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A Comparative Study of Aromatic Pollutants Degradation Using Two Different Peroxidases

Aysha Hamad Muftah Al Neyadi

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United Arab Emirates University

College of Science

Department of Biology

A COMPARATIVE STUDY OF AROMATIC POLLUTANTS
DEGRADATION USING TWO DIFFERENT PEROXIDASES

Aysha Hamad Muftah Al Neyadi

This thesis is submitted in partial fulfilment of the requirements for the degree of
Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Professor Syed Salman Ashraf

April 2016

Declaration of Original Work

I, Aysha Hamad Muftah Al Neyadi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “*A Comparative Study of Aromatic Pollutants Degradation Using Two Different Peroxidases*”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Sayed Salman Ashraf, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Advisory Committee

1) Advisor: Syed Salman Ashraf

Title: Professor

Department of Chemistry

College of Science

2) Co-advisor: Synan Abu-Qamar

Title: Associate Professor

Department of Biology

College of Science

Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

- 1) Advisor (Committee Chair): S. Salman Ashraf

Title: Professor of Biochemistry

Department of Chemistry

College of Science

Signature Salman Ashraf


Date 28 April 2016

- 2) Member: Dr. Ranjit Vijayan

Title: Assistant Professor

Department of Biology

College of Science

Signature 

Date 28/4/2016

- 3) Member: Dr. Mohammed Meetani

Title: Associate Professor

Department of Chemistry

College of Science

Signature 

Date 28/4/2016

- 4) Member (External Examiner): Prof. Firdos Khan

Title: Professor and Chairperson, School of Life Sciences

Department of Biotechnology

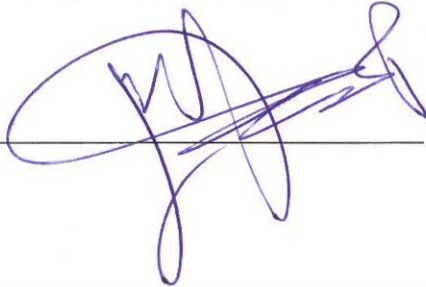
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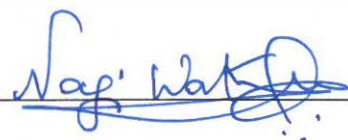
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This Master Thesis is accepted by:

Dean of the College of Science Dr. Ahmed Murad

Signature  Date 31/5/2016

Dean of the College of the Graduate Studies: Professor Nagi T. Wakim

Signature  Date 31/5/2016

Abstract

Enzyme based degradation of organic pollutants is a promising new remediation approach due to the promiscuous nature of the technique, the wide range of available enzymes, and the scalability of the process. Oxidoreductases, specifically, peroxidases are the most popular class of enzymes that have been used for the degradation of organic pollutants. It is generously assumed that all peroxidases behave similarly and produce similar degradation products. Our main objective was to test the hypothesis that related peroxidases may act differentially towards the degradation of aromatic pollutants. Therefore, in this study, we have carried out detailed degradation studies on three model of aromatic pollutants using two of the most commonly used peroxidases – Soybean peroxidase (SBP) and Chloroperoxidase (CPO). Our studies show that these two enzymes have very different optimum conditions when degrading these aromatic pollutants. For example, SBP had a pH optimum of 4, whereas CPO is most active at pH 2-3. Additionally, SBP and CPO had very different thermal stabilities, with SBP showing full activity up until 80°C, which was very different than CPO, which was almost completely inactive at 60°C. HPLC analyses confirmed that both SBP and CPO transformed the all the aromatic pollutants into different compounds, which was further confirmed by LC-MS-MS studies. Furthermore, toxicological evaluation showed that SBP-based treatment was able to reduce the toxicity, while CPO treatment failed to eliminate the toxicity of the compounds. Our results show that related peroxidases may behave very differently when used for remediation purposes and points to the need for toxicity analysis of peroxidase degraded pollutants, as well as to carry out detailed mechanistic studies to identify the intermediates produced.

Keywords: Bioremediation, pollutant degradation, peroxidases, soybean peroxidase, chloroperoxidase.

Title and Abstract (in Arabic)

دراسة تفصيلية لمعالجة المركبات الأروماتية باستخدام صنفين من البيروكسيديزات

الملخص

استخدام الإنزيمات لمعالجة وتحليل الملوثات العضوية هو نهج جديد وواعد في مجال المعالجة نظراً لطبيعة تقنية المعالجة المستخدمة، ومجموعة الإنزيمات المتاحة، وقابلية هذه التقنية للتوسع. إنزيمات الأكسدة-الاختزال وخصوصاً البيروكسيديزات هي الأكثر شيوعاً من بين الإنزيمات التي استخدمت لمعالجة وتحليل الملوثات العضوية، حيث أنه من المفترض أن جميع البيروكسيديزات تتصرف على نحو متماثل، وتنتج نواتج متماثلة. لذا كان هدفنا الرئيسي لهذه الدراسة هو اختبار فرضية أن البيروكسيديزات المتصلة تحلل الملوثات العطرية بشكل مختلف. وبالتالي فقد قمنا بدراسة مفصلة لثلاثة نماذج من الملوثات العطرية باستخدام اثنين من أكثر البيروكسيديزات شيوعاً وهما لبيروكسيديز فول الصويا (Soybean peroxidase (SBP) والكوروبيروكسيديز Chloroperoxidase (CPO). تظهر دراستنا أن الظروف الملائمة لهذه الإنزيمات مختلفة جداً عند تحليل هذه الملوثات العطرية. على سبيل المثال، كان الرقم الهيدروجيني الأمثل لل SBP هو 4، في حين كان ال CPO أكثر نشاطاً عند الرقم الهيدروجيني 2-3. تقنية ال HPLC أكدت أن كلا من ال SBP وال CPO حولوا جميع الملوثات العطرية إلى مركبات مختلفة، وهو ما أكدته تقنية ال LC-MS-MS. وعلاوة على ذلك، فقد أظهر تقييم السموم أن المعالجة باستخدام ال SBP كانت قادرة على الحد من سمية الملوثات، في حين فشلت المعالجة باستخدام ال CPO في القضاء على سمية الملوثات. تشير النتائج التي توصلنا إليها إلى أن البيروكسيديزات المتصلة قد تتصرف بشكل مغاير عند استخدامها لمعالجة الملوثات، كما نشير إلى الحاجة لتقييم السموم الناتجة عن معالجة الملوثات بالبيروكسيديزات.

مفاهيم البحث الرئيسية: المعالجة، الملوثات العطرية، البيروكسيديزات.

Acknowledgements

This thesis owes its existence to the help, support and inspiration of several people who were involved directly or indirectly throughout my MSc journey and made these years a treasured and extraordinary experience for me.

First and foremost, my deepest gratitude is to my supervisor, my greatest teacher, Prof. Salman Ashraf. I have been amazingly fortunate to have an advisor who gave me the continuous support, motivation, and immense knowledge that helped me to overcome many situations in my academic and personal life. He was always in the lab, even during his vacations, to listen to my difficulties and give advice whenever I needed them. He has been supportive since the day I began working in his lab and throughout the rough road to finishing this thesis. He would often say "Hang in there, Aysha!" to encourage and support me when things didn't work well. During my tenure, he always treated me as a colleague by giving me intellectual freedom in my work, supporting me to participate and attend various conferences, engaging me in new ideas and projects that further enriched my knowledge. His guidance helped me throughout the time of research and writing of this thesis. I really appreciate all his effort to make me a good student. I could not have imagined having a kindest and better advisor and mentor for my MSc study and this accomplishment would not have been achieved without him. I hope that one day I would become as good as him. Thanks for believing in me and for being a constant source of knowledge and inspiration!

I would also like to acknowledge my co-supervisor, Dr. Synan Abu-Qamar for all his help in this thesis, I'm grateful for his help in the toxicity part of my thesis.

I would like to thank Prof. Firdos Khan, Dr. Mohammed Meetani and Dr. Ranjit Vijayan for agreeing to serve as examiners for my MSc thesis. I am deeply grateful to them for the long discussions that helped me sort out the technical details of my work. I really appreciate all their valuable comments and suggestion which made this thesis even better.

I am also thankful to United Arab Emirates University, the College of Graduate Studies and the College of Science for giving me the opportunity to obtain this MSc degree. Also, I would like to extend my thanks and appreciation to all member of Department of Biology for all their kind support during my MSc studies. Special thanks and appreciation go to Dr. Rabah Iratni “our real teacher”, who taught me almost all of my MSc courses. I learned so many things from him and I will be always grateful for all his support and kindness. Thanks also to Mr. Arjun Sham for his help in toxicity part. I would like to express my sincere thanks also to all member of Department of Chemistry, especially Mr. Bassam Hindawi and Dr. Altaf Shah. Also, I should not forget UAEU Libraries for providing me with the relevant reference material. I am also grateful to all my instructors and teachers who taught me through my undergraduate and graduate years.

A very special thanks to Mrs. Raja Al-Muskari and Miss Afra Al-Blooshi, whom I considered as my older sisters. I will be always grateful for all your advices, care, concerns and support.

I would never forget all the chats and beautiful moments I shared with my friends. I greatly value their friendship. I have to give a special mention for the support given by Halima Al Sammri, my unique sister, she was with me all the time and I would never forget all her support and kindness. Also, a special thanks to Khawlah

Athamneh for her care and encouragement. Many thanks are also extended to Fatima Saleh, Esraa Saleh, Badriya Baig, Aysha Al Kaabi, Shamma Al Shamisi and Asma Al Rashedi. Millions of thanks also to my high school friends, Mariam Al Shamisi, Safeya al Senani, Fatima Saleh, Noura, Laila Al Neyadi, Maitha Al Zarooni, Fatima Ali, Arwaa Al Neyadi and Ghuwaya Al Neyadi.

Thanks Also to my lab mates; Khadega, Manal and Ghadah. I wish you a very nice and successful MSc journey.

Most importantly, none of this would have been possible without the love and patience of my family. They have been a constant source of love, concern, support and strength throughout my years of study and through the process of researching and writing this thesis and my life in general. I would like to express my heart-felt gratitude to my mother as she aided and encouraged me throughout this endeavor. I have to give a special mention for the support given by all my brothers and my sisters.

Dedication

To my late father, whom I still miss everyday...

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List of Abbreviation

AOPs	Advance Oxidative Processes
CPO	Chloroperoxidase
CR	Congo Red
E1	Estrone
E2	17B-Estradiol
E3	Estriol
EE2	17a-Ethinylestradiol
HOBT	1-Hydroxybenzotriazole
HRP	Horseradish Peroxidase
LiP	Lignin Peroxidase
MnP	Manganese Peroxidase
PPCPs	Pharmaceuticals And Personal Care Products
RM	Redox Mediator
RO	Reverse Osmosis
SBP	Soybean Peroxidase
SRB	Sulforhodamine B
ThT	Thioflavin T

Chapter 1: Introduction

The search for efficient and novel approaches for remediation and clean-up of contaminated water bodies is attracting a lot of attention by environmental scientists (Gu et al., 2016; Jeon and Chang, 2013; Petrie et al., 2015; Rocha-Martin et al., 2014; Rodrigues et al., 2013; Wang et al., 2015). One of the most significant classes of pollutants are organic aromatic compounds and due to their potentially carcinogenic nature, they pose a very serious health risk to humans as well as other aquatic organisms (Martins et al., 2015b; Richardson et al., 2007; Zubair Alam et al., 2010). For example, aromatic textile dyes (which are abundantly present in textile industrial waste) have been linked to increased incidence of cancer textile industry workers (Alves de Lima et al., 2007; De Roos et al., 2005).

Likewise, the increasing presence of pharmaceuticals and personal care products (PPCPs) in the environment is becoming a serious environmental issue (Evgenidou et al., 2015; Li et al., 2015; Zhang et al., 2014a). PPCPs are a growing family of chemicals that consist of non-prescription as well as prescription drugs that are widely consumed by the general public, either as drugs or as part of other consumer products. Most common examples of PPCPs are antibiotics, anti-inflammatory drugs, oral contraceptives, etc. These PPCPs can be introduced into surface and ground water through direct human consumption and subsequent excretion as well as improper disposal of these compounds.

Although some PPCPs may be degraded during various wastewater treatment systems, it is suspected that a significant amount enters various drinking water sources. Table 1 shows the disturbing trend that significant amounts (and perhaps physiologically active concentrations) of various antibiotics, hormones and other

PPCPs can be readily found in drinking water in different countries. Obviously, these biologically active and potent chemicals can have wide-ranging effects in various organisms and bioaccumulation of hormones, antimicrobials, and antidepressants have been shown to occur in a variety of aquatic organisms (Gojkovic et al., 2015; Harmon, 2015; Silva et al., 2015; Czekalski et al., 2015; Fernández Fuentes et al., 2014; Na et al., 2014; Yang et al., 2016). Moreover, PPCPs have also been found to have serious adverse and harmful effects on larger organism such as aquatic animals (Burkina et al., 2015) and humans (Fernández-Rodríguez et al., 2015; Gilbert et al., 2015). One of the major aims of environmental scientists is to destroy or remove these pollutants from the water supply. In this regard, many stages of removal are often employed; these include simple filtration (Amini et al., 2011; Capar et al., 2006; Lin et al., 2014), adsorption (Ali, 2012; Tan et al., 2015; Wang and Chen, 2015; Yagub et al., 2014), coagulation (Daud et al., 2015; Liang et al., 2014; Sudoh et al., 2015), chemical (Mahmoudian et al., 2015; Qian et al., 2015; Yang et al., 2015) or biological treatments (Ali et al., 2013; Ali et al., 2014; Kalsoom et al., 2015; Santini et al., 2015). However, in many cases the methods are either costly or cannot be fully integrated into the removal procedures because they generate secondary waste, or the pollutants are recalcitrant to chemical treatment.

Among the various approaches mentioned, bioremediation is gaining a lot of attention as a good alternative to handle the removal of harmful substances from wastewaters as it is environmentally friendlier (Chaalal et al., 2015; García-Delgado et al., 2015; Helbling, 2015; Liu et al., 2015; Paniagua-Michel, 2015). This process takes advantage of microorganisms, plants, or enzymes from different sources to degrade and metabolize the various pollutants into less toxic forms (Ahemad, 2015; Li and Yu, 2011; Malik et al., 2015; Ullah et al., 2015).

Table 1: Summary of some pharmaceuticals and emerging contaminants detected in drinking water supply

PPC		Representative concentration, ng/L	Reference
NSAIDs and analgesics	Paracetamol	10000	(Kolpin <i>et al.</i> , 2002)
	Diclofenac	15000	(Jux <i>et al.</i> , 2002)
	Ibuprofen	1000	(Kolpin <i>et al.</i> , 2002)
	Valsartan	1300	(Huerta-Fontela <i>et al.</i> , 2011)
Antibiotics	Sulfamethoxazole	700	(Vanderford and Snyder, 2006)
	lincomycin	750	(Kolpin <i>et al.</i> , 2002)
	Erythromycin	450	(Managaki <i>et al.</i> , 2007)
Hormones	Estrone (E1)	1	(Huerta-Fontela <i>et al.</i> , 2011)
	Diethylstilbestrol	5.3	(Caban <i>et al.</i> , 2015)
	Estriol (E3)	72	(Huerta-Fontela <i>et al.</i> , 2011)
Pesticides	Carbofuran	25	(Papadakis <i>et al.</i> , 2015)
	Lindane	34	(Papadakis <i>et al.</i> , 2015)
Personal care product	Caffeine	14.60	(García-Vaquero <i>et al.</i> , 2014)
	Bisphenol A	4500	(Erickson <i>et al.</i> , 2014)
	Triclosan	35	(Azzouz and Ballesteros, 2013)
	Cotinine	88.5	(Sun <i>et al.</i> , 2015)

Most microbial bioremediation systems use aerobic conditions, but anaerobic conditions also permit facultative anaerobes to degrade various organic pollutants, presumably through different metabolic pathways (Chen and Yien Ting, 2015). In addition, various enzyme-based systems have been successfully used for the degradation of these different kinds of aromatic pollutants. Figure 1 shows the different approaches that have been used to remove or degrade various organic pollutants.

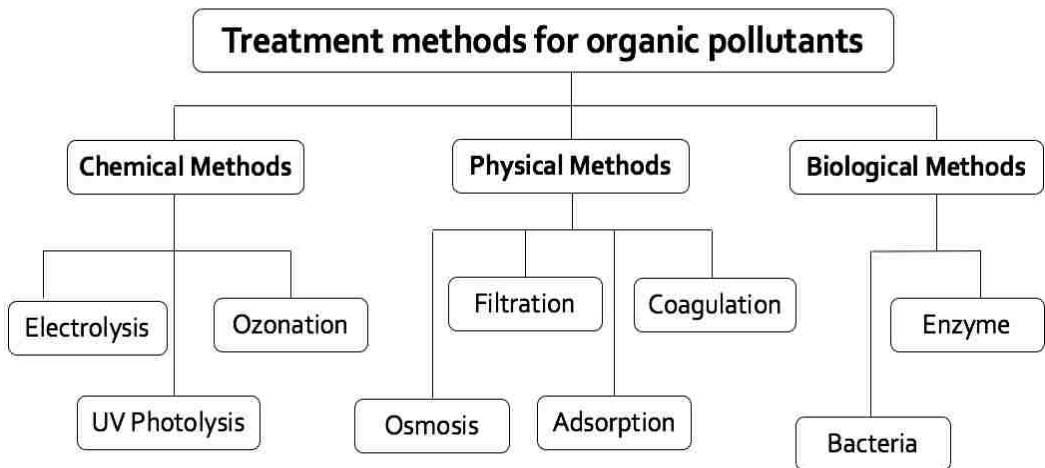


Figure 1: Approaches used to degrade organic pollutants

1.2 Removal of organic pollutants by various methods

1.2.1 Physical processes

Several physical methods such as filtration, specific coagulation, adsorption, chemical flocculation etc have been used widely in dye removal processing. In case of membrane filtration, several membrane types have been developed such as reverse osmosis (RO) membranes (Ciardelli and Ranieri, 2001). RO membrane showed a 90%

retention of most of the reactive dyes and other chemical compounds. Additionally, ultrafiltration can also be used as a pre-treatment and then followed by any of the others techniques (Van der Bruggen et al., 2005). These techniques are found to be effective but are quite expensive to use, besides producing secondary waste. On the other hand, adsorption technique depends on the adsorbent characteristics such as pore size and regeneration of the adsorbent. The adsorbents could be activated carbon, peat, wood chips, fly ash, and coal (Robinson et al., 2001). For soluble dyes, activated carbon showed a great efficiency (Robinson et al., 2002). However, adsorption methods have the major disadvantages related to the regeneration of the adsorbents and disposal of potential sludge. Coagulation, sedimentation and flocculation techniques are selective towards the type of contaminants present in wastewater (Gautami and Khanam, 2012).

1.2.2 Chemical methods

These include methods such as electrochemical destruction, use of NaOCl and advanced oxidation processes (AOP's) (Ahmed et al., 2015; Brillas and Martínez-Huitle, 2015; Rojas et al., 2015; Särkkä et al., 2015). The electrochemical techniques have been shown to be effective for the degradation of reactive, acid and disperse dyes. The combination of UV with sonication has also been employed to remove other organic pollutants (Furgal et al., 2015; Kiai et al., 2014), however, the method can be quite expensive and resource-intensive.

The advanced oxidation processes (AOP's) are a promising approach, which have been effectively used for the degradation of various kinds of organic compounds. The use of various AOPs for waste water remediation has been extensively reviewed and reported in scientific literature (Robinson et al., 2001). All AOP techniques primarily rely on the generation of reactive hydroxyl radicals for

subsequent reactions with organic pollutants leading their degradation to less harmful intermediates. However, it should be noted that these AOPs can be costly, as the process demands a continuous input of expensive (and reactive/corrosive) chemicals and large amounts of electricity. Moreover, these processes may not be able to treat large amounts of wastewater; instead, they are ideally suited for final “polishing” step of the remediation process.

1.3 Bioremediation/biodegradation

Bio-treatment offers a potentially cheaper and environmentally friendlier alternative for removing organic pollutants from contaminated wastewater. A number of microorganisms such as bacteria, fungi, and yeasts have been found to decolorize organic pollutants (Behnood et al., 2014; Evgenidou et al., 2015; Jasińska et al., 2015; Qu et al., 2015). Most of the microbial remediation of organic pollutants is due to the presence of promiscuous oxidoreductase enzymes and thus they show relatively wide substrate specificities and are able to degrade various kinds of pollutants. For example, various microbes have been reported that carry out efficient enzymatic decolorization and mineralization of azo dyes (Schückel et al., 2011) (Rauf and Ashraf, 2012). Therefore, in cases where the target molecule or additives inhibit growth, isolated enzyme (*in vitro*) systems may be preferred. Furthermore, the presence of redox mediator (RM) in enzyme-based systems can dramatically enhance the rate of reaction and increase the range of substrates (Adelaja et al., 2015; Martins et al., 2015a; Pereira et al., 2014).

1.3.1 Enzymatic assisted removal of organic pollutants

Various enzyme systems have been employed for the efficient degradation of diverse organic pollutants and have shown to oxidize and degrade the pollutants into smaller intermediates. Amongst the various advantages offered by enzymatic degradation approach, the most important ones are the mild and less toxic reagents and conditions that are normally employed in their use as well as their ability to degrade a wide range of substrates. The main potential disadvantage with the use of enzymes are their relatively high cost, however, this can be ameliorated through the use of recombinant DNA technology to mass-produce enzymes. Literature survey shows that various types of pollutants have been degraded by two different classes of enzymes which are laccases (Bautista *et al.*, 2015) and peroxidases such as soybean peroxidase (SBP), manganese peroxidase (MnP), lignin peroxidase (LiP) and horseradish peroxidase (HRP) (Bautista *et al.*, 2015; Coconi-Linares *et al.*, 2015; Husain, 2010). Additionally, peroxidases from other plant sources such as cauliflower, white radish, and turnip, have been used for the degradation of various organic compounds (Chang *et al.*, 2015; Kalsoom, 2010; Satar and Husain, 2011; Silva *et al.*, 2012). Besides this, peroxidases from bamboo shoots and lemon peel have also been used for degrading dyes (Hsu *et al.*, 2012; Nouren and Bhatti, 2015). The addition of redox mediator (RM) to the system has shown to enhance the degradation process to produce less toxic substances (Bibi *et al.*, 2011; Husain and Husain, 2007; Shiraishi *et al.*, 2013; Zhang *et al.*, 2014b).

Not surprisingly, numerous studies have reported the effective and extensive use of peroxidases in dye degradation (Rauf and Ashraf, 2012; Singh *et al.*, 2015). However, there are only a very few published studies that have reported on the use of

peroxidases for the degradation of PPCPs such as NSAIDs and analgesics, hormones, antibiotics and pesticides. An exhaustive literature search has indicated that hormones are the most common PPCPs that were degraded efficiently by various peroxidases.

1.3.1.1 Classification of pollutant-degrading enzymes (peroxidases)

Peroxidases are oxidoreductase enzymes, which are extensively distributed in nature, especially in microbes and plant. These enzymes (in the presence of hydrogen peroxide or other peroxide) have the ability to oxidize a wide range of substrates (Dunford and Stillman, 1976). The biological functions of peroxidases vary within organisms, in plant they play an important role in defense against pathogens, cell wall formation and lignification process (Biles and Martyn, 1993). Peroxidases have unique properties such as high redox potential and high thermal stability (Demarche et al., 2012; Regalado et al., 2004). Peroxidases can be classified on two broad groups, which are heme-peroxidases and non-heme peroxidases (Zamocky et al., 2008).

Heme-peroxidases have been recently classified based on their enzymatic activities into four main superfamilies, which are peroxidase–catalase superfamily, peroxidase cyclooxygenase, peroxidase–chlorite dismutase superfamily and peroxidase–peroxygenase superfamily (Figure 2A and Figure 2B). Table 2 summarizes the main characteristics of the four superfamilies including their subfamilies, members, the conserved sequence of active site and their oxidation reactions (Zámocký et al., 2015). Peroxidase–catalase superfamily is the most abundant superfamily that includes bacterial, fungal and plant peroxidases and it is further divided into three families. Family 1 represents intracellular bacterial catalase–peroxidases, while family 2 include secretory fungal peroxidases such as lignin and manganese peroxidases (MnP). In contrast, family 3 is plant peroxidases composed of

secreted plant peroxidases and the most common member of this class are horseradish peroxidase (HRP) and soybean peroxidase (SBP) (Battistuzzi et al., 2010). It is interesting to note that although, both SBP and HRP belongs to the same family (#3), their primary structures are very different, having only 58.7% amino acid identity (Figure 3A). However, in spite of very little sequence homology, they both have very similar structure (Figure 3B). Interestingly, SBP and CPO which belong to different families (and have very low sequence alignment) appear to have similar tertiary structure (~46% similarity) (Figure 3C). Peroxidase cyclooxygenase superfamily was annotated as animal peroxidases. It has seven subfamilies and they are mainly involved in innate immune response (Zamocky et al., 2008). On the other hand, peroxidase–chlorite dismutase superfamily contains three families, which are Dyp-type Peroxidases, Chlorite dismutases and Chlorite dismutases like proteins. Dyp-type Peroxidases are peroxidases that mainly found in bacterial that are involved in the dye degradation mechanisms while their physiological functions are not known (Yoshida and Sugano, 2015). Chlorite dismutases are responsible for degrading chlorite efficiently to chloride and molecular oxygen (Schaffner et al., 2014). Lastly, peroxidase–peroxygenase superfamily is still not well known and cannot be further divided into subfamilies. For a long time, Chloroperoxidase (CPO) from *Caldariomyces fumago* was the only known enzyme from this superfamily. Many of these peroxidases have been used widely in the organic pollutant degradation especially soybean peroxidase (SBP), horseradish peroxidase (HRP) Manganese peroxidases (MnP), Chloroperoxidase (CPO) and lignin peroxidases (LiP).

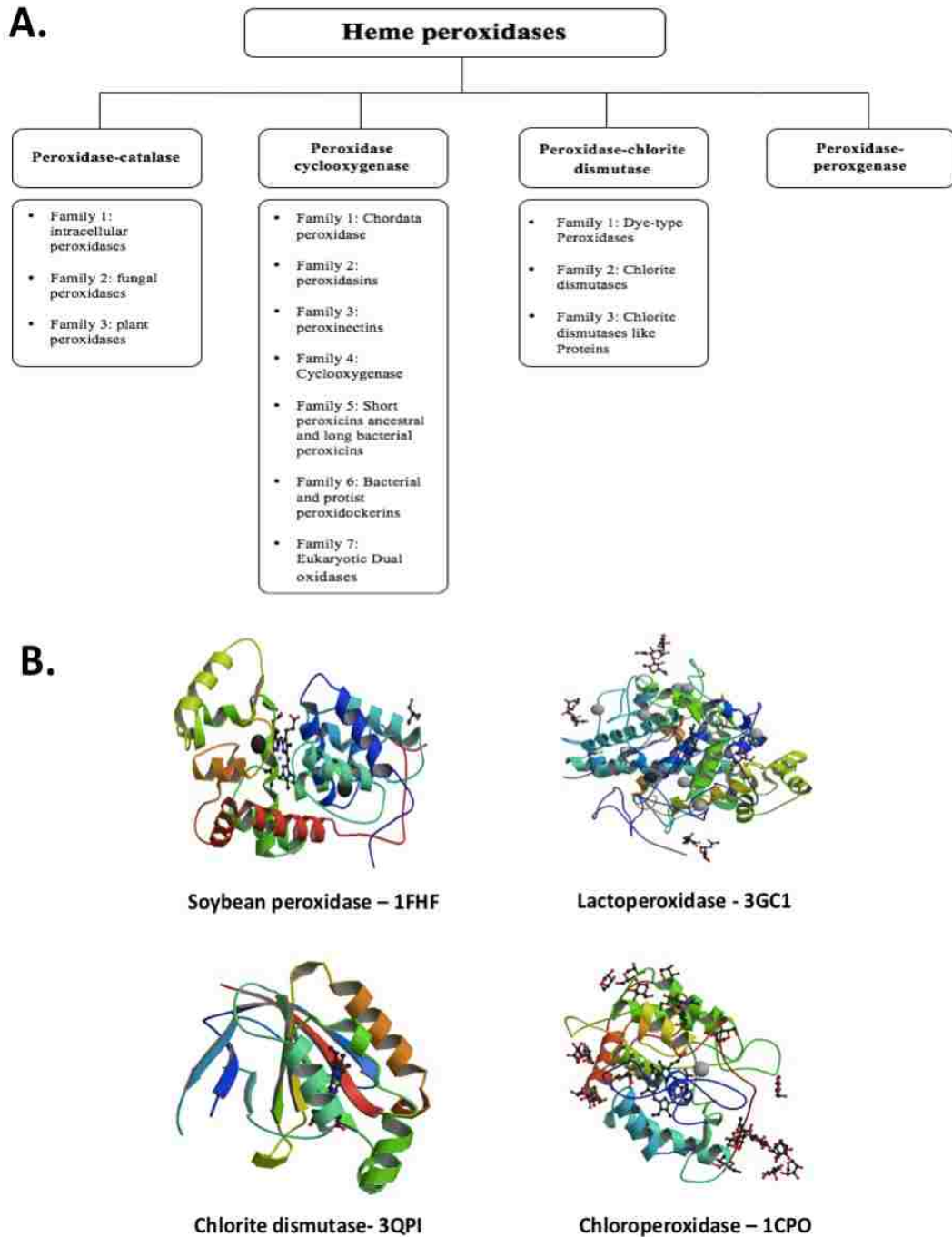


Figure 2: Peroxidases classification. (A) Diagram shows the four main peroxidases superfamilies and their subfamilies. (B) 3D structure of members from each peroxidase superfamily. Soybean peroxidase (Peroxidase–catalase superfamily), Lactoperoxidase (Peroxidase cyclooxygenase), Chlorite dismutases (Peroxidase–chlorite dismutase superfamily) and Chloroperoxidase (Peroxidase–peroxygenase superfamily).

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 (304 letters)

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Length = 308

Score = 286 bits (732), Expect = 4e-82

Identities = 156/305 (51%), Positives = 189/305 (61%), Gaps = 2/305 (0%)

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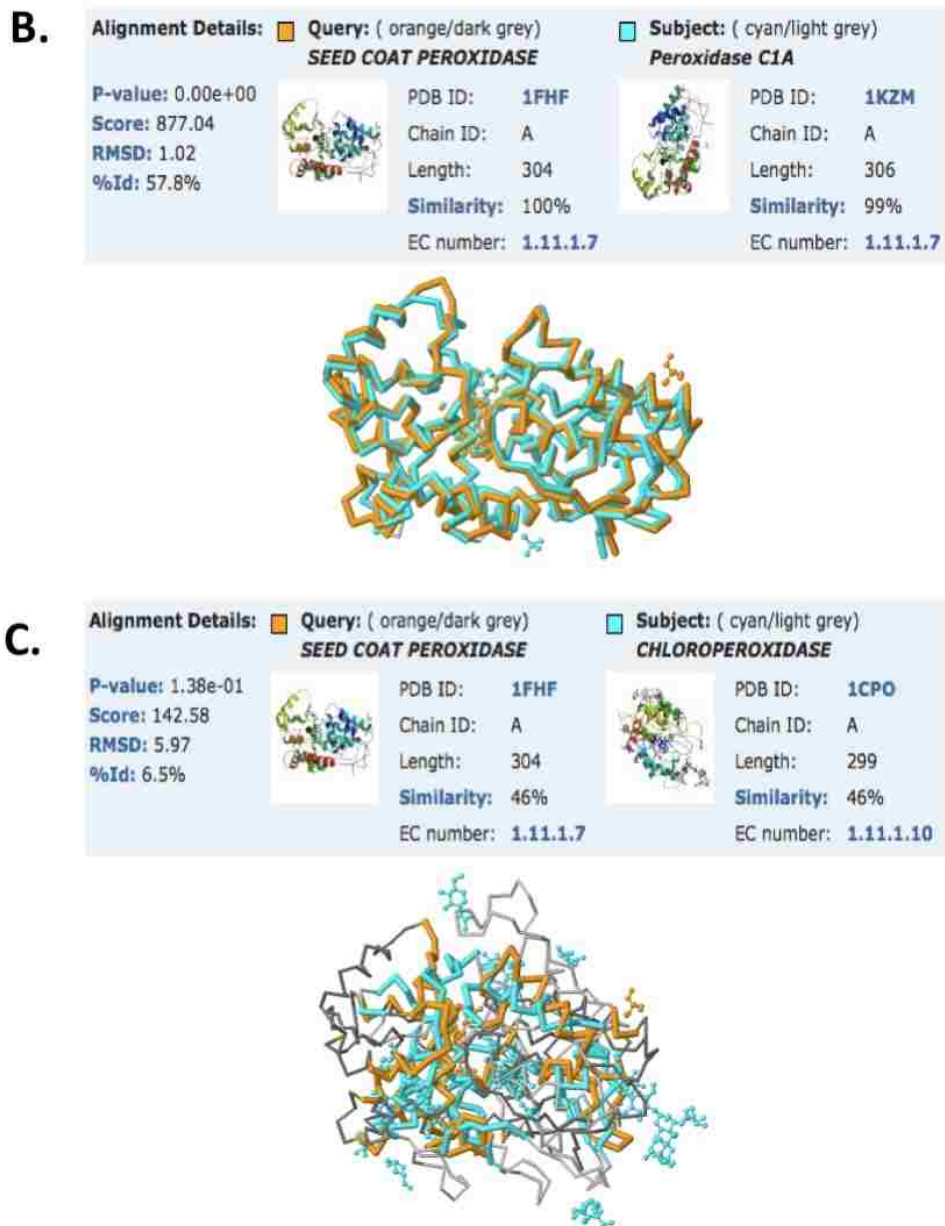


Figure 3: Bioinformatics analyses of SBP, HRB and CPO. (A) The pairwise alignment of SBP (PDB: 1FHF) and HRP (PDB: 1KZM) using EMBOSS-water algorithm (http://www.ebi.ac.uk/Tools/psa/emboss_water/) showing only 58.7 amino acid sequence identity. (B) The tertiary structure alignment of SBP and HRP shows 99% amino acid sequence identity. Panel (C) shows the tertiary structure similarity (~46%) between SBP and CPO enzymes.

Table 2: Summary of the main characteristics of the four superfamilies including their subfamilies, members, the conserved sequence of active site and their oxidation reaction

Superfamily	Families	Examples/members	Active site Conserved sequence ^a	Type of redox reaction catalyzed ^b
Peroxidase–catalase superfamily	Family 1: intracellular peroxidase	Cytochrome c peroxidases	XR-XX-W/F-H-X-	R3
	Family 2: fungus secreted peroxidases	Lignin, manganese, versatile and generic peroxidases		R1 and R2
	Family 3: Plant peroxidase	Plant secretory peroxidases		R1
Peroxidase cyclooxygenase	Family 1: Chordata peroxidase	Lactoperoxidase (LPO)	-X-G-Q-X-X-DH-D-X-	R1 and R2
	Family 2: peroxidasins			
	Family 3: peroxinectins	Myeloperoxidase		
	Family 4: Cyclooxygenase	Cyclooxygenase		
	Family 5: Short peroxicins ancestral and long bacterial peroxicins		-X-G-Q-X-X-DH-D-X-	
	Family 6: Bacterial and protist peroxidockerins			
	Family 7: Eukaryotic Dual oxidases			
Peroxidase–chlorite dismutase superfamily	Family 1: Dyp-type Peroxidases	Dyp-type Peroxidases	-X-I-P-V/I-K/ R-K-X-	R1
	Family 2: Chlorite dismutases	Chlorite dismutases		
	Family 3: Chlorite dismutases like Proteins	Chlorite dismutase-like Protein		
Peroxidase–peroxygenase superfamily		Chloroperoxidase	-X-P-C-P-X-	R1, R2 and R4

^a:R=Arginine, W= Tryptophan, F= Phenylalanine, H=Histidine, G= Glycine, Q= Glutamine, D= Aspartate, I= Isoleucine, V= Valine, K= Lysine, P= Proline, C= Cysteine and X: any amino acid

^b:

R1: $\text{H}_2\text{O}_2 + 2\text{AH}_2 \longrightarrow \text{H}_2\text{O} + 2\text{AH}$,

R2: $\text{H}_2\text{O}_2 + \text{X}^- + \text{H}^+ \longrightarrow \text{H}_2\text{O} + \text{HOX}$

R3: $\text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \longrightarrow 2\text{H}_2\text{O} + \text{O}_2$

R4: $\text{H}_2\text{O}_2 + \text{RH} \longrightarrow \text{H}_2\text{O} + \text{ROH}$

1.3.1.2 Examples of pollutants degradation by different peroxidases

1.3.1.2.1 Pollutants degradation by Soybean Peroxidase (SBP)

SBP is a plant peroxidase that is extracted from the seed coat of the soybean plant (Gijzen et al., 1993). It can oxidize a wide variety of organic and inorganic substrates using hydrogen peroxide as shown in Figure 4A. If a redox mediator is used in the reaction, the schematics will be slightly different as shown in Figure 4B.

There are many recent published examples of pollutant degradation using SBP, some of which will be summarized here. It has been previously reported that Trypan Blue dye could be efficiently degraded using the soybean peroxidase (SBP) catalyzed enzymatic reactions (Kalsoom et al., 2013). The enzymatic based degradation has been found to be affected by several factors such as the concentrations of the dye, enzyme and H_2O_2 , buffer pH and reaction time. Moreover, the immobilization of SBP in polyacrylamide matrix allowed the use of the enzyme for the degradation several times which served as an advantage to the enzymatic degradation. The detailed mechanism of Trypan Blue degradation (determined using LC-MS and tandem MS-MS analyses) revealed that several aromatic compounds were produced which were then degraded into smaller compounds.

The same group also carried a similar study in which they used SBP to degrade Crystal Ponceau 6R (CP6R) (Ali et al., 2013). After optimizing the reaction conditions, it was found that the CP6R could be completely degraded by SBP, as confirmed using Total Organic Carbon and HPLC analyses. Additionally, LC-MS-MS analyses predicted two different pathways for the degradation of CP6R, either symmetric or asymmetric cleavage of the azo bond followed by several reactions such as desulfonation, deamination, and oxidation reactions. Moreover, SBP was also found

to be able to efficiently (by almost 86%) degrade the textile dye Remazol Brilliant Blue R (RBBR) (Silva et al., 2013). Interestingly, toxicological tests using two model plants, *Artemia salina* and *Lactuca sativa*, showed that the products of the SBP degradation were more toxic than the RBBR, thus highlighting importance of performing toxicological tests after enzymatic based degradation.

In another study, SBP was used to degrade Remazol Turquoise Blue G 133 (RTB), and the degradation was monitored and confirmed using LC-MS, which also showed that the dye was degraded mainly into sulfophthalimide and NH_4^+ ions (Marchis *et al.*, 2011).

Soybean peroxidase has been used to degrade other non-dye pollutants as well, such as phenolic compounds present in coffee (Chagas et al., 2015). In this study, free SBP as well as chitosan-immobilized SBP were tested for their abilities to treat coffee processing wastewater containing caffeic acid and other phenolic compounds. Interestingly, contrary to previously published reports on the pH optimum of SBP to be around 3-4, the authors showed that SBP appeared to have a pH optimum of 6 for the degradation of caffeic acid (Chagas et al., 2015).

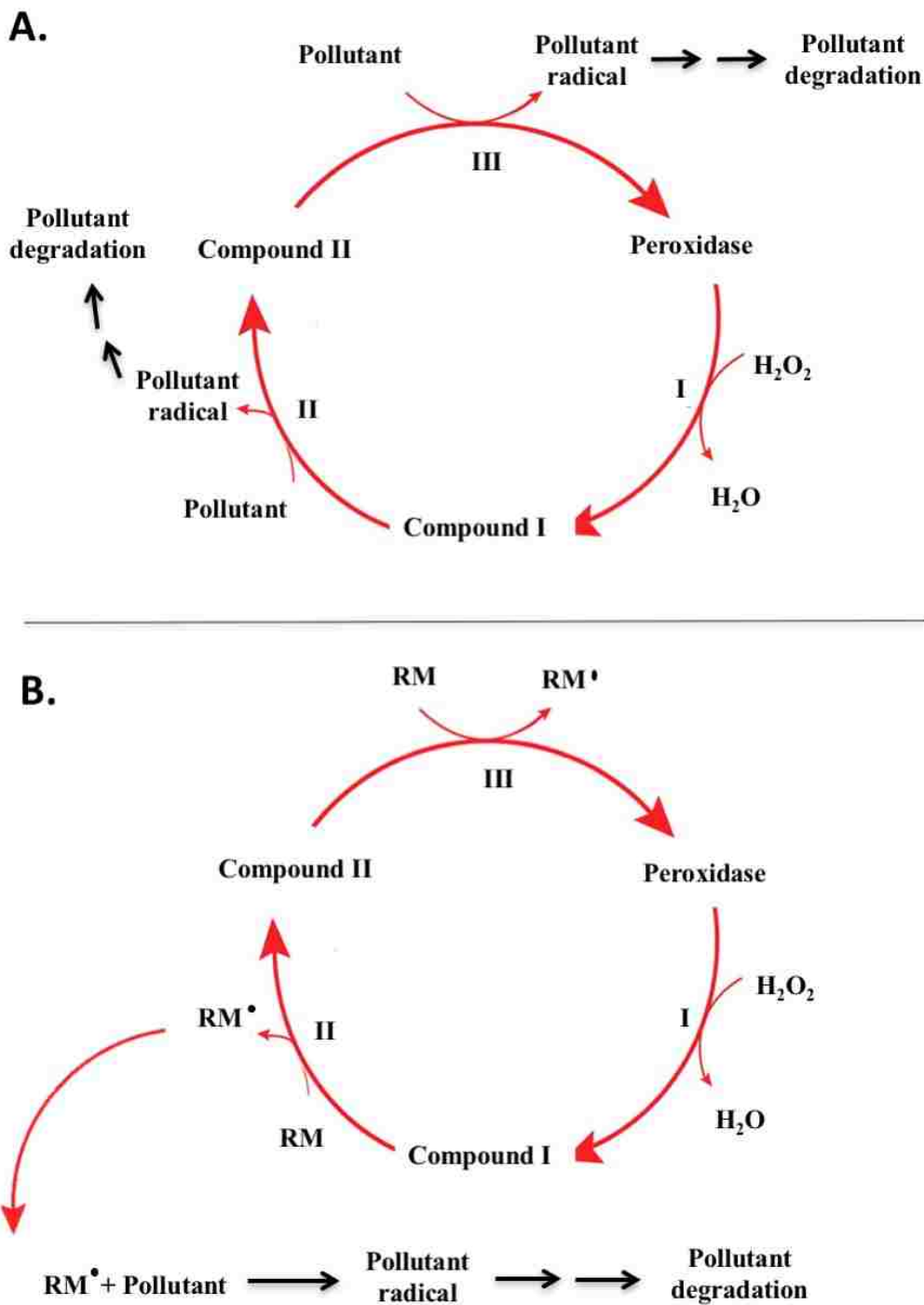


Figure 4: Peroxidases catalytic cycle. (A) Peroxidases catalytic cycle involves the formation of Compound I and Compound II during pollutants degradation. (B) In presence of redox mediators (RM), RM radicals are produced which then react with the pollutants leading to their subsequent degradation.

1.3.1.2.2 Pollutants degradation by Horseradish Peroxidase (HRP)

Literature survey shows that numerous groups have successfully used HRP for pollutant degradation as well. In a detailed study, HRP have been used to remove 10 different synthetic and natural dyes, and various parameters such as the concentration of the dyes and the presence of redox mediators were optimized. HRP caused more than 70 % degradation of most of the dyes used in the study (Abdel-Aty et al., 2013). Several studies have used different techniques to immobilize HRP in order to get enhanced degradation. One of these techniques is immobilizing HRP in phospholipid-templated titania and then exposing it to wastewater containing different phenolic compounds and dyes (Jiang et al., 2014). This immobilization technique provided several advantages to HRP properties such as increasing the thermal stability, expanding the pH range and providing resistance against several chemicals such as BaCl_2 and acetone. The degradation of phenol, 2-chlorophenol and Direct Black-38 by immobilized HRP and free HRP was examined and interestingly, the immobilized HRP was more effective for the removal of the aromatic compounds as compared to the free HRP (Jiang et al., 2014). Another technique is immobilizing HRP into β -cyclodextrin (β -CD)–chitosan to degrade textile effluents. It was found that the cross-linked HRP had great stability and was more active where it showed complete removal of the textile effluent compared with the others. Furthermore, the used of the cross-linked HRP in a reactor as well as the reusing it showed an efficient degradation of the textile effluent (Karim et al., 2012). Furthermore, free and immobilized HRP was used to degrade Acid Blue 113, and they both showed an efficient degradation with more than 80% removal. However, immobilized HRP could be used again up to three times with the same degradation efficiency (Malani et al., 2013; Preethi et al., 2013). Interestingly, a recent study showed that HRP treatment could be combined with

sonochemical technique to produce an even more efficient degradation of dyes (Malani et al., 2013; Preethi et al., 2013).

HRP has also been used with great potential for the degradation of PCPPs, such as estrogenic hormones. A recent study has shown that HRP could efficiently degrade estrone (E1), 17 β -estradiol (E2), estriol (E3), and 17 α -ethinylestradiol (EE2) (Auriol et al., 2008). Just like the dye degradation studies reported above, pH and temperature were important parameters that affected the efficacy of the degradation. The authors showed that under optimized conditions within an hour of treatment HRP was able to remove about 92% of E1, E2, E3 and 100% of EE2. However, when HRP was used to treat real wastewater containing these hormones (E1, E2, E3, and EE2) it showed only 29%, 19%, 28%, and 37% degradation, respectively. The inefficient degradation was suggested to be due to the presence of other organic pollutants which interfere with the ability of HRP to degrade these hormones (Auriol et al., 2006). Subsequent studies carried by the same group to overcome the inhibition of hormone degradation in the real wastewater samples showed that additional optimization and higher doses of the HRP and H₂O₂ helped to completely degrade all the hormones present (Auriol et al., 2007).

1.3.1.2.3 Pollutants degradation by Manganese Peroxidase (MnP)

Manganese peroxidase was one of the earliest peroxidase used in degrading organic pollutants. For example, MnP was able to degrade Congo Red, Orange IV, and Orange G more efficiently than laccase and lignin peroxidases (LiP). Optimizing the reaction conditions led to enhance degradation of Orange IV and Orange G by MnP with 76 and 57% degradation, respectively (Yao et al., 2013).

Manganese peroxidases have also been shown to completely degrade steroidal estrogenic hormones (such as E2 and EE2) (Suzuki et al., 2003). Moreover, MnP was found to be comparable to laccases in its ability to completely remove E1 after 1h (Tamagawa et al., 2006). A recent study has also reported on a MnP-dependent system to degrade imidazole fungicide, miconazole (MCZ) and the antidepressant agent, sertraline (SER). This system helped to eliminate 88% of MCZ and 85% of SER after 24 h of treatment (Inoue et al., 2015). Moreover, the efficiencies of MnP, laccase and laccase-mediator system with the redox mediator, 1-hydroxybenzotriazole (HOBT), were compared to degrade the antimicrobial agent, triclosan. The result showed that MnP was the most effective and it could completely degrade triclosan within one hour (Inoue et al., 2015). Furthermore, a separate group has shown that tetracycline can also be degraded (by almost 73%) by MnP after 4hr incubation (Wen et al., 2010).

1.3.1.2.4 Pollutants degradation by Chloroperoxidase (CPO)

Chloroperoxidase, another member of the peroxidase superfamily, has also been recently used for the degradation of organic pollutants. CPO has been shown to decolorize Sunset Yellow and Orange G efficiently by 77.25% and 98.72%, respectively (Zhang et al., 2012).. Furthermore, CPO have been used also in degrading Crystal Violet and Alizarin Red with 97.68% and 98.23%, respectively. Interestingly, it was suggested that CPO in the presence of Cl^- was able to decolorize more efficiently by producing a strong oxidizing complex HOCl (Liu et al., 2014) which annotated as R2 in Table 2.

1.3.1.2.5 Pollutants degradation by Lignin Peroxidase (LiP)

Lignin peroxidase is one of the earliest peroxidases that has been used for dye degradation. A comparative study showed that LiP was able to degrade Methylene Blue and Azure B dyes with high reaction kinetics and with less H₂O₂ concentration compared with HRP (Ferreira-Leitão et al., 2003). Moreover, it was found that LiP by-products were less toxic compared to those produced by HRP. In addition, LiP purified from *Acinetobacter calcoaceticus*, was able to degrade ten different textile dyes with almost 90% efficient for most of tested dyes (Ghodake et al., 2009).

Lignin peroxidases have also been tested in their abilities to degrade Endocrine-Disrupting Compounds such as Bisphenol A (BPA), E1, E2 and EE2. A study investigated the efficiency of LiP enzymes extracted from white-rot fungi *Phanerochaete sordida* and *Phanerochaete chrysosporium* for the removal of these compounds. HPLC analyses showed that LiP from *Phanerochaete sordida* treatment achieved complete degradation of BPA, E1, E2 and EE2. Interestingly, LiP extracted from *P. chrysosporium* showed a significantly lower degradation with 52.5%, 23.9%, 38.2%, and 45.0%, respectively (Wang et al., 2012). In addition to hormones removal, several studies have shown the ability of LiP to degrade antibiotics, for example Wen *et al* have shown that about 95% of tetracycline could be degraded by LiP within 5 minutes (Wen et al., 2009).

1.4 Factors affecting enzymatic degradation of organic pollutants

1.4.1 Choice of enzyme (peroxidases)

It is generally accepted that most commonly used pollutant-degradable enzymes are oxidoreductases, especially peroxidases. Furthermore, as reviewed above, various research groups have used different peroxidases for degrading different

pollutants, thus leading to the impression that all peroxidase are equivalent in their abilities to degrade pollutants. Interestingly, comparative degradation studies showing the behavior of different peroxidases on the same compound have not been extensively reported, and there are only a very few published data on such comparative studies. For example, oxidation of phenol has been studied using CPO, LPO (Lactoperoxidase), SBP, HRP, catalase and microperoxidase-11 (which was obtained from the cleavage of cytochrome c and has peroxidase activity) (van de Velde et al., 1999). They found a slight variation in the rate of phenol oxidation by CPO, LPO, SBP, HRP, however, catalase and microperoxidase-11 showed inefficient oxidation. Moreover, they carried out additional experiments to examine the ability of CPO and HRP to oxidize/degrade indole and dihydroxyfumaric acid (DHF), and it was observed that CPO could oxidize indole more efficiently than DHF and the verse versa in case of HRP (van de Velde et al., 1999).

1.4.2 pH

Enzymes, especially peroxidases, are highly sensitive to solution pH because changes in the pH cause changes in the ionic state of amino acids in the active site and may affect substrate binding and catalysis (Hawes, 1991). Thus, pH optimization studies are critical for achieving maximum enzyme-mediated degradation of pollutants. Additionally, different dyes may have different pH optima. For examples, HRP degrades Disperse Red 343 most efficiently at pH 5 (Schmitt et al., 2012), whereas Lanaset Blue 2R reached the maximum degradation by HRP at pH 4 (Ulson de Souza et al., 2007). Another study using Citrus *limon* peroxidase to degrade Basic Violet 3 found the optimum pH to be 4.5 with 56.85% degradation. However, the degradation percentage dramatically declined to 36% at pH 6 (Nouren and Bhatti,

2015). Additionally, about 96% of Remazol Turquoise Blue G 133 was decolorized by SBP at pH 3 while only 5% decolorization was observed at pH 2 and pH 8 (Marchis et al., 2011). Similarly, SBP degraded Trypan Blue completely at pH 4, but at pH 2 and pH 9 SBP showed inefficient degradation (Kalsoom et al., 2013). These studies suggest that peroxidases from peroxidase–catalase superfamily such as HRP and SBP are more active and efficient in pH range 3.5-5. On the other hand, several studies with Chloroperoxidase (CPO) from peroxidase–peroxygenase superfamily have shown that pH 2 is the optimum pH for the enzymatic degradation of various dyes. For instance, Orange G and Sunset Yellow degradation by CPO reached the maximum at pH 2 and decreased significantly in pH range 4-9 (Zhang et al., 2012). Another study used CPO to degrade Alizarin Red and Crystal Violet and found that the optimum pH was between 2-3 (almost 100% degradation for both dyes), while the degradation decreased sharply after pH 3 (Liu et al., 2014). This seems to indicate that SBP and CPO may have different pH optimum ranges, 3.5-5 versus 2-3, respectively.

1.4.3 Organic pollutant structure

Organic pollutants mainly dyes are classified into several categories such as anthraquinone, acidic, basic, azo and diazo dyes based on their functional groups (Langhals, 2004). Since efficient degradation of organic compounds by various peroxidase enzymes depends on a proper binding and orientation of these compounds within the active sites of the enzymes, it is expected that the substrates' structure would be a very important parameter. For example, study published by Jamal et al showed that *Trichosanthes dioica* peroxidase was able to degrade azo dyes (Congo Red, Reactive Red 2, Reactive Red 120 and Reactive Black 5) much more efficiently than anthraquinone dyes (Remazol Brilliant Blue R and Reactive Blue 4)

(Jamal et al., 2011b). In addition, it has been reported that mono-azo dye was degraded by *P. australis* peroxidase more easily as compared with di-azo dye (Haddaji et al., 2014). These studies have suggested a relationship between dyes structure and their enzymatic degradation. However, this does not appear to be universally true

1.4.4 Temperature

Temperature also has a significant effect on enzymatic reaction. Increasing the temperature speeds up the enzymatic reactions by providing the activation energy that is needed for the reaction. However, further increase in the temperature will denature the enzyme (Shuler and Kargi, 1992). Plant peroxidases are known to be thermally stable at high temperature. For example, SBP can degrade Trypan Blue efficiently even up to 50 °C (Kalsoom et al., 2013). Similarly, HRP can degrade Reactive Blue 52, Reactive Blue 198, Reactive Red 55 and Reactive Blue 19 completely at 45 °C (da Silva et al., 2010). Likewise, White Radish Peroxidase showed efficient degradation of Reactive Red 120 and Reactive Blue 171 at 40 °C (Satar and Husain, 2009). In contrast, studies carried out with CPO showed that the dye degradation decreases after 45 °C (Liu et al., 2014). Similarly, decolonization of Orange G and Sunset yellow carried out at 20, 30, 40, 50 and 60 °C showed that the optimum temperature for Sunset yellow and Orange G degradation was in range of 20-45 °C (Zhang et al., 2012). Similarly, Liu *et al.* found that CPO could degrade Alizarin Red in temperature range from 20 °C to 60 °C. However, surprisingly they found that Crystal Violet could be degraded by 85% even at 90 °C (Liu et al., 2014). The authors have explained this unusual observation by suggesting that this “degradation” of Crystal Violet may not necessarily be due to the enzyme, but rather due to a yet-unidentified high-temperature chemical oxidation involving the chloride ion from the dye and hydrogen peroxide.

1.4.5 Presence of redox mediators

As reviewed above, peroxidases show efficient degradation of a wide range of organic pollutants. However, some of organic pollutants cannot be readily degraded by peroxidases and require the presence of small diffusible compounds known as redox mediators (RM) (Van der Zee and Cervantes, 2009). These RM can be reduced and oxidized readily which help peroxidases to degrade the recalcitrant compounds (Adelaja et al., 2015; Martins et al., 2015a; Pereira et al., 2014). The most widely used RM in peroxidase-based degradation are veratryl alcohol, 1-hydroxybenzotriazole (HOBT), acetosyringone and syringaldehyde. Several studies have suggested that their efficiency mainly depends on their redox potential, such that redox mediators with high redox potential increase the efficiency of the degradation. A recent study has examined the role of several RMs such as HOBT, vanillin, guaiacol and bromophenol in degrading Disperse Red 19 (DR19) and Disperse Black 9 (DB9) by *Trichosanthes diocia* peroxidase (Jamal et al., 2010). It was found that HOBT was the best RM where it enhanced the degradation to 79 % and 60% for DR19 and DB9, respectively (Jamal et al., 2011a). Furthermore, another comparison study was performed to examine the role of HOBT and vanillin in textile and non-textile dyes degradation by *Trichosanthes diocia* peroxidase. The results indicated that HOBT supplementation leads to more efficient degradation than Vanillin (Jamal et al., 2012). Furthermore, HOBT was also found to be an efficient RM in the degradation of Reactive Black 5, Reactive Red 2, Reactive Blue 4, Disperse Black 9, and Disperse Orange 25 by cauliflower bud peroxidase with 83%, 78%, 76%, 73% and 72% degradation, respectively (Jamal et al., 2011a).

Chapter 2: Objectives

Objectives of the current work:

1. Establish a comparative degradation study using two closely-related classes of peroxidases (SBP and CPO) to degrade three different aromatic pollutants—Thioflavin T (ThT), Sulforhodamine B (SRB) and Congo Red (CR).
2. Optimize the degradation conditions such as requirement for Redox Mediator, pH optimization, temperature effect and H₂O₂ concentration optimization.
3. Study the degradation pathway of each model pollutant via either SBP or CPO and to identify a set of intermediates that will be produced.
4. Perform toxicity tests using model plant (*Lactuca stavia* seeds) to analyze the potential toxicity of the enzymes-based degradation of pollutants.

Chapter 3: Materials and Methods

3.1 Enzymes and chemicals

Thioflavin T, Sulforhodamine B and Congo Red were purchased from AnaSpec (California, USA) with high purity, herein abbreviated as ThT, SRB and CR, respectively, and their properties are shown in Table 3. Soybean peroxidase (SBP) and Chloroperoxidase (CPO) were purchased from Bio-Research Products (Iowa, USA). The specific activity of SBP was 2700 IU/mg (1mg/ml), 26 μ M, while CPO activity was 1296 IU/mg (17mg/ml), 405 μ M and was used as such. Hydrogen peroxide (30% w/v) was purchased from Sigma-Aldrich (USA). Universal buffers were prepared using 0.1 M citric acid and 0.2 M K₂HPO₄. LC-MS grade of acetonitrile, methanol, ammonia formate and formic acid were obtained from Sigma-Aldrich chemical Co., USA.

3.2 Dye degradation

Dyes decolorization assay by either SBP or CPO along with buffer and hydrogen peroxide was examined using 4 ml quartz cuvette in a Spectrophotometer (Cary 60, USA) as previously described in other work (Ali et al., 2013; Kalsoom et al., 2013). When needed, 0.05 mM 1-hydroxybenzotriazole (HOBT), a redox mediator was added in the reaction mixture of the dyes that didn't show any decolorization in the absence of HOBT. Different experiments were performed in order to optimize the reaction conditions such as HOBT concentration, pH, enzymes concentrations, concentration of dyes, H₂O₂ concentration and temperature effect using one factor at a time (OFAT) methodology. The effect of each factor was investigated by changing one factor while keeping the parameters constant. All the optimization experiments

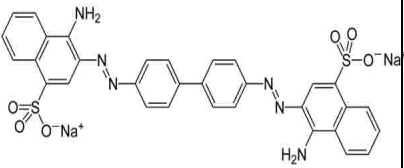
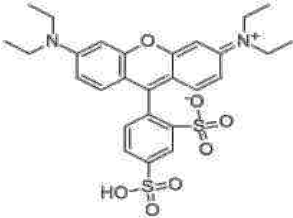
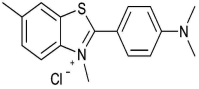
were carried out using a 96-well plate in a BioTek Epoch plate reader (BioTek, USA) by monitoring the λ_{max} of each dye. Each optimization experiment were replicated at least three times under identical conditions.

For thermal stability experiments, SBP and CPO were pre-heated in water bath at 95 °C for 10 min and then their activities were examined. In all experiments, reaction were stopped by adding 33mM NaOH and the % decolorization were calculated as the following:

$$\% \text{ decolorization} = \frac{A_0 - A_t}{A_0} \times 100 \quad (1)$$

where A_0 refers to the initial absorbance of dyes solutions and A_t refers to the absorbance of the solutions at any given time.

Table 3: Classification of dyes and their structures

Dyes	Class	Molecular Formula	FW	λ_{\max}	Structure
Congo Red (CR)	Azo (di)	$C_{32}H_{22}N_6O_6$ S_2Na_2	696.7	498	
Sulforhodamine B (SRB)	Triphenyl-methane	$C_{27}H_{30}N_2O_7$ S_2	558.6	565	
Thioflavin T (ThT)	Thiazole	$C_{17}H_{19}N_2S$ Cl	318.9	412	

3.3 HPLC and LCMS analyses

The biodegradation intermediates produced after the decolorization of the dye by SBP and CPO were analyzed by LC-MS. The dyes samples before and after the enzymatic treatments were filtered using 0.45µm syringe filter preceding the analyses. The HPLC was fitted with a ZORBAX Extend-C18 column (3.5 µm particle size, 4.6 mm× 250mm length, Agilent Technologies, USA) maintained at 35°C, coupled to a tunable UV-Vis detector (Agilent Technologies, USA) and 6420 Triple Quadrupole LC/MS System (Agilent Technologies, USA). For ThT and CR analyses, the mobile phases used were A= 10mM Ammonium formate and B= acetonitrile and a gradient of 0% B to 100% B in 15 minutes at the flow rate of 1 mL/min was used to obtain the chromatographs. However, for SRB analyses, the mobile phases used were A= water with 0.1% formic acid and B= acetonitrile and the gradient was: 0-5 min: 10% B, 5-20 min: 10%-100% B, 20-25 min: 100% B and 25-30 min: 10% B with 1 ml/min.

For MS and MS/MS analyses, the same mobile phases and gradient were used as the HPLC methods (described above), but at a slower flow rate of 0.2 ml/min. Electrospray ionization (ESI) source was used in LC-MS (6420 Triple Quadrupole, Agilent Technologies) system in positive polarity. LC-MS operating conditions were as follows: capillary voltage: 4 kV; the nebulizer pressure was 45 psi; drying gas flow was 11 L/min and drying temperature was 325 °C. The mass range monitored was from 100 to 1000 Da. Tandem MS experiments were done using the product ion mode wherein Nitrogen gas was used as a collision gas with different collision energies.

3.4 Phytotoxicity assay

The toxicity of the dyes before and after the degradation were determined by measuring the phytotoxicity effect of these samples on the germination of *Lactuca sativa* by following previously described protocol (Garcia et al., 2009) with slight modifications. Briefly, Whatman filter paper (No. 3, sterilized) was placed on Petri dish and then saturated with 4 ml of the sample avoiding bubble formation. Twenty *L. sativa* seeds were placed on the filter paper with enough space to allow it to grow properly. After that, the petri dishes were incubated for 5 days in a humidified chamber at 22 ± 2 °C to avoid moisture loss. Distilled water was used as negative control and each sample was tested at least in quadruplicates. The effect of untreated and treated dye samples on germination was calculated according to the following equation:

$$\% \text{ germination} = \frac{\text{number of germinated seeds}}{\text{total number of seeds}} \times 100 \quad (2)$$

Moreover, the effect of the original dyes and the degraded dye samples by either SBP or CPO was also examined by measuring the length of the root of the germinated seeds using a ruler and % of root inhibition were calculated as illustrated below:

$$\% \text{ root inhibition} = \frac{\text{mean of control root growth} - \text{mean of sample root growth}}{\text{mean of control root growth}} \times 100 \quad (3)$$

Chapter 4: Results and Discussion

4.1 Degradation optimization of dyes

4.1.1 The requirement for redox mediator (RM)

It is well-known that some organic compounds cannot be readily degraded by peroxidases alone, rather, they need small, diffusible and readily oxidizable molecules called redox mediators which act as “middle-men” in peroxidase-mediated oxidation and degradation of organic compounds (Husain and Husain, 2007; Van der Zee and Cervantes, 2009). Our initial experiments with SBP and Thioflavin T dye (ThT) showed that the ThT could not be degraded by SBP and H₂O₂ alone. However, as can be seen in Figure 5, addition of 1-hydroxybenzotriazole (HOBT) to the reaction mixture caused the ThT dye to start decolorizing very quickly, as judged by the decrease in absorbance maxima. Interestingly, when ThT decolorization was studied using CPO, the reaction proceeded very quickly without any need for redox mediator, in fact, addition of HOBT caused a slight decrease in the reaction rate. We believe this may be because redox mediators themselves can also act as substrates for peroxidases and compete with ThT molecules, thus leading to a lower degradation rate.

In contrast to what was seen for ThT, SRB showed an absolute requirement of HOBT when treated by SBP and CPO. The optimum concentrations of HOBT needed for efficient SRB degradation was found to be 0.05mM and 0.165mM, respectively (Figure 6A and 6B).

Unlike ThT, CR was degraded efficiently by SBP without HOBT, however, it required HOBT when treated by CPO (Figure 7). These results, collectively with previously reported literature review, indicated that redox mediators may not be

required for all enzymatic-mediated degradation reactions. Moreover, its requirement might be influenced by other factors such as the structure of the substrate and the enzyme used (Mendoza et al., 2011; Tilli et al., 2011). This remarkable difference between SBP and CPO clearly highlights the difference between these two peroxidases.

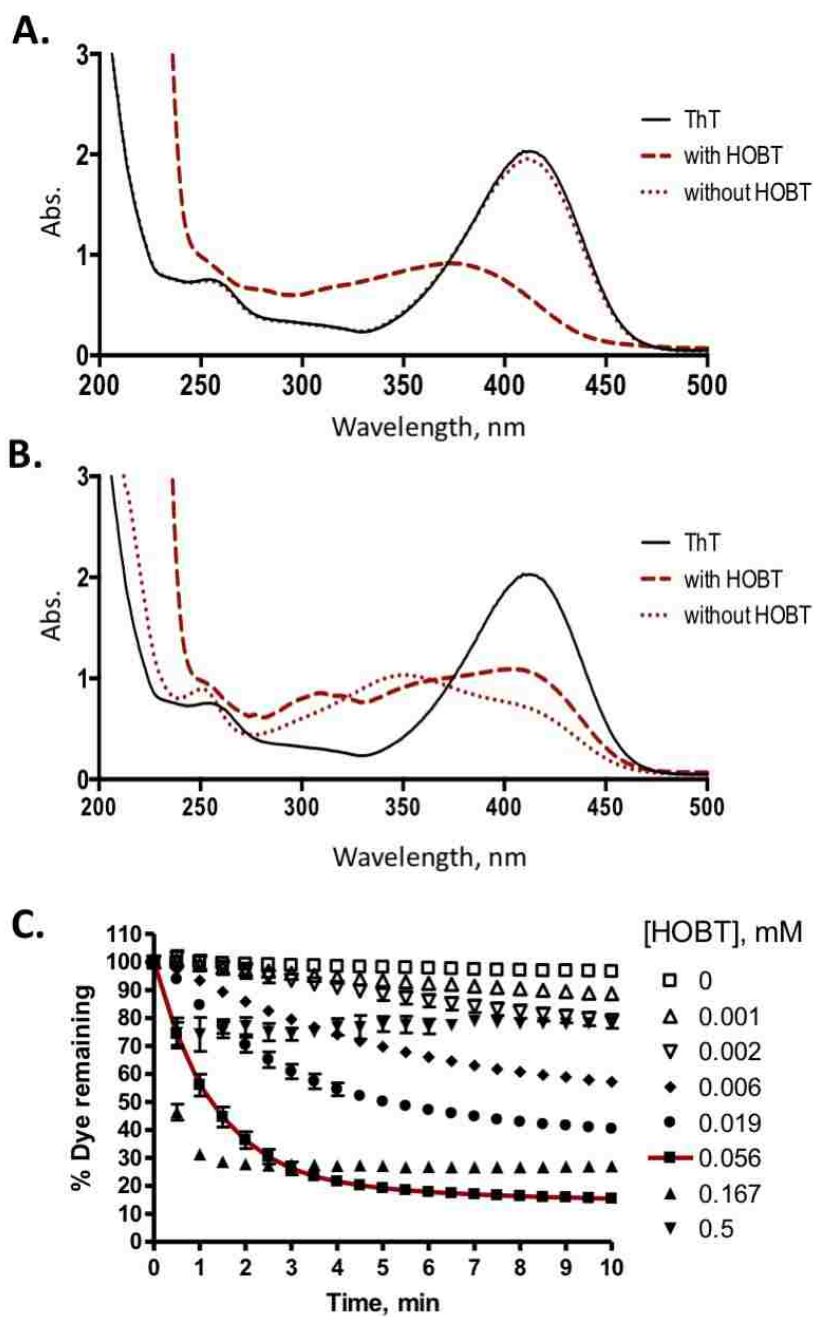


Figure 5: UV/Vis absorbance spectra of ThT. Degraded by (A) SBP and (B) CPO in the present and absent of HOBT. (C) HOBT concentration optimization for ThT degradation by SBP.

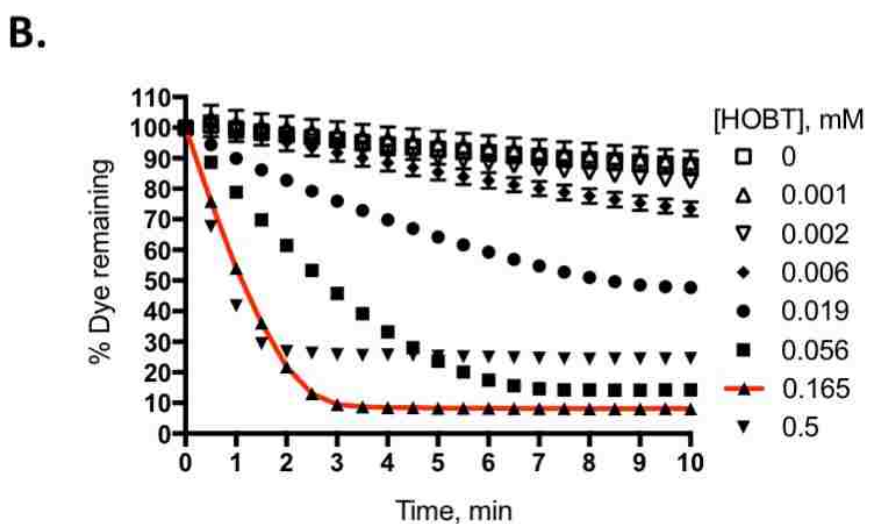
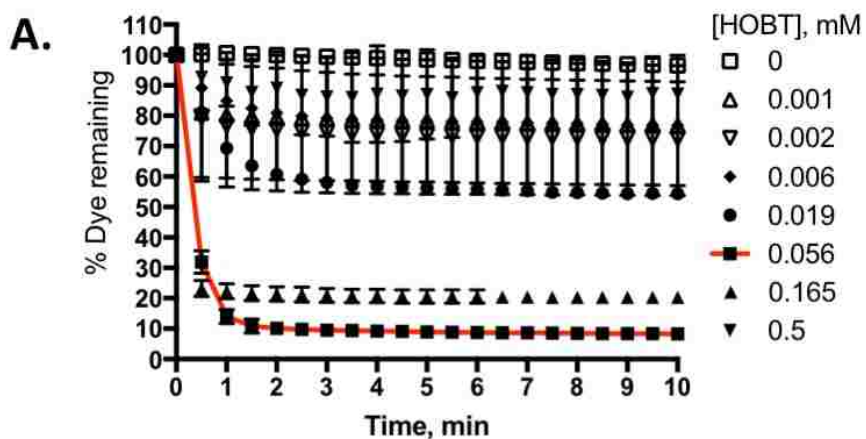


Figure 6: HOBT concentration optimization for SRB. Degraded by (A) SBP and (B) CPO.

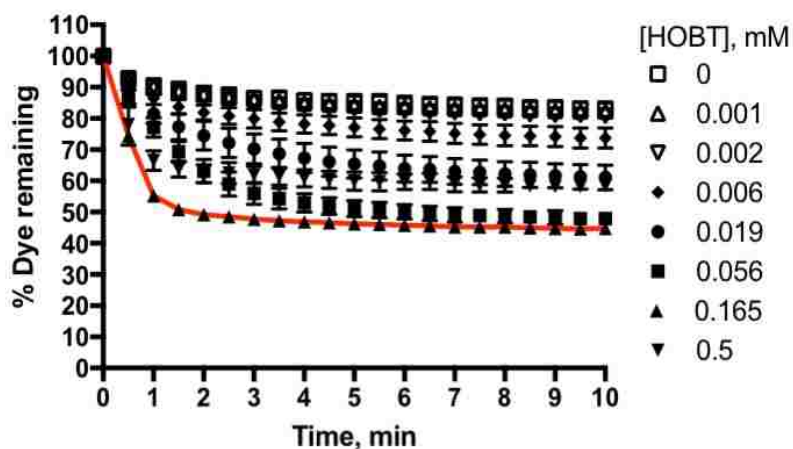


Figure 7: HOBT concentration optimization for CR. Degraded by CPO.

4.1.2 pH effect

Enzyme's activity is strongly influenced by the pH of the solution because changing the pH might affect the amino acids interaction which might lead to change the active site of the enzyme, thus affecting its activity (Hawes, 1991). SBP and CPO mediated dyes degradation were examined at pH range from 2-9, while keeping the other parameters constant. Figure 8 showed the pH profile of ThT, SRB and CR when degraded by SBP and CPO. Both enzymes were active in the acidic conditions, while they were totally inactive in pH range 6-9. In SBP-based treatment, there were no significant differences in the decolorization percentages of SRB and ThT at pH values of 2, 3 and 4. However, SRB and ThT showed an optimum degradation by CPO at pH 2 and increase in pH value caused the degradation to decrease dramatically until it reached almost 10% degradation at pH 4. Interestingly, CR showed a slightly different trend than the other dyes, where it didn't show any noticeable degradation by CPO at pH 2, followed by sharp increase in the degradation at pH 3, suggesting that the structure of the organic pollutant may have an influence on the activity of the enzyme. These results are in agreement with the previously published studies where SBP reached the maximum degradation of Trypan blue, Amido black, Remazol Turquoise Blue G 133 and CP6R at pH 4 (Ali et al., 2013; Kalsoom et al., 2013; Liu et al., 2014; Ashraf, 2013; Zhang et al., 2012), whereas CPO activity was at maximum level in pH range 2-3 when degrading Alizarin Red, Crystal violet, Orange G and Sunset yellow (Ali et al., 2013; Kalsoom et al., 2013; Liu et al., 2014; Ashraf, 2013; Zhang et al., 2012). Collectively, these results indicate that enzymatic treatment is significantly influenced by the pH of the reaction mixture, and SBP and CPO have different optimum pH which are pH 4 and pH 2-3, respectively.

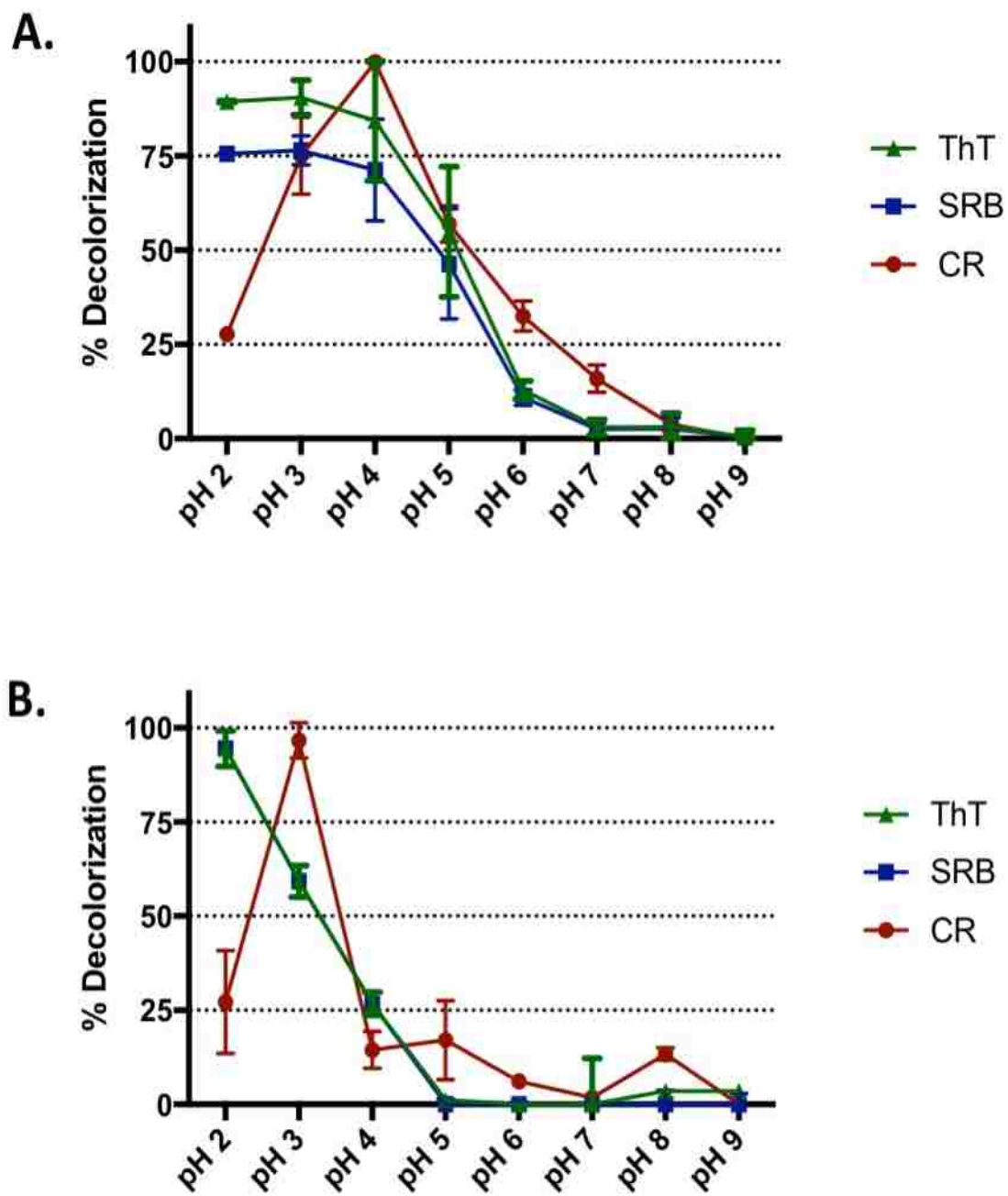


Figure 8: The effect of pH. pH profile of ThT, SRB and CR degradation by (A) SBP and (B) CPO.

4.1.3 Enzyme concentrations

Enzyme concentration is another factor that play a significant role in enzymatic-based degradation. Also, it's important to find the least enzyme concentration that is able to degrade the organic pollutant efficiently in order to make the enzymatic approach economically viable. Generally, the results showed that SBP was somehow more efficient in degrading the three dyes when compared with CPO (Figure 9). For example, at 50 pM enzyme concentration, SBP showed almost efficient degradation of CR, SRB and ThT with 45%, 65% and 75% degradation, respectively, however the degradation percentages were 25%, 10% and 70% using the same concentration of CPO. The optimum concentrations of SBP were 50 pM, 50 pM and 150 pM, while CPO optimum concentration were 50 pM, 440 pM and 50 pM when treating ThT, SRB and CR, respectively. It is worth noting that high concentrations of enzymes caused less efficient degradation in all the three tested compounds. This might be due to the excess enzymes reacting with and consuming all the hydrogen peroxide present in the reaction mixture and which leads to a lower observed degradation of the substrates. Several studies showed that the optimum CPO concentration was found to be 150 nM, 30 nM, 250 nM and 50 nM to efficiently degrade Alizarin Red, Crystal Violat, Orange G and Sunset yellow, respectively (Ali et al., 2013; Kalsoom et al., 2013; Liu et al., 2014; Ashraf, 2013; Zhang et al., 2012).

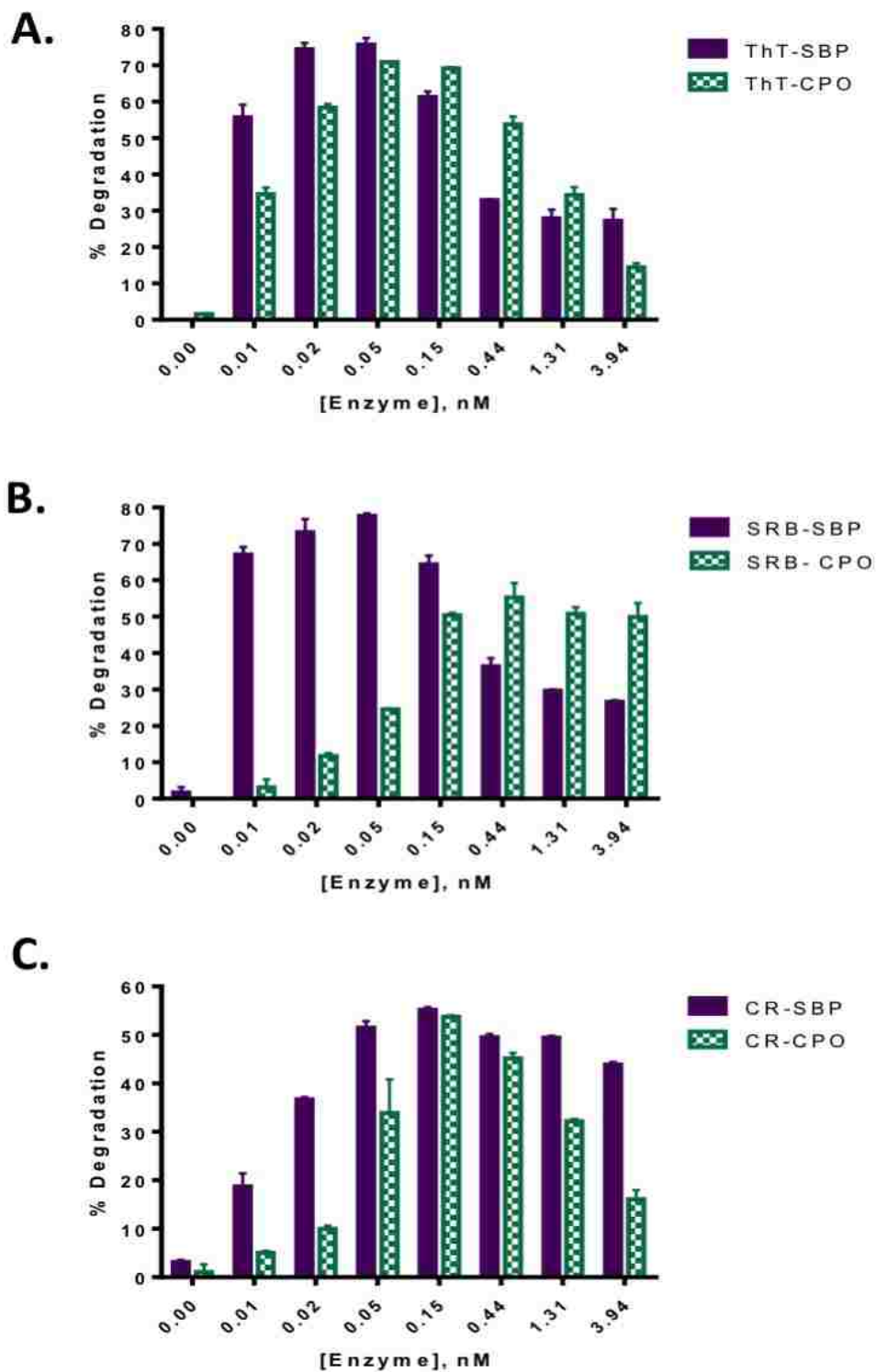
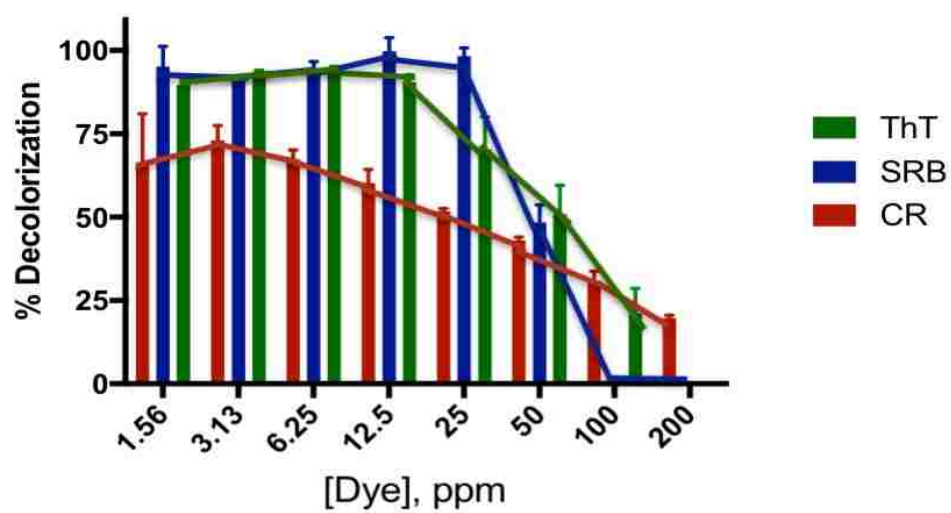


Figure 9: Optimizing enzyme concentration. (A) ThT, (B) SRB and (C) CR decolorization.

4.1.4 Optimization of dye concentration

For enzymatic remediation methods to be useful, it is important to find out the maximum concentration of organic pollutants that can be degraded. Since different pollutants have very different structures, it is quite possible that they may show differential degradation profiles with a given peroxidase. In this study, we examined the abilities of SBP and CPO to degrade increasing concentrations of the three chosen organic pollutants ThT, SFR, and CG). As can be seen in Figure 10, SBP was able to degrade up to 25ppm CR and 10 ppm SRB as opposed to 12ppm CR and 6.25 ppm SRB when CPO was used. Interestingly, CPO was able to effectively decolorize a much higher concentration of ThT where 50 ppm appeared to be the optimum dye concentration, and even 75% decolorization can be achieved when higher concentration (100 ppm ThT) were used. In contrast, the optimum concentration of SBP-mediated degradation of ThT was only 25ppm. Neither CPO nor SBP were able to degrade the three dyes at concentration of 200 ppm and above.

A.



B.

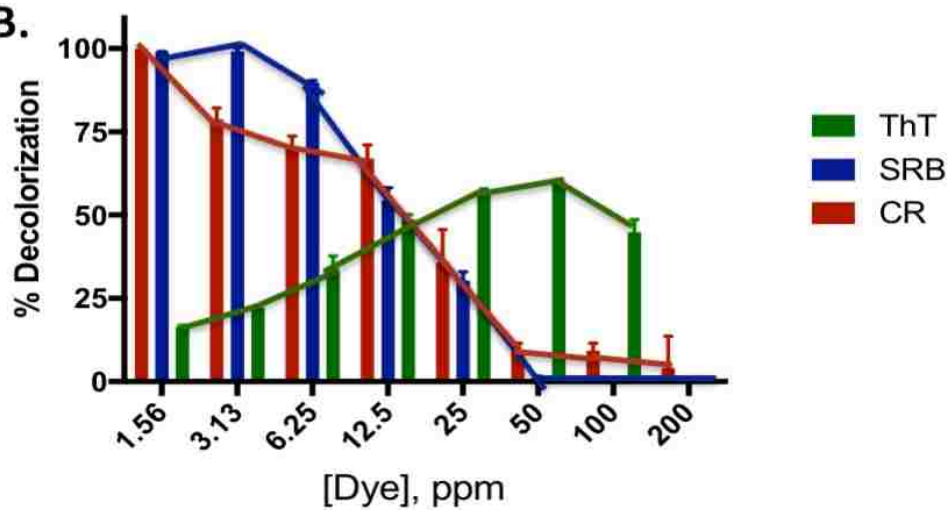


Figure 10: ThT, SRB and CR concentrations optimization. (A) SBP- and (B) CPO-based degradation

4.1.5 H₂O₂ optimization

As illustrated previously in Figure 4, hydrogen peroxide is essential for peroxidase-based degradation since it acts as co-substrate and leads to the generation of the radical form of the enzymes “compound I”. However, excess H₂O₂ amount might lead to irreversible oxidation of these enzymes and their subsequent denaturation and loss of activity. Conversely, low and sub-optimal concentrations of H₂O₂ would restrict the enzymatic activity of these enzymes and lead to poor degradation of organic pollutants. This was clearly seen in case of CPO mediated degradation of the three chosen dyes. There were no observed degradation in the absence of H₂O₂ and then the degradation percentage increased with increasing H₂O₂ concentration until it reached its maximum with 0.2 mM, however, the degradation decreased dramatically with further increase in H₂O₂ concentration to reach almost 15% degradation at 16.5mM H₂O₂. This was also reported by Zhang et al in which 5 mM H₂O₂ exposure to CPO caused the dye decolorization percentage to be reduced to half from almost 75% to 35% (Zhang et al., 2012). Interestingly, SBP enzyme showed a very different profile, and appears to be very resistant to damage by high concentration of H₂O₂. As can be seen from Figure 11, there were no significant changes in the degradation of all the three dyes at H₂O₂ concentration ranging from 0.2mM until 16.5 mM. Similar results have also been reported in previous studies (Ali et al., 2013; Kalsoom et al., 2013; Silva et al., 2013). These results suggest that SBP is a much more stable enzyme when compared to CPO as it can tolerate high concentrations of strong oxidants, such as H₂O₂.

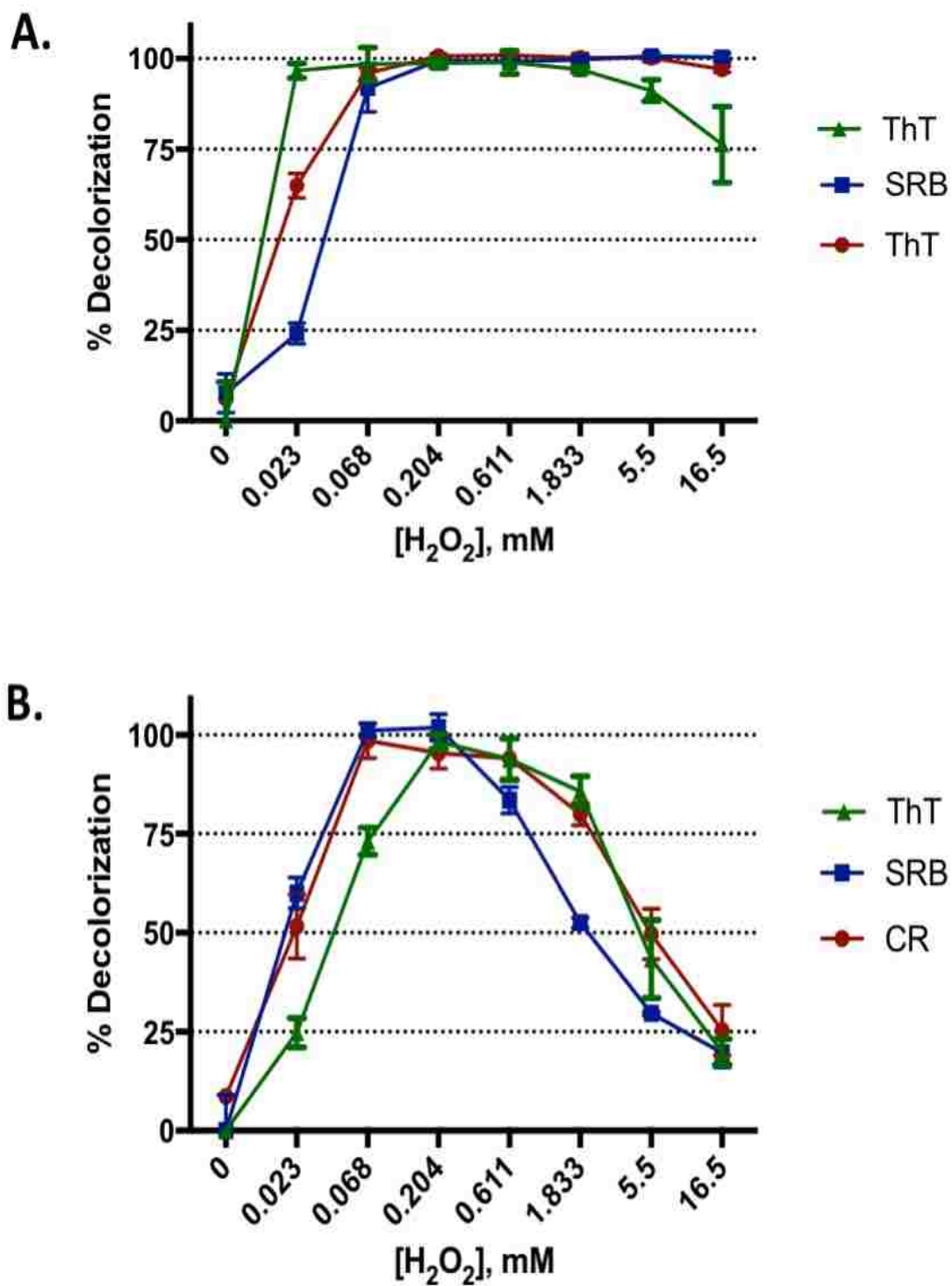


Figure 11: H₂O₂ concentration optimization. The effect of H₂O₂ on ThT, SRB and CR degradation by (A) SBP and (B) CPO

4.1.6 Effect of temperature on dye degradation

It is well known that temperature has a huge effect on enzyme-based reactions, as each enzyme has its optimum operating temperature. Due to thermodynamic factors, increase in temperature would initially increase enzyme activity since it provides more energy to speed up the reaction. However, after the reaction reaches the optimum temperature where the enzyme has maximum activity, increasing in the temperature starts to denature the enzyme and thus changing the active site that will impair substrate to binding and catalysis and thus lead to decreased activity (Shuler and Kargi, 1992). This was clearly seen in CPO-mediated degradation of SRB (Figure 12B), where the degradation increased from 55% until 90% degradation with increasing the temperature from 20°C to 40°C (Figure 12B). However, the degradation of SRB dramatically decreased to approximately 25% when the temperature increased to 60°C. In the case of CPO-mediated degradation of CR (Figure 12B), increasing the temperature from 20°C to 40°C caused also an increase on its degradation from almost 60% until 98%, while there was no significant difference in ThT degradation in same temperature range. Interestingly, CPO didn't show any degradation of these three dyes at temperatures beyond 60°C, suggesting that CPO was inactive and had completely denatured. On the other hand, there were no significant differences on SBP-based degradation of the three dyes when increasing the temperature from 20 °C-80 °C, indicating that SBP is a very robust and stable enzyme and it is not affected by high temperature (Figure 12A). This observation is consistent with previous studies which have shown that SBP is a very thermostable enzyme (McEldoon and Dordick, 1996). Moreover, another study showed that SBP exhibited almost 66% of its enzymatic activity even after heated for 90 minutes at 80 °C (Wright and Nicell, 1999).

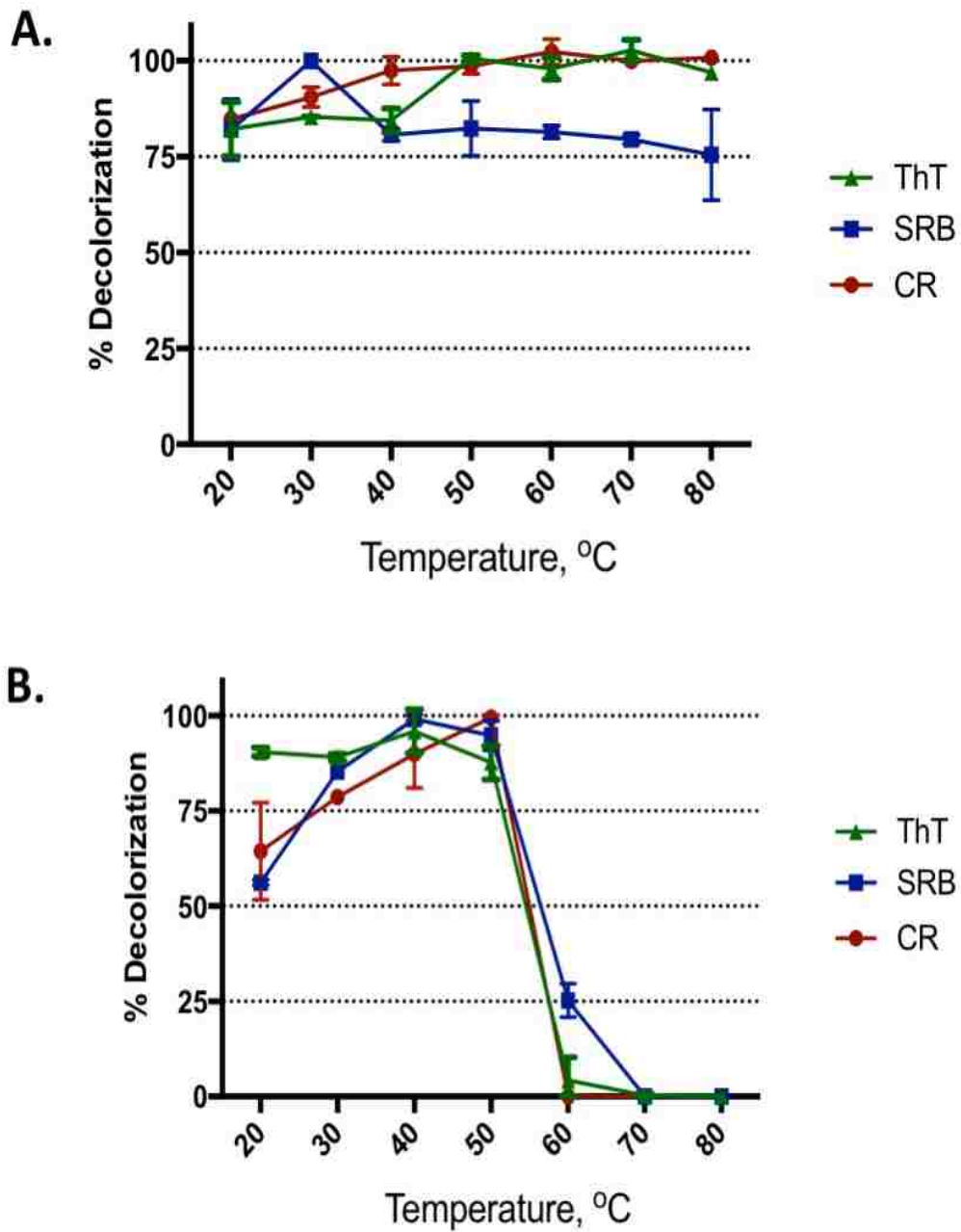


Figure 12: Temperature effect. The effect of various temperatures on degradation of ThT, SRB and CR when treated by (A) SBP or (B) CPO

4.2 Thermal stability

Due to the interesting results obtained from temperature optimization experiments, we further examined the thermal stabilities of SBP and CPO. In this experiment, enzymes were pre-heated for 10 min at 95 °C and then their activities were evaluated (Figure 13). Interestingly, the results were consistent with the temperature effect experiments in which SBP was active even when heated at 95 °C (for ten minutes), while CPO was totally inactive at temperature above 50 °C. This thermal stability experiment showed that SBP is also very different than CPO, which again indicates a major difference between these two peroxidases.

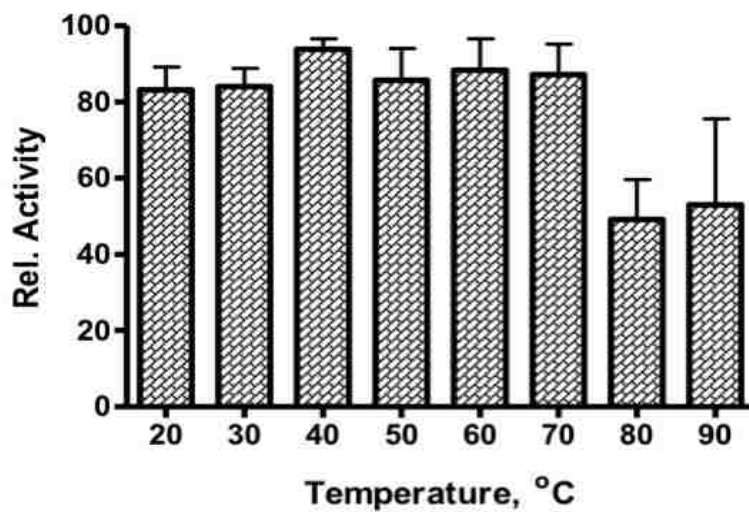
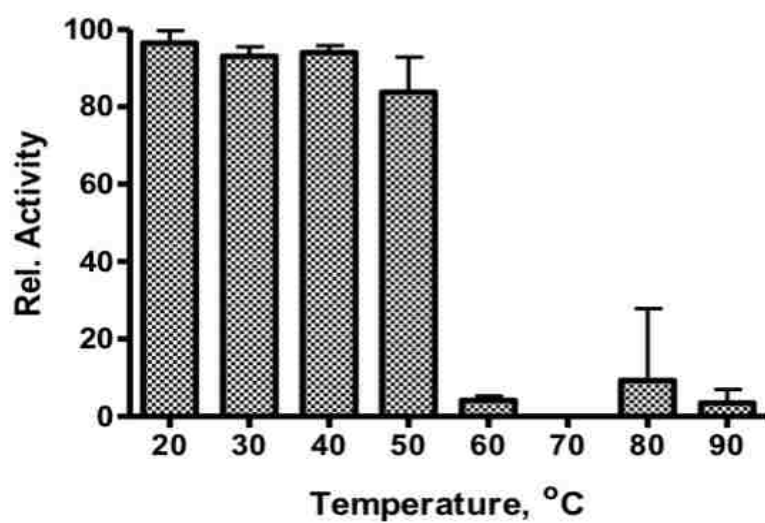
A.**B.**

Figure 13: Thermal stability of (A) SBP and (B) CPO.

4.3 HPLC and LCMS analyses

Although decrease in the absorbance maximum of a dye is indicative of degradation of the compound, we wanted to confirm this using a different technique and also identify some of the intermediates produced during SBP and CPO treatments of the three dyes. Hence, for this part of the study, extensive HPLC and LC-MS-MS analyses were carried out on pure and CPO- and SBP-degraded dyes.

4.3.1 Thioflavin T

Figure 14 shows the HPLC chromatographic profile for ThT alone, ThT treated with SBP together with HOBT and H₂O₂, as well as ThT treated with CPO+ H₂O₂. As can be seen in the HPLC profiles, SBP and CPO treatment of ThT led to the complete elimination of ThT peak, as indicated by the dashed line and produced new peaks, suggesting that ThT was being transformed into new compounds.

Detailed and extensive LC-MS analyses were subsequently carried out to identify these new products and to possibly elucidate their most probable structures. Figure 15 shows the chemical structure of ThT dye (MW = 318 Da), which in LC/MS analysis would show up as a cation (after losing the chloride ion), with a molecular weight of 283 Da. However, it is known that depending on the pH, ThT can exist in a deprotonated form as well, with a molecular weight of 282 Da. In our MS analyses we consistently see a *m/z* of 283 for the ThT cation, which is consistent with a protonated form of the 282 Da ThT species $[M-Cl+H]^+$, as shown in Figure 15B. The inset in Figure 15B also shows the daughter ions observed with fragmentation of the 282 Da form of ThT (*m/z* = 283), which further allowed the confirmation of the structure of ThT.

We were able to confirm the generation of two degradation intermediates of ThT upon treatment by SBP+HOBT+ H₂O₂ with the masses of 268 Da and 128 Da. Interestingly, the 268 Da was observed in only 30% and 50% ThT degradation samples and was completely missing from the 100% ThT degradation samples. Furthermore, a small amount of 128 Da intermediate could be seen forming in the 50% ThT degradation sample, which was abundantly present in the 100% ThT degradation sample. This seems to imply that ThT is initially converted to 268 Da intermediate by the demethylation of any of the four methyl groups, and then this intermediate is further broken down to the 128 Da aliphatic carboxylic acid (3-methylhex-4-enoic acid). Surprisingly, contrary to the SBP-treated samples, analysis of the CPO-treated ThT samples failed to show any lower molecular weight intermediates; rather only one main intermediate with the molecular mass of 316 Da was being generated. Further investigation showed that the 316 Da product was in fact the chlorinated form of ThT. The structures of all detected intermediates were confirmed by MS-MS analyses, the products generated by SBP (Figure 16A and 16B) and the chlorinated ThT produced by CPO (Figure 17). The chlorination of ThT by CPO is consistent with published literature, which shows that CPO can in fact chlorinate organic molecules (Vázquez-Duhalt et al., 2001). However, interestingly, such chlorination of organic pollutants or dyes is not widely published. For example, study by Liu et al, showed that CPO was able to efficiently degrade two different organic dyes into smaller products (Liu et al., 2014). Figure 18 shows the summary of the SBP- and CPO-mediated decolorization of ThT and the structures of the intermediates produced.

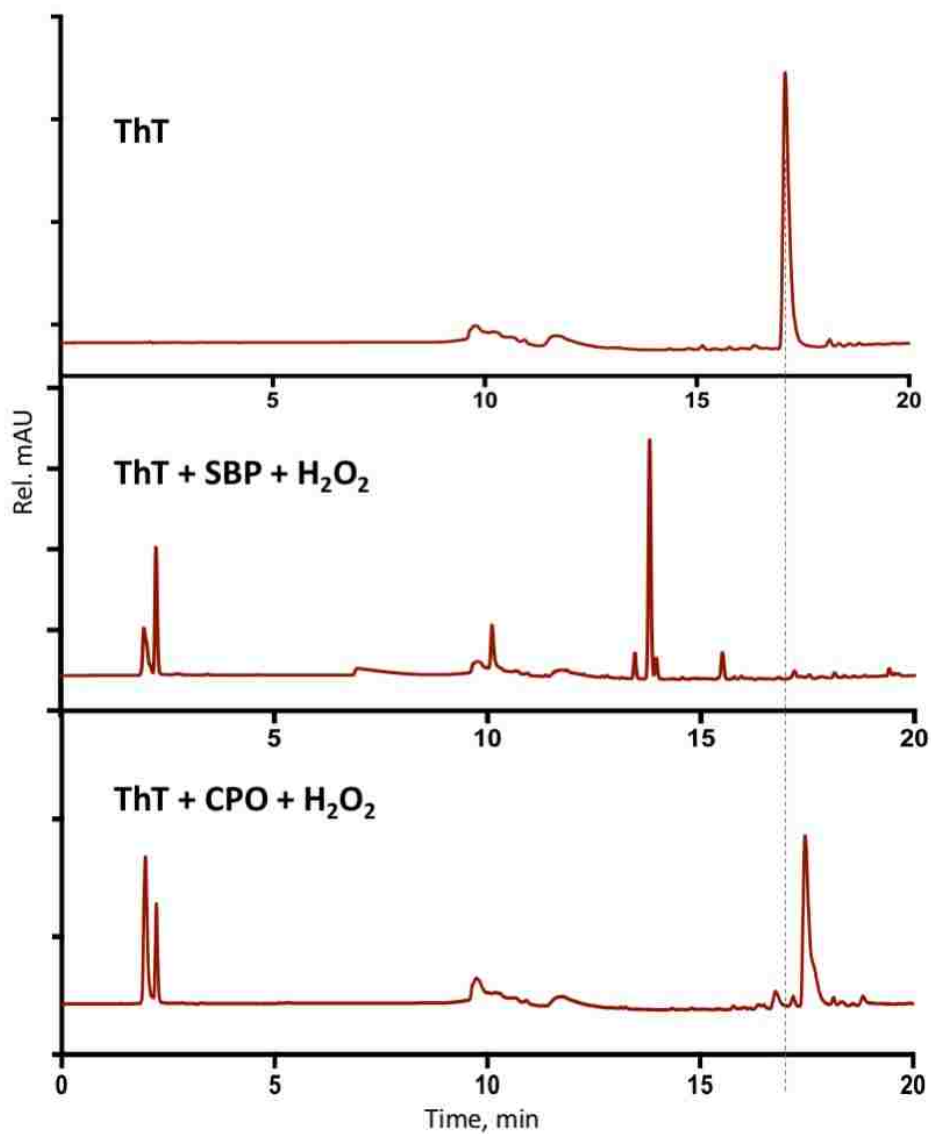


Figure 14: HPLC analyses of ThT. HPLC chromatograms of ThT degradation by SBP and CPO at their optimum conditions.

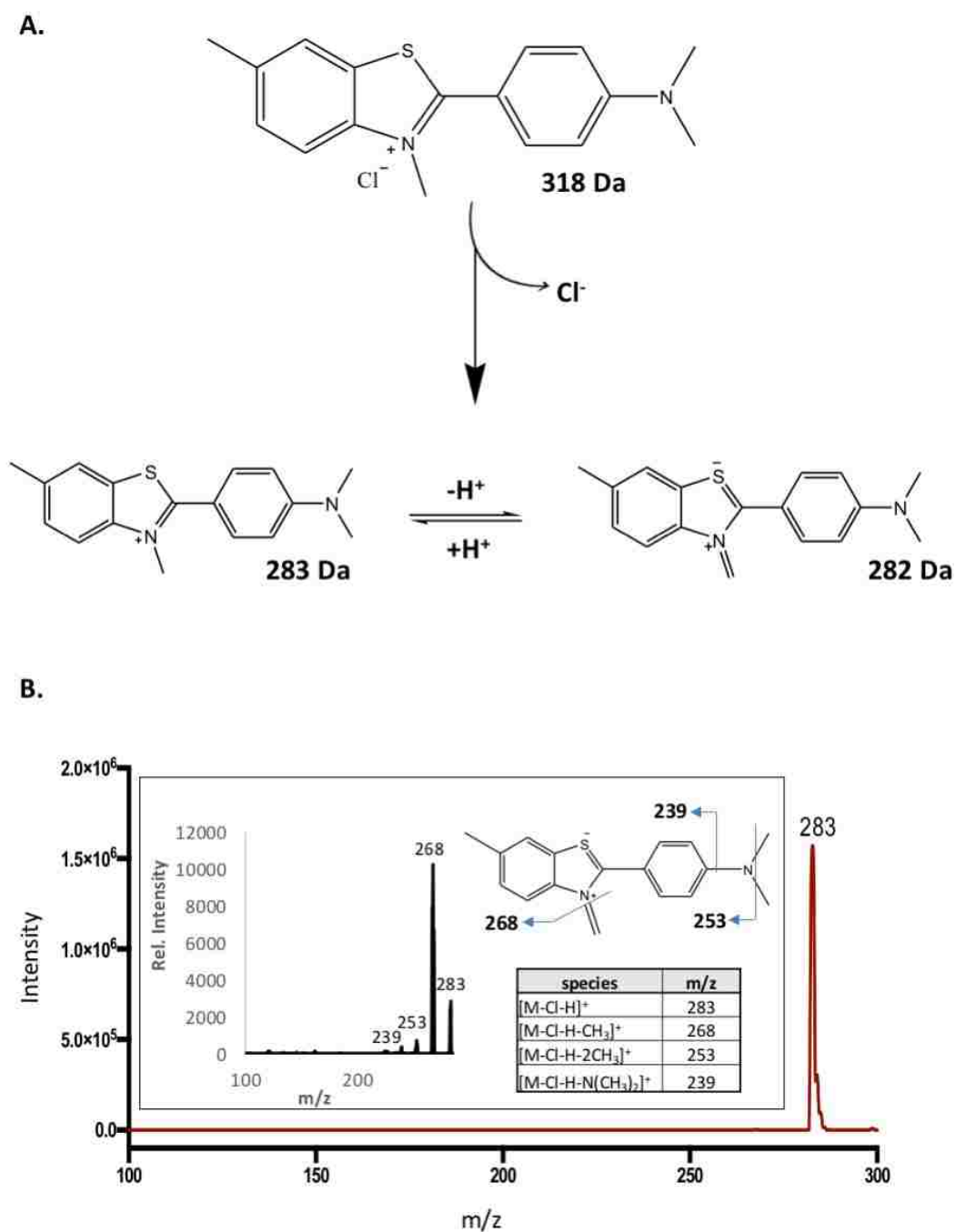


Figure 15: LC-MS analyses of ThT. (A) The chemical structure of ThT detected by LCMS (282 Da). (B) The mass of ThT obtained and its tandem mass analyses (inset).

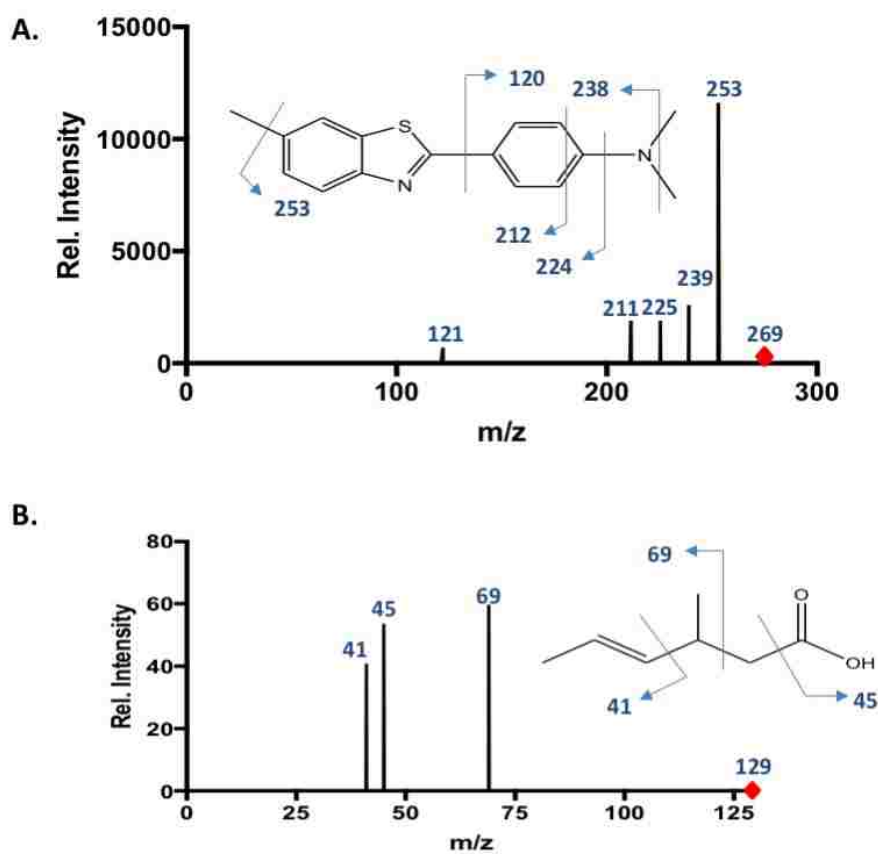


Figure 16: LC-MS analyses of ThT-SBP products. Tandem mass spectrometry fragmentation analyses of the intermediates produced after ThT degradation by SBP. (A) 269 m/z and (B) 129m/z

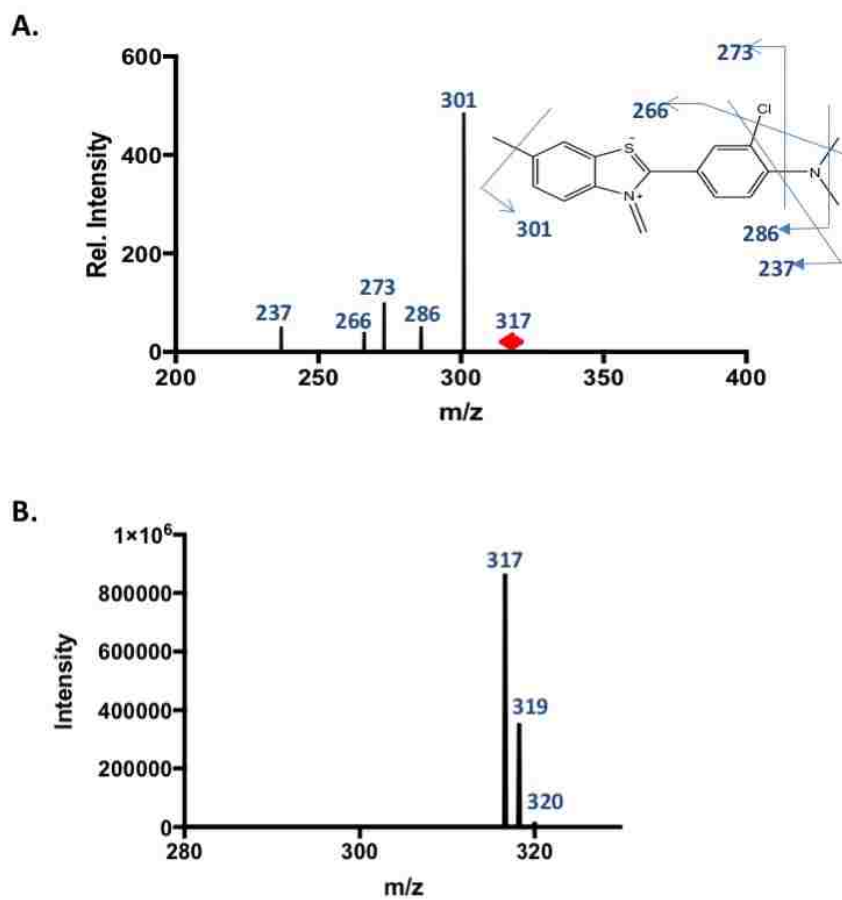


Figure 17: LC-MS analyses of Cl-ThT. Tandem mass analysis for the chlorinated ThT produced by CPO, 317 m/z .

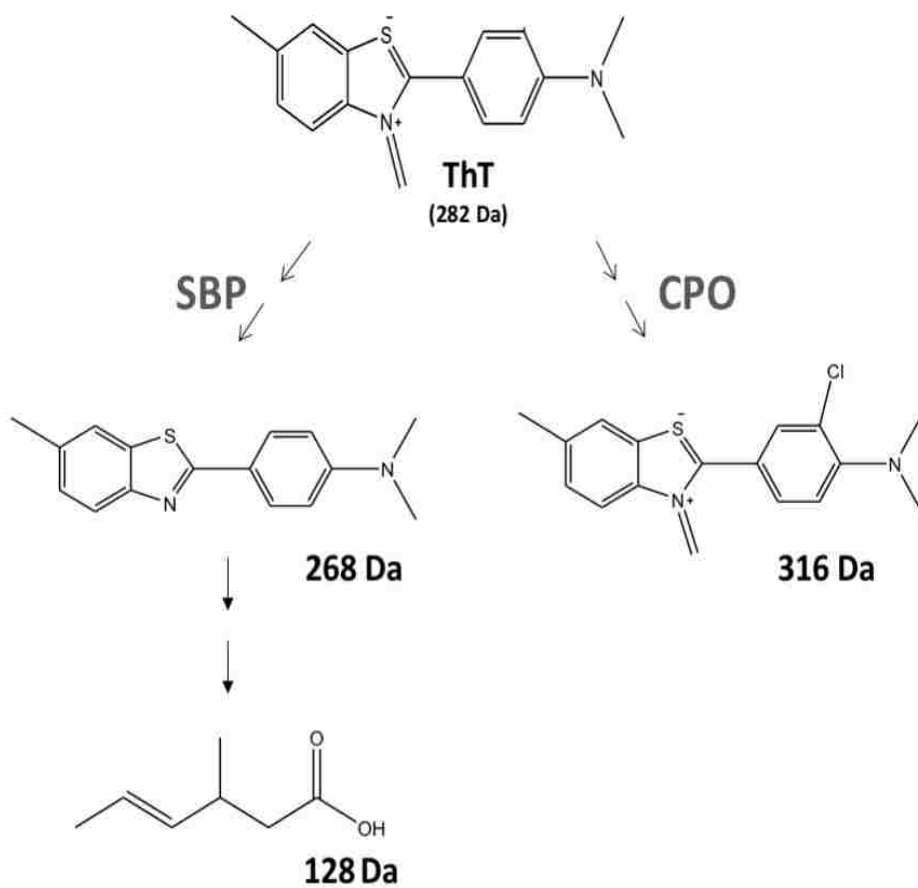


Figure 18: Degradation pathway of ThT degradation by SBP and CPO.

4.3.2 Sulforhodamine B

HPLC and LCMS analyses were also performed to confirm SRB degradation by both SBP and CPO. HPLC analyses of SRB, SRB treated by either SBP or CPO along with their optimum conditions are illustrated in the Figure 19. It is worth noting that SRB chromatogram showed SRB main peak and another small peak indicating a minor impurity. Interestingly, both peaks disappeared completely upon SBP and CPO treatments as noted by the dash-lines. These samples were further investigated using LCMS analyses to identify the degradation products. Figure 20 shows the total ion chromatogram of neat SRB sample, as can be seen from the spectrum, there is a major peak at 559 m/z, which represents the SRB dye. In addition, there is another peak at 531 m/z, which correspond to the de-ethylated form of SRB. Furthermore, MS/MS fragmentation experiments were carried out to confirm the structure of SRB as illustrated in the Figure 21.

As observed with ThT, further analyses of the enzyme-mediated SRB degradation samples showed that SBP and CPO produced different intermediates after degrading SRB. In addition, the structures of most of the intermediates produced after 100% degradation by either SBP or CPO were confirmed using LCMSMS. Interestingly, only two of the SRB degradation products, (123 m/z and 120 m/z), were detected during degradation of SRB by both enzymes (Figure 22 and Figure 23)

SBP-mediated degradation of SRB produced other high mass intermediates: 233.3 m/z and 206 m/z. On the other hands, CPO produced more intermediates which are 272 m/z, 102 m/z and 101 m/z (Figure 23). Unfortunately, the structures of 233.3 m/z and 272 m/z could not be confirmed due to the low molecular weight species produced during fragmentation. Based on the structures of the intermediates, the

degradation pathway of SRB degradation by SBP and CPO is illustrated in the Figure 24. In close agreement with our results, other researchers working on the photocatalytic degradation of Rhodamine B (Luan et al., 2011; Natarajan et al., 2011; Ravi Dhas et al., 2015) as well as those working on Triphenylmethane dyes, such as Crystal Violat (Liu et al., 2014), Malachite Green (Bibi et al., 2011) and Basic Violet 3 (Nouren and Bhatti, 2015) also have reported similar N-demethylation, desulfonation, as well as chromophoric ring openings.

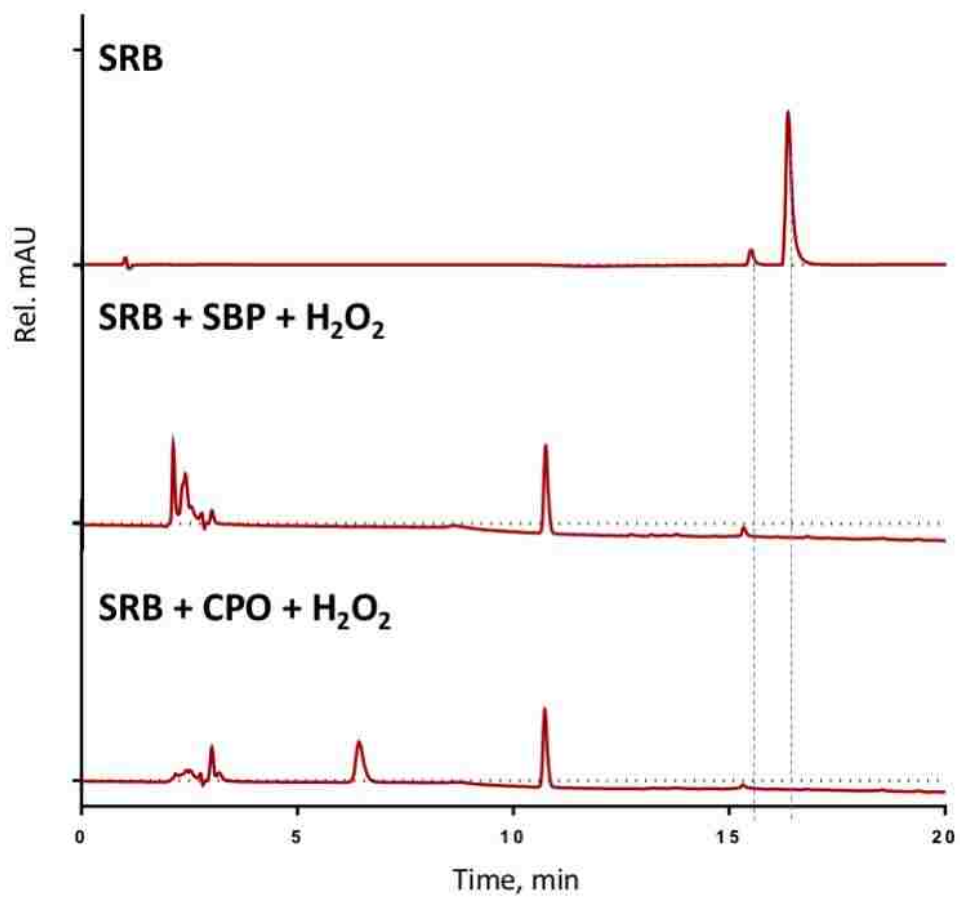


Figure 19: UV-Vis chromatograms of SRB. Degradated by SBP and CPO.

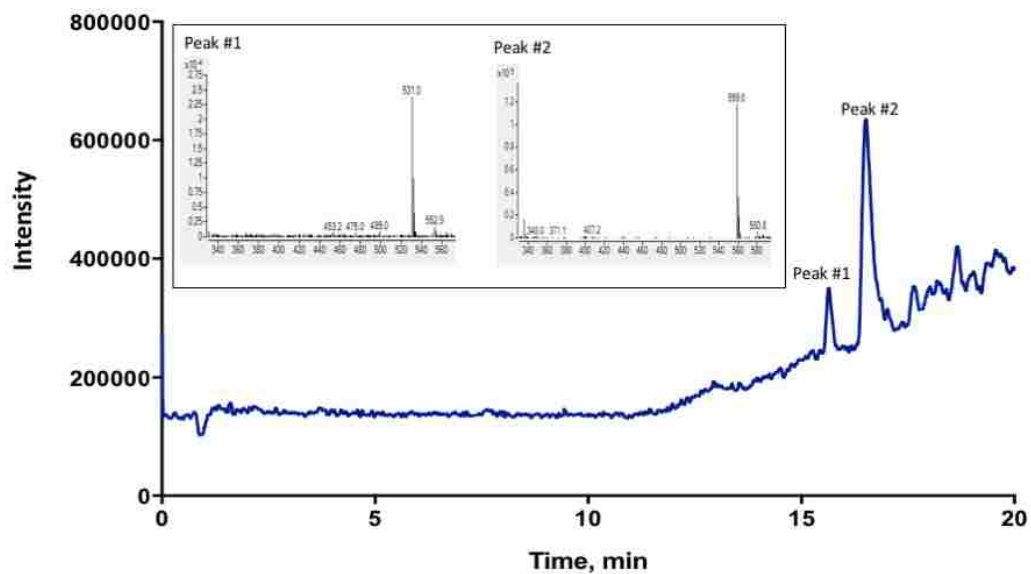


Figure 20: Total ion chromatogram (TIC) of SRB. The insets show the m/z for the SRB peak (peak #2) and the minor contaminant (peak #1).

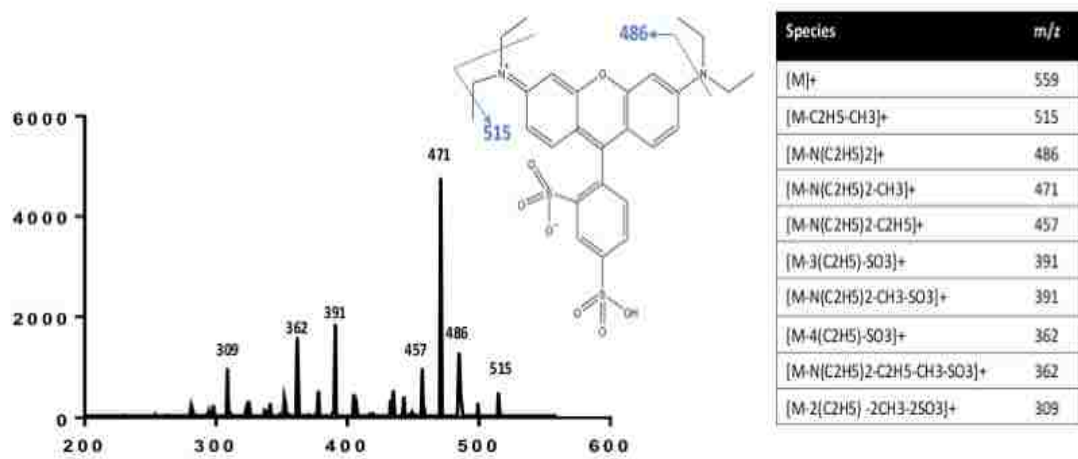


Figure 21: Product ion analysis of SRB at 559 m/z .

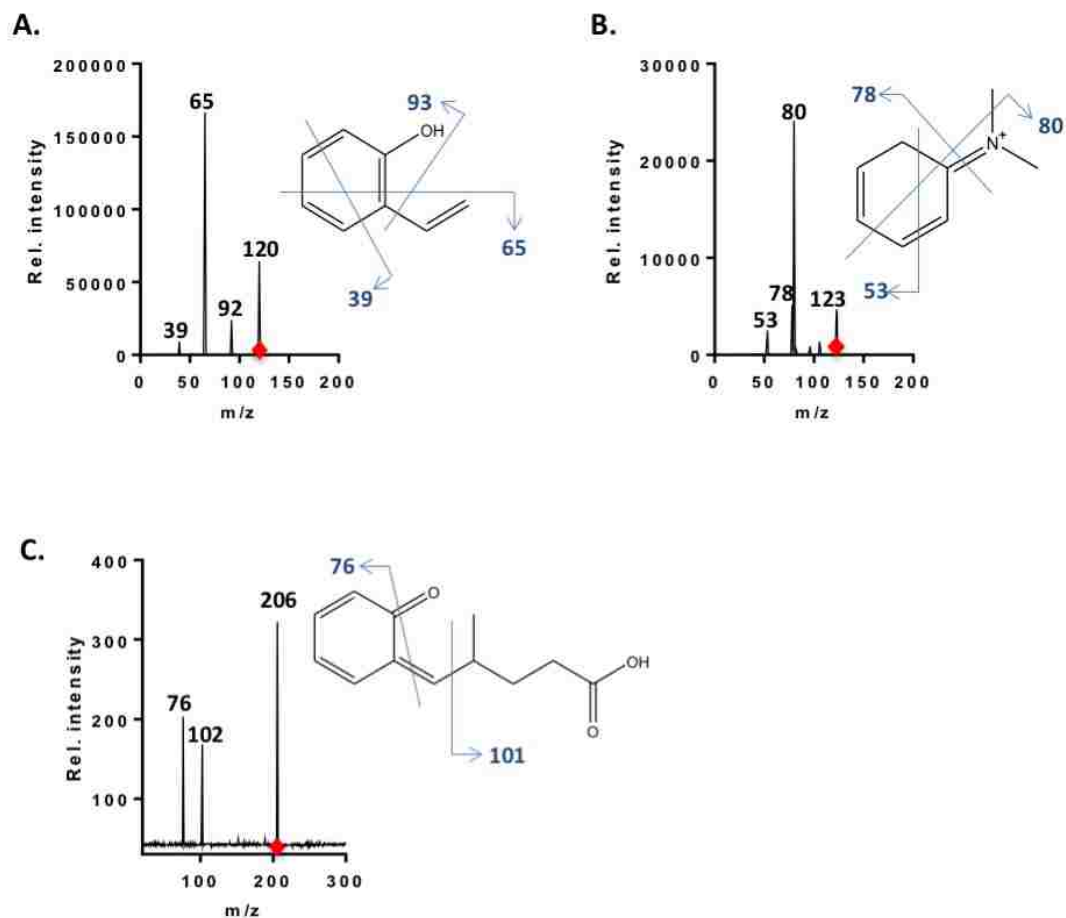


Figure 22: LC-MS analysis of SRB-SBP intermediates. Tandem mass spectrometry fragmentation analyses of intermediates (panel A = intermediate with m/z of 120; panel B = intermediate with m/z of 123; panel C = intermediate with m/z of 206) produced upon SRB treatment by SBP.

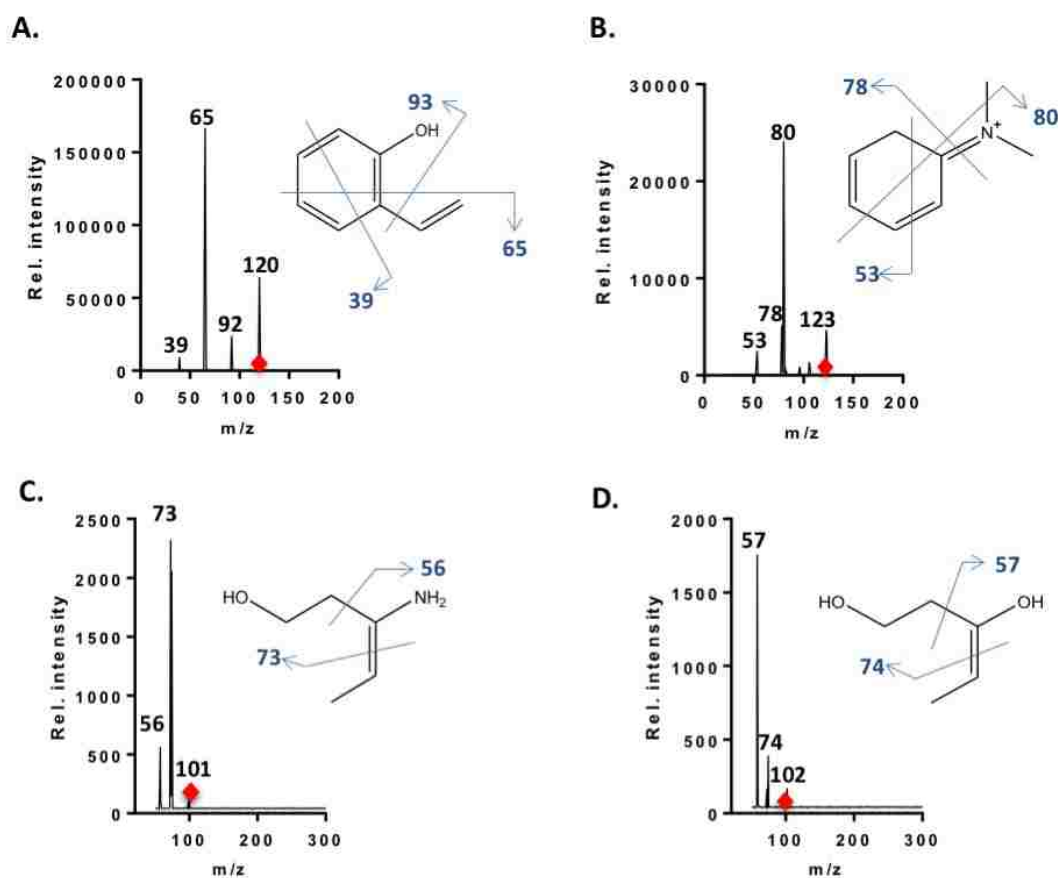


Figure 23: LC-MS analysis of SRB-CPO intermediates. Tandem mass spectrometry fragmentation analyses of intermediates (panel A = intermediate with m/z of 120; panel B = intermediate with m/z of 123; panel C = intermediate with m/z of 101 and panel D = intermediate with m/z of 102) produced upon SRB treatment by CPO.

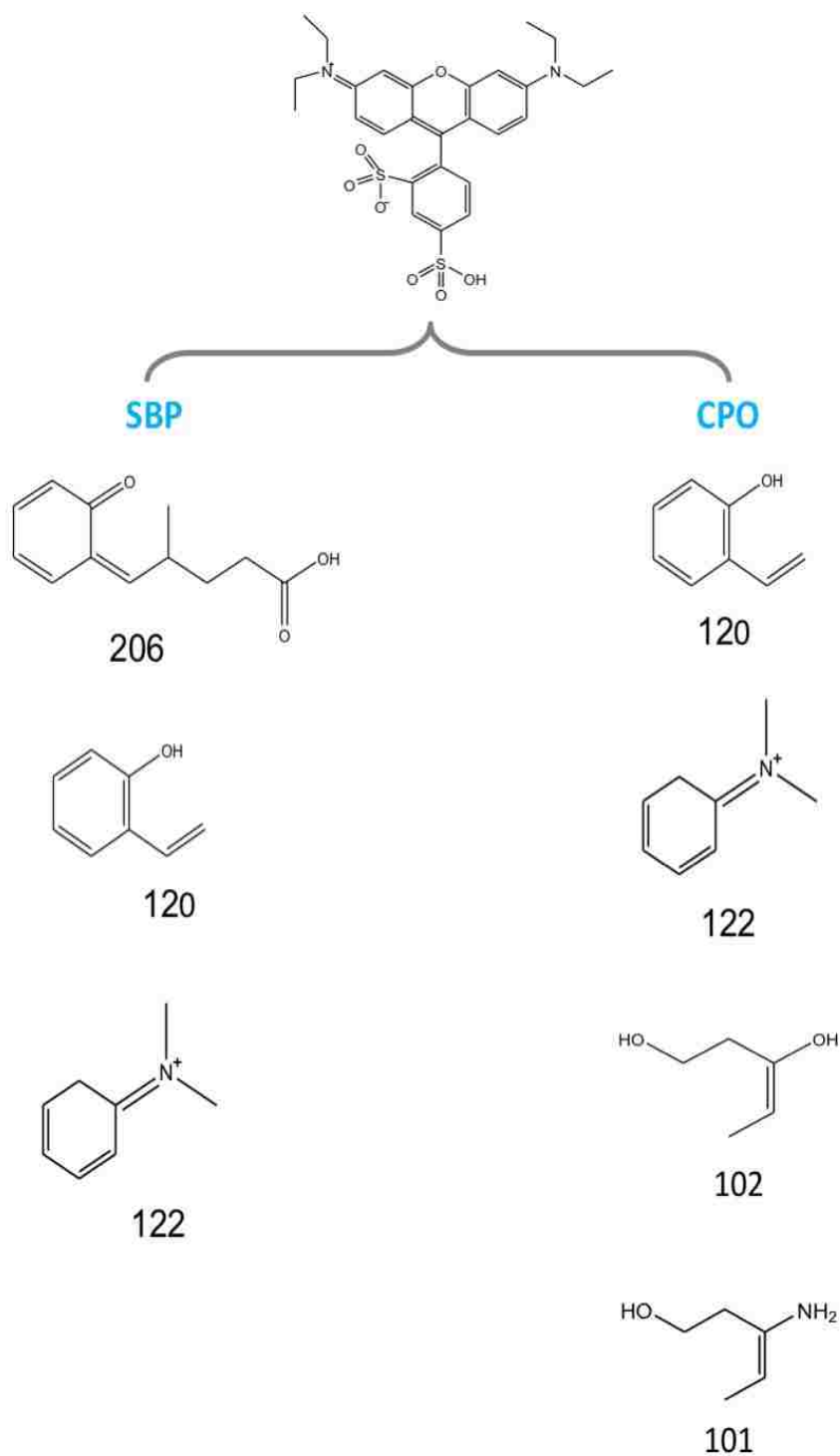


Figure 24: proposed degradation pathway of SBP- and CPO- based degradation of SRB.

4.3.3 Congo Red

The HPLC analyses of untreated CR sample showed a single peak at 16 min and as expected this peak disappeared completely upon the treatment by either SBP or CPO while new peaks were produced (Figure 25).

LCMSMS experiments were also carried out to further investigate the intermediates formation, several samples of CR degradation by SBP and CPO. Figure 26 shows the CR structure confirmation by tandem MS analysis. Figures 27 and 28 show the LC-MS analyses of partially degraded CR samples (different degradation percentages). As can be seen from the figures, as the dye degradation progressed, the emergence of additional peaks were observed, most likely due to the degradation of the parent CR peak into smaller intermediates. Interestingly, most of the intermediates produced appeared to be more hydrophobic as indicated by higher retention time when compared with original CR. Table 4 shows the molecular weights of all the end-products produce after CR degradation by both SBP and CPO. Interestingly, several masses were detected in both SBP- and CPO- mediated degradation such as 570 m/z, 425 m/z, 363 m/z and 267 m/z.

Table 4: Intermediates produced upon CR degradation by SBP and CPO

CR-SBP, m/z	CR-CPO, m/z
608	611
570	570
550	522
425	425
395	371
363	363
340	343
308	312
267	271
240	267
224	258

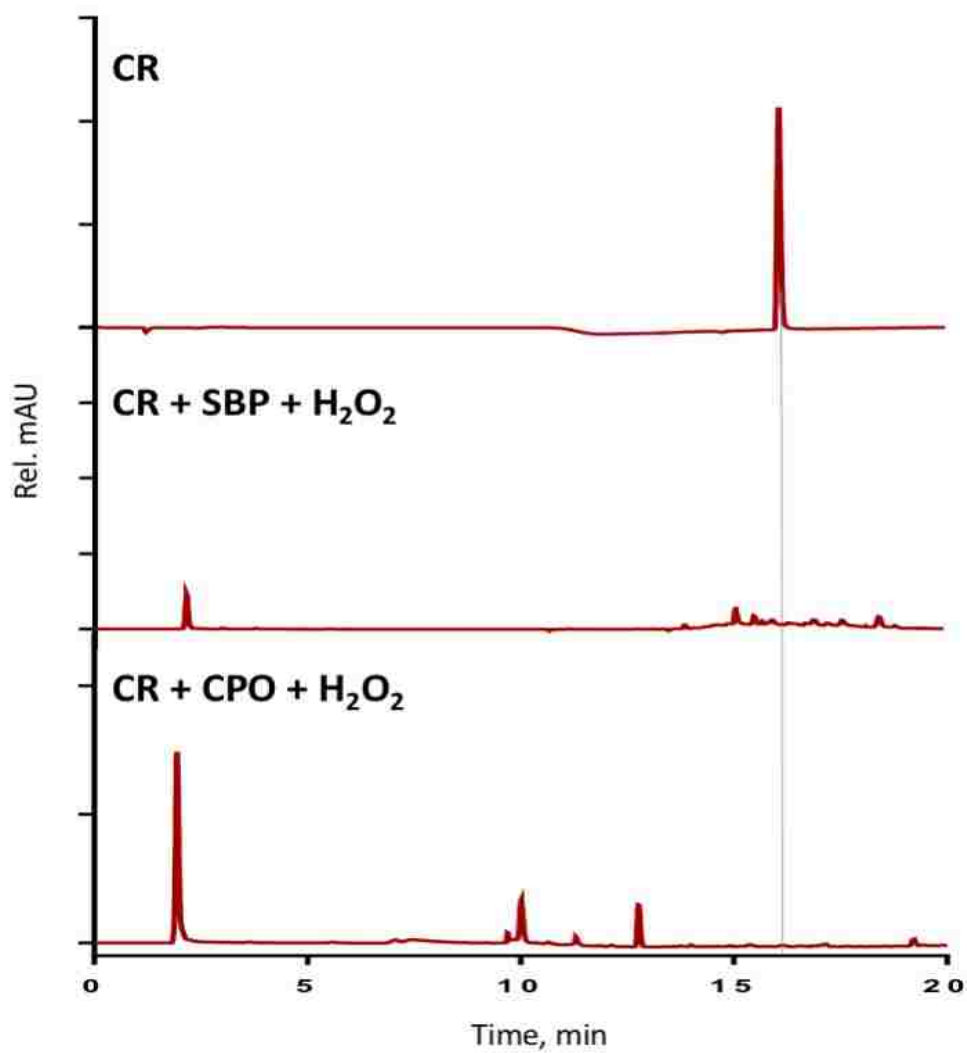


Figure 25: UV-Vis chromatograms of CR. Degraded by SBP and CPO at their optimum conditions

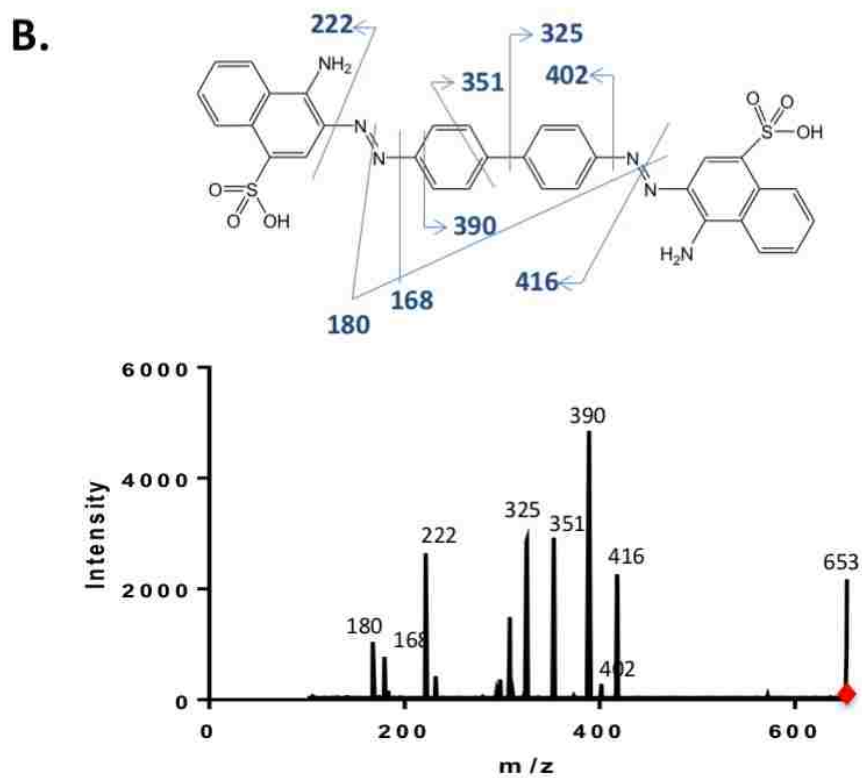
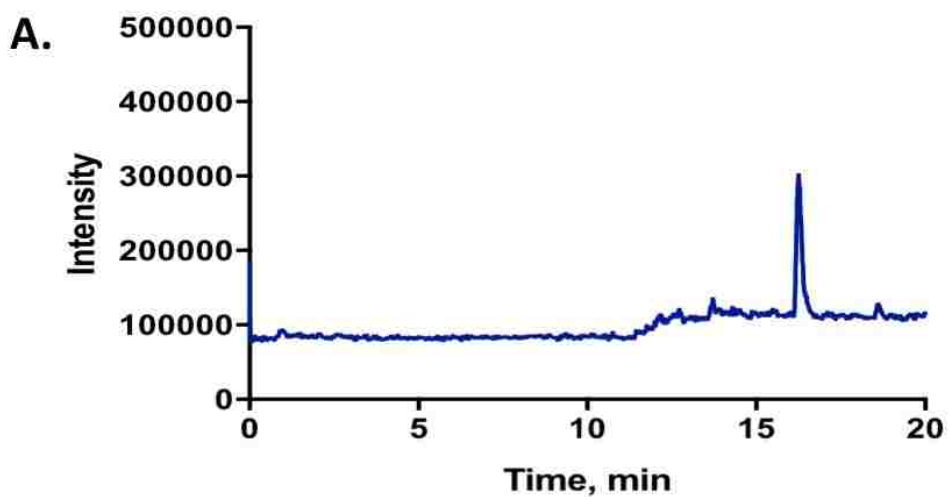


Figure 26: LC-MS analyses of CR. (A) Total ion chromatogram of CR. (B) The fragmentation pattern of SRB obtained by tandem mass analyses.

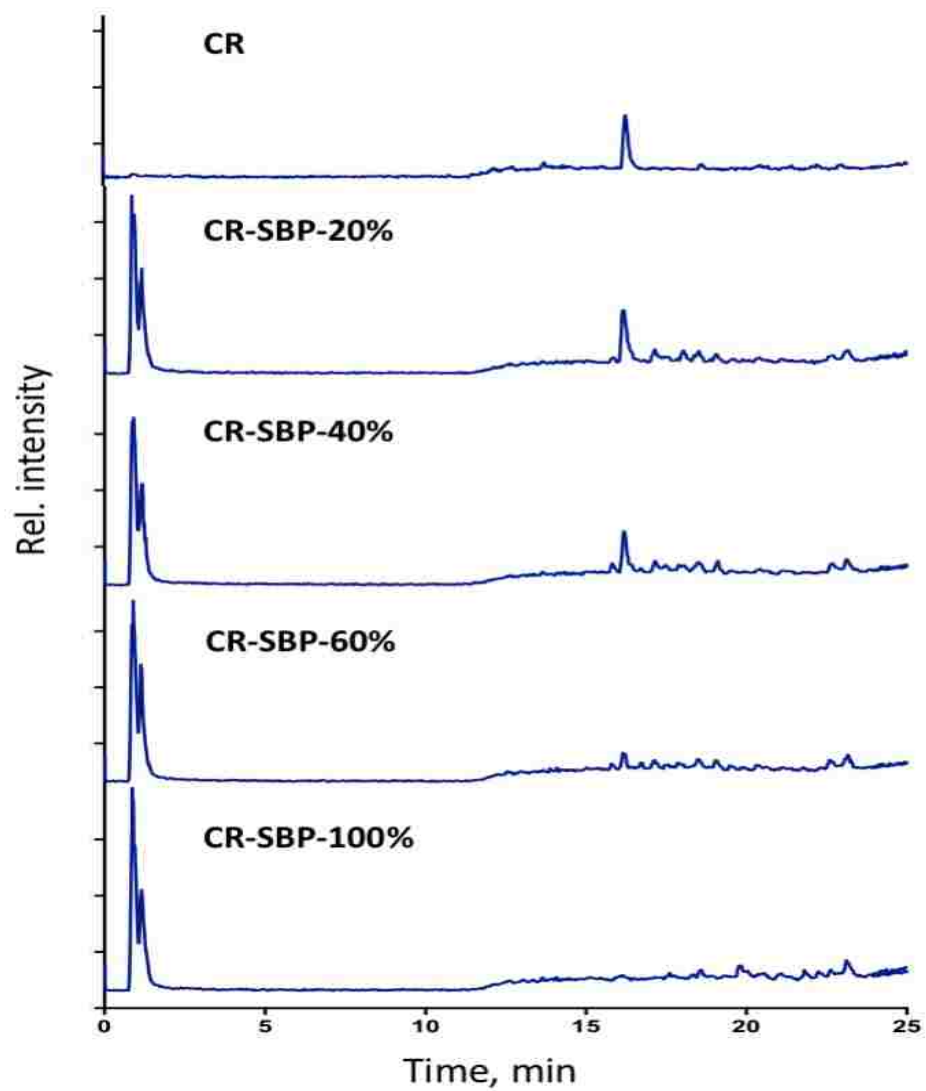


Figure 27: LC-MS chromatogram of CR-SBP with different percentage degradations

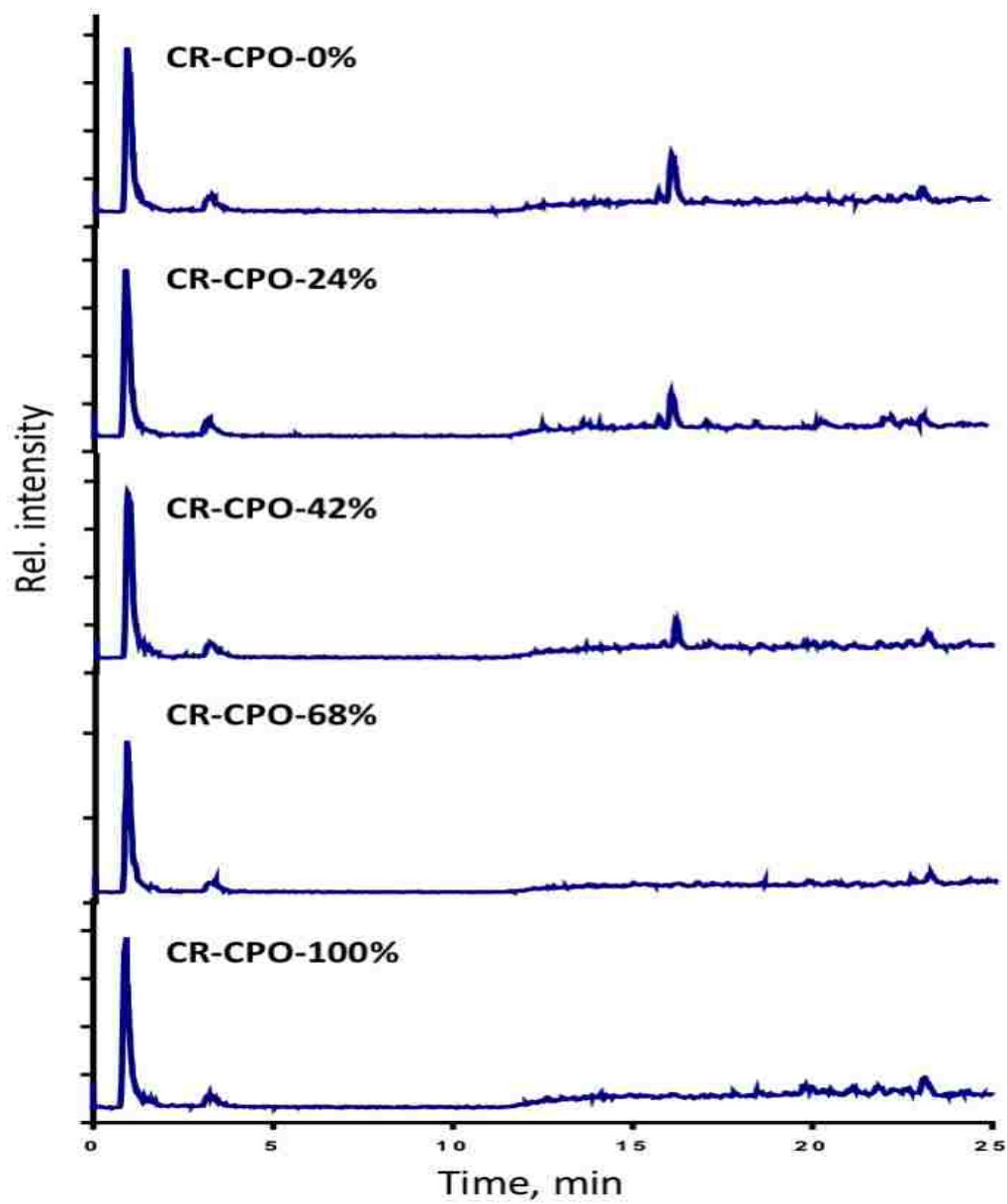


Figure 28: LC-MS chromatogram of CR-CPO with different percentage degradations

4.4 Toxicity test

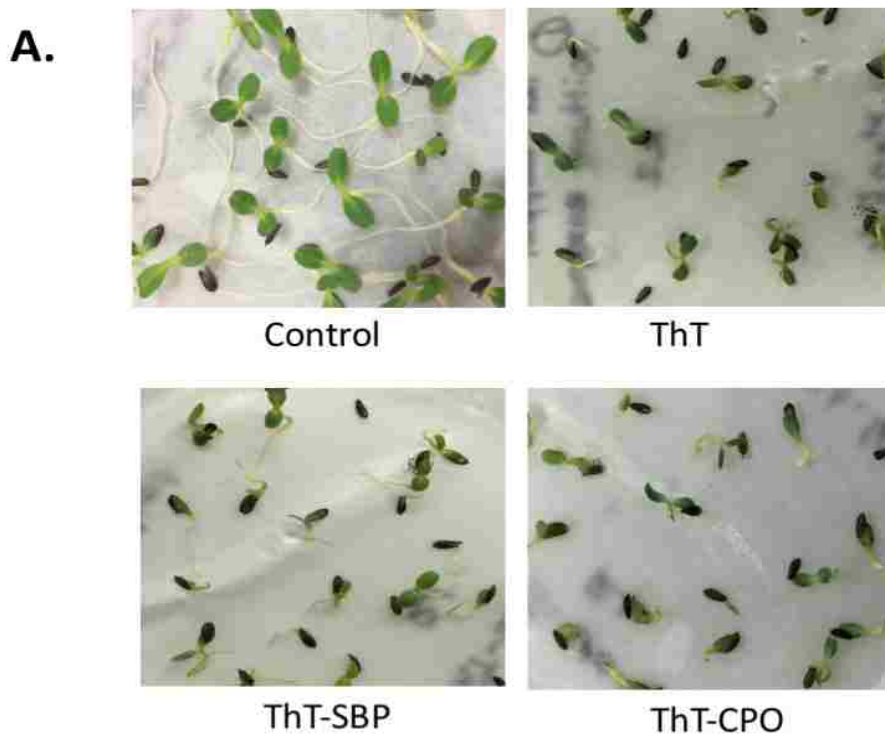
It has been well established that most of the aromatic dyes (and other aromatic pollutants) can be toxic to various organisms [Refs], specifically to aquatic species as well as plants. In addition, one of the hopes of bioremediation is that this process will lead to the decrease or elimination of the toxicities associated with the compounds being treated. In order to test this hypothesis, phytotoxicity studies of untreated dyes and the dyes after the enzymatic treatment were carried out using lettuce seeds (*L. sativa seeds*). Our initial studies to test the toxicity of neat (untreated) dyes showed that only ThT and SRB were toxic to *L. sativa* seeds (prevented their germination), and CR did not show any noticeable toxicity, even at the highest concentration tested (2000 ppm). Therefore, all the subsequent tests were performed using only ThT and SRB dyes.

Table 5 shows the toxicity of the ThT and SRB dyes before and after enzymatic treatments by SBP and CPO, as can be seen from the table, the untreated dyes were clearly toxic to *L. sativa* seeds. Not only these dyes prevented germination at high concentrations, even at low concentrations, they had a dramatic effect on the shoot and root lengths of the seedlings. The toxicity of the dyes before and after the enzymatic treatments was clearly seen by the root length and the % of root inhibition, while there was no significant change on the % germination of the seeds. Interestingly, after the SBP based treatment, the toxicity of both dyes have been reduced and it can be clearly seen on Figures 29, where the root inhibition percentage of SRB-SBP sample was only 31% as opposed to 96% of the untreated SRB. Surprisingly, CPO-based treatment of both dyes failed to eliminate the toxicity of the original dyes Figures 30. Interestingly, other researchers have also reported in some cases, enzymatic degradation of some

dyes resulted in increased toxicity (Kunz et al., 2002; Silva et al., 2012). These results again highlight the dramatic differences between SBP and CPO enzymes and also stresses the importance of toxicological evaluation of the degraded dye solutions after enzymatic treatment. The potential application of peroxidases for color removal and the increase in the products' toxicity reinforce the need for continued research in this field.

Table 5: Toxicity analysis. Mean percent germination and % relative root growth inhibition of lettuce seeds for ThT and SRB before and after the degradation by either SBP or CPO.

	% of germination	Mean root growth	% relative root growth inhibition
water	99	2.36125	-
ThT	90	0.34125	86
ThT-SBP	91	0.67	72
ThT-CPO	93	0.2925	88
SRB	93	0.09375	96
SRB-SBP	98	1.63625	31
SRB-CPO	96	0.1425	94



B.

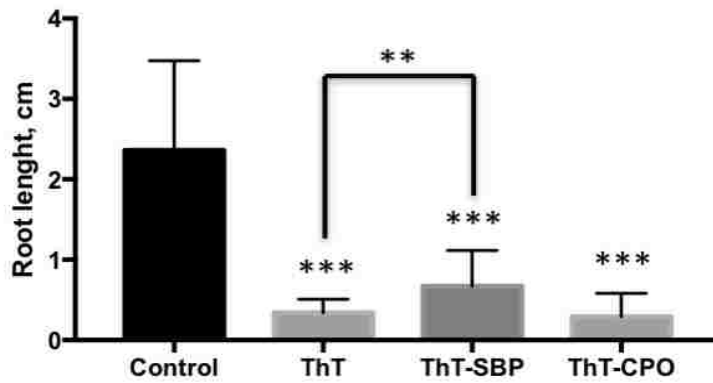


Figure 29: ThT toxicity on *L. stavia* seed. (A) representative sample of *L. stavia* seed treated by ThT (25ppm), ThT decolorized by SBP (ThT-SBP) and ThT treated by CPO (ThT-CPO). Statistical analysis was performed using one-way ANOVA (** $p < 0.005$, *** $p < 0.001$).

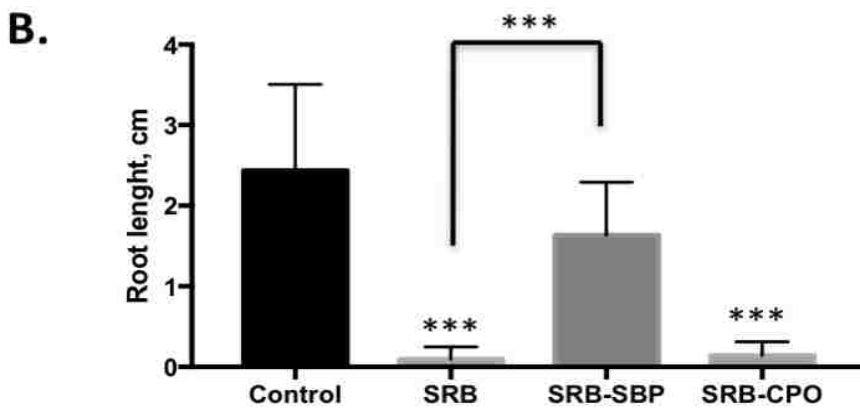


Figure 30: SRB toxicity on *L. stavia* seed. (A) representative sample of *L. stavia* seed treated by SRB (10 ppm), SRB decolorized by SBP (SRB-SBP) and SRB treated by CPO (SRB-CPO). Statistical analysis was performed using one-way ANOVA (***) $p < 0.001$).

Chapter 5: Conclusion

In summary, the data presented here showed that peroxidases are a versatile group of enzymes that have tremendous potential for organic pollutants degradation and wastewater remediation. However, peroxidase-based pollutant degradation can be affected by various factors such as the enzyme used, pH, temperature, dye structure and the presence of redox mediators, and they must be optimized for efficient degradation. Interestingly, we also showed that closely related peroxidases, SBP and CPO behaved very differently and had very different pH optima, thermal stabilities, and H₂O₂ tolerance. Furthermore, LC-MS-MS studies showed that treatment of ThT, SRB and CR by these two enzymes led to very different products being generated. As its clearly seen on ThT degradation, where SBP was able to breakdown the parent molecule into smaller intermediate and eventually to an aliphatic carboxylic acid, CPO treatment produced a higher molecular weight chlorinated ThT. Moreover, the present study highlighted the importance of toxicological evaluation of the degraded dye solutions after enzymatic treatment and showed that enzyme-mediated treatment of aromatic organic pollutants can lead to almost complete elimination of their toxicity. However, one has to be careful, as different enzymes might produce various compounds with different toxicity – some with increased toxicity!

All these results further emphasize the point that commonly used peroxidases may not all be “equal” when used for remediation of organic pollutants. It is clear that additional research is needed to further enhance our understanding of how these versatile and powerful enzymes can be used for efficient environmental and wastewater bioremediation.

Chapter 6: Further Studies

The data presented here shows the power and the limitations of two different peroxidases for organic pollutant degradation. However, it is obvious that additional research is needed to further enhance our understanding of how these versatile and powerful enzymes can be used for efficient environmental and wastewater bioremediation processes.

A few suggestions for further studies in this field are presented below:

1. Combination of both enzymes (SBP & CPO) in order to treat organic pollutant and investigate how they will interact together.
2. Combination of two different approaches such as chemical and enzymatic based degradation in order to efficiently degrade different pollutants.
3. Use different model organisms (such as water fleas, daphnia, etc) to further evaluate the toxicity of the organic pollutants before and after the enzymatic remediation processes.

All of these might enhance our understanding of how these powerful enzymes could be used efficiently for organic pollutants degradation.

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