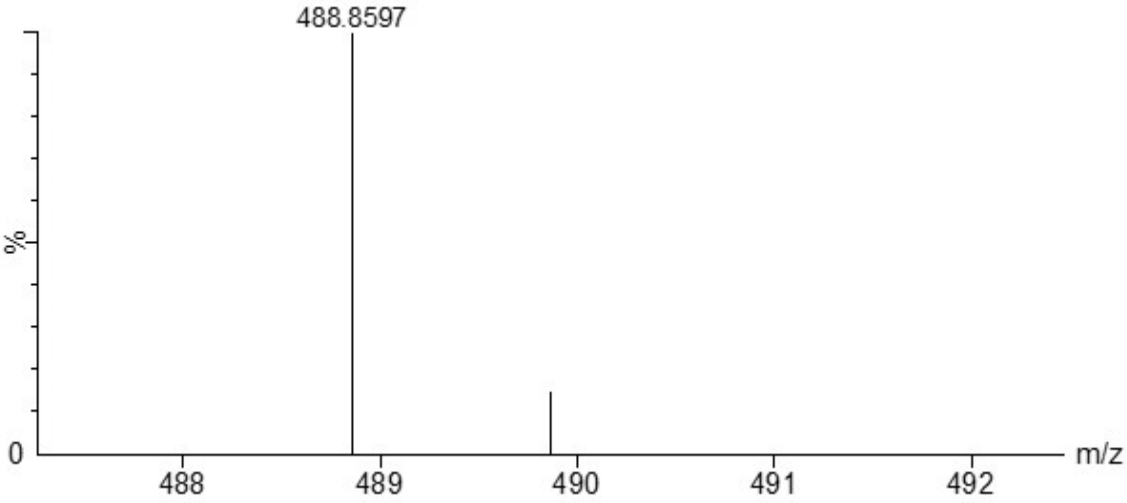


Figure 4.24 ASAP-MS (+) of Ioxynil oxidative dimer with 2I loss. Molecular formula $C_{14}H_7N_2O_2I_2$ (M_2H-2I, m/z = 488.8597, ^{13}C- isotope m/z = 489.8634).

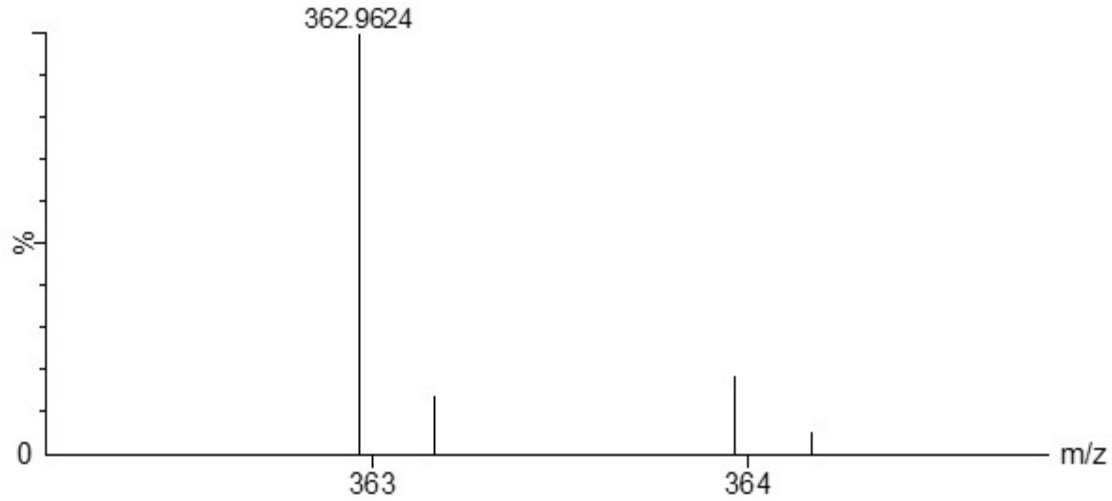


Figure 4.25 ASAP-MS (+) of Ioxynil oxidative dimer with 3I loss. Molecular formula $C_{14}H_8N_2O_2I$ (M_2H-3I, m/z = 362.9624; ^{13}C- isotope m/z = 363.9678).

Hydroxylation was also observed in the residual Ioxynil monomer (MH-I+OH, m/z = 261.9372) and one oxidative dimer (M_2H-3I+OH, m/z = 378.9567), the corresponding formulae are C_7H5NO_2I and $C_{14}H_8N_2O_3I$, respectively, see Figures 4.26 and 4.27. An iodine atom was substituted by a hydroxyl group, as for hydroxylated Bromoxynil and its oligomeric products. Furthermore, the occurrence of hydroxylation found in reaction mixture sample (not in the solid standards) supports the conclusion that hydroxylation was happening because of enzymatic treatment.

