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LACCASE CATALYZED REMOVAL OF DIPHENYLAMINE FROM
SYNTHETIC WASTEWATER

by

Beeta Saha

A Thesis

Submitted to the Faculty of Graduate Studies and Research
through Environmental Engineering
in Partial Fulfillment of the Requirements for
the Degree of Master of Applied Science at the
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ABSTRACT

DPA, a contaminant found in the wastewater of various industries, is a priority pollutant in the US EPA's and the EU's list of priority pollutants. Experiments were conducted to investigate the potential of using laccase from *Trametes villosa* to remove DPA from buffered synthetic wastewater as an alternative treatment method. In continuously stirred batch reactors, effects of pH, laccase concentration, molecular mass and concentration of polyethylene glycol (PEG) were investigated to achieve 95% substrate conversion in three hours. After successful enzymatic treatment, colored end-products were removed via adsorptive micellar flocculation (AMF) by using sodium dodecyl sulfate (SDS) and alum.

For 0.19 mM substrate concentration and at optimum pH of 7.0, optimum enzyme concentration varied from 0.0025 U/mL to 0.0075 U/mL. Except for PEG₄₀₀, the presence of PEG reduced the optimum enzyme requirement. Optimum enzyme and PEG concentration decreased with an increase in PEG molecular mass. Optimum AMF conditions were: pH 3.0 to pH 6.5, 200mg/L of SDS, and 150 mg/L of alum.

DEDICATION

I dedicate this thesis to my family members for their unconditional love and support which has inspired me to pursue my goals.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere thanks to my advisors Dr. N. Biswas, Dr. J.K. Bewtra and Dr. K.E. Taylor for their guidance, support and constructive criticism throughout the course of research without which it would not have been possible to complete this work successfully.

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LIST OF NOMENCLATURE

Abbreviations

AMF	Adsorptive micellar flocculation
AOP	Advanced oxidation process
ARP	<i>Arthromyces ramosus</i> peroxidase
BOD	Biological oxygen demand
COD	Chemical oxygen demand
DPA	Diphenylamine
HRP	Horseradish peroxidase
PEI	Polyethyleneimine
PEG	Polyethylene glycol
SBP	Soybean peroxidase
SDS	Sodium dodecyl sulfate or Sodium lauryl sulfate
TOC	Total organic carbon

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CHAPTER I

INTRODUCTION

1.1 Background

Diphenylamine (DPA) is an impurity found in the effluent of various industries. The main industrial applications of DPA include use as an agrochemical, corrosion inhibitor, explosive stabilizer for nitrocellulose-containing explosives and a major intermediate for production of various dyes, pharmaceuticals, photography chemicals, etc. It is also used in the rubber industries. It has been applied to prevent the post-harvest deterioration of the apple and pear crops. The US EPA lists DPA as a pesticide (EPA: Substance, 2006). DPA is a priority pollutant in the EPA's TRI (Toxic Release Inventory) list (EPA, 2006) and in the third European Union (EU) list of Priority Pollutants (Drzyzga, 2003).

DPA is a high volume chemical with production exceeding one million pounds (454,545 kg) annually in the U.S (Scorecard, 2005). According to the US EPA TRI (2005), the total release of DPA was 414,097 pounds (188,226 kg) per year. Of this, 76,386 pounds (34,721 kg) were disposed of onsite as underground injection, surface water discharge, air release, etc. The remainder accounted for the offsite disposal which includes landfill, transfer to wastewater treatment plants, etc. In Europe, the average DPA production per annum was 89,600,000 pounds (40,633,600 kg) (Murin et al., 1997). Of this, 134,400 pounds (60,950 kg) were released to the environment (Murin et al., 1997). Reports show that, in Europe, DPA was found in soil and ground

water (Drzyzga, 2003). The chemical has significant industrial application and it is anticipated that the production and use of this chemical will increase significantly worldwide (Drzyzga, 2003).

Studies show that, in Europe, a wastewater treatment stream can have an average DPA concentration of 60 mg/L (Murin et al., 1997). The conventional wastewater treatment plants were successful in removal of 30 % of the initial DPA concentration on an average effluent concentration as high as 42 mg/L, which resulted in 10 mg/L of DPA in the river Vah stream (Murin et al., 1997). But, GDCh (1988) reported that as little as 0.048 mg/L of DPA can result in toxic effects to algae (*S. subspicatus*).

1.2 Problems with DPA

In a workplace, exposure to DPA dust is possible if it is in powder or granular form and is mixed with air. Heating or burning of DPA can produce toxic fumes including nitrogen oxides. It reacts with strong oxidants and strong acids (International Chemical Safety Card, 1994).

DPA can be absorbed into the body by inhalation of its aerosol, through the skin, and by ingestion (IPCS, CEC 1999). Short-term exposure to DPA can irritate eyes, skin and the respiratory tract. Long-term exposure to DPA can have adverse effects on the kidneys and blood. High concentrations of DPA can have mutagenic effects on humans (Drzyzga, 2003). Exposure of cultured human lymphocytes to DPA increased the sister chromatid exchange (Ardito et al., 1996). DPA exposure can result in methaemoglobin formation in human blood (International Chemical Safety Card, 1994).

DPA treatment in short-term studies and during long-term expositions in animal experiments with dogs, mice and rats showed an increase of organ weights, methemoglobinemia, and liver, spleen and kidney damaging effects (Drzyzga, 2003). The USEPA declared DPA as “not likely carcinogenic” to humans in 1997 (EPA: Federal Register, 1999). However, the nitrosamine impurity present in DPA is considered to be a carcinogen by the USEPA. DPA and some of its derivatives (nitro- and amino-substituted compounds and the stabilizers) have mutagenic and carcinogenic potential (Greim et al., 1998; Lachance et al., 1999). Products derived from anaerobic conversion of DPA have more carcinogenic effects than DPA itself. According to EU classification criteria (cf. Annex IV of Council Dir. 93/21/EEC), aquatic hazard and risk assessment was proposed for DPA by Murin et al., (1997). According to their study, DPA was classified as a “very toxic” chemical (Acute toxicity < 1 mg/L, not readily biodegradable, bioconcentration factor > 100 and $\log P_{ow} \geq 3$). In that study, they have also found that DPA is a high-risk chemical for aquatic environments, having a risk quotient (R_Q) of 5.3. Germany also classifies DPA as strongly water-endangering (water endangering class 3) (Drzyzga, 2003). The ecotoxicological properties of DPA are presented in Table 1.2. DPA shows an inhibitory effect on photosynthesis of the phototrophic bacteria (Drzyzga, 2003). Goodwin (1980) has reported on the inhibition of the carotenoid synthesis of a few photosynthetic bacteria by DPA (Drzyzga, 2003).

Table 1.2: Ecotoxicological Properties for DPA (Murin et al., 1997)

Property	Information
CAS No.	122-39-4
Sum Formula	C ₁₂ H ₁₁ N
Molecular Mass	169.77 g/mol (7)
Water Solubility	40 mg/L (10)
Vapour Pressure	0.021 Pa (20°C) (10)
Henry's Law Constant	0.09 Pa·m ³ /mol (10)
log P _{ow}	3.5 (8)
	3.42-3.62 (10)
Ready Biodegradability	0 % (2)
Bioaccumulation, BCF	51-253 (<i>Cyprinus carpio</i>) (2)
	30 (<i>Pimephales promelas</i>) (10)
	70 (10)
Toxicity (microorganisms)	Inhibition > 10 mg/L (<i>Saprophytic microflora</i>) (10)
	NOEC = 100 mg/L (<i>Nitrosomonas sp.</i>) (10)
	NOEC = 1000 mg/L (<i>Pseudomonas fluorescens</i>) (10)
Toxicity (algae)	EC ₅₀ = 0.048 mg/L (<i>Scenedesmus subspicatus</i>) (10)
Toxicity (crustaceans)	EC ₅₀ = 2.3 mg/L (<i>Daphnia magna</i>) (10)
	EC ₀ (21 days) = 0.16 mg/L (<i>Daphnia magna</i>) (10)
	EC ₇₀ (21 days) = 0.5 mg/L (<i>Daphnia magna</i>) (10)

TABLE 1.2: Ecotoxicological Properties for DPA (Murin et al., 1997)(contd.)

Property	Information
Toxicity (fish)	LC ₅₀ (96 h) = 3.8 mg/L (<i>Pimephales promelas</i>)(6)
	LC ₅₀ = 1-100 mg/L (3)
	LC ₅₀ (48 h) = 2.2 mg/L (<i>Oryzias latipes</i>) (6)
<p>The numbers in the table refer to the following sources: (1) Verschueren (1983), (2) MITI(1992), (3) Roth (1993), (4) Nikunen et al. (1990), (5) Howard (1989), (6) US-EPA, (7) CHEMBANK, (8) LOGKOW (1994), (9) IUCLID (1995), (10) GDCh (1988).</p>	

1.3 Regulations

The EPA's Universal Treatment Standards (UTS) set the regulatory limit for the most prohibited hazardous wastes present in the 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, 0.92 mg/L DPA is an acceptable treatment standard for a wastewater treatment stream. For non-wastewater the UTS for DPA is 13 mg/kg (EPA. 2005).

The Environment Protection Act, Ontario (R.R.O. 1990, REGULATION 347, Amended to O. Reg. 461/05) has listed the sources of DPA in the environment and regulatory concentration. According to it, DPA can occur in the environment due to leachate (liquids that have percolated through land disposed wastes) resulting from the disposal of more than one hazardous waste. It is also present in distillation bottom tars from the production of phenol/acetone from cumene and in the distillation

bottoms in the aniline production. This act also complies with the EPA's UTS as a regulatory concentration for discharge or disposal.

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) for DPA is 10 µg/L (MISA, 1999).

The Washington State Department of Ecology has outlined certain discharges which are subjected to specific treatment/discharge methods (TDM) and effluent limitations. This regulation was originally issued on February 10, 1994 and was revised on June 15, 2004 particularly for the fresh fruit packing industries operating in the state of Washington (Washington State Department of Ecology, 2004). This guideline enforces six TDMs which aim to achieve the permissible effluent limits. These TDMs are: 1) lined evaporative lagoons, 2) dust abatement, 3) publicly owned treatment works (POTW), 4) land application, 5) percolation systems, and 6) surface waters. According to the regulation, DPA is prohibited for discharge into the POTW at a concentration higher than 10mg/L, since it interferes with the POTW. This concentration limit will not be applicable to the discharges in lined evaporative lagoons. These lined evaporative lagoons are imperviously lined, engineered structures which depend entirely upon evaporation for water removal. For this reason, priority pollutants, dangerous wastes or toxics in toxic amounts, are only permitted to be discharged to lined evaporative lagoons. The maximum concentration limit for the dust abatement and land applications is 2,200 mg/L.

According to the American Conference of Industrial Hygienists (ACGIH) (2005), occupational exposure limit for DPA is threshold limit values (TLV): 10 mg/m³ as a time-weighted average (TWA) (ICSC, 1999). The Occupational Safety and Health Administration (OSHA) have stated an 8-hour time weighted average of 10 mg/m³ as the permissible exposure limit (Occupational Safety & Health Administration, 1998). The National Institute for Occupational Safety and Health (NIOSH) recommends a maximum exposure level of diphenylamine as a 10- hour time-weighted average 10 mg/m³ (NIOSH, 2005).

1.4 Conventional Treatment Methods

The most commonly used techniques for DPA removal are physical methods (adsorption), chemical methods (advanced oxidation, photocatalysis, etc.) and biological methods. Very few studies have been done on biodegradability of DPA. Strategies involved in the removal of such recalcitrant organic compounds are mostly phase-transfer and oxidation.

1.4.1 Physical Methods

Adsorption technologies are used to remove DPA. Activated carbon has been reported as an adsorption medium for DPA removal (Rao et al., 2001). According to the study, 100% of DPA having an initial concentration of 100 µg/mL was adsorbed on the activated carbon easily, though the desorption process was not successful (Rao et al., 2001). These technologies are becoming unpopular because they only cause a displacement of the pollutants (Bolduc and William, 1997).

1.4.2 Chemical Methods

Photocatalytic conversion and oxidation are two major chemical methods to remove DPA. Advanced oxidation processes (AOP) are also involved to remove DPA.

The US EPA's Re-registration Eligibility Decision (RED) states that both aerobic soil metabolism and aqueous photolysis play an important role in DPA degradation. According to RED, DPA has a half-life less than one day under aerobic soil conditions. If, it is exposed to light, the water transformation half-life is 4.39 hours (EPA, 1998). DPA undergoes rapid degradation in the presence of ultraviolet (UV) light and air. In unamended soil, DPA has a half life of approximately 30 days (EPA, 1998). Bolduc and William, (1997) applied photocatalytic pre-oxidation for DPA prior to biodegradation. They measured the biodegradability of the wastewater in terms of BOD₁/TOC. The results of the study showed that, the photocatalytic pre-oxidation enhanced the biodegradability of DPA solution by mineralizing the parent compound.

However, 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).

1.4.3 Biological Methods

Both aerobic and anaerobic conditions are used to remove DPA biologically from wastewater. Gardner et al., (1982) used sewage sludge for biodegradability of DPA

under aerobic conditions. They reported an oxidative cleavage of the ^{14}C -labeled DPA occurred after an incubation time of 6-24 hours resulting in 4-hydroxy-DPA, aniline, indole and an unidentified isomeric compound. But their studies showed that 35% of the initial DPA concentration remained unchanged after 6 hours of incubation in the sewage sludge.

