

Enzymatic Biobleaching of Recalcitrant Paper Dyes

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LIST OF ABBREVIATIONS

ABTS	2-2' azinobis (3-ethylbenzthiazoline-6-sulfonate)
cs	Consistency (wt% pulp in slurry)
FAS	Formamidine sulfinic acid
HBT	1-Hydroxybenzotriazole
HRP	Horseradish Peroxidase
LMS	Laccase-mediator system
o.d.	Oven-Dried
VA	Violuric acid
VtA	Veratryl Alcohol
SBP	Soybean Peroxidase

1 ABSTRACT

Modern manufacturing processes assume efficient utilization and recycling of natural resources whenever possible. Over the past decade paper recycling has progressed from 33.5% in 1990 to just above 48% in 2002.¹ Indeed, for certain select grades, (newspaper and old corrugated containers) greater than 70% is currently being recycled. In contrast, mixed office waste and colored directory papers are often underutilized. A major difficulty in recycling these grades of paper is the problems associated with decolorizing the dyes present in the paper.² Of the commonly used paper dyes, the stilbene dye Direct Yellow 11³ and methine dye Basazol 46L are notorious⁴ for poor bleachability with the commonly used chemical bleaching agents including chlorine dioxide, oxygen, hydrogen peroxide and sodium dithionite.

The ability of white-rot fungi to decolorize colored effluents containing textile dyes is currently the subject of intensive research efforts. The secreted enzymes involved in dye decolorization include manganese peroxidase, lignin peroxidase and laccase. Laccase, a lignolytic enzyme, has also been studied for many years for the biobleaching of wood pulps. The ability of laccase to delignify pulp is greatly enhanced by the addition of small molecule mediators such as 2-2' azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT).

This research project focused on applying laccase combined with a mediator to decolorize C.I. Direct Yellow 11 and Basazol 46L. Three mediators were tested: ABTS, HBT and violuric acid. Laccase/ABTS was most effective with 60% of the color being removed. The level of color removal was maintained at 60% even when ABTS concentration was lowered from 5 mM to 0.01 mM. When laccase/1 mM ABTS was applied to Direct Yellow 11 in solution, the majority of color loss occurred within 60 minutes.

The ability of soybean (SBP) and horseradish (HRP) peroxidases and laccase to decolorize Direct Yellow 11 and Basazol 46L in solution was also examined. The results demonstrated that these two recalcitrant dyes could be effectively decolorized by enzymatic treatments by horseradish peroxidase, soybean peroxidase, and laccase with

ABTS as mediator. SBP is effective from pH 4.5 to 8.5. The stilbene dye Direct Yellow 11 responded to both SBP and laccase/ABTS. For the methine dye Basazol 46L, SBP was a more effective treatment than HRP or laccase/ABTS. Basazol 46L responded quickly to SBP treatment with 74% reduction in signal intensity within 5 minutes.

To evaluate the effectiveness of laccase/ABTS treatment, pulp dyed with Direct Yellow 11 and three commercial colored pulps were subjected to seven different bleaching treatments. These treatments consisted of 1)laccase/ABTS; 2)laccase/ABTS followed by alkaline extraction; 3)laccase/ABTS followed by bleaching with sodium dithionite; 4)oxygen bleaching; 5)oxygen bleaching followed by dithionite treatment; 6)alkaline hydrogen peroxide bleaching; and 7)alkaline peroxide bleaching followed by dithionite treatment. The best results were obtained by including reductive bleaching with sodium dithionite. For Direct Yellow 11 dyed pulp, laccase/ABTS followed by dithionite yield comparable reduction in color to oxygen or peroxide followed by dithionite.

2 INTRODUCTION

Many factors converge to spur increases in the recycling of paper. Managers of municipal solid waste systems⁵ prefer recycling because recycling reduces the volume of material they must handle. Paper mills prefer to lower their fiber cost by incorporating as much low cost fiber as the desired product quality will allow. Many consumers set aside their newspapers, magazines and loose papers to be recycled because they wish to help the environment. However, the different types of wastepaper are not equally valuable,

From the viewpoint of wastepaper recyclers, colored ledger paper and mixed office waste contain desirable high-quality chemical pulp fibers. For environmental, landfill management and public policy reasons, the large number of telephone directories printed each year warrant the collection and recycling of outdated directories. However, the dyes in these types of wastepaper complicate their recycling and limit both the recycling of these types of wastepaper and the quality of fiber that can be produced from them.