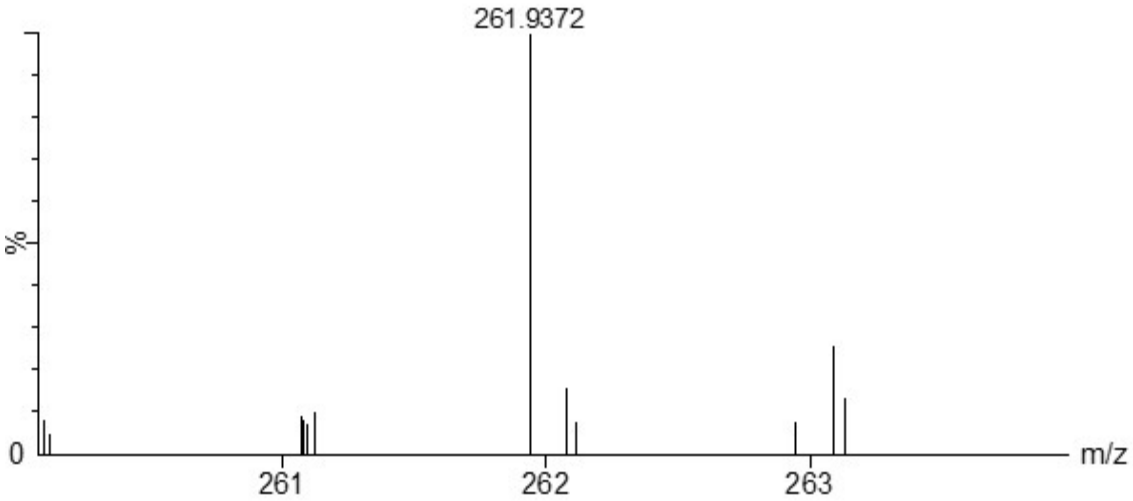


Figure 4.26 ASAP-MS (+) of Ioxynil monomer with 1I loss and hydroxylation. Molecular formula $C_7H_5NO_2I$ (MH-I+OH, m/z = 261.9372, ^{13}C- isotope m/z = 262.9401).

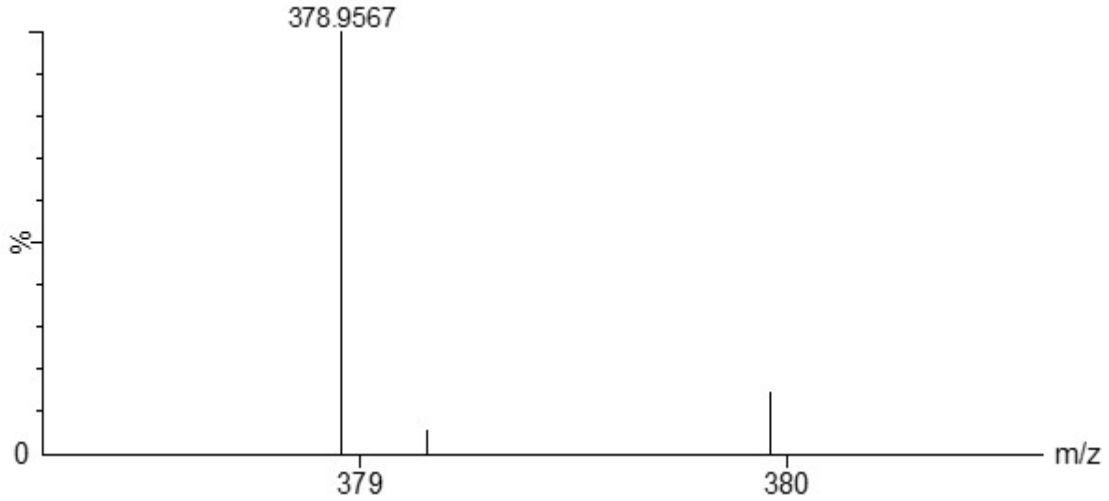


Figure 4.27 ASAP-MS (+) of Ioxynil oxidative dimer with 3I loss and hydroxylation. Molecular formula $C_{14}H_8N_2O_3I$ (M_2H-3I+OH, m/z = 378.9567; ^{13}C- isotope m/z = 379.61).

CHAPTER 5 SUMMARY AND CONCLUSIONS

In this thesis, the feasibility of halogenated benzonitrile removal by soybean peroxidase was studied. The results obtained from preliminary studies illustrated Bromoxynil and Ioxynil are substrates for SBP, then the second objective: operational conditions were optimized for >95% removal of these two pollutants. The optimum pH for both compounds was in the acidic range, with maximum removal efficiency at pH 4.0, which is very close to their pK_a value. The optimum H_2O_2-to-substrate ratio 1.0 was higher than the theoretical value 0.5, indicating the higher peroxide demand due oligomer formation, or due to catalase activity of soybean peroxidase. The minimum SBP concentration with more than 95% substrate conversion were achieved for both compounds. The normalized half-lives of SBP for Bromoxynil and Ioxynil 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 reaction, then the degradation rate slowed down due to reduced enzyme activity. The optimized conditions, the reaction rate constants and half-lives for the two pollutants are summarized in Table 5.1.

Table 5.1 Summary of optimized conditions for SBP-catalyzed process and rate constants and half-lives for two compounds

Parameters	Optimized Conditions	
	Bromoxynil	**Ioxynil**
Initial concentration (mM)	0.5	0.1
pH	4.0	4.0
H_2O_2-to-substrate ratio	1.0	1.0
SBP concentration (U/mL)	0.9	0.3
Rate constant, k (min^{-1} per U/mL SBP)	0.26±0.01	1.51±0.06
Half-life, $t_{1/2}$ (min per U/mL SBP)	3.30±0.02	1.70±0.03

The possible reaction products were identified using ASAP-MS in positive ion mode. The summarized results are listed in Tables 5.2 and 5.3. For Bromoxynil, oxidative dimers and trimers with loss of 1 to 3 bromine atoms were present after SBP-catalyzed treatment under optimized conditions. For Ioxynil, only oxidative dimers with loss of 2 or 3 iodine atoms were found after reaction, but in the standard loss of one iodine was observed. Since the positions for oxidative couplings were all occupied, the dehalogenation was inevitable with the generation of oxidative oligomers. In addition, hydroxylation was found in residual monomer and some of the oxidative oligomers, where halogens (bromine or iodine) were replaced by a hydroxyl group. All these generated oligomeric products confirm the fact that SBP-catalyzed treatment is viable for removing Bromoxynil and Ioxynil from aqueous solution.