Christodoulatos et al. (1997) used three pure *Pseudomonas* species namely *P. cepacia*, *P. putida*, and *P. resinovorans* and mixed activated sludge for biodegradation of DPA under aerobic conditions. The initial DPA concentration in this study varied between 45 and 60 mg/L. DPA was consumed by all cultures as a carbon source and use of glucose as co-substrate did not improve the degradation process.

Co-metabolism is a process where a substrate is modified but not utilized for the growth of the organism. This organism grows on or metabolizes another substrate. Co-metabolic degradation of DPA under anoxic conditions was observed with sulfate reducing bacteria (*Desulfovibrio sp.*) (Drzyzga and Blotevogel, 1997). In this study, the bacteria used acetone and lactate for growth and DPA was co-metabolized. 75% of the initial DPA was converted into the major reaction product, aniline.

Most of these biological treatments were unable to reach the regulatory limit. DPA has been reported as one of the chemicals which are recalcitrant or inhibitory to the microorganisms used in conventional biological treatment processes (Bolduc and William, 1997).

1.5 Enzymatic Treatment Method

In conventional biological treatment processes, microorganisms produce enzymes, which degrade the chemical compounds. In enzymatic treatment, isolated enzymes are used instead of the microorganisms. Enzymes are specific biological catalysts which increase rates of reactions without undergoing any overall change.

Use of isolated enzymes in the wastewater treatment is not new. This concept was first introduced in 1930s (Munnecke, 1976). Due to advancements in biotechnology, wide varieties of enzymes are produced and cheaper purification and extraction processes are also available (Karam and Nicell, 1997). It should be noted that the use of enzymes in the enzymatic treatment works in the opposite sense to conventional biological treatment; the latter involves the breakdown of the target compounds while the enzymic method involves the build up of the target compound through polymerization.

1.5.1 Advantages of Enzymatic Treatment

Today the government laws and regulations are designed to remove specific substrates to the regulatory limits. In many cases, the conventional chemical and biological processes are not capable of pollutant removal to the desired level, especially the bio-refractory chemicals. Since enzymes are substrate specific, they may perform better on remediation of certain substrates.

Caza (1999) has noted the following advantages of enzymatic treatment over conventional physical and chemical process:

- Operation under milder, less corrosive conditions.
- Operation in a catalytic manner.

- Operation on trace levels of organic compounds and on organics not removed by existing chemical/physical processes.
- Reduced consumption of oxidants.
- Reduced amount of adsorbent media for disposal.

The enzymatic treatment has the following advantages over conventional biological treatment processes (Taylor et al., 1996):

- Enzymes can work on a broad, but specific range of chemicals.
- They have the capability of treating bio-refractory chemicals.
- They operate over wide temperature, pH and salinity ranges.
- Applicable in treating wastes containing very dilute concentrations of substrate.
- Sludge volume is reduced as no biomass is produced in the system.
- No shock loading effects.
- No delays associated with shutdown and startup of the system.
- Simpler process control.
- Short reaction time and less energy consumption.
- Low capital cost.

1.5.2 Disadvantages of Enzymatic Treatment

The main drawback of enzymatic treatment is the cost of enzyme itself. A large amount of enzyme is required to treat wastewater on a large scale. Another drawback can be enzyme inactivation. Some inactivation mechanisms for peroxidase enzymes have been studied (Hinter et al., 1996). But little information is available on the inactivation mechanism of laccase.

1.6 Use of Enzyme in DPA Removal

Peroxidase enzymes, horseradish peroxidase (HRP) and *A. ramosus* peroxidase (ARP), demonstrated the efficient removal of DPA from water. Removal of DPA using HRP was first studied by Klibanov et al. (1980). They reported that, removal

efficiency was 80.5% with pH 7.0 and hydrogen peroxide concentration of 1 mM. However, Verschueren, (2001) has reported 99.6% removal of DPA using HRP. DPA is used as an intermediate in dye manufacturing. During treatment of these dyes, DPA is produced which is an environmentally hazardous chemical itself. While the removal of reactive azo dyes from water by Fe^0 reduction followed by peroxidase-catalyzed polymerization has been studied in this laboratory, the treatability of diphenylamine with *A. ramosus* peroxidase (ARP) was studied for a better understanding of the breakdown products of two reactive azo-dyes (Biswas, et al., In press). Since DPA is an aromatic amine, zero-valent iron treatment was tried for its breakdown, but only 12% of the initial DPA was converted (Biswas, 2004). Subsequently, studies were conducted to determine whether DPA itself was a substrate for the enzyme. DPA proved to be a good substrate for ARP and 90-95% removal was achieved by varying different reaction parameters (Biswas, 2004).

1.7 Proposed Treatment Method

An alternative treatment method for removal of DPA from synthetic wastewater using an oxidase enzyme was studied. Suitable coagulant aid was investigated to remove the reaction products from the solution by coagulation and filtration.

A fungal laccase from *Trametes villosa* was of particular interest for this study. Polyethylene glycol (PEG) was reported to have a protective effect on oxidative enzymes in treatment processes. The effect of PEG on DPA removal had also been studied.

1.8 Research Objectives

The main objectives of this research were to:

- Determine whether laccase is capable of catalyzing DPA removal from water and develop optimum reactor conditions by optimizing reaction parameters.
- Determine the protective effect of PEG on the enzyme and develop optimum reaction conditions in the presence of PEG.
- Investigate methods to remove the color in the batch reactor solutions after enzymatic treatment.

1.9 Scope of the Study

The scope of this study was to assess the effectiveness of laccase to remove DPA from synthetic wastewater in laboratory-scale batch reactor studies under the following conditions:

- The synthetic wastewater was comprised of DPA in buffered solution.
- All reactions were conducted at room temperature ($21^{\circ} \pm 2^{\circ} \text{C}$).
- Substrate concentration was kept at 0.19 mM for most of the studies. But some experiments were also conducted at a substrate concentration of 0.16 mM.
- The pH of the synthetic waste water varied from 3.0 to 9.0.
- Reactions were conducted without and with PEG₄₀₀, PEG₁₄₅₀, PEG₃₅₀₀, PEG₈₀₀₀ and PEG₃₅₀₀₀.

- Colored end products were removed by adsorptive micellar flocculation (AMF), using sodium dodecyl sulfate (SDS) and alum.
- For the color removal process, pH of the study ranged from 3.0 to 8.0.

CHAPTER II

LITERATURE REVIEW

This work aims to achieve satisfactory removal of DPA from synthetic waste water by using enzymatic treatment. Enzymatic treatment, which is another form of oxidation, can be an innovative and effective way of treating a recalcitrant chemical like DPA. After enzymatic treatment coagulation and precipitation aids are required to precipitate the reaction products out of the aqueous solution. This chapter presents the literature review on the reaction mechanism and the process parameters involved in the DPA removal process.

2.1 Diphenylamine

Diphenylamine is the simplest secondary amine of pure aromatic structure and is a highly reactive compound, which is mainly due to amine hydrogen atom, which can be easily replaced electrophilically. This is the reason why numerous DPA derivatives are formed when the N-hydrogen is replaced by alkyl-, aryl- or acyl- groups (Drzyzga, 2003). Deprotonation of DPA produces a highly reactive radical named “diphenylamidogen”, which exhibits its property as a highly effective anti-oxidant (Sugihara et al., 1993). The physical and chemical properties of diphenylamine (CAS NO. 122-39-4) and its application along with sources in the environment are discussed in the following sections.

2.2 Physical and Chemical Properties of Diphenylamine

Diphenylamine ((C₆H₅)₂NH) is a colorless, crystalline solid with a pleasant floral odor (NTP:ChemIDplus/HSDB, 2006). It has a molecular mass of 169.2 g/mol, boiling point of 302°C and a melting point of 53°C. It is freely soluble in benzene, ether, glacial acetic acid, carbon disulfide; very soluble in ethanol, acetone, benzene, carbon tetrachloride, pyridine, ethyl acetate; soluble in ether, acetic acid and slightly soluble in chloroform (NTP:ChemIDplus/HSDB, 2006). Depending on temperature DPA shows a water solubility of 35-45 mg/L (Drzyzga, 2003). DPA has a pKa of 0.78 at 24°C (NTP:ChemIDplus/HSDB, 2006). This chemical is stable towards the hydrolysis at pH 5.0, 7.0 and 9.0 (US EPA, 1998). The physical and chemical properties of DPA are listed in Table 2.1.1.

**Table 2.1.1: Physical and Chemical Properties of DPA
(NTP: ChemIDplus/ HSDB, 2006)**

Property	Information
Color/Form	Crystals Colorless, tan, amber, or brown crystalline solid
Odor	Pleasant, floral odor
Taste	Unknown
Boiling Point	302° C
Melting Point	53-54° C
Molecular Mass	169.23 g/mol
Corrosivity	Unknown
Critical Temperature & Pressure	Unknown

Property	Information
Specific Gravity	1.16
Disassociation Constants	$pK_a = 0.78$ at 24°C
Heat of Combustion	$-16.300\text{ BTU/lb} = -9.060\text{ cal/g} = -379 \times 10^5\text{ J/kg}$
Heat of Vaporization	Unknown
log P (octanol-water)	$\log K_{ow} = 3.50$
pH	Unknown
Solubilities	<ul style="list-style-type: none"> • 1 g dissolves in 2.2 mL of alcohol, 4 mL propyl alcohol; freely sol in benzene, ether, glacial acetic acid, carbon disulfide. • Very soluble in ethanol, acetone, benzene, carbon tetrachloride, pyridine, ethyl acetate; soluble in ether, acetic acid; slightly soluble in chloroform • Soluble in oxygenated and aromatic solvents • In water, 35 mg/liter at 20°C
Spectral Properties	<ul style="list-style-type: none"> • Maximum Absorption (Alcohol) : 208 nm (Log E = 4.33); 286 nm (Log E = 4.29); Sadtler reference number: 68 (IR, Prism); 8009 (IR, Grating) • Intense mass spectral peaks: 169 m/z (100%), 168 m/z (47%), 167 m/z (28%), 51 m/z (14%) • IR: 4833 (Coblentz Society Spectral Collection) • UV: 30 (Sadtler Research Laboratories Spectral Collection) • NMR: 11 (Sadtler Research Laboratories Spectral Collection) • MASS: 60820 (NIST/EPA/MSDC Mass Spectral Database, 1990 Version); 4272 (National Bureau of Standards) • RAMAN:241 (Sadtler Research Laboratories spectral collection)
Surface Tension	$39.3\text{ dynes/cm} = 0.0393\text{ N/m}$ at 60°C
Surface Tension	5.82 (Air = 1)

Property	Information
Vapor Pressure	6.70×10^{-4} mm Hg at 25° C
Viscosity	Unknown
Other Properties	<ul style="list-style-type: none"> • Forms salts with strong acids • Henry's Law constant: 2.69×10^{-6} atm-cu m/mole at 25° C (Estimated)

2.3 Industrial Application of DPA and its Effluent Sources

DPA is widely used as a stabilizer for single- or multi-base propellants and nitrocellulose-containing explosives. These explosives produce degradation products like NO, NO₂, and HNO₃ during long storage periods. These nitric degradation products further enhance the degradation of explosives. DPA binds with these nitric degradation products and is converted to its nitrate derivatives (Espinoza and Thornton.1994). This process prevents the explosive degradation by nitric degradation products and prolongs the storage period. Studies show DPA from ammunition waste causes soil and water contamination (Drzyzga, 2003). A former ammunition plant in Lower Saxony, Germany, where nitrocellulose had been produced and mixed with DPA during the Second World War contaminated the site. A large fraction of DPA could still be detected in 1990 (Drzyzga, 2003).

DPA is also used to prevent the post-harvest deterioration of apple and pear crops. According to FAO and WHO (1985), the annual worldwide application of DPA only for this purpose was 85 tonnes. DPA prevents the storage scald of these fruits. Storage scald is detected by the irregular brown patches on the dead skin of the fruit. There are several theories accounting for the scalding but it has been proven that DPA prevents this

problem. Hence, a large amount of DPA is present in the effluent from fresh fruit packing industries. Discharge of this waste water is prohibited into the publicly owned treatment works (POTW). DPA effluents having a concentration higher than 10mg/L interfere with the POTW. EPA lists DPA as a pesticide (Pc code 038501). It is both herbicide and fungicide.

DPA is extensively used as an antioxidant for various polymers and elastomers. It is used as condensate in insulation of rubber. DPA is used as an intermediate in production of dyes, pharmaceutical products, and photography chemicals. Other than these applications, Drzyzga (2003) has listed some other important industrial applications of this chemical:

- Precursor for the chemical synthesis of azo-dyes namely Metanil Yellow and Orange IV.
- Stabilizer in perfumery products.
- Oxidizer detection.
- DNA detection.
- Biocide against chiggers and houseflies.
- Precursor of non-steroidal anti-inflammatory drugs.

Some of the DPA derivatives are used by the US Navy as fuel and explosive. For example, 2, 2', 4, 4', 6, 6'-hexanitro-DPA is used in underwater explosives like marine bombs, torpedoes, marine mines, etc.

DPA can occur in the environment as leachate (liquids that have percolated through land-disposed wastes) resulting from the disposal of more than one hazardous waste. It is also present in distillation bottom tars from the production of phenol/acetone from cumene and in the distillation bottoms from aniline production.

2.4 Reason for Choosing Enzymatic Treatment

When a waste water treatment process is chosen, its applicability should be studied based on certain factors, such as chemical constituent of the waste water stream, permissible discharge limit to be achieved, difficulties in process control, chances of producing toxic by-products, treatment methods for the by-products, economic feasibility, etc. With more strict 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).

