REMOVAL OF BENZENE FROM
WASTEWATER BY ENZYME-CATALYZED
OXIDATIVE POLYMERIZATION
COMBINED WITH A MODIFIED FENTON
REACTION

Beeta Saha
University of Windsor

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

Recommended Citation
Saha, Beeta, "REMOVAL OF BENZENE FROM WASTEWATER BY ENZYME-CATALYZED OXIDATIVE POLYMERIZATION COMBINED WITH A MODIFIED FENTON REACTION" (2011). Electronic Theses and Dissertations. 408.
https://scholar.uwindsor.ca/etd/408
REMOVAL OF BENZENE FROM WASTEWATER BY
ENZYME-CATALYZED OXIDATIVE POLYMERIZATION
COMBINED WITH A MODIFIED FENTON REACTION

by

BEETA SAHA

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Civil and Environmental Engineering
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada

2011

© 2011 Beeta Saha
Removal of Benzene from Wastewater by Enzyme-Catalyzed Oxidative Polymerization Combined with a Modified Fenton Reaction

by

Beeta Saha

APPROVED BY:

______________________________________________
Dr. Stefan J. Grimberg
Clarkson University

______________________________________________
Dr. Nihar Biswas, Co-Advisor
Department of Civil and Environmental Engineering

______________________________________________
Dr. Keith E. Taylor, Co-Advisor
Department of Chemistry and Biochemistry

______________________________________________
Dr. Paul Henshaw, Department Reader
Department of Civil and Environmental Engineering

______________________________________________
Dr. Rajesh Seth, Department Reader
Department of Civil and Environmental Engineering

______________________________________________
Dr. Andrew Hubberstey, Outside Department Reader
Department of Biological Sciences

______________________________________________
Dr. Michael Crawford, Chair of Defense
Faculty of Graduate Studies

Date: 12th May, 2011
DECLARATION OF ORIGINALITY

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

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

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

A two-step process for the removal of benzene from wastewater, pretreatment by modified Fenton reaction coupled with enzyme-catalyzed polymerization of the resulting phenolic compounds, is presented. Two oxidoreductase enzymes, namely laccase and soybean peroxidase (SBP), were investigated for their capacity to catalyze the oxidative polymerization of the phenolic compounds generated during Fenton pre-treatment.

In the benzene pre-treatment step, the effect of pH, hydrogen peroxide and ferrous iron concentrations and reaction time for the Fenton reaction were studied to maximize the conversion of benzene to phenolic compounds without causing significant mineralization. Under optimum Fenton reaction conditions, conversion of benzene generated a mixture containing phenol, benzenediols (hydroquinone, catechol and resorcinol), biphenyl and benzoquinone. Most of the identified products generated after benzene pre-treatment are priority pollutants themselves. Biphenyl and benzoquinone were outside the scope of enzymatic treatment due to their chemical structure. In order to remove the rest of the Fenton products by the enzymatic process, their individual treatabilities by enzymes were explored.

The effectiveness of removing 1 mM phenol and benzenediols by using a laccase from Trametes villosa, was investigated. Factors of interest were pH, enzyme concentration, effect of polyethylene glycol (PEG), effect of substrate concentration on enzyme demand, and enzyme inactivation over reaction period. Previous studies have demonstrated that SBP could also be used to treat phenol and benzenediols. As phenol and benzenediols can co-exist in wastewater, treatability of a composite wastewater containing an equimolar mixture of phenol and benzenediols was examined as well.
During the enzymatic treatment of phenolic products from benzene, both laccase and SBP were successful in polymerizing the phenolic compounds. Factors of interest for the three-hour enzymatic step were pH, enzyme and hydrogen peroxide concentration. Biphenyl was removed from the solution due to its poor solubility. The benzoquinone generated was removed by employing additives like chitosan or polyethyleneimine (PEI). Alum was used for color removal of the reaction mixture.
DEDICATION

This work is dedicated to

My Proud Parents- Nani Gopal Saha and Debi Saha

My Loving Husband - Siddhartha Banerjee

My Little Sister- Deepa Saha

For being a continuous source of support, assistance and encouragement throughout this journey.
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere thanks to my advisors, Dr. N. Biswas, Dr. K.E. Taylor and Dr. J.K. Bewtra, for their guidance, support, encouragement and constructive criticism throughout the course of research without which it would not have been possible to complete this work. I am grateful for the time they have taken to actively participate in all my endeavours. Words cannot express my appreciation for the opportunities you have given me.

I would like to extend my thanks to Dr. P. Henshaw, Dr. R. Seth, Dr. A. Hubberstey and Dr. Stefan J. Grimberg for taking the time to review my work and participate in the examination committee.

I am also greatly appreciative of the guidance and valuable advice received from Dr. Henshaw and Dr. Seth during my academic years in the University of Windsor.

My thanks go to our laboratory technicians, B. Middleton and U. Lee, for extending their help whenever needed.

My sincere gratitude goes to my colleagues for all the day to day support they have provided. Special thanks to Mohammad Mousa Al-Ansari and Sailesh Kumar Singh for their assistance.
# TABLE OF CONTENTS

ABSTRACT...................................................................................................................... iv
DEDICATION................................................................................................................ vi
ACKNOWLEDGEMENTS............................................................................................ vii
LIST OF TABLES........................................................................................................ xiv
LIST OF FIGURES....................................................................................................... xv

## CHAPTER 1  INTRODUCTION................................................................. 1

1.1 Background ........................................................................................................... 1
1.2 Benzene .................................................................................................................. 2
1.3 Benzene Exposure and Health Effects ................................................................. 3
1.4 Regulations .......................................................................................................... 6
1.5 Conventional Treatment Methods ..................................................................... 10
   1.5.1 Physical Methods ........................................................................................... 10
   1.5.1.1 Adsorption ................................................................................................ 10
   1.5.1.2 Air Stripping ............................................................................................. 11
   1.5.1.3 Reverse Osmosis ...................................................................................... 12
   1.5.2 Chemical Methods ....................................................................................... 13
   1.5.2.1 Ozonation ................................................................................................ 13
   1.5.2.2 Photo-catalytic Oxidation ....................................................................... 14
   1.5.2.3 Electron Beam Radiation ....................................................................... 15
   1.5.2.4 Other Advanced Oxidation Processes ................................................... 15
   1.5.3 Biological Methods ....................................................................................... 16
1.6 Enzymatic Treatment ......................................................................................... 22
1.7 Proposed Treatment Method ............................................................................. 26
1.8 Research Objectives ......................................................................................... 27
1.9 Scope of the Study ............................................................................................. 27

## CHAPTER 2  REVIEW OF THE LITERATURE........................................ 30

2.1 Benzene as a Pollutant ......................................................................................... 30
2.1.1 Physical and Chemical Properties of Benzene (Table 2-1) ......................... 30
2.1.2 Industrial Application of Benzene ................................................................. 32
2.1.3 Sources of Benzene in the Environment ..................................................... 34
2.2 Fenton Reaction Products as Pollutants .......................................................... 35
   2.2.1 Physical and Chemical Properties of Fenton Reaction Products (Table 2-2) ..................................................................................................................... 36
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1.1</td>
<td>Phenol</td>
<td>36</td>
</tr>
<tr>
<td>2.2.1.2</td>
<td>Benzenediols</td>
<td>37</td>
</tr>
<tr>
<td>2.2.1.3</td>
<td>Benzoquinone</td>
<td>37</td>
</tr>
<tr>
<td>2.2.1.4</td>
<td>Biphenyl</td>
<td>37</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Industrial Applications</td>
<td>40</td>
</tr>
<tr>
<td>2.2.2.1</td>
<td>Phenol</td>
<td>40</td>
</tr>
<tr>
<td>2.2.2.2</td>
<td>Benzenediols</td>
<td>41</td>
</tr>
<tr>
<td>2.2.2.3</td>
<td>Benzoquinone</td>
<td>42</td>
</tr>
<tr>
<td>2.2.2.4</td>
<td>Biphenyl</td>
<td>42</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Sources in the Environment</td>
<td>42</td>
</tr>
<tr>
<td>2.2.3.1</td>
<td>Phenol</td>
<td>42</td>
</tr>
<tr>
<td>2.2.3.2</td>
<td>Benzenediols</td>
<td>43</td>
</tr>
<tr>
<td>2.2.3.3</td>
<td>Benzoquinone</td>
<td>43</td>
</tr>
<tr>
<td>2.2.3.4</td>
<td>Biphenyl</td>
<td>44</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Adverse Effects</td>
<td>44</td>
</tr>
<tr>
<td>2.2.4.1</td>
<td>Phenol</td>
<td>44</td>
</tr>
<tr>
<td>2.2.4.2</td>
<td>Benzenediols</td>
<td>45</td>
</tr>
<tr>
<td>2.2.4.3</td>
<td>Benzoquinone</td>
<td>45</td>
</tr>
<tr>
<td>2.2.4.4</td>
<td>Biphenyl</td>
<td>46</td>
</tr>
<tr>
<td>2.2.5</td>
<td>Conventional Treatment Methods</td>
<td>46</td>
</tr>
<tr>
<td>2.3</td>
<td>Fenton Reaction</td>
<td>46</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Background</td>
<td>47</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Mechanism of the Fenton Reaction</td>
<td>50</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Effect of Reaction Parameters on the Fenton Process</td>
<td>56</td>
</tr>
<tr>
<td>2.3.3.1</td>
<td>Effect of pH</td>
<td>56</td>
</tr>
<tr>
<td>2.3.3.2</td>
<td>Effect of Iron</td>
<td>60</td>
</tr>
<tr>
<td>2.3.3.3</td>
<td>Effect of Hydrogen Peroxide</td>
<td>63</td>
</tr>
<tr>
<td>2.3.3.4</td>
<td>Effect of the Structure of Hazardous Compound</td>
<td>65</td>
</tr>
<tr>
<td>2.3.3.5</td>
<td>Influence of Inorganic Ions</td>
<td>67</td>
</tr>
<tr>
<td>2.4</td>
<td>Enzyme-based Wastewater Treatment</td>
<td>68</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Use of Enzymes in Wastewater Treatment</td>
<td>68</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Laccase</td>
<td>70</td>
</tr>
<tr>
<td>2.4.2.1</td>
<td>Active Site of Laccase</td>
<td>71</td>
</tr>
<tr>
<td>2.4.2.2</td>
<td>Laccase Reaction Mechanism</td>
<td>72</td>
</tr>
<tr>
<td>2.4.3</td>
<td>Soybean Peroxidase</td>
<td>75</td>
</tr>
<tr>
<td>2.4.3.1</td>
<td>Active Site of SBP</td>
<td>76</td>
</tr>
<tr>
<td>2.4.3.2</td>
<td>Peroxidase Reaction Mechanism</td>
<td>77</td>
</tr>
<tr>
<td>2.5</td>
<td>Coagulation and Precipitation</td>
<td>80</td>
</tr>
</tbody>
</table>
3.4.5 Process Parameter Optimization for Color Removal Using Alum..........................................................95
3.5 Estimation and Minimization of Errors .................................95

CHAPTER 4

RESULTS AND DISCUSSION ...........................................97

4.1 Process Parameter Optimization for Laccase-Catalyzed Removal of Phenolic Compounds........................................97
4.1.1 Effect of pH on Conversion of Phenol and Benzenediols........98
4.1.2 Effect of Laccase Concentration on the Conversion of Phenol and Benzenediols ........................................102
4.1.3 Effect of PEG in Conversion of Phenol and Benzenediols....103
4.1.4 Time Course of Substrate Removal and Enzyme Inactivation........................................................................107
4.1.5 Effect of Laccase Concentration on a Composite Synthetic Wastewater Containing Phenol and the Benzenediol Mixture.............................................................................................................110
4.1.6 Effect of Substrate Concentration........................................112
4.1.7 Color Removal .........................................................................115

4.2 Process Parameter Optimization for SBP Catalyzed Removal of Phenolic Compounds .........................................................118
4.2.1 Effect of Process Parameters on Conversion of 1mM Phenol and Benzenediols .........................................................118
4.2.2 Effect of Reaction Parameters on a Composite Synthetic Wastewater Containing Phenol and the Benzenediol Mixture.............................................................................................................119
4.2.2.1 Effect of pH ...........................................................................120
4.2.2.2 Effect of Hydrogen Peroxide and SBP Concentration on a Composite Wastewater Containing Phenol and the Benzenediol Mixture.............................................................................................................125
4.2.2.2.1 Single Addition of Hydrogen Peroxide and SBP ...............125
4.2.2.2.2 Step addition of Hydrogen peroxide and SBP ..................131

4.3 Process Parameter Optimization for Removal of Benzoquinone Using Additives.................................................................138
4.3.1 Effect of pH ...........................................................................139
4.3.2 Effect of additive concentration and contact time...............143

4.4 Process Parameter Optimization for Removal of Product Generated from Enzymatic Treatment of Hydroquinone by Using Additives...........................................................................149
4.4.1 Optimum pH ...........................................................................150
4.4.2 Minimum Polyamine Concentration and Contact Time to Achieve ≥95% Product Removal.................................151
4.4.3 TOC Analysis .................................................................................................................. 154
4.5 Process Parameter Optimization for Fenton Reaction on Benzene (Single Step Reactant Addition) .................................................................................................................. 157
  4.5.1 Optimum pH for Benzene Conversion .......................................................................... 158
  4.5.2 Optimum Fe$^{+2}$ Concentration for Benzene Conversion ........................................ 163
  4.5.3 Optimum H$_2$O$_2$ concentration for Benzene Conversion ........................................ 165
  4.5.4 Optimum Reaction Time for Benzene Conversion ...................................................... 166
  4.5.5 Identification of Reaction Products ............................................................................ 167
  4.5.6 Quantification of Reaction Product ............................................................................ 169
4.6 Effect of Multiple Step of Reactant Addition on the Fenton System ................................. 170
  4.6.1 Gradual Addition of Hydrogen Peroxide in the Fenton Reaction ................................. 170
  4.6.2 Step Addition of Fe$^{+2}$ ............................................................................................. 173
  4.6.3 Step Addition of H$_2$O$_2$ .......................................................................................... 174
  4.6.4 Step Addition of Fe$^{+2}$ and H$_2$O$_2$ ......................................................................... 176
4.7 Process Parameter Optimization for Enzymatic Removal of the Fenton Reaction Products (Two-Stage, Two-Reactor System) .................................................................................. 177
  4.7.1 Optimum Reaction Conditions for Laccase-Catalyzed Treatment ............................. 178
    4.7.1.1 Effect of pH on conversion of phenolic compounds ............................................ 178
    4.7.1.2 Effect of enzyme concentration on the conversion of phenolic compounds .......... 178
  4.7.2 Optimum Reaction Conditions for SBP Catalyzed Treatment .................................... 181
    4.7.2.1 Effect of pH on Conversion of Phenolic Compounds ........................................... 181
    4.7.2.2 Effect of enzyme and hydrogen peroxide concentration on the conversion of phenolic compounds ................................................................. 181
  4.7.3 Removal of Benzoquinone and Biphenyl generated ................................................. 185
4.8 Simultaneous Fenton and Enzymatic Reaction (Single Batch Reactor) ............................. 187
  4.8.1 Simultaneous Fenton and Enzymatic Reaction Using Laccase ................................... 189
  4.8.2 Simultaneous Fenton and Enzymatic Reaction Using SBP ....................................... 194

CHAPTER 5

SUMMARY OF RESULTS ........................................................................................................ 205

  5.1 Process Parameter Optimization for Laccase-Catalyzed Removal of Phenolic Compounds ................................................................................................................................. 205
  5.2 Process Parameter Optimization for SBP-Catalyzed Removal of Phenolic Compounds ..................................................................................................................................... 208
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>Process Parameter Optimization for Removal of Benzoquinone Using Additives</td>
<td>210</td>
</tr>
<tr>
<td>5.4</td>
<td>Process Parameter Optimization for Removal of Product Generated from Enzymatic Treatment of Hydroquinone by Using Additives</td>
<td>211</td>
</tr>
<tr>
<td>5.5</td>
<td>Process Parameter Optimization for Fenton Reaction on Benzene (Single-Step Reactant Addition)</td>
<td>213</td>
</tr>
<tr>
<td>5.6</td>
<td>Effect of Multiple Step of Reactant Addition on the Fenton System</td>
<td>213</td>
</tr>
<tr>
<td>5.7</td>
<td>Process Parameter Optimization for Enzymatic Removal of the Fenton Reaction Products (Two-Stage, Two-Reactor System)</td>
<td>215</td>
</tr>
<tr>
<td>5.8</td>
<td>Simultaneous Fenton and Enzymatic Reaction (Single Batch Reactor)</td>
<td>217</td>
</tr>
</tbody>
</table>

**CHAPTER 6**

CONCLUSIONS ................................................................. 220

**CHAPTER 7**

ENGINEERING SIGNIFICANCE AND RECOMMENDATIONS ................................. 223

REFERENCES ................................................................................. 223

APPENDICES .................................................................................. 238

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase Activity Assay</td>
<td>239</td>
</tr>
<tr>
<td>SBP Activity Assay</td>
<td>241</td>
</tr>
<tr>
<td>HPLC Standard Curves for Aromatic Compounds</td>
<td>244</td>
</tr>
</tbody>
</table>

VITA AUCTORIS .............................................................................. 252
LIST OF TABLES

TABLE 1-1: REGULATIONS AND GUIDELINES APPLICABLE TO BENZENE.............................. 7

TABLE 2-1: PHYSICAL AND CHEMICAL PROPERTIES OF BENZENE.................................. 31

TABLE 4-1: OPTIMUM REACTION CONDITIONS FOR LACCASE CATALYZED REMOVAL OF
PHENOL AND BENZENEDIOLS...................................................................................... 101

TABLE 4-2: OPTIMUM REACTION CONDITIONS FOR SBP CATALYZED REMOVAL OF PHENOL
AND BENZENEDIOLS................................................................................................. 119
LIST OF FIGURES

FIGURE 2-1: MAJOR CHEMICALS AND POLYMERS DERIVED FROM BENZENE ..................... 33

FIGURE 2-2: ACTIVE SITE OF LACCASE ........................................................................ 72

FIGURE 2-3: PROPOSED MECHANISM FOR 4E- REDUCTION OF OXYGEN TO WATER BY MULTI COPPER OXIDASE ...................................................................................................................... 73

FIGURE 2-4: CATALYTIC CYCLE OF LACCASE ................................................................. 74

FIGURE 2-5: STRUCTURE OF THE HEME IN PEROXIDASES ........................................... 76

FIGURE 2-6: PROPOSED MECHANISM FOR PEROXIDASE ENZYME ............................... 78

FIGURE 2-7: STRUCTURE OF CHITOSAN MOLECULE ................................................... 81

FIGURE 2-8: PEI STRUCTURE ............................................................................................ 82

FIGURE 4-1: EFFECT OF pH ON SUBSTRATE CONVERSION IN THE ABSENCE OF ENZYME ...... 99

FIGURE 4-2: EFFECT OF pH ON SUBSTRATE CONVERSION IN THE PRESENCE OF LACCASE ......................................................................................................................................................... 100

FIGURE 4-3: CONVERSION OF 1 mM CATECHOL IN THE PRESENCE AND ABSENCE OF 200 mg/L OF PEG .......................................................................................................................... 104

FIGURE 4-4: CONVERSION OF 1 mM RESORCINOL IN THE PRESENCE AND ABSENCE OF 200 mg/L OF PEG .......................................................................................................................... 105

FIGURE 4-5: CONVERSION OF 1 mM HYDROQUINONE IN THE PRESENCE AND ABSENCE OF 200 mg/L OF PEG .......................................................................................................................... 106

FIGURE 4-6: TIME COURSE OF SUBSTRATE REMOVAL .................................................. 108

FIGURE 4-7: LACCASE ACTIVITY IN BATCH REACTORS OVER TIME ............................... 109

FIGURE 4-8: EFFECT OF LACCASE CONCENTRATION ON SUBSTRATE CONVERSION IN COMPOSITE SYNTHETIC WASTEWATER ..................................................................................... 111
FIGURE 4-9: EFFECT OF PHENOL CONCENTRATION ON ENZYME REQUIREMENT .................. 113
FIGURE 4-10: EFFECT OF RESORCINOL AND CATECHOL CONCENTRATIONS ON ENZYME REQUIREMENT .................................................................................................................. 114
FIGURE 4-11: EFFECT OF HYDROQUINONE CONCENTRATION ON ENZYME REQUIREMENT ................................................................................................................................. 115
FIGURE 4-12: COAGULANT CONCENTRATION OPTIMIZATION FOR COLOR REMOVAL ........................................................................................................................................ 117
FIGURE 4-13: EFFECT OF pH ON COMPOSITE WASTEWATER CONTAINING PHENOL AND THE BENZENEDIOL MIXTURE IN PRESENCE OF DIFFERENT CONCENTRATION OF HYDROGEN PEROXIDE .................................................................................................................................................. 121
FIGURE 4-14: EFFECT OF pH ON COMPOSITE WASTEWATER CONTAINING PHENOL AND THE BENZENEDIOL MIXTURE IN PRESENCE OF DIFFERENT CONCENTRATION OF HYDROGEN PEROXIDE AND 0.5 U/mL OF SBP .................................................................................................................................................. 123
FIGURE 4-15: EFFECT OF 8 mM H₂O₂ AND 0.8 U/mL SBP ON COMPOSITE WASTEWATER CONTAINING PHENOL AND THE BENZENEDIOL MIXTURE ............................................................................................................ 126
FIGURE 4-16: EFFECT OF 12 mM H₂O₂ AND 1.2 U/mL SBP ON COMPOSITE WASTEWATER CONTAINING PHENOL AND THE BENZENEDIOL MIXTURE ............................................................................................................ 127
FIGURE 4-17: EFFECT OF 12 mM H₂O₂ AND 1.5 U/mL SBP ON COMPOSITE WASTEWATER CONTAINING PHENOL AND THE BENZENEDIOL MIXTURE ............................................................................................................ 128
FIGURE 4-18: EFFECT OF 15 mM H₂O₂ AND 1.8 U/mL SBP ON COMPOSITE WASTEWATER CONTAINING PHENOL AND THE BENZENEDIOL MIXTURE ............................................................................................................ 129
FIGURE 4-19: EFFECT OF STEP ADDITION OF HYDROGEN PEROXIDE ON COMPOSITE WASTEWATER CONTAINING PHENOL AND THE BENZENEDIOL MIXTURE ............................................................................ 132
Figure 4-20: Effect of step addition of 8 mM H₂O₂ and 0.8 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture........................ 133

Figure 4-21: Effect of step addition of 12 mM H₂O₂ and 1.2 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture...................... 134

Figure 4-22: Effect of step addition of 12 mM H₂O₂ and 1.5 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture............... 135

Figure 4-23: Effect of step addition of 15 mM H₂O₂ and 1.5 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture............... 136

Figure 4-24: Effect of pH on 1mM benzoquinone conversion in the absence of additives........................................................................................................ 139

Figure 4-25: Effect of pH on 1mM benzoquinone conversion in the presence of chitosan solution ................................................................................................ 141

Figure 4-26: Effect of pH on 1mM benzoquinone conversion in the presence of chitosan flakes .................................................................................................. 142

Figure 4-27: Effect of pH on 1mM benzoquinone conversion in the presence of PEI solution ...................................................................................................... 143

Figure 4-28: Chitosan Flakes Concentration Optimization for Benzoquinone Removal ........................................................................................................ 145

Figure 4-29: Color Generated during Chitosan Flakes Concentration Optimization for Benzoquinone Removal.......................................................... 146

Figure 4-30: PEI Optimization for Benzoquinone Removal .................................................. 147

Figure 4-31: Color Generated during PEI Optimization for Benzoquinone Removal ................................................................. 149
FIGURE 4-32: EFFECT OF CHITOSAN FLAKES CONCENTRATION ON PRODUCT REMOVAL FROM LACCASE TREATED HYDROQUINONE SAMPLE ................................................. 151

FIGURE 4-33: EFFECT OF CHITOSAN FLAKES CONCENTRATION ON REACTION MIXTURE COLOR ........................................................................................................ 152

FIGURE 4-34: EFFECT OF PEI CONCENTRATION ON PRODUCT REMOVAL FROM LACCASE TREATED HYDROQUINONE SAMPLE ........................................... 153

FIGURE 4-35: EFFECT OF PEI CONCENTRATION ON REACTION MIXTURE COLOR ......................................................... 154

FIGURE 4-36: TOC ANALYSIS ON LACCASE-TREATED HYDROQUINONE MIXTURE AFTER CHITOSAN TREATMENT ........................................................................ 155

FIGURE 4-37: TOC ANALYSIS ON LACCASE-TREATED HYDROQUINONE MIXTURE AFTER PEI AND ALUM TREATMENT ....................................................................... 157

FIGURE 4-38: EFFECT OF pH ON BENZENE CONVERSION ................................................................................................................................. 158

FIGURE 4-39: SOLUBLE IRON CONCENTRATION IN THE FENTON REACTION MIXTURE AT DIFFERENT pH ......................................................................................... 159

FIGURE 4-40: EFFECT OF pH ON FORMATION OF IDENTIFIABLE AROMATIC PRODUCTS ................................................................. 160

FIGURE 4-41: TOC ANALYSIS ON FENTON REACTION MIXTURE ................................................................................................. 162

FIGURE 4-42: OPTIMUM Fe$^{2+}$ CONCENTRATION FOR BENZENE CONVERSION AT pH 5.0 ................................................................................................................ 164

FIGURE 4-43: OPTIMUM H$_2$O$_2$ CONCENTRATIONS FOR BENZENE CONVERSION AT pH 5.0 .......................................................................................................................... 165

FIGURE 4-44: BENZENE DEGRADATION PATHWAY ................................................................................................................................. 167

FIGURE 4-45: ANALYSIS OF REACTION MIXTURE AFTER 1 HR OF FENTON PRE-TREATMENT UNDER OPTIMUM CONDITION ............................................................................. 169
FIGURE 4-46: Fenton Reaction on 6mM Benzene under Optimum Conditions ..... 171

FIGURE 4-47: Gradual Addition of 10.8 mM Hydrogen Peroxide in the Fenton Reaction

FIGURE 4-48: Step Addition of Fe²⁺ in the Fenton Reaction ...................... 174

FIGURE 4-49: Step Addition of H₂O₂ in the Fenton Reaction .......................... 175

FIGURE 4-50: Step Addition of Fe²⁺ and H₂O₂ in the Fenton Reaction .............. 177

FIGURE 4-51: Laccase Optimization of Fenton Reaction Mixture .................. 179

FIGURE 4-52: Laccase Activity in the Batch Reactors Containing Fenton Reaction Products .......................................................... 180

FIGURE 4-53: Effect of SBP Concentration on the Fenton Reaction Mixture in Absence of Additional Hydrogen Peroxide ..................................... 182

FIGURE 4-54: Hydrogen Peroxide Optimization for the Reaction Mixture at the End of the Fenton Reaction .......................................................... 183

FIGURE 4-55: SBP Optimization of Fenton Reaction Mixture ....................... 184

FIGURE 4-56: Effect of PEI Concentration and Reaction Time on Removal of Benzoquinone Generated from Two-Stage Fenton-Enzymatic Treatment .......................................................................................................................... 186

FIGURE 4-57: Alum Treatment on PEI Treated Sample ................................. 187

FIGURE 4-58: Simultaneous Fenton and Laccase Catalyzed Oxidation of Benzene ............................................................................................................. 189

FIGURE 4-59: Simultaneous Fenton and Laccase-Catalyzed Oxidation of Benzene in Presence of Higher Dissolved Oxygen ......................................... 192
FIGURE 4-60: LACCASE ACTIVITY IN THE SIMULTANEOUS FENTON AND ENZYMATIC SYSTEM
......................................................................................................................... 193

FIGURE 4-61: FENTON OXIDATION OF BENZENE IN ABSENCE OF SBP ....................... 195

FIGURE 4-62: SIMULTANEOUS FENTON AND SBP CATALYZED OXIDATION OF BENZENE IN
PRESENCE OF 0.5 U/mL SBP .................................................................................... 196

FIGURE 4-63: SIMULTANEOUS FENTON AND SBP CATALYZED OXIDATION OF BENZENE IN
PRESENCE OF 1.0 U/mL SBP .................................................................................... 197

FIGURE 4-64: SIMULTANEOUS FENTON AND SBP CATALYZED OXIDATION OF BENZENE IN
PRESENCE OF 1.5 U/mL SBP .................................................................................... 198

FIGURE 4-65: SIMULTANEOUS FENTON AND SBP CATALYZED OXIDATION OF BENZENE IN
PRESENCE OF 2.0 U/mL SBP .................................................................................... 199

FIGURE 4-66: SIMULTANEOUS FENTON AND SBP CATALYZED OXIDATION OF BENZENE IN
PRESENCE OF 5 U/mL SBP ....................................................................................... 200

FIGURE 4-67: SIMULTANEOUS FENTON AND SBP CATALYZED OXIDATION OF BENZENE IN
PRESENCE OF 10 U/mL SBP ..................................................................................... 201

FIGURE 4-68: SBP ACTIVITY IN THE SIMULTANEOUS FENTON AND ENZYMATIC SYSTEM
................................................................................................................................. 203

FIGURE C-1: HPLC STANDARD CURVE PLOT FOR BENZENE .................................... 245

FIGURE C-2: HPLC STANDARD CURVE PLOT FOR PHENOL ..................................... 246

FIGURE C-3: HPLC STANDARD CURVE PLOT FOR CATECHOL.................................. 247

FIGURE C-4: HPLC STANDARD CURVE PLOT FOR RESORCINOL............................ 248

FIGURE C-5: HPLC STANDARD CURVE PLOT FOR HYDROQUINONE..................... 249

FIGURE C-6: HPLC STANDARD CURVE PLOT FOR BENZOQUINONE...................... 250
FIGURE C-7: HPLC STANDARD CURVE PLOT FOR BIPHENYL ........................................ 251
CHAPTER 1
INTRODUCTION

1.1 Background

“BTEX” represents a group of volatile organic compounds (VOCs) comprised of benzene, toluene, ethylbenzene and xylenes. These EPA priority pollutants frequently co-occur at hazardous waste sites and contaminate different media including air, water, and soil (U.S. Department of Health and Human Services, 2004). The contamination occurs as a result of incomplete combustion of fossil fuels, leakage in pipelines and underground storage, oil spills and as a byproduct of petroleum refining and other industrial processing (Nadarajah et al., 2002). Generally, these chemicals are carcinogenic, mutagenic to humans and other animals, and are capable of bio-accumulation in the food chain (U.S. Department of Health and Human Services, 2004).

The methods used to remove such aromatic contaminants from the environment include volatilization, photo- and chemical oxidation, adsorption, bioaccumulation and biodegradation (Health Canada, 2009). However, many of these treatment methods do not result in complete destruction of the chemical unless followed by catalytic oxidation (Xu et al., 1995). Microbial degradation using both pure and mixed cultures has been studied (Nadarajah et al., 2002). BTEXs and PAHs (polycyclic aromatic hydrocarbons) can be degraded by the highly reactive hydroxyl (OH•) radicals in the natural environment (Martens et al., 1995). These hydroxyl radicals can be generated from photolysis of \( \text{H}_2\text{O}_2 \), or by mixing FeSO\(_4\) and \( \text{H}_2\text{O}_2 \) (modified Fenton reaction), etc. (Nadarajah et al., 2002).
Fenton’s reagent was discovered about 100 years ago; however, its application as an oxidizing agent for destroying toxic organics was not applied until the late 1960s (Huang et al., 1993). The Fenton reaction in wastewater treatment processes is known to be very effective in the removal of many hazardous organic pollutants from water, since the process results in the complete destruction of contaminants to harmless compounds, e.g. CO₂, water and inorganic salts (Neyens and Baeyens, 2003).

In situ remediation of contaminated soils is more cost-effective than on-site and off-site treatment, but it depends on the quantity and location of the soil to be treated (EPA, 1998). Advanced oxidation processes (e.g. modified Fenton reactions) and biodegradation are promising in situ remediation techniques (Neyens and Baeyens, 2003). The extensive time needed for the destruction of the substrate in biological processes, is considered a detrimental factor as it requires larger capital cost (Xu et al., 1995). Another problem with traditional biological process is the water solubility of these aromatic compounds. As BTEXs are not very soluble in water, often these chemicals are not available to the microbes to carry out the mineralization process (Palmroth et al., 2006). The Fenton reaction to remove BTEXs is performed under harsh conditions which is harmful for the environment (Palmorth et al., 2006).

1.2 Benzene

Benzene is a widely used chemical formed from both natural processes and human activities (ASTDR, 2007). It is used in the production of rubbers, lubricants, dyes, detergents, drugs, pesticides and other chemicals which are used to make plastics, resins, nylon and other synthetic fibers (ASTDR, 2007). Natural sources of benzene include
emissions from volcanoes and forest fires (ASTDR, 2007). It also occurs naturally as a part of crude oil, gasoline and cigarette smoke (ASTDR, 2007).

Benzene ranks in the top 20 in production volume for chemicals produced in the United States (U. S. Department of Health and Human Services, 2007). Benzene is a priority pollutant in the EPA’s Toxics Release Inventory (TRI) (EPA, 2008) and Environment Canada’s National Pollutant Release Inventory (NPRI) (Environment Canada, 2008) list. According to the US EPA TRI (2008), the total release of benzene in 2008 was 5,519,649 pounds (2,503 tones). Out of these 5,311,576 pounds (2,409 tones) were disposed of onsite through underground injection, surface water discharge and air releases. The remainder accounted for the total offsite disposal which included landfill, transfer to the treatment plants, etc. According to the NPRI (2008) data provided by Environment Canada for the year 2006, 915 tonnes was accounted for onsite releases in air, water and land, 155 tonnes were disposed onsite, 529 tonnes were disposed offsite and only 40 tonnes went for offsite recycling.

The majority of the environmental releases in all cases were air releases. However, benzene can also be found in water and soil. In most of cases, benzene in air can be smelled at as low as 60 ppm and identified as benzene at 100 ppm (U. S. Department of Health and Human Services, 2007). It can be tasted in water as little as 0.5 mg/L (ppm) concentration (U. S. Department of Health and Human Services, 2007).

1.3 Benzene Exposure and Health Effects

Benzene is a highly toxic chemical which can cause serious health effects. Everyone is exposed to a small amount of benzene every day (U. S. Department of Health and Human Services, 2007). The exposure mainly occurs through breathing air containing benzene.
The major sources of benzene exposure are tobacco smoke, automobile service stations, exhaust from motor vehicles, and industrial emissions and vapors (or gases) from products that contain benzene, such as glues, paints, furniture wax, and detergents (U. S. Department of Health and Human Services, 2007). Auto exhaust and industrial emissions account for about 20% of the total exposure to benzene in the U. S. (U. S. Department of Health and Human Services, 2007). About half of the exposure to benzene in the United States results from smoking tobacco or from exposure to tobacco smoke (U. S. Department of Health and Human Services, 2007). Brief exposure (5–10 minutes) to very high levels of benzene in air (10,000–20,000 ppm) can result in death (U. S. Department of Health and Human Services, 2007). Lower levels (700–3,000 ppm) can cause drowsiness, dizziness, rapid heart rate, headaches, tremors, confusion, and unconsciousness (ASTDR, 2007). Literature suggests that in outdoor air benzene concentration can vary between 0.02 to 34 ppb (U. S. Department of Health and Human Services, 2007). In the urban atmosphere the benzene level is higher than in rural areas. At the same time, proximity to hazardous waste sites, petroleum refining operations, petrochemical manufacturing sites, or gas stations results in higher levels of benzene exposure (U. S. Department of Health and Human Services, 2007).

Other than inhalation, people can be exposed to benzene through food, beverages, or drinking water. Drinking water typically contains less than 0.1 ppb benzene (U. S. Department of Health and Human Services, 2007). Leakage from underground gasoline storage tanks, landfills and hazardous waste sites that contain benzene can result in contamination of well water (U. S. Department of Health and Human Services, 2007). In addition, exposure can result from breathing in benzene while showering, bathing, or
cooking with contaminated water (U. S. Department of Health and Human Services, 2007). Eating or drinking foods containing high levels of benzene can cause vomiting, irritation of the stomach, dizziness, sleepiness, convulsions, and death (ASTDR, 2007).

The major chronic effects of benzene exposure occur through the blood (ASTDR, 2007). It causes harmful effects on the bone marrow leading to a decrease in red blood cells and finally resulting in anemia (ASTDR, 2007). It can also cause excessive bleeding and depress the immune system, increasing the chance of infection (ASTDR, 2007).

Exposure to benzene may be harmful to the reproductive organs (U. S. Department of Health and Human Services, 2007). Women who breathed high levels of benzene for many months had irregular menstrual periods and a decrease in the size of their ovaries (ASTDR, 2007). However, exposure effects on the developing fetus in pregnant women or fertility in men are not yet certain (ASTDR, 2007). Animal studies have shown low birth weights, delayed bone formation, and bone marrow damage when pregnant animals breathed benzene (U. S. Department of Health and Human Services, 2007).

The US Department of Health and Human Services and the US EPA classify benzene as a human carcinogen. The Department of Health and Human Services determined that benzene is a known carcinogen based on human evidence showing a causal relationship between exposure to benzene and cancer (U. S. Department of Health and Human Services, 2007). Two studies classify benzene in Group 1 (carcinogenic to humans) based on sufficient evidence in both humans and animals (U. S. Department of Health and Human Services, 2007). The EPA classified benzene in Category A (known human carcinogen) based on convincing evidence in humans supported by evidence from animal studies (U. S. Department of Health and Human Services, 2007). Under the EPA’s most
recent guidelines for carcinogen risk assessment, benzene is characterized as a known human carcinogen for all routes of exposure based on convincing human evidence as well as supporting evidence from animal studies. The carcinogenicity of benzene is well documented in exposed workers (U. S. Department of Health and Human Services, 2007). Long-term exposure to high levels of benzene in the air can cause leukemia. It is also known to have some mutagenic effects. Data from both humans and animals indicate that benzene and/or its metabolites are genotoxic. Chromosomal abnormality is the predominant effect seen in humans (U. S. Department of Health and Human Services, 2007). In case of high-level exposure to benzene, neurological effects have been commonly reported in humans (U. S. Department of Health and Human Services, 2007). Fatal inhalation exposure has been associated with vascular congestion in the brain (U. S. Department of Health and Human Services, 2007). Chronic inhalation exposure has been associated with distal neuropathy, difficulty in sleeping, and memory loss (U. S. Department of Health and Human Services, 2007).