Table 5.2 Summary of mass spectrometry results for standard and identified reaction products of Bromoxynil after SBP-catalyzed process

Bromoxynil		Molecular Formula	Symbols	m/z
Standard (protonated)		$C_7H_4NOBr_2$	MH	277.8644
Identified products* after reaction (protonated)	oxidative dimer with 1Br loss	$C_{14}H_6N_2O_2Br_3$	$^{79}Br_2{}^{81}Br$-M_2H-1-Br	472.7954
	oxidative dimer with 2Br loss	$C_{14}H_5N_2O_2Br_2$	$^{79}Br{}^{81}Br$-M_2H-2-2Br	392.8736
	oxidative trimer with 2Br loss	$C_{21}H_8N_3O_3Br_4$	$^{79}Br_2{}^{81}Br_2$-M_3H-2-2Br	669.7259
	oxidative trimer with 3Br loss	$C_{21}H_7N_3O_3Br_3$	$^{79}Br_2{}^{81}Br$-M_3H-3-3Br	589.8096
	residual monomer with 1Br loss and hydroxylation	$C_7H_5NO_2Br$	^{79}Br-MH-Br+OH	213.9501
	oxidative dimer with 2Br loss and hydroxylation	$C_{14}H_7N_2O_3Br_2$	$^{79}Br{}^{81}Br$-M_2H-1-2Br +OH	410.8794
	oxidative dimer with 3Br loss and hydroxylation	$C_{14}H_6N_2O_3Br$	^{81}Br-M_2H-2-3Br +OH	330.9563
	oxidative trimer with 3Br loss and hydroxylation	$C_{21}H_9N_3O_4Br_3$	$^{79}Br{}^{81}Br_2$-M_3H-2-3Br +OH	607.8113

* In this table only one of the bromine-isotopic combinations is listed, but all possible combinations were found according to the (n+1)-rule for number of bromines in the molecule and each was accompanied by the corresponding ^{13}C peak.

Table 5.3 Summary of mass spectrometry results for standard and identified reaction products of Ioxynil after SBP-catalyzed process

Ioxynil		Molecular Formula	Symbols	m/z
Standard (protonated)		$C_7H_4NOI_2$	MH	371.8387
Standard with 1I loss		C_7H_5NOI	MH-I	245.9422
Identified products after reaction (protonated)	oxidative dimer with 2I loss	$C_{14}H_7N_2O_2I_2$	M_2H-2I	488.8597
	oxidative dimer with 3I loss	$C_{14}H_8N_2O_2I$	M_2H-3I	362.9624
	residual monomer with 1I loss and hydroxylation	$C_7H_5NO_2I$	MH-I+OH	261.9372
	oxidative dimer with 3I loss and hydroxylation	$C_{14}H_8N_2O_3I$	M_2H-3I+OH	378.9567

In conclusion, this study provides an alternative wastewater treatment method for removing pesticides. SBP extracted from an agricultural by-product is not only cost-effective but a robust enzyme, having >95% removal efficiency on Bromoxynil and Ioxynil. Bromine or iodine release from Bromoxynil and Ioxynil during oxidative coupling of the enzymatic treatment process in aqueous solution provides valuable insights to the occurrence of dehalogenation and hydroxylation by SBP.

CHAPTER 6 FUTURE WORK

This study confirms the potential of SBP as a halogenated benzonitrile removal method from wastewater. But the following investigations are suggested for the real application of SBP-based treatment.

1. Other halogenated benzonitriles and their corresponding metabolites can be investigated, thus the effect of halogens on optimized conditions can be determined.

2. Further studies for the generated polymeric products are recommended, such as their environmental fate and toxicity. In this way, a proper method can be developed for safe disposal and avoiding further contamination.

3. Development of a sedimentation system for the suspended solids generated will be needed in real practice.

4. Feasibility of SBP for treating other pesticides and real agricultural runoff should be studied. The current parameters may be re-optimized or adjusted.

5. Cost and benefit analysis for the combination of SBP-catalyzed treatment with wastewater treatment plants or constructed wetlands should be interesting to clarify the real cost for full-scale application.

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APPENDICES

Appendix A Isosbestic Point and Maximum Wavelength

The maximum wavelength (λ_{max}) and isosbestic point of two herbicides were shown in the UV-vis spectroscopy. The extinction coefficients were calculated through equation 7:

Equation 7) $$\varepsilon = \frac{A}{Cl} \ (l = 1cm)$$

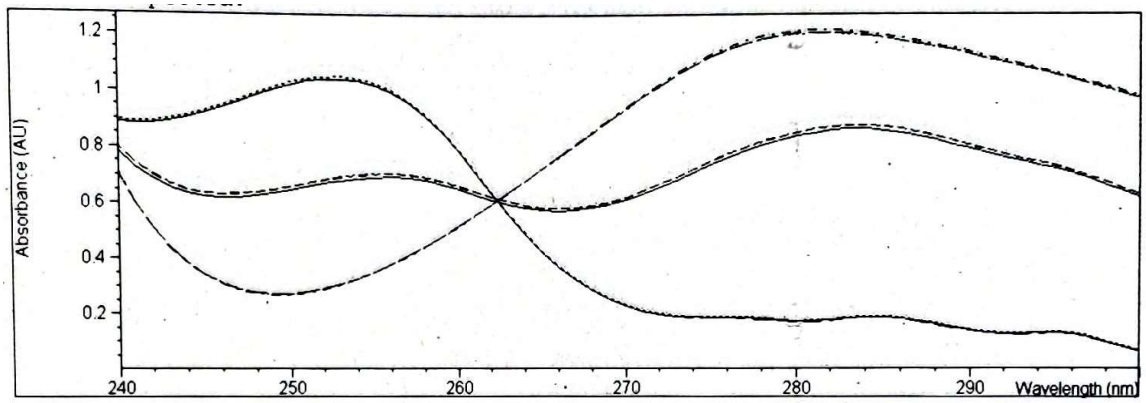

Figure A.1 0.1mM Bromoxynil at pH 2.0, 4.0 and 6.0
(Isosbestic point at 262.5 nm, ε = 6052 $M^{-1}cm^{-1}$; λmax at 281 nm for phenoxide form).

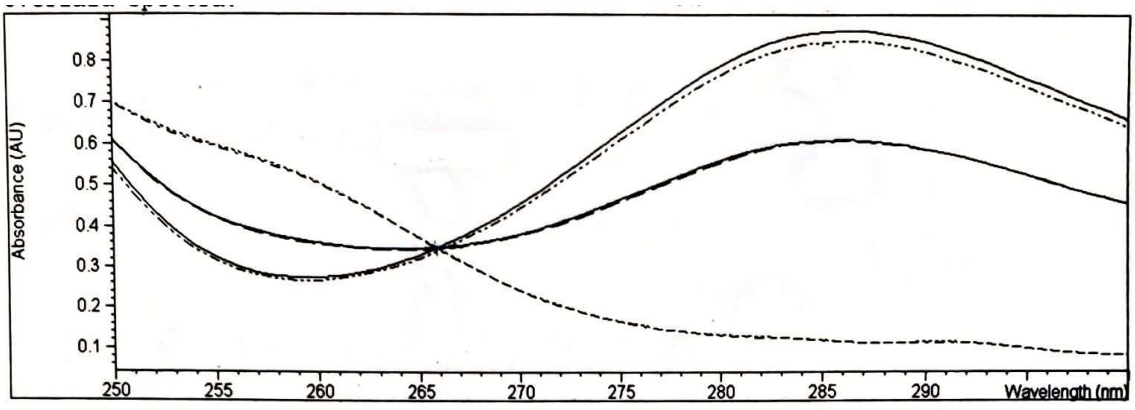

Figure A.2 0.05mM Ioxynil at pH 2.0, 4.0 and 6.0
(Isosbestic point at 266 nm ε = 6936 $M^{-1}cm^{-1}$; λ_{max} at 284 nm for phenoxide form).

Appendix B SBP Activity Assay

A colorimetric assay was performed to determine SBP activity. The activity was obtained by monitoring the formation of pink chromophore at 510 nm.

Reagents:

5 mL 10 × concentrate (0.9400g phenol, 1.3105 g monobasic and 3.7479 g dibasic sodium phosphate, made up to 47.5 ml with distilled water).

100 µL of 100 mM H_2O_2

0.025g of 4-AAP

42.5 mL distilled water

Multiplication Value Calculation

$$\text{SBP Activity } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{initial\ rate\ \left(\frac{AU}{S}\right) \times 60\left(\frac{sec}{\text{min}}\right) \times \left(\frac{1000\mu L}{50\mu L}\ dilution\ in\ cuvette\right)}{6mM^{-1}cm^{-1}}$$

$$= 200 \times initial\ rate\ (\frac{U}{mL})$$

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

Procedure

1) Blank the instrument with 950 µL reagent and 50 µL distilled water;
2) Inject a 50 µL of diluted SBP sample then a 950 µL of reagent into the cuvette;
3) Quickly place cuvette into UV-vis and lock the vessel;
4) Record the activity value of SBP, calculated by software.

Appendix C Calibration Curves

Standard curves are presented below as the average value of triplicate experiments. Error bars are based on calculated standard deviations. One standard curve for Bromoxynil at the isosbestic point and 9 separate standard curves (pH 2.0, 2.6, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) for Ioxynil at its λ_{max} at 284 nm are shown. There are no separate calibration curves of Ioxynil from pH 6.0 to 8.0 due to no change in absorbance observed.