Enzymes are highly specific biological catalysts which can remove specific target pollutants up to the satisfactory limit. The conventional treatment facilities include physical, chemical and biological methods. Physical and chemical methods are substrate-specific. Hence, the desired removal limit cannot be achieved by using these technologies in many cases. Chemical treatment methods are expensive and are ideal for wastes having low pollutant concentrations (Caza et al., 1999). As opposed to conventional biological treatment method, micro-organisms are not used in enzymatic treatment. This reduces the process timing as the individual enzyme does not need the acclimatizing time which is essential for microbes. The process is not affected by shock loading and the process control is simpler (Taylor et al., 1996).

A considerable amount of work has been done in past few decades to find out the applicability of enzymes in industrial waste water treatment. Karam and Nicell (1997) have reported the reasons behind the growing interest in enzymatic treatment. These are:

- Conventional chemical and biological treatment processes are not successful in achieving the pollutant removal limit in many cases.
- Enzymes are successful in removing specific pollutants.

- Due to advancement in biotechnology and cheaper purification and extraction methods, cheaper enzymes are available now.

Though enzymatic treatment is advantageous in many ways, the main disadvantage of the process is the cost of enzyme. The production cost of the enzyme itself can be high, but with advancements in biotechnology, bulk production of enzymes from cheap sources is possible, which can overcome the associated cost factor (Karam and Nicell, 1997).

A second drawback of enzymatic treatment is the enzyme's fairly short catalytic life, attributed to enzyme inactivation. This may be improved in some cases by using immobilized enzyme or by using additives which exert protective effects on the enzyme (Nakamoto and Machida, 1992).

2.4.1 Use of Enzyme in Wastewater Treatment

Enzymatic treatment of waste water was first proposed in 1930 (Munnecke, 1976). Since then, many researchers have studied the applicability of enzymatic treatment technology to remove aromatic pollutants from aqueous mixture (Bollag et al., 1980; Kilbanov and Morris. 1981; Aitken. 1993; Taylor et al., 1996). Mainly oxidoreductases have been used for this purpose. 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 oxygen source, while oxidases, like laccase, use molecular oxygen for the same purpose.

Peroxidases have been successful in removing aromatic compounds such as phenols, anilines, naphthols, benzidines, biphenol, diphenylamine, naphthylamine, etc. from waste water (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 waste water 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, waste water 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 (Arnao et al., 1990; Nakamoto and Machida, 1992; Nicell and Wright, 1997; Villalobos and Buchanan, 2002; Masuda et al., 2002).

Laccase was first described by Yoshida in 1883 as a component of the resin ducts of the lacquer tree *Rhus vernicifera* (Riva, 2006). Typical substrates for these enzymes are amines and phenols. These chemicals produce reactive radical intermediates after oxidation and these intermediates undergo chemical coupling to generate dimeric and oligomeric derivatives (Baratto et al., 2006). Removal of phenolic compounds using laccases has been studied (Torres et al., 2003) and it was found that polymers resulting from the laccase oxidation process were generally insoluble in water and could be removed by filtration or sedimentation. Industrial application of laccases is relatively a new concept as compared to peroxidases. This is mainly due to the fact that they were not commercially available previously. 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 (Bajpai, 1999; Claus, 2002; Duran et al., 2002; Riva, 2006). They are also used for the color removal in the dye and printing industries (Abadulla et al., 2000).

2.5 Choice of Enzyme Laccase

Laccase (E.C.1.10.3.2, p-benzenediol:oxygen oxidoreductase) is a cuproprotein belonging to a small group of enzymes denoted as blue oxidases (Duran et al., 2002).

This oxidoreductase is capable of catalyzing the oxidation of various organic compounds with the simultaneous reduction of oxygen to water (Thurston, 1994). Interest in these essentially 'eco-friendly' enzymes, which work with air and produce water as the only by-product, has grown significantly in recent years (Riva, 2006). Their effectiveness in removal of phenolic pollutants has been established (Torres et al., 2003).

The multi-copper oxidases are a family of enzymes that couple the four-electron reduction of oxygen to water with four one-electron oxidations of substrates (Solomon et al., 1996). In the laccase-mediated oxidation, the phenolic substrate undergoes a one-electron oxidation which produces aryloxy radical. This active radical can be converted to quinone in the second stage of oxidation (Duran et al., 2002). This quinone as well as the radical undergo non-enzymatic coupling to produce polymers. Laccases have low substrate specificity. Simple diphenols, substituted polyphenols, aromatic amines, benzenethiols are good substrates of laccase (Yaropolov, 1994).

A typical laccase molecule is 60-80 kDa, of which 15-20% is carbohydrate comprised 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).

2.5.1 Sources of Laccase

It has been observed that 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). They are also present in insects (Thomas et al., 1989).

Currently *Trametes* species are keenly researched for laccase production (Ikehata et al., 2006). This specie is a natural wood decomposer which is usually available in most parts of the world. Large amount of laccase production has been reported from the *T. versicolor* which has already been marketed by several companies (Ikehata et al., 2006). But the current price of laccase is still high for large-scale applications (Duran and Esposito 2000). The main goal for current studies on laccase production is improving productivity and reducing the production cost (Ikehata et al., 2006).

For this study, laccase SP504 has been used. This is a developmental preparation from commercial enzyme producer, Novozymes North America, Inc. (Franklinton, NC), who described it as a fungal laccase from *Trametes villosa*.

2.5.2 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 adsorption 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 histidine 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 catalytic and active site of laccase is presented in

Figure 2.5.1.

Figure 2.5.1 : Active and Catalytic Site of Laccase (Riva, 2006)

2.5.3 Catalytic Site of Laccase

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 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 2.5.2 represents the proposed mechanism for oxygen reduction to water by multicopper oxidases.

Figure 2.5.2: Proposed mechanism for 4e⁻ reduction of oxygen to water by multi copper oxidase (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 2.5.3 provides a summary of the catalytic cycle of laccase. First the electron from substrate reduces the T1 site of the native intermediate state of enzyme. At 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 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 sequentially gets 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).

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).

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).

Figure 2.5.3: Catalytic Cycle of Laccase (Solomon et al., 1996)

2.6 Reaction Mechanism

2.6.1 Enzyme as Catalyst

A catalyst is a substance that increases the rate of a reaction without modifying the overall standard Gibbs-energy change in the reaction. Enzymes are proteins which act as catalysts and speed up the reaction rate, which otherwise would be too slow.

Some of the reactions proceed spontaneously whereas some do not. The reactions which proceed spontaneously are known to be energetically favorable reactions. But if a reaction proceeds in a forward direction does not ensure that it will be energetically favorable under all reaction conditions. For a reaction to take place, the participating reactant molecules should overcome a potential-barrier known as the energy of activation. Transition state theory, developed by Eyring, gives a good explanation for the requirement of the activation energy in a chemical reaction (Palmer, 1995). According to this theory, every chemical reaction passes through an unstable state during its course. This unstable transition state breaks down to generate more stable compounds. It can be stated that the energy needed to form the transition state from the reactants is the activation energy.

A catalyst enhances the reaction rate by reducing the energy of activation needed for the reaction. It combines with the reactants to generate a transition state complex which has lower energy than that in an uncatalyzed reaction. Figure 2.6.1 shows the free energy change of an energetically favorable reaction in presence and absence of catalyst (Palmer, 1995).

Figure 2.6.1: Free energy changes for an energetically favorable reaction and the effect of catalyst (Palmer, 1995)

During a one-substrate enzymatic reaction, substrate binds with the active site of the enzyme producing an enzyme-substrate complex. Though the enzyme-substrate complex is relatively stable, formation of this complex proceeds through an unstable transition state. The catalyzed reaction takes place by formation of an unstable state to generate the product. At this stage, the reaction product can still be bound to the enzyme which creates another relatively stable reaction intermediate namely, enzyme-product complex. The energy profile of such equation is presented in Figure 2.6.2 (Palmer, 1995).

Figure 2.6.2: Free energy profile of an enzyme-catalyzed reaction (Palmer, 1995)

2.6.2 Kinetics of Enzyme-catalyzed Reactions

It has been observed that there is an effect of substrate concentration on enzyme kinetics. In the presence of single or multiple substrates, when one substrate is kept constant at lower concentration, the reaction is a first-order reaction with respect to the substrate. But at higher substrate concentration, the reaction becomes zero-order. As shown in Figure 2.6.3, a graph of initial velocity (v_o) against the initial substrate concentration ($[S_o]$) at a

constant total enzyme concentration ($[E_0]$), generally appears as a rectangular hyperbola (Palmer, 1995).

Figure 2.6.3: Plot of initial velocity against initial substrate concentration at constant initial enzyme concentration for a single substrate enzyme-catalyzed reaction.

Since initial reaction conditions of an enzyme-catalyzed reaction are easy to identify, initial velocity is considered to be an important variable in exploring the enzyme kinetics. It minimizes the complicating factors such as reversible reaction, inhibition of the enzyme by-products, progressive inactivation of enzyme, etc. Initial velocity is usually defined as the measured velocity before more than ten percent of substrate has been converted to product.

Figure 2.6.4 : Initial velocity calculation using a progress curve

Figure 2.6.4 represents a progress curve using which, initial velocity is calculated. The plot of substrate conversion or product formation with time gives the initial velocity of the reaction. For example, if at time t_1 substrate concentration was $[S_1]$ and after enzymatic reaction, the substrate concentration became $[S_2]$ at time t_2 , then the rate of substrate conversion between time interval, $t_1 \rightarrow t_2$ was,

$$\frac{\Delta S}{\Delta t} = \frac{S_2 - S_1}{t_2 - t_1} \quad 2.1$$

If the time interval is really small, then, the slope of the tangent can be expressed as dS/dt . If dS/dt is measured at time t_0 , then it would represent initial velocity v_0 .

2.6.2.1 Michaelis-Menten Equation for single substrate steady-state kinetics

Kinetic models to explain the relationship between the initial velocity and the maximum velocity was proposed by Michaelis and Menten (Palmer, 1995). The simplest reaction for a single substrate enzyme catalyzed reaction, where there is only one substrate binding site is available per enzyme, would be

Where,	E	=	Enzyme Concentration
	S	=	Substrate Concentration
	ES	=	Enzyme-Substrate complex Concentration
	P	=	Product Concentration
	k_1, k_{-1}, k_2	=	Reaction rate constants.

For this reaction, Michaelis and Menten assumed that there was an instantaneous equilibrium between enzyme, substrate and enzyme-substrate complex (also known as Michaelis-Menten complex), which was maintained throughout the reaction and the breakdown of enzyme-substrate complex to form products was too slow to disturb the equilibrium. One more assumption made by Michaelis and Menten is that the substrate is generally present in much greater concentration than the enzyme.

The plot of initial substrate concentration against initial velocity of this enzymatic reaction gives a rectangular hyperbola. Figure 2.6.3 represents the plot between initial velocity V_0 and initial substrate concentration $[S_0]$ for a enzyme catalyzed reaction. This

hyperbola can be expressed using the Michaelis-Menten equation, which is given in the following form,

$$v_0 = \frac{V_{\max} [S_0]}{K_M + [S_0]} \quad 2.3$$

Here, v_0 = initial velocity
 $V_{\max}$ = $k_2[E]$ = limiting initial velocity
 K_M = $(k_{-1}+k_2)/k_1$ = Michaelis-Menten constant
 $[S]_0$ = Initial substrate concentration

For enzyme reactions following Michaelis-Menten type equation, when $v_0 = V_{\max}/2$, the substrate concentration becomes numerically equal to K_M .

One of the key features of the Michaelis-Menten plot is that when substrate concentration is really low, the reaction becomes first-order. In this situation,

$$K_M \gg [S]_0, \quad v_0 = \frac{V_{\max} [S]_0}{K_M + [S]_0} \approx \frac{V_{\max} [S]_0}{K_M} \propto [S]_0 \quad 2.4$$

When the substrate concentration $[S]_0$, is equal to K_M , the reaction is of mixed order and $v_0 = V_{\max}/2$. Finally when the substrate concentration is really high, the reaction is independent of the substrate concentration and it follows zero-order kinetics. Then the equation becomes,

$$v_0 = \frac{V_{\max} [S]_0}{K_M + [S]_0} \approx \frac{V_{\max} [S]_0}{[S]_0} = V_{\max} \quad 2.5$$

2.6.2.2 Significance of the Michaelis-Menten Equation

The Michaelis-Menten equation was obtained by considering a single-substrate enzyme-catalyzed reaction with one substrate binding site per enzyme and involving one intermediate complex ES. But in reality, these types of reaction are rare. There could be multi-substrate reaction where enzymes have more than one binding site. Finally, for a single substrate reaction, there could be more than one intermediate complex generated and the reaction of such kind has the following form (Equation 2.6),

Here, conversion of ES to EP becomes the rate-limiting step for the overall reaction.

Despite all these additional factors, equations of the same form as the Michaelis-Menten equation were derived and certain terms like, turnover number, catalytic efficiency, etc. appear which are of significance.

- Turnover Number (k_{cat}) is defined as the maximum number of moles of substrate that can be converted to product per mole of catalytic site per unit time. This term is related to $V_{\max}$, where $V_{\max} = k_{\text{cat}} [E]_0$. For single-substrate reactions as discussed above, $k_{\text{cat}} = k_2$. For more complex reactions, it becomes a function of several rate constants. Enzyme activity is measured by turnover number (McMurry and Castellion, 1992).