1.4 Regulations

The major environmental sinks for benzene, due to its relatively high vapor pressure, moderate water solubility and low octanol/water partition coefficient, are the atmosphere and the surface waters (Health Canada, 2009). This priority pollutant has been identified and marked as a human carcinogen by International Agency for Research on Cancer (IARC), Environmental Protection Agency (EPA), National Toxicology Program (NTP) and Environment Canada. Hence the release of benzene is regulated both in air and water. The guidelines and regulations are summarized in Table 1-1.
Table 1-1: Regulations and Guidelines Applicable to Benzene

<table>
<thead>
<tr>
<th>AGENCY</th>
<th>DESCRIPTION</th>
<th>INFORMATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>World Health Organization (WHO)</td>
<td>Air quality</td>
<td>$6 \times 10^{-6}$ unit risk</td>
</tr>
<tr>
<td>Environmental Protection Agency (EPA)</td>
<td>Hazardous air pollutant</td>
<td>---------</td>
</tr>
<tr>
<td>American Conference of Industrial Hygienists (ACGIH)</td>
<td>Threshold limit value (TLV), Time Weighted Average (TWA) Short Term Exposure Limit (STEL)</td>
<td>0.5 ppm$^1$ 2.5 ppm$^1$</td>
</tr>
<tr>
<td>National Institute for Occupational Safety and Health (NIOSH)</td>
<td>Recommended exposure limit (REL) (10 hour TWA) STEL</td>
<td>0.1 ppm$^2$ 1.0 ppm$^2$</td>
</tr>
<tr>
<td>Occupational Safety and Health Administration (OSHA)</td>
<td>Permissible exposure limit (PEL) for industry (8-hour TWA)</td>
<td>1.0 ppm</td>
</tr>
<tr>
<td>WATER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPA</td>
<td>Hazardous substance Section 311 (2)(b) (a) of Clean Water Act Drinking water standard</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maximum contaminant level goal (MCLG)</td>
<td>Zero</td>
</tr>
<tr>
<td></td>
<td>Maximum contaminant level (MCL)</td>
<td>0.005 mg/L</td>
</tr>
<tr>
<td></td>
<td>Drinking water equivalent level (DWEL)</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td></td>
<td>Wastewater stream standard Universal Treatment Standard (UTS)</td>
<td>0.14 mg/L</td>
</tr>
<tr>
<td></td>
<td>Non-wastewater stream standard UTS</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td>Health Canada</td>
<td>Maximum acceptable concentration (MAC)</td>
<td>0.005 mg/L</td>
</tr>
<tr>
<td>Municipal Industrial Strategy for Abatement (MISA)</td>
<td>Regulatory Method Detection Limit (RMDL)/ Limit of characterization (LOC)</td>
<td>0.5 µg/L</td>
</tr>
<tr>
<td>WHO</td>
<td>Drinking Water</td>
<td>0.01 mg/L$^3$</td>
</tr>
</tbody>
</table>
### Table 1-1: Regulations and Guidelines Applicable to Benzene (continued.)

<table>
<thead>
<tr>
<th>FOOD</th>
<th>International Agency for Research on Cancer (IARC)</th>
<th>American Conference of Industrial Hygienists (ACGIH)</th>
<th>EPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Food and Drug Administration (FDA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bottle drinking water</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 1 carcinogen</td>
<td>A1 carcinogen</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 1: human carcinogens</td>
<td>A1: confirmed human carcinogen</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Designated substance under Comprehensive</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Environmental Response, Compensation, and Liability</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Act (CERCLA) (Section 311(b)(2) and 307(a) of Clean</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water Act, Section 112 of Clean Air Act, Resource</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conservation and Recovery Act (RCRA) section 3001.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reportable quantity</td>
<td>10 lbs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RCRA hazardous waste number</td>
<td>U019</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Effective date of toxic chemical release reporting</td>
<td>01/01/87</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>National Toxicology Program (NTP)</td>
<td>Known human carcinogen</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Potential significant contribution to the overall exposure by cutaneous route, including mucus membranes and eyes, either by contact with vapors or, of probable greater significance, by direct skin contact.
2. NIOSH potential occupational carcinogen
3. The guideline value is the concentration in drinking water associated with an upper-bound excess lifetime cancer risks of $10^{-5}$ (one additional cancer per 100,000 of the population ingesting drinking water containing the substance at the guideline value for 70 years).

The U.S. EPA recognizes benzene as “hazardous substance” under section 311 (2)(b) (a) of the Clean Water Act. According to the EPA guideline, the maximum contaminant level goal (MCLG) of benzene in water should be zero. However, the maximum contaminant level (MLC) in drinking water should not be more than 0.005 mg/L. Similar regulation is also in effect in Canada. According to Health Canada (2009), the maximum acceptable concentration (MAC) of benzene in drinking water is 0.005 mg/L. According to World Health Organization (WHO), benzene concentration in drinking water should not exceed 0.01 mg/L. The U.S. Food and Drug Administration (FDA) regulate the benzene concentration in bottled water as well to not be more than 0.005 mg/L.

The EPA’s Universal Treatment Standards (UTS) set the regulatory limit for the most prohibited hazardous wastes present in non-wastewater and wastewater streams. These treatment standards should not be exceeded. Compliance with these treatment standards is measured by grab sample analysis. According to UTS, 0.14 mg/L of benzene is acceptable treatment standard for a wastewater treatment stream. For non-wastewater, i.e., solids/soil, the UTS for benzene is 10 mg/kg (EPA, 1994).

The Municipal Industrial Strategy for Abatement (MISA) program in Ontario has provided the guidelines for the grab-sampling used for the determining the compliance with the regulatory standards provided by the Environment Protection Act. According to MISA guidelines, the Regulatory Method Detection Limit (RMDL) or the Limit of characterization (LOC) for benzene is 0.5 µg/L (MISA, 1999). The LOC represents the value above which organic compounds or elements must be identified and their approximate concentration determined in open characterization analyses ATGs 28a, 28b and 29 (MISA, 1999).
1.5 Conventional Treatment Methods

In general, municipal drinking water treatment plants rely on conventional treatment methods such as coagulation, sedimentation, filtration and chlorination. However, studies indicate that these methods are ineffective in reducing benzene concentration (Love et al., 1983, Health Canada, 2009). Two common treatment technologies reported to be effective for the reduction of benzene in water are granular activated carbon (GAC) adsorption and air stripping (Health Canada, 2009). The most common methods used to remove benzene from the environment include volatilization, photo- and chemical oxidation, adsorption and biodegradation (Health Canada, 2009).

1.5.1 Physical Methods

1.5.1.1 Adsorption

Adsorption is considered to be an effective technology for removing contaminants from water. The adsorption efficiency depends on the presence of other contaminants in the waste stream and adsorptive competition, influent concentrations, preloading of natural dissolved organic matter, humic interactions, microbial growth, pH, physical and chemical properties of the chemical in question and the carbon used (Speth, 1990). GAC is widely used to reduce benzene concentration in water. Effectiveness of GAC filtration is also a function of the empty bed contact time (EBCT), flow rate, and filter run time (Health Canada, 2009).

Studies by Koffskey and Brodtmann have demonstrated that a GAC filter adsorber having a bed volume of 23.8 m$^3$, a flow rate of 1.5 ML/day and an EBCT of 23.7 minutes were successful in reducing the influent benzene concentration of 10 µg/L to the finished
water concentration of 0.1 µg/L (Health Canada, 2009). During the 180-day study period, no breakthrough of benzene was observed (Health Canada, 2009). Another study reported by AWWA demonstrated that three parallel GAC adsorbers with a flow rate of 5 ML/day, EBCT of 21 minutes, bed life of 12 months were capable in achieving 99% removal efficiency (reduction of benzene concentrations from 20 µg/L to 0.2 µg/L) (Health Canada, 2009).

Studies by Yue et al., (2001) demonstrated that fibreglass-supported activated carbon filters have a higher BTEX adsorption capacity than conventional activated carbon process. Synthetic carbonaceous resins also have shown better removal efficiency than activated carbon (Shih et al., 2005). A combination of photocatalysis, using platinum and titanium dioxide catalyst, and adsorption processes also have shown higher removal efficiency and prolonged adsorbent bed life (Crittenden et al., 1997).

One of the major drawbacks of adsorption methods is that they do not actually treat benzene but simply shift it from the aqueous phase to the solid phase. The benzene remains unaltered in the process but gets more concentrated in the solid. Moreover, the removed concentrated benzene and the spent carbon need to be disposed of. This adds to the overall cost of this treatment technology.

1.5.1.2 Air Stripping

The physical process of transferring volatile organic compounds (VOCs) from water into air is known as air stripping (Eckenfelder, 2000). Generally this is accomplished by injection of water into air via spray systems (e.g., spray towers or packed towers) or injection of air into water through diffused or mechanical aeration systems (Eckenfelder, 2000).
The most effective air stripping system for benzene removal is packed tower aeration (PTA) (Health Canada, 2009). However, treatment of the stripping tower off-gas is necessary as it contains a high concentration of benzene. Design considerations for PTA are the temperature of the air and water, physical and chemical characteristics of the contaminant, air-to-water ratio, contact time, and available surface area for mass transfer (Health Canada, 2009).

Studies completed at a full-scale drinking water treatment plant indicates that countercurrent flow PTA using an air-to-water ratio of 75, an air stripper length of 5.50 m, and a packed column diameter of 1.52 m was successful in reducing an influent benzene concentration of 30 µg/L to 1.5 µg/L (Health Canada, 2009). A report (Report No. 0033986) published by the American Water Works Association (AWWA) in 1991, demonstrates that in a full-scale drinking water treatment plant the influent benzene concentration of 200 µg/L was reduced to less that 2 µg/L by using a PTA having an air-to-water ratio of 100, an air stripper length of 10.05 m, and a packed column diameter of 3.05 m (Health Canada, 2009).

1.5.1.3 Reverse Osmosis

Reverse osmosis has shown some promise for its potential to remove VOCs from drinking water (Clark et al., 1988). However, Clark et al., (1988) observed that for benzene, removal efficiency with reverse osmosis process varied and was poor (0- 29% removal). In a pilot plant reverse osmosis study, Al-Bastaki, (2003) used a FilmTec SW30 membrane made from polyamide thin-film composite to show that, from an influent benzene concentration of 100 ppm (mg/L) , 82.3% removal was possible at 30
bar (29.61 atm). However, the process showed a negative permeability at an operating pressure of 20 bar (19.74 atm).

Poor (less that 20%) benzene removal efficiency has been reported in the reverse osmosis process when cellulose, polyamide, and thin film composite membranes were used (Health Canada, 2009). This was mainly because, for removal of benzene using reverse osmosis depended on the type of material used, solubility of chemical, molecular weight etc (Health Canada, 2009).

1.5.2 Chemical Methods

Oxidation and advanced oxidation processes (AOPs) have been reported to be effective for the reduction of benzene in water.

1.5.2.1 Ozonation

For ozonation of benzene in the source water, the presence of natural organic matter can be a determining factor (Health Canada, 2009). Studies indicate that such organic matter generally reacts with ozone to generate hydroxyl radical and the reaction rate between benzene and hydroxyl radical is much higher than that between benzene and ozone (Health Canada, 2009). Hence, depending on the influent benzene and natural organic matter concentrations in the influent, the ozone dose, contact time and pH of the water must be varied in order to achieve a satisfactorily low benzene effluent concentration (Health Canada, 2009).

In a pilot scale study on distilled water, 6 mg/L of ozone was successful in achieving 81% removal of benzene (from 50 µg/L to 10 µg/L; Health Canada, 2009). In 1987, another study performed on both distilled water and groundwater was successful in
achieving 94% reduction of benzene by using 12 mg/L of ozone (Fronk, 1987). In 1997, another pilot scale study reported 75% removal of benzene by using 0.8-1.5 mg/L of benzene (Kang et al., 1997).

1.5.2.2 Photo-catalytic Oxidation

Photocatalytic oxidation systems were also reported to be successful in benzene concentration reduction. These processes generally utilize UV light to supply energy to the semiconductor titanium dioxide. The process generates a free electron, which is taken up by the oxygen molecule to generate superoxide. The superoxide radicals react with the water molecules to produce hydroxyl radicals (Al-Bastaki, 2003). The release of the free electron generates a positively charged catalyst. At this positively charged point, water molecules or hydroxyl ions react to generate additional reactive hydroxyl radicals (Al-Bastaki, 2003). Hence the benzene can be oxidized either at the catalyst surface or in the solution by the hydroxyl radicals.

In one pilot scale study, 123 µg/L of influent benzene concentration was reduced to 0.5 µg/L by utilizing ultraviolet (UV) light with titanium dioxide, 70 mg/L of hydrogen peroxide and 0.4 mg/L of ozone (Topudurti et al., 1998). A commercial scale photocatalytic oxidation system developed by Matrix Photocatalytic Inc. was used for the above mentioned study. In a pilot scale study, Al-Bastaki (2003) was able to achieve more than 99% removal of benzene from an influent having 100 ppm of benzene concentration.
1.5.2.3 Electron Beam Radiation

When electrons are injected into water, short-lived highly reactive hydroxyl radicals are generated (Lubicki et al., 2001). These radicals initiate rapid reactions with organic contaminants and break the contaminants into harmless products.

In an electron beam study by Lubicki et al., (2001), an initial benzene concentration of 10 mg/L was exposed to a beam voltage of 100–175 kV and less than 0.5 mA beam current. The water flow rate was kept at 1 kg/min. At a dose of 15 kJ/kg, a 99% or better benzene removal was achieved under the above mentioned condition. However, the process generated carboxylic acids, phenols, and aldehydes as reaction products. An additional 20 kJ/kg was necessary to remove the reaction products from the system. In another study, 95 kJ/kg energy was required to achieve 99% removal of 1.3 mg/L of influent benzene (Nickelsen et al., 1994).

1.5.2.4 Other Advanced Oxidation Processes

Ollis et al., (1991) reported complete mineralization of 279 µmol of benzene in a UV-assisted photo-Fenton process within a 30 min period. Tiburtius et al., (2005) also reported complete destruction of benzene in 5 min and destruction of phenolic compounds generated from benzene oxidation in 30 min when a UV-A photo-Fenton system was employed.

Oliveira et al., (2007) studied electrochemical oxidation of benzene on boron-doped diamond (BDD) electrodes. BDD can produce a large amount of adsorbed hydroxyl radicals from water oxidation during the electrolysis process. Electro-oxidation of 1 mM benzene was performed at 2.5 V versus Ag/AgCl for 5 hours on the rotating (2000 rpm) BDD electrode in 0.5M sulfuric acid. Hydroquinone, resorcinol, catechol, benzoquinone
and phenol were identified as reaction products. Some of these products are priority pollutants.

One of the main concerns for the application of advanced oxidation processes is byproduct formation. Potential byproducts of benzene oxidation processes have been identified as phenolic compounds, aldehydes, ketones, and carboxylic acids (Health Canada, 2009). Some of these byproducts are known toxic priority pollutants. Another major concern for advanced oxidation processes would be cost due to higher energy consumption. In general, photocatalysis is an energy-intensive method since oxidation of organics is proportional to the electrical energy input. Complete mineralization of pollutants by photocatalysis can be very expensive (Bolduc and William, 1997). Oxidation methods like ultraviolet light and ozonation are expensive and in many cases they only cause partial destruction of the target chemical (Ollis, 1985).

1.5.3 Biological Methods

In-situ natural or enhanced biodegradation is a common mechanism for benzene applied at contaminated sites (Edwards and Grbic-Galic, 1992). Both aerobic and anaerobic processes have been employed to accomplish benzene mineralization. The rate and extent of biodegradation of BTEXs can be influenced by several factors, such as active biomass concentration, temperature, pH, availability of inorganic nutrients and electron acceptors, and microbial adaptation (Alvarez and Vogel, 1991). BTEX degradation by bacterial consortia from sewage, indigenous soil, groundwater microorganisms or pure cultures, either in batch microcosms or in continuous-flow reactors has been investigated (De Nardi et al., 2002).
When it comes to anaerobic in situ degradation, out of all BTEX compounds, benzene is generally the most persistent (Reinhard et al., 1997). However, studies demonstrate that benzene was successfully degraded under methanogenic, sulfate-reducing, and iron-reducing conditions. The literature reported that for benzene under anaerobic conditions, degradation is slow and in many cases incomplete and long lag times preceded the degradation process (Edwards and Grbic-Galic, 1992). In many cases, if any other BTEXs were present, anaerobic degradation of benzene was not realizable (Edwards and Grbic-Galic, 1992).

Vogel and Grbic-Galic, (1986) used a mixed methanogenic culture derived from sewage sludge to degrade benzene via phenol to methane and carbon dioxide. Prior to the initiation of experiments, the culture was maintained on benzene as sole carbon and energy source for a year. The acclimated seed from these cultures was then inoculated into defined mineral salts medium with reducing agents (ferrous chloride and sodium sulfide), ammonium phosphate as the nitrogen source, and vitamins (Vogel and Grbic-Galic, 1986). A 40-day incubation period was allowed to utilize 3 mM benzene.

Edwards and Grbic-Galic, (1992) studied complete anaerobic mineralization of benzene to carbon dioxide by utilizing aquifer-derived microorganisms. They reported that benzene degradation began after at least a 30 day lag-time. They also noted that the degradation increased up to a benzene concentration of 140 µM. But, longer lag phases and slower degradation rates, indicating substrate toxicity, were observed at a benzene concentration of 200 µM. In about 31 days’ incubation, complete mineralization of benzene was observed.
Alvarez and Vogel, (1991), studied the substrate interaction during benzene, toluene, and p-xylene degradation by two pure cultures, *Pseudomonas sp.* strain CFS-215 and *Arthrobacter sp.* strain HCB, and a mixed culture indigenous to a shallow sandy aquifer. Initial benzene concentration for this study was 50 mg/L. Results of this study indicate that, when benzene was used as carbon source the lag periods for mixed culture, *Pseudomonas sp.* CFS-215 and *Arthrobacter sp.* HCB were 2 days, 6 days and 2 days, respectively. The pseudo-zero-order biodegradation rate for mixed culture, *Pseudomonas sp.* CFS-215 and *Arthrobacter sp.* HCB were 25, 7 and 52 (mg/L/d) respectively. In all cases, within 6 weeks incubation the benzene concentration decreased from 50 mg/L to below 0.01 mg/L.

Yadav and Reddy (1993) reported that *Phanerochaete chrysosporium*, a naturally occurring wood-degrading white-rot fungus, was able to degrade benzene under non-ligninolytic culture conditions when no lignin peroxidases (LIPs) and manganese-dependent peroxidases (MNPs) were produced. The initial benzene concentration in their study was 10 mg/L. Results of their study indicate that only 13.2% of initial benzene was converted within a 2-week period.

Lovley et al., (1996) studied anaerobic benzene oxidation with a variety of chelated Fe(III) forms and reported its applicability to aquifer remediation. In this study petroleum-contaminated sediment was incubated under anaerobic conditions. Ferric sodium EDTA (Fe (III)-EDTA), nitrilotriacetic acid (NTA), N-methyliminodiacetic acid (MIDA), ethanol diglycine (EDG), humic acids and phosphate glass (calgon) were used in this study. The initial benzene concentration was 10 µM. In the presence of 10 mM Fe(III)-EDTA, 10 µM benzene was significantly degraded within 40 days of incubation.
In the presence of 2 mM NTA, 10 mM EDG 10 mM MIDA, 20 mg humic acid or 1 mM Calgon achieved comparable levels of benzene degradation within 60, 70, 68, 65 and 60 days, respectively. These findings suggest that it should be possible to find a suitable chelated Fe(III) form that can stimulate aromatic hydrocarbon degradation \textit{in situ} without significant negative environmental impact (Lovley et al., 1996).

Reinhard et al., (1997) studied \textit{in situ} biodegradation of BTEX compounds under nitrate- and sulfate-reducing conditions. Under sulfate-reducing conditions, hydrogen sulfide was created. Hydrogen sulfide generated inhibited the BTEX degradation process. However, if ferric or ferrous iron was introduced to the system, it removed the free hydrogen sulfide. Removal of the inhibiting hydrogen sulfide prevents sulfide toxicity and aids in initiating or accelerating BTEX degradation. Field studies also indicate that under anaerobic conditions, nitrate can enhance BTEX removal in the contaminated sites. Reinhard et al., (1997) conducted this study at a gasoline-contaminated site located on the premises of the Seal Beach Naval weapons station in southern California. The redox conditions at the site varied and were more reducing toward the center of the plume. In the test zone, nitrate and sulfate concentrations were 5 mg/L and 85 mg/L, respectively. Slug test methodology was adopted in this study, where 470-1700 L of groundwater was incubated in an unconfined test zone. The initial concentration of benzene in the test zone was 750 µg/L. Under nitrate reducing conditions with 209 mg/L of nitrate added, the benzene concentration in the test well remained stable and relatively unchanged in an 80-day period. Under sulfate-reducing conditions, in an 80-day period, about 25% of initial benzene was removed. Reinhard et al., (1997) indicated that their method for BTEX
removal can be applicable only for a slow moving aquifer having groundwater flow of 1 cm/day.

Anderson et al., (1998) studied the potential for anaerobic benzene oxidation in the Fe(III)-reduction zone of petroleum-contaminated aquifers. The potential for benzene to be oxidized with the reduction of Fe(III) in petroleum-contaminated aquifers was of interest because petroleum-contaminated aquifers often contain extensive anaerobic zones and Fe(III) is generally the most abundant electron acceptor for organic matter oxidation in these systems (Anderson et al., 1998). In order to simulate the in situ conditions without any alteration, the sediment were incubated under strict anaerobic conditions. The results of this study illustrated that benzene metabolism was not possible in all aquifers. The limited zones of anaerobic benzene degradation were associated with microorganisms in the family Geobacteraceae. They also indicated that, in the previous studies with aquifer material, anaerobic benzene degradation was only observed after long lag periods and/or after making various amendments to the aquifer material to increase electron acceptor and/or nutrient availability. In their opinion, benzene degradation under such artificial conditions will not be representative of in situ conditions.

BTEXs are generally rapidly degraded under aerobic conditions (Reinhard et al., 1997). However, aerobic processes are limited by the slow oxygen supply rate to the contaminated zone (Reinhard et al., 1997). Hence, aerobic degradation of BTEXs is only effective at the fringes of the contaminated zones (Reinhard et al., 1997). Alvarez and Vogel (1995) conducted a study on BTEX and their aerobic metabolites’ degradation under nitrate-reducing conditions and in the presence of microorganisms found in four
separate aquifer materials. The initial benzene concentration for this study was 30 mg/L. They reported that benzene was not significantly degraded under anoxic conditions (DO<0.1 mg/L) and the presence of nitrate under anoxic conditions did not have any effect on the conversion. However, benzene degradation was observed under limited aerobic enrichments. Nitrate-free aerobic enrichments degraded 7 mg/L of benzene in a seven-day period. The smallest amount of nitrate removal (63.7 mg/L of nitrate removed) was observed when the residual benzene concentration was highest (22.3 mg/L). Whereas, when the highest nitrate removal (121 mg/L) was achieved, the residual benzene concentration was the lowest (6 mg/L). They observed that even though toluene and xylene were degraded in the presence of aquifer microorganisms and nitrate reducing conditions, benzene and ethylbenzene, two of the BTEXs, did not get converted within four months of incubation. Benzene degradation under denitrifying conditions was reported unlikely (Alvarez and Vogel, 1995).

Yeom and Daugulis (2001) studied benzene degradation in a two-phase (aqueous-organic) partitioning bioreactor. The two-phase partitioning bioreactor consisted of a 1L aqueous phase and 500 mL hexadecane. A. xylosoxidans Y234 isolated from oil-contaminated soil was used in this study. It was ensured that the selected microorganism utilized benzene as the sole carbon source. The initial cell concentration was 96.5 mg/L. An initial loading of 7000 mg benzene was introduced in the hexadecane phase (14,000 mg/L), which partitioned into the aqueous phase to around 100 mg/L. As the cells were pre-adapted to benzene, no lag period was observed and after 20 hours, benzene was exhausted in the aqueous phase. Over the course of 24 h, 63.8% of the benzene was degraded by the microorganism, and 36.2% was stripped by aeration. In order to reduce
the effect of stripping and to encourage more biological removal of benzene, a condenser was installed on the exit gas line of the two-phase bioreactor, and instead of air pure oxygen was used at a lower air flow rate. In the new configuration, 99% of initial benzene was degraded by microorganisms within a 24 hour period.

A bench-scale horizontal-flow anaerobic immobilized biomass (HAIB) reactor study was performed to observe BTEX degradation under anaerobic conditions in the presence of two co-solvents, ethanol and linear alkylbenzene sulphonate (LAS) (De Nardi et al., 2002). The 138 mL HAIB reactor was filled with polyurethane foam matrices. The matrices were previously inoculated with a mixture of sludges taken from up-flow anaerobic sludge blanket (UASB) reactors treating recycled paper industry wastewater, domestic sewage and poultry slaughterhouse wastewater. When ethanol (848 mg/L) was used as co-solvent, the reactor was operated for 75 days. When LAS (0.70 g of commercial detergent/L) was used, the reactor was operated for 72 days. In the appropriate environment, initial benzene concentrations (varied between 3.6 and 27 mg/L) were reduced to less that 0.1 mg/L in the effluent.

Though extensive work has been done on biodegradation of benzene, in most of the cases the biological processes did not meet the regulatory limit. In many cases, complete mineralization of benzene was not achieved. In such cases toxic intermediates and byproducts were identified.

1.6 Enzymatic Treatment

When a wastewater treatment process is chosen, its applicability should be studied based on certain factors, such as chemical constituents of the wastewater stream, permissible discharge limit to be achieved, difficulties in process control, chances of producing toxic
byproducts, treatment methods for the byproducts, economic feasibility, etc. With stricter standards for effluent discharge, the need for more effective treatment technology is recognized. Enzymatic treatment represents one method by which selective removal of pollutants may be accomplished (Aitken, 1993).

The conventional treatment methods for phenolic wastewaters can be classified into physical processes (i.e., adsorption, etc.), chemical processes (i.e., advanced oxidation, etc.) and biological processes (aerobic and anaerobic). However, these conventional treatment methods may suffer from limitations such as high cost, poor removal efficiency and/or hazardous byproduct formation (Klibanov et al., 1980). For example, adsorption by activated carbon, a commonly used phase-transfer technology, is expensive due to the high cost of the medium regeneration and hence only effectively applicable in specific cases (Shakir et al., 2008). In addition, the adverse effects associated with the target pollutants still exist since the pollutants remain unaltered in this process. Other physical/chemical methods like adsorption by ion-exchange resin and adsorptive micellar flocculation suffer from similar drawbacks and require post-treatment of the effluent and the sludge generated (Saha, et al., 2011). The effectiveness of biological treatment depends on the health and activity of microbial population. In order to maintain optimum efficiency, these microorganisms need sufficient food, oxygen and stable environmental conditions such as pH, temperature, etc. These processes require a larger foot-print and time. In many cases, these methods are unable to produce consistent effluent quality (Mandal et al., 2004).

An innovative and effective alternative for removing aromatic pollutants from aqueous solution is enzymatic treatment (Ibrahim et al., 2001). Enzymes are specific biological
catalysts which increase rates of reactions without undergoing overall change. Enzymatic treatment has several advantages over conventional biological treatment processes including the capability of working over a range of specific chemicals, treating bio-refractory chemicals, operating over wide temperature, pH, salinity and substrate concentration ranges, reducing sludge volume, having no shock loading effect, no delays associated with start up and shut down, simpler process control, less energy requirement and low capital cost, etc. (Taylor et al., 1996; Caza et al., 1999; Mantha et al., 2002).

In enzymatic treatment, isolated enzymes are used instead of microorganisms. The idea of using isolated enzymes to remove toxic pollutants from wastewater was first proposed in the 1930s (Munnecke, 1976). Many researchers have subsequently studied the applicability of various oxidoreductases, such as laccases and peroxidases, in the removal of aromatic pollutants from aqueous solution (Bollag et al., 1980, Aitken, 1993, Masuda et al., 2001, Biswas et al., 2007, Modaressi et al., 2005). By using enzymatic treatment, many phenols and amines could be removed from water with an efficiency of 99% or higher.

In this treatment strategy, oxidoreductase enzymes like laccases or peroxidases catalyze oxidation of phenols or amines to generate aryloxy or arylinium radicals. Laccase-catalyzed oxidation takes place in the presence of dissolved molecular oxygen, whereas peroxidase-catalyzed oxidation occurs in the presence of hydrogen peroxide. The aryloxy or arylinium radicals undergo chemical coupling to generate dimeric derivatives. These dimeric derivatives can undergo subsequent enzyme cycles to generate oligomeric products. The resulting polymers are generally less soluble or insoluble in water and can be removed by filtration or sedimentation, often aided by different coagulants (Torres et
Thus, enzyme-catalyzed oxidative polymerization actually transforms water-soluble toxic organic compounds into less water-soluble or insoluble polymers without any apparent degradation. Hence, the enzymatic approach is the reverse of conventional biological treatment: enzymatic treatment involves buildup of target compounds through oxidative polymerization, whereas the biological treatment breaks down the target compound (Saha et al., 2008).

Enzymes are substrate-specific, easy to handle and store, and their concentration is easier to control than microbes (Mantha et al., 2002). Effectiveness of an enzymatic reaction depends on enzyme and substrate concentrations, pH, temperature, reaction time, and susceptibility to inhibition (Wynn, 1979). The major limitations to application of enzymatic treatment are the cost of enzyme and its susceptibility to inactivation.

In order to perform a cost comparison between conventional treatment methods and the proposed enzymatic method, an overall system cost analysis should be done. This cost estimation should be based on the results of the continuous operation of a pilot plant (Ibrahim et al., 2001). This is outside the scope of the present study and hence, a cost analysis is not yet possible for the proposed method. However, feasibility studies performed (Ibrahim et al., 2001; Steevensz et al., 2009) in the past for phenolic wastewater indicated that the estimated total cost for enzymatic treatment using soybean peroxidase would be comparable to the treatment costs for activated carbon or biological treatments. Since the method of production and the formulation of the laccase-catalyzed reactions are analogous to those for peroxidases, it is expected that laccase-catalyzed conversion of phenolic compounds will have similar process costs as well. The enzymatic method has very low capital costs; hence, a major portion of the total cost is the cost of
enzyme. This high enzyme cost may be reduced significantly by improving the treatment efficiency (enzyme turnovers) or by using a less expensive enzyme. Advancements in biotechnology have made mass production of enzymes, as well as cheaper purification and extraction processes, possible (Karam et al., 1997).

Kilbanov et al., (1983) suggested that during enzymatic treatment, inactivation occurs due to the interaction of the phenoxy radicals with the enzyme active site. On the other hand, Nakamoto and Machida (1992), reported that enzyme inactivation is a result of the polymeric end-product, which adsorbs the enzyme and hinders the access of substrate to the active site of the enzyme. They demonstrated that the treatment cost can be reduced by using additives like polyethylene glycol (PEG), gelatin, etc. Such additives can suppress the enzyme inactivation and reduce enzyme requirement for complete conversion (Nakamoto and Machida, 1992). PEG has been successfully used with oxidoreductases such as horseradish peroxidase (HRP) (Wu et al., 1998), soybean peroxidase (SBP) (Caza et al., 1999) and laccase (Modaressi et al., 2005, Saha et al., 2008). This non-toxic (Harris, 1992) chemical is the additive of choice since it can significantly reduce the amount of enzyme needed (Cooper et al., 1996). The mechanism of the protection is not yet fully understood; however, previous work in this laboratory has shown that a certain amount of PEG precipitates with the polymeric phenolic products (Wu et al., 1998, Modaressi et al., 2005).

1.7 Proposed Treatment Method

Enzyme-catalyzed coupling is an effective strategy for removal of phenols and anilines from water (Mantha, et al. 2002). However, benzene is outside the scope of enzyme catalysis. In order to remove benzene by enzymatic treatment, its partial oxidation is
required to produce corresponding phenolic compounds which are excellent substrates of oxidoreductase enzymes.

There is substantial literature available on the potential for using the Fenton reaction to soil and water contaminated with various polycyclic aromatic hydrocarbons (PAHs) and BTEXs. In such treatment, complete mineralization of PAHs and BTEXs is performed under harsh conditions (i.e., high hydrogen peroxide concentration, reactive radical generated etc.). However, as a pre-treatment, controlled Fenton reaction could cause partial oxidation of BTEXs (Zeng et al., 2000) to generate the corresponding phenolic compounds (Xu et al., 1995) which could be removed by enzymatic treatment. Literature on using the Fenton reaction as a pre-treatment is very limited.

The proposed method consists of a hybrid process for treatment of benzene using a chemical-enzymatic technique. This process employs a controlled Fenton reaction as a pre-treatment method to cause partial oxidation (Zeng et al., 2000) to generate the corresponding phenolic compounds. These phenolic compounds are then removed by enzyme-catalyzed polymerization using laccase or SBP as the oxidative enzyme. Such a combined chemical-enzymatic treatment would be a more environmentally benign process to treat BTEXs.

1.8 Research Objectives

The primary objectives of this study were to:

- Explore the feasibility of an efficient pre-treatment process based on the modified Fenton reaction to maximize the conversion of benzene to corresponding phenolic compounds without causing significant mineralization, and
• Explore the feasibility of laccase- and peroxidise-catalyzed oxidative coupling and precipitation of the phenolic compounds generated in the modified Fenton reaction process.

1.9 Scope of the Study

The scope of the study included:

• Investigating the potential of a modified Fenton reaction to oxidize benzene present in the millimolar range in wastewater;

• Identifying the reaction conditions such as pH, hydrogen peroxide and ferrous iron concentrations and reaction time to maximize the conversion of benzene to phenolic compounds;

• Identifying and quantifying the products generated in the Fenton pre-treatment process;

• Investigating the feasibility of using laccase and SBP-catalyzed polymerization to remove the phenolic products (phenol and benzenediols) generated in the benzene pre-treatment;

• Determining the optimum reaction conditions for more than 95% conversion (an arbitrary benchmark for comparison) of phenol and benzene diols (hydroquinone, catechol, and resorcinol) with laccase with respect to pH, enzyme concentration and substrate concentration;

• Investigating the effect of an additive, PEG, in improving the conversion efficiency;
• Exploring the feasibility of using additives like chitosan flakes and polyethyleneimine (PEI) to remove quinone generated in the pre-treatment and enzymatic treatment;

• Identifying optimum pH and PEI or chitosan concentration for quinone removal;

• Studying the effect of alum as a coagulant aid to remove the colored products generated from enzyme-catalyzed polymerization;

• Evaluating the proposed two-step process on benzene in laboratory-scale batch reactors; and

• Conducting studies on the two steps operated together in the laboratory-scale batch reactor.

All discussions of optima in this work refer to local optima as determined for the parameter in question within the respective ranges specified. In most of the cases, the system is optimized for 95% conversion of the pollutant, an arbitrary benchmark for comparison purposes. At this removal efficiency, it is recognized that the pollutant concentration in the treated effluent might be above the discharge limit and need to be addressed.
CHAPTER 2
REVIEW OF THE LITERATURE

2.1 Benzene as a Pollutant

Benzene, the simplest of the aromatic hydrocarbons, first isolated by Michael Faraday (Encyclopedia Britannica, 2010; U.S. Department of Health and Human Services, 2007), has become a huge item of commerce. In the year 2004, 3.1 billion gallons of benzene were produced in the U.S, about 45% from catalytic reformats, 30% from toluene and xylene hydrodealkylation, 23% from pyrolysis gasoline and less than 2% from coke ovens (U.S. Department of Health and Human Services, 2007). A limited quantity of benzene was also produced from destructive distillation of coal.

2.1.1 Physical and Chemical Properties of Benzene (Table 2-1)

Benzene, C₆H₆, is a colorless liquid with a characteristic sweet odor (Health Canada, 2009) and is both volatile and flammable (EPA, 1988). It is miscible with polar solvents such as chloroform, acetone, alcohol, and carbon tetrachloride (EPA, 1988). It is relatively soluble in water (Health Canada, 2009). Even though benzene is a highly stable hydrocarbon, it reacts with other chemicals primarily by hydrogen atom substitution (EPA, 1988).
<table>
<thead>
<tr>
<th>Property</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Mass</td>
<td>78.11 g/gmol (92.3% carbon, 7.7% hydrogen)</td>
</tr>
<tr>
<td>CAS Registry Number</td>
<td>71-43-2</td>
</tr>
<tr>
<td>Molecular Structure</td>
<td>![Molecular Structure Image]</td>
</tr>
<tr>
<td>Color</td>
<td>Clear colorless liquid</td>
</tr>
<tr>
<td>Physical State</td>
<td>Colorless to light yellow liquid</td>
</tr>
<tr>
<td>Melting point</td>
<td>5.5˚C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>80.1˚C</td>
</tr>
<tr>
<td>Density at 15˚C</td>
<td>0.8787 g/cm³</td>
</tr>
<tr>
<td>Odor</td>
<td>Aromatic</td>
</tr>
<tr>
<td>Odor Threshold</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>2.0 mg/L</td>
</tr>
<tr>
<td>Air</td>
<td>Detection Range: 34 to 119 ppm, recognition: 97 ppm</td>
</tr>
<tr>
<td>Taste threshold</td>
<td>0.5~4.5 mg/L</td>
</tr>
<tr>
<td>Solubility</td>
<td></td>
</tr>
<tr>
<td>Water at 25˚C</td>
<td>1.79 g/L</td>
</tr>
<tr>
<td>Organic Solvents</td>
<td>Alcohol, chloroform, ether, carbon disulphide, acetone, oils</td>
</tr>
<tr>
<td>Partition coefficient</td>
<td></td>
</tr>
<tr>
<td>Log $K_{ow}$</td>
<td>2.13</td>
</tr>
<tr>
<td>Log $K_{oc}$</td>
<td>1.8~1.9</td>
</tr>
<tr>
<td>Vapor pressure at 20˚C</td>
<td>75 mm Hg (0.098 atm)</td>
</tr>
<tr>
<td>Henry’s law constant at 25˚C</td>
<td>$5.5 \times 10^{-3}$ atm-m$^3$/mol</td>
</tr>
<tr>
<td>Auto ignition temperature</td>
<td>498˚C</td>
</tr>
<tr>
<td>Flashpoint</td>
<td>-11˚C (closed cup)</td>
</tr>
<tr>
<td>National Fire Protection Association (NFPA) hazard classification</td>
<td></td>
</tr>
<tr>
<td>Health</td>
<td>2.2</td>
</tr>
<tr>
<td>Flammability</td>
<td>3.3</td>
</tr>
<tr>
<td>Reactivity</td>
<td>0.0</td>
</tr>
<tr>
<td>Explosive limits in air</td>
<td>LEL = 1.2%, UEL = 7.8%</td>
</tr>
</tbody>
</table>
2.1.2 Industrial Application of Benzene

Historically benzene had been used extensively as a solvent and as a synthetic intermediate for numerous chemicals such as paint strippers, carburetor cleaners, denatured alcohol, rubber cement, carpet glue, textured carpet, liquid detergent and furniture wax (U. S. Department of Health and Human Services, 2007). It has been widely used as a gasoline additive to increase the octane rating.

However, benzene is a known human carcinogen and it has been identified as hazardous air and water pollutant (EPA 1994). Because of its adverse health effects, widespread use of benzene has decreased significantly in recent years, until many of the above-mentioned formulations having it replaced by other chemicals (U. S. Department of Health and Human Services, 2007).

In recent years, the consumer products safety commission (CPSC) found that benzene was no longer used as an intentional ingredient and the benzene levels remaining in consumer products were unlikely to result in significant exposures (U. S. Department of Health and Human Services, 2007). According to the law, any merchandise containing more than 5% benzene, and less than 10% of petroleum distillates (such as benzene), are required to meet specified labeling requirements (U. S. Department of Health and Human Services, 2007). Under the food, drug, and cosmetics act (FDCA), use of benzene in articles intended for packaging, transport, or holding foods is restricted to its being a component of adhesives (FDA, 1977).

Nowadays, benzene is primarily used as a synthetic intermediate (U. S. Department of Health and Human Services, 2007). About 55%, 24%, 12% and 5% of the total benzene
production volume respectively is used to produce ethylbenzene, cumene, cyclohexane and nitrobenzene (U. S. Department of Health and Human Services, 2007). Figure 2-1 illustrates the major commodity and chemicals that are generated from benzene.