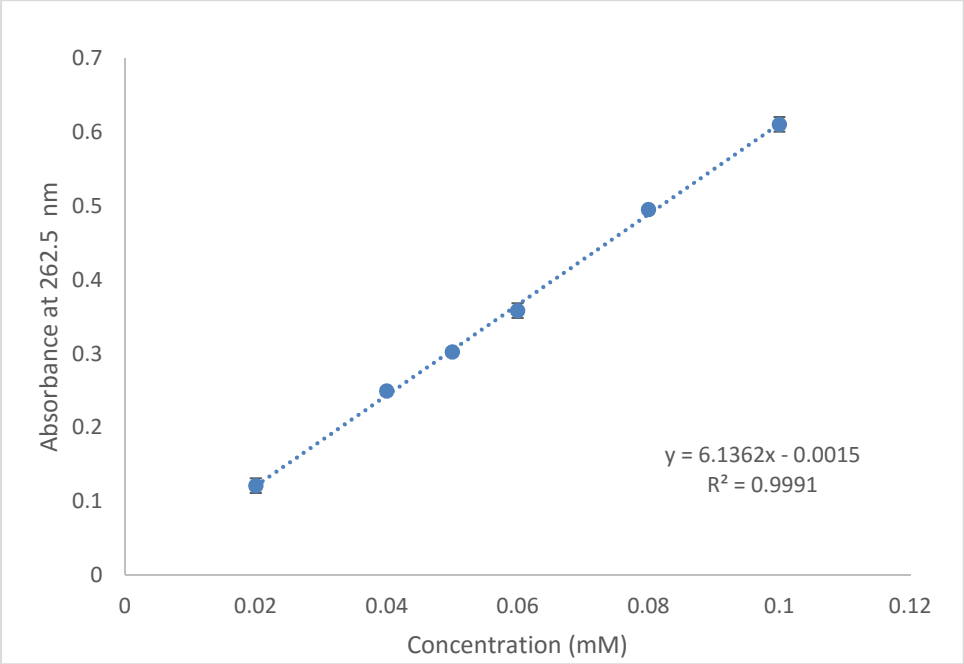

Figure C.1 Bromoxynil standard curve at 262.5 nm at pH 4.0

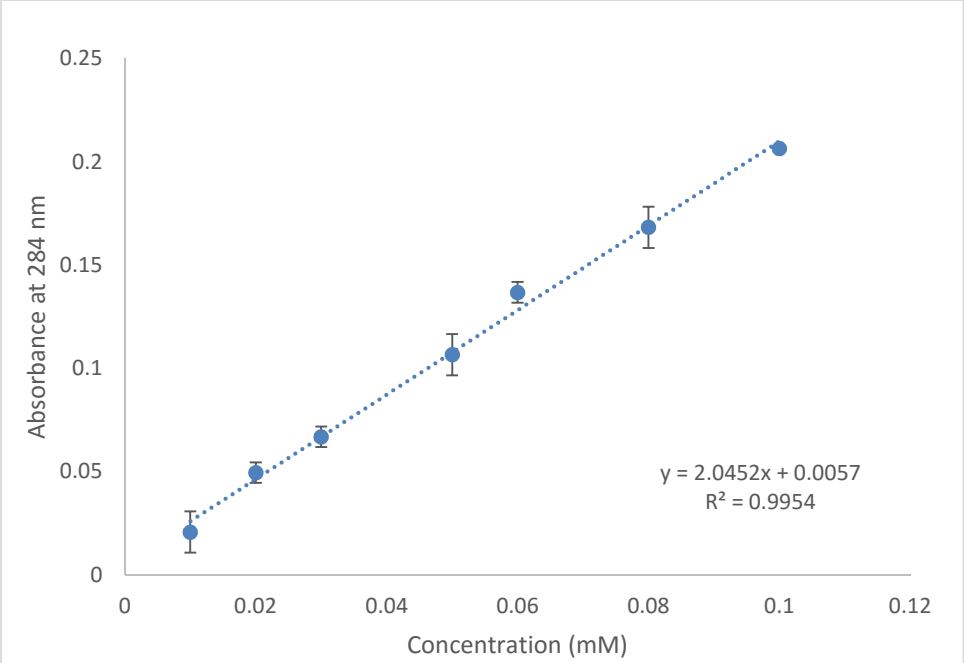

Figure C.2 Ioxynil standard curve at 284 nm at pH 2.0

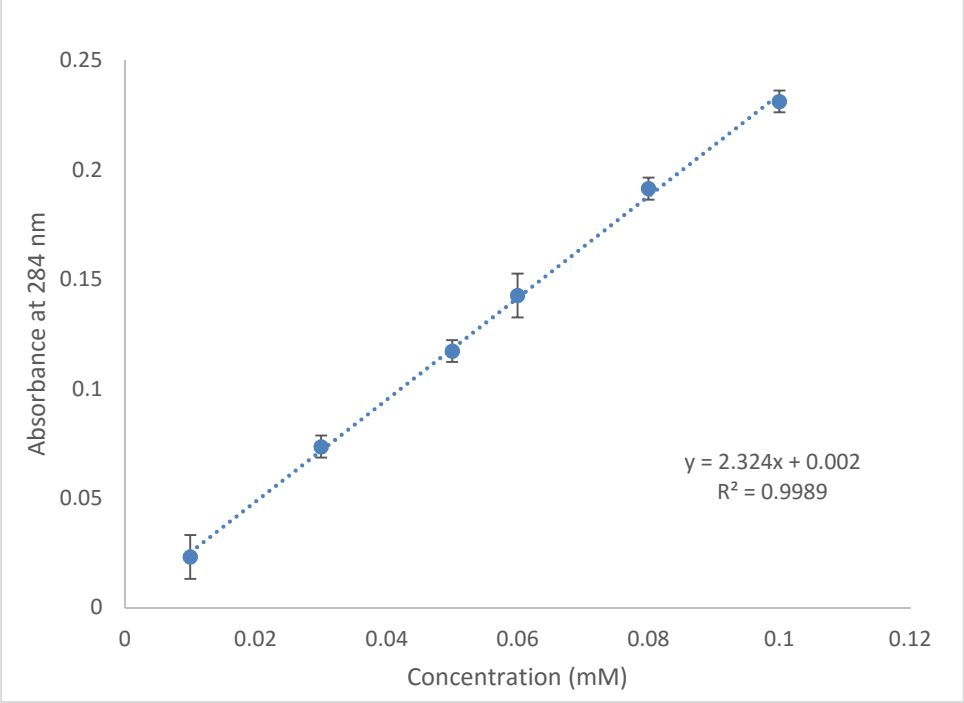