Paper dyes are highly colored and have a strong affinity to cellulose. Recycling mills often use both oxidative and reductive bleaching stages to remove or reduce the color of wastepaper fibers. Some dyes respond very poorly to the bleaching chemicals. This project focused on two dyes that are considered unbleachable by current methods. One of these dyes is C.I. Direct Yellow 11, which is applied to fine papers with chemical pulps. The other dye is Basazol Yellow 46L (C.I. Basic Yellow 96), which the dye most commonly used to produce yellow directory paper.

White-rot fungi and their lignolytic enzymes such as manganese peroxidase, lignin peroxidase and laccase have been studied for their ability to decolorize textile dyes.⁶ These same enzymes have been scrutinized for delignifying wood pulp. When the ability of laccase to bleach kraft pulps was examined, a minimal delignification response resulted due to the size of the enzyme and its inability to diffuse into the secondary wall of a pulp fiber.⁷ The addition of a small molecule mediator has been found to greatly enhance the ability of laccase to delignify wood pulp. Bourbonnais and Paice⁸ introduced the first mediator, ABTS (2-2' azinobis-(3-ethyl benzthiazodine) in 1990. Their studies

found that a laccase-ABTS system could selectively remove as much as 32% of the residual lignin in both softwood and hardwood kraft pulps. Later, a new class of mediators containing the N-OH moiety was introduced. Call found 1-hydroxybenzotriazole (HBT) to be the most effective one.⁹ With HBT as mediator, delignification levels as high as 60% were obtained for both softwood and hardwood kraft pulps in a single stage.

The goal of this project was to determine whether the laccase-mediator system or peroxidases similar to the peroxidases in lignolytic fungi could effectively decolorize the recalcitrant dyes Direct Yellow 11 and Basazol 46L.

3 LITERATURE REVIEW

First, wastepaper recycling and problems due to color from dyed papers will be discussed. Next, the basics of color and dye chemistry will be examined. Enzymes, their types and requirements will then be discussed. Peroxidase and laccase enzymes will be described followed by a brief review of the laccase-mediator system applied to bleaching virgin pulps. Finally, decolorization of dyes by intact fungi and isolated enzymes will be discussed.

3.1 OVERVIEW OF WASTEPAPER RECYCLING

3.1.1 METHODS

A number of processes are used during paper recycling to remove undesirable material and produce clean fibers. The necessary actions are listed in Table 3-1. To remove large and/or dense contaminants, mechanical screens and centrifugal cleaners are used.

The removal of ink and other small particles can be done by washing and/or floatation deinking. Washing removes hydrophilic particles around 1-100 microns in size.¹⁰ Floatation removes larger, hydrophobic particles, around 10-1000 microns in size. The washing and floatation deinking processes may also be combined.

Table 3-1 Actions Performed During Paper Recycling

Removal of contaminants (ex: sand, plastic, styrofoam)
Repulping/swelling of cellulose fibers
Deinking
Bleaching to remove color (for fine paper products)

In addition to washing and floatation, ink may also be removed enzymatically. Low concentrations of cellulases are used to attack the fiber and release attached ink¹¹ Other enzymes have also been used.¹² Xylanases attack hemicellulose that may be on the fiber surface. Lipases attack fats and can release vegetable oil-based ink from fibers.

To reduce the size of remaining visible ink specks after deinking, a hot dispersion system is also commonly used. To obtain high quality fiber, deinking may be followed by bleaching. To benefit from bleaching, the furnish must contain a low percentage of mechanical pulp.¹³

3.1.2 TYPES OF FURNISH

Wastepaper for recycling is divided into many different grades.¹⁴ The grade classification information (Table 3-2) can include the original use, fiber type, quality, source and materials applied. The numerous grades of wastepaper can be classified into two broad groups: News Grades and High or Ledger Grades (Table 3-3).

Table 3-2 Wastepaper Grade Information According to Garbutt¹⁴

The original use of the wastepaper (News, Computer Printout).
The fiber type contained in the wastepaper (Mixed Groundwood Shavings, Printed Bleached Sulfate Cuttings).
The quality of the wastepaper (Special News, Deink Quality, Sorted White Ledger).
Materials applied to the wastepaper (Coated Groundwood Sections, Coated Book Stock).
The source of the wastepaper (Over-issue News, Hard White Envelope Cuttings).