Figure 2-1: Major Chemicals and Polymers Derived from Benzene

Styrene, produced from ethylbenzene, is used to make plastics and rubber (HSDB, 2007). Generally, phenol and acetone are produced from cumene. Phenols are widely used in manufacturing of pharmaceuticals, phenolic resins, nylon and rubber production (HSDB, 2007). Acetone is used as a solvent. The cyclohexane generated from benzene is used to produce nylon. Nitrobenzene is used in the production of aniline, urethanes, linear alkylbenzene sulfonates, chlorobenzene, and maleic anhydride (HSDB, 2007). Benzene in also used in the manufacturing of rubbers, lubricants, dyes, detergents, drugs and pesticides (U. S. Department of Health and Human Services, 2007).
2.1.3 Sources of Benzene in the Environment

Benzene has been identified in about 1000 of the 1684 hazardous sites that have been proposed for inclusion on the EPA national priority list (NPL) (HazDat, 2006). It can be present in the environment both from natural and industrial sources. However, natural sources only account for a small amount of benzene released into the air, water, and soil.

Natural sources of benzene in air include forest fires, gas emissions from volcanoes, crude oil leaks, plant volatiles, etc. Anthropogenic benzene emission can result from burning coal and oil, storage and waste operation, gasoline vapors, auto exhaust, chemical production and user facilities and tobacco smoke (U. S. Department of Health and Human Services, 2007). Benzene is also released from hazardous waste sites which have been contaminated by benzene (U. S. Department of Health and Human Services, 2007). Out of 1,684 NPL sites, benzene has been detected in the air of 200 such sites (HazDat, 2006). Other contributing sources of benzene in air include petrochemical and petroleum industries and wastewater treatment plants (Edgerton and Shah, 1992).

Discharge of treated and untreated industrial wastewater, gasoline leaks from underground storage tanks, accidental spills during marine transportation of chemical products, landfill leachate, runoff and seepage from contaminated soils are some major sources of benzene released to water (U. S. Department of Health and Human Services, 2007). Benzene can also be released in water from hazardous waste sites. Out of 1,684 NPL sites, benzene has been detected in the groundwater of 832 and surface water of 208 such sites (HazDat, 2006).
Benzene release to soil can occur due to industrial discharges, land disposal of benzene wastes, gasoline leaks from underground storage tanks, etc. (U. S. Department of Health and Human Services, 2007). Out of 1,684 NPL sites, benzene has been detected in the soil of 145 such sites (HazDat, 2006).

In general, the atmospheric residence time of benzene is only a few days due to chemical degradation reaction with hydroxyl radicals (U. S. Department of Health and Human Services, 2007). Benzene in air can also be deposited on the ground by snow or rain. However, benzene in soil and water breaks down more slowly. In general, benzene in water and air is subject to volatilization, photo-oxidation and biodegradation (U. S. Department of Health and Human Services, 2007). For water-associated benzene, biodegradation under aerobic conditions is an important environmental fate process (U. S. Department of Health and Human Services, 2007).

2.2 Fenton Reaction Products as Pollutants

The controlled Fenton reaction, as a pretreatment, can cause partial oxidation of BTEXs (Zeng et al., 2000) to generate corresponding phenolic compounds (Xu et al., 1995) which can then be removed by enzymatic treatment.

In most of the cases, Fenton reaction on benzene was performed to achieve complete mineralization of the starting material. Studies on partial oxidation of benzene to form the corresponding phenolic compounds are very limited. In many cases the Fenton reaction products are themselves hazardous pollutants. Xu et al. (1995) studied partial oxidation of benzene by employing Fenton reaction and identified phenol and biphenyl as major reaction products. In a separate study, Bremner et al. (2000) also identified phenol as one
of the benzene hydroxylation products. In their study, they also found that some of the phenol generated underwent hydroxylation to produce substituted diphenols like hydroquinone. The hydroquinone generated underwent further oxidation to benzoquinone. Later studies on hydroxylation of phenol generated benzenediols, benzoquinone and some low molecular mass organic acids as reaction products (Zazo et al., 2005; Bremner et al., 2006). The benzenediols are phenol derivatives, the ortho-, meta- and para-hydroxyphenols, commonly known as catechol, resorcinol and hydroquinone, respectively. Among these reaction products, phenol, catechol, hydroquinone, benzoquinone and biphenyl are priority pollutants (EPA, 2010). The Toxics Release Inventory (TRI) of the United States Environmental Protection Agency (US EPA) reported onsite disposal of 5,116,781 lb (2,325,810 kg) of phenol; 15,640 lb (7,109 kg) of catechol; 457,737 lb (208,062 kg) of hydroquinone; 74 lb (33 kg) of benzoquinone and 367,663 lb (167,120 kg) of biphenyl in the year 2009 (EPA, 2010).

2.2.1 Physical and Chemical Properties of Fenton Reaction Products (Table 2-2)

2.2.1.1 Phenol

Phenol (CAS Registry Number 108-95-2) is a mono-substituted aromatic hydrocarbon (EPA, 2002). It is a manufactured chemical as well as a natural substance (ATSDR, 2008). It naturally occurs in some foods, human and animal wastes and decomposing organic materials (EPA, 2002). It is produced endogenously in the digestive tract from the metabolism of aromatic amino acids (EPA, 2002). Phenol is a solid at room temperature and normal atmospheric pressure consisting of clear crystals that turn pink or red when exposed to air and light (WHO, 1994). It has a sweet and tarry distinct odor,
melting point of 43° C and boiling point of 183° C (ATSDR, 2008). The compound is soluble in water and very soluble in most organic solvents (WHO, 1994).

2.2.1.2 Benzenediols

Benzenediols are aromatic compounds in which two hydroxyl groups are substituted onto a benzene ring. There are three isomers of benzenediol. The ortho-isomer (1,2-benzenediol) is known as catechol (CAS Registry Number 120-80-9). The meta-isomer (1,3-benzenediol) is commonly known as resorcinol (CAS Registry Number 108-46-3), and the para-isomer (1,4-benzenediol) is commonly known as hydroquinone (CAS Registry Number 123-31-9). All these isomers are white granular solids at room temperature and pressure.

2.2.1.3 Benzoquinone

Benzoquinone (CAS Registry Number 106-51-4) is an oxidation derivative of hydroquinone. Under normal temperature and pressure, this chemical is in solid yellow crystal form (TOXNET, 1996) with a pungent odour. Benzoquinone is sensitive towards both strong mineral acids and alkali, which cause condensation and decomposition of the compound.

2.2.1.4 Biphenyl

Biphenyl (CAS Registry Number 92-52-4) is an aromatic hydrocarbon having a peculiar characteristic odor (TOXNET, 2005). It appears as colorless leaflets that are practically insoluble in water.
**Table 2-2: Physical and Chemical Properties of Phenol, Benzenediols, Benzoquinone and Biphenyl**

<table>
<thead>
<tr>
<th>Property</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Phenol</strong></td>
</tr>
<tr>
<td>Molecular Mass</td>
<td>94.11 g/mol</td>
</tr>
<tr>
<td>Molecular Structure</td>
<td><img src="image" alt="OH" /></td>
</tr>
<tr>
<td>Color</td>
<td>Colorless to light pink</td>
</tr>
<tr>
<td>Melting point</td>
<td>41 ºC</td>
</tr>
<tr>
<td>Boiling point</td>
<td>181.1 ºC</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>1.071</td>
</tr>
<tr>
<td>Odor</td>
<td>Distinct aromatic, somewhat sickening</td>
</tr>
<tr>
<td>Odor Threshold</td>
<td>7.9 ppm (w/v)</td>
</tr>
<tr>
<td></td>
<td>1 ppm (w/v)</td>
</tr>
</tbody>
</table>
### Table 2-2: Physical & Chemical Properties of Phenol, Benzenediols, Benzoquinone, Biphenyl (continued)

<table>
<thead>
<tr>
<th>Property</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solubility</strong></td>
<td></td>
</tr>
<tr>
<td>Water at 25°C</td>
<td>87 g/L&lt;br&gt;Very soluble in alcohol, chloroform, ether, benzene</td>
</tr>
<tr>
<td>Organic Solvents</td>
<td>430 g/L&lt;br&gt;Soluble in alcohol, ether, acetate, benzene</td>
</tr>
<tr>
<td></td>
<td>1400 g/L&lt;br&gt;Soluble in alcohol, ether, acetic acid, freely sol in ether, glycerol; slightly sol in chloroform</td>
</tr>
<tr>
<td></td>
<td>70 g/L&lt;br&gt;Soluble in ethanol, acetone, carbon tetrachloride, ethyl ether ether</td>
</tr>
<tr>
<td></td>
<td>11.3 g/L&lt;br&gt;(at 25°C)&lt;br&gt;Soluble in alkalies, hot petroleum ether, ethanol</td>
</tr>
<tr>
<td></td>
<td>Not soluble&lt;br&gt;Soluble in ethanol, ethyl ether; very soluble in benzene, carbon tetrachloride, carbon disulfide, and methanol.</td>
</tr>
<tr>
<td><strong>Partition coefficient</strong></td>
<td></td>
</tr>
<tr>
<td>Log $K_{ow}$</td>
<td>1.46&lt;br&gt;1.21-1.96</td>
</tr>
<tr>
<td>Log $K_{oc}$</td>
<td>0.88&lt;br&gt;2.07</td>
</tr>
<tr>
<td></td>
<td>0.80&lt;br&gt;1.81</td>
</tr>
<tr>
<td></td>
<td>0.59&lt;br&gt;1.70</td>
</tr>
<tr>
<td></td>
<td>0.20&lt;br&gt;0.20</td>
</tr>
<tr>
<td></td>
<td>4.01</td>
</tr>
<tr>
<td><strong>Vapor pressure</strong></td>
<td>0.35 mmHg&lt;br&gt;at 25°C&lt;br&gt;0.03 mm Hg at 20°C</td>
</tr>
<tr>
<td></td>
<td>4.89 X 10^{-4}&lt;br&gt;mm Hg at 25°C&lt;br&gt;1.9 X 10^{-3}&lt;br&gt;mm Hg at 25°C</td>
</tr>
<tr>
<td></td>
<td>0.1 mm&lt;br&gt;Hg at 25°C&lt;br&gt;8.93 X 10^{-3}&lt;br&gt;mm Hg at 25°C</td>
</tr>
<tr>
<td><strong>Henry’s law constant at 25°C</strong></td>
<td>4.0 X 10^{-7}&lt;br&gt;atm-m^3/mol&lt;br&gt;1.2 X 10^{-7}&lt;br&gt;atm-m^3/mol</td>
</tr>
<tr>
<td></td>
<td>9.9 X 10^{-11}&lt;br&gt;atm-m^3/mol&lt;br&gt;3.82 X 10^{-11}&lt;br&gt;atm-m^3/mol</td>
</tr>
<tr>
<td></td>
<td>4.79 X 10^{-4}&lt;br&gt;atm-m^3/mol&lt;br&gt;3.08 X 10^{-3}&lt;br&gt;atm-m^3/mol</td>
</tr>
<tr>
<td><strong>Auto ignition temp</strong></td>
<td>715°C&lt;br&gt;510°C</td>
</tr>
<tr>
<td></td>
<td>608°C&lt;br&gt;516°C</td>
</tr>
<tr>
<td></td>
<td>560°C&lt;br&gt;540°C</td>
</tr>
<tr>
<td><strong>Flashpoint</strong></td>
<td>79°C (closed cup)&lt;br&gt;127 °C (closed cup)&lt;br&gt;127 °C</td>
</tr>
<tr>
<td></td>
<td>165 °C&lt;br&gt;38-93 °C (closed cup)&lt;br&gt;113 °C (closed cup)</td>
</tr>
</tbody>
</table>
### Table 2-2: Physical & Chemical Properties of Phenol, Benzenediols, Benzoquinone, Biphenyl (continued)

<table>
<thead>
<tr>
<th>Property</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenol</td>
</tr>
<tr>
<td>National Fire Protection Association (NFPA) hazard classification</td>
<td></td>
</tr>
<tr>
<td>Health</td>
<td>4</td>
</tr>
<tr>
<td>Flammability</td>
<td>2</td>
</tr>
<tr>
<td>Reactivity</td>
<td>0</td>
</tr>
<tr>
<td>Special</td>
<td>corrosive</td>
</tr>
<tr>
<td></td>
<td>LEL = 1.7%, UEL = 8.6%</td>
</tr>
</tbody>
</table>

Phenol information adopted from EPA, 2002; ATSDR, 2008
Catechol Information adopted from EPA, 2007; TOXNET, 2006
Resorcinol Information adopted from TOXNET.2001
Hydroquinone information adopted from TOXNET, 2009
Benzoquinone information adopted from TOXNET, 1996
Biphenyl information adopted from TOXNET, 2005; WHO, 1999

### 2.2.2 Industrial Applications

#### 2.2.2.1 Phenol

Phenol has been used in industry since the 1860s (WHO, 1994). Originally isolated from coal tar, phenol is now produced either by oxidation of cumene or toluene, by vaporphase hydrolysis of chlorobenzene, or by distillation from crude petroleum (WHO, 1994). Currently, phenol is primarily used as an intermediate in the production of phenolic resins (ATSDR, 2008), which are used in the plywood, adhesive, construction, automotive, and
appliance industries (EPA, 2002). It is also widely used in the production of synthetic fibers such as nylon and for epoxy resin precursors such as bisphenol A (EPA, 2002). Due to its toxicity towards bacteria and fungi, it is used as a slimicide, disinfectant and antiseptic (ATSDR, 2008). It is used in medicinal preparations, such as mouth wash, ointments, antiseptic lotions, ear and nose drops, cold sore lotions, throat lozenges and sprays etc (EPA, 2002; ATSDR, 2008). It is found in a number of consumer products as well (ATSDR, 2008).

2.2.2.2 Benzenediols

Catechol is a high production volume (HPV; production or import volume of more than 1 million pounds/yr) chemical; US production volume in 2002 being 10 -50 million pounds (4, 536 – 22, 680 metric tons) TOXNET, 2006). Catechol is widely used in the synthetic intermediates, rubber, chemical, photographic, dye, and oil industries (TOXNET, 2006). It is also used in cosmetics and hair dyes (TOXNET, 2006). Approximately 50% of the total catechol production is used as starting material for insecticides, 35-40% for perfumes and drugs and 10-15% for polymerization inhibitors and other chemicals (TOXNET, 2006).

Resorcinol is used in tanning; manufacturing resins, resin adhesives, hexylresorcinol, p-amino salicyclic acid, explosives, and dyes (TOXNET, 2001). It is used primarily in the rubber industry for tires and reinforced rubber products (conveyer belts, driving belts) and in high-quality wood adhesives (TOXNET, 2001). It is also used in the preparation of dyes and pharmaceuticals (TOXNET, 2001).
Like catechol, hydroquinone is also a HPV chemical. It is used as a reducing agent, antioxidant, polymerization inhibitor, and chemical intermediate (TOXNET, 2009). Many over-the-counter drugs and plant-derived products, including vegetables, fruits, grains, coffee, tea, beer, and wine can contain hydroquinone (TOXNET, 2009). Hydroquinone is used in photographic applications, dyes and pigments and agricultural chemicals production (TOXNET, 2006).

### 2.2.2.3 Benzoquinone

Benzoquinone is primarily used for hydroquinone production (TOXNET, 1996). It is also used as a chemical intermediate, a polymerization inhibitor, an oxidizing agent, a photographic chemical, a tanning agent, and a chemical reagent (TOXNET, 1996).

### 2.2.2.4 Biphenyl

Biphenyl is used in organic syntheses, heat transfer fluids, dye carriers and as an intermediate for polychlorinated biphenyls (WHO, 1999). It is used in plant disease control (TOXNET, 2005). Paper impregnated with biphenyl is used in citrus packing to reduce fruit damage by fungus during shipment and storage (TOXNET, 2005).

### 2.2.3 Sources in the Environment

#### 2.2.3.1 Phenol

Phenol has been found in at least 595 of the 1,678 National Priority List (NPL) sites identified by the Environmental Protection Agency (EPA) (ATSDR, 2008). It is a component of oil refinery wastes. It is also produced in the conversion of coal into
gaseous or liquid fuels and in the production of metallurgical coke from coal (NPI, 2010). Phenol may enter the environment from oil refinery discharges, coal conversion plants, municipal waste treatment plant discharges, industrial effluents or spills (NPI, 2010).

### 2.2.3.2 Benzenediols

The benzenediols are widely produced and/or consumed by various industrial processes (Masuda et al., 2001, Kumar et al., 2003) and these are generally present in the process water of such industries as chemical intermediates, petroleum refineries, pulp and paper mills, pharmaceutical, cosmetic, dye and resin manufacturing plants (Shakir et al., 2008). Depending on the type of industry, raw material used and process conditions employed, the effluent concentration of benzenediols can vary from hundreds to thousands of mg/L (Kinsley et al., 2000). For example, effluents generated from synthetic coal fuel conversion processes may contain catechol and resorcinol concentrations up to 1000 mg/L (Phutdhawong et al., 2000). However, these industries are required to meet a maximum allowable discharge limit, ranging from 0.1 to 5.0 mg/L, in their effluents (Cooper et al., 1996). Therefore, removal of such organic pollutants from wastewater is of great importance.

### 2.2.3.3 Benzoquinone

Benzoquinone is released to the environment during commercial use and production (TOXNET, 1996). Wasterwaters from the coal industry also contain this chemical (TOXNET, 1996). Benzoquinone can be present in the environment from the natural sources as well. It occurs naturally in a variety of arthropods (TOXNET, 1996). Many insects also synthesize this chemical (TOXNET, 1996).
2.2.3.4 Biphenyl

Biphenyl can end up in the environment from both natural and anthropogenic sources. Certain plants and algae can synthesize it (TOXNET, 2005). It is directly released to the environment during the combustion of biomass, coal, oil, plastics, refuse, rubber, and wood (TOXNET, 2005). It can also end up in the environment due to its various uses. It is a product of coal gasification, natural gas production and textile mill processes (TOXNET, 2005). It has been detected in water from Lake Ontario, Mississippi River and Merrimack River, MA (TOXNET, 2005). It was also identified in the ground water samples adjacent to wood-preserving chemical manufacturing facilities, underground coal gasification units, coal-tar distillation units etc (TOXNET, 2005).

2.2.4 Adverse Effects

2.2.4.1 Phenol

Phenol may enter the body by inhalation, ingestion or skin exposure (NPI, 2010). Exposure to high levels of phenol can cause skin burns, liver damage, dark urine, irregular heartbeat, and even death. Ingestion of concentrated phenol can produce internal burns (ATSDR, 2008). The International Agency for Research on Cancer (IARC) and the EPA have determined that phenol is not classifiable as to its carcinogenicity to humans (ATSDR, 2008). Chronic exposure to phenol can cause vertigo, digestive difficulties, skin eruptions, nervous problems and headaches (NPI, 2010). It can cause birth defects (ATSDR, 2008). It is toxic for aquatic organisms (WHO, 1994). Acute exposure to phenol can cause death of animals, birds, or fish, and death or low growth rate in plants (NPI, 2010). Reduced lifespan, reproductive disorders, lower fertility, changes in
appearance or behavior is caused by long term exposure to phenol (NPI, 2010). Phenol does not bioaccumulate significantly (WHO, 1994). The environmental concern level for phenol in the aquatic environment is 0.02 µg/liter (WHO, 1994).

### 2.2.4.2 Benzenediols

These phenolic compounds are toxic and, in many cases, bio-refractory (Kumar et al., 2003) and pose carcinogenic or mutagenic potential and/or act as endocrine disruptors (Steevensz et al., 2009). The benzenediols like catechol and hydroquinone are more toxic than phenol. As little as 50 µg/L of catechol can induce changes in functionality of red blood cells in humans, whereas it takes 250 µg/L of phenol to cause a similar effect (Bukowska and Kowalaska, 2004). Like phenol, catechol and hydroquinone are also responsible for inducing cell transformation and causing genotoxic effects (TOXNET, 2009). All three benzenediols can cause DNA damage (TOXNET, 20009). Catechol has been identified as a possible human carcinogen (Group 2B) (TOXNET, 2006). Hydroquinone has been identified as “immediately dangerous to life or health”. As low as 50 mg/m$^3$ hydroquinone in air can cause severe health damage (TOXNET, 2009).

### 2.2.4.3 Benzoquinone

Benzoquinone can be absorbed into the body by inhalation, or by ingestion. It can irritate eyes, skin and respiratory tract (TOXNET, 1996). Chronic dermal contact to benzoquinone in humans may result in skin ulceration, while chronic inhalation exposure may result in visual disturbances (TOXNET, 1996). EPA has not yet established a carcinogenicity of this compound. No information is available on the reproductive or developmental effects of this quinone in humans or animals (TOXNET, 1996).
2.2.4.4 Biphenyl

Biphenyl exposure can be by inhalation, skin exposure and ingestion. Acute exposure to high levels of biphenyl can cause eye and skin irritation. It can result in toxic effects on the liver, kidneys, and central and peripheral nervous systems (TOXNET, 2005). Exposure to this chemical can cause headache, gastrointestinal pain, nausea, indigestion, numbness and aching of limbs, and general fatigue (TOXNET, 2005). The USEPA classifies biphenyl in Group D. This means it is not classifiable as to human carcinogenicity due to no human data and inadequate studies in mice and rats (TOXNET, 2005).

2.2.5 Conventional Treatment Methods

The methods used for the treatment of these phenolic compounds include aerobic and anaerobic biodegradation, chemical oxidation with ozone, adsorption by ion-exchange resin and activated carbon and adsorptive micellar flocculation (Kumar et al., 2003, Shakir et al., 2008). In recent years, government regulations require removal of specific substrates down to the specific limits. Conventional biological processes may not be capable of pollutant removal to the desired level, especially for bio-refractory chemicals.

2.3 Fenton Reaction

Oxidative destruction of organic pollutants, an apt solution for the treatment of hazardous wastes, can be achieved by biological, chemical and physical/thermal means (Huang et al., 1993). However, in the last two decades chemical oxidation processes have become more prominent due to their ability to destroy toxic, recalcitrant and biologically
refractory organic pollutants in aqueous solution (Duesterberg et al., 2008). These oxidation processes utilize reactive oxygen species, mainly in the form of highly reactive and nonselective hydroxyl radical (HO•), to oxidize organic pollutants (Duesterberg et al., 2008). Such processes are commonly known as advanced oxidation processes (AOPs). The Fenton reaction, one of the common AOPs used today, encompasses reaction of hydrogen peroxide with iron (II) to form the active oxygen species (Huang et al., 1993; Pignatello, et al., 2006). It is known to be very effective in destruction of many hazardous organic pollutants in water (Neyens and Baeyens, 2003).

2.3.1 Background

The history of Fenton chemistry dates back to 1894 (Pignatello, et al., 2006). In that year, Henry J. Fenton reported that ferrous iron strongly promotes oxidation of tartaric acid by hydrogen peroxide (Walling, 1975, Pignatello, et al., 2006). His subsequent work demonstrated that the combination of hydrogen peroxide and a ferrous salt, commonly known as “Fenton’s reagent”, is an efficient oxidant for extensive array of organic substrates (Walling, 1975). However, due to the unselective nature of the Fenton process, it was not accepted widely in organic chemistry until fifty years after its discovery (Huang et al., 1993). Since then, Fenton and related chemical processes have become of great interest for their relevance to biological chemistry, synthesis, chemistry of natural waters and the treatment of hazardous wastes (Pignatello, et al., 2006).

In 1934, Haber and Weiss proposed that the actual oxidant generated in the Fenton system was the hydroxyl radical (Walling, 1975; Pignatello, et al., 2006). The potency of an oxidant is reflected in its oxidation-reduction potential, $E^\circ$. The hydroxyl radical has a
high standard oxidation potential ($E^0 = 2.73 \text{ V}$) and demonstrates higher reaction rates as compared to other conventional oxidants (Huang, 1993, Pignatello, et al., 2006, Bautista et al., 2008). In the early 1950s, Barb, Baxendale, George and Hargrave revised and expanded upon the original mechanism proposed by Haber and Weiss on the decomposition of hydrogen peroxide (Pignatello, et al., 2006). They proposed the “classical” or “free radical” Fenton chain reaction which was initiated by the hydroxyl radical production (Pignatello, et al., 2006). Shortly after this, it was proposed that high-valent oxoiron complexes also might take part in the Fenton reaction (Pignatello, et al., 2006). However, the work published by Walling (1975) reinforced the free radical pathway concept over others and renewed interest in the Fenton process (Pignatello, et al., 2006).

The treatment of hazardous pollutants by using the Fenton reaction began in the late sixties (Huang et al., 1993). However, the use of the Fenton reaction during that time was limited to small scale applications (Pignatello, et al., 2006). Academic research on the application of the Fenton chemistry to waste treatment started in 1990 (Pignatello, et al., 2006). The Fenton reagent has been tested on a wide array of synthetic wastewaters containing different target pollutants. Some of these organic pollutants include phenol, chlorophenols, 2,4-dinitrophenol, 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, chlorobenzene, tetrachloroethylene, halomethanes, amines, etc. (Bautista et al., 2008). In addition to synthetic wastewater studies, the Fenton process has been successfully applied to chemical, pharmaceutical, textile, pulp and paper, cosmetics, food and cork processing industry effluents (Bautista et al., 2008). It has also been used to decontaminate soil,
landfill leachate and sludge (Bautista et al., 2008). The major advantages of treating hazardous wastes by the Fenton reagent are:

- Both hydrogen peroxide and iron are considered as inexpensive, nontoxic, easy to handle and safe (Jiang et al., 2010),
- Due to the homogeneous catalytic nature of the process, mass transfer limitation is not an issue (Huang et al., 1993),
- In the case of mineralization, complete destruction of contaminants to harmless compounds such as carbon dioxide, water and inorganic salts is achieved (Neyens and Baeyens, 2003),
- In the case of pretreatment, significantly reduced toxicity, better biodegradability and color and odor removal are achieved in the resulting effluent (Bautista et al., 2008),
- The reactor design is much simpler than for other AOPs (Huang et al., 1993),
- As the reaction takes place at ambient temperature and atmospheric pressure, there is no energy input required to activate the hydrogen peroxide (Bautista et al., 2008), and
- Requires relatively short reaction time (Bautista et al., 2008).

However, there are certain disadvantages associated with the unmodified (i.e. -mineralizing) Fenton reaction as well:

- Limited control over the reaction due to the unselective nature of the hydroxyl radical (Neyens and Baeyens, 2003),
• Recycling of catalyst (i.e., ferrous iron) is not always successful. Hence, further addition of ferrous iron is required due to continuous loss of soluble iron from the system (Bremner et al., 2000),
• There is the necessity of an aqueous medium in which solubility of many of the organic contaminants is limited (Bremner et al., 2000), and
• Removal of iron after the treatment process adds to the cost (Bautista et al., 2008).

2.3.2 Mechanism of the Fenton Reaction

The Fenton reaction is a homogeneous process which is based on generation of hydroxyl radicals from hydrogen peroxide and iron ions at acidic pH (at around pH 3.0) and ambient conditions (Bautista et al., 2008). The mechanism for decomposing hydrogen peroxide in acidic solution, involves a complex reaction sequence (Neyens and Baeyens, 2003), starting with production of hydroxyl radical in the presence of ferrous iron (Equation 1). This reaction is also known as the chain initiation reaction.

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^- \text{ (chain initiation)} \]  \hspace{1cm} (1)

The hydroxyl radical oxidizes a second ferrous iron molecule (Equation 2), a chain termination reaction (Huang et al., 1993).

\[ \text{OH}^\cdot + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{Fe}^{3+} \text{ (chain termination)} \]  \hspace{1cm} (2)

The newly formed ferric irons may consume more hydrogen peroxide in a reaction (Equation 3) referred to as “Fenton-like reaction” (De Laat and Gallard, 1999) to form ferrous iron and superoxide radicals (\( \text{HO}_2^\cdot \) or \( \text{O}_2^- + \text{H}^+ \)).
Fe^{3+} + H_{2}O_{2} \rightarrow Fe^{2+} + HO_2^\cdot + H^+ \tag{3}

In the Fenton system, even hydrogen peroxide itself can act as hydroxyl radical scavenger (Equation 4) and generate water and superoxide radical.

OH^\cdot + H_{2}O_{2} \rightarrow H_2O + HO_2^\cdot \tag{4}

This superoxide (HO_2^\cdot) radical participates in radical chain reactions by reducing ferric to ferrous ion (Equation 5) or by oxidizing ferrous iron to ferric ion in absence of organic compounds (Equation 6).

Fe^{3+} + HO_2^\cdot \rightarrow Fe^{2+} + O_2 + H^+ \tag{5}

Fe^{2+} + HO_2^\cdot \rightarrow Fe^{3+} + H_2O_2 \tag{6}

If an organic substrate (RH) is present in the system, then the decomposition mechanism competes with the organic substrate for available active oxidant, hydrogen peroxide (Bishop et al., 1968). The reactions of hydroxyl radical and organic compounds lead to the formation of carbon centered radicals (Pignatello et al., 2006). The oxidation of the organic compound itself can proceed via addition of hydroxyl radical (Equation 7) or via abstraction of hydrogen atom (Equation 8) (Huang et al., 1993). In general, the addition of hydroxyl group produces hydroxylated products. Whereas, products generated by hydrogen atom abstraction are oxidized products (Huang et al., 1993). Here, Reaction 8 is irreversible reaction whereas Reaction 7 is a reversible one. In this reaction (Equation 7), a hydroxycyclohexadienyl radical (RHOH^\cdot) is formed as a result of hydroxyl radical attack on an aromatic ring (Pignatello, et al., 2006).

RH + OH^\cdot \Leftrightarrow RHOH^\cdot \rightarrow \text{hydroxylated products} \tag{7}
RH + OH′ → R′ + H₂O → oxidized products .........................................................(8)

If air is present in the solution, the aromatic radicals (for example, R′) generated in Reactions 7-8 may react with the dissolved molecular oxygen and generate HO₂ ′ (or O₂ ′−) (Equation 9), peroxyl radicals (R-OO′) or oxyl radicals (R-O′) (Equation 10) (Pignatello, et al., 2006).

R′ + O₂ → →RH′ + HO₂ ′ .................................................................(9)

R′ + O₂ → R-OO′→ → R-O′ ..............................................................(10)

The radicals generated (R′, R-OO′, R-O′) may couple (Equation 11), disproportionate to stable molecules or may react with iron ions (Equations 12-13) (Huang et al., 1993; Pignatello, et al., 2006).

R′ + R′→ R-R (dimerization) .............................................................(11)

Fe³⁺ + R′→ Fe²⁺ + R⁺ (oxidation) ...........................................................(12)

Fe²⁺ + R′ + H⁺ → Fe³⁺ + RH (reduction) ..............................................(13)

In the conventional Fenton process, organic intermediates generated in the first stage of the oxidation process can further react with hydroxyl radicals and eventually produce harmless products such as carbon dioxide (CO₂) and water (H₂O) (Neyens and Baeyens, 2003; Pignatello, et al., 2006). If the target pollutant contains heteroatoms, then along with CO₂ and H₂O some inorganic acids might also be generated (Pignatello, et al., 2006). Studies indicate that the rate of disappearance of initial compound is faster than
production of CO$_2$ and the reaction rate generally decreases as the products/intermediates become less and less reactive with hydroxyl radical (Pignatello, et al., 2006).

The sequence of Equations 1,2,7,8 and 12 comprise the currently accepted scheme for the conventional Fenton reaction (Neyens and Baeyens, 2003). These equations are repeated in the section below for convenience, with R replaced by A (notation for aromatic compound) since that the focus of this study.

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\bullet \quad \text{(chain initiation)} \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \q
mostly on the hydroxyl radical, the non-chain reaction takes place (Huang et al., 1993). In
the non-chain oxidation process, a considerable amount of hydroxyl radical is lost by
reaction between ferrous ion and hydroxyl radical (Equation 14) (Huang et al., 1993).

\[ \text{Fe}^{2+} + \text{OH}^- \rightarrow (\text{Fe-OH})^2+ \]

The reaction between ferrous iron and hydroxide ions can form ferric hydroxo complexes
as well (Walling and Kato, 1971; Lin and Lo, 1997, Neyens and Baeyens, 2003). These
iron complexes account for the coagulation capability of the Fenton reaction (Neyens and
Baeyens, 2003). Large amounts of small flocs are normally visible in the Fenton
oxidation steps which at times take overnight to settle out (Neyens and Baeyens, 2003).
In such cases, chemical coagulant is necessary to achieve good coagulation. Between pH
3 and 7, the number of iron flocs increases with the pH increase.

It should be noted that most of the literature on understanding the Fenton reaction
mechanism was done under low pH (at around pH 3.0) and reagent conditions which are
substantially different from those frequently required in the real wastewater treatment
(Yoon et al., 2001). Furthermore, most of the literature covers the conventional Fenton
process which focuses on successful chain reactions and aims for complete
mineralization of the substrate. Literature on the controlled Fenton reaction, which seeks
to cause partial oxidation of aromatic compound and limit the process to generation of
phenolic compounds only, is rare. The forgoing synopsis serves to illustrate the potential
complexity of the Fenton reaction in chain- vs. non-chain reaction, aromatic vs. aliphatic
compound, between substrate mineralizing vs limited reaction, etc. The following section
will focus on situations pertaining to limited Fenton reaction of aromatic substrates under non-chain conditions.

The only literature which reported conversion of benzene to the corresponding phenolic compounds using a conventional Fenton system (ferrous salt and hydrogen peroxide) reported that the best phenolic compound yield was achieved at a pH of 5.4 and \([\text{benzene}]_{\text{initial}} : [\text{Fe}^{2+}]_{\text{initial}} : [\text{H}_2\text{O}_2]_{\text{initial}}\) of 1:1:1 (Xu et al., 1995). According to this study, the products resulting under these conditions were phenol and biphenyl. Based on this result, it can be concluded that production of phenolic compounds is favored at a higher pH range which is outside the optimum pH range (at around pH 3.0) of the conventional Fenton reaction when mineralization of substrate is attempted. The stoichiometric iron requirement also indicates that the predominating conversion of iron occurs from the ferrous to the ferric state and most likely the oxidation of benzene was carried out by a non-chain Fenton reaction. As discussed in the previous section, in a non-chain reaction regeneration of ferrous iron is negligible and all oxidation process mostly depends on the hydroxyl radical (Huang et al., 1993). Hence, the predominant reactions in the non-chain Fenton reaction are Reactions 1, 2, 4, 7, 8 and 14.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \quad (\text{chain initiation}) \quad (1)
\]

\[
\text{OH}^- + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{Fe}^{3+} \quad (\text{chain termination}) \quad (2)
\]

\[
\text{OH}^- + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{HO}_2^- \quad (4)
\]

\[
\text{AH} + \text{OH}^- \rightarrow \text{AHOH}^- \rightarrow \text{hydroxylated products} \quad (7)
\]

\[
\text{AH} + \text{OH}^- \rightarrow \text{A}^- + \text{H}_2\text{O} \rightarrow \text{oxidized products} \quad (8)
\]
Based on the results of Xu et al., (1995) and current preliminary studies, it is expected that the limited oxidation of benzene to phenolic compounds will follow a non-chain Fenton reaction pathway.

2.3.3 Effect of Reaction Parameters on the Fenton Process

In the Fenton process, the overall reaction efficiency is determined by its reagent conditions and reaction characteristics. The production and consumption of OH•, the key feature of the Fenton process, is influenced by reagent conditions which include Fe^{2+}, Fe^{3+} and H_{2}O_{2} concentrations, reaction characteristics like pH, quantity of organic and inorganic constituents and the mutual relationships among these parameters (Neyens and Baeyens, 2003).

2.3.3.1 Effect of pH

For peroxide oxidation or polymerization systems, pH is the most important variable (Bishop et al., 1968). Hydroxyl radical is identified as the active oxidizing species in the commonly accepted mechanism for the Fenton process at low pH (Duesterberg et al., 2008) (Equation 1). The hydroxyl radical can react with ferrous iron, hydrogen peroxide or any aromatic compound present in the solution at that pH (Duesterberg et al., 2008). The reaction between ferrous iron and hydrogen peroxide extends the reaction process by producing superoxide and its conjugate acid (Equation 4) or by reducing ferric ion to ferrous iron (Equation 5). It can terminate the chain reaction by oxidizing ferrous ion (Equations 2 and 6). It can also terminate the chain reaction by oxidizing ferrous iron or
scavenging available hydroxyl radical (Walling, 1975). Ferric ion can also produce ferrous iron and superoxide (Equation 3) (De Laat and Gallard, 1999; Duesterberg et al., 2008). However, the reaction rate for Equation 3 is generally much smaller than the reaction rates for Equations 4 and 5 under acidic conditions (Duesterberg et al., 2008). In a non-chain reaction system, Equations 15-17 can act as additional minor chain termination pathways which result in unproductive decomposition of superoxide or hydroxyl radicals (Duesterberg et al., 2008).

\[
{\text{HO}_2^\cdot + \text{HO}_2^\cdot} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]  
\[\text{15}\]

\[
{\text{OH}^\cdot + \text{HO}_2^\cdot} \rightarrow \text{H}_2\text{O} + \text{O}_2
\]  
\[\text{16}\]

\[
{\text{OH}^\cdot + \text{OH}^\cdot} \rightarrow \text{H}_2\text{O}_2
\]  
\[\text{17}\]

The hydroxyl radical may be generated stoichiometrically via Reaction 1. However this also produces stoichiometric amount of Fe (III) which may precipitate from the solution. As the pH of the Fenton process increases from acidic to neutral pH, Fe (III) removal increases and recycling of iron decreases.

From the mechanism of the Fenton reaction, it is evident that the efficiency of the Fenton system in generating active hydroxyl radical oxidant largely depends on the catalytic cycling of iron between ferrous and ferric states for which pH plays a very important role (Duesterberg et al., 2008). For the Fenton oxidation process, it essentially means that there has to be sufficient dissolved iron present in the solution to carry out the chain propagation and the reaction between hydroxyl radical and other solutes should be able to continue process as well (Kwan and Voelker, 2002). If there is not sufficient dissolved
iron present in the solution the Fenton reaction will most likely go through a non-chain reaction process in order to carry out the oxidation process and a stoichiometric amount of reagents will be required. Even though the Fenton reaction has been studied extensively, there is still ongoing debate about the pH effects on iron catalysis and oxidation performance.

The accepted Fenton reaction (chain reaction process) takes place at pH of 3.0 or lower. Oxidative degradation of organic contaminants in the Fenton process usually gives optimal results below pH 3.0 (Pignatello et al., 2006). The decomposition rate for hydrogen peroxide reaches its maximum at pH 3.5 (Huang et al., 1993). Studies show that the decomposition rates in the ferric/hydrogen peroxide system decrease dramatically at pH values greater than 3.0 (De Laat and Gallard, 1999; Kwan and Voelker, 2002). According to Pignatello et al. (2006), this type of result is dictated by speciation of ferric ion. Pignatello et al. (2006) also expressed the opinion that the presence of ligands can influence the pH dependence of the Fenton reaction considerably. Below pH 3.0, the majority of the ferrous iron will be Fe$^{2+}$ (Pignatello et al., 2006). Ferrous salts are quite soluble in water even at the neutral pH (Pignatello et al., 2006). However, if the solution contains both ferrous and ferric hydroxo ions together and the pH of the solution is raised above pH 3.0, the ferrous ion tends to co-precipitate with the ferric hydroxo ion (Pignatello et al., 2006). The presence of hydrolyzed species can be identified by the turbidity and/or slightly yellow-orange color of the solution (Pignatello et al., 2006).