Figure C.3 Ioxynil standard curve at 284 nm at pH 2.6

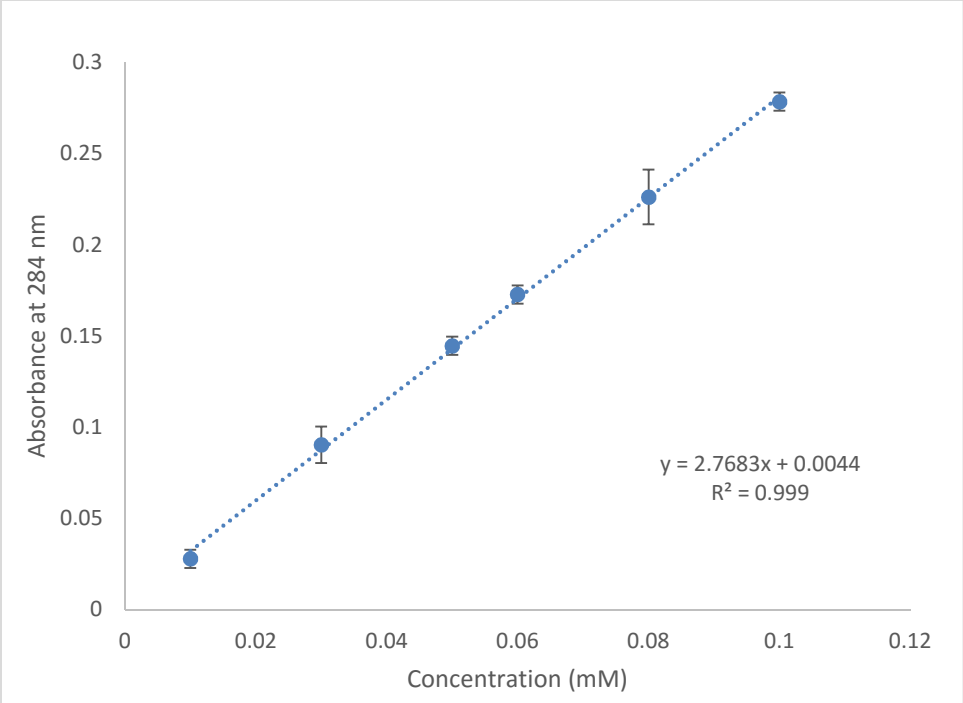

Figure C.4 Ioxynil standard curve at 284 nm at pH 3.0

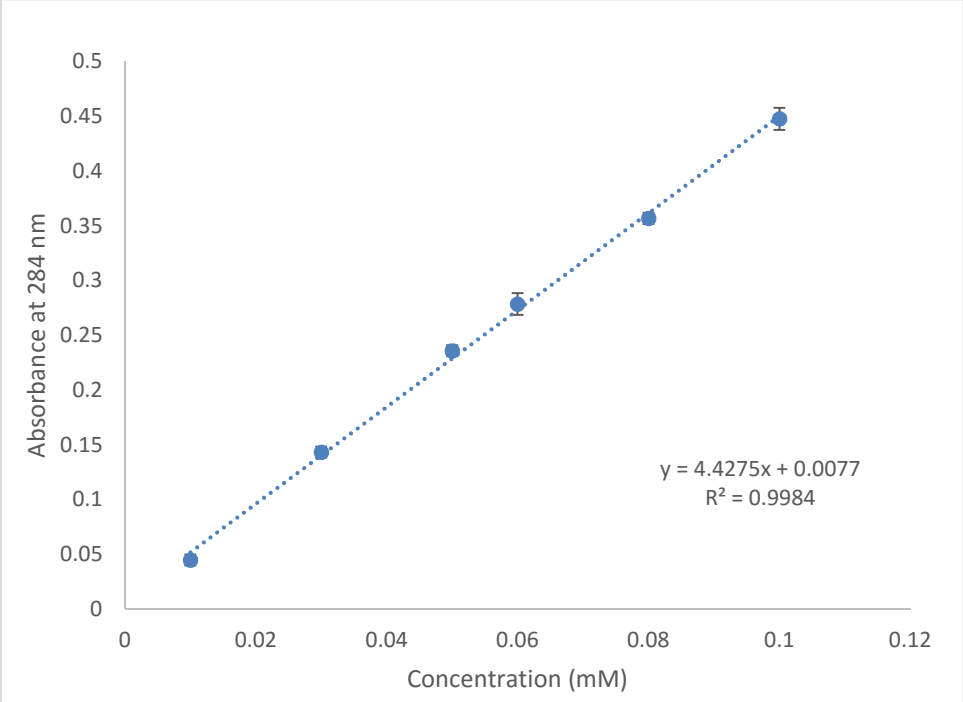

Figure C.5 Ioxynil standard curve at 284 nm at pH 3.5

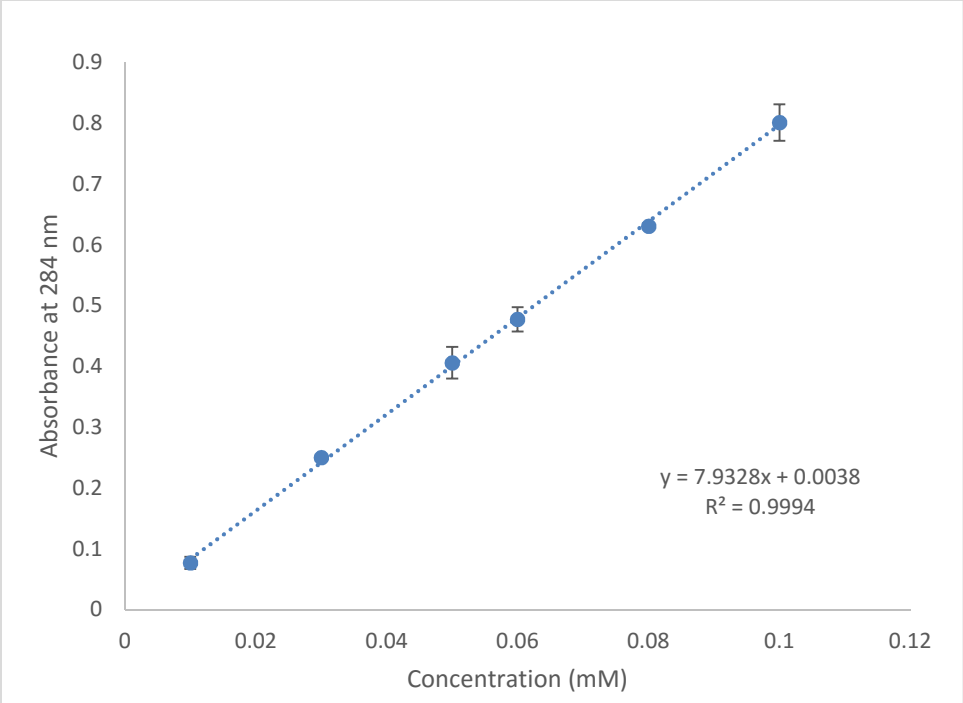