Table 3-3 Major Wastepaper Categories According to Garbutt¹⁴

1. News Grades – wastepaper grades typically consumed in deinking plants producing deinked pulp for newsprint manufacture – usually high percentage of groundwood
2. High Grade or Ledger Grades – wastepaper grades typically consumed in deinking plants producing deinked pulp for manufacture of printing and writing papers or tissue products – varies considerably in quality but usually has high percentage chemical pulp

Each grade also has a specified maximum percentage of outthrows and prohibitive materials. Prohibitive materials include high-density contaminants, (*e.g.* rocks, tin cans, glass), wood (*e.g.* wood chips, plywood pieces, studs) low density contaminants (*e.g.* styrofoam, rubber bands) and adhesives (*e.g.* pressure sensitive, hot melt and plastic adhesives). The market value of different wastepaper grades depends on a number of factors including the amount of contaminant materials and the fiber quality.

Table 3-4 Market Value Factors for Wastepaper According to Garbutt¹⁴

The optical properties of the fibers in the wastepaper (brightness, color, etc.)
The types of fiber contained in the wastepaper (mechanical fibers, chemical fibers)
The types of materials applied to the fibers contained in the wastepaper

(clay-based coatings, laser inks, etc.)
The presence and amount of various other types of wastepaper.
The presence and amount of contaminant materials in the wastepaper.

3.2 RECYCLING OF COLORED WASTEPAPER

The demand for recycled paper continues to grow. However, a significant amount of high quality wastepaper is contaminated with colored dyes that are difficult to remove.¹⁵ In a 1998 survey of industrial professionals, bleaching and color stripping was identified as the most problematical area.¹⁶

3.2.1 PROCESS OF COLOR STRIPPING AND BLEACHING

Removing colored dyes from wastepaper consists of two processes. (Table 3-5)

Table 3-5 Dye Removal Processes

1. Color stripping – release of attached colored material from the fibers into the solution
2. Bleaching or decolorization – destruction of the dye's ability to absorb visible light

Color stripping allows dyes to be washed away. However, color-stripping alone is usually not enough. Most paper dyes have a high tinctorial strength (high molar absorptivity). Removal by simple dilution and washing would require an excessive amount of water.

Bleaching and decolorization both refer to the destruction of light-absorption capacity. This process is commonly called bleaching when dealing with pulp suspensions. In effluent treatment, the destruction of light-absorption capacity is referred to as decolorization. Decolorization can occur while dye molecules are still associated with the fiber. The fragmentation of large dye molecules can result in the formation of smaller compounds that do not absorb visible light but which are still attached to fiber. Chemicals that improve the brightness of recycled pulp can color-strip, bleach or both.

3.2.2 CURRENT BLEACHING METHODS IN WASTEPAPER RECYCLING

A number of different bleaching agents are commonly used in recycling mills to remove color. (The oxidative potentials for bleaching agents are given in Table 3-6 and the reactions are shown in Table 3-8, page 9). The majority of recycle mills process old

corrugated containers and newsprint so their major color source is chromophores generated from lignin. The color from dyed papers cause problems for recycle mills producing high brightness papers from furnishes that include mixed office waste and directory papers.

Table 3-6 Bleaching Agents for Recycled Fiber

Oxidizing Agents	
D	Chlorine dioxide
H	Sodium hypochlorite
O	Oxygen bleaching
P	Hydrogen peroxide bleaching
Z	Ozone bleaching
Reducing Agents	
FAS	Formamidine sulfinic acid (thiourea dioxide)
Y	Sodium hydrosulfite (dithionite)

Table 3-7 Oxidation Potentials of Bleaching Agents as per Bierman and Kronis¹⁷

Species	Structure	Standard Oxidation Potential (Volts)	Potential Relative to Chlorine (Cl₂ = 1.00)
<i>Acidic solution (or pH is not relevant)</i>			
Ozone	O ₃	2.07	1.52
Permanganate	MnO ₄ ⁻	1.77	1.30
Hypochlorous acid	HOCl	1.63	1.20
Chlorine dioxide	ClO ₂	1.57	1.15
Peroxymonosulfate	HSO ₅ ⁻	1.44	1.06
Chlorine	Cl ₂	1.36	1.00
Peracetic acid	CH ₃ CO ₃ H	1.06	0.78
<i>Basic solution</i>			
Hypochlorite	ClO ⁻	0.89	0.65
Hydrogen peroxide	H ₂ O ₂	0.88	0.65
Oxygen	O ₂	0.41	0.30
<i>Reducing agent</i>			
Hydrosulfite	S ₂ O ₄ ²⁻	-1.12	---