- Catalytic efficiency is given by the term k_{cat}/K_M . A high value for this term signifies that the limiting factor for the overall reaction is the frequency of collisions between S and E molecules, whereas a low value indicates the equilibrium condition. A comparison between catalytic efficiency of different substrates gives a measure of the specificity of the enzyme.
- K_M is the substrate concentration corresponding to the initial velocity when initial velocity becomes half of maximum velocity.
- Enzyme's affinity for the substrate can be defined by K_s , where,

$$K_s = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} \quad 2.7$$

In general, a low K_s value indicates high affinity of enzyme for substrate whereas a high value indicates low affinity. Since K_s is often hard to determine, K_M is frequently taken as a surrogate for affinity of enzyme and substrate.

2.6.3 Two-substrate Enzyme Catalyzed Reaction

Most biochemical reactions involve more than one substrate. The simplest form of such a reaction would be a two-substrate, two-product reaction which is also known as bi-bi reactions. These reactions may be sequential ones, where before first product is formed, both the substrates bind to the enzyme and form a ternary complex. It can also be non-sequential where products are generated before binding of new substrates. There are two mechanisms for two substrate reactions, ping-pong mechanism and ternary complex mechanism.

2.6.3.1 Ping-Pong Mechanism

This mechanism is also known as double-displacement mechanism. This mechanism suggests that, before the second substrate is bound first product is generated and released, usually as a modified enzyme as intermediate.

2.6.3.2 Ternary-Complex Mechanism

This is a sequential mechanism which is also known as single-displacement mechanism. It involves formation of a ternary complex before the product is formed and released.

2.6.4 Ping-Pong Bi-Bi Mechanism

Laccase follows a “two-site ping-pong bi-bi” mechanism (Piontek et al., 2002). The reaction for bi-bi ping-pong mechanism type enzymatic reaction is:

In this, X is generally a small group which generally does not contribute in the reaction as a free molecule. AX first binds to the enzyme to generate E.AX. Then the restructuring of the E.AX produces EX.A by breaking the A-X bond and generating E-X bond. The first product A is generated which leaves the EX form of enzyme. EX is the modified enzyme. Now the second substrate B comes into the picture. B does not bind with E, but binds with EX, generating EX.B. The intermolecular rearrangement of EX.B generates E.BX by breaking E-X bond and generating B-X bond. Finally, the product BX is produced which leaves the enzyme in original form E (Palmer, 1995).

2.7 Enzyme Stability

Stability of an enzyme is important to maintain its catalytic activity. In the presence of certain factors, enzymes lose their tertiary structures and become a random structure, which is inactive. This phenomenon is commonly known as denaturation. Factors that can hamper enzyme stability are temperature, pH, organic solvents and ionic strength of solutions. The effect of these parameters on enzyme stability is discussed in the following sections.

2.7.1 Effect of pH

Enzyme activity is greatly dependent on pH. Each enzyme is active over a relatively small pH range. Most enzymes show a bell-shaped plot of activity against pH, specifying an optimum pH value. This pH-dependence of enzymes is due to the ionizable side chains of the amino acid residues in the polypeptide which only function properly at a certain pH value. At extremes of pH, enzymes lose their tertiary structures and become denatured. Extreme pH values (typically, below pH 4.0 and above pH 9.0) should be avoided for most enzymes (Vermette, 2000). Even at moderate pH values, enzyme activity varies depending on the degree of ionization of the side chains (Palmer, 1995).

2.7.2 Effect of Temperature

Above 40-50° C thermal agitation causes denaturation of many enzymes (Palmer, 1995). Mode of enzyme preparation along with freezing and thawing can cause loss of enzyme activity.

2.7.3 Effect of Organic Solvents

Water-miscible organic solvents, like ethanol, may affect enzyme activity adversely leading to denaturation (Palmer, 1995).

2.7.4 Effect of Ionic Strength of Salt

Presence of certain ionic salts in high concentrations can also reduce enzyme activity (Palmer, 1995).

2.8 Enzyme Inhibition

An inhibitor is a substance that reduces the rate of an enzyme-catalyzed reaction. Though inhibitors can bind with enzyme itself or act on the substrate or the cofactor, they mostly bind with the enzyme, either reversibly or irreversibly.

2.8.1 Reversible Inhibition

In reversible inhibition, inhibitors bind to the enzyme in a reversible manner so that they can be removed to restore full enzyme activity. Decreasing the concentration of the inhibitor reverses the equilibrium and regenerates active free enzyme. Reversible inhibition can be categorized into three types; competitive, uncompetitive and mixed inhibition (Cornish-Bowden and Wharton, 1988).

- **Competitive Inhibition:** Competitive inhibitors are often structurally similar to the substrate structure, whose reaction they inhibit. In this type of inhibition, both substrate and inhibitor compete for the active site of enzyme. The enzyme-bound

inhibitor then either lacks appropriate reactive groups, or it is held in an unsuitable position with respect to the enzyme catalytic site for the reaction to occur. In all these cases, a dead-end complex is formed. If the inhibitor concentration is kept constant and substrate concentration is increased, then it provides a possible solution to reverse the competitive inhibition.

- **Uncompetitive Inhibition:** Instead of binding with the free enzyme, inhibitors that cause this type of inhibition, bind with the enzyme-substrate complex. In this type of inhibition, substrate binding to the enzyme can modify the enzyme structure and makes the inhibitor binding site available or, inhibitors bind directly to the enzyme-substrate complex. In either case, it produces a dead-end complex.
- **Mixed Inhibition:** In mixed inhibition, inhibitors compete with the substrate to bind with the enzyme active site and they bind with the enzyme-substrate complex as well. In this type of inhibition, both competitive and uncompetitive inhibition takes place simultaneously.

2.8.2 Irreversible Inhibition

In this type of inhibition, the inhibitors bind to the enzyme active site in an irreversible manner, generally via a covalent bond. Irreversible inhibitors can act by preventing substrate binding or it may destroy some parts of catalytic site (Palmer, 1995). The latter type of inhibition is also known as “suicide inhibition” if a reactive intermediate is generated as a part of normal catalytic cycle of enzyme. This intermediate then either modifies the enzyme or attaches to the enzyme in such a manner that the enzyme itself becomes a poor catalyst (Palmer, 1995).

2.8.3 Use of Additives to Suppress Inactivation

One of the major disadvantages of the enzymatic treatment is the relatively short catalytic life of enzyme due to inactivation. Aitken and Heck (1998) have identified the susceptibility of laccase to inactivation as a key limitation for its industrial application. Several mechanisms for inactivation of peroxidase enzymes have been studied (Hinter et al., 1996) but little information is available on the inactivation mechanism of laccase.

Klibanov et al., (1983) proposed that, during enzymatic reaction, phenoxyl radicals interact with active site of the enzyme, which results in the inactivation. Irreversible, time-dependent and mechanism-based inactivation was observed in horseradish peroxidase when reductant substrates were present (Baynton et al., 1994). Nakamoto and Machida (1992) have suggested that enzyme inactivation is due to the adsorption of enzyme onto the end-product polymer and the obstruction caused by this for substrate access to the active site.

The apparent inactivation of peroxidase during phenol polymerization reaction was mainly caused by end-product polymers (Wu et al., 1998). Nakamoto and Machida (1992) have pointed out that the cost of the enzymatic treatment cannot be reduced if the enzyme is inactivated by the phenoxyl radicals. However, they demonstrated that the required amount of enzyme was reduced by introducing hydrophilic synthetic polymers like polyethylene glycol (PEG) or by adding proteins like gelatin. These additives suppressed the enzyme adsorption which alleviated the apparent enzyme inactivation (Wu et al., 1998).

PEG was effective in suppressing the inactivation of horseradish peroxidase (HRP) (Wu et al., 1998), soybean peroxidase (Caza et al., 1999), and laccase (Modaressi et al., 2005). PEG is the additive of interest as it significantly reduces the cost of treatment by reducing amount of enzyme needed (Cooper and Nicell, 1996). It is also a non-toxic compound which is considered to be fit for human consumption by the United States Food and Drug Administration (Harris, 1992).

The mechanism of the protection is not yet fully understood, but the effectiveness of PEG is a function of its concentration and molecular mass (Kinsley and Nicell, 1999). Several researchers have proposed different working mechanism for PEG protection. Nakamoto and Machida (1992) have observed that PEG having molecular mass of 400 or less had no protective effect on enzyme and the protective effect increased with increasing the molecular mass above 600. Researchers have attributed this phenomenon to the interaction of PEG with water. They have found that PEG interacts with water and binds with water molecule, which creates a relatively large hydrated volume (Osterberg et al, 1995). Antonsen and Hoffman (1992) suggested that PEG was able to fold upon itself forming a “water-structure”, which enabled it to bind with more water molecules between the fold lengths. The number of water molecules attached per monomer unit is defined as hydration number (Kjellander and Florin, 1981). It has been reported that basic hydration of PEG is not satisfied until hydration number reaches two (Kjellander and Florin, 1981). PEG is able to bind with more water molecules only when the molecular mass reaches approximately 600 (Kinsley and Nicell, 1999). This explanation agrees with the fact that at lower molecular mass no increase in protective effect was seen (Kinsley and Nicell, 2000).

Another explanation proposed for the protective effect of PEG states that hydroxyl end groups of PEG competitively bonds with the hydrogen bonding sites (Nakamoto and Machida, 1992). But, Kinsley and Nicell (2000) argued that if this theory was right then PEG having a lower molecular mass would have been able to exert a greater protective effect than higher molecular mass PEG, as lower molecular mass PEGs have more hydroxyl groups per unit mass than PEGs of higher molecular mass. Studies show that higher molecular mass PEGs were successful in protecting enzymes than those of lower molecular mass. Kinsley and Nicell (2000) concluded that, it is unlikely that the hydroxyl bonding sites of PEG are responsible for quenching the bonding sites on polymeric end products.

Gombotz et al., (1992) demonstrated that PEG attached to a surface had the capability to reject proteins. Based on this fact, Buchanan and Nicell (1997) suggested that, PEG attached to the polymeric end products could be acting the same way to repel enzymes, which are essentially protein molecules. They hypothesized that, PEG attaches to the phenolic polymers as they form. Thus, inactivation resulting from the adsorption of enzyme to the polymeric end product could be reduced (Kinsley and Nicell, 2000).

2.9 Coagulation and Precipitation

Coagulation and flocculation are the processes where compounds such as metal salts are added to effluents in order to destabilize the colloidal material and cause the aggregation of small particles into larger, more easily removed flocs (Stephenson and Duff, 1996). Coagulants are used to remove color and COD from wastewater (Nemerow, 1978). The

effectiveness of the process depends on factors like coagulating agent, coagulant dosage, 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, polyethylene imine (PEI), chitosan, anionic, nonionic or cationic polymers (Al-Kassim et al., 1994; Wada et al., 1995; Caza et al., 1999; Mantha et al., 2001). In the present work, color was removed using surfactant mediated separation using both alum and sodium dodecyl sulfate (SDS).

2.9.1 Surfactant-Mediated Separation

Surfactant-mediated separation processes have been studied for several years though there are issues related to the practical application of these processes in the waste water treatment. But surfactant-mediated separations have potential to recover and recycle the target pollutant (Talens-Alessen et al., 2006).

The main drawback of surfactant-mediated separation process is that it introduces a new chemical (surfactant) to the system. This violates the conventional process design rule, which encourages avoidance of any new species in the system (Seider et al., 1999). In these processes, surfactant is used in excess to remove a target chemical which is more hazardous and toxic. If the surfactant fails to remove the pollutant up to the required limit, then the pollutant along with a new chemical becomes the point of concern. As long as the environmental cost of the pollutant is higher than the environmental cost of the surfactant and the flocculant, the process is significant (Talens-Alessen et al., 2004). So far, this process has been successful in removing the toxic pollutants.

Surfactants are organic compounds having both hydrophobic and hydrophilic groups attached to them. In bulk solution surfactants can remain in organized or in disorganized fashion. The organized array of surfactants is known as a micelle. The surfactant concentration at which micelles are formed is known as critical micelle concentration (CMC). When micelles are in water, their hydrophilic end or ionic end organizes themselves into a spherical or ellipsoid structure keeping the hydrophobic ends in the center.

Depending on the charges on the surfactant head, they can be classified into three main categories, ionic, nonionic and zwitterionic. If surfactants have no charge on the head, then it is a nonionic surfactant. Ionic surfactants carry charge in their head. They can be further subdivided into anionic (having a negative charge) and cationic (having a positive) surfactants. If a surfactant contains a head with both anionic and cationic charge group then it is known as zwitterionic.

The first application of the surfactant mediated separation was micellar-enhanced ultra filtration (MEUF). In MEUF, solutes bind onto the surface of the surfactant micelles or they get dissolved into the micellar cores. This process creates a liquid waste which needs further processing using overpressured membrane filtration. The main drawbacks of MEUF are membrane fouling, relatively high volume ratio between retentate and permeate (generally 0.2 under continuous operation) and removal of the surfactant present in the retentate (Porrás-Rodríguez and Talens-Alesson, 1999).

The second and more recent application of the surfactant mediated separation is adsorptive micellar flocculation (AMF). AMF is a method for removal of pollutants from water involving the attraction of cationic species to the surface of the anionic surfactant

micelles and flocculation of the said micelles as their mutual electrostatic repulsion is neutralized (Talens-Alession et al., 2006). The flocculates formed in this process can be removed easily from water by inexpensive methods. AMF has been used to remove the color generating pollutants in the present work.

2.9.2 Mechanism of Adsorptive Micellar Flocculation

Sodium lauryl sulfate (SDS), also known as sodium dodecyl sulfate (NaDS) is an anionic surfactant. It is widely used in cosmetic products like toothpastes, shampoos, shaving foams, bubble baths, etc. SDS ($(\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na})$) has a hydrophilic end consisting of a negative charge and a hydrophobic tail consisting of twelve carbons. Figure 2.9.1 presents the structure of SDS molecule.