Figure C.6 Ioxynil standard curve at 284 nm at pH 4.0

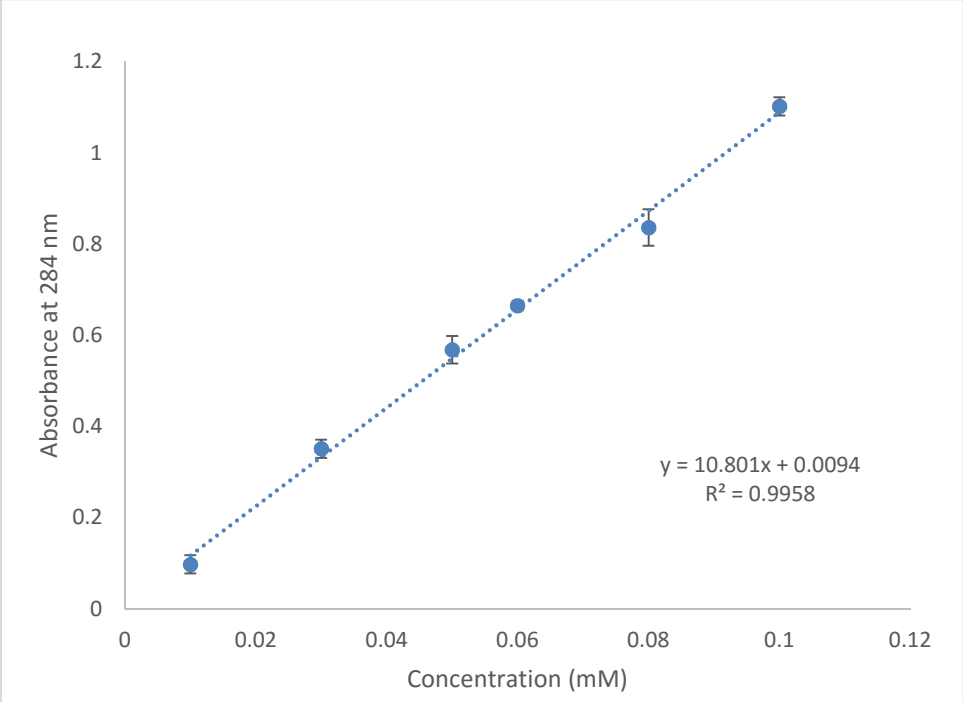

Figure C.7 Ioxynil standard curve at 284 nm at pH 4.5

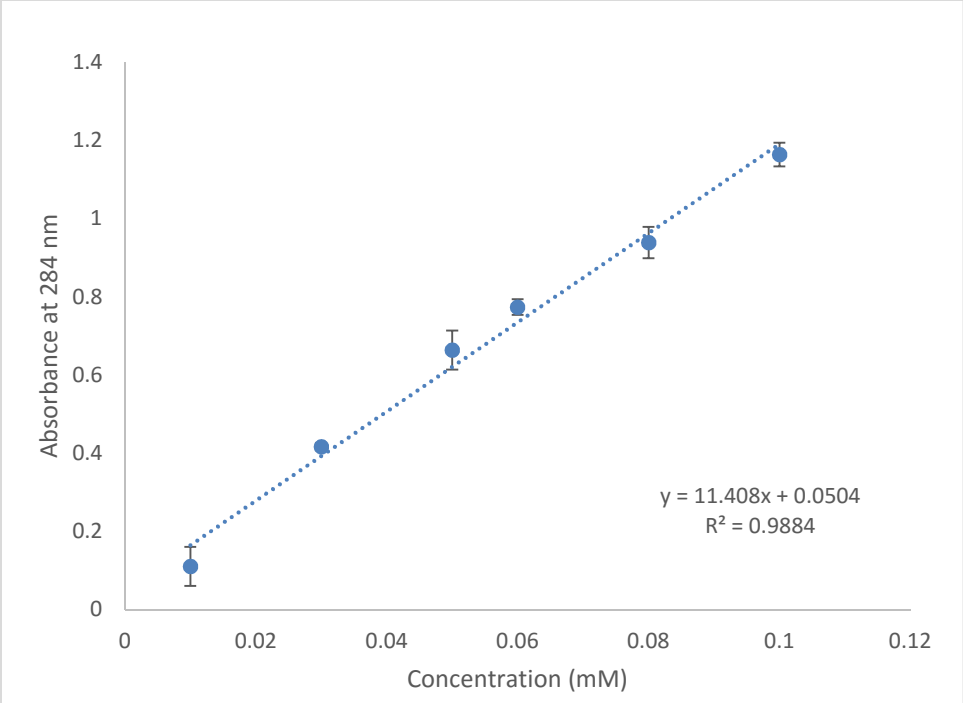

Figure C.8 Ioxynil standard curve at 284 nm at pH 5.0