Hypochlorite has been the major bleaching agent in recycling mills using chemical pulps. Sodium hypochlorite is relatively effective and inexpensive. However, the use of hypochlorite is being phased out because hypochlorite bleaching creates chloroform. Chlorine dioxide can replace sodium hypochlorite,¹⁸ but chlorine dioxide

generators are expensive and the pulp can not be labeled 'environmentally friendly' or 'chlorine-free'.⁴

Table 3-8 Reactions of Bleaching Agents as per Bierman and Kronis^{17 a}

Oxidizing Agents				
Chlorine	$\text{Cl}_2 + 2\text{e}^-$	\rightarrow	2Cl^-	$E^\circ = 1.36 \text{ V}$
Chlorine Dioxide	$\text{ClO}_2 + 4\text{H}^+ + 5\text{e}^-$	\rightarrow	$\text{Cl}^- + 2\text{H}_2\text{O}$	$E^\circ = 1.57 \text{ V}$
	<i>pH 4^{b,c}</i>			$E^\circ = 1.38 \text{ V}$
(Na) Hypochlorite	$\text{ClO}^- + \text{H}_2\text{O} + 2\text{e}^-$	\rightarrow	$\text{Cl}^- + 2\text{OH}^-$	$E^\circ = 0.89 \text{ V}$
Oxygen (<i>basic</i>) ^b	$\text{O}_2 + 2\text{H}_2\text{O} + 4\text{e}^-$	\rightarrow	4OH^-	$E^\circ = 0.41 \text{ V}$
Oxygen (<i>acidic</i>)	$\text{O}_2 + 4\text{H}^+ + 4\text{e}^-$	\rightarrow	$2\text{H}_2\text{O}$	$E^\circ = 1.230 \text{ V}$
Ozone (<i>acidic</i>) ^b	$\text{O}_3 + 2\text{H}^+ + 2\text{e}^- + \text{O}_2$	\rightarrow	H_2O	$E^\circ = 2.07 \text{ V}$
Ozone (<i>basic</i>)	$\text{O}_2 + \text{H}_2\text{O} + 2\text{e}^-$	\rightarrow	$\text{O}_2 + 2\text{OH}^-$	$E^\circ = 1.24 \text{ V}$
Hydrogen peroxide				
	<i>(acidic)</i> ^b	$\text{H}_2\text{O}_2 + 2\text{H}^+ + 2\text{e}^-$	\rightarrow	$2\text{H}_2\text{O}$ $E^\circ = 1.78 \text{ V}$
	<i>(basic)</i>	$\text{H}_2\text{O}_2 + 2\text{e}^-$	\rightarrow	2OH^- $E^\circ = 0.88 \text{ V}$
Peroxymonosulfuric acid (Caro's Acid)				
	$\text{HSO}_5^- + 2\text{H}^+ + 2\text{e}^-$	\rightarrow	$\text{HSO}_4^- + \text{H}_2\text{O}$	$E^\circ = 1.44 \text{ V}$
Peracetic acid				
	$\text{CH}_3\text{COOOH} + 2\text{H}^+ + 2\text{e}^-$	\rightarrow	$\text{CH}_3\text{COOH} + \text{H}_2\text{O}$	$E^\circ = 1.06 \text{ V}$
K Permanganate				
	$\text{MnO}_4^- + 8\text{H}^+ + 5\text{e}^-$	\rightarrow	$\text{Mn}^{2+} + 4\text{H}_2\text{O}$	$E^\circ = 1.77 \text{ V}$
Reducing Agents				
Hydrosulfite (Dithionite)				
	$2\text{SO}_3^{2-} + 2\text{H}_2\text{O} + 2\text{e}^-$	\rightarrow	$\text{S}_2\text{O}_4^{2-} + 4\text{OH}^-$	$E^\circ = -1.12 \text{ V}$
	<i>pH 5-6^b</i>	\rightarrow		$E^\circ = -0.88 \text{ V}$
Formamidine Sulfinic Acid (FAS or thiourea dioxide)				
	$\text{NH}_2\text{CONH}_2 + \text{H}_2\text{O} + \text{HSO}_3^- + 2\text{e}^-$	\rightarrow	$\text{NH}_2\text{C}(=\text{NH})\text{SO}_2\text{H} + 3 \text{OH}^-$	
	At pH 10, 20°C, vs. Pt//Ag/AgCl, $v = -0.80$			

^a E° is standard reduction potential relative to hydrogen

^b Reaction conditions normally used

^c Cell potential depends on relative concentrations of Cl species, which varies with pH

In theory, oxygen bleaching can produce a bright 'chlorine-free' pulp. However, a pressure vessel capable of 25-115 psig is required. High concentrations of groundwood result in 10-20% yield loss and insufficient bleaching.¹⁹ Air Products and Chemicals² is marketing the technology under the trade name OXYPRO O_R.

Ozone continues to be explored for bleaching recycled fibers.^{20,21,22,23,24} In some applications, ozone is desirable because it can both bleach and disinfect recycled (secondary) pulps.^{25,26} Ozone bleaching has been limited by the prohibitive cost of the ozone generator and other ancillary equipment. The poor selectivity of ozone bleaching is another limiting factor. Ozone reacts with many compounds present in recycled furnish such as kraft lignin, uncooked lignin, dyes and other oxidizable material. Since ozone reacts with lignin and reduces yield, it is not used to treat wastepaper furnishes with high mechanical fiber content.¹³ Yamamoto *et al.*²⁷ found that bleaching waste copy paper with ozone reduced the halogen content of the pulp when compared to pulp deinked by standard methods. However, they also report a significant decrease in the pulp viscosity during ozone treatment. Economou²⁸ applied ozone based TCF bleaching sequences to a recycled wastepaper mixture containing about 12% mechanical pulp. He did obtain a color stripped high brightness pulp, but he also limited ozone consumption to less than 1.3% to limit fiber damage. Ozone has been reported to be effective²⁹ for decolorizing spent whitewaters from papermachines producing strongly colored papers. However, Archibald and Roy-Arcand did also note that of the 15 direct dyes they tested,²⁹ "a few of the yellow stilbene and azo dyes were quite resistant."

Hydrogen peroxide is widely used, especially when recycling wastepaper containing mechanical pulp. Peroxide can be added at the pulping stage, in the disperser after deinking,³⁰ or later, in a bleaching tower. Peroxide will brighten pulp, but it is poor at dye stripping.⁴ To remove color, a reductive bleaching stage is often added. Any residual peroxide present will consume reducing agents. Sulfur dioxide or sulfuric acid is often added between bleaching stages to destroy residual peroxide.⁴

Most dyes do not react with oxidative reagents.¹³ Consequently furnishes with significant amounts of colored ledger paper or yellow directory paper require the addition of a reductive bleaching stage. The most common reducing agent is sodium hydrosulfite (sodium dithionite). This compound easily reduces oxygen. Appreciable amounts of oxygen must be absent for effective application.³¹ Hydrosulfite bleaching is reasonably flexible and can be used with or without prior peroxide addition.⁴

Another common reducing agent is FAS. Formamidine sulfinic acid (FAS) is also called thiourea dioxide. It requires the presence of caustic. FAS bleaching can replace hydrosulfite bleaching or be used in addition to it. FAS is a relatively powerful reducing agent. It is capable of disrupting and decolorizing the chromogens in most dyes. It should be noted that bleaching with FAS or sodium dithionite alone has been reported³² to increase the yellowness of the pulp even as it improved the lightness. In a direct comparison, based on optical properties, Ackerman³³ *et al.* report ozone and oxygen treatments offered no additional benefits when compared to peroxide combined with sodium dithionite or FAS.

New bleaching methods continue to be developed and tested such as direct borohydride injection.^{34,35} In this process, solutions of sodium bisulfite and sodium borohydride are added sequentially and directly into a recycled paper pulp stream in medium or high consistency mixing equipment at high temp. Tests at recycle mills have been positive³⁶ or inconclusive³⁷ Other bleaching processes recently patented include reductive bleaching with hydroxymethanesulfinic acid,³⁸ and bleaching with a peroxyacid³⁹ followed by treatment with a reducing agent such as sodium dithionite. The peroxyacid is generated in situ by reaction of a peroxygen bleaching agent, such as hydrogen peroxide, with a bleach activator, such as an acylated amine, (e.g., tetraacetylenediamine). Recently developed improvements for peroxide bleaching include adding aldehyde donors,⁴⁰ metal substituted xerogels⁴¹ or polymers.⁴² The continued development of new bleaching technologies suggests that color removal continues to be a problem for recycling paper.

3.3 PROBLEMS WITH RECYCLING COLORED WASTEPAPER

Yellow directory and colored ledger (office) paper are two high-quality fiber grades that are difficult to recycle.¹⁵ The major difficulty with these grades is not the removal of ink but the removal of dye. Generally, basic dyes are used for mechanical and unbleached pulp. Acid, cationic, and direct dyes are used for bleached chemical pulps.¹⁵ In terms of one specific color, yellow directory is probably the most studied grade.⁴³

3.3.1 MEASUREMENT OF COLOR

The perception of color is a human phenomenon. It depends on the brain's interpretation of signals received from the eyes. To enable quantitative comparison between samples, quantitative testing methods had to be developed. Absorption, reflection, transmission and scattering of light are physical phenomena that can be measured quantitatively. To relate the physical measurements to the human perception of color, the International Commission on Illumination (Commission Internationale de l'Eclairage, CIE) developed the tristimulus system. Individuals were shown light of a specific wavelength and asked to mix red, blue and green light to match the color. The results of these tests were averaged and became the basis for CIE 10° Standard Observer color matching functions.

The CIE color matching functions relate the human perception of color to the spectral reflectance curve of objects. The reflectance or transmittance spectrum of a sample are combined with emission spectrum of a standard illuminant and the CIE Standard Observer color matching functions to calculate the tristimulus values X, Y, and Z (Equation 1). $P(\lambda)$ is the value of the spectral power distribution of the light source at wavelength λ . $R(\lambda)$ is the reflectance factor of the sample at wavelength λ . $\bar{x}(\lambda)$, $\bar{y}(\lambda)$, $\bar{z}(\lambda)$ are the CIE color matching functions for the Standard Observer at wavelength λ . The factor κ normalizes the tristimulus value so that Y will have a value of 100 for the perfect white diffuser (a theoretical material that reflects or transmits 100% of the incident light). Most modern colorimeters automatically calculate the tristimulus values X, Y, and Z.

Equation 1 Calculation of Tristimulus Values as per Marcus⁴⁴

$$\begin{aligned} X &= \kappa \sum P(\lambda) \bar{x}(\lambda) R(\lambda) & Y &= \kappa \sum P(\lambda) \bar{y}(\lambda) R(\lambda) \\ Z &= \kappa \sum P(\lambda) \bar{z}(\lambda) R(\lambda) & \kappa &= 100 / \sum P(\lambda) \bar{y}(\lambda) \end{aligned}$$

The CIE color space is complex and hard to understand. Consequently, other, more easily understood color spaces have been developed based on transformations of CIE tristimulus values. Richard Hunter developed the L, a, b color space (Figure 1, page 14). This system has been improved to produce the CIE L^*, a^*, b^* system. The calculation of CIE L^*, a^*, b^* values are shown in Equation 2.

Equation 2 Calculation of CIE L^*, a^*, b^* Coordinates from Sharpe⁴⁵

$$\begin{aligned} L^* &= 116 (Y/100)^{1/3} - 16 & \Delta E &= \{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2\}^{1/2} \\ a^* &= 500 [(X/98.04)^{1/3} - (Y/100)^{1/3}] & b^* &= 200 [(Y/100)^{1/3} - (Z/118.1)^{1/3}] \end{aligned}$$

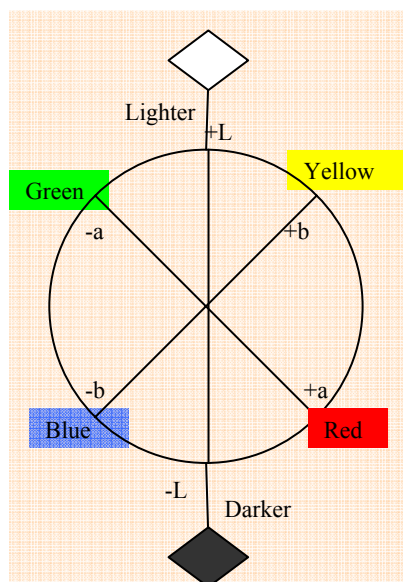
When testing a sample, a colorimeter will measure the sample reflectance, determine the tristimulus values and calculate the CIE L^*, a^*, b^* values. The CIE L^*, a^*, b^* values quantitatively describe the appearance of a reflecting surface. However, the observed color is the result of absorbance of *all* materials in the surface. Identical CIE L^*, a^*, b^* values may result from different combinations of absorbing chemicals.

3.3.2 MEASUREMENT OF DYE REMOVAL

The TAPPI brightness measurement (commonly used to test virgin fibers) does not measure color because it depends on the blue portion of the visible spectrum. To accurately characterize colored fibers and sheets, a colorimeter that determines the tristimulus values should be used. Color measurements in the paper industry are usually based on the CIE L^*, a^*, b^* system (Figure 1). L^* is a measure of the lightness/darkness of a paper sheet ($L^*=100$ = maximum lightness). The a^* value quantifies how red/green the sheet is while b^* describes the relative blue/yellowness of the sheet. The geometric distance, R , between the $L^*a^*b^*$ values for a sheet and the ideal bleach point (where $L^*=a^*=b^*=0$) is measure of the intensity of color in the sheet. The dye removal index⁴⁵ (DRI) for a bleaching process is based on the change in R that occurs during the bleaching process. The dye removal index is expressed as a percentage of the original

distance from the ideal bleach point. The DRI does not give the direction of the color change, but rather measures how much color was removed. Calculation of the dye removal index is discussed more fully in the experimental procedure section.

Figure 1 L, a, b Color Space Based on Sharpe⁴⁵



3.3.3 RECYCLING STUDIES OF TELEPHONE DIRECTORIES

About 750,000 tons of old telephone directories are available each year for collection and recycling.⁴⁶ Directory paper is a specialty lightweight uncoated paper. The majority of the pulp furnish is mechanical pulp, but clay (for opacity) and chemical pulp (for strength) are included. The yellow color is achieved either by adding dyestuff in pulp during production, or by using color ink to tint white paper yellow during printing.⁴⁶ Although other dyestuffs are available that are more bleachable,¹⁵ the dyestuff most commonly used is Basazol Yellow 46L⁴⁷ The commonly used dye dosage is 0.2 to 0.4% wt (4 to 8 lb./ton.)⁴⁸ Sutman in 1992,⁴⁸ Dumont *et al.* in 1994,¹⁵ and Marchildon *et al.* in 1996,⁴⁹ report moderately effective color removal from yellow directory paper by reductive bleaching with sodium hydrosulfite (Sutman, Dumont) or with formamidine sulfinic acid (Marchildon). However, Cao *et al.* reported at the TAPPI 2000 Recycling Symposium⁴⁶ that they were unable to bleach old telephone directories with FAS or hydrosulfite. The reaction conditions and results from these papers are summarized in Table 3-9.

3.3.3.1 Study of recycling complete telephone directories by Sutman⁴⁸

Sutman's⁴⁸ experiments were designed to examine the quality of recovered pulp from recycling complete old telephone directories. In addition to bleachability, Sutman assessed stock deinking, binder glue repulpability, and cover stock repulpability. Wash deinking and combination flotation/wash deinking were investigated on the laboratory and pilot plant scale, using the directory white pages.

Sutman's bleachability study was performed at the laboratory scale only. Sodium hydrosulfite at 1% wt (20 lb. /ton) was added to 1% consistency pulp at pH 5, 60° C (140° F) and allowed to react for 60 minutes. Most color stripping happened within the first 15 minutes. Both printed and unprinted yellow pages were treated. Sutman reported "excellent removal of the yellow tint." The Hunter "b" value is a measure of 'yellowness' of paper. For unprinted yellow pages, Sutman reported that hydrosulfite bleaching reduced the Hunter b value from +26.2 to +11.4. The Hunter "b" value for unbleached groundwood stock is normally about +10. Sutman reported that yellow directory samples from several sources were tested and produced similar results. Based on his laboratory tests with unspecified yellow directory samples, Sutman concluded, "Color removal from deinked yellow pages stock should not be a concern, as long as a properly designed reductive bleaching system is used."

3.3.3.2 Study of dyes to allow recycling of directory paper by Dumont¹⁵

The publication by Dumont¹⁵ and her colleagues at Hoechst Celanese was based on work performed at the request of three different paper mills. The work was focused on using sodium hydrosulfite to remove dyes during recycling yellow directory broke (Mill 1 and 2) and colored ledger paper (Mill 3). Unlike Sutman's study, the dyes were specified.

Mill 1 wanted to be able to easily color-strip their yellow directory broke and asked Hoechst Celanese for help. Two dyes were under consideration: Basazol Yellow 46L and Calcozine Yellow 9G. Bleaching tests were performed under laboratory conditions. Enough colorant was added to 2.5% consistency pulp to produce a b* value

close to 35 points. The hydrosulfite dosage, retention time, temperature and pH were varied. Under the mill's conditions, (pH 4.6, 38°C), 1% dosage of hydrosulfite reduced b^* only 5 points, (from b^* 35 to 30), in 30 minutes for Basazol dyed pulp. To reduce b^* values from 35 to 15 with 1% hydrosulfite required 120 minutes at 70°C if Basazol Yellow 46L was used and less than 30 minutes at 60°C if Calcozine 9GL was used.

The second mill used Astra Yellow 4GN dye for producing a yellow directory paper. They wished to reduce changeover time when switching from the yellow grade to newsprint. Dumont *et al.* report how mixed pulp, broke and whitewater responded to different dosages of hydrosulfite at 50°C and 60°C. With 1.2% sodium hydrosulfite at pH 5.0, the b^* value could be reduced from 33 to 15 in 120 minutes. The consistency of the blended pulp was 2.8% and the temperature 60°C. Dumont's results indicate that, when optimized, reductive bleaching can remove the color from yellow directory. However, of the three dyes mentioned, the one hardest to bleach, Basazol Yellow 46L, is also the most commonly used dye for yellow directory.⁴⁷

3.3.3.3 Study of bleaching yellow directory paper with FAS by Marchildon⁴⁹

Sutman and Dumont both used sodium hydrosulfite at acidic pHs. Marchildon *et al.*⁴⁹ used formamidine sulfinic acid (FAS) in alkaline solution to remove color. The pulp was 8% consistency and the bleaching equipment was laboratory scale. The publication date and location of the yellow telephone directories are specified, but the dye in the furnish is not. Marchildon *et al.* performed a statistical experiment where FAS concentration and the sodium hydroxide charge were independently varied. The data was used to generate three dimensional response surfaces and to calculate coefficients for second order regression equations. Four response variables were tested: change in ISO brightness, change in b^* value, burst index and breaking length. Since only the changes in b^* values are reported, it unknown whether Marchildon's initial deinked unbleached pulp was as strongly colored as the furnishes used by Sutman and Dumont. Sutman and Dumont report conditions where b^* values about 35 were reduced to b^* 15, a decrease of 20 points. Marchildon's maximum reported change in b^* values was a decrease of 8 points.

3.3.3.4 Study of recycling telephone directories with floatation cell by Cao⁴⁶

To gain operational experience with a new generation laboratory flotation cell, Cao *et al.*⁴⁶ investigated the properties of old telephone directories from three different printings. The telephone directories were separated into white pages, yellow pages, cover, inserts, and binder. The weight percent, ash content, and fiber quality were determined for each type. Since the basis weight of directory paper is lower than newsprint, Cao calculated that directories would contain 20% more ink per weight than newsprint. The new flotation cell was able to removal ink, but the higher ink concentration required more calcium ions (harder water) than newsprint requires.

The brightness and color of unprinted white and yellow pages were determined for each of the three printings. The b* values found ranged from 24.65 to 30.27 for the yellow pages and 7.52 to 8.50 for the white pages. When complete telephone directories were pulped, the calculated weight-averaged b* value for the feed furnish was 19.7. Cao calculated that 90% of the ink was removed by flotation deinking. The deinking process decreased the b* value from 19.7 to 9.7. The deinked pulp was then reductively bleached with hydrosulfite or FAS For the hydrosulfite bleaching, a 1% dosage was added to 12% consistency pulp at pH 6.6, 140°F (60°C), and allowed to react 45 minutes. For FAS bleaching, the initial pH was 10, temperature 177-188°F (80-87°C) and reaction time 30 minutes. Dosages of 1% or 2% FAS were applied to 15% consistency pulp. No brightness gain was observed after bleaching. Cao *et al.* concluded that the yellow color in old telephone directories “cannot be stripped with current bleaching chemicals.”⁴⁶ According to the 1996 environmental council action plan of the Yellow Pages Publishers’ Association, publishers throughout the industry have been switching from dyed yellow pages to printed yellow pages. Printing inks are often based on insoluble pigments rather than soluble dyes. Cao *et al.* suggest that they observed no effect from reductive bleaching because the 1998 telephone directories they used may have been printed yellow rather than dyed.

Table 3-9 summarizes the bleaching conditions used and the results obtained for Sutman, Dumont *et al.*, Marchildon *et al.* and Cao *et al.* The extent of bleachability of yellow directory paper is not clear. Dumont’s work shows that the basic yellow dyes

differ in bleachability. It appears that reductive bleaching has worked in the past when optimized, but the dyes in the paper are unspecified. Basazol Yellow 46L is considered unbleachable.⁵⁰ Since this dye is cheap and tinctorially strong, it is still the most common dye for directory paper.⁴⁷ Therefore, bleaching directory paper remains problematic. Whether the