Figure 2.9.1: Structure of Sodium lauryl sulfate

Use of alum in waste water treatment is not new. In this case, alum provides the cationic charge needed for the charge neutralization of the micelles and to enhance floc formation. Talens-Alession et al., (2004) suggested that, the AMF mechanism is based on the adsorption of an organic pollutant on an amorphous substrate formed by the flocculation of micelles formed by SDS with trivalent cations like Al^{3+} . Micellar flocculation occurs in two steps: in first step, the cations bind themselves onto the micellar surface, which causes the charge neutralization of micelles. In the second step, suppression of

intermicellar repulsion leads to the aggregation of micelles into a filterable aggregate with amorphous structure (Talens-Alession et al., 1998). The flocculate serves as an adsorption media for the organic pollutant (Porrás-Rodríguez and Talens-Alession, 1999, 2000).

Figure 2.9.2: Color Removal Using Adsorptive Micellar Flocculation
(Black circles represent organic pollutants)

The polymeric end products of enzymatic treatment are hydrophobic. It is expected that, some of these hydrophobic end products are capable of partitioning into the micelles. When alum is added into the micellar solution, the Al^{3+} bind onto the micelles and neutralizes them. The reduction in intermicellar repulsion causes the micelles to floc

together and to serve as an adsorption medium for the organic pollutants. These flocs can be easily removed from solution by means of filtration. Figure 2.9.2 presents the mechanism of AMF.

Micellar flocculation takes place between pH 2.0 and pH 6.0 (Talens-Alession et al., 2004) which is a broad range of pH for industrial applications. This process releases into the effluent the non-flocculated surfactant on the order of the magnitude of CMC of the system and Al^{3+} . For SDS to be flocculated with alum, the $\text{CMC}_{\text{AL}(\text{DS})_3}$ has been reported to be 5×10^{-4} M (Talens-Alession et al., 1998). The nature of AMF makes it suitable for processes that recycle compounds found in effluents from the chemical industries (Talens-Alession et al., 2002). As it is an adsorption process, it is expected to be more effective in solutions having high concentrations of pollutants (Talens-Alession et al., 2002).

CHAPTER III

MATERIALS AND METHODS

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

3.1 Materials

A fungal laccase from *Trametes villosa* (SP-504; EC 1.10.32, 200 LACU/mL. batch # 1999-00091) was provided by Novozymes North America, Inc. (Franklinton, NC). It had a nominal activity of 200 U/mL at pH 5.5 and at room temperature. A unit of activity is defined as the number of micromoles of substrate converted per minute under standard conditions. The enzyme was stored at -15°C. A sub-stock solution was prepared from it and was stored at 4°C. The specific activity of the aqueous laccase solution was determined using the laccase activity assay described later in this chapter.

Diphenylamine (DPA) was purchased from Aldrich Chemical Co. (Milwaukee, WI). It had a purity of 99% or better. The DPA stock solution was stored at 4°C.

PEG was purchased from Sigma Chemical Co. (St. Louis, MO). For this study, PEG having molecular masses of 400 g/mol, 1450 g/mol, 3350 g/mol, 8000 g/mol and 35000 g/mol were used.

MES buffer and syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) for the laccase activity test was purchased from Sigma Chemical Co. (St Louis, MO). Analytical grade monobasic and dibasic sodium phosphate was purchased from BDH, Toronto, ON. Alum, as aluminum sulphate ($\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$), was obtained from BDH, Toronto, ON. All other chemicals used for this study were of analytical grade and were purchased from Fisher Scientific, Fair Lawn, NJ and BDH, Toronto, ON.

Plastic syringes were purchased from Becton Dickinson & Co, Clifton, NJ. For coarse filtration, Whatman (No 42) filter paper was used. For microfiltration, 0.2 μm HT Tuffryn membrane filters from Gelman Labs (Mississauga, ON) were used.

3.2 Equipment

Solution absorbances were measured with 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.

For standardization of the chemicals, high performance liquid chromatography (HPLC) was carried out on a system obtained 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.

The pH was measured with an IQ 200 pH meter which was purchased from IQ Scientific, London, ON. It was fitted with ISFET probe (p=205, pH 15-ss, 57084).

A Shimadzu TOC-V CSH Total Carbon Analyzer was used to measure the residual PEG in the solution. This analyzer used oxygen as an oxidizing agent.

3.3 Experimental Procedure

Batch reactors were set up to study the effect of pH, enzyme concentration, and molecular mass and concentration of PEG over a fixed reaction period. Experiments were conducted at room temperature, approximately 22°C. The study was designed to achieve at least 95% removal of DPA by optimizing pH, PEG concentration and enzyme concentration.

Batch reactors used for the study, volumes of 20 mL or 100 mL, contained a buffered solution of DPA, with or without one of the additives PEG₄₀₀, PEG₁₄₅₀, PEG₃₃₅₀, PEG₈₀₀₀ or PEG₃₅₀₀₀. Enzyme was added to initiate the reaction and the batch reactors were kept open and were mixed vigorously using Teflon-coated stir bars and a magnetic stirrer.

After an appropriate reaction period, 900 µL of reaction mixture was quenched with 100 µL of 0.5 M hydrochloric acid to stop the reaction. Samples were filtered using 0.2 µm HT Tuffryn membrane filters and analyzed for residual DPA by HPLC.

3.3.1 pH

The pH was optimized first among all reaction parameters. The reaction mixture consisted of DPA, buffer and laccase. Effect of pH was also monitored in the presence of PEG₄₀₀, PEG₁₄₅₀, PEG₃₃₅₀, PEG₈₀₀₀ or PEG₃₅₀₀₀. Substrate concentration in all the batch reactors was 0.19 mM. Laccase concentration in these batch reactors was varied from 0.0015 U/mL to 0.005 U/mL both in the presence and absence of PEG. When used, PEG

concentration in the batch reactors was 200 mg/L. The pH was adjusted within a range of 3.0 to 9.0 using different buffer solutions. The reaction time in all cases was three hours.

3.3.2 Laccase Concentration

A sub-stock was prepared from the laccase stock solution daily and was stored at 4°C. Whenever a new sub-stock was prepared, the activity was measured using the laccase activity assay.

The effect of enzyme concentration was studied at the optimum pH obtained in the previous experiments to achieve 95% DPA conversion in a three-hour reaction period. Laccase concentration in the batch reactors varied from 0.001 U/mL to 0.01U/mL with a substrate concentration of 0.19 mM. Optimum laccase concentration was measured both in the presence and absence of PEG. A concentration of 200 mg/L of PEG₄₀₀, PEG₁₄₅₀, PEG₃₃₅₀, PEG₈₀₀₀ or PEG₃₅₀₀₀ was used to determine the optimum enzyme concentration in the presence of PEG.

3.3.3 PEG Concentration

The last parameter which was optimized was PEG concentration. Stock solutions of PEG₄₀₀, PEG₁₄₅₀, PEG₃₃₅₀, PEG₈₀₀₀ and PEG₃₅₀₀₀ were prepared at 4 g/L and stored at room temperature. Batch reactors had optimum pH and optimum enzyme concentration determined from the previous experiments and an initial DPA concentration of 0.19 mM. PEG concentration in these experiments varied from 20 mg/L to 240 mg/L.

3.3.4 Coagulation

After enzymatic reaction, several coagulant aids such as anionic polymers, cationic polymers, polyethyleneimine (PEI), alum and sodium lauryl sulfate (SDS) were individually used to remove the color. None of these produced satisfactory results. Finally, sodium lauryl sulfate (SDS) and alum were used together to remove the polymers by micellar flocculation (Porrás-Rodríguez and Talens-Alessón, 1999). After a three-hour reaction period, first SDS was added to the batch reactors. Rapid mixing was allowed for five minutes. Then alum was added to the mixture and rapid mixing was carried out for another five minutes. This rapid mixing was followed by slow mixing for 10 minutes. Samples were allowed to settle for an hour by gravity and the settled particles were removed by filtration.

3.4 Analytical Techniques

3.4.1 Laccase Activity Assay

Syringaldazine, a unique substrate for laccase, was used to measure the enzyme activity. Under aerobic conditions, 19 μM syringaldazine (4-hydroxy-3, 5-dimethoxybenzaldehyde azine) is oxidized to the corresponding quinone. Figure 3.4. represents the oxidation of syringaldazine by laccase to form the corresponding quinone.

Figure 3.4.1: Oxidation of Syringaldazine

All components were provided in sufficient quantity so that the rate of reaction became directly proportional to enzyme activity (Felby, 1998). The assay mixture consisted of 850 μL of MES Buffer (pH 5.5), 50 μL of 0.38 mM syringaldazine solution in ethanol and 100 μL of laccase solution. The rate of reaction was measured by measuring the rate of formation of colored products. These colored products absorbed light at a peak wavelength of 530 nm (Felby, 1998). Increase in absorbance at 530 nm determined the enzyme activity.

In a semi-micro cuvette, the reagents and laccase sample were mixed in the same order and in the same quantity as mentioned previously. Total volume of the mixture was 1 mL. Immediately after addition of laccase sample, the cuvette was shaken to ensure proper mixing and placed in the spectrophotometer to monitor the change in absorbance at 530 nm. The change in absorbance was measured at 15 s and 75 s. Absorbance was noted at the mentioned time interval and the following calculations were carried out to measure the activity of laccase.

$$\begin{aligned}\text{Activity in the cuvette (LACU/mL)} &= (\Delta A * 1.0 \text{ mL} * 10^{-3} * D) / (0.065 * 0.1 \text{ mL}) \\ &= \Delta A * 1.538 * D\end{aligned}$$

Where,

$$\Delta A = \text{Change in absorbance per minute} = A_{75 \text{ s}} - A_{15 \text{ s}}$$

(Range of absorbance should be, 0.1 to 0.4 $\Delta A/\text{min}$)

$$1.0 = \text{Total volume in the cuvette (mL)}$$

$$0.065 = \text{Micro-molar extinction coefficient } (\mu\text{M/L})$$

10^{-3} = Conversion factor for LACU/mL to LACU/L

D = Dilution factor

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

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

= Activity in the cuvette (LACU/mL) * reactor volume (mL) /
enzyme solution added to the reactor (mL)

3.4.2 DPA Concentration Assay

HPLC was used to measure the concentration of DPA. Peak areas were measured for different concentrations of DPA at 280 nm (80:20 = methanol: distilled water was used for elution). A standard curve was developed from the data obtained and was used to measure the concentrations of DPA in sample solutions.

3.4.3 Buffer Preparation

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

3.4.4 Total Organic Carbon (TOC)

TOC analysis of the samples was done to measure the PEG remaining in the solution. 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 ppm TC and IC carbon, respectively.

100 μ L of the sample solution was injected into the analyzer to determine the TOC of the solution. Separate standard curves were prepared for PEG₄₀₀, PEG₁₄₅₀, PEG₃₃₅₀, PEG₈₀₀₀, PEG₃₅₀₀₀ and DPA. The difference between the measured TOC of the samples and TOC caused by DPA was used to calculate the TOC due to PEG remaining in the samples. All the samples were micro-filtered prior to TOC analysis to prevent clogging.

3.4.5 Optimum SDS and Al⁺³ Concentrations for Color Removal

The pH and SDS to alum ratio were varied to achieve optimum conditions for color removal. The optimum condition was determined by comparing the direct absorbance of the reaction products immediately after enzymatic reaction and reduction in absorbance of the reaction products after the SDS and Al⁺³ addition. Direct absorbance was measured at 440 nm.

3.5 Estimation and Minimization of Errors

In any experiment, reliability of results can be affected by occurrence of errors. Mainly two types of errors, namely systematic and random errors, can affect 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 are caused due to human inaccuracy such as measurement errors, sampling errors, observation errors. Inaccurate results can be caused due to aging of experiments as well. All sets of reactions were done 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 IV

RESULTS AND DISCUSSION

This study demonstrates oxidative polymerization of DPA using laccase followed by removal of those polymers via adsorptive micellar flocculation (AMF) by using sodium lauryl sulfate (SDS) and alum. The main drawback of enzymatic treatment is the high cost of enzyme. Additives like PEG decrease the cost of the process by reducing the amount of enzyme required (Nakamoto and Machida, 1992). The protective effect of PEG, which is a function of its molecular mass and concentration, was studied for DPA removal. The optimal conditions needed to obtain over 95% conversion of DPA, both with and without PEG addition, were determined. Factors of particular interest included optimal pH, optimal enzyme concentration, effective PEG concentration for PEG of different molecular masses and change in enzyme activity over the reaction period. Effectiveness of a color removal process depends on factors like coagulating agent, coagulant concentration, pH and concentration of the organic compound, etc. (Randtke, 1988). These factors were considered while using AMF for color removal.

4.1 Effect of pH

The optimum pH for the enzyme-catalyzed removal of DPA was determined by using a pH range of 3.0 to 9.0. Different buffers were prepared to obtain each pH of interest. Acetate buffers were used for the pH range from of 3.0-5.5. Monobasic and dibasic

sodium phosphate buffers were used for pH 5.6-7.5. For higher pH, 8.0-9.0, bicarbonate buffers were used.

Initial substrate concentration was 0.19 mM in the batch reactors, which is approximately the solubility limit for DPA to determine the optimum pH. The enzyme concentration was varied within a range of 0.0015 U/mL to 0.005 U/mL both in the presence and absence of PEG. Initial substrate and enzyme concentrations were kept constant in each batch reactor to ensure that the conversion of DPA in each batch reactor depended on pH. The pH was also optimized in presence of 200 mg/L of PEG₄₀₀, PEG₁₄₅₀, PEG₃₅₀₀, PEG₈₀₀₀ or PEG₃₅₀₀₀ to check if optimum pH changed in the presence of PEG or not. Optimum pH was determined at stringent condition with respect to enzyme concentration.

The reaction was run at room temperature and reaction time was kept at three hours which was considered to be sufficient based on the previous similar enzymatic reactions (Klibanov et al., 1980).

Neutral pH was found to be the optimum pH for DPA conversion. The presence of different PEGs did not change the optimum pH. Conversion efficiencies as a function of pH are shown in Figure 4.1.1 and 4.1.2.

—◆— 0.0045 U/mL
 —■— 0.004 U/mL

Reactor Conditions		
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h

Figure 4.1.1: Optimum pH for DPA Conversion in the Absence of PEG

—■— With PEG₄₀₀
 —◆— With PEG₁₄₅₀
 —●— With PEG₃₃₅₀
 —●— With PEG₈₀₀₀

Reactor Conditions		
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Enzyme Concentration	:	0.0015 U/mL
PEG Concentration	:	200 mg/L

Figure 4.1.2: Optimum pH for DPA Conversion in the Presence of PEG

4.2 Effect of Enzyme Concentration

Optimum enzyme concentration for DPA removal was determined at the optimum pH found in the previous experiment. A three-hour reaction period was chosen for this study. Initial substrate concentration remained at 0.19 mM. The enzyme concentration which was successful in removing 95% of the initial substrate concentration, at the optimum pH, was considered as the optimum enzyme concentration.

Laccase concentration in the batch reactor varied from 0.001 to 0.01U/mL. Experiments were run both in the presence and absence of PEG. PEG₄₀₀, PEG₁₄₅₀, PEG₃₅₀₀, PEG₈₀₀₀ or PEG₃₅₀₀₀ were used in excess, 200mg/L, to ensure that the conversion efficiency at the optimum pH was only a function of enzyme concentration.

Figure 4.2.1 represents the effect of enzyme concentration in the absence of PEG. Figures 4.2.2 to 4.2.6 represents the effect of enzyme concentration on DPA conversion in the presence of PEG. Table 4.2.3 summarizes the optimum enzyme requirement for the reaction conditions considered.

0.0075 U/mL of laccase was needed to achieve 95% conversion of the initial substrate without PEG. The optimum enzyme concentration decreased with an increase in PEG molecular mass except for PEG₄₀₀. In the presence of PEG₄₀₀, the enzyme required to achieve 95% conversion was about the same as that in the absence of PEG.

The optimum enzyme requirement, in the absence of PEG was almost twice as that needed in the presence of PEG₃₃₅₀. Figure 4.2.7 represents the comparison between the removal efficiency in batch reactors with PEG₃₃₅₀ and without PEG at the same enzyme concentration.

—◆— Without PEG

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h

Figure 4.2.1: Effect of Laccase (SP-504) Concentration on DPA Conversion

—◆— With PEG₄₀₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
PEG Concentration	:	200 mg/L
Molecular Mass of PEG	:	400 g/ mole

Figure 4.2.2: Effect of Laccase (SP-504) Concentration in DPA Conversion in the Presence of PEG₄₀₀

—◆— With PEG₁₄₅₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
PEG Concentration	:	200 mg/L
Molecular Mass of PEG	:	1450 g/ mole

Figure 4.2.3: Effect of Laccase (SP-504) Concentration in DPA Conversion in the Presence of PEG₁₄₅₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
PEG Concentration	:	200 mg/L
Molecular Mass of PEG	:	3350 g/ mole

Figure 4.2.4: Effect of Laccase (SP-504) Concentration in DPA Conversion in the Presence of PEG₃₃₅₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
PEG Concentration	:	200 mg/L
Molecular Mass of PEG	:	8000 g/ mole

Figure 4.2.5: Effect of Laccase (SP-504) Concentration in DPA Conversion in the Presence of PEG₈₀₀₀

—◆— With PEG₃₅₀₀₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
PEG Concentration	:	200 mg/L
Molecular Mass of PEG	:	35000 g/ mole

Figure 4.2.6: Effect of Laccase (SP-504) Concentration in DPA Conversion in the Presence of PEG₃₅₀₀₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
PEG Concentration	:	200 mg/L
Molecular Mass of PEG	:	3350 g/ mole

Figure 4.2.7: Comparison between the Conversion Efficiency of Laccase (SP-504) in the Presence of PEG₃₃₅₀ and in the absence of PEG.

Table 4.2.1 : Optimum Enzyme Concentration

PEG Used	Optimum Enzyme Concentration (U/mL)
Without PEG	0.0075
PEG ₄₀₀	0.0075
PEG ₁₄₅₀	0.0040
PEG ₃₅₀₀	0.0035
PEG ₈₀₀₀	0.0032
PEG ₃₅₀₀₀	0.0020

4.3 Effect of Molecular Mass and Concentration of Polyethylene Glycol (PEG)

The effectiveness of PEG is a function of its concentration and molecular mass (Kinsley and Nicell, 1999). PEG₄₀₀, PEG₁₄₅₀, PEG₃₅₀₀, PEG₈₀₀₀ and PEG₃₅₀₀₀ were used in the batch reactors to determine the effect of molecular mass of PEG. These batch reactors were set up at the optimum pH and with optimum enzyme concentration obtained earlier. PEG concentration varied from 20mg/L to 240 mg/L for all molecular masses of PEG. Initial substrate concentration in all the batch reactors was 0.19 mM and a three-hour reaction period was chosen for the study.

Figures 4.3.1 to 4.3.5 represent DPA conversion in the presence of PEG of different molecular masses and different concentrations. These figures show that PEG₄₀₀ did not

improve the conversion of DPA. The enzyme required for this particular PEG was same as that needed when PEG was not used. However, when higher molecular mass PEG was used, the enzyme requirement decreased with an increase in mass. This suggests that PEG₄₀₀ does not have any protective effect on the enzyme. This result agrees with the observation of Nakamoto and Machida (1992) who observed that PEG of molecular mass 400 or less had no protective effect on the enzyme and the protective effect increased with increasing the molecular mass above 600.

With PEGs of molecular mass 1450 g/mol and above, it was observed that its addition improved the conversion efficiency up to the optimum additive concentration. Beyond this optimum concentration, excess PEG neither increased nor decreased the removal efficiency. The results also suggest that a higher molecular mass of PEG provided greater protection to the enzyme in lower concentrations, i.e. with the increase in molecular mass of PEG, both the optimum enzyme concentration needed was lower and optimum PEG concentration needed was lower as well. These findings match the observation of Nicell et al., (1995) who found that higher molecular mass PEG provided the greatest protection of enzyme with the lowest concentrations of PEG.

→ With PEG₁₄₅₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Molecular Mass of PEG	:	1450 g/mole
Enzyme Concentration	:	0.004 U/mL

Figure 4.3.1: Effect of PEG₁₄₅₀ in DPA Conversion

—◆— With PEG₃₃₅₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
Molecular Mass of PEG	:	3350 g/mole
Enzyme Concentration	:	0.0035 U/mL

Figure 4.3.2: Effect of PEG₃₃₅₀ in DPA Conversion

◆ With PEG₈₀₀₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
Molecular Mass of PEG	:	8000 g/mole
Enzyme Concentration	:	0.0032 U/mL

Figure 4.3.3: Effect of PEG₈₀₀₀ in DPA Conversion

◆ With PEG₃₅₀₀₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 hours
Molecular Mass of PEG	:	35000 g/ mole
Enzyme Concentration	:	0.002 U/mL

Figure 4.3.4: Effect of PEG₃₅₀₀₀ in DPA Conversion

4.4 Fate of PEG

Although PEG is a non-toxic compound considered to be fit for human consumption (Harris, 1992), it has an environmental impact because of its oxygen demand (Nakamoto and Machida, 1992). It has been observed that the benefits of PEG addition level off after the threshold concentration and the amount of PEG above the threshold concentration remains in the solution (Modaressi et al., 2005; Wu et al., 1998). The threshold PEG concentration is defined by the minimum effective PEG concentration to achieve certain amount of removal at a particular enzyme and substrate concentration. TOC measurement was used to estimate the PEG remaining in the solution.

Batch reactors were set up with varying concentrations of each PEG type. Different concentrations of PEG₄₀₀, PEG₁₄₅₀, PEG₃₅₀₀, PEG₈₀₀₀ and PEG₃₅₀₀₀ were used for this study. Three-hour reactions were carried out with excess enzyme to ensure complete conversion of DPA. Triplicate samples were filtered and used for TOC measurement after a three-hour reaction period.

Separate standard curves were prepared for DPA and for each type of PEG involved. The TOC contributed by DPA was calculated by using the standard curve. The difference between the measured TOC of the samples and TOC caused by remaining DPA was used to calculate the TOC due to PEG remaining in the samples.

Figures 4.4.1 to figure 4.4.5 show the TOC results for different types of PEG at different concentrations. It can be seen that PEG₄₀₀ did not contribute to the system and remained in the solution. But for PEGs of higher molecular mass, the amount of PEG consumed

increased with increasing PEG concentration and reached a threshold value. Excess PEG remained in the solution beyond this threshold value.

Different threshold values were obtained with different molecular masses of PEG. It is noted that, with an increase in molecular mass, a lower PEG concentration was required to reach the threshold value. The correlation between the molecular mass of PEG and threshold PEG concentration needed to achieve 95% DPA conversion is presented in Figure 4.4.6. The threshold values for each PEG type are summarized in table 4.4.1.

▲ TOC Standard Curve for PEG₄₀₀

—■— Samples With PEG₄₀₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Molecular Mass of PEG	:	400 g/mole

Figure 4.4.1: Fate of PEG₄₀₀ in DPA Conversion

▲ TOC Standard Curve for PEG₁₄₅₀

■ Samples With PEG₁₄₅₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Molecular Mass of PEG	:	1450 g/mole

Figure 4.4.2: Fate of PEG₁₄₅₀ in DPA Conversion

◆ TOC Standard Curve for PEG₃₃₅₀

■ Samples With PEG₃₃₅₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Molecular Mass of PEG	:	3350 g/mole

Figure 4.4.3: Fate of PEG₃₃₅₀ in DPA Conversion

◆ TOC Standard Curve for PEG₈₀₀₀
 ■ Samples With PEG₈₀₀₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Molecular Mass of PEG	:	8000 g/mole

Figure 4.4.4: Fate of PEG₈₀₀₀ in DPA Conversion

◆ TOC Standard Curve for PEG₃₅₀₀₀
 ■ Samples With PEG₃₅₀₀₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Molecular Mass of PEG	:	35000 g/mole

Figure 4.4.5: Fate of PEG₃₅₀₀₀ in DPA Conversion

◆ Threshold Concentration of PEG

— Trendline

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h

Figure 4.4.6: Co-relation between Threshold PEG Concentration and Molecular Mass in DPA Conversion

Table 4.4.1 : Threshold PEG Concentration

PEG Used	Threshold PEG Concentration (mg/L)
PEG ₄₀₀	N/A
PEG ₁₄₅₀	90
PEG ₃₅₀₀	80
PEG ₈₀₀₀	55
PEG ₃₅₀₀₀	40

4.5 Laccase Stability under Reactor Conditions

The enzymatic reactions in this study were mostly conducted for three hours. It is known that laccase can undergo inactivation if it is exposed to high temperatures for a long time. Denaturation of enzymes can also result from fast mixing in the batch reactors. To check enzyme stability over time in the presence of mixing, enzyme activity was monitored over a three-hour period at room temperature without adding the substrate. Laccase was added in buffered water and mixing was provided with stirring bars for a three-hour period. As optimum pH for conversion of DPA was found to be 7.0, laccase was incubated with buffered water which had a pH of 7.0. After the appropriate time intervals, samples were withdrawn and activity was measured by using the standard activity test.

The results are presented in Figure 4.5.1. There was about 3% loss in enzyme activity over time for the laccase sample having initial activity of 0.0075 LACU/mL. But the samples having initial activity of 0.0035 LACU/mL lost about 6.5% of activity over a three hour period. These laccase concentrations were chosen based on the optimum enzyme concentration for DPA conversion without PEG and with PEG₃₃₅₀, respectively. These are not considered substantial losses and thus, laccase is considered to be stable at room temperature and the mixing speeds at which the reactions took place.

—●— Enzyme Activity Starting at 0.0075 LACU/mL

—■— Enzyme Activity Starting at 0.0035 LACU/mL

Conditions		
Reactor Volume	:	100 mL
Observation Time	:	3 h
Temperature	:	Room Temperature (21°C)

Figure 4.5.1: Stability of Laccase Over time

4.6 Additive Effect on Enzyme Activity

Two sets of experiments were designed to monitor the additive effect on enzyme activity. In the first set of experiments, laccase activity was measured both in the presence and absence of PEG₃₅₀₀ without the enzymatic reaction. In second set of experiments, enzyme activity was monitored over time with the enzymatic reaction.

4.6.1 Laccase Activity as a Function of Incubation Time

To investigate the effect of PEG on laccase activity in the absence of substrate, laccase was pre-incubated with PEG for different time intervals. A high concentration (400 mg/L) of PEG₃₅₀₀ was used to incubate 0.0066 U/mL of laccase SP-504. This enzyme concentration was chosen based on the dilution requirements to obtain good absorbance values for standard laccase activity test.

Laccase activity was measured as a function of incubation time. After the incubation period, samples were taken in triplicate and were diluted ten-fold and used in the standard activity test. The results are presented in Figure 4.6.1.1. Average activities are expressed as percentage increase over control (without PEG).

In the presence of PEG there was about a 10% instantaneous increase in activity. This increasing trend in activity was observed for 20 minutes. After that time, there was no significant increase in activity.

—◆— Activity of Laccase Incubated with 400 mg/L of PEG₃₃₅₀

Reactor Conditions		
Reactor Volume	:	100 mL
Observation Time	:	30 min
Molecular Mass of PEG	:	3500 g/mole
PEG Concentration	:	400 mg/L
Laccase Concentration	:	0.0066 U/mL

Figure 4.6.1.1 : Laccase Activity as a Function of Incubation Time

4.6.2 Laccase Activity as a Function of PEG Concentration

To determine the effect of PEG concentration on laccase activity in the absence of substrate, the same amount of laccase was pre-incubated for 20 minutes with different concentrations of PEG₃₅₀₀. A 20-minute incubation time was selected based on the results of the previous experiment. Laccase activity as a function of PEG₃₅₀₀ concentration is presented in Figure 4.6.2.1. The results show that, with increase in PEG concentration, laccase activity increased by as high as much as 25%. This increasing trend in activity is similar to that observed by Modaressi et al., (2005).

—●— Activity of Laccase Incubated with PEG₃₃₅₀

Reactor Conditions		
Reactor Volume	:	100 mL
Observation Time	:	30 minutes
Molecular Mass of PEG	:	3500 g/mole
PEG Concentration	:	400 mg/L
Laccase Concentration	:	0.0066 U/mL

Figure 4.6.2.1 : Laccase Activity as a Function of Incubation Concentration of PEG

4.6.3 Effect of PEG on Enzyme Activity during Enzymatic Reaction

To investigate the effect of PEG on enzyme activity during enzymatic reaction, enzyme activity was monitored over a three-hour reaction period under optimum reactor conditions in the presence and absence of PEG₃₅₀₀.

Figure 4.6.3.1 represents the enzyme activity test results during enzymatic reaction. It is observed that, in the presence of PEG, after a three-hour reaction period, about 20% of the initial activity was still remaining, whereas without PEG, after a three-hour reaction period, about 10% of the initial enzyme activity was remaining. As the batch reactors were run under optimum reaction conditions, the amounts of enzyme used in the batch reactors were different.

Both in the presence and absence of PEG, there was a substantial drop in enzyme activity after a one-hour reaction period. But the activity drop was higher in case of samples with no PEG. This could be due to reaction product inactivation. During the laccase-catalyzed oxidation of DPA, radicals are generated which undergo non-enzymatic coupling to form polymers and oligomers. The polymers which are formed after a certain reaction period have an affinity to attach to the enzyme, causing enzyme inactivation and resulting in a drop in enzyme activity. It is hypothesized that PEG attaches to the polymers as they form. Gombotz et al., (1992) demonstrated that PEG attached to a surface has the capability to reject proteins. Considering this fact, one rationale is that PEG attached to the polymeric end products is capable of repelling the enzyme. Thus, inactivation resulting from the adsorption of the enzyme to the polymeric end product could be reduced (Kinsley and Nicell, 2000).

◆ With PEG₃₃₅₀ (Enzyme concentration 0.0035 U/mL)

■ Without PEG (Enzyme concentration 0.0075 U/mL)

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Molecular Mass of PEG	:	3500 g/mole
PEG Concentration	:	80 mg/L

Figure 4.6.3.1: Laccase (SP-504) Activity during DPA conversion in Presence of PEG₃₅₀₀ and in absence of PEG.

4.7 Progress Curve for DPA Removal

Progress curves of percent DPA remaining for 0.19 mM substrate concentration were determined under optimum reactor conditions with and without PEG₃₃₅₀ over a three-hour reaction period. 100 mL batch reactors were run in triplicate to ensure consistency of the results. After selected time intervals, 0.9 mL samples were withdrawn from the batch reactors and 0.1 mL of 50 mM HCl was added to each sample to stop the reaction. Then, it was filtered and the filtrate was used for HPLC analysis to determine the percent removal.

These experimental data points were fitted with exponential curves as shown in Figures 4.7.1 and 4.7.2. The data points collected from the samples having PEG were more consistent with the fitted curve than those without PEG.

In order to obtain kinetic parameters for DPA removal, another set of experiments was run in triplicate to determine the initial rates of DPA removal both in the presence and absence of PEG.

Due to the low solubility of DPA in water and inaccurate HPLC data at lower substrate concentrations, the initial rates of DPA removal were determined only for two substrate concentrations, 0.16mM and 0.19mM. 100 mL batch reactors were set up in triplicate both in the presence and absence of PEG. The results are shown in Figures 4.7.3 to 4.7.7. The initial velocity for both the substrate concentrations is presented in Table 4.7.1.

— Trendline
 ◆ Without PEG

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Enzyme concentration	:	0.0075 U/mL

Figure 4.7.1: Progress Curve for DPA Removal Without PEG

— Trendline
 ◆ With PEG₃₃₅₀

Reactor Conditions		
pH	:	7.0
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
Buffer	:	50 mM
Reaction Time	:	3 h
Enzyme concentration	:	0.0035 U/mL

Figure 4.7.2: Progress Curve for DPA Removal in the Presence of PEG₃₃₅₀

◆ 0.19 mM Initial Substrate Concentration

■ 0.16 mM Initial Substrate Concentration

Reactor Conditions			
pH	:	7.0	
Reactor Volume	:	100 mL	
Buffer	:	50 mM	
Enzyme concentration	:	0.0025 U/mL	

Figure 4.7.3: Progress Curve for First 20 Minutes of Reaction Without PEG

◆ 0.19 mM Initial Substrate Concentration

■ 0.16 mM Initial Substrate Concentration

Reactor Conditions		
pH	:	7.0
Reactor Volume	:	100 mL
Buffer	:	50 mM
PEG Concentration	:	90 mg/L
Enzyme concentration	:	0.0025 U/mL

Figure 4.7.4: Progress Curve for First 20 Minutes of Reaction With PEG₁₄₅₀

◆ 0.19 mM Initial Substrate Concentration

■ 0.16 mM Initial Substrate Concentration

Reactor Conditions		
pH	:	7.0
Reactor Volume	:	100 mL
Buffer	:	50 mM
PEG Concentration	:	80 mg/L
Enzyme concentration	:	0.0025 U/mL

Figure 4.7.5: Progress Curve for First 20 Minutes of Reaction With PEG₃₃₅₀

◆ 0.19 mM Initial Substrate Concentration

■ 0.16 mM Initial Substrate Concentration

Reactor Conditions		
pH	:	7.0
Reactor Volume	:	100 mL
Buffer	:	50 mM
PEG Concentration	:	55 mg/L
Enzyme concentration	:	0.0025 U/mL

Figure 4.7.6: Progress Curve for First 20 Minutes of Reaction With PEG₈₀₀₀

◆ 0.19 mM Initial Substrate Concentration

■ 0.16 mM Initial Substrate Concentration

Reactor Conditions		
pH	:	7.0
Reactor Volume	:	100 mL
Buffer	:	50 mM
PEG Concentration	:	40 mg/L
Enzyme concentration	:	0.0025 U/mL

Figure 4.7.7: Progress Curve for First 20 Minutes of Reaction With PEG₃₅₀₀₀

Table 4.7.1: Initial Rate of Reaction (mM/ min)

Initial Substrate Concentration (mM) \ PEG Molecular Mass (g/mol)	Without PEG	PEG ₁₄₅₀	PEG ₃₃₅₀	PEG ₈₀₀₀	PEG ₃₅₀₀₀
0.16	0.0020	0.0021	0.0022	0.0025	0.0029
0.19	0.0018	0.0020	0.0021	0.0023	0.0027

4.8 Color Removal

Products resulting from enzymatic treatment of DPA were soluble in water and resulted in a yellow-colored solution. This solution showed a peak at 440 nm and the absorbance of the solution at that wavelength was 0.537 at room temperature and neutral pH. This was considered as 100% color. This color is not acceptable for effluent discharge. Common coagulation and precipitation aids like alum, PEI, anionic, nonionic or cationic polymers were applied separately or in combination to remove the reaction end-products from the solution, but none of these were able to remove the color. Hence, surfactant-mediated separation was adopted to remove the unacceptable color by using both alum and sodium lauryl sulfate (SDS). This technique is known as adsorptive micellar flocculation (AMF). The effectiveness of a color removal process depends on factors like coagulating agent, coagulant concentration, pH, etc. (Randtke, 1988). Experiments were carried out to identify the optimum parameters for effective removal of the colored products.

4.8.1 Optimum pH

SDS was added at a concentration of 200 mg/L (≈ 0.7 mM) to the batch reactors having reaction end products. Fast mixing was provided to the system and, after five minutes, 150 mg/L (≈ 0.4 mM) of alum was added to the solution. The pH of this mixture was adjusted to a desirable pH by adding acid or base. After 10 minutes of rapid mixing, slow mixing was provided and flocs were observed. The floc was allowed to settle for 30 minutes, the mixture was filtered and the filtrate was used for spectrophotometric analysis.

The absorbance of the filtrate was measured at 440 nm and was compared with the original reaction end product absorbance, which was considered as 100%. The results are shown in Figure 4.8.1. From pH 3.2 to pH 6.6, color removed was more than 95% of the initial color. Therefore, this range of pH is considered as the optimum pH based on 95% color removal. The best result was obtained at pH 3.4 when all the color was removed. Below this pH, the filtrate was not clear and this may have resulted from re-solubilization of the micelles formed.

4.8.2 Optimum Coagulant Concentration

For optimal coagulant concentration at optimum pH, a set of batch reactors was set up by using varying SDS concentration between 0.5 mM (144 mg/L) and 1mM (288 mg/L) and also aluminum ion molar ratio. In each set of experiments, SDS concentration was kept constant while the aluminum ion concentration was varied. It was observed that batch reactors having 200mg/L (0.7mM) SDS were performing better than other SDS

concentrations. For that SDS concentration, the optimum SDS to Al^{+3} molar ratio was observed to be 0.65. The results are shown in Figure 4.8.2.

At this concentration and at optimum pH, the supernatants after settling were filtered. The coarse filtered samples had only 3% color remaining and microfiltered samples were colorless. Results are shown in Figure 4.8.3.

—◆— Color Removal

Reactor Conditions

Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
SDS Concentration	:	200 mg/L (≈ 0.7 mM)
Alum Concentration	:	150 mg/L (≈ 0.4 mM)

Figure 4.8.1 : pH Optimization for Color Removal Process

Reactor Conditions		
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
SDS Concentration	:	200 mg/L (0.7 mM)

Figure 4.8.2 : Coagulant Concentration Optimization for Color Removal Process

Reactor Conditions		
Initial Substrate Concentration	:	0.19 mM
Reactor Volume	:	20 mL
SDS Concentration	:	200 mg/L (0.7 mM)
Alum Concentration	:	154 mg/L (0.9 mM Al ⁺³)

Figure 4.8.3: Comparison between Coarse-filtered and Micro-filtered Samples at Optimum Coagulant Concentration at Varying pH

4.9 Possible Mechanism for DPA Radical Coupling

Enzymatic treatment is an alternative oxidation method to remove aromatic compounds from water. Aromatic compounds are converted to radicals as a result of this process. These radicals undergo non-enzymatic coupling to produce dimers and subsequently, oligomers. Some of these dimers and oligomers are substrates of the enzyme and are, in turn, converted to radicals which couple non-enzymatically to generate oligomers and polymers. This process continues until the polymers reach their solubility limit or they are no longer substrates of the enzyme. This study demonstrates oxidative polymerization of diphenylamine using laccase. Based on the literature survey, possible products generated from the enzymatic polymerization of DPA are predicted.

The T1 site of laccase extracts an electron from the substrate DPA in laccase-catalyzed DPA removal. The radical generated from DPA oxidation can have three possible resonance structures, shown in Figure 4.9.1. (Pankratov et al., 1984). Lui and Lund (2005) have optimized the geometry of $\text{Ph}_2(\text{H})\text{N}^+$ by using by the B3LYP/6-31 + G(d,p) computational method (Figure 4.9.2).

DPA polymerization was anticipated to be similar to that of aniline or N-substituted anilines as they consist of aromatic structure and amine group. However, studies show that mechanism of polymerization of DPA is different from other N-substituted anilines (Nagarajan et al., 2005).

Figure 4.9.1: Radical Generation from DPA Oxidation

Figure 4.9.2: Geometry Optimization of $\text{Ph}_2(\text{H})\text{N}^+$ (Liu and Lund, 2005).
(The unit of bond length is Å)

Laha and Luthy, (1990) have studied the oxidation mechanism for aniline. According to their study, aniline loses one electron as a result of the oxidation process to form a cation radical. This cation radical can have three possible resonance structures (Figure 4.9.). These three resonance structures of the aniline radical can provide coupling sites at nitrogen or at the ortho- and para- positions of the ring. But radical coupling was not noticed in the ortho- position of the ring (Sharma et al, 1982).

Figure 4.9.3: Radical Generated from Oxidation of Aniline (Laha and Luthy, 1990)

Aniline cation radical can undergo coupling through the possible radical structures generating p-aminodiphenylamine, benzidine, and hydrazobenzene as intermediate products (Figure 4.9.). These intermediate products are more readily oxidized than parent substrate (Laha and Luthy, 1990; Hambitzer and Stassen, 1993). Oxidation of these intermediates generates azobenzene as a stable end product. Polymerization of aniline and N-substituted anilines proceed through C-C, N-N and N-C coupling (Nagarajan et al., 2005).

Figure 4.9.4: Products Generated from Aniline Radical Coupling

However, DPA polymerization proceeds through 4,4'-phenyl-phenyl coupling mechanism and shows no N-C coupling (Nagarajan et al., 2005). Two DPA radicals couple to generate diphenylbenzidine (Figure 4.9.). Diphenylbenzidine can further get oxidized and form diphenylbenzidine radical (Nagarajan et al., 2005). This radical can undergo further polymerization and form poly-DPA.

Figure 4.9.5: DPA Radical Coupling

4.10 Error Estimation

Two sets of statistical experiments were carried out to estimate the errors due to human, equipment and other factors. Error analysis was done both for short term and long term data. Data collected over a short term was collected within few minutes interval in the same day. Error analysis on the short-term data will mostly account for human errors and instrumental errors. Long term data was collected on different days having a few months' interval. Error analysis on long-term data will include variation due to human error, instrumental reliability and chemical stability.

These experiments were carried out for enzyme activity test, absorbance measured for color removal and TOC data. In all these cases, first experimental data set represents short-term data and the second set of data consists of long-term data.

4.10.1 Error Analysis on Enzyme Activity Test

The first set of statistical experiments was run for enzyme activity test and the data in Table 4.10.1 was collected within a short time interval. The variations in data are really negligible. The standard deviation estimated is 0.62% of the observed mean value. The 95% confidence interval is 0.0649 ± 0.0011 ("t" statistics with degree of freedom= 5) of observed value.

For the second set of statistical experiments run on the enzyme activity test, data were collected over a longer span of time and the data collected are presented in Table 4.10.2. The standard deviation (including all human and spectrophotometer factors) was estimated as 0.000792 and is only 1.2 % of the observed mean value. The 95% confidence interval is 0.06534 ± 0.0023 ("t" statistics with degree of freedom= 5) of observed value.

Table 4.10.1: Error Analysis for Enzyme Activity Test over Small Time Interval

Sample Number	Time	Enzyme Activity (LACU/mL)	Mean (LACU/mL)	Standard Deviation
1	10:10	0.0651	0.0649	0.62%
2	10:11	0.0642		
3	10:12	0.0654		
4	10:13	0.0647		
5	10:14	0.0647		
6	10:15	0.0651		

Table 4.10.2: Error Analysis for Enzyme Activity Test over Long Time Span

Sample Number	Time	Enzyme Activity (LACU/mL)	Mean (LACU/mL)	Standard Deviation
1	Aug	0.0651	0.0654	1.2 %
2	Aug	0.0642		
3	Aug	0.0654		
4	Feb	0.0663		
5	Feb	0.0650		
6	Feb	0.0662		

4.10.2 Error Analysis on Direct Absorbance Measurement for Color Removal

Statistical analysis was done on the absorbance measured for color removal processes.

For shorter periods of time, the data in Table 4.10.3 show smaller variations. The observed values show a standard deviation of 0.000409 which is 2.4% of the observed mean value. The 95% confidence interval is 0.0169 ± 0.0012 ("t" statistics with degree of freedom= 5) of observed value.

Absorbance was measured over a longer time span after coagulation, precipitation and filtration. The data collected are presented in Table 4.10.4. The standard deviation was estimated as 0.000576. This value is only 3.4 % of the observed mean value. The 95% confidence interval is 0.0171 ± 0.0016 ("t" statistics with degree of freedom= 5) of observed value.

Table 4.10.3 : Error Analysis for Color Absorbance for Small Time Interval

Sample Number	Time	Absorbance at 440 nM	Mean Absorbance at 440 nM	Standard Deviation
1	12:15	0.0175	0.0169	2.4 %
2	12:16	0.0172		
3	12:17	0.0169		
4	12:18	0.0170		
5	12:19	0.0168		
6	12:20	0.0163		

Table 4.10.4 : Error Analysis for Color Absorbance over Longer Time Span

Sample Number	Time	Absorbance at 440 nM	Mean Absorbance at 440 nM	Standard Deviation
1	July	0.0175	0.0171	2.4%
2	July	0.0179		
3	July	0.0172		
4	Sep	0.0166		
5	Sep	0.0169		
6	Sep	0.0163		

4.10.3 Error Analysis on TOC data

Error analysis was also performed for the TOC data. It was observed that data collected over a short time span (Table 4.10.5) had a standard deviation of 0.36 which is only 1.2% of the mean value. This deviation accounted for factors like human error, etc. The 95% confidence interval is 30.24 ± 1.03 (“t”statistics with degree of freedom= 5) of observed value.

When TOC data were collected over a longer time span the standard deviation observed was 0.48 over the observed data as shown in Table 4.10.6. The observed standard deviation is 1.6% of the mean value. The 95% confidence interval is 30.32 ± 1.36 (“t”statistics with degree of freedom= 5) of observed value.

Table 4.10.5 : Error Analysis for TOC of DPA over Short Time Span

Sample Number	Time	TOC of 0.2mM DPA (mg/L)	Mean TOC (mg/L)	Standard Deviation
1	3:30	29.99	30.24	3.4 %
2	3:45	29.96		
3	4:00	30.26		
4	4:15	30.78		
5	4:30	30.55		
6	4:45	29.87		

Table 4.10.6 : Error Analysis for TOC of DPA over Long Time Span

Sample Number	Time	TOC of 0.2mM DPA (mg/L)	Mean TOC (mg/L)	Standard Deviation
1	Nov	29.86	30.32	1.2 %
2	Nov	29.69		
3	Nov	30.26		
4	Mar	30.53		
5	Mar	30.97		
6	Mar	30.62		

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The results of this study demonstrate the applicability of using laccase to treat waste water contaminated with diphenylamine. The reaction parameters optimized to achieve a removal efficiency of at least 95%, were pH, laccase concentration in the presence and absence of PEG and PEG concentration. Effects of molecular weight and concentration of PEG on pH and enzyme concentration were observed. Stability of laccase activity at room temperature over time was determined. Laccase activity was also monitored for incubation time with PEG and incubation concentration of PEG.

The study established the following:

- The optimum pH for DPA conversion was determined to be 7.0. Stringent condition were used to determine the optimum pH. Addition of PEG did not change the optimum pH. There was no effect of molecular weight of PEG on optimum pH.
- Optimum enzyme concentration for DPA conversion was more when PEG was not used in the batch reactors, than when PEG was used. But PEG₄₀₀ was an exception to this.
- Addition of PEG had an effect on optimum enzyme required to achieve 95% of initial DPA concentration. It was observed that the optimum enzyme requirement in the presence of PEG was a function of molecular weight of PEG. With increase in

molecular weight of PEG, optimum enzyme concentration to achieve the same amount of substrate conversion decreased. But PEG₄₀₀ was an exception to this as well.

- A threshold concentration of PEG was sufficient to achieve the maximum substrate conversion. An excess of PEG neither increased nor decreased the conversion efficiency. TOC results show that, any amount exceeding the threshold PEG concentration remained in the solution. PEG₄₀₀ did not contribute to the removal efficiency and totally remained in solution.
- Laccase was stable for the duration of all the experiments conducted.
- Laccase activity was a function of incubation time with PEG. It was observed that laccase activity had an instantaneous 10% increase when incubated with PEG. Significant increase in activity was observed till 20 minute of incubation time, to about 25%.
- Laccase activity increased with an increase of PEG concentration.
- Alum, PEI, cationic, anionic and non ionic polymers individually were unable to remove the colored solution generated from enzyme treatment. PEI and above mentioned polymers, in presence of alum were not able to remove the color as well.
- Adsorptive micellar flocculation was used to remove the colored enzyme treatment products. SDS and alum was used for this process. Optimum SDS concentration for color removal was 0.7 mM with presence of 0.45 mM Al³⁺. Optimum pH for this process had a broad range of 3.0 to 6.5. But at a pH of 3.4 100% color was removed. Below this pH, the solution was not clear due to dissolving of micelles into the solution.

- Experiments were carried out to determine the kinetic parameters of enzymatic removal of DPA in presence and absence of PEG. But the experiments were not successful due to very low solubility of DPA in water.

5.2 Recommendations

The results of this experiment show that laccase is a viable alternative to remove recalcitrant chemical, DPA. In order to implement the enzymatic method of treatment to full scale industrial application, several other factors must be considered.

- The nature of the end-products resulting from this process must be determined. Knowledge on potential toxicity of the end products could be useful for determining a suitable sludge disposal method.
- Initial substrate concentration in this study was low due to the solubility limit of DPA in water at room temperature. But, industrial effluent has been reported to have higher concentrations of DPA (Murin et al., 1997). At this concentration, the conventional biological treatment processes were unable to remove DPA up to UTS limit (Murin et al., 1997). A real industrial wastewater matrix should be treated to check the effectiveness of the laccase-catalyzed removal. Other components present in the waste water stream can contribute or interfere with DPA conversion.
- As AMF is used in color removal process, SDS and alum are introduced to the system. SDS is a surfactant and can be reused if recycled. In real industrial implementation, recycling of SDS should be considered to optimize the cost of the system and to generate less waste.

- A cost analysis should be carried out to determine the economic effectiveness of laccase catalyzed removal over conventional treatment process.

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APPENDICES

APPENDIX A

Laccase Activity Assay

1. General:

The purpose of 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. Increase in absorbance at 530 nm determined the enzyme activity.

2. Reagents

I. 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

II. 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

3. Procedure

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

850 μ L MES buffer
50 μ L Syringaldazine solution
100 μ L 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.

4. Calculation

$$\begin{aligned}\text{Activity in the cuvette (LACU/mL)} &= (\Delta A * 1.0\text{mL} * 10^{-3} * D) / (0.065 * 0.1\text{mL}) \\ &= \Delta A * 1.538 * D\end{aligned}$$

Where,

$$\Delta A = \text{Change in absorbance per minute} = A_{75\text{ s}} - A_{15\text{ s}}$$

(Range of absorbance should be, 0.1 to 0.4 $\Delta A/\text{min}$)

$$1.0 = \text{Total volume in the cuvette (mL)}$$

$$0.065 = \text{Micro-molar extinction coefficient } (\mu\text{M/L})$$

$$10^{-3} = \text{Conversion factor for LACU/mL to LACU/L}$$

$$D = \text{Dilution factor}$$

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

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

$$\begin{aligned}&= \frac{\text{Activity in the cuvette (LACU/mL)} * \text{reactor volume (mL)}}{\text{enzyme solution added to the reactor (mL)}}\end{aligned}$$

APPENDIX B

Standard Curve for DPA

1.General

HPLC was used to identify and quantify DPA. A standard curve was prepared which was used to determine the concentration of DPA in the reaction end-product.

1.1 Preparation of HPLC Standard Curve

Different known concentrations of DPA were prepared varying from 0.05 to 0.2 mM after proper dilution. The samples were run in HPLC for 10 minutes. Peak areas were measured for these concentrations of DPA at 280 nm (80:20 = methanol: distilled water was used for elution). The peak area vs. concentration was plotted to get the standard curve for DPA.

◆ 280 nm

— Linear (280nm)

Figure B.1: HPLC Standard Curve for DPA with Methanol

SAMPLE INFORMATION			
Sample Name:	DPA	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	07/07/05 4:54:23 AM
Vial:	56	Acquired Method:	80% A 20% B
Injection #:	1	Date Processed:	07/07/05 10:06:45 AM
Injection Volume:	10.00 μ l	Run Time:	10.00 Minutes

Figure B.2: Chromatogram of DPA (4.004 min) observed at 280 nm

APPENDIX C

Unsuccessful Color Removal Processes

Alum, PEI, cationic, anionic and non-ionic polymers individually were unable to remove the colored solution generated from enzyme treatment. PEI and the above-mentioned polymers, in the presence of alum were not able to remove the color as well. The properties of the polymers used for color removal are presented in Table C.1.

Table C.1: Properties of Polymers Used as Coagulant Aid

Commercial Name	Polymer Number	Property
Magnafloc	120L	Anionic, Low charge.
Magnafloc	110L	Anionic, Medium charge.
Zetag	7873	Cationic, Low charge.
Zetag	7875	Cationic, Medium charge.
Zetag	7888	Cationic, High charge.

For both anionic polymers, the maximum color removal efficiency achieved was around 3% (Figure C.1 and C.2). At higher concentration of these anionic polymers, the solution became turbid; hence the direct absorbance value obtained at 442 nm by spectrophotometric analysis was higher.

Maximum possible color removal using the cationic polymers were about 1% in all the cases (Figure C.3, C.4 and C.5) and at higher concentrations the solutions became turbid. Alum alone could remove approximately 25% of the color (Figure C.6). PEI removed 7% of the color at 250 mg/L concentration (Figure C.7).

150 mg/L of alum was used with various concentrations of Magnafloc (120L), Zetag (7888) and PEI. None of these combinations was successful in achieving good color removal efficiency (Figure C.8, C.9 and C.10).

Figure C.1: Color Removal Using Magnafloc (120 L)

Figure C.2: Color Removal Using Magnafloc (110 L)

Figure C.3: Color Removal Using Zetag 7873

Figure C.4: Color Removal Using Zetag 7875

Figure C.5: Color Removal Using Zetag 7888

Figure C.6: Color Removal Using Alum

Figure C.7: Color Removal Using PEI

Figure C.8: Color Removal Using Alum and Magnafloc (120L)

Figure C.9: Color Removal Using Alum and Zetag 7888

Figure C.10: Color Removal Using Alum and PEI

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