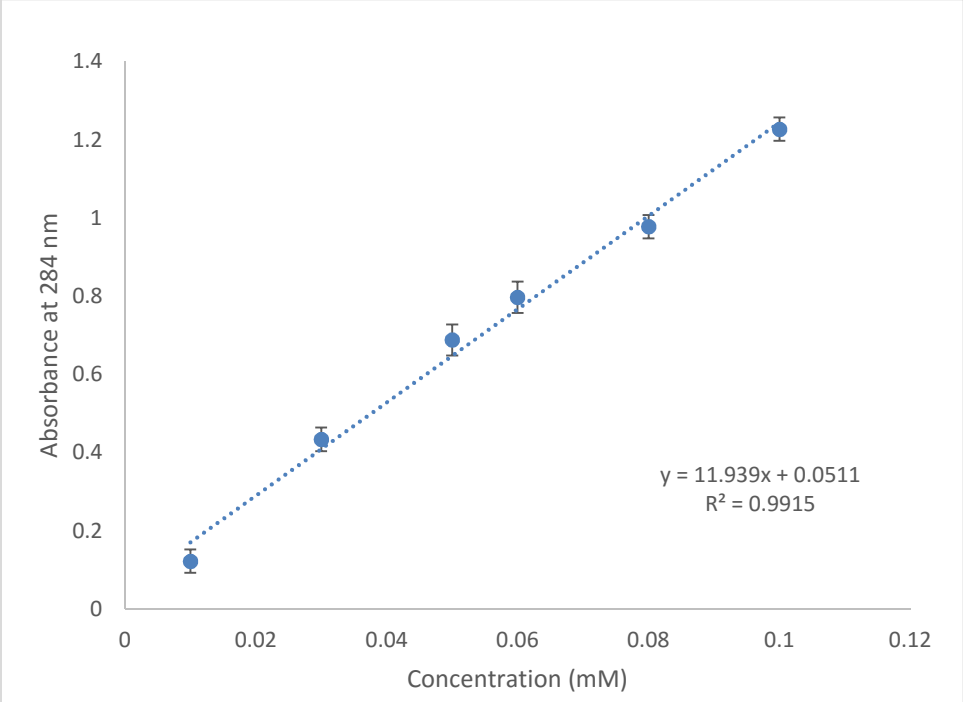

Figure C.9 Ioxynil standard curve at 284 nm at pH 5.5

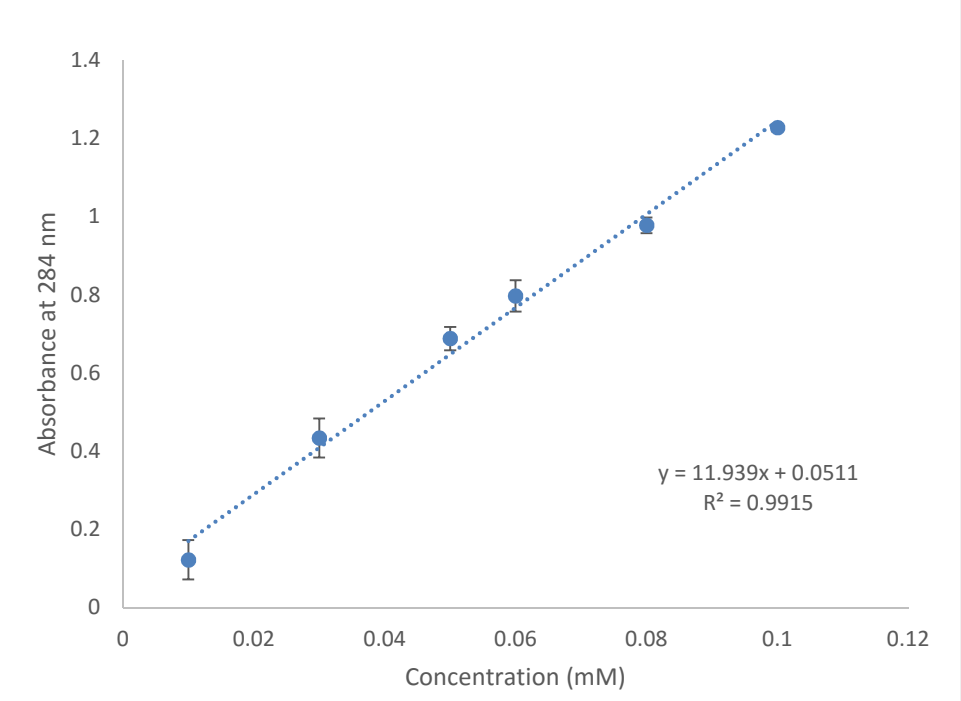

Figure C.10 Ioxynil standard curve at 284 nm at pH 6.0

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