Contrary to popular claim, the initiation reaction (Equation 1), is not optimum at pH 3.0 (Pignatello et al., 2006). It is independent of pH below pH 3.0 and at about pH 4.0 the reaction rate is seven times greater than that at pH 3.0 (Pignatello et al., 2006). Pignatello
et al. (2006), attributed this phenomenon to the formation of Fe(OH)$_2$ at that pH, which is roughly ten times more reactive than Fe$^{2+}$. The commonly claimed pH optimum of the Fenton process is due to the pH effects on Fe(III) speciation (Pignatello et al., 2006) and formation of colloids (De Laat and Gallard 1999).

Studies done by Kwan and Voelker (2002) demonstrated that sufficient dissolved Fe(III) may be present at pH 3.0 – 5.0 range. This essentially raises the possibility of effectively propagating the chain reaction at pH range of 4.0- 5.0. For this reason, it is possible to achieve decomposition of hydrogen peroxide and organic compounds in that pH range. They speculated that, at higher pH range, Fe(III)- hydroperoxy complexes became more important contributors towards the chain reaction. Bishop et al, (1968), also demonstrated that the maximum oxidation efficiency occurred in a pH range of 3.0 to 5.0. However, they also mentioned that, as the dissolved iron continued to precipitate at higher pH range, additional reactions might have taken place to interfere with the chain propagation.

In their study, Jiang et al. (2010) found pH sensitivity for both Fenton reaction (ferrous iron added to initiate the reaction) and Fenton-like reaction (ferric iron added to initiate the reaction). However, their findings indicated that the Fenton-like processes were more pH sensitive than the Fenton process itself. The optimum pH values for the Fenton process were in the pH range of 2.5-6.0. Whereas, for the Fenton-like process, the optimum pH occurred within the pH range of 2.8-3.8. As the pH is raised above 3.0, the ferric ion generated starts precipitating and around neutral pH, hydrogen peroxide breaks down to water and oxygen (Szpyrkowicz et al., 2001). Formation of Fe (II) complexes at high pH values leads to a drop of available ferrous concentration in the system (Benitez
et al., 2001). But the regeneration of the ferrous iron by reaction of ferric ion and hydrogen peroxide becomes more inhibited at these pH values (Bautista et al., 2008).

The applicability of the Fenton reaction can be increased by broadening the pH range of the reaction. Studies indicate that iron oxide/hydrogen peroxide systems can effectively oxidize pollutants at pH values ranging from 3.0 to circumneutral (Kwan and Voelker, 2002; Watts et al., 1997). Though a consensus on the mechanism of such process does not exist, it is expected that the reactions like Equations 1 and 3, take place on the iron oxide surface (Kwan and Voelker, 2002).

2.3.3.2 Effect of Iron

Ferrous iron is the catalyst used in the classic Fenton’s procedure. However, many other materials, both soluble and particulate, have been used to catalyze hydrogen peroxide decomposition (Watts and Teel, 2005). Soluble ferrous iron is the most effective catalyst when hydrogen peroxide concentration is not high. On the other hand, ferric catalyst system (Fenton-like system) is more suitable when hydrogen peroxide concentration is fairly high (Watts and Teel, 2005). Inter-conversion of ferrous and ferric species in the Fenton system drives the catalytic process.

Even though soluble iron forms are the most efficient catalysts for the Fenton system, there are certain disadvantages as well:

- An acidic pH is necessary to keep the iron in solution. This is generally done by addition of sulfuric acid (Watts and Teel, 2005).
• Shortly after the contact with hydrogen peroxide, the soluble iron precipitates as an amorphous iron oxide floc which is an inefficient form of catalyst (Tyre et al., 1991).

To overcome the pH dependence on the iron solubility, numerous other catalysts have been studied. Some of the common forms of the alternative catalysts are iron-bearing solid heterogeneous catalysts such as, iron-chelate complexes, iron oxides, zeolites, pillared clays, alumina, silica, mesoporous molecular sieves, niobia, activated carbon, etc. (Bautista et al., 2008). Some of the common iron chelate complexes are: iron (III)-nitrilotriacetic acid (NTA), iron (III)-hydroxyethyliminodiacetic acid (HEIDA), iron (II)-ethylenediamine tetraacetic acid (EDTA), iron (II)-hydroxyethylethylenediamine triacetic acid (HEDTA), etc. (Watts and Teel, 2005; Pignatello et al., 2006). It has been reported that, if iron powder is used in place of iron salts in the Fenton process, 50% reduction in the iron sludge can be achieved (Lucking et al., 1998). However, this study explored only application of soluble iron salts in the Fenton process. Hence, this section is limited to soluble iron systems.

In general, when ferrous iron was added as the soluble iron form, Fe(II) was converted to Fe(III) rapidly and the degradation of the aromatic compound was also fast (Jiang et al., 2010). Results of this study indicated that the Fenton system was much faster than a Fenton-like system. Researchers believe that this phenomenon is mostly due to the fact that in a classical Fenton system, Fe(II) reacts with hydrogen peroxide directly to generate hydroxyl radical (Equation 1). Thus a rapid release of hydroxyl radical occurs, which causes the initial rapid degradation of pollutants (Pignatello et al., 2006).
The concentration of hydrogen peroxide and ferrous ion are two relevant, closely related factors affecting the Fenton process (Bautista et al., 2008). Depending on the ferrous iron and hydrogen peroxide ratio, the Fenton reaction can either have effects of chemical coagulation or oxidation (Neyens and Baeyens, 2003). When the concentration of ferrous ion exceeds the amount of hydrogen peroxide, the process tends to have effects of coagulation. Whereas, when hydrogen peroxide is used in higher concentration than ferrous iron, the reactions work as a chemical oxidation process.

When aromatic pollutants (RH) are present in the system, they only influence the behavior of ferrous iron. The aromatic compounds compete with excess ferrous iron for hydroxyl radical (Neyens and Baeyens, 2003). So, Equation 2 competes with Equations 7 and 8; and at the high Fe(II) concentration, the iron acts as a stoichiometric reactant, not as a catalyst (Yoon et al., 2001). The presence of excess Fe(II) also prevents secondary reactions involving Fe(III) (Gallard and De Laat, 2001).

When the concentrations of Fe(II) and hydrogen peroxide are almost equal in the Fenton system, the reaction greatly depends on the oxidation state of iron initially added and the major oxidation state of iron present in the system (Neyens and Baeyens, 2003). Under these reaction conditions, there is a sudden drop in the concentration of ferrous ion due to the initiation reaction (Equation 1) (Yoon et al., 2001). This reaction also produces a considerable amount of ferric ion which now takes part in the subsequent reactions. Hence the Fenton reaction in the ferric phase proceeds via ferric ion induced reaction (Equation 3) in order to produce hydroxyl radical (Equation 1) (Yoon et al., 2001).
2.3.3.3 Effect of Hydrogen Peroxide

Hydrogen peroxide (H$_2$O$_2$) is a strong oxidant having standard potential of 1.80 and 0.87V respectively at pH of 0 and 14 (Neyens and Baeyens, 2003). Because of this phenomenon, acidic conditions are applied for oxidative treatment of wastewaters using hydrogen peroxide (Lucking et al., 1998). The concentration of hydrogen peroxide mainly depends on the initial pollutant concentration (Bautista et al., 2008). It is a common practice to use an amount of hydrogen peroxide corresponding to the theoretical stoichiometric hydrogen peroxide to chemical oxygen demand ratio (Lucking et al., 1998). However, the concentration and ratio should also depend on the objective pursued in terms of the reduction of contaminant load and the response of the target pollutant towards the oxidation process (Bautista et al., 2008).

Another important parameter for the effectiveness of hydrogen peroxide is the temperature. The reaction rates generally increase when the temperature increases. However, it also favors degradation of hydrogen peroxide to water and oxygen. The rate of such decomposition within 20-100°C range increases about 2.2 times with each 10°C increase in temperature (Bautista et al., 2008). This indicates that, even though Fenton reaction rates might increase with temperature, the availability of hydrogen peroxide in the system might diminish.

Typically, the concentration of ferrous and ferric ion at any instant depends on the hydrogen peroxide concentration (Pignatello et al., 2006). In general, complete mineralization or pre-treatment of pollutants is attempted in the Fenton process. In such cases, significant amounts of hydrogen peroxide are used. In water treatment, typical
peroxide-to-iron molar ratios lie in the range of 100 to 1000 (Pignatello et al., 2006). Studies on low hydrogen peroxide concentration in the Fenton system are limited.

When the hydrogen peroxide is present in the Fenton system in excess, all initially added Fe(II) is rapidly oxidized to Fe(III) and a large amount of hydroxyl radicals are generated from the initiation reaction (Pignatello et al., 2006). When hydrogen peroxide is present in large stoichiometric excess, the Fenton system displays two-stage kinetics: a) a fast stage which is attributed to the initiation reaction (Equation 1) and b) a rate-limiting step due to Equation 3 (Gallard and De Laat, 2000). The contribution of the fast stage depends on the molar ratio of target compound to the starting Fe(II) concentration (Pignatello et al., 2006). At high hydrogen peroxide concentration, reduction of iron by \( \text{HO}_2^- \) (\( \text{O}_2^- \)) is more favourable (Pignatello et al., 2006). Thus \( \text{HO}_2^- \) propagates the Fenton reaction by generating either Fe(II) or hydrogen peroxide (Pignatello et al., 2006).

For moderate hydrogen peroxide concentration in the Fenton system, the presence of aromatic compound impacts the decomposition of hydrogen peroxide. There is a rapid decrease in hydrogen peroxide concentration due to the production of hydroxyl radical via Equation 1 (Neyens and Baeyens, 2003). After that, no further degradation of hydrogen peroxide is observed since the aromatic compound present in the solution reacts with the hydroxyl radical and reaction between hydroxyl radical and hydrogen peroxide occurs (Yoon et al., 2001). If aromatic pollutant is present at higher concentration than the concentration of hydrogen peroxide, the reaction between ferrous ion and hydroxyl radical (Equation 2) can be hindered (Yoon et al., 2001).
2.3.3.4 Effect of the Structure of Hazardous Compound

In the Fenton system, fates of the target organic compound and its reaction byproducts depend on their reaction with the hydroxyl radicals (Pignatello et al., 2006). Hydroxyl radicals mainly react with the target pollutant by abstracting hydrogen atom or adding to the unsaturated bonds (Equations 7-9). Based on their behavior, Walling and El-Taliawi (1973) categorized the organic radicals produced as a result of reaction between hydroxyl radical and organic compounds in three distinct classes: a) those oxidized by Fe$^{3+}$ (Equation 13); b) those which are inert and dimerize (Equation 12); and c) those which undergo reduction by Fe$^{2+}$ (Equation 14).

Highly reactive and indiscriminate hydroxyl radical appears to be weakly electrophilic (Anbar et al., 1966). Other than the electrophilic nature of hydroxyl radical, there are some other factors that can influence the process. Pignatello et al. (2006) listed them as: strength of the C-H bond, stability of the organic radicals generated, number of equivalent H atoms or positions of attack and steric effects. Some of these factors are interrelated. For example, the strength of C-H bond is generally inversely related to the stability of organic radicals (Pignatello et al., 2006). In the case of hydroxyl radical reaction, effect of pH, solvent, etc. are expected to be minor (Pignatello et al., 2006).

The chemistry of hydroxyl radical reaction adducts to the aromatic compound is complicated (Walling and Johnson, 1975). Anbar et al. (1966) concluded that, the mechanism of hydroxyl radical attack on aromatic compound is analogous to that of an electrophilic substitution. They also concluded that, the rate-determining step in such attacks is similar to the addition of the electrophilic reactants on the aromatic ring.
However, the substituent effects on the Fenton reaction are not always consistent with electron-withdrawing ability of the substituent or the electrophilic nature of the hydroxyl radical (Pignatello et al., 2006). In most of the cases, hydroxyl radical attack on aromatic compound occurs via addition, which yields to hydrooxycyclohexadienyl radicals (Walling and Johnson, 1975).

For substituted aromatics, it is possible that the hydroxyl radical can attack the substituent itself and not the ring (Pignatello et al., 2006). The Fenton reaction on aromatic compounds has revealed side chain cleavage reactions as well (Walling and Johnson, 1975). Studies by Snook and Hamilton (1974) indicated that the reaction between hydroxyl radical and aromatic ring was much faster than hydrogen abstraction reaction from the side chain.

The organic radical generated by reaction between hydroxyl radical and aromatic compound can undergo oxidation, dimerization or reduction (Walling and Johnson, 1975). Studies have indicated that in a Fenton system, the concentration and nature of the intermediate radical generated can greatly depend on the pH of the reaction (Walling and Johnson, 1975). In order to obtain ring cleavage products from the Fenton system, acidic conditions are a must (Walling and Johnson, 1975).

In several cases, it has been found that the rate of reaction depends on the substituent pattern on the ring as well. Eisenhauer (1964) observed that in general the greater the degree of substitution the slower was the reaction rate. Under Fenton reaction conditions, hydroxylation easily occurred in the ortho- and para- positions of the ring with the lowest hydroxylation yield in the meta- position (Chen and Pignatello, 1997). The distribution of
phenolic isomers (generated from the hydroxylation of the aromatic compounds), depends on reaction conditions (Walling and Johnson, 1975).

The intermediates generated in the Fenton process can also influence the behavior of the reaction parameters. The reaction of hydroxyl radicals with aromatic rings generates hydroxycyclohexadienyl radicals. These radicals can potentially form quinone molecules (Chen and Pignatello, 1997). By using an electron-shuttle mechanism, the quinone can take part in the hydroxylation of aromatic compounds (Equation 18) (Pignatello et al., 2006).

\[ R \cdot H + H_2O_2 \xrightarrow{\text{Fe(III)}} \text{quinone} \rightarrow R \cdot OH + H_2O \]

(18)

2.3.3.5 Influence of Inorganic Ions

The Fenton system is extremely sensitive to the presence of anions (Lu et al., 1997). Fenton oxidations of organic compounds were inhibited by phosphate, sulfate, organosulfonate, fluoride, bromide, and chloride ions (Pignatello et al., 2006). Inhibition by these anions could be due to precipitation of iron, scavenging of hydroxyl ion or formation of a less reactive iron complex (Pignatello et al., 2006).

Phosphate ions in particular seriously suppress the oxidation capacity of the Fenton system by producing a less-reactive complex with iron. For example, Lu et al. (1997) found that in the presence of 0.2 M phosphate, the chain initiation reaction (Equation 1) remained unaltered, but the ferric ion catalyzed reaction (Equation 3) completely stopped due to the Fe(III)-phosphate ion complex formation.
Even though sulfate ions are poor scavengers of hydroxyl radical, they too potentially can inhibit the Fenton reaction (Pignatello et al., 2006). The reasons for sulfate inhibition could be: a) sulfate ions forming complexes with both Fe(II) and Fe(III); b) Fe(II), Fe(III) sulfato complexes might have different chemical properties than free iron species, etc. For example, a spectrophometric study revealed that hydrogen peroxide did not react with the Fe(III) sulfato complex (De Laat and Le, 2005). However, Pignatello et al., (2006) concluded that the millimolar concentrations of sulfate resulting from the iron sulfate salt had a small effect on the Fenton reaction system.

2.4 Enzyme-based Wastewater Treatment

2.4.1 Use of Enzymes in Wastewater Treatment

In wastewater treatment, isolated enzymes were first used in the 1930s (Munnecke, 1976). Many researchers have subsequently studied the applicability of various oxidoreductases, such as laccases and peroxidases, in the removal of aromatic pollutants from aqueous solution (Bollag et al., 1980, Aitken, 1993, Masuda et al., 2001, Biswas et al., 2007, Modaressi et al., 2005). These oxidoreductases can be classified into two groups: peroxidases and oxidases. Peroxidases like horseradish peroxidase (HRP), soybean peroxidase (SBP), and Arthromyces ramosus peroxidase (ARP) catalyze the oxidation of aromatic pollutants in the presence of hydrogen peroxide as the oxidant, while oxidases, like laccase, use molecular oxygen for the same purpose.

In enzymatic treatment, the enzyme works in reverse of conventional biological treatment: enzymatic treatment involves buildup of target compounds through oxidative polymerization, whereas the biological treatment breaks down the target compound (Saha
et al., 2008). In enzyme-catalyzed oxidation, the phenolic substrate undergoes oxidation, which produces an aryloxy radical. These aryloxy radicals undergo chemical coupling to generate dimeric and thence oligomeric derivatives through subsequent enzyme cycles (Baratto et al., 2006). The resulting polymers are generally insoluble in water and can be removed by filtration or sedimentation (Torres et al., 2003).

Peroxidases have been successful in removing aromatic compounds such as phenols, anilines, naphthols, benzidines, biphenol, diphenylamine, napthylamine, etc. from wastewater (Klibanov et al., 1980; Taylor et al., 1998). Klibanov et al. (1980) had first proposed removal of thirty different phenols and aromatic amines from water using HRP. The study showed good removal efficiency of the pollutants. Today peroxidases have proven to be successful in removing pollutants from wastewater including the pollutants which have large complex structure. They can be used in industrial sectors as well. For example, they can be used for biocatalysis, on-site waste destruction, wastewater treatment, solid remediation and for bleaching in the pulp and paper industry (Ikehata et al., 2006). Economical production of these enzymes is possible because of their wide availability among organisms and large-scale industrial applications. Extensive work has been done on peroxidase enzyme application and their reaction mechanism (Nakamoto and Machida, 1992; Nicell and Wright, 1997; Taylor et al., 1998; Villalobos and Buchanan, 2002; Masuda et al., 2001).

Industrial application of laccases is a relatively new concept as compared to peroxidases. Increasing availability of these biocatalysts and improved biochemical knowledge about this type of enzyme has been useful in initiating new technological applications. Three large industrial processes, dye bleaching in the textile industry, bio-bleaching of lignin in
the pulp and paper industry and the bleaching of cork for bottled wine, were using laccases at the end of 2005 (Claus, 2002; Duran et al., 2002; Riva, 2006).

2.4.2 Laccase

Laccase (E.C.1.10.3.2, p-benzenediol: oxygen oxidoreductase) is a cuproprotein belonging to a small group of enzymes identified as blue copper oxidases (Duran et al., 2002). It is a green catalyst that requires oxygen and produces water as the only byproduct (Riva, 2006). This class of cupro-proteins performs four-electron reduction of oxygen to water along with one-electron oxidations of four substrate molecules (Solomon et al., 1996). It has low substrate specificity and can remove simple phenols, diphenols, substituted polyphenols, aromatic amines and benzenethiols (Yaropolov et al., 1994). Their effectiveness in removal of phenolic pollutants has been established (Torres et al., 2003). A typical laccase molecule is 60-80 kDa, of which 15-20% is carbohydrate comprised of mannose, galactose, hexoseamine, glucose, arabinose, and fucose residues (Shaw and Freeman, 2004). The protein constituent of these enzymes contains 520-550 amino acid residues (Thurston, 1994).

The catalytic lifetime of laccase depends on the source (Duran et al., 2002). The first reported source of laccase was the resin ducts of the liquor tree Rhus vernicifera (Riva, 2006). Today, laccase has been discovered in many other sources. Depending on the source type, laccase can be classified mainly into two categories, namely, plant and fungal laccases (Ikehata et al., 2006). Laccases are commonly present in higher plants and fungi (Thurston, 1994). Recently some bacterial strains like Azospirillum lipoferum, Alteromonas sp. have been reported as sources of laccase (Alexandre and Zhulin, 2000). Currently Trametes species are keenly researched for laccase production (Ikehata et al.,
This specie is a natural wood decomposer which is usually available in most parts of the world. A large amount of laccase production has been reported from *T. versicolor* which has already been marketed by several companies (Ikehata et al., 2006). For this study, fungal laccase from *Trametes villosa*, a developmental preparation from commercial enzyme producer, Novozymes, has been used.

### 2.4.2.1 Active Site of Laccase

Spectroscopy along with crystallography has provided the details of the active site of laccase (Duran et al., 2002). Four copper atoms are the central redox feature. The classification of these copper atoms based on their electron paramagnetic resonance (EPR) is: Type 1 (T1) or blue Cu, Type 2 (T2) or normal Cu and Type 3 (T3) or coupled binuclear Cu sites (Quintanar et al., 2005).

The mononuclear T1 site extracts electrons from the reducing substrate and mediates their transfer to the trinuclear T2/T3 center where molecular oxygen is reduced (Bertrand et al., 2002). X-ray absorption spectroscopy and magnetic circular dichroism (MCD) of laccase have shown that Type 2 and Type 3 coppers act as a trinuclear copper cluster during exogenous ligand interaction and reaction interaction with dioxygen (Cole et al., 1990). Type 1 copper is three-coordinate, with two histidines and a cysteine ligand (Bertrand et al., 2002). The Type 2 center is three-coordinate with two histidines and water as ligands (Duran et al., 2002). Type 3 coppers are each four-coordinate, with three histidine ligands and bridging hydroxide (Sundaram et al., 1997; Palmer et al., 1999). The active site of laccase is presented in Figure 2-2.
2.4.2.2 Laccase Reaction Mechanism

The proposed reaction mechanism for laccase is a “two-site ping-pong bi-bi” reaction mechanism, which suggests that products are released before the binding of new substrates (Piontek et al., 2002). After a complete catalytic cycle, one molecule of oxygen is reduced to form two molecules of water. During the formation of water molecules, simultaneous oxidation of substrates produces four radicals, which might undergo non-enzymatic coupling to produce dimers, oligomers or polymers (Riva, 2006).

Reduction of dioxygen by laccase occurs in two 2e⁻ steps. First, the fully reduced laccase site reacts with oxygen to produce a peroxide level intermediate, which is also known as
a bridged hydroperoxide species, bridging the T2 and one of the T3 coppers (Solomon et al., 1996). In the second step, further reduction of this peroxide-level intermediate generates the native “intermediate”, which is also described as a hydroxide product (Solomon et al., 1996). The first step is rate-determining whereas the second step is fast. Reduction of this native intermediate state generates the resting state of the enzyme. Figure 2-3 represents the proposed mechanism for oxygen reduction to water by multicopper oxidases.

Figure 2-3: Proposed mechanism for 4e- reduction of oxygen to water by multicopper oxidase (adopted from, Solomon et al., 1996)
The transfer of an electron from the substrate to the initial electron acceptor site (Type 1 site) is rate-determining for the turnover. The reduction mechanism for oxidized laccase is complex (Solomon et al., 1996). Figure 2-4 provides a summary of the catalytic cycle of laccase.

**Figure 2-4: Catalytic Cycle of Laccase** (adopted from, Solomon et al., 1996)

First the electron from substrate reduces the T1 site of the native intermediate state of enzyme. In this state, the trinuclear copper cluster can access the electron by two possible
mechanisms (Solomon et al., 1996). The first mechanism proposes that electron transfer happens from the T1 site to T2 site and the T1 site gets rereduced. The T1 and T2 transfer two electrons to T3 and T1 gets rereduced. The T1 transfers its electron to T2 and gets rereduced. This generates a fully reduced form of enzyme. The second mechanism proposes that the trinuclear copper cluster gets sequentially reduced by three one-electron transfer steps from the T1 site. But the sequence by which the coppers get reduced is not known (Quintanar et al., 2005).

In short, according to the first mechanism, the T3 pair is reduced by T1 and T2 copper; whereas, the second mechanism suggests that there is a sequential one-electron transfer to the three coppers of the trinuclear site from the T1 site. But the latter mechanism suggests that the T3 site does not work as a two-electron acceptor. The native intermediate state of enzyme is slowly transformed into the fully oxidized resting form, in which the T1 site can still be reduced by the substrate but the electron transfer to the trinuclear site is too slow to be of catalytic importance (Solomon et al., 1996).

### 2.4.3 Soybean Peroxidase

Soybean peroxidase (SBP) belongs to the superfamily of class III plant peroxidases that can oxidize a wide variety of organic pollutants (Henriksen et al., 2001). Soybean seed coats are a rich source of this peroxide (Gillikin and Graham, 1991). Since the seed coat of the soybean is a byproduct of the food industry, soybean hulls could be a cheap source of SBP (Kinsley and Nicell, 2000). SBP is very stable at high temperature, pH extremes and in organic solvents (Welinder and Larsen, 2004). It is also very reactive towards organic and inorganic substrates (Welinder and Larsen, 2004). Because of its high
stability and activity, it has attracted considerable biotechnological attention in recent years.

SBP, a heterogeneous glycoprotein, has a molecular mass of ≈ 37 KDa (Henriksen et al., 2001). The protein consists of 306 residues (Welinder and Larsen, 2004). It also contains four disulfide bonds, two calcium-ion binding sites located distal and proximal to the active site, eight glycans and a single tryptophan (Trp 117) residue (Kamal and Behere, 2002). The secondary structure of the enzyme consists of 13 α-helices and 2 β-strands (Henriksen et al., 2001; Welinder and Larsen, 2004).

2.4.3.1 Active Site of SBP

The active site of SBP contains three polymer chains, each of which has a Fe(III) protoporphyrin IX (heme) as the prosthetic group (Kamal and Behere, 2003). This prosthetic group (Figure 2-5) is common for all peroxidases (Dunford, 1999).

![Figure 2-5: Structure of the heme in peroxidases](image)

Figure 2-5: Structure of the heme in peroxidases (adopted from Al-ansari et al, 2010)
The iron in the active site of peroxidase can have six possible ligands (Dunford, 1999). Positions 1 to 4 are occupied by four pyrrole nitrogen atoms. Position 5 is located on the proximal side of heme. It is occupied by the imidazole side chain of a histidine residue (His 169). The histidine residue is linked to the Fe(III) by a covalent bond. Peroxidase reaction occurs in position 6. It is actually located on the distal side of heme. The histidine residue (His 42) located at this site acts as a proton acceptor from hydrogen peroxide. The Arg 38 residue acts as a charge stabilizer. In native resting enzyme, except for position 6, all other positions are filled up (Dunford, 1999).

2.4.3.2 Peroxidase Reaction Mechanism

Peroxidase-catalyzed oxidation of phenolic compounds with hydrogen peroxide generates aryloxl radicals which diffuse from the active site of the enzyme into the solution and react non-enzymatically to form higher-order polymers. Peroxidases follow a modified bi-bi ping-pong mechanism (Figure 2-6; Dunford, 1999) according to which the native form of the enzyme (E₀) is oxidized by hydrogen peroxide. The active enzyme resulting from this process is commonly known as Compound I (E₁) (Equation 27). Compound I is capable of oxidizing aromatic compounds (AH) like phenols and amines and converting those compounds to free radicals (A•) (Equation 28). During this process, Compound I gets converted to Compound II, another active form of enzyme. In the subsequent step of the cycle, Compound II oxidizes another aromatic compound to the free radical (Equation 29). In this process, Compound II gets reduced to the native enzyme form. The free radicals formed in this cycle can react with each other to form dimers which are further
oxidized by peroxidase to form higher order oligomers and polymers until these polymers are no longer enzyme substrates as the solubility limit is reached (Wu et al, 1998).

Figure 2-6: Proposed Mechanism for Peroxidase Enzyme (adopted from Dunford, 1999)

Native peroxidase (E₀) + H₂O₂ → Compound I (E₁) + H₂O .............................. (19)

Compound I (E₁) + AH → Compound II (E₂) + A• ................................. (20)
\textbf{Compound II (E_2)} + AH $\rightarrow$ Native peroxidase (E_0) + A$^\cdot$ + H$_2$O

\[ \text{(21)} \]

Compound I formation follows an “an electron push-pull mechanism” (Figure 2-6; Dunford, 1999, Al-Ansari et al., 2010). The histidine and arginine residues on the distal side of the heme (position 6) play a significant role in the formation of Compound I. In the active site of SBP, the distal histidine accepts a proton from the hydrogen peroxide and the arginine acts as a charge stabilizer (Dunford, 1999). The negative charge on the peroxide and protonation of the proximal histidine results in the formation of the Fe-OOH intermediate. The proton transfer from the distal histidine to the departing hydrogen peroxide facilitate the formation of the ferryl (Fe$^{IV}$=O) group.

In the formation of Compound II, the reducing substrate acts as a hydrogen atom donor. The electron of the donated hydrogen atom goes to the porphyrin ring, whereas, the proton is accepted by the imidazole side chain of distal His42. Thus, this process also generates a free radical (A$^\cdot$) from the oxidized substrate and Compound II (Dunford, 1999).

The reduction process of Compound II to the native enzyme state is complicated (Dunford, 1999). In this process, both the distal histidine residue and the reducing substrate provide one proton each. The electron transfer from the ferryl bond reduces Fe(IV) to Fe(III). The ferryl oxygen and the proton from the reducing substrate form a
new bond and in the process another free radical (A\textbullet) and a water molecule formed (Dunford, 1999). This brings the enzyme back to the native state.

**2.5 Coagulation and Precipitation**

Coagulation and flocculation are the processes wherein compounds such as metal salts are added to the effluent to destabilize the colloidal material and cause the aggregation of small particles into larger, more easily removble floc. Coagulants are used to remove color and particular COD from the wastewater (Nemerow, 1978). The effectiveness of the process depends on factors like coagulating agent, coagulant concentration, pH, nature and concentration of the organic compound (Randtke, 1988).

Common coagulation and precipitation aids used to remove enzyme-catalyzed reaction end products are alum, polyethyleneimine (PEI), chitosan, anionic, nonionic or cationic polymers (Wada et al., 1995; Caza et al., 1999; Mantha et al., 2002). At the same time, surfactants such as sodium dodecyl sulfate (SDS) also have been proven efficive in removing reaction end products via adsorptive micellar flocculation (Saha et al, 2008). In the present work, quinone generated from the Fenton and the enzymatic reaction was removed by using PEI and chitosan. The quinones can be chemisorbed onto these sorbents (Sun and Payne, 1996). The polymeric end products generated from the enzymatic treatment were removed by alum treatment. In all cases, the coagulation process is expected to perform best at an optimum pH or pH range which mainly depends on nature of coagulat aid.
2.5.1 Chitosan

Chitosan, natural polymer of glucosamine, is made from chitin (Wada et al., 1993). Chitin, a straight-chain polysaccharide composed of β-1, 4-linked N-acetyl-D-glucosamine residues, is a constituent of the hard shells of crustaceans. So, chitosan is abundantly available in shellfish waste (Takahashi et al., 2005). Chitosan demonstrates interesting characteristics such as biocompatibility, biodegradability and its degradation products are non-toxic, non-immunogenic and non-carcinogenic (Alves and Mano, 2008). It has a number of applications in the commercial, biomedical and wastewater treatment area (Wada et al., 1993; Takahashi et al., 2005; Alves and Mano, 2008). Figure 2-7 represents a typical chitosan molecule.

![Structure of Chitosan Molecule](adopted from Takahashi et al., 2005)

Wada et al., (1995) have demonstrated that quinones in wastewater can undergo a nucleophilic condensation with chitosan. In this process, the lone electron pair from nitrogen in an amino group of chitosan can easily attack the quinone and form a C-N double bond. Chitosan is normally not soluble in water (Alves and Mano, 2008), thus, the quinone removal efficiency greatly depends on the surface chemistry (Sun and Payne, 1996). However, quinone-containing flakes can be easily separated by sedimentation and
filtration. Chitosan solution (made in the presence of acid) is more efficient in quinone removal (Wada et al., 1995; Sun and Payne, 1996), however, an appropriate coagulant aid is required to remove the colored condensation products.

### 2.5.2 Polyethyleneimine (PEI)

PEI is a synthetic cationic coagulant aid. This highly branched chemical contains primary, secondary and tertiary amine groups. The repeating chemical unit is -(CH₂CH₂NH)-. A typical PEI structure is presented in Figure 2-8 (degree of protonation depends on pH). PEI is used in protein purification from feed stock, immobilization of biocatalysts, as a soluble carrier for enzymes and affinity legands, color removal from wastewater etc. (Andersson and Hatti-Kaul, 1999).

![PEI Structure](image)

**Figure 2-8: PEI Structure (Adopted from Biswas, 2004)**

Like the chitosan-quinone reaction, PEI also reacts with quinones by forming a carbon-nitrogen double bond (Wada, et al., 1995). Alum is generally used to remove the quinine-PEI complex. Alum in water forms a gel which entraps these substrate PEI complexes and
precipitates them from solution (Biswas, 2004). PEI demand may vary depending on the substrate structure.

2.5.3 Alum

The polymeric products generated from the enzymatic treatment of phenolic compounds are generally hydrophobic in nature and in many cases can be removed by sedimentation and filtration. However, in other cases enzymatic treatment of phenolic compounds generates colored effluent which is unacceptable for discharge. It is speculated that the color generated in these cases may result from quinone-like products remaining in the solution (Nicell et al, 1993). It is also possible that the products generated as a result of radical coupling would not precipitate due to the presence of high number of hydrophilic and polar functional groups. Alum can remove a wide range of water contaminants because it can remove the pollutants by “charge neutralization” or “adsorption on aluminum hydroxide gel”. The effectiveness of this process is pH, pollutant type and concentration dependent.
CHAPTER 3
MATERIALS AND METHODS

The experimental procedures and analytical techniques used in the study are presented in this chapter.

3.1 Materials

3.1.1 Aromatic compounds

The aromatic compounds, benzene, phenol, catechol, resorcinol, hydroquinone, p-benzoquinone and biphenyl, having purity of 98% or better, were purchased from Aldrich Chemical Co. (Milwaukee, WI).

3.1.2 Enzymes

Laccase SP504 (EC 1.10.32, batch # 1999-00091-03 and -04, 200 LACU/mL), a fungal laccase from Trametes villosa, and ARP (SP-502, activity 2000 U/mL, Rz value of ≈0.5), a developmental preparation, were gifts from Novozymes North America, Inc. (Franklinton, NC). SBP (E.C. 1.11.7, Industrial Grade lot #18541NX, Rz value of 0.75 ± 0.10; activity of ≈ 5 U/mg) was obtained from Organic Technologies (Coshocton, OH). The Rz value can be described as the optical purity index of the peroxidase. It is the absorbance ratio of A403/A275. It is a measure of hemin content of the peroxidase, not enzyme activity. Catalase from bovine liver (E.C. 1.11.1.6, lot #120H7060, 19,900 U/mg) was purchased from Sigma Chemical Company Inc. (St. Louis, MO).

A unit of activity is defined as the number of micromoles of substrate converted per minute under standard conditions (given in Sections 3.3.1 and 3.3.2, below). The
enzymes were stored at -15°C. A sub-stock solution was prepared from it and was stored at 4°C.

3.1.3 Colorimetric Assay Reagents

Syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine), 4-aminoantipyrine (4-AAP) and potassium ferricyanide, were purchased from Sigma Chemical Co. (St. Louis, MO). Syringaldazine was stored in a desiccator at 2-4 °C. Hydrogen peroxide (30. % w/v) was purchased from BDH Inc (Toronto, ON) and stored at 4.0°C.

3.1.4 Additives

Polyethylene glycol (average molar mass of 3350 g) was purchased from Sigma Chemical Co. (St. Louis, MO). Polyethyleneimine (PEI) (50 wt % solution in H₂O) having an average MM of 750,000 (lot no: 14520PR) and chitosan (practical grade, ≥75% deacetylated from shrimp shell) were obtained from Sigma Aldrich (Milwaukee, WI). Sodium dodecyl sulphate (SDS) was obtained from Sigma Chemical Company Inc. (St. Louis, MO).

3.1.5 Buffers and solvents

Analytical grade monobasic and dibasic sodium phosphate, sodium acetate were purchased from BDH (Toronto, ON). Glacial acetic acid, hydrochloric acid, sulphuric acid and 95% ethanol were purchased from ACP Chemicals Inc. (Montreal QC). Sodium borate, boric acid crystals were obtained from Fisher Scientific Co. (Fair Lawn, NJ). MES (2-(N-morpholino) ethanesulfonic acid) > 99.5 % purity (pH range 5.5-6.7) was purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific Co. (Fair Lawn, NJ).
3.1.6 Other Chemicals

Iron (II) sulfate heptahydrate (FeSO₄•7H₂O, 99%) and alum as aluminum sulphate (Al₂(SO₄)₃.16H₂O), lot no:14238, were obtained from BDH (Toronto, ON). Iron standard for atomic absorption spectroscopy was made from the stock solution (made from 1025 µg/mL in 1 wt. % HCl) purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used for this study were of analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ) and BDH (Toronto, ON).

3.2 Equipment

3.2.1 UV-VIS Spectroscopy

Solution absorbances were measured by a Hewlett Packard Diode Array Spectrophotometer (Model 8452A), wavelength range 190-820 nm, with 2 nm resolution. The spectrophotometer was controlled by a Hewlett Packard Vectra ES/12 computer. A quartz cell with 10 mm path length was purchased from Hellma Limited, Concord, ON., to measure the absorbance.

3.2.2 HPLC Analysis

For standardization of the chemicals, high performance liquid chromatography (HPLC) was carried out on a system from Waters Co. (Milford, MA). It had a model 2487 dual wavelength absorbance detector, model 1525 binary HPLC pump and model 717 autosampler. A C₁₈ (5 µm, 4.6 X 150 mm) column was used for this study. The Waters System was operated by Breeze software.
3.2.3 Total Organic Carbon (TOC) Analysis

A Shimadzu TOC-V CSH Total Carbon Analyzer, purchased from Shimadzu Scientific Instruments (Columbia, MD), was used to measure the carbon content of the solution. This analyzer used oxygen as an oxidizing agent. The TOC-V CSH Total Carbon Analyzer was calibrated following the procedures mentioned in the operation manual. Potassium hydrogen phthalate (2125 mg/L) was used as the standard for the Total Carbon (TC) solution. For Inorganic Carbon (IC) standard solution, a mixture of sodium hydrogen carbonate (3500 mg/L) and sodium carbonate (4410 mg/L) was used. The carbon concentration in these solutions corresponds to 1000 mg/L TC and IC carbon, respectively.

3.2.4 Atomic Absorption Spectroscopy

Iron concentration was analyzed with a Varian- Spectra AA 55B atomic absorption spectrometer. The lamp current was 5 mA, oxidant was air and acetylene was used as fuel for the samples.

3.2.5 pH Measurement

The pH was measured with an IQ 200 pH meter obtained from IQ Scientific (London, ON). It was fitted with ion-sensitive field-effect transistor (ISFET) probe (p=205, pH 15-ss, 57084). Calibration buffers (pH 4.0, 7.0, 10.0) were obtained from BDH Inc. (Toronto, ON.).
3.2.6 Other Equipment

Plastic syringes were purchased from Becton Dickinson & Co, (Clifton, NJ). Whatman (No 42) filter paper was used for coarse filtration. For microfiltration, 0.2 μm HT Tuffryn membrane filters from Gelman Labs (Mississauga, ON) were used. Micro V magnetic stirrers (0-1100 rpm, model 4805-00) and VWR MAGSTIRRER (100-1500 rpm, model 82026-764) were purchased from VWR International Inc. (Mississauga, ON.). Magnetic stir bars were obtained from Cole-Parmer (Chicago, IL.).

3.3 Analytical Techniques

3.3.1 Laccase Activity Assay

Syringaldazine, a unique substrate for laccase, was used to measure the enzyme activity. Under aerobic conditions, syringaldazine (4-hydroxy-3, 5-dimethoxybenzaldehyde azine) was oxidized to the corresponding quinone. One unit (LACU) of laccase activity at pH 5.5 is defined as the amount of enzyme required for the conversion of 1.0 μmol of syringaldazine/minute. All components were provided in sufficient quantity so that the rate of reaction became directly proportional to enzyme activity (Felby, 1998). The rate of reaction was measured by measuring the rate of formation of colored products. These pink colored products absorbed light at a peak wavelength of 530 nm (Felby, 1998; Vermette et al., 2000). Increase in absorbance at 530 nm determined the enzyme activity. Details of this assay are presented in Appendix A.

3.3.2 SBP Activity Assay

Catalytic activity (U) of SBP is defined as number of micromoles of H₂O₂ utilized in one minute at pH 7.4 and at temperature of 25°C. The SBP activity was determined by
monitoring the initial rate of color formation resulting from the oxidative coupling of phenol and 4-AAP in the presence of hydrogen peroxide. In order to ensure that the initial rate of reaction was directly proportional to the concentration of the enzyme present, phenol, hydrogen peroxide and 4-AAP were used in excess (at saturating concentrations where possible) and SBP was used in limited quantity. The pink chromophore generated in the reaction had an absorption maximum at 510 nm and an extinction coefficient of 6000 M⁻¹cm⁻¹ relative to hydrogen peroxide. A detailed description of this assay is presented in Appendix B.

3.3.3 Aromatic Compound Concentration Assay

HPLC was used to measure the concentration of the aromatic compounds and standard curves were constructed for each compound. Isocratic elution with 20:80 (v/v) acetonitrile: 0.1% acetic acid was monitored at 280 nm for phenol, resorcinol, catechol, hydroquinone and benzoquinone. Isocratic elution with 37:63 (v/v) acetonitrile: 0.1% acetic acid was monitored at 254 nm for benzene. For biphenyl, isocratic elution with 70:30 (v/v) acetonitrile: 0.1% acetic acid was monitored at 280 nm. For the concentration of phenolic compounds in a reaction mixture, a gradient elution of acetonitrile: 0.1% acetic acid ranging from 20:80 (v/v) to 37:63 (v/v) at dual wavelengths of 254 nm and 280 nm was used. Flow rate and injection volumes for all the samples were 1.0 mL/min and 10µL, respectively. The column was not heated.

3.3.4 Analysis of Insoluble Products

Most of the Fenton reaction products were soluble in water. However, biphenyl had very limited solubility in water and hence precipitated out of solution easily. A 60:40 (v/v)
mixture of acetonitrile and water was used to identify it. This mixture was stirred vigorously to dissolve the biphenyl dissolved. A control study was performed first to ensure that biphenyl is dissolvable in the 60:40 (v/v) mixture of acetonitrile and water. This 60% acetonitrile solution was used for identification and quantification of biphenyl.

3.3.5 Buffer Preparation

Buffers were prepared according to Gomori (Gomori 1955). The pH values ranged from 3.0-9.0 for this study. Acetic acid - sodium acetate buffer were used for the pH range from 3.0-5.5. Monobasic -dibasic sodium phosphate buffer was used for pH 5.6-7.5. Bicarbonate buffers were used for higher pH (8.0-9.0).

3.3.6 Total Organic Carbon (TOC)

TOC analysis was performed on the Fenton reaction samples to measure the carbon content of the solution. Samples were acidified and microfiltered prior to making the 100 µL sample injection. Separate standard curves were prepared for all the aromatic compounds, chitosan and PEI.

3.4 Experimental Procedure

The experimental procedures for the current study are presented in the following sections. The batch reactors were set up in triplicates and the results of triplicate analyses are presented in chapter four as averages, with error bars representing the standard errors.
3.4.1 Process Parameter Optimization for Laccase and SBP-Catalyzed Removal of Phenolic Compounds

Batch reactors were set up to study the effect of pH, laccase concentration and substrate concentration over a fixed reaction period of three hours, unless noted, at approximately 22°C. The study was designed to achieve at least 95% removal of aromatic compounds (phenol, catechol, resorcinol and hydroquinone) by optimizing pH and enzyme concentration. The effect of PEG on the removal of these substrates was also investigated. The exact values for the appropriate reaction parameters have been provided in the appropriate sub-sections under section 4.1 and 4.2. Twenty-millilitre, open, stirred batch reactors contained synthetic samples in 40 mM buffer. Laccase was added to initiate the reaction. After the reaction period, each reaction mixture was quenched with 0.5 M sulphuric acid to lower the pH to 2.0. Samples were filtered through 0.2 μm HT Tuffryn membrane filters and analyzed for residual aromatic compound by HPLC.

Previous studies had already determined the optimum conditions for SBP-catalyzed removal of 1 mM phenol, catechol, resorcinol and hydroquinone individually (Caza et al., 1999, Al-Ansari et al., 2009). When appropriate, the findings of those studies were utilized.
3.4.2 Process Parameter Optimization of Fenton Reaction on Benzene

Batch reactors were set up to study the effect of pH, [Fe2+], [H2O2] and reaction time at room temperature, approximately 22°C. Sealed 35-mL batch reactors used for the study contained a buffered solution of benzene (6 mM) along with various concentrations of Fe2+. Hydrogen peroxide was added to initiate the reaction and the reactors were mixed vigorously with Teflon-coated stir bars and a magnetic stirrer. After an appropriate reaction period, the reaction mixture was quenched with sodium hydroxide and catalase. The addition of base brought the pH to 7.0 and most of the Fe2+ present in the solution was converted to Fe3+ which precipitated out of the solution. The samples were quenched with excess catalase to a concentration of 250 U/mL to ensure that there was no residual H2O2 left in the samples. Samples were filtered through 0.2 μm HT Tuffryn membrane filters and analyzed for residual benzene and production of phenolics by HPLC. The exact values for the appropriate reaction parameters have been described in the sub-sections under section 4.5.

3.4.3 Different Batch Reactor Configurations for the Fenton Pre-treatment and Enzymatic Treatment

Different types of reactor configuration were studied to achieve the best possible condition to convert the starting benzene to corresponding phenolics and to remove those phenolics using enzyme.
3.4.3.1 Process Parameter Optimization for Enzymatic Removal of the Fenton Reaction Products (Two-Stage, Two-Reactor System)

Two separate reactors were used to carry out the reactions. In the first, sealed batch reactor, the Fenton reaction was carried out under optimum conditions. After the Fenton pre-treatment reaction was stopped as described in section 3.4.2. The iron sludge was allowed to settle and the supernatant of the mixture was used for the enzymatic treatment. The supernatant of this batch reactor was used for the enzymatic treatment. The pH adjustment was done according to the optimum pH of the enzyme of choice.

For laccase-catalyzed removal of the Fenton reaction products, the effects of pH and laccase concentration over a fixed reaction period was monitored at room temperature. Enzyme was added to initiate the reaction in the open batch reactors. Whereas, for SBP-catalyzed removal of the Fenton reaction products, the effects of pH and SBP and hydrogen peroxide concentrations over a fixed reaction period were monitored at room temperature. Hydrogen peroxide was added to initiate the reaction. The exact values for the reaction conditions and parameters are provided in the section 4.7.

The reactants were mixed with Teflon-coated stir bars and a magnetic stirrer. After an appropriate reaction period, the laccase reaction mixtures were quenched with sulfuric acid and SBP reaction mixtures were quenched with excess catalase to a concentration of 250 U/mL to ensure that there was no residual H$_2$O$_2$ left in the samples. Samples were filtered as above and analyzed for residual phenolics, benzoquinone and biphenyl concentrations by HPLC.
3.4.3.2 Simultaneous Fenton and Enzymatic Reactions (Single Batch Reactor)

Both Fenton and enzymatic reactions were carried out simultaneously in the same sealed batch reactor. Other than single addition of all the reactants required for both Fenton and enzymatic reactions, in this reactor configuration step addition of hydrogen peroxide, ferrous iron and enzyme was also attempted. After a three-hour reaction period, the reaction was quenched for Fenton reaction and both the enzymes by following the procedures described in Section 3.4.3.1.

To facilitate presence of sufficient oxygen for laccase reaction, water with dissolved oxygen (by bubbling air for 24 h) was used in the laccase-containing batch reactors. The appropriate reaction conditions and reaction parameter values are presented in section 4.8.

3.4.4 Process Parameter Optimization for Removal of Benzoquinone Using Additives

Although benzoquinone is not an enzyme substrate, it can be removed by using additives like chitosan and PEI. After enzymatic reaction, chitosan flakes, chitosan solution and PEI were individually used to remove the benzoquinone generated. The effects of pH, additive concentration and reaction time were monitored to determine the optimum conditions for use of these additives. After appropriate reaction period, the samples were withdrawn and filtered through 0.2 μm HT Tuffryn membrane filters and analyzed for residual benzoquinone concentration by HPLC. The direct absorbances of the reaction mixture were monitored at 424 nm. The appropriate reaction parameter values are presented in section 4.3 and 4.7.3.
3.4.5 Process Parameter Optimization for Color Removal Using Alum

After the enzymatic treatment on phenol, catechol, resorcinol, hydroquinone and the equimolar (1.0 mM each) mixture of phenol and three benzenediols, the colorless solutions turned into dark brown, reddish brown, brownish yellow, light yellow and dark brown colored solutions, respectively. Enzymatic treatment on the supernatant generated from Fenton reaction mixture also resulted in a brown colored solution. The absorbance maxima for the reaction products were between 420-440 nm. Optimum alum concentration was determined by comparing the reduction in absorbance of the reaction products before and after the alum treatment. The appropriate reaction conditions and parameter values are presented in section 4.1.7 and 4.7.

3.5 Estimation and Minimization of Errors

In any experiment, reliability of results can be affected by the occurrence of errors. Mainly two types of errors, namely systematic and random errors, can happen in any experimental study. Systematic or determinate errors occur due to improper experimental design, analytical techniques and instruments. Random or human errors occur due to human or equipment inaccuracy. The major portion of the determinate errors can be minimized by maintaining experimental protocols such as calibrating instruments regularly, keeping time, using proper amount of reactants, etc. In the current study, calibration curves were done at regular intervals and were compared to confirm accuracy of the data. Instruments such as the TOC analyzer were calibrated regularly by following the procedure provided in the operation manual to minimize errors. Inappropriate experimental design also contributes
to the systemic or determinate errors. Hence, proper care was taken while designing the experiments.

Random or indeterminate errors cannot be controlled directly but can be estimated. Random errors can be due to human inaccuracy such as measurement errors, sampling errors, and observation errors. Inaccurate results can be caused due to aging of experiments as well. All sets of reactions were carried out in triplicate to minimize random errors. Some experiments were repeated over a time interval and results were compared to check the reliability of experimental results.
CHAPTER 4
RESULTS AND DISCUSSION

In order to determine the most effective system for benzene removal via the proposed method, optimum conditions must be obtained for both the Fenton reaction and enzymatic reaction.

4.1 Process Parameter Optimization for Laccase-Catalyzed Removal of Phenolic Compounds

The limited Fenton reaction on benzene is expected to produce phenolic compounds (i.e., phenol, catechol, resorcinol, hydroquinone etc.) without causing significant mineralization. These phenolic compounds can then be removed by enzyme-catalyzed polymerization. Two enzymes were investigated for this purpose; namely, laccase and SBP. Hence, it is important to identify the optimum conditions for enzymatic removal of these components. Previous studies have demonstrated that the phenolic reaction products from the Fenton system most likely will be phenol and benzenediols, namely, catechol, resorcinol and hydroquinone. The optimum conditions for removal of these priority pollutants by using SBP have already been investigated by Caza et al., (1999) and Al-Ansari et al., (2009).

This study seeks to demonstrate oxidative polymerization of phenol and each of the three benzenediols in the presence of laccase followed by removal of products via coagulation and flocculation with alum. As the first step of the treatment process, the optimal conditions for \( \geq 95\% \) conversion (an arbitrary benchmark for comparison) of these aromatic compounds were determined. All discussions of optima in this section refer to
local optima as determined for the parameter in question within the respective ranges specified.

In the second step of this treatment process, effectiveness of the color removal process was investigated for its dependence on factors such as coagulating agent, coagulant concentration, pH, and concentration of the substrate.

4.1.1 Effect of pH on Conversion of Phenol and Benzenediols

Batch reactors were set up in the pH range of 3.0 to 11.5, to determine the effect of pH in the laccase-catalyzed polymerization of phenol and benzenediols (1 mM). Reactions were run for three hours at room temperature under “stringent” conditions with respect to enzyme concentration (insufficient to achieve complete conversion of substrate). The pH was also optimized in the presence of 200 mg/L of PEG. Previously, it was found that the presence of PEG can reduce the laccase requirement substantially (Modaressi et al., 2005, Saha et al., 2008, Steevensz, 2008).

The effect of pH on these substrates was monitored first in the absence of enzyme, under the same conditions as for enzymatic treatment, to use as a control. The results are presented in Figure 4-1. In the control experiments, a change in pH did not result in the conversion of any phenol and about 5% of resorcinol was converted above pH 7.0. However, catechol and hydroquinone had a more pronounced pH effect. Between pH 3.5 and 6.1, 5-10% of hydroquinone conversion occurred (Figure 4-1) and it significantly increased at pH values above 6.5. Similarly, the conversion of catechol in the pH range 5.0 to 7.8 was 5-10% and above pH 7.8 the conversion increased with an increase in pH. This phenomenon can be explained by chemical transformation of catechol and hydroquinone (confirmed for hydroquinone by HPLC analysis) in those pH ranges. It is
surmised that at higher pH, catechol and hydroquinone were chemically oxidized to quinone or semi-quinone structures (Al-Ansari et al., 2009).

Figure 4-1: Effect of pH on substrate conversion in the absence of enzyme [Batch reactors containing 1mM substrate and 40 mM buffer (acetate for pH 3.0 to 5.6, phosphate for pH 5.7 to 7.5, and bicarbonate for pH range 8.0-11.5) mixed for a three-hour period and analyzed with HPLC].

In the presence of laccase, optimum pH for enzyme catalyzed conversion of each substrate was determined from the conventional bell-shaped curve of pH-dependence (Figure 4-2). The optimum pH values for phenol and the benzenediols were in the range of 5.0-5.6 and hydroquinone showed a broad pH range (Figure 4-2). Other studies with laccase from *Trametes villosa* have shown similar optimum pH ranges (Steevensz et al., 2009). Those studies also revealed that lower substrate conversion occurred below pH 3.5
and above pH 7.0. From the literature, it is evident that laccase undergoes conformational changes below pH 3.5 and above pH 7.0, which result in reduced enzyme stability and/or efficiency (summarized by Steevensz et al., 2009). The presence of PEG had no effect on optimum pH as reported later in Table 4-1.

Figure 4-2: Effect of pH on substrate conversion in the presence of laccase [Batch reactors containing 1mM substrate and 40 mM buffer (acetate for pH 3.0 to 5.6, phosphate for pH 5.7 to 7.5, and bicarbonate for pH range 8.0-11.5) mixed for a three-hour period and analyzed using HPLC. The batch reactors containing phenol, catechol, resorcinol and hydroquinone had laccase concentrations of 0.045 U/mL, 0.0014 U/mL, 0.004 U/mL and 0.0001 U/mL respectively].
Figure 4-2 also reveals that higher conversions of catechol and hydroquinone were achieved in the basic pH range, which is analogous to the conversion achieved when no laccase was present (Figure 4-1). Thus, it is concluded that higher conversion was not enzyme-catalyzed but due to chemical transformation of those substrates. Catechol and hydroquinone can easily be transformed into quinones because of the respective ortho- and para-positions of the hydroxyl groups. Conversely, the meta-position of hydroxyl groups in resorcinol, prevent its conversion to a quinone. In any case, laccase is not capable of oxidative polymerization of such quinone structures (results not shown). Therefore, experiments with laccase-catalyzed conversion were conducted at lower pH, well below the zone of chemical conversion of catechol and hydroquinone.

Table 4-1: Optimum Reaction Conditions for Laccase Catalyzed Removal of Phenol and Benzenediols

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Without PEG*</th>
<th>With PEG*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optimum pH</td>
<td>Minimum Laccase needed for ≥ 95% conversion (U/mL)</td>
</tr>
<tr>
<td>Phenol</td>
<td>5.0- 6.1</td>
<td>0.085</td>
</tr>
<tr>
<td>Catechol</td>
<td>4.5-5.0</td>
<td>0.002</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>5.0- 6.1</td>
<td>0.007</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>4.8- 6.1</td>
<td>0.00016</td>
</tr>
</tbody>
</table>

*The laccase concentration ranges studied for 1mM concentrations of phenol, catechol, resorcinol and hydroquinone were 0.10 – 0.30 U/mL, 0.0002- 0.002 U/mL, 0.0001- 0.008 U/mL and 0.00001- 0.0002 U/mL, respectively.
4.1.2 Effect of Laccase Concentration on the Conversion of Phenol and Benzenediols

The minimum enzyme concentration at which 95% conversion of substrate was achieved at optimum pH is defined as the optimum enzyme concentration. In order to determine those optimum values for phenol and benzenediols, experiments were run at previously determined pH optima. The optimum enzyme requirement for phenol, catechol, resorcinol and hydroquinone were 0.085 U/mL, 0.002 U/mL, 0.007 U/mL and 0.00016 U/mL respectively (Table 4-1). Previous studies with the same laccase had reported a similar enzyme requirement for ≥ 95% conversion of 1 mM phenol (Steevensz et al., 2009). However, conversion of catechol, resorcinol and hydroquinone using this fungal laccase has never been studied. Among these four compounds, the parent compound, phenol, required the most enzyme, the \textit{m-}, \textit{o-}, and \textit{p-} substituted compounds followed in decreasing order of enzyme requirement. These results are analogous to the findings of Xu (1996), Smirnov et al., (2001), and Steevensz (2008).

The relative laccase requirement for the above-mentioned substrates can be explained based on a qualitative ranking of the respective radical reactivity. The general hypothesis is that ‘the more reactive the radical, the more enzyme inactivation caused’. The relative reactivities are determined by estimating corresponding homolytic bond dissociation energies (O-H for phenols) (Bordwell and Cheng, 1991). The higher the bond dissociation energy is, the more reactive the radical becomes and the more detrimental it is to the enzyme (Al-Ansari et al., 2009). Hence, higher enzyme concentrations are required for the removal of those substrates with more reactive radicals. The \textit{ortho-} and \textit{para-} oxyl substituents on the phenoxy radical are strongly stabilizing, whereas the \textit{meta-} substituted radical is less stabilizing (Al-Ansari et al., 2009). As the \textit{meta}-substituted
radical is more reactive than the ortho- and para- substituted radicals, it is expected to require more enzyme than the ortho- and para- isomers, consistent with these observations.

4.1.3 Effect of PEG in Conversion of Phenol and Benzenediols

The hydrophilic synthetic polymer PEG might assist in reducing the amount of laccase SP504 needed for removal of certain phenolic compounds without compromising the conversion efficiency and reaction time (Modaressi et al., 2005; Saha et al., 2008; Steevensz, 2008). The mechanism of the protective effect of PEG is not understood, but one hypothesis is the “sacrificial polymer theory”, which suggests that some of the insoluble products are attracted to PEG thereby preventing those polymers from adsorbing the free enzyme and settling out of the solution, or from the radicals themselves attacking the active site of the enzyme (Steevensz, 2008). Experiments were run in the presence of 200 mg/L of PEG at optimum pH, room temperature and under stringent conditions with respect to enzyme to determine whether PEG could assist in reducing the enzyme requirement for achieving more than 95% conversion of phenol, catechol, resorcinol and hydroquinone. The presence of PEG had no effect on the conversion of parent phenol (in accord with previous studies (Steevensz et al., 2009). Also, the presence of PEG did not significantly reduce the enzyme requirement for conversion of catechol (Figure 4-3), resorcinol (Figure 4-4) and hydroquinone (Figure 4-5), (Table 4-1). Hence, it is concluded that PEG had no significant effect on the removal of the four substrates by laccase SP504.
Figure 4-3: Conversion of 1 mM catechol in the presence and absence of 200 mg/L of PEG [Batch reactors containing 1mM catechol, 40 mM acetate buffer at pH 5.6, at room temperature and a three-hour reaction period.]
Figure 4-4: Conversion of 1 mM resorcinol in the presence and absence of 200 mg/L of PEG [Batch reactors containing 1mM resorcinol, 40 mM acetate buffer at pH 5.6, at room temperature and a three-hour reaction period.]
Figure 4-5: Conversion of 1 mM hydroquinone in the presence and absence of 200 mg/L of PEG [Batch reactors containing 1 mM hydroquinone, 40 mM acetate buffer at pH 5.6, at room temperature and a three-hour reaction period]

It appears that the PEG effect also depends on the functional group of the substrate, the intermediates involved, as well as the enzyme involved. Hydrophobic substrates like cresols (Steevensz, 2008), bisphenol A (Modaressi et al., 2005), diphenylamine (DPA) (Saha et al., 2008) and 2,4-dimethylphenol (Ghosh et al., 2008) showed a significant PEG effect with laccase SP504. However, it showed no significant PEG effect on phenol, aniline and the toluidines (Steevensz, 2008). Similarly, in the presence of peroxidases such as soybean peroxidase (SBP) (Caza et al., 1999, Kinsley et al., 2000), horseradish peroxidase (HRP) (Wu et al., 1998, Ikehata K. et al., 2002) and Arthromyces ramosus peroxidase (ARP) (Taylor et al., 1996), phenol conversion showed significant PEG
effects. Limited work has been done on the PEG effect on different classes of substrates, thus it is difficult to predict which substrate would be a more suitable candidate for PEG effect. However, in general, products from phenolic compounds have higher affinity towards the hydrophilic PEG (Steevensz, 2008).

The “sacrificial polymer theory” for PEG hypothesizes that PEG prevents the adsorption of free enzyme on insoluble polymers, formed as a result of enzymatic reactions, by attaching to the polymers themselves. In order to act as a “sacrificial polymer”, there should be insoluble polymers generated. Since there were no precipitate generated during the enzymatic reaction with diols, PEG did not work as a “sacrificial polymer” and hence no PEG effect was observed. It can be speculated that even though visible precipitate were generated as a result of enzymatic reaction on phenol, the product generated did not associate with laccase. As a result, no PEG effect was seen on laccase-catalyzed oxidation on phenol either. Previous studies done in the lab also confirm a similar finding (Steevensz, 2008).

**4.1.4 Time Course of Substrate Removal and Enzyme Inactivation**

Reaction time is one of the important parameters in treatment plant design which determines the volume and thus the economics of an enzyme reactor (Wu et al., 1993). Therefore, it is important to determine the minimum time required to achieve a specified conversion of these substrates (chosen as $\geq 95\%$). In order to observe substrate conversion over a three-hour period, batch reactors containing 1 mM substrate were run at the optimum pH and enzyme concentration. Samples were withdrawn at various time intervals, quenched with acid, micro-filtered and analyzed by HPLC (Figure 4-6).
Figure 4-6: Time course of substrate removal [Batch reactors containing 1 mM substrate, 40 mM acetate buffer at pH 5.6, at room temperature, three-hour reaction period. Initial laccase concentrations for 1 mM phenol, catechol, resorcinol and hydroquinone were 0.085 U/mL, 0.002, 0.007 and 0.00016 U/mL, respectively. The equations for exponential fit for phenol, catechol, resorcinol and hydroquinone are: \( y = 95.5e^{-0.018x} \) (R² = 0.99); \( y = 104.35e^{-0.017x} \) (R² = 0.99); \( y = 95.6e^{-0.015x} \) (R² = 0.99); \( y = 112.3e^{-0.017x} \) (R² = 1.00), respectively.]

For all four substrates, ≥80% conversion was achieved in the first two hours of reaction but a three-hour reaction time was needed to achieve ≥ 95% conversion. In enzymatic treatment, enzyme inactivation and diminishing substrate concentration can slow down the conversion of substrates. In order to determine the time course of enzyme activity in batch reactors, samples were withdrawn periodically and the laccase activity tested
(Figure 4-7). For all four substrates, low levels of enzyme inactivation occurred during the three-hour reaction period. About 30% and 70% of laccase inactivation was observed for benzenediols and phenol, respectively. If enzyme inactivation occurs as a result of product generation, then rapid product generation should cause higher inactivation, according to Wu et al. (1998) in a study with a peroxidase. They demonstrated that, by increasing enzyme concentration, the rate of reaction can be increased. However, the inactivation also increased proportionally.

Figure 4-7: Laccase activity in Batch Reactors over Time [Batch reactors containing 1 mM substrate, 40 mM acetate buffers at pH 5.6, at room temperature, three-hour reaction period. Initial laccase concentrations for 1 mM phenol, catechol, resorcinol and hydroquinone were 0.085 U/mL, 0.002 U/mL, 0.007 U/mL and 0.00016 U/mL, respectively.]
4.1.5 Effect of Laccase Concentration on a Composite Synthetic Wastewater Containing Phenol and the Benzenediol Mixture

Phenol and benzenediols can be present simultaneously in an effluent (Phutdhawong et al., 2000, Kumar et al., 2003). Pollutants like catechol, resorcinol and hydroquinone have also been identified in the Fenton oxidation products of phenol (Zazo et al., 2005). Thus, experiments were conducted on a solution containing 1 mM each of phenol and the three benzenediols to determine the minimum enzyme requirement for more than 95% conversion of these phenolics in a reaction mixture. As phenol had the highest enzyme requirement of the four substrates, the pH of this study was kept at the optimum pH for phenol conversion (pH 5.6). Figure 4-8 reveals that the optimum enzyme required to achieve more than 95% conversion of all the substrates was not equal to the sum of optimum enzyme requirements for individual substrates (0.94 U/mL), rather, more enzyme was required to achieve similar conversion. It is anticipated that each substrate in the reaction mixture, competed with the others for conversion. At the same time, due to the presence for four substrates, it is possible that the radicals generated in this process are different in nature than those generated when only one substrate is present. The nature of these radicals could also have an impact on the higher enzyme demand. The quantity of radical generated is more when four substrates are present. More radicals could cause higher enzyme inactivation, which could also add to the enzyme requirement.

As mentioned in Section 4.1.2 above, a better substrate required lesser enzyme and was converted faster. In this case, the benzenediols required less enzyme than phenol and were converted more efficiently than phenol.
Figure 4-8: Effect of laccase concentration on substrate conversion in composite synthetic wastewater [A batch reactor containing a mixture of 1mM each of phenol, catechol, resorcinol and hydroquinone, 40 mM acetate buffers at pH 5.6, room temperature, three-hour reaction period.]

The higher laccase requirement could also be due to possible enzyme inactivation. Previous studies have demonstrated that, under the similar reaction conditions, enzyme inactivation was higher when starting substrate concentration was higher (Dasgupta et al., 2007). The decrease in enzyme activity with higher substrate concentration was attributed to the effect of products formed during the oxidation of phenol. Under the optimum enzymatic reaction conditions, 1 mM benzenediol and phenol individually caused 30 to 70% reduction in laccase activity (Section 4.1.4). Hence, in principle, a wastewater containing 4 mM of such substrate should cause larger amount of laccase inactivation. This will increase the optimum enzyme requirement of the solution.
In an enzymatic cycle, the products generated from the starting substrate can themselves become the substrates of the enzyme. It is possible that the nature of products generated from 1 mM phenol and benzenediols under the individual optimum enzymatic reaction condition was different than the products generated in the composite mixed synthetic wastewater; hence, the enzyme demand of the individual substrate enzymatic reaction product was different from that of the composite wastewater reaction products. This could be another reason for the higher laccase demand.

4.1.6 Effect of Substrate Concentration

Phenolic compounds in wastewater can range from a few milligrams to thousands of milligrams per liter. Experiments were conducted for three hours at optimum pH and room temperature to determine the enzyme requirement to achieve more than 95% conversion over a substrate concentration of 0.5 to 2.5 mM. Linear relationships were observed as shown in Figure 4-9, Figure 4-10 and Figure 4-11. The laccase requirements for ≥ 95% conversion of o-, m- and p- cresols within a three-hour reaction period and 0.5-2.5 mM concentration range also showed linear dependence (Steevensz, 2008).
Figure 4-9: Effect of phenol concentration on enzyme requirement [Batch reactors containing 0.5-2.5 mM substrate, 40 mM acetate buffer at pH 5.6, at room temperature, three-hour reaction period. The equation for laccase concentration vs. substrate concentration for phenol is; y = 0.083 x + 0.0035 (R² = 0.99)]
Figure 4-10: Effect of resorcinol and catechol concentrations on enzyme requirement [Batch reactors containing 0.5-2.5 mM substrate, 40 mM acetate buffer at pH 5.6, at room temperature, three-hour reaction period. The equations for laccase concentration vs. substrate concentration for catechol and resorcinol are; $y = 0.0024x - 0.0004$ ($R^2 = 0.98$) and $y = 0.008x - 0.0009$ ($R^2 = 0.99$), respectively.]
Figure 4-11: Effect of hydroquinone concentration on enzyme requirement [Batch reactors containing 0.5-2.5 mM substrate, 40 mM acetate buffer at pH 5.6, at room temperature, three-hour reaction period. The equation for laccase concentration vs. substrate concentration hydroquinone is; \( y = 0.0003x + 1E^{-19} (R^2 = 0.99) \)].

4.1.7 Color Removal

The products generated from the enzymatic treatment of phenol, catechol, resorcinol, hydroquinone and their equimolar mixture resulted in dark brown, reddish brown, brownish yellow, light yellow and dark brown coloured solutions, respectively. The colored solutions resulting from enzymatic reaction of benzenediols did not have any visible precipitate formation. It is speculated that the color generated in these cases may have resulted from quinone-like products remaining in the solution (Nicell et al., 1993). However, it is also possible that the products generated as a result of radical coupling did
not precipitate due to the high number of hydrophilic and polar functional groups they might have (Al-Ansari et al, 2009). A dimer resulting from enzymatic reaction on benzenediols could have 3 or 4 hydroxyl groups for a O to C or C to C coupling, respectively (Al-Ansari et al, 2009).

This residual color is not acceptable and removal of soluble products (colored or not) is necessary before effluent discharge, therefore alum was used as a coagulant. Alum (as aluminum sulphate) concentration was varied within the range of 10-300 mg/L in these post-enzymatic reaction mixtures after pH was adjusted to 7.0 with sodium hydroxide. The optimum pH for alum coagulation occurs within the pH range of 6.5 to 7.5, a range that ensures that the floc generated would have little or no electrical charge and thus lowest solubility (Edzwald and Kaminski 2007). The results show (Figure 4-12) that 12 mg/L alum was able to remove more than 95% of the residual colored product generated from laccase-catalyzed oxidation of catechol. For a similar amount of color removal for products generated by phenol and the mixture of diols plus phenol, respectively, 100 mg/L and 150 mg/L of alum were required. However, alum could remove only 60% and 80% of coloured products from reaction mixtures of hydroquinone and resorcinol, respectively. Increasing alum concentration did not improve the color removal for these two benzenediols.
Figure 4-12: Coagulant Concentration Optimization for Color Removal [Enzymatic reaction was carried out with 1 mM substrate, 40 mM acetate buffer, at pH 5.6 and optimum enzyme concentrations (Table 4-1) for three hours. The absorbance of the reaction products of phenol, catechol and mixture of phenol and benzenediols were measured at 424 nm. The absorbance of resorcinol reaction products was measured at 440 nm. Alum was added along with the acid or base to achieve the desired pH.]

The products generated from the laccase-catalyzed oxidation of hydroquinone consisted of mostly benzoquinone (HPLC analysis). Benzoquinone was also identified as the major product when SBP was used on hydroquinone (Al-Ansari et al., 2009). In order to determine whether the alum-aided color removal reduced the carbon content, TOC analysis was done after coagulation and flocculation using alum (Al-Ansari et al., 2009). The results of their TOC analysis demonstrated that about 80% of the carbon was
removed for catechol and resorcinol reaction mixtures. However, only 20% of the carbon was removed for the hydroquinone reaction sample. The study concluded that, even though alum was effective in color removal, it was not effective in removing the reaction products which are at the monomer stage, most likely as semiquinone and quinone. Based on the similarity of product generated in SBP-catalyzed removal of benzenediols (Al-Ansari et al., 2009) and that of this study, it is likely that even though alum was fairly successful in removing most of the color generated as a result of laccase-catalyzed oxidation of hydroquinone, it was limited in reducing the total carbon content.

4.2 Process Parameter Optimization for SBP Catalyzed Removal of Phenolic Compounds

4.2.1 Effect of Process Parameters on Conversion of 1mM Phenol and Benzenediols

Previous studies indicated that when SBP was used to remove 1 mM of phenol, catechol and resorcinol individually, the optimum pH range was between pH 6.5 and 7.5 (Caza et al., 1999, Al-Ansari et al., 2009). However, for 1 mM hydroquinone optimum pH occurred in the pH range of 4.0-6.5 (Al-Ansari et al., 2009). The optimum hydrogen peroxide demands for conversion of 1mM benzenediols were higher than that for phenol. However, among these four compounds, the parent compound, phenol, required the most SBP, the m-, o-, and p- substituted compounds followed in decreasing order of SBP requirement. This finding is analogous to that of laccase requirement of these four substrates. Among these four substrates, only phenol showed limited amount of PEG effect. The optimum conditions for removal of these phenolic compounds using SBP are listed in Table 4-2.
Table 4-2: Optimum Reaction Conditions for SBP Catalyzed Removal of Phenol and Benzenediols

<table>
<thead>
<tr>
<th>Aromatic Compound (1.0 mM)</th>
<th>Optimum pH</th>
<th>Optimum H₂O₂ concentration</th>
<th>Minimum SBP concentration required for 95% conversion of substrate</th>
<th>PEG Effect</th>
<th>Data Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>6.0-7.2</td>
<td>1.5</td>
<td>1.2</td>
<td>Observed (Presence of 600 mg/L of PEG reduced SBP requirement)</td>
<td>Caza et al., 1999</td>
</tr>
<tr>
<td>Catechol</td>
<td>6.5-7.5</td>
<td>2.5</td>
<td>0.025</td>
<td>No effect</td>
<td>Al-Ansari et al., 2009</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>7.5-8.25</td>
<td>2.0</td>
<td>0.2</td>
<td>No effect</td>
<td>Al-Ansari et al., 2009</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>4.0-6.5</td>
<td>1.5</td>
<td>0.005</td>
<td>No effect</td>
<td>Al-Ansari et al., 2009</td>
</tr>
</tbody>
</table>

4.2.2 Effect of Reaction Parameters on a Composite Synthetic Wastewater Containing Phenol and the Benzenediol Mixture

SBP catalyzed removal of 1 mM phenol and three benzenediols have been studied before. However, these priority pollutants can be present simultaneously in an industrial effluent (Phutdhawong et al., 2000, Kumar et al., 2003) or they can coexist in the Fenton oxidation products of phenol (Zazo et al., 2005). SBP catalyzed removal of these compounds from a composite wastewater has never been attempted. Experiments were conducted on a solution containing 1 mM each of phenol and the three benzenediols to determine the optimum pH, minimum SBP and hydrogen peroxide requirements for more than 95% conversion of these phenolics in a reaction mixture.
4.2.2.1 Effect of pH

When SBP catalyzed removal of 1 mM phenol and benzenediols were examined, the optimum pH for most of these substrates occurred at around neutral pH (Table 4-2). However, the pH effect on the composite wastewater containing 1mM of phenol and benzenediols has not been studied. In earlier experiments to observe the pH effect on 1 mM phenol and benzenediols individually in the absence of laccase, it was observed that a change in pH did not result in significant change in the conversion of phenol and resorcinol (Figure 4-1). On the other hand, catechol and hydroquinone had a pronounced pH effect and underwent significant chemical transformation above pH 7.8 and 6.5 respectively (Figure 4-1). It was inferred that at higher pH, catechol and hydroquinone were chemically oxidized to quinone or semi-quinone structures (Al-Ansari et al., 2009).

In an SBP catalyzed enzymatic system, hydrogen peroxide is required to initiate the enzymatic process. As hydrogen peroxide is an oxidant, the effect of different concentration of hydrogen peroxide alone on the composite wastewater containing phenol and the benzenediol mixture was monitored at different pH values. The results are presented in Figure 4-13.
Figure 4-13: Effect of pH on composite wastewater containing phenol and the benzenediol mixture in presence of different concentration of hydrogen peroxide [Batch reactors containing composite wastewater consisting of 1mM of phenol, hydroquinone, catechol and resorcinol and 40 mM buffer (acetate for pH 3.0 to 5.6, phosphate for pH 5.7 to 7.5, and bicarbonate for pH range 8.0-11.5) and (a) 2mM H₂O₂, (b) 4mM H₂O₂, (c) 6mM H₂O₂, mixed for a three-hour period and analyzed with HPLC].
The results of this study indicate that the presence of hydrogen peroxide in the system aids in the chemical conversion of the benzenediols. However, conversion of phenol was not significant in the presence of hydrogen peroxide. In the presence of 2, 4 and 6mM hydrogen peroxide, at a pH range of 3.0 to 6.6, hydroquinone conversion was about 20%, 30% and 40% respectively. Hydroquinone conversion increased significantly above this pH range. However, higher conversion was achieved in the presence of higher concentrations of hydrogen peroxide. In presence of hydrogen peroxide, catechol and resorcinol also showed significant conversion above pH 6.5 and conversion of these benzenediols too increased with increasing amount of hydrogen peroxide. It can also be inferred from the results that the conversion of benzenediols in the presence of hydrogen peroxide was more pronounced at higher pH values.

This phenomenon can be explained by instability of hydrogen peroxide. The stability of hydrogen peroxide is also affected by pH. Decomposition of hydrogen peroxide generates water and oxygen (Equation 4-1).

\[ 2H_2O_2 \rightarrow 2H_2O + O_2 \]  

The instability at lower pH is not normally large. Hydrogen peroxide stability is normally the best in the region of neutral pH (Solvay Chemicals, 2005). However, as the pH increases, the decomposition of hydrogen peroxide increases rapidly which yields a large amount of oxygen. It is possible that higher concentrations of oxygen aided in greater conversion of the benzenediols at high pH values.

In order to determine the optimum pH for SBP-catalyzed removal of phenolic compounds from the composite wastewater, batch reactors were run under the similar reaction
conditions as presented in Figure 4-13, but in the presence of 0.5U/mL of SBP. The results of this study are presented in Figure 4-14.

**Figure 4-14:** Effect of pH on composite wastewater containing phenol and the benzenediol mixture in presence of different concentration of hydrogen peroxide and 0.5 U/mL of SBP [Batch reactors containing composite wastewater consisting of 1mM of phenol, hydroquinone, catechol and resorcinol and 40 mM buffer (acetate for pH 3.0 to 5.6, phosphate for pH 5.7 to 7.5, and bicarbonate for pH range 8.0- 11.5), 0.5 U/mL SBP and (a) 2 mM H$_2$O$_2$, (b) 4mM H$_2$O$_2$, (c) 6 mM H$_2$O$_2$, mixed for a three-hour period and analyzed with HPLC].
The results indicate that in the presence of 0.5 U/mL of SBP higher phenolic compound conversion was observed. This higher conversion is due to enzymatic reaction and not due to chemical conversion of phenolic compounds. In the presence of SBP, over a pH range of 5.5 to 7.0, better enzymatic conversion of hydroquinone was observed. However, previous studies have indicated that above pH 7.0, a significant amount of hydroquinone undergoes chemical conversion (Saha et al., 2011). Hence, enzymatic treatment at pHs below 7.0, will eliminate the possibility of significant chemical conversion of hydroquinone. The best enzymatic conversion for catechol occurred at a pH range of 5.5 to 7.0. The higher catechol conversion above pH 7.0 is also not due to enzymatic conversion, but due to chemical conversion of the compound. Though resorcinol showed less pH sensitivity than other benzenediols, in the presence of SBP, best enzymatic conversion of resorcinol was observed at a pH range of 6.0 to 7.5. Even though higher resorcinol conversion was observed above pH 7.5, that conversion is expected to be chemical conversion. At a pH range of 6.5- 7.5, best enzymatic conversion of phenol was observed. Except for hydroquinone, the optimum pH range for the rest of the compounds were close to the findings of Al-ansari et al. (2009) and Caza et al. (1999) (Table 4-2). Based on the result of the current study, a pH range of 6.5 to 7.0 was considered as optimum pH range for SBP catalyzed oxidative polymerization of the composite wastewater.
4.2.2.2 Effect of Hydrogen Peroxide and SBP Concentration on a Composite Wastewater Containing Phenol and the Benzenediol Mixture

4.2.2.2.1 Single Addition of Hydrogen Peroxide and SBP

In order to determine the optimum hydrogen peroxide and SBP concentration for the composite wastewater, batch reactors were run at previously determined optimum pH of 7.0. In previous studies, individual optimum reaction conditions for 1mM phenol, catechol, resorcinol and hydroquinone were determined (Caza et al., 1999, Al-Ansari et al., 2009). Based on the results of those studies (Table 4-2), it can be speculated that to achieve more than 95% removal of the phenolic compounds from the composite wastewater containing 1mM of each substrate, about 1.5 U/mL of SBP and 7.5 mM of hydrogen peroxide will be required. In order to determine the optimum SBP and hydrogen peroxide concentrations for the composite wastewater, SBP concentration was varied from 0.8 U/mL to 2.0 U/mL and hydrogen peroxide concentration was varied from 8 mM to 16 mM. The results of this study are presented in Figure 4-15, Figure 4-16, Figure 4-17 and Figure 4-18.
Figure 4-15: Effect of 8 mM H$_2$O$_2$ and 0.8 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture [Batch reactors containing composite wastewater consisting of 1mM of phenol, hydroquinone, catechol and resorcinol and 40 mM phosphate buffer at pH 7.0 and mixed for a three-hour period and analyzed with HPLC].
Figure 4-16: Effect of 12 mM H$_2$O$_2$ and 1.2 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture [Batch reactors containing composite wastewater consisting of 1mM of phenol, hydroquinone, catechol and resorcinol and 40 mM phosphate buffer at pH 7.0 and mixed for a three-hour period and analyzed with HPLC].
Figure 4-17: Effect of 12 mM H$_2$O$_2$ and 1.5 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture [Batch reactors containing composite wastewater consisting of 1mM of phenol, hydroquinone, catechol and resorcinol and 40 mM phosphate buffer at pH 7.0 and mixed for a three-hour period and analyzed with HPLC].
The results of this study indicate that, the minimum SBP and hydrogen peroxide requirements to achieve more than 95% conversion of all phenolic compounds in the composite wastewater were 1.8 U/mL and 15 mM, respectively. Hence, the optimum SBP and hydrogen peroxide required to achieve more than 95% conversion of all the substrates were not equal to the sum of optimum SBP and hydrogen peroxide requirements for individual substrates (1.5 U/mL of SBP and 7.5 mM of hydrogen peroxide), rather, more enzyme and more hydrogen peroxide were required to achieve
similar conversion. This finding is analogous to that of Section 4.1.5. The results of that study demonstrated that the optimum laccase requirement for the composite wastewater was more than the sum of laccase requirements for the individual substrates.

In all cases, catechol and hydroquinone were converted quickly while resorcinol and phenol took longer time. Conversion efficiency of the substrates in this study follows the same trend as their individual optimum SBP requirement. The results presented in Table 4-2 demonstrate that, among these four compounds, the parent compound, phenol, required the most enzyme, the m-, o-, and p- substituted compounds followed in decreasing order of enzyme requirement. These results are analogous to the findings of Xu (1996), Smirnov et al., (2001), and Steevensz (2008). In the composite wastewater the o-, and p- substituted compounds, catechol and hydroquinone, were more efficiently converted than the m- substituted compound, resorcinol (Figure 4-15, Figure 4-16, Figure 4-17 and Figure 4-18). In all the figures, the parent compound phenol took longer time and required a higher concentration of enzyme to reach the optimum removal. It is anticipated that each substrate in the reaction mixture, competed with the others for conversion. As reported in Section 4.1.2, a better substrate required lesser enzyme and was converted faster. In this case, the benzenediols required less enzyme than phenol and were converted more efficiently.

In general, benzenediols demonstrated higher hydrogen peroxide demand than the theoretical hydrogen peroxide demand (Table 4-2). Theoretically, one mole of hydrogen peroxide is required for two moles of the aromatic functional group. However, the soluble dimers produced as a result of enzymatic cycle can become substrates of the enzyme and undergo further cycles of polymerization. These additional cycles will cause
additional hydrogen peroxide demand. At the same time, it is likely that the dimers generated from the composite wastewater were different from those of individual substrates. Due to the probable dissimilar nature of the dimers, the enzyme and hydrogen peroxide demand could be different too. Hence, it is likely that the dimers generated in the composite wastewater were the reason for higher hydrogen peroxide demand.

4.2.2.2.2 Step addition of Hydrogen peroxide and SBP

The broad pH and thermal stability, lower susceptibility to irreversible inactivation by hydrogen peroxide and potentially low price makes SBP a more suitable enzyme choice for wastewater treatment than other peroxidases and laccases (Al-Ansari et al., 2009; Steevensz et al., 2009). However, enzyme inactivation still remains as one of the major challenges of the enzymatic treatment, which adds to the cost of the treatment. Three possible ways of SBP inactivation are: a) inactivation by excess hydrogen peroxide, b) free-radical generation during the enzymatic cycle and c) adsorption and precipitation of free enzyme with the polymeric end products (Klibanov et al., 1983; Wright and Nicell, 1999; Nakamoto and Machida, 1992). However, inactivation pathways that will dominate in a process, will depend on reaction conditions such as concentrations of hydrogen peroxide, SBP and the substrate in question (Al-Ansari et al., 2010).

In addition to the use of additives like PEG (discussed in Section 4.1.3), step feeding of hydrogen peroxide has been found effective in reducing peroxidise inactivation (Ibrahim et al., 2001, Al-Ansari et al., 2010). Generally, step addition of hydrogen peroxide is considered to minimize the instantaneous radical formation, which aids in preventing SBP inactivation. Step additions of both SBP and hydrogen peroxide have been found to
be more effective in reducing the overall SBP requirement than step addition of hydrogen peroxide (Al-Ansari et al., 2010). In order to examine whether step addition of SBP and hydrogen peroxide will help in reducing overall enzyme and hydrogen peroxide demands for the composite wastewater, reactions were run at the previously determined optimum pH of 7.0, at a hydrogen peroxide concentrations of 8-16 mM and SBP concentrations of 0.4-1.8 U/mL. Results are shown in Figure 4-19 (step addition of only hydrogen peroxide) and Figure 4-20, Figure 4-21, Figure 4-22 and Figure 4-23.

**Figure 4-19: Effect of step addition of hydrogen peroxide on composite wastewater containing phenol and the benzenediol mixture** [Batch reactors contained composite wastewater consisting of 1mM each of phenol, hydroquinone, catechol and resorcinol and 40 mM phosphate buffer at pH 7.0; 4, 2 and 2 mM H$_2$O$_2$ were added at 0, 30 and 60 min, respectively; 0.4 U/mL SBP was added at 0 min, mixed for a three-hour period and analyzed with HPLC].
Figure 4-20: Effect of step addition of 8 mM H$_2$O$_2$ and 0.8 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture [Batch reactors contained composite wastewater consisting of 1mM each of phenol, hydroquinone, catechol and resorcinol and 40 mM phosphate buffer at pH 7.0, 4, 2 and 2 mM H$_2$O$_2$ were added at 0, 30 and 60 min, respectively; 0.4, 0.2 and 0.2 U/mL SBP were added at 0, 30 and 60 min, respectively; reaction mixture mixed for a three-hour period and analyzed with HPLC].
Figure 4-21: Effect of step addition of 12 mM H$_2$O$_2$ and 1.2 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture. [Batch reactors contained composite wastewater consisting of 1mM each of phenol, hydroquinone, catechol and resorcinol and 40 mM phosphate buffer at pH 7.0. 4, 4 and 4 mM H$_2$O$_2$ were added at 0, 10 and 20 min, respectively; 0.4, 0.4 and 0.4 U/mL SBP were added at 0, 10 and 20 min, respectively; reaction mixture mixed for a three-hour period and analyzed with HPLC].
Figure 4-22: Effect of step addition of 12 mM H$_2$O$_2$ and 1.5 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture [Batch reactors contained composite wastewater consisting of 1mM each of phenol, hydroquinone, catechol and resorcinol and 40 mM phosphate buffer at pH 7.0, 4, 4 and 4 mM H$_2$O$_2$ were added at 0, 10 and 20 min, respectively; 0.5 , 0.5 and 0.5 U/mL SBP were added at 0, 10 and 20 min, respectively; reaction mixture mixed for a three-hour period and analyzed with HPLC].
Figure 4-23: Effect of step addition of 15 mM H$_2$O$_2$ and 1.5 U/mL SBP on composite wastewater containing phenol and the benzenediol mixture [Batch reactors contained composite wastewater consisting of 1mM each of phenol, hydroquinone, catechol and resorcinol and 40 mM phosphate buffer at pH 7.0, 5, 5 and 5mM H$_2$O$_2$ were added at 0, 10 and 20 min, respectively; 0.5, 0.5 and 0.5 U/mL SBP were added at 0, 10 and 20 min, respectively; reaction mixture mixed for a three-hour period and analyzed with HPLC].

The results of this study indicate that step addition of SBP and hydrogen peroxide aided in efficient conversion of the phenolic compounds. For example, when 0.8 U/mL of SBP and 8 mM hydrogen peroxide were added to the batch reactors at 0 min, about 75% resorcinol and 40% of phenol conversion was achieved (Figure 4-15). However, when the same total concentrations of SBP and hydrogen peroxide were added as 4, 2 and 2 mM H$_2$O$_2$ at 0, 30 and 60 min, respectively and 0.4, 0.2 and 0.2 U/mL SBP at 0, 30 and 60 min, respectively to the reaction mixture by step addition, conversion of the phenolic
compounds increased to about 80% for resorcinol and 60% for phenol (Figure 4-20). Similarly when 1.2 U/mL of SBP and 12 mM hydrogen peroxide were added at the beginning of the reaction, about 84% of resorcinol and 60% of phenol conversion were observed (Figure 4-16). But when the same concentrations of SBP and hydrogen peroxide were added as 4, 4 and 4 mM H₂O₂ at 0, 10 and 20 min, respectively and 0.4, 0.4 and 0.4 U/mL SBP at 0, 10 and 20 min, respectively to the reaction mixture by step addition, about 90% resorcinol and 75% phenol conversion efficiency was achieved (Figure 4-21). In all cases, step addition of hydrogen peroxide and SBP aided in better conversion efficiency when compared to single addition of hydrogen peroxide and SBP of same respective concentrations. The single addition of SBP and hydrogen peroxide at the beginning of the reaction is expected to cause a rapid burst of free phenoxy radicals. However, if hydrogen peroxide and SBP are added over time at a lower instantaneous concentration, the resulting concentration of free phenoxy radicals will be lower. It has been suggested that single addition will cause more SBP inactivation by these free radicals than the step addition (Klibanov et al., 1980; Wu et al., 1998, Al-Ansari et al., 2010).

However, the optimum SBP and hydrogen peroxide requirements for single- and step addition were not significantly different. In the case of single addition of SBP and hydrogen peroxide, 15 mM hydrogen peroxide and 1.8 U/mL of SBP were required (Figure 4-18). In case of step addition, 15 mM hydrogen peroxide and 1.5 U/mL of SBP (5, 5 and 5 mM H₂O₂ added at 0, 10 and 20 min, respectively and 0.5, 0.5 and 0.5 U/mL SBP added at 0, 10 and 20 min, respectively) were required to achieve the optimum phenolic compound conversion (Figure 4-23). So, the step addition reduced the optimum SBP
requirement by only 0.3 U/mL. Thus, it can be concluded that for the composite wastewater, step addition of SBP and hydrogen peroxide was not significantly effective.

4.3 Process Parameter Optimization for Removal of Benzoquinone Using Additives

Benzenediols, namely catechol, resorcinol and hydroquinone have been identified in the Fenton oxidation products of phenol and further oxidation of some of these benzenediols produced quinones (Zazo et al., 2005). At the same time, benzoquinone is also generated from enzymatic treatment of hydroquinone. This priority pollutant is not an enzyme substrate. Removal of this chemical by using alum aided coagulation is not very successful (as described in section 4.1.7). However, additives like chitosan and PEI have been successfully used to remove quinones from wastewater (Wada et al., 1995). Hence batch reactors were set up to determine the optimum reaction conditions to remove benzoquinone by using the coagulant aids chitosan and PEI. Both chitosan and PEI reacts with quinone by forming a carbon-nitrogen bond. The nitrogen from the amino groups of chitosan or PEI condenses with a carbonyl carbon of quinone molecules (Wada, et al., 1995).

Quinone removal efficiency will greatly depend on the surface chemistry of chitosan flakes (Sun and Payne, 1996), as chitosan is insoluble in aqueous solution at pH≥ 6, except for low molecular-weight samples. (Alves and Mano, 2008). However, the quinone-containing flakes can be easily separated by sedimentation and filtration. Studies indicate that chitosan solution (made in the presence of acid) is more efficient in quinone removal (Wada et al., 1995; Sun and Payne, 1996). Along with this process, however, an appropriate coagulant aid is required to remove the colored products. Similarly, to remove the quinone-PEI complex alum is generally used.
4.3.1 Effect of pH

Batch reactors were set up in the pH range 3.0 to 11.5, to determine the effect of pH in the chitosan- and PEI-aided removal of 1 mM benzoquinone. Reactions were run for three hours at room temperature and under limited coagulant concentration so that only pH had an effect in removal of benzoquinone.

In order to determine whether pH has an effect on benzoquinone stability, a set of batch reactors were set up in the above-mentioned pH range containing only 1mM benzoquinone in the absence of any additives. The results of this study are presented in Figure 4-24.

Figure 4-24: Effect of pH on 1mM benzoquinone conversion in the absence of additives [Batch reactors containing 1mM substrate and 40 mM buffer (acetate for pH 3.0 to 5.6, phosphate for pH 5.7 to 7.5, and bicarbonate for pH range 8.0-11.5) and analyzed after 3 h at room temperature with HPLC].
The results of this study reveal that benzoquinone is fairly stable within a pH range of 3.5 to 7.2. Above this pH range, benzoquinone undergoes some form of transformation and this phenomenon increases with increase in pH. In their study, Dawson and Nelson (1938) demonstrated that, the rate of disappearance of benzoquinone in aqueous solutions containing no increases with decrease in hydrogen ion concentration. They claimed that there might be a definite relationship between the pH of the system and the rate of disappearance of the benzoquinone. In their study, they found that disappearance of benzoquinone increased with increasing pH. The results of the current study appear to be analogous with their findings. At the basic pH range, the colorless benzoquinone solution turned orangish, indicating chemical transformation.

Removal of 1mM benzoquinone was also studied in the presence of chitosan solution (100 mg/L of 75% deacetylated chitosan), chitosan flakes (100 mg/L of 75% deacetylated flakes) and PEI solution (100 mg/L) over a three-hour reaction period in the above-mentioned pH ranges. The results of these studies are presented in Figure 4-25, Figure 4-26 and Figure 4-27, respectively. In all cases, higher benzoquinone conversion was observed above neutral pH. However, as benzoquinone itself undergoes chemical transformation above the neutral pH, additive effects at this pH range was not considered successful. In all cases, better removal was achieved at a pH range of 6.0-7.5. Hence, this pH range was considered optimum for benzoquinone removal. Benzoquinone removal depended on the contact time as well. Between chitosan solution and chitosan flakes, the solution was slightly more effective. Among all the additives, PEI was the most effective one. Both chitosan solution and PEI resulted in an orange-colored solution. However,
above pH 6.5, after a two-hour reaction period, blackish particles were visible in PEI-containing batch reactors.

Figure 4-25: Effect of pH on 1mM benzoquinone conversion in the presence of chitosan solution [Batch reactors containing 1mM substrate and 40 mM buffer (acetate for pH 3.0 to 5.6, phosphate for pH 5.7 to 7.5, and bicarbonate for pH range 8.0-11.5) and chitosan solution containing 100 mg/L of 75% deacetylated chitosan; mixed and monitored over three hours and analyzed with HPLC].
Figure 4-26: Effect of pH on 1mM benzoquinone conversion in the presence of chitosan flakes [Batch reactors containing 1mM substrate and 40 mM buffer (acetate for pH 3.0 to 5.6, phosphate for pH 5.7 to 7.5, and bicarbonate for pH range 8.0- 11.5) and 100 mg/L of 75% deacetylated chitosan flakes; mixed and monitored over three hours and analyzed with HPLC].
4.3.2 Effect of additive concentration and contact time

In order to determine the effect of chitosan solution, chitosan flakes and PEI concentrations to remove 1mM benzoquinone at the previously-determined optimum pH of 7.0, batch reactors were set up containing 0-3000 mg/L of chitosan flakes or 0-1000 mg/L of PEI. To determine the effect of contact time on benzoquinone removal, reactions were monitored over three hours. It was noticed in previous experiments that, when chitosan solution and PEI was introduced to the benzoquinone solution, it produced
lightly orange-colored solution. The direct absorbance of the reaction mixture was also monitored to account for the color generated.

The effect of chitosan flakes concentration at neutral pH is presented in Figure 4-28. The optimum concentration occurred at 2700-3000 mg/L. A three-hour contact time was successful in removing ≥ 95% of the initial benzoquinone. Hence, this concentration and 3 h contact time were considered optimum conditions for removal of 1 mM benzoquinone. Wada et al., (1993) and Sun et al., (1992) investigated adsorption on chitosan to remove quinone type colored products formed from phenols by tyrosinase. Wada et al., (1995), found that in neutral pH to completely remove products generated from 0.5 mM phenol, 1.4 mg/mL of chitosan flakes were required. It can be concluded that, even though the chitosan adsorption is an effective way to remove quinones, the amount of chitosan required to achieve the removal is always very high (Wada et al., 1995).

After a three-hour reaction time, the chitosan flakes turned brown in color. These flakes were allowed to settle by gravity and the filtered supernatant was used for absorbance measurements. These solutions had really low absorbance at 424 nm. The results of this study (Figure 4-29) indicate that, the absorbance of the solution decreased with increasing amount of chitosan flakes.
Figure 4-28: Chitosan Flakes Concentration Optimization for Benzoquinone Removal [Batch reactors containing 1 mM benzoquinone and 40 mM phosphate buffer at pH 7.0 along with varying amount of 75% deacetylated chitosan flakes; mixed and monitored over three hours and analyzed with HPLC]
Figure 4-29: Color Generated during Chitosan Flakes Concentration Optimization for Benzoquinone Removal [Batch reactors containing 1 mM benzoquinone and 40 mM phosphate buffer at pH 7.0 along with varying amount of 75% deacetylated chitosan flakes; mixed and monitored over three hours; direct absorbance was measured at 424 nm after three hour reaction]

To determine the effect of PEI concentration and reaction time to remove benzoquinone from solution at neutral pH, PEI concentration in the batch reactors was varied from 0-1000 mg/L. As soon as PEI was introduced to the benzoquinone solution, it turned orange in color and some turbidity was visible. Above 550 mg/L of PEI, the turbidity disappeared and the solutions turned bright orange in color. The results of this study are presented in Figure 4-30. At about 150-200 mg/L of PEI, ≥ 95% of benzoquinone removal was achieved. The reactions were monitored over a three-hour period. However, removal of benzoquinone did not change significantly over this time period. Based on this, it can be concluded that longer contact time for PEI-benzoquinone reaction was not
required. Based on the results (Figure 4-30), one hour contact time was considered sufficient to achieve more than 95% removal of benzoquinone at neutral pH.

![Graph showing PEI Optimization for Benzoquinone Removal](image)

**Figure 4-30: PEI Optimization for Benzoquinone Removal** [Batch reactors containing 1mM substrate and 40 mM buffer phosphate at pH 7.0 and varying concentration of PEI; mixed and monitored over three hour and analyzed with HPLC].

In PEI-benzoquinone reaction, the quinone is easily attacked by the lone electron pair from the nitrogen in the amino group in PEI. It undergoes a nucleophilic reaction followed by dehydration to form a carbon-nitrogen double bond (an imine or Schiff base; Wada et al., 1995). There is an optimum concentration range for both chitosan and PEI.
which contributes to maximum amount of removal. Quinones react with individual PEI molecules. When insufficient PEI is present, the products cannot aggregate (Wada et al., 1995). May be this is the reason why, turbidity was visible at lower PEI concentration; however, those particles were unable to settle. On the other hand, when an excess amount of PEI is present in the solution, quinones react with PEI molecules, but the “bridging” between molecules is minimized (Wada et al., 1995). So, coagulation does not occur and particles do not precipitate. This could be the reason why at high PEI concentration, the solution turned bright orange and all the turbidity disappeared.

At the same time, PEI is a highly branched polyamine containing primary and secondary amine functions. The reaction between polyfunctional PEI and quinone carbonyl proceeds through imine (-C=N-) linkage formation. Though such adducts are not always strictly stoichiometric, the literature reports that precipitates form when a particular C/N mass ratio is attained (Land and Ellis, 1982). Hence, one of the major requirements for precipitate formation is the presence of an optimum ratio of carbonyls to amines. However, if excess PEI is present in the solution, such colloids undergo a restabilisation process (Land and Ellis, 1982). This could be the reason for visible turbidity at low PEI concentration and disappearance of turbidity at high PEI concentrations.

After a three-hour reaction time, the filtered samples were taken for absorbance measurements. In order to determine whether alum could be effective in settling PEI-benzoquinone products, 150 mg/L of alum (as aluminum sulfate) was added in another set of batch reactors after three hours of PEI treatment. The results, presented in Figure 4-31, demonstrate that the absorbance of these solutions at 424 nm increased with increasing PEI concentration. However, at low PEI concentrations (50-250 mg/L), alum
was successful in removing all the color. Alum was able to reduce color at higher PEI concentrations as well.

Figure 4-31: Color Generated during PEI Optimization for Benzoquinone Removal

![Graph showing color absorbance at 424 nm against concentration for PEI and Alum + PEI]

[Batch reactors containing 1mM benzoquinone, 40 mM buffer phosphate at pH 7.0 and varying concentration of PEI; mixed and monitored over three, hoursthen of alum (as aluminum sulfate) was added to 150 mg/L, samples were mixed and filtered, then absorbance was measured at 424 nm.]


Previous studies have indicated that the products generated from the laccase- and SBP-catalyzed oxidation of hydroquinone consisted of mostly benzoquinone (from HPLC analysis). (Al-Ansari et al., 2009; Saha et al., 2011). The alum-aided color removal on
the hydroquinone reaction sample could only reduce the carbon content 20%. The study concluded that, even though alum was effective in color removal, it was not effective in removing the reaction products generated from enzyme-catalyzed hydroquinone oxidation. These products are believed to be at the monomer stage, most likely as semiquinone and quinone.

Chitosan and PEI were successful in removing authentic benzoquinone. Hence, it was expected that chitosan and PEI would be successful in removing hydroquinone reaction product as well. In order to determine optimum reaction conditions to remove benzoquinone generated from laccase-catalyzed oxidation of hydroquinone, enzymatic reaction on 1mM hydroquinone was run at the previously determined optimum enzymatic reaction conditions (presented in Table 4-1) in the presence of excess laccase (0.001 U/mL) for three hours to ensure complete conversion of hydroquinone. HPLC analysis of the reaction mixture at that time revealed that it consisted of about 0.85 mM benzoquinone: absorbance at 424 nm at this time was 0.15, considered as 100% color. These post-enzymatic reaction mixtures were used below to determine the optimum benzoquinone removal conditions using chitosan and PEI.

4.4.1 Optimum pH

The optimum pH for 1mM authentic benzoquinone removal using chitosan and PEI occurred at near neutral pH (section 4.3.1). Hence for this study, the pH of the post-enzymatic reaction mixtures was adjusted to 7.0 with sodium hydroxide and was considered optimum for the current study.
4.4.2 Minimum Polyamine Concentration and Contact Time to Achieve ≥95% Product Removal

Enzymatic reaction on hydroquinone yielded benzoquinone as the major reaction product. The effect of chitosan flakes at the optimum pH (neutral) to remove these products is presented in Figure 4-32. A chitosan concentration around 2800-3000 mg/L was able to remove 95% of the reaction product, but only with a three-hour contact time.

![Figure 4-32: Effect of Chitosan Flakes Concentration on Product Removal from Laccase Treated Hydroquinone Sample](image)

**Figure 4-32: Effect of Chitosan Flakes Concentration on Product Removal from Laccase Treated Hydroquinone Sample** [Batch reactors containing post-enzymatic reaction mixture generated from laccase-catalyzed oxidation of 1 mM hydroquinone (under optimum reaction conditions (Table 4-1) and in the presence of 0.001 U/mL of laccase); at pH 7.0 (adjusted to 7.0 by using base) along with varying amounts of 75% deacetylated chitosan flakes; mixed and monitored over three hours; and analyzed with HPLC]
After three hours contact time the resulting solutions were almost colorless (Figure 4-33). The color removal efficiency of chitosan was also examined during this procedure. At 2800-3000 mg/L concentration, adsorption of reaction product on chitosan was able to remove 93% of the color (Figure 4-33) generated during enzymatic reaction.

![Figure 4-33: Effect of Chitosan Flakes Concentration on Reaction Mixture Color](image)

[Enzymatic reaction was carried out with 1 mM hydroquinone, at pH 5.6, and 0.001 U/mL laccase concentration for 3hr. The pH of this post-enzymatic reaction mixture was adjusted to pH 7.0 by using base; varying amounts of 75% deacetylated chitosan flakes added mixed and monitored over three hours; absorbance was measured at 424 nm, samples were filtered prior to analysis]

To determine effect of PEI concentration at neutral pH to remove the primary product of enzyme-catalyzed hydroquinone reaction, PEI concentrations in the batch reactors were
varied from 0-400 mg/L, Figure 4-34. At about 140-200 mg/L of PEI concentration, ≥ 95% removal of benzoquinone was achieved. The reactions were monitored over a three-hour period. However, removal of benzoquinone did not change significantly in the second and third hours. Based on this, it can be concluded that a two hour contact time for the PEI-benzoquinone reaction was sufficient to achieve more than 95% removal of the reaction product at neutral pH.

Figure 4-34: Effect of PEI Concentration on Product Removal from Laccase Treated Hydroquinone Sample [Enzymatic reaction was carried out with 1 mM hydroquinone, at pH 5.6, and 0.001 U/mL laccase concentration for 3hr. The pH of this post-enzymatic reaction mixture was adjusted to pH 7.0 by using base; varying amounts of PEI were added, mixed and monitored over three hours; analyzed by HPLC]
Figure 4-35: Effect of PEI Concentration on Reaction Mixture Color [Enzymatic reaction was carried out with 1 mM hydroquinone, at pH 5.6, and 0.001 U/mL laccase concentration for 3hr. The pH of this post-enzymatic reaction mixture was adjusted to pH 7.0 by using base; varying amount of PEI added mixed and monitored over three hour; 200 mg/L of alum as aluminum sulfate was added (when appropriate); direct absorbance was measured at 424 nm, samples were filtered prior to analysis]

4.4.3 TOC Analysis

TOC analysis was done both on chitosan- and PEI-treated samples in order to determine the remaining carbon in the solution. The results of the TOC analysis of the chitosan samples are presented in Figure 4-36. After the chitosan treatment, the final carbon content of the resulting solution varied from about 30 to 40 mg/L. The contributing factors for this carbon concentration are the carbon from the remaining product (quinone)
and the carbon from the chitosan itself. Even though chitosan flakes are not water soluble, it is possible that there are some impurities in the product itself which contributed the carbon in the solution. At a chitosan concentration of 2.8 to 3 mg/mL (2800-3000 mg/L) the TOC resulting from the benzoquinone was the least. At this concentration range, about 5-8% of the TOC was due to the remaining quinone in the solution.

![Figure 4-36: TOC Analysis on Laccase-treated Hydroquinone Mixture after Chitosan Treatment](image)

[Enzymatic reaction was carried out with 1 mM hydroquinone, at pH 5.6, and 0.001 U/mL laccase concentration for 3hr. The pH of this post-enzymatic reaction mixture was adjusted to pH 7.0 by using base; varying amount of 75% deacetylated chitosan flakes added mixed and monitored over three hours; samples were filtered prior to TOC analysis]
TOC analysis on laccase-treated hydroquinone mixture was done after PEI treatment and PEI followed by alum treatment. The results of that study are presented in Figure 4-37. After only PEI treatment, the TOC of the resulting colored mixture varied from 100 – 270 mg/L. The TOC increased with increasing amount of PEI. There was some visible turbidity but no precipitate at this PEI concentration range. In order to remove the quinone-PEI products, 200 mg/L of alum was added to the solution. It generated visible flocs and those were removed by sedimentation and filtration. The TOC analysis of the PEI followed by alum treated samples (Figure 4-37) show that, about 83-88% of the TOC was removed by the alum treatment. After the alum treatment, the TOC content of the samples varied between 20 and 35 mg/L. There were two contributors to the remaining TOC: the remaining quinone and remaining PEI. After PEI and alum treatment the TOC due to the remaining quinone was less that 5 mg/L (HLPC results) above 140 mg/L of PEI. However, it is possible that even after alum treatment, there was some PEI remaining in the solution which contributed towards the TOC content of the solution. At a PEI concentration range of 140-200 mg/L, the TOC possibly associated with remaining PEI was less than 20 mg/L.
Figure 4-37: TOC Analysis on Laccase-treated Hydroquinone Mixture after PEI and Alum Treatment [Enzymatic reaction was carried out with 1 mM hydroquinone, at pH 5.6, and 0.001 U/mL laccase concentration for 3hr. The pH of this post-enzymatic reaction mixture was adjusted to pH 7.0 by using base; varying amount of PEI added mixed and monitored over three hour; 200 mg/L of alum as aluminum sulfate was added (when appropriate); samples were filtered prior to TOC analysis]

4.5 Process Parameter Optimization for Fenton Reaction on Benzene (Single Step Reactant Addition)

To determine the most efficient system to convert benzene into corresponding phenolics without causing any mineralization, batch reactors were set up to study the effect of pH, [benzene], [Fe$^{2+}$], [H$_2$O$_2$], and reaction time. In this study, all the reactants were added in the system at the beginning of the reaction.
4.5.1 Optimum pH for Benzene Conversion

In order to maximize formation of phenolic compounds from benzene via Fenton pre-treatment, conversion of benzene was studied over a pH range 3.0-6.0 for a 3-h reaction period. Higher benzene conversion was achieved in the pH range of 3.0-4.0 (Figure 4-38).

![Graph showing effect of pH on benzene conversion](image)

**Figure 4-38: Effect of pH on benzene conversion** [Initial benzene concentration 6 mM; at different pHs; [Benzene]_{initial}: [H_2O_2]_{initial}: [Fe^{2+}]_{initial} = 1:1.8:1 added at 0 min of reaction, mixed and monitored over three hours; analyzed by HPLC]

It is speculated that the decreased conversion at higher pHs is due to loss of soluble iron. In order to determine the iron loss from solution, atomic absorption analysis was done on the Fenton reaction mixture generated at different pHs. The results of that analysis (Figure 4-39) reveal that, soluble iron is lost at all the pHs. However, as the pH of the
reaction mixture increases, the amount of soluble iron decreases. The reaction time also has an effect on the iron solubility. As the reaction progresses, the amount of soluble iron decreases. This phenomenon is more pronounced at higher pHs. Hence it is possible that the conversion of benzene decreased with increasing pH due to the iron loss. At pH around 5.5 and higher, the conversion of benzene was reduced significantly due to significant iron precipitation from the system.

![Graph showing soluble iron concentration in the Fenton reaction mixture at different pHs.](image)

**Figure 4-39: Soluble iron concentration in the Fenton reaction mixture at different pH**

[Initial benzene concentration 6 mM; at different pHs; [Benzene]_initial: [H_2O_2]_initial: [Fe^{2+}]_initial = 1:1.8:1 added at 0 min of reaction, mixed and monitored over three hours; soluble iron concentration analyzed by atomic absorption spectrophotometer, samples filtered prior to analysis]

On the other hand, the formation of identifiable products increased with increasing pH (Figure 4-40). These identifiable products will be discussed in detail in sections 4.5.5 and
Even though conversion of benzene was higher at a pH range of 3.0-4.0, there were some unidentified products were generated along with phenolic compounds.

![Figure 4-40: Effect of pH on formation of identifiable aromatic products](image)

**Figure 4-40: Effect of pH on formation of identifiable aromatic products** [Initial benzene concentration 6 mM; at different pHs; \(\text{[Benzene]}_{\text{initial}}\): \(\text{[H}_2\text{O}_2\)\text{]}_{\text{initial}}\): \(\text{[Fe}^{2+}\)\text{]}_{\text{initial}} = 1:1.8:1 added at 0 min of reaction, mixed and monitored over three hours; analyzed by HPLC; samples filtered prior to analysis; identifiable products include phenol, catechol, resorcinol, hydroquinone, benzoquinone and biphenyl]

Preliminary analysis of these unidentified products suggests these to be ring-opened products. The main focus of the Fenton-pretreatment was to limit the conversion of benzene to aromatic compounds, preferably to phenolic compounds, so that these phenolic compounds can be removed by the enzymatic treatment. Hence, the presence of the ring-opening products in the reaction mixture is not desirable. However most of the benzene conversion at pH 5.0 produced identifiable aromatic compounds by HPLC
analysis (Figure 4-40). Most of these aromatic compounds can be removed by enzymatic treatment. Hence, pH 5.0 was considered the most suitable pH for the production of aromatic compounds from the Fenton reaction.

In order to ensure that there was no significant carbon loss from the system due to mineralization, TOC analysis of reaction mixture at different pHs was performed. The results of the TOC analysis reveal that (Figure 4-41), after one hour of reaction, at pH 5.0 only 0.4% carbon was lost while about 3% and 10% starting carbon was lost at pH 4.0 and pH 3.0, respectively. As the reaction progresses, the amount of unaccounted for carbon also increases in all cases. However, at pH 3.0 and 4.0 the unaccounted carbon is significantly higher than that at pH 5.0. It is speculated that this carbon loss is due to the mineralization of benzene. The mass balance at pH 5.0 also indicates that (Figure 4-38, Figure 4-40), most of the benzene conversion resulted in aromatic compound production without causing significant ring opening. Hence for this study, pH 5.0 was considered as optimum pH for phenolic compound production from Fenton reaction.
Figure 4-41: TOC Analysis on Fenton Reaction Mixture [Initial benzene concentration 6 mM; [Benzene]_{initial}; [H_2O_2]_{initial}; [Fe^{2+}]_{initial} = 1:1.8:1 added at 0 min of reaction, mixed and monitored over three hours; analyzed by HPLC; samples filtered prior to TOC analysis; (a) at pH 3.0, (b) at pH 4.0 and (c) at pH 5.0]
4.5.2 Optimum Fe$^{+2}$ Concentration for Benzene Conversion

The overall reaction efficiency of Fenton reaction is determined by its reagent conditions and the reaction characteristics (Neyens and Baeyens, 2003). The mutual relationships among pH, [Fe$^{2+}$], [H$_2$O$_2$], and [substrate] have profound effects on hydroxyl radical generation. To determine the effect of [Fe$^{+2}$] on benzene conversion, the molar ratios of Fe$^{+2}$ to benzene in the Fenton reaction mixture were varied from 0.1 to 2.0. The reactions were carried out at the previously determined optimum pH of 5.0 over a two-hour period. The results of this study (Figure 4-42) indicate that the maximum amount of phenolic product generation occurs at [Fe$^{+2}$]/[benzene] of 1.0. This phenomenon can be explained by the nature of the Fenton reaction. The Fenton reaction begins by producing OH• from the reaction between ferrous ion and hydrogen peroxide (Equation 1).

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \quad (\text{chain initiation}) \quad (1)$$

$$\text{OH}^- + \text{Fe}^{2+} \rightarrow \text{OH}^- + \text{Fe}^{3+} \quad (\text{chain termination}) \quad (2)$$

$$\text{RH} + \text{OH}^- \rightarrow \text{H}_2\text{O} + \text{R}^- \rightarrow \text{further oxidation} \quad (3)$$

As aromatic compounds (RH) compete with ferrous ion for OH• (Equation 2 and 3), the presence of RH influences the behaviour of the ferrous ion (Neyens and Baeyens, 2003). Since, at high [Fe$^{+2}$], ferrous iron acts as a major reactant, not as a catalyst (Yoon et al., 2001), the conversion of benzene to phenolic compound decreases.

The H$_2$O$_2$ decomposition and OH• generation is low (Equation 1) when [Fe$^{+2}$] is low. At this situation, OH• reacts to a greater extent with H$_2$O$_2$ and generates HO$_2$• (Equation 4).

$$\text{H}_2\text{O}_2 \rightarrow \text{HO}_2^- + \text{H}^+ \quad (4)$$

This additional HO$_2$• participates in radical chain reactions by reducing ferric to ferrous ion [Equation 5] (Neyens and Baeyens, 2003). In equation 5, H$_2$O$_2$ acts as an OH•
scavenger. These could be the possible reasons for poor phenolic compound production efficiency when $[\text{Fe}^{+2}]/[\text{benzene}] < 1$.

\[
\text{OH}^\bullet + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{HO}_2^\bullet \]

...(4)

\[
\text{Fe}^{3+} + \text{HO}_2^\bullet \rightarrow \text{Fe}^{2+} + \text{O}_2 + \text{H}^+ \]

...(5)

When sufficient Fe$^{+2}$ is present, equation 3 overpowers equation 4 (Yoon et al., 2001). The presence of RH hinders the reaction between OH$^\bullet$ and the ferrous ion (equation 2), which is another route of OH$^\bullet$ depletion (Neyens and Baeyens, 2003). Therefore, a larger amount of OH$^\bullet$ remains available for benzene conversion and phenolic compound generation. Hence, $[\text{Fe}^{+2}]/[\text{benzene}] = 1$, was considered optimum for 6 mM benzene conversion.

Figure 4-42: Optimum Fe$^{+2}$ Concentration for Benzene Conversion at pH 5.0 [Initial benzene concentration 6 mM; $[\text{Benzene}]_{\text{initial}}$: $[\text{H}_2\text{O}_2]_{\text{initial}}$ = 1:1; $[\text{Fe}^{2+}]_{\text{initial}}$: $[\text{Benzene}]_{\text{initial}}$ varied from 0.0 to 2.0; added at 0 min of reaction, mixed and monitored over two hours; analyzed by HPLC]
4.5.3 Optimum H$_2$O$_2$ concentration for Benzene Conversion

To determine the effect of H$_2$O$_2$ concentration on benzene conversion, the molar ratios of H$_2$O$_2$ to benzene in the Fenton reaction mixture were varied from 0.1 to 3.0. The reactions were carried out at optimum pH and [Fe$^{2+}$], over two hour period. The effect of varying H$_2$O$_2$ is of particular interest, since the ratio of benzene to H$_2$O$_2$ determines the final conversion of benzene. The results show (Figure 4-43) that the best ratio to achieve maximum conversion of benzene to phenolic compounds is 1:1.8. It was considered the optimum ratio for [benzene]:[H$_2$O$_2$].

![Graph showing the effect of H$_2$O$_2$ concentration on benzene conversion](image)

**Figure 4-43:** Optimum H$_2$O$_2$ concentrations for Benzene Conversion at pH 5.0

[Initial benzene concentration 6 mM; [Benzene]$_{initial}$: [Fe$^{2+}$]$_{initial}$ = 1:1; [H$_2$O$_2$]$_{initial}$:[Benzene]$_{initial}$ varied from 0.1 to 3.0; added at 0 min of reaction, mixed and monitored over two hours; analyzed by HPLC]
At lower H$_2$O$_2$ concentration, production of phenolic compounds was low due to insufficient H$_2$O$_2$ concentration. At high H$_2$O$_2$ concentration, higher conversion of benzene was observed. However the concentration of phenolic compounds produced during the reaction decreased. This phenomenon can be attributed to the non-selective nature of OH•. The loss of phenolic compounds is due to the fact that hydroxyl radical reacts with these chemicals and generates further oxidation products. The H$_2$O$_2$ concentration in a Fenton reaction depends on the initial pollutant concentration, the response of the pollutant to oxidation and the objective pursued in terms of reduction of the contaminant load (Bautista et al., 2008). The purpose of this study was to define the optimum conditions for which the maximum conversion of benzene to phenolic compounds could be achieved. The complete understanding of the reaction between benzene and H$_2$O$_2$, at high levels of H$_2$O$_2$ has not been attempted here.

4.5.4 Optimum Reaction Time for Benzene Conversion

The hydroxyl radical generated in the Fenton reaction is non-selective in nature. Hence, these radicals can cause mineralization of the generated phenolic compounds as well. For that purpose the formation of the phenolics from benzene was monitored over a three hour reaction period to find a suitable reaction time. The results presented in Figure 4-40 indicate that at pH 5.0, 60 min was sufficient to convert benzene to the corresponding phenolics. After 1hr, the concentration of phenolic compounds did not increase significantly and after 2 hr, the products started to decrease indicating further oxidation. Hence a reaction period of 1 hr was considered as the optimum reaction period.
4.5.5 Identification of Reaction Products

Xu et al., (1995) proposed that benzene undergoes hydroxylation and first forms an unstable hydroxycyclohexadienyl radical intermediate which undergoes oxidation to generate phenol or dimerization and dehydration to form biphenyl (Figure 4-44). The second mechanism (Nickelsen et al., 1994 and McIntyre, 1999), suggests that the OH• reacts with benzene to generate phenol. This phenol undergoes further hydroxylation to generate corresponding diols.

![Benzene Degradation Pathway](image)

**Figure 4-44: Benzene Degradation Pathway**

The analysis of the Fenton reaction mixture generated in this study revealed that benzene follows mostly the oxidation pathway presented in Figure 4-44. The Fenton reaction
product analysis revealed that at pH 5.0, about 80% of the initial benzene was converted to a mixture of phenol, catechol, resorcinol, hydroquinone, benzoquinone and biphenyl (Figure 4-45). It is speculated that OH• reacted with benzene to generate phenol which underwent further hydroxylation to generate the corresponding diols and quinones. Along with benzenediols and benzoquinone, Zazo et al., (2005) identified maleic, acetic, formic and muconic acid in the reaction mixture generated during the Fenton oxidation of 100 mg/L of phenol at pH 3 ([hydrogen peroxide]_{\text{initial}}/[phenol]_{\text{initial}} = 5:1). In their study, maleic, acetic, oxalic and formic acids were present in high concentrations whereas muconic acid was found in low concentration. The dicarboxylic acids, namely muconic and maleic acids, are believed to be the primary products from ring-opening of the aromatic compounds. These acids give rise to the short-chain acids and carbon dioxide in the reaction pathway.

TOC analysis of the reaction mixture at pH 5.0 indicates that there is no substantial carbon loss from the batch reactors at this pH (Figure 4-41). However, due to the non-selective nature of OH•, it is possible that some of the aromatic compounds formed in the process underwent further oxidation to produce lower molecular weight organic acids. The resulting reaction mixtures were analyzed for the presence of low molecular weight organic acids. The possible presence of maleic, succinic and acetic acid in the reaction mixture were identified. Unfortunately, their individual peaks could not be separated and quantified from the reaction mixture due to poor separation in the HPLC. On the other hand, muconic acid could be identified and quantified in trace amounts ($\approx 0.2\%$ of initial benzene).
4.5.6 Quantification of Reaction Product

After the Fenton pre-treatment process, the reaction mixture was analyzed using HPLC in order to determine its composition (Figure 4-45). Previous studies have established that phenol and benzenediols (catechol, resorcinol and hydroquinone) are good substrates of laccase (Saha et al., 2011) and SBP (Caza et al., 1999, Al-Ansari et al., 2009). Based on these studies it is expected that enzymatic treatment would be successful in reaction product removal.

![Figure 4-45: Analysis of Reaction Mixture after 1 Hr of Fenton Pre-treatment under Optimum Condition](Image)

The results indicate that about 4% of initial benzene was converted to biphenyl. This product, having limited solubility in aqueous solution, is expected to precipitate out of the reaction mixture. A large amount of benzoquinone is also generated in the process. The pH study on hydroquinone indicates that above pH 6.5, it undergoes chemical oxidation (just in the presence of ambient oxygen) to form quinone or semi-quinone structures.
As the Fenton reaction is stopped at pH 7.0, it is possible that a large amount of hydroquinone generated in the Fenton reaction is converted to benzoquinone during the iron removal process. On the other hand, it is also possible that benzoquinone is generated in the Fenton reaction itself as an oxidation product of hydroquinone. It should be noted that both benzoquinone and biphenyl are outside the scope of enzyme-catalyzed removal due to their chemical structure.

4.6 Effect of Multiple Step of Reactant Addition on the Fenton System

The foregoing section demonstrates that when all the reactants are introduced to the system at the beginning of the reaction, the best benzene removal efficiency without causing significant mineralization occurs at pH 5.0 (Figure 4-45). However, under this condition, about 23% of benzene still remains in solution. The conversion of benzene and production of aromatic compounds (considering phenol, catechol, resorcinol, hydroquinone and benzoquinone only) under this optimum condition (benzene: Fe$^{2+}$:H$_2$O$_2$= 1: 1: 1.8, pH 5.0, 1 hr reaction) is presented in Figure 4-46. It is of interest to examine different modes of Fe$^{2+}$ and H$_2$O$_2$ addition and to determine whether those would improve the benzene conversion efficiency and phenolic product yield.

4.6.1 Gradual Addition of Hydrogen Peroxide in the Fenton Reaction

In the Fenton system, hydrogen peroxide concentration plays a vital role in the product yield and benzene conversion. In order to determine the effect of gradually increasing hydrogen peroxide in the Fenton system, previously determined optimum concentration of hydrogen peroxide for 6 mM benzene was added at a rate of 1.08 mM H$_2$O$_2$/min,
Figure 4-46. To avoid the possibility of mineralization, hydrogen peroxide concentration in this study was kept at the previously determined optimum concentration.

Figure 4-46: Fenton Reaction on 6mM Benzene under Optimum Conditions [Initial benzene concentration 6 mM; [Benzene]\text{initial}; [Fe}^{2+}]_{\text{initial}}; [H_2O_2]_{\text{initial}} = 1:1:1.8; added at 0 min of reaction, at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; product concentration represents of phenol, catechol, resorcinol, hydroquinone and benzoquinone only]

Gradual addition of hydrogen peroxide (Figure 4-47) is not beneficial compared to single addition of hydrogen peroxide at 0 min (Figure 4-46). For example, when all the reactants are added at 0 min, about 76% of benzene conversion is achieved at the end of a 1hr reaction period (Figure 4-46), whereas, with gradual addition of hydrogen peroxide over
the first 10 minutes (Figure 4-47), the benzene conversion efficiency after a 1-hr reaction is 70%.

---

**Figure 4-47: Gradual Addition of 10.8 mM Hydrogen Peroxide in the Fenton Reaction**

[Initial benzene concentration 6 mM; [Benzene]_initial: [Fe^{2+}]_initial: [H_2O_2]_initial = 1:1: 1.8; benzene and Fe^{2+} added at 0 min of reaction; H_2O_2 added at a rate of 1.08 mM/min; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; product concentration represents of phenol, catechol, resorcinol, hydroquinone and benzoquinone only]

The product yield is also adversely affected by the gradual addition of hydrogen peroxide. When hydrogen peroxide is added at the beginning of the reaction, about 67% initial benzene gets converted to aromatic compounds (Figure 4-46), whereas with gradually added hydrogen peroxide, only 50% of the initial benzene could be accounted
for as a mixture of phenol, catechol, resorcinol, hydroquinone and benzoquinone (Figure 4-47).

4.6.2 Step Addition of Fe$^{2+}$

Under the optimum Fenton reaction condition (all reactants added at 0 min), after 10 minutes of reaction, the soluble iron concentration in the reaction mixture depletes to 30% (Figure 4-39). Thus, the conversion of benzene could be limited by absence of Fe$^{2+}$. However, the iron optimization study indicates that if a larger quantity of iron is introduced at the beginning of the Fenton reaction, the product yield of benzene is adversely affected (Figure 4-42). Hence, it is speculated that if a smaller quantity of Fe$^{2+}$, is introduced at a later stage of the reaction, it might aid in improving benzene conversion and product yield. For this reason, 2 mM of additional Fe$^{2+}$ was introduced to the previously determined optimum Fenton reaction condition.

The addition of Fe$^{2+}$, at the 5-minute point, did neither significantly alter the benzene removal efficiently, nor the product yield (Figure 4-48). After one hour of reaction, in the current study, 62% product yield and 20% remaining benzene was observed, this product yield is only 5% less than that of the single addition of iron study (Figure 4-46). Thus, step addition of Fe$^{2+}$ did not significantly improve the product yield or benzene conversion efficiency.
Figure 4.48: Step Addition of Fe$^{2+}$ in the Fenton Reaction [Initial benzene concentration 6 mM; [Benzene]$\text{initial}$: [Fe$^{2+}$]$\text{initial}$: [H$_2$O$_2$]$\text{initial}$ = 1:1: 1.8; added at 0 min of reaction, after 5 min of reaction additional 2 mM Fe$^{2+}$ added; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; product concentration represents of phenol, catechol, resorcinol, hydroquinone and benzoquinone only]

4.6.3 Step Addition of H$_2$O$_2$

The conversion efficiency and product yield in a Fenton system can be regulated by the amount of hydrogen peroxide in the system. As hydrogen peroxide is the main source of oxidant in the Fenton reaction, it is possible that addition of hydrogen peroxide might improve the benzene conversion efficiency. However, the hydrogen peroxide optimization study indicates that if larger quantity of hydrogen peroxide is introduced at the beginning of the Fenton reaction, the aromatic products generated might undergo
further oxidation (Figure 4-43). This is not desirable as the aim of this study is to generate maximum possible concentrations of phenolic products from benzene. Hence, it is speculated that if a smaller quantity of hydrogen peroxide, is introduced at a later stage of the reaction, it might aid in improving benzene conversion without adversely impacting phenolic product yield. For this reason, 3.6 mM of additional H₂O₂ was introduced after 5 minutes to the previously determined optimum Fenton reaction condition.

Figure 4-49: Step Addition of H₂O₂ in the Fenton Reaction [Initial benzene concentration 6 mM; [Benzene]initial: [Fe²⁺]initial: [H₂O₂]initial = 1:1: 1.8; added at 0 min of reaction, after 5 min of reaction additional 3.6 mM H₂O₂ added; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; product concentration represents of phenol, catechol, resorcinol, hydroquinone and benzoquinone only]
The results of this study demonstrate that the addition of hydrogen peroxide decreases the total aromatic compound concentration (Figure 4-49). After one hour of reaction under these conditions, the concentration of aromatic products accounts for about 55% of the initial benzene (Figure 4-49). This concentration of products is less than that under the optimum condition (Figure 4-46) and step addition of Fe+2 (Figure 4-48), by 12% and 7%, respectively. However, the additional hydrogen peroxide did not alter the remaining benzene conversion. The results indicate that the additional hydrogen peroxide most likely aided in further mineralization of the aromatic products.

4.6.4 Step Addition of Fe$^{+2}$ and H$_2$O$_2$

In order to determine whether step addition of Fe$^{+2}$ and H$_2$O$_2$ could improve the benzene conversion efficiency and aromatic product yield, an additional 2 mM Fe$^{+2}$ and 3.6 mM H$_2$O$_2$ were introduced to the Fenton reaction at the 5-min reaction time.

The addition of Fe$^{+2}$ and H$_2$O$_2$ slightly increased the benzene conversion efficiency (Figure 4-50). After one h of reaction under the current condition, 17% benzene was remaining in the solution. However, the aromatic product concentration was only 40% of the initial benzene. It is possible that the aromatic compounds underwent further oxidation under the current condition. Thus the resulting concentration of products in the reaction mixture was smaller than that under the optimum conditions.
Figure 4-50: Step Addition of Fe$^{2+}$ and H$_2$O$_2$ in the Fenton Reaction [Initial benzene concentration 6 mM; [Benzene]$_{initial}$: [Fe$^{2+}$]$_{initial}$: [H$_2$O$_2$]$_{initial}$ = 1:1:1.8; added at 0 min of reaction, after 5 min of reaction an additional 2 mM Fe$^{2+}$ and 3.6 mM H$_2$O$_2$ added; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; product concentration represents of phenol, catechol, resorcinol, hydroquinone and benzoquinone only]

4.7 Process Parameter Optimization for Enzymatic Removal of the Fenton Reaction Products (Two-Stage, Two-Reactor System)

When all the reactants of the Fenton system are added together at the beginning of the reaction, the best benzene to aromatic compound conversion efficiency occurs at a pH of 5.0, benzene:Fe$^{2+}$:H$_2$O$_2$ of 1:1:1.8 and a reaction time of 1hr. These reactant concentrations and reaction conditions are considered as the first step of the two-stage, two-reactor configuration. In the first stage, the Fenton reaction stage, about 72% of initial benzene gets converted to a mixture of biphenyl, hydroquinone, benzoquinone,
resorcinol, catechol and phenol (Figure 4-45). Though benzoquinone and biphenyl are outside the scope of enzymatic treatment due to their chemical structure, these two compounds can be removed by other means. Among the other products, phenol, catechol, resorcinol and hydroquinone, which account for about 35% of the initial benzene concentration, are good substrates for enzymatic treatment.

As the second step of the treatment process, the optimal conditions needed to obtain ≥ 95% conversion (an arbitrary benchmark for ease of comparison) of the aromatic compounds generated in the Fenton reaction were determined. All discussions of optima in this paper refer to local optima as determined for the parameter in question within the respective ranges specified. The factors of interest include pH, minimum enzyme requirement to achieve ≥ 95% removal, and reaction time. Optimum enzymatic reaction conditions were determined for both laccase and SBP.

4.7.1 Optimum Reaction Conditions for Laccase-Catalyzed Treatment

4.7.1.1 Effect of pH on conversion of phenolic compounds

Previous studies have established that, when laccase was used as enzyme on equimolar mixture of phenol and the benzenediols, the best removal was achieved at pH 5.6 (Saha et al., 2011). Hence this pH was considered as optimum pH.

4.7.1.2 Effect of enzyme concentration on the conversion of phenolic compounds

In order to determine the optimum enzyme requirement for the phenolic compound mixture generated from Fenton reaction, experiments were run at pH 5.6 in batch reactors containing 0- 3 U/mL laccase. The results (Figure 4-51) indicate that, in order to achieve ≥ 95% of substrate conversion, 2.2- 2.4 U/mL of laccase was required. This amount is
higher than the laccase requirement of equimolar mixture of phenol and benzenediols. The high enzyme requirement could be a result of enzyme requirement of other unidentified phenolic compounds that might be present in the reaction mixture.

Figure 4-51: Laccase optimization of Fenton reaction mixture [Batch reactors containing a mixture of known phenolic compounds (phenol, catechol, resorcinol and hydroquinone) generated from Fenton reaction (reaction conditions and phenolic concentrations as presented in Figure 4-45); laccase treatment performed at pH 5.6, room temperature and three-hour reaction period; samples analyzed by HPLC.]

It is possible that the amount of dissolved iron present in the solution is causing enzyme inactivation and as a result the enzyme requirement is higher. Laccase activity analysis confirms this possibility (Figure 4-52). It is also possible that at this pH range the dissolved iron is demonstrating the coagulation capability (Neyens and Baeyens, 2003).
During laccase optimization of the composite wastewater containing equimolar concentration of phenol and benzenediols, no visible precipitates were observed (section 4.1.5). However, when the reaction mixture generated from the Fenton reaction was treated with laccase, some small flocs were observed.

Figure 4-52: Laccase activity in the batch reactors containing Fenton reaction products [Batch reactors containing a mixture of known phenolic compounds (phenol, catechol, resorcinol and hydroquinone) generated from Fenton reaction (reaction conditions and phenolic concentrations as presented in Figure 4-45); laccase treatment performed at pH 5.6, room temperature and three-hour reaction period; initial laccase concentrations 1U/mL; samples were withdrawn at appropriate time, micro filtered and enzyme activity measured by using standard laccase activity assay]
The literature demonstrates that as a coagulant iron captures the dissolved suspended solids and precipitates them. The iron present in the solution aids in the coagulation and precipitation of such flocs, which are most like enzymatic reaction products. The polymers formed during the enzyme-catalyzed oxidation have an adverse effect on enzyme activity (Dasgupta et al., 2007). Previous studies have demonstrated that enzymes tend to have an affinity towards such polymeric products. The free enzyme in the solution gets attached to the polymeric end products and settles out of the solution. The loss of active enzyme in such manner could also add to the enzyme demand.

4.7.2 Optimum Reaction Conditions for SBP Catalyzed Treatment

4.7.2.1 Effect of pH on Conversion of Phenolic Compounds

Previous studies indicate that, when SBP was used to remove 1 mM of phenol, catechol and resorcinol individually, the optimum pH range was between pH 6.5 to 7.5 (Caza et al., 1999, Al-Ansari et al., 2009). However, for 1 mM hydroquinone optimum pH occurred in the pH range 4.0-6.5. As optimum pH for most of the substrates occurred close to neutral pH, pH 7.0 was considered as optimum pH for enzymatic removal of phenolic compounds generated.

4.7.2.2 Effect of enzyme and hydrogen peroxide concentration on the conversion of phenolic compounds

To determine the SBP and H$_2$O$_2$ concentrations required to accomplish removal of the phenolic compounds, three-hour enzymatic treatment was allowed at neutral pH. At first SBP catalyzed removal of the phenolic compounds were attempted without addition of any additional hydrogen peroxide. It was speculated that, there might be some hydrogen
peroxide remaining in the post-Fenton treatment reaction mixture, which would be sufficient to meet the hydrogen peroxide demand of SBP catalysis. The SBP concentration was varied from 1.5 to 4.0 U/mL. The results of the study indicate that hydroquinone, catechol, resorcinol and phenol underwent about 45%, 30%, 25% and 7% conversion at the best (Figure 4-53). This conversion efficiency did not improve with increasing SBP. This indicates that the remaining hydrogen peroxide in the reaction mixture was inadequate to meet the hydrogen peroxide demand of peroxidase-catalyzed treatment.

Figure 4-53: Effect of SBP concentration on the Fenton Reaction Mixture in absence of additional hydrogen peroxide [Batch reactors containing a mixture of known phenolic compounds (phenol, catechol, resorcinol and hydroquinone) generated from Fenton reaction (reaction conditions and phenolic concentrations as presented in Figure 4-45); 0-4 U/mL SBP added without any additional H₂O₂; enzymatic reaction at pH 7.0, room temperature, three-hour reaction period; samples analyzed by HPLC]
In order to determine the minimum hydrogen peroxide concentration for more than 95% removal of the phenolic compounds, SBP and H₂O₂ concentrations were varied from 1 to 4 U/mL and 0 to 8 mM, respectively, at neutral pH. The batch reactor study indicates that, for ≥ 95% substrate conversion, 2.0 U/mL SBP and 5 mM hydrogen peroxide were required (Figure 4-54, Figure 4-55).

Figure 4-54: Hydrogen peroxide optimization for the reaction mixture at the end of the Fenton reaction [Batch reactors containing a mixture of known phenolic compounds (phenol, catechol, resorcinol and hydroquinone) generated from Fenton reaction (reaction conditions and phenolic concentrations as presented in Figure 4-45); Enzymatic reaction in the presence of 1 U/mL SBP with additional H₂O₂, at pH 7.0, room temperature, three-hour reaction period; samples analyzed by HPLC]
**Figure 4-55: SBP Optimization of Fenton Reaction Mixture** [Batch reactors containing a mixture of known phenolic compounds (phenol, catechol, resorcinol and hydroquinone) generated from Fenton reaction (reaction conditions and phenolic concentrations as presented in Figure 4-45); Enzymatic reaction in the presence of 0-4 U/mL SBP with additional 5 mM H\(_2\)O\(_2\), at pH 7.0, room temperature, three-hour reaction period; samples analyzed by HPLC]

This enzyme and hydrogen peroxide requirements are higher than those prorated hydrogen peroxide and SBP requirements. Based on previous results (Caza et al., 1999, Al-Ansari et al., 2009), the reaction mixture generated after the Fenton reaction (Table 4-2) should require 4.0 mM hydrogen peroxide and 1.5 U/mL SBP. The reason for higher SBP and hydrogen peroxide demand could potentially be due to presence of any unknown products in the solution. However, it is also possible that, like laccase, the iron and the polymeric end products in the solution have an impact on the SBP-catalyzed conversion.
4.7.3 Removal of Benzoquinone and Biphenyl generated

Enzymatic removal of biphenyl and benzoquinone was not possible as they were outside the scope of such process. Biphenyl, accounting for 4% of the initial benzene, precipitated from the aqueous solution. Benzoquinone, accounting for 30% of the initial benzene, cannot be removed by the enzymatic process. In addition, hydroquinone, accounting for 15% of the initial benzene, is most likely to be converted to benzoquinone in the enzymatic step. Thus, other methods should be explored to remove this product.

After the Fenton pre-treatment under the optimum reaction conditions and enzymatic treatment, the total benzoquinone concentration in the reaction mixture was about 2.4 mM. This was considered 100% benzoquinone concentration for this study.

Previous studies have demonstrated that additives like chitosan flakes and PEI can remove quinone from the solution by forming a carbon-nitrogen bond. The amino groups of chitosan or PEI react with the carbonyls of quinone molecules (Wada et al., 1995). These additives can selectively remove quinones from solution without affecting the removal of phenolic compounds (Edwards et al., 1999; Takasashi et al., 2005). Studies by others (Wada et al., 1995) as well as the current study (section 4.3) have also shown that PEI is more effective than chitosan. Hence, in order to remove the benzoquinone generated from the two-stage Fenton and enzymatic system, only the effect of PEI was investigated.

The results indicate that (Figure 4-56), at a PEI concentration of 375-425 mg/L, more than 95% of the benzoquinone was removed after one-hour contact time.
Figure 4-56: Effect of PEI concentration and reaction time on removal of benzoquinone generated from two-stage Fenton-Enzymatic treatment [Batch reactors containing post-Fenton and enzymatic reaction mixtures at pH 7.0, along with varying concentrations of PEI, samples mixed and monitored for three hours, analyzed with HPLC]

However, after the PEI treatment, the light brown colored solution turned light orange in color. There was some visible turbidity along with precipitates. This residual color is not acceptable and removal of soluble products (colored or not) is necessary for effluent discharge, thus alum was used as a coagulant. At alum (as aluminum sulphate) concentration of 200 mg/L, about 85% of this color was removed (Figure 4-57). The resulting solution was almost colorless.
Figure 4-57: Alum Treatment on PEI treated sample [Batch reactors containing post-Fenton and enzymatic reaction mixtures at pH 7.0; along with varying concentrations of PEI; mixed for two hours; after two hours, 200 mg/L of alum as aluminum sulfate concentration added, samples filtered prior to analysis]

4.8 Simultaneous Fenton and Enzymatic Reaction (Single Batch Reactor)

The hydroxyl radical generated in the Fenton process is non-selective in nature. The products of the Fenton reaction on benzene are mostly aromatic compounds which can further react with the available oxidizing agent and compete with benzene for the available hydroxyl radicals. However, if these competing aromatic products can be removed from the system immediately, then it might improve overall benzene conversion efficiency.

Under the optimum Fenton reaction conditions (as described in section 0), about 80% benzene conversion efficiency is achieved. About 37% of the starting benzene generates phenolic compounds which are very good candidates for enzyme catalyzed polymerization.
However, about 35% of the starting benzene gets converted to aromatic compounds like benzoquinone and biphenyl which are outside the scope of enzymatic process. It is hypothesized that the simultaneous Fenton and enzymatic reactions will remove the phenolic compounds from the system and thus will increase the possibility of improved benzene conversion.

It should be noted that the larger polymeric products generated in the enzymatic system are hydrophobic in nature and can be removed from solution by using simple coagulant aids. Iron (III) which is a regular coagulant in water treatment facilities which improves the settling characteristics of such products. Atomic absorption analysis on the Fenton reaction mixture (Figure 4-39) demonstrated that, at the optimum Fenton reaction pH of 5.0, within one hour reaction period, about 80% of the initial iron starts contributing as coagulant as it gets converted to insoluble iron(III). Hence, there is a good possibility that if the enzymatic treatment is successful in the simultaneous system, the iron will aid in the polymeric product removal as well.

On the other hand, if the simultaneous system fails to improve the benzene conversion efficiency, but removes the phenolic products efficiently, then in principle, there will be only one reactor required for both the Fenton and enzymatic reaction. This will reduce the footprint and cost of the proposed system as well.

In order to determine the effect of simultaneous Fenton and enzymatic reactions, batch reactors were set up at with varying amounts of laccase or SBP at previously determined optimum Fenton-reaction conditions. The conversion and appearance/disappearance of aromatic products were monitored at over a three-hour reaction time. The results of this study are presented in the following sections.
4.8.1 Simultaneous Fenton and Enzymatic Reaction Using Laccase

Both Fenton and enzymatic reactions were carried out simultaneously in the same sealed batch reactor. In these experiments, single additions of all the reactants were required for both Fenton and enzymatic reaction. In order to determine the effect of laccase in this reactor configuration, laccase concentration was varied from 0.2 to 4.0 U/mL. After a three-hour reaction period, the reaction was quenched for Fenton reaction and laccase. The results of this study are presented in Figure 4-58.

![Graph showing phenolic compounds and remaining benzene vs. laccase concentration](image)

**Figure 4-58: Simultaneous Fenton and Laccase Catalyzed Oxidation of Benzene**

[Initial benzene concentration 6 mM; \([\text{Benzene}]_{\text{initial}} : [\text{Fe}^{2+}]_{\text{initial}} : [\text{H}_2\text{O}_2]_{\text{initial}} = 1:1:1.8\); added at 0 min in the presence of varying laccase concentration in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]
It was hypothesized that the simultaneous Fenton and enzymatic reaction will remove the phenolic compounds from the system and thus will increase the possibility of improved benzene conversion. As the phenolic compounds will be removed from the solution, there will be less competition in the system for the available hydroxyl radicals. Thus, this will make more hydroxyl radical available for benzene conversion. This study indicates that with increasing laccase concentration the simultaneous reactor configuration decreased the remaining benzene concentration. In absence of any laccase in the system, about 23% of initial benzene remains in the solution (Figure 4-45) under optimum Fenton reaction condition. However, in presence of 3 U/mL laccase, the remaining benzene in the solution comes down to 16% of the initial benzene concentration. This indicates that simultaneous Fenton and laccase system can increase the benzene conversion by 7% over that of a two stage-two step system.

The increase in laccase concentration in the simultaneous Fenton and enzymatic system also decreases the phenolic substrate (phenol, catechol, resorcinol and hydroquinone) concentration in the system. It is possible that the phenolic compound concentration in the current system is a combination of unconverted phenolic compounds from the enzymatic system and newly formed phenolic compound from additional benzene conversion in the Fenton system. The reduction in phenolic compound concentration in the simultaneous system required much more laccase than the two stage-two reactor system. In the two stage-two reactor system, at a laccase concentration of 2.2 U/mL most of the phenolic compounds were converted to end products. However, in the simultaneous one-stage reactor configuration, even twice the amount of laccase was
unable to achieve such conversion efficiency. This possibly poor conversion efficiency could be due to unavailability of dissolved oxygen or inactivation of laccase.

In order to oxidize the phenolic products, laccase utilizes molecular oxygen. Generally, in an open reactor, the molecular oxygen is supplied by natural diffusion from the atmosphere. However, in a sealed batch reactor, diffusion from the atmosphere will not be possible, which might lead to limiting oxygen conditions. In the past, researchers have attempted to provide the necessary oxygen for the laccase catalyzed reaction by a) solution aeration (i.e., water was aerated by bubbling air over extended period of time) and (2) adding hydrogen peroxide to the solution (Vermette et al., 2000). Normally, when hydrogen peroxide is added as a dissolved oxygen source in an enzymatic system, catalase is also added so that the hydrogen peroxide can dissociate to molecular oxygen. However, in case of the simultaneous Fenton and enzymatic system, adding catalase would affect the hydroxyl radical yield in the Fenton process.

The stoichiometric calculation indicates that the amount of dissolved oxygen present in the distilled water should be sufficient for laccase catalyzed oxidation of phenolic compounds generated in the Fenton reaction. However, to ensure the presence of enough oxygen in the laccase containing batch reactors, water saturated with oxygen (by air bubbling for 24 hrs) was used.

The results of the study reveal that (Figure 4-59) the presence of higher dissolved oxygen did improve the benzene or phenolic compound conversion efficiency. In the presence (Figure 4-59) and absence (Figure 4-58) of additional dissolved oxygen, benzene conversion efficiency remained almost the same. In the presence of additional dissolved oxygen and 4.0 U/mL laccase, the phenolic compound conversion efficiency increased
only by 3%. This phenomenon suggests that the dissolved oxygen is not the main factor adversely affecting the removal efficiency.

Figure 4-59: Simultaneous Fenton and Laccase-Catalyzed Oxidation of Benzene in presence of higher dissolved oxygen [Water saturated with oxygen (by passing air bubble for 24 hrs) was used for sample preparation; Initial benzene concentration 6 mM; [Benzene]initial: [Fe^{2+}]_{initial}: [H_2O_2]_{initial} = 1: 1: 1.8; added at 0 min in the presence of varying laccase concentration in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]

In order to determine laccase activity in the simultaneous system, laccase activity in the simultaneous Fenton and enzymatic system was monitored over a one-hour reaction period. In the simultaneous system laccase becomes inactivated very fast (Figure 4-60). Within the
first 10 min of reaction, laccase loses about 60% of its initial activity. Within first 20 minutes, about 80% of laccase activity is lost. This could be the main reason for poor phenolic conversion efficiency in the simultaneous system.

![Graph showing laccase activity in the simultaneous Fenton and enzymatic system](image)

**Figure 4-60: Laccase activity in the simultaneous Fenton and enzymatic system**

[Initial benzene concentration 6 mM; [Benzene]$_{initial}$: [Fe$^{2+}$]$_{initial}$: [H$_2$O$_2$]$_{initial}$ = 1:1: 1.8; added at 0 min in the presence of 1U/mL laccase concentration in a sealed batch reactor; at pH 5.0; mixed and monitored for 1 hr, after appropriate time period, samples were withdrawn, filtered and activity measured by standard activity test]

The simultaneous Fenton and enzymatic system is operated at pH 5.0. It is very close to the optimum pH range of most of the laccase substrates. Hence, inactivation due to pH is unlikely. However, it is very likely that the highly reactive hydroxyl radical generated in the Fenton system is contributing towards the rapid decrease of laccase activity. Previous studies have demonstrated that the presence of dissolved iron and the coagulation capacity
of iron can also contribute towards decreasing laccase activity (Figure 4-52). In the simultaneous system, dark-brown precipitate starts forming within the first hour of reaction. The free available laccase can also precipitate alongside these particles. The iron present in the solution also starts to precipitate (Figure 4-39). This iron precipitate helps in larger floc formation and better settling of the particulates, which might aid in further loss of laccase.

In the first few minutes of reaction in the simultaneous system, the enzyme converted phenolic compounds to corresponding radicals up to its maximum capacity. As the laccase concentration increases in the batch reactor, the amount of active enzyme also increases which contributes to the removal of phenolic compounds. However, presences of hydroxyl radical, organic particulates formation and iron sludge are probably the main contributing factors in inactivating laccase in the simultaneous system.

4.8.2 Simultaneous Fenton and Enzymatic Reaction Using SBP

Simultaneous Fenton and SBP-catalyzed oxidation was carried out in the same sealed batch reactor. In these experiments, single additions of all the reactants were required for both Fenton and enzymatic reaction. In order to determine the effect of SBP in this reactor configuration, SBP concentration was varied from 0.0 to 10.0 U/mL and monitored over a three-hour reaction period. During this time, disappearance of benzene from the system and concentration of phenolic substrates (phenol, catechol, resorcinol and hydroquinone) were monitored. The results of this study are presented in Figure 4-61 to Figure 4-67.

The presence of SBP did not significantly improve the disappearance of benzene from the system (Figure 4-61 to Figure 4-67). In the absence of SBP, after three hours of Fenton
reaction the benzene concentration in the reactor accounts for about 22% of the initial benzene (Figure 4-61).

**Figure 4-61: Fenton Oxidation of Benzene in absence of SBP** [Initial benzene concentration 6 mM; \( [\text{Benzene}]_{\text{initial}} : [\text{Fe}^{2+}]_{\text{initial}} : [\text{H}_2\text{O}_2]_{\text{initial}} = 1:1:1.8 \); added at 0 min without any SBP in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]
Figure 4-62: Simultaneous Fenton and SBP Catalyzed Oxidation of Benzene in presence of 0.5 U/mL SBP [Initial benzene concentration 6 mM; [Benzene]_{initial}; [Fe^{2+}]_{initial}; [H_2O_2]_{initial} = 1:1: 1.8; added at 0 min with 0.5U/mL SBP in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]
Figure 4-63: Simultaneous Fenton and SBP Catalyzed Oxidation of Benzene in presence of 1.0 U/mL SBP [Initial benzene concentration 6 mM; \([\text{Benzene}]_{\text{initial}}\); \([\text{Fe}^{2+}]_{\text{initial}}\); \([\text{H}_2\text{O}_2]_{\text{initial}}\) = 1:1: 1.8; added at 0 min with 1.0 U/mL SBP in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]
Figure 4-64: Simultaneous Fenton and SBP Catalyzed Oxidation of Benzene in presence of 1.5 U/mL SBP [Initial benzene concentration 6 mM; [Benzene]_{initial}: \[\text{Fe}^{2+}\]_{initial}: [H_{2}O_{2}]_{initial} = 1:1: 1.8; added at 0 min with 1.5 U/mL SBP in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]
Figure 4-65: Simultaneous Fenton and SBP Catalyzed Oxidation of Benzene in presence of 2.0 U/mL SBP [Initial benzene concentration 6 mM; [Benzene]$_{\text{initial}}$; [Fe$^{2+}$]$_{\text{initial}}$; [H$_2$O$_2$]$_{\text{initial}}$ = 1:1: 1.8; added at 0 min with 2.0 U/mL SBP in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]
Figure 4-66: Simultaneous Fenton and SBP Catalyzed Oxidation of Benzene in presence of 5 U/mL SBP [Initial benzene concentration 6 mM; [Benzene]_{initial}: [Fe^{2+}]_{initial}: [H_2O_2]_{initial} = 1:1: 1.8; added at 0 min with 5.0 U/mL SBP in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]
Figure 4-67: Simultaneous Fenton and SBP Catalyzed Oxidation of Benzene in presence of 10 U/mL SBP [Initial benzene concentration 6 mM; [Benzene]_{initial}: [Fe^{2+}]_{initial}: [H_2O_2]_{initial} = 1:1: 1.8; added at 0 min with 10.0 U/mL SBP in a sealed batch reactor; at pH 5.0; mixed and monitored for 3 hours; analyzed by HPLC; phenolic products represents total quantified phenol, hydroquinone, catechol and resorcinol in the resulting solution]

However, in the presence of 1 U/mL and 10 U/mL of SBP, the remaining benzene concentration in the reactor accounts for 16% and 18% of the initial benzene concentration, respectively. As the Fenton reaction and SBP-catalyzed oxidation, both utilize hydrogen peroxide, it is possible that there was not sufficient hydrogen peroxide left in the reactor to transform benzene. However, it is also likely that during the three-hour reaction period, there was not enough soluble iron to carry forward the Fenton system.
Another interesting observation in these experiments was the rate of benzene disappearance. If there is no SBP present in the reactor, the benzene disappearance in the early period of reaction (first 10 minutes), is rapid and after that time period, benzene concentration slowly decreases over time (Figure 4-61). However, when SBP is present in the solution, the disappearance of benzene during the early reaction period is slower (Figure 4-62 to Figure 4-67). However, this phenomenon is more pronounced in the batch reactors containing more than 2 U/mL of SBP (Figure 4-65 to Figure 4-67). The Fenton system and the SBP catalyzed polymerization both utilize hydrogen peroxide in their system. It is possible that this phenomenon is due to the competition between SBP and Fenton reagent for available hydrogen peroxide.

The presence of SBP in this reactor configuration aids in reducing the phenolic substrate concentration (Figure 4-61 to Figure 4-67). If there is no SBP present in the reactor (Figure 4-61), then after 3 hrs of Fenton reaction at previously determined optimum condition (benzene: Fe+2; H2O2= 1: 1: 1.8; pH 5.0), the phenolic substrate concentration in the reactor accounts for about 27% of the initial benzene concentration. The overall reduction in the phenolic substrate concentration indicates removal of the phenolic compounds.

The concentration of phenolic substrates decreases with increasing SBP concentration. For example, after three hour of simultaneous reaction in the presence of 1U/mL of SBP (Figure 4-62), the phenolic concentration in the reaction mixture is about 18% of initial benzene concentration. When 2U/mL of SBP (Figure 4-65) is present in the system, that concentration becomes about 9.5%. However, in the presence of 10 U/mL of SBP (Figure 4-66), the phenolic compound concentration becomes about 4% of the initial benzene...
concentration. Previously, it has been observed that the disappearance of phenolic compounds increases linearly with increasing enzyme concentration. On the contrary, based on the results of the current study, it can be suggested that the phenolic compound concentration does not decrease linearly with increasing SBP concentration. Such phenomenon is possibly due to SBP inactivation.

![SBP Activity](image)

**Figure 4-68: SBP activity in the simultaneous Fenton and enzymatic system** [Initial benzene concentration 6 mM; [Benzene]$_{initial}$: [Fe$^{2+}$]$_{initial}$: [H$_2$O$_2$]$_{initial}$ = 1:1: 1.8; added at 0 min with 2.0 U/mL SBP in a sealed batch reactor; at pH 5.0; mixed and SBP activity monitored for 1 hr using standard SBP activity assay]

The activity analysis on a simultaneous sample containing 2U/mL of SBP, indicate that within first 30 minutes of reaction, about 55% of initial activity is lost (Figure 4-68). This could be the reason for the poor phenolic compound removal efficiency. The simultaneous Fenton and enzymatic system is operated at pH 5.0. However, SBP has
shown unusual pH stability (Al-Ansari et al., 2011). Hence, at pH 5.0, inactivation due to pH is unlikely. However, it is possible that the highly reactive hydroxyl radical generated in the Fenton system is contributing towards the inactivation of SBP.
CHAPTER 5
SUMMARY OF RESULTS

Based on the results obtained from the batch reactor studies, the following summary can be made.

5.1 Process Parameter Optimization for Laccase-Catalyzed Removal of Phenolic Compounds

The limited Fenton reaction on benzene is expected to produce phenolic compounds without causing significant mineralization. These phenolic compounds will then be removed by enzyme-catalyzed polymerization. This study sought to demonstrate oxidative polymerization of phenol and each of the three benzenediols, namely catechol, resorcinol and hydroquinone, in the presence of laccase followed by removal of products via coagulation and flocculation with alum.

As the first step of the treatment process, the optimal conditions needed to obtain ≥ 95% conversion of these aromatic compounds were determined. The factors of interest were effect of pH, laccase concentration, substrate concentration, and PEG effect.

- In the absence of enzyme, pH above 6.5 and 7.8 had a pronounced effect on hydroquinone and catechol conversion, respectively. At higher pH, catechol and hydroquinone were chemically oxidized to quinone or semi-quinone structures. Catechol and hydroquinone can easily be transformed into quinones because of the respective ortho- and para-positions of the hydroxyl groups. Conversely, the meta-position of hydroxyl groups in resorcinol, prevent its conversion to a quinone. Elevated pH did not result in the conversion of any phenol while about 5% of resorcinol was converted above pH 7.0.
In the presence of laccase, optimum pH for enzyme-catalyzed conversion of each substrate was determined from the conventional bell-shaped curve of pH-dependence. The optimum pHs for phenol and the benzenediols were in the range of 5.0-5.6 with the exception of hydroquinone, which showed a broad pH range.

The minimum enzyme concentration at which 95% conversion of substrate was achieved at optimum pH is defined as the optimum enzyme concentration. The optimum enzyme requirements for phenol, catechol, resorcinol and hydroquinone are 0.085, 0.002, 0.007 and 0.00016 U/mL, respectively. Among these four compounds, the parent compound, phenol, required the most enzyme, the \textit{m}, \textit{o}-, and \textit{p}- substituted compounds followed in decreasing order of enzyme requirement. The relative laccase requirement for the above-mentioned substrates can be explained based on a qualitative ranking of the respective radical reactivity. The general hypothesis is, the more reactive the radical, the more enzyme inactivation caused. As the \textit{meta}-substituted radical is more reactive than the \textit{ortho}- and \textit{para}- substituted radicals, it would be expected to require more enzyme than the \textit{ortho}- and \textit{para}- isomers, consistent with our observations.

The presence of PEG could not assist in reducing the laccase requirement for achieving more than 95% conversion of phenol, catechol, resorcinol and hydroquinone. It appears that the PEG effect depends on the functional group of the substrate, the intermediates involved, as well as the enzyme involved. However, limited work has been done on the PEG effect of different classes of substrates, thus at this moment, it is difficult to comment which substrate would be a more suitable candidate for PEG effect.
• Reaction time is one of the important parameters in treatment plant design which determines the volume and thus the economics of an enzyme reactor. For all four substrates, ≥80% conversion was achieved in the first two hours of reaction but a three-hour reaction time was needed to achieve ≥ 95% conversion. For all four substrates, low levels of enzyme inactivation occurred during the three-hour reaction period. About 30% and 70% laccase inactivation was observed for benzenediols and phenol, respectively.

• The optimum enzyme required to achieve more than 95% conversion of all the substrates in the composite wastewater is not equal to the sum of optimum enzyme requirements for individual substrates (0.94 U/mL), rather, more enzyme is required to achieve similar conversion.

• The enzyme requirement to achieve more that 95% conversation over a substrate concentration range of 0.5 to 2.5 mM demonstrated linear relationships for phenol, hydroquinone, catechol and resorcinol.

In the second step of this treatment process, effectiveness of the color removal process was investigated for its dependence on factors such as coagulating agent, coagulant concentration, pH, and concentration of the substrate.

• The results of the color removal study show that 12 mg/L alum (as aluminum sulfate) was able to remove more than 95% of the residual colored product generated from laccase-catalyzed oxidation of catechol. For a similar amount of color removal for products generated by phenol and the equimolar mixture of diols and phenol, respectively, 100 mg/L and 150 mg/L of alum were required. Use of alum could only remove 60% and 80% of coloured products from reaction
mixtures of hydroquinone and resorcinol, respectively. Increasing alum concentration did not improve the color removal for these two benzenediols.

- The products generated from the laccase-catalyzed oxidation of hydroquinone consisted of mostly benzoquinone. The TOC analysis indicated that alum was successful in removing only 20% of the carbon from the hydroquinone reaction sample. Hence, alum was not a successful additive in removing laccase-catalyzed hydroquinone reaction products. It also indicated that a more effective additive should be explored to achieve these reaction products. (See Section 5.3, below, for more on benzoquinone removal.)

5.2 Process Parameter Optimization for SBP-Catalyzed Removal of Phenolic Compounds

The optimum conditions for removal of 1 mM phenol and benzenediols by using SBP have already been identified by Caza et al. (1999) and Al-Ansari et al. (2009). When appropriate, these previously determined optimum conditions were used in the study. However, SBP-catalyzed removal of 1 mM phenol and benzenediols from a composite wastewater has never been attempted. Experiments were conducted on a solution containing 1 mM each of phenol and the three benzenediols to determine the optimum pH, minimum SBP and hydrogen peroxide requirement for more than 95% conversion of these phenolics in a reaction mixture.

- In the SBP-catalyzed enzymatic system, hydrogen peroxide is required stoichiometrically for the enzymatic process. Previous studies have indicated that, in the absence of laccase, the chemical oxidation of benzenediols can be facilitated by pH change. As hydrogen peroxide is an oxidant and can further aid
in such chemical conversion, the effect of different concentration of hydrogen peroxide on the composite wastewater containing phenol and the benzenediol mixture was monitored at different pHs. The presence of hydrogen peroxide in the system aids in the chemical conversion of the benzenediols above pH 6.5. However, conversion of phenol was not significant in the presence of hydrogen peroxide.

- In the presence of SBP, higher conversions of phenol and benzenediols were observed in the pH range 6.5 to 7.0. This was considered as optimum pH range for SBP catalyzed oxidative polymerization of the composite wastewater.

- The minimum SBP and hydrogen peroxide requirement to achieve more than 95% conversion of all phenolic compounds in the composite wastewater are 1.8 U/mL and 15 mM, respectively. Hence, the optimum SBP and hydrogen peroxide required to achieve more than 95% conversion of all the substrates in the composite wastewater, is not equal to the sum of optimum SBP and hydrogen peroxide requirements for individual substrates (1.5 U/mL SBP and 7.5 mM hydrogen peroxide), rather, slightly more enzyme and much more hydrogen peroxide are required to achieve similar conversion. This result is similar to optimum laccase requirement of the composite wastewater. In all cases, catechol and hydroquinone get quickly converted. Resorcinol and phenol take longer to get converted. Relative conversion efficiencies of these substrates in this study follow the same trend as their individual optimum SBP requirement.

- Step addition of hydrogen peroxide and SBP aided in better conversion efficiency when compared to single addition of hydrogen peroxide and SBP of same
concentration. However, the optimum SBP and hydrogen peroxide requirements for single and step addition were not significantly different. In the case of single addition of SBP and hydrogen peroxide, 15 mM hydrogen peroxide and 1.8 U/mL of SBP were required. In case of step addition, 15 mM hydrogen peroxide and 1.5 U/mL of SBP (5, 5 and 5mM H₂O₂ was added at 0, 10 and 20 min, respectively; 0.5, 0.5 and 0.5 U/mL SBP was added at 0, 10 and 20 min, respectively) were required to achieve the optimum phenolic compound conversion.

5.3 Process Parameter Optimization for Removal of Benzoquinone Using Additives

The laccase- and SBP-catalyzed oxidation of hydroquinone generates benzoquinone as the major reaction product. This priority pollutant is not an enzyme substrate. Removal of it by using alum-aided coagulation was not successful. Hence, the additives chitosan and PEI were explored to remove quinone from wastewater. Factors of interest were pH, additive concentration and contact time.

- In order to determine whether pH has an effect on benzoquinone stability, a set of batch reactors were set up in the pH range of 3.5-11.3 containing only 1mM benzoquinone in the absence of any additives. The results of this study reveal that benzoquinone is fairly stable within the pH range 3.5 - 7.2. Above this pH range, the colorless benzoquinone solution turned orangish, indicating chemical transformation. This phenomenon increases with increase in pH.

- In the presence of chitosan solution, chitosan flakes and PEI, better benzoquinone removal was achieved in the pH range 6.0 - 7.5. Hence, this pH range was considered optimum for benzoquinone removal.
Benzoquinone removal depended on the contact time as well. For chitosan-aided benzoquinone removal, the removal increased with increase in contact time.

Between, chitosan solution and chitosan flakes, chitosan solution was slightly more effective. Among all the additives, PEI was the most effective one.

The observed optimum chitosan flakes concentration occurred at a chitosan concentration of 2700-3000 mg/L. A three-hour contact time was sufficient for removing ≥ 95% of the initial benzoquinone.

At about 150-200 mg/L PEI, ≥ 95% of benzoquinone removal was achieved after a one-hour contact time.

Both chitosan solution and PEI resulted in an orange-colored solution which needed subsequent alum treatment.

5.4 Process Parameter Optimization for Removal of Product Generated from Enzymatic Treatment of Hydroquinone by Using Additives

Previous studies have indicated that the products generated from the laccase and SBP-catalyzed oxidation of hydroquinone consisted of mostly benzoquinone. (Al-Ansari et al., 2009; Saha et al., 2011). The alum-aided color removal from the hydroquinone reaction sample could only reduce the carbon content 20%. The study concluded that, even though alum was effective in color removal, it was not effective in removing the reaction products generated from enzyme-catalyzed hydroquinone reaction. These products are believed to be at the monomer stage, most likely as semiquinone and quinone. Chitosan and PEI were successful in removing authentic benzoquinone. Hence, it is expected that chitosan and PEI would be successful in removing hydroquinone reaction product as well. After a three-hour reaction, HPLC analysis of the reaction mixture revealed that it
consisted of about 0.85 mM benzoquinone. These post-enzymatic reaction mixtures were used to determine the optimum benzoquinone removal conditions using chitosan and PEI.

- The chitosan- and PEI-aided removal of hydroquinone reaction product(s) was performed at the previously determined optimum pH of 7.0.
- The observed optimum chitosan concentration was around 2800-3000 mg/L, which was able to remove 95% of the reaction product after a three-hour reaction.
- At 2800-3000 mg/L, chemisorption of reaction product on chitosan was able to remove 93% of the color generated during enzymatic reaction.
- At a chitosan concentration of 2800-3000 mg/L the TOC resulting from the benzoquinone was the least. In this chitosan concentration range, about 5-8% of the TOC was due to the remaining quinone in the solution.
- At about 140-200 mg/L PEI, ≥ 95% removal of benzoquinone was achieved after a two-hour reaction at neutral pH.
- The color intensity of the PEI-treated solution increased with increasing PEI concentrations. Above 20 mg/L PEI, the color intensity of the solution was more than that of the post-enzymatic solution. There was some visible turbidity, but no precipitate was observed. In order to aid in particle settling 200 mg/L of alum was added as coagulant aid.
- At 150-250 mg/L PEI, 200 mg/L alum was able to remove 80% of the color generated. The resulting solution was colorless after alum treatment.
- After PEI (above 140 mg/L) and alum (200 mg/L) treatment, the TOC due to the remaining quinone was less that 5 mg/L.
5.5 Process Parameter Optimization for Fenton Reaction on Benzene (Single-Step Reactant Addition)

To determine the most efficient system for conversion of benzene into corresponding phenolics without causing any mineralization, batch reactors were set up to study the effect of pH, substrate concentration, [Fe$^{2+}$], [H$_2$O$_2$] and reaction time. In this study, all the reactants were added in the system at the beginning of the reaction.

- The optimum reaction conditions for conversion of 6mM benzene without causing significant mineralization were: pH 5.0, [benzene]:[Fe$^{2+}$]:[H$_2$O$_2$] of 1:1:1.8, reaction time 1 hr at room temperature.
- The TOC analysis revealed that under the optimum condition only 0.4% of the initial benzene was lost.
- The reaction products mostly consisted of phenol, hydroquinone, resorcinol, catechol, benzoquinone and biphenyl.
- Under the optimum condition, after a 1-hr reaction, the mixture consisted of 4% biphenyl, 15% hydroquinone, 4.5% resorcinol, 31.5% benzoquinone, 0.7% catechol, 17.3% phenol, 23% benzene and 4% unknown product.

5.6 Effect of Multiple Step of Reactant Addition on the Fenton System

In the Fenton reaction system, when all the reactants are introduced at the beginning of the reaction, best benzene removal efficiency without causing significant mineralization occurs at pH 5.0. However, under this condition, about 23% of benzene still remains in solution. It is of interest to examine different modes of reactant addition and to determine
whether those would improve the benzene conversion efficiency and phenolic product yield. For this purpose, various modes of Fe$^{+2}$ and H$_2$O$_2$ addition were explored.

- At pH 5.0 and [benzene]: [Fe$^{2+}$] of 1:1, continuous addition of hydrogen peroxide ([benzene]:[H$_2$O$_2$] = 1:1.8) over the first 10 minutes of the Fenton reaction resulted in lower benzene conversion and phenolic product yield.

- The step addition of Fe$^{+2}$ did neither significantly alter the benzene removal efficiently, nor the product yield. (Batch reactors containing 6 mM benzene, 6 mM Fe$^{2+}$, 10.8 mM H$_2$O$_2$ at 0 min; after 5 min of reaction, additional 2 mM Fe$^{+2}$ was added; reaction carried out at pH 5.0).

- In order to determine the effect of step addition of hydrogen peroxide in the Fenton system, batch reactor studies were initiated at the optimum Fenton reaction conditions (pH 5.0, 6 mM benzene, [benzene]:[Fe$^{2+}$]:[H$_2$O$_2$] of 1:1:1.8) and after 5 min of reaction 3.6 mM of additional hydrogen peroxide was added. The results of this step-addition study demonstrate that the addition of hydrogen peroxide decreases the total aromatic compound concentration, most likely aiding in further mineralization of the aromatic products. However, the additional hydrogen peroxide did not alter the remaining benzene conversation.

- In order to determine whether step addition of both Fe$^{+2}$ and H$_2$O$_2$ could improve the benzene conversion efficiency and aromatic product yield, additional 2 mM Fe$^{+2}$ and 3.6 mM H$_2$O$_2$ were introduced to the optimum Fenton reaction conditions (pH 5.0, 6 mM benzene, [benzene]:[Fe$^{2+}$]:[H$_2$O$_2$] of 1:1:1.8) after 5 minutes’ reaction time. The combined step addition of Fe$^{+2}$ and H$_2$O$_2$ slightly increased the benzene conversion efficiency. However, the aromatic product
concentration was only 40% of the initial benzene. It is possible that the aromatic compounds underwent further oxidation under these conditions. Thus the resulting concentration of aromatic products in the reaction mixture was smaller than that under the optimum conditions.

5.7 Process Parameter Optimization for Enzymatic Removal of the Fenton Reaction Products (Two-Stage, Two-Reactor System)

When all the reactants of the Fenton system are added together at the beginning of the reaction, the best benzene to aromatic compound conversion efficiency occurs at a pH of 5.0, benzene: Fe$^{2+}$: H$_2$O$_2$ of 1:1:1.8 and at a reaction time of 1 hr. These reactant concentrations and reaction conditions are considered as the first step of the two-stage, two-reactor configuration. In the first stage, the Fenton reaction stage, of the reaction, about 72% of initial benzene gets converted to a mixture of biphenyl, hydroquinone, benzoquinone, resorcinol, catechol and phenol. Though benzoquinone and biphenyl are outside the scope of enzymatic treatment due to their chemical structure, these two compounds can be removed by other means. The other products, phenol, catechol, resorcinol and hydroquinone, which account for about 35% of the initial benzene concentration, are good substrates for enzymatic treatment.

As the second step of the treatment process, the optimal conditions needed to obtain ≥ 95% conversion of the aromatic compounds generated in the Fenton reaction were determined. The factors of interest include pH, minimum enzyme requirement to achieve ≥ 95% removal, and reaction time. Optimum enzymatic reaction conditions were determined for both laccase and SBP.
• The optimum reaction conditions for laccase-catalyzed removal of the Fenton reaction products are: pH 5.6, 2.2-2.4 U/mL laccase, three-hour reaction time. This amount is higher than the laccase requirement for an equimolar mixture of phenol and benzenediols.

• The optimum SBP-catalyzed reaction conditions for removal of the phenolic compounds from the mixture generated after the Fenton reaction are: pH 7.0, 2.0 U/mL of SBP, 5 mM of hydrogen peroxide and a three-hour reaction. These enzyme and hydrogen peroxide requirements are higher than those prorated from the individual requirements.

• The reason for higher SBP and laccase requirement could potentially be due to presence of any unknown phenolic products in the solution, enzyme inactivation, due, for example to the iron present in the solution, could also impact the enzyme activity.

• Benzoquinone, accounting for 30% of the initial benzene, cannot be removed by the enzymatic process. In addition, hydroquinone, accounting for 15% of the initial benzene, is most likely to be converted to benzoquinone in the enzymatic step. At a PEI concentration of 375-425 mg/L, more than 95% of the benzoquinone was removed after a one-hour contact time.

• After the PEI treatment, the light-brown-colored solution turned light orange in color. There was some visible turbidity along with precipitates. This residual color is not acceptable and removal of soluble products (colored or not) is necessary for effluent discharge, thus alum was used as a coagulant. At an alum (as aluminum
sulphate) concentration of 200 mg/L, about 85% of this color was removed. The resulting solution was almost colorless.

5.8 Simultaneous Fenton and Enzymatic Reaction (Single Batch Reactor)

The products from the Fenton reaction of benzene are mostly aromatic compounds which can further react with the available oxidizing agent and compete with benzene for available hydroxyl radicals. However, if these competing aromatic products can be removed from the system immediately, then it might improve overall benzene conversion efficiency. Under the optimum Fenton reaction conditions about 37% of the starting benzene generates phenolic compounds which are very good candidates for enzyme-catalyzed polymerization. It is hypothesized that the simultaneous Fenton and enzymatic reaction could remove the phenolic compounds from the system and thus would increase the possibility of improved benzene conversion. In order to determine the effect of simultaneous Fenton and enzymatic reactions, batch reactors were set up with varying amounts of laccase or SBP at the previously-determined optimum Fenton reaction conditions. The conversion and appearance/disappearance of aromatic products were monitored at over a three-hour reaction time.

- Increased laccase concentrations in the simultaneous Fenton and enzymatic system decreased the phenolic substrate (phenol, catechol, resorcinol and hydroquinone) and remaining benzene concentration in the system. The reduction in phenolic compound concentration in the simultaneous system required significantly more laccase than the two-stage, two-reactor system. This possibly poor conversion efficiency could be due to unavailability of dissolved oxygen or inactivation of laccase.
In order to oxidize the phenolic products, in an open reactor, laccase utilizes molecular oxygen. This oxygen is supplied by natural diffusion from the atmosphere. However, in a sealed batch reactor, diffusion from the atmosphere will not be possible, which might lead to limiting oxygen conditions. The theoretical calculation indicates that the dissolved oxygen present in the batch reactor will be sufficient for laccase-catalyzed polymerization of the phenolic compounds. However, to ensure the presence of enough oxygen in the laccase-containing batch reactors, water saturated with oxygen was used. The presence of this dissolved oxygen improved the phenolic conversion efficiency marginally. This phenomenon suggests that the dissolved oxygen is not the main factor adversely affecting the removal efficiency.

In the simultaneous system, within the first 20 minutes of reaction, about 80% of laccase activity is lost. This could be the main reason for poor phenolic conversion efficiency in the simultaneous system.

In the simultaneous Fenton and enzymatic system, the presence of SBP did not significantly improve the disappearance of benzene from the system. As the Fenton reaction and SBP-catalyzed enzymatic oxidation both utilize hydrogen peroxide, it is possible that there was not sufficient hydrogen peroxide left in the reactor to transform benzene. However, it is also likely that during the three-hour reaction period, there was not enough soluble iron to carry forward the Fenton system.

The concentration of phenolic substrates decreases with increasing SBP concentration in the simultaneous Fenton and enzymatic reactor system.
However, the phenolic compound removal efficiency did not show a linear relationship with increasing SBP concentrations.

- In the simultaneous Fenton and enzymatic system, SBP inactivation was observed. Enzyme activity analysis on a simultaneous reaction sample containing 2U/mL SBP, indicated that within the first 30 minutes of reaction, about 55% of the initial activity is lost which could be the reason for the poor phenolic compound removal efficiency.
CHAPTER 6
CONCLUSIONS

The results of this study demonstrate the feasibility of the two-step hybrid chemical-enzymatic method to remove benzene from water. Both single- and step-addition of the reactants in the Fenton system were explored. However, a better yield of identifiable aromatic products was achieved when all the reactants of the Fenton system were added at the same time. Recycling of sub-stoichiometric Fe$^{2+}$ was not beneficial.

In the benzene pre-treatment phase, the optimum pH, H$_2$O$_2$ and Fe$^{2+}$ concentrations and reaction time for the Fenton reaction were determined to maximize the conversion of benzene to phenolic compounds without causing significant mineralization. At pH 5.0 and [benzene]:[Fe$^{2+}$]:[H$_2$O$_2$] = 1:1:1.8, about 77% of the initial benzene was converted into aromatic compounds. The pre-treatment process was followed by oxidative polymerization of the phenolic compounds catalyzed by a laccase from *Trametes villosa* or a peroxidase from soybean seed coat. Under optimum Fenton reaction conditions, the reaction mixture contained the oxidative dimerization product (biphenyl) and hydroxylation products (phenol, catechol, resorcinol, benzoquinone and hydroquinone). Biphenyl and benzoquinone were not substrates of the enzyme. However, both laccase and soybean peroxidase were successful in removing the rest of the identified phenolic compounds from the Fenton reaction mixture. The biphenyl generated was removed from the solution due to its poor solubility in the aqueous media. In addition to the benzoquinone generated in the Fenton reaction of benzene, enzymatic reaction on hydroquinone also yielded benzoquinone as major reaction product. Benzoquinone was
removed from the solution by using polyethylenimine (PEI). A subsequent alum treatment was successful in generating a colorless reaction mixture.

A simultaneous Fenton and enzymatic system was also explored to determine the effectiveness of such co-existing systems in the benzene conversion. This study revealed that enzyme requirement for simultaneous system was much higher than the two-step system. In this study, effectiveness and stability of SBP were higher than those of laccase.

The applicability of removal of the phenolic compounds (at 1 mM) namely phenol, catechol, resorcinol and hydroquinone by using laccase-catalyzed oxidation was studied. Among these four compounds, the parent compound, phenol, required the greatest amount of enzyme, the m-, o-, and p-substituted compounds followed in decreasing order of enzyme requirements. Enzyme requirement to achieve 95% removal increased linearly with an increase in substrate concentration. The presence of the additive polyethyleneglycol showed insignificant effect on phenol and benzenediol conversion. In the second stage of the treatment, alum was effective in removing the soluble colored products generated from enzymatic treatment for all substrates except hydroquinone. The main reaction product generated from enzymatic reaction on hydroquinone is benzoquinone. Additives like chitosan or PEI were successful in removing benzoquinone from post-enzymatic reaction mixtures.

Phenol and benzenediols might coexist in industrial effluents. The removal efficiency of the benzenediols and phenol combined in an equimolar reaction mixture demonstrate that the optimum laccase or SBP requirement to achieve more than 95% conversion of all the
substrates is more than the sum of optimum enzyme requirements for individual substrates.

As noted at the outset, optima in this work refer to local optima as determined for the parameter in question within the respective ranges specified. To achieve the arbitrary benchmark, 95% conversion of the pollutant. At this removal efficiency, in most of the cases, the pollutant concentration in the treated effluent might be above the discharge limit. However, as the current treatment method is proposed at the source, it is expected that subsequent treatment techniques will be able to remove the remaining chemical in polishing the effluent. Alternatively, the remaining pollutant could also be removed completely by simply increasing the enzyme concentration.
CHAPTER 7
ENGINEERING SIGNIFICANCE AND RECOMMENDATIONS

The results of this dissertation demonstrate the feasibility of the enzymatic treatment preceded with a limited Fenton reaction, as a possible alternative to remove the priority pollutant, benzene, from wastewater. This study also indicates that enzymatic treatment can be a viable alternative to treat wastewater containing phenol and benzenediols.

The conventional treatment methods to treat benzene include air stripping, adsorption by activated carbon, microbial degradation, etc. Unfortunately, methods like air stripping and adsorption just cause phase transfer of the pollutant from one medium to another and do not actually remove it from the environment. Because of the phase transfer, for example, spent carbon in the activated carbon process, a large quantity of waste is generated which needs further treatment or special disposal.

Another important factor is the concentration of benzene in the waste stream. In most of the cases, the conventional methods are effective only when benzene concentration is low (as presented in section 1.5). The biological processes are mostly used as a polishing technique and are successful in treating benzene only in low concentrations (≤ 100 mg/L in many cases). However, in this process, the concentration of phenolic compounds has to be monitored so phenolic compounds do not have toxic effects on the microbes. The biological process may also require time for acclimatization.

The proposed hybrid Fenton-Enzymatic process shows promise in treating benzene at a very high concentration (treatment of 6 mM benzene is demonstrated in this study). The current treatment method is suitable for treating benzene at the source, before dilution.
So, this method has the capacity of treating a high strength wastewater containing benzene.

Enzymes are green catalysts which utilize oxygen (in case of laccase) or hydrogen peroxide (in case of SBP) to catalyze oxidative polymerization of phenolic compounds. As mentioned earlier, this process has several advantages over conventional treatment methods, including: capability of treating chemical which are toxic/refractory to microbes; capability of treating pollutant in both high and low concentrations; operating on a broad range of compounds and reaction conditions (wide pH, temperature and salinity ranges); simpler process control; elimination of the acclimatization period; reduction in sludge volume, small footprint, etc. (Ibrahim et al., 2001).

The conventional biological process utilizes the pollutant. In many cases, incomplete mineralization results in products which are toxic in nature. These by-products need further treatment. In the enzymatic process, the enzyme causes polymerization of the pollutant. Though limited work has been done on the toxicity and mobility of these polymers, some preliminary results indicate that these polymeric products are less toxic than the initial substrate, and due to their limited solubility, most likely are suitable for disposal in a landfill (Steevensz, 2008).

In order to consider and implement enzymatic treatment using laccase or soybean peroxidase to full-scale practical industrial application, several other factors must be considered.

1. The current study demonstrates the feasibility of using a hybrid Fenton-Enzyme system to treat high strength benzene effluent. In this study, process was optimized (local optima) for identified key process parameters. At this stage, in order to obtain the global
optimum of this process, a more systematic experimental design, i.e., factorial design should be attempted.

2. The proper experimental design could also potentially open the possibility of development of a model which would be a useful tool in predicting desired outcome.

3. In the conventional Fenton reaction system, shortly after the contact with hydrogen peroxide, the soluble iron precipitates as an amorphous iron oxide floc. Additionally, in this study, the best pH identified for limited Fenton reaction was pH 5.0. This pH also adversely affects the solubility of iron. The insoluble form of iron also has to be disposed of properly. The loss of iron also possibly affects the conversion of benzene. In order to retain and recycle the iron in the Fenton system, applicability of other forms of iron (chelated iron, iron power, etc.) should be assessed.

4. In the current study, under optimum Fenton pre-treatment conditions, about 23% of initial benzene remains unaltered in the reaction mixture. Applicability of a benzene recycling system should be explored in order to improve efficiency.

5. Preliminary study on the Fenton reaction products indicates the possible presence of low molecular-weight organic acids, as well as some unidentified products in the solution. An appropriate analytical technique should be developed to identify these products.

6. The nature of the end-products, resulting from enzyme-catalyzed oxidation of individual compound or composite wastewater containing a mixture of phenol and benzenediols, must be determined. Knowledge of potential toxicity of the end products is necessary for determining a suitable disposal method.
7. The enzymatic treatment generates higher order polymers as reaction end products. However, these polymers could be useful as feedstocks in other industries. Thus, feasibility of recycling of such polymers should be explored.

8. The enzyme cost could be one of the major operational costs associated with enzymatic treatments. Using a cheaper enzyme source could reduce the cost of enzymatic processes as well. A cost analysis should be carried out to determine the economic effectiveness of enzyme-catalyzed removal over conventional treatment processes.

9. In order to assess the applicability of the proposed Fenton-enzymatic treatment system in a larger set-up, it should be tested in a continuous-flow reactor system.
REFERENCES


Gallard, J., De Laat, J. (2001) Kinetics of oxidation of chlorobenzenes and phenyl-ureas byFe(II)/H₂O₂ and Fe(III)/H₂O₂. Evidence of reduction and oxidation reactions of intermediates by Fe(II) or Fe(III), *Chemospher.*, 42 405-413.


APPENDICES
The purpose of an enzyme activity assay is to determine the amount of active enzyme present in a solution. The rate of reaction was measured by measuring the rate of formation of colored products under saturating conditions of syringaldazine. These colored products absorbed light at a peak wavelength of 530 nm. The rate of increase in absorbance at 530 nm determined the enzyme activity.

One unit, "U," of laccase activity at pH 5.5 is the amount of enzyme required for the conversion of 1 micromole of syringaldazine/min.

1. Reagents

1.1. MES buffer (23 mM, pH 5.5 ± 0.05)

2.66 g of MES
1.0 mL of 2M sodium hydroxide
Distilled water to 1.0 L

1.2. Syringaldazine solution (0.38 mM)

6.8 mg of syringaldazine in flask
25 ml of 96% ethanol dissolved for 1.5 hours
Distilled water to 50 mL
Store in dark

2. Procedure

In a semi-micro cuvette, combine in the following order;

850 μL MES buffer
50 μL Syringaldazine solution
100 μL Laccase solution
The sample volume must be 1 mL and the rate of color formation must be measured before substrate depletion becomes significant. Immediately after the addition of the sample, shake the cuvette and then place it in the spectrophotometer to monitor the absorbance change with time at 530 nm. The change in absorbance should be measured at 15s and 75s.

3. Estimation of Laccase Activity

Activity in the cuvette (U/mL) = \( \frac{\Delta A \cdot 1.0 \text{mL} \cdot 10^{-3} \cdot D}{0.065 \cdot 0.1 \text{mL}} \)

\( = \Delta A \cdot 1.538 \cdot D \)

Where,

\( \Delta A \) = Change in absorbance per minute = \( A_{75 \text{s}} - A_{15 \text{s}} \)  
(Range of absorbance should be, 0.1 to 0.4 \( \Delta A/\text{min} \))

1.0 = Total volume in the cuvette (mL)

0.065 = Micro-molar extinction coefficient (µM/L)

10\(^{-3}\) = Conversion factor for U/mL to U/L

\( D \) = Dilution factor

The activity was measured in terms of micromoles of syringaldazine converted per minute at 20°C and pH 5.5.

Activity in the enzyme sample added to the reactor (U/mL)

\( = \) Activity in the cuvette (U/mL) \( \times \) reactor volume (mL) / enzyme solution added to the reactor (mL)
SBP enzyme activity assay is carried out to determine the amount of active enzyme present in the sample. This assay uses saturation concentrations of phenol, 4-aminoantipyrine (4-AAP) and an appropriate concentration of hydrogen peroxide such that the initial reaction rate is proportional to the enzyme activity. The reaction between phenol and hydrogen peroxide, catalyzed by the enzyme is such that it forms a pink colored solution. The rate of reaction is measured by observing the rate of color formation in the reaction solution. This colored solution absorbs light at a peak wavelength of 510 nm. Based on peroxide, the pink colored solution has an extinction coefficient of 6000 M\(^{-1}\)cm\(^{-1}\).

One unit of activity is defined as number of micromolecules of hydrogen peroxide utilized in one minute at pH 7.4 and at 20°C in an assay mixture containing 10 mM phenol, 2.4 mM 4-AAP and 0.2 mM hydrogen peroxide.

1. Reagents

1.1. Phosphate Buffer (0.5 M, pH 7.4)

In a 1000 mL volumetric flask,
- 13.796 g monobasic sodium phosphate
- 56.78 g dibasic sodium phosphate
- Distilled water to make a 1000 mL solution

1.2. Phenol (0.1 M) in Phosphate Buffer (0.5 M, pH 7.4)

9.411 g phenol in 1000 mL of 0.5 M phosphate buffer
1.3. Hydrogen Peroxide (100 mM)

567 µL of 30% (w/v) hydrogen peroxide diluted to 50 mL using distilled water. This needs to be made fresh each time an activity assay is performed.

1.4. Assay Mixture

In a 50.0 mL volumetric flask,
- 100.0 µL of 100.0 mM of H2O2.
- 25.0 mg of 4-AAP.
- 5.0 ml of 100.0 mM phenol in 0.5 M phosphate buffer
- Distilled water to make 50.0 mL solution

2. Procedure

In a semi-micro cuvette, combine in the following order;

950 µL of the assay mixture
50 µL SBP solution

The sample volume must be 1 mL and the rate of color formation must be measured before substrate depletion becomes significant. Immediately after the addition of the sample, shake the cuvette and then place it in the spectrophotometer to monitor the absorbance change with time at 510 nm. The change in absorbance should be measured at 5 second interval for the time duration of 35 seconds.

4. Estimation of SBP Activity

The activity of SBP is obtained from the average slope of the data within linear range, the dilution factor of reaction and the extinction coefficient of the product. Average slope over the linear range of the data is calculated in terms of absorbance units per unit time (AU/min).

\[
\text{Activity in the cuvette (U/mL)} = \frac{slope \ (AU/min)}{6000 \ AU.L/mol} \times 10^6 \ \frac{\mu mol}{mol} \times \frac{1L}{1000 \ mL}
\]
The activity is in terms of micromolecules of hydrogen peroxide converted per minute at pH 7.4 and at 20°C.

Activity in the enzyme sample (U/mL) = Activity in the cuvette (U/mL) \times \frac{1000 \text{ (μL)}}{enzyme solution \text{ (μL)}}
APPENDIX C

HPLC STANDARD CURVES FOR

AROMATIC COMPOUNDS

1. General

HPLC was used to identify and quantify aromatic compounds. Individual standard curves were prepared to determine the concentration of benzene, phenol, catechol, resorcinol, hydroquinone, benzoquinone and biphenyl.

2. Preparation of HPLC Standard Curves

2.1. Benzene

Different known concentrations of benzene solutions were prepared varying from 0.5 to 6.0 mM. Isocratic elution with 37:63 (V/V) acetonitrile: 0.1% acetic acid was monitored at 254 nm for benzene. Under these conditions, retention time for benzene was 24.6 min. The peak area vs. concentration was plotted to get the standard curve for benzene. The HPLC standard curve plot for benzene is presented in Figure C-1. The equation of the best fit line was, \( y = 41259x + 16348 \) and it had a \( R^2 \) value of 0.996.
2.2. Phenol

Different known concentrations of phenol were prepared varying from 0.1 to 1.0 mM after proper dilution. Isocratic elution with 20:80 (V/V) acetonitrile: 0.1% acetic acid was monitored at 280 nm for phenol. Under these conditions, retention time for phenol was 9.36 min.

The peak area vs. concentration was plotted to get the standard curve. The HPLC standard curve plot for phenol is presented in Figure C-2. The equation of the best fit line was, $y = 424853x - 198.93$ and it had a $R^2$ value of 0.998.
Different known concentrations of catechol were prepared varying from 0.1 to 1.0 mM after proper dilution. Isocratic elution with 20:80 (V/V) acetonitrile: 0.1% acetic acid was monitored at 280 nm for catechol. Under these conditions, retention time for catechol was 4.7 min.

The peak area vs. concentration was plotted to get the standard curve. The HPLC standard curve plot for catechol is presented in Figure C-3. The equation of the best fit line was, $y = 845775x - 26951$ and it had a $R^2$ value of 0.999.
Different known concentrations of resorcinol were prepared varying from 0.1 to 1.0 mM after proper dilution. Isocratic elution with 20:80 (V/V) acetonitrile: 0.1% acetic acid was monitored at 280 nm. Under these conditions, retention time for resorcinol was 3.35 min. The peak area vs. concentration was plotted to get the standard curve. The HPLC standard curve plot for resorcinol is presented in Figure C-4. The equation of the best fit line was, $y = 857343x + 3138.7$ and it had a $R^2$ value of 1.00.
Different known concentrations of hydroquinone were prepared varying from 0.1 to 1.0 mM after proper dilution. Isocratic elution with 20:80 (V/V) acetonitrile: 0.1% acetic acid was monitored at 280 nm. Under these conditions, retention time for hydroquinone was 2.30 min.

The peak area vs. concentration was plotted to get the standard curve. The HPLC standard curve plot for hydroquinone is presented in Figure C-5. The equation of the best fit line was, \( y = 1\times10^6x - 81394 \) and it had a \( R^2 \) value of 0.997.
2.6. Benzoquinone

Different known concentrations of benzoquinone were prepared varying from 0.1 to 1.0 mM after proper dilution. Isocratic elution with 20:80 (V/V) acetonitrile: 0.1% acetic acid was monitored at 280 nm. Under these conditions, retention time for benzoquinone was 4.04 min.

The peak area vs. concentration was plotted to get the standard curve. The HPLC standard curve plot for benzoquinone is presented in Figure C-6. The equation of the best fit line was, \( y = 102541x - 2565.6 \) and it had a \( R^2 \) value of 0.994.
Biphenyl has very limited solubility in water. In order to identify and quantify it, a 60:40 (v/v) mixture of acetonitrile and water was used. This mixture was stirred vigorously to ensure biphenyl was completely dissolved. This 60% acetonitrile solution was used for identification and quantification of biphenyl. Different known concentrations of biphenyl were prepared varying from 0.1 to 1.0 mM after proper dilution. Isocratic elution with 70:30 (V/V) acetonitrile: 0.1% acetic acid was monitored at 254 nm. Under these conditions, retention time for biphenyl was 7.04 min.

The peak area vs. concentration was plotted to get the standard curve. The HPLC standard curve plot for biphenyl is presented in Figure C-7. The equation of the best fit line was, $y = 993,661.76x - 4,300.47$ and it had a $R^2$ value of 1.00.
Figure C-7: HPLC Standard Curve Plot for Biphenyl
VITA AUCTORIS

Name
Beeta Saha

Education
PhD, Environmental Engineering
University of Windsor,
Windsor, Ontario, Canada
2011

M. A. Sc, Environmental Engineering
University of Windsor,
Windsor, Ontario, Canada
2006

Bachelor of Technology (Honors), Civil Engineering
Indian Institute of Technology,
Kharagpur, WB 721302, India
2003

Publications

Journal Article


Book Chapter

Conference Proceedings and Presentations

“Catalyzed Polymerization”, IWA World Water Congress and Exhibition, 19–24 September 2010. Montréal, Quebec, Canada


**Awards**


2. Graduate Tuition Scholarship, the Faculty of Graduate Studies of University of Windsor, May 2010 – August, 2010.


4. International graduate student scholarships (IGSS), the Faculty of Graduate Studies of University of Windsor, September 2004-April, 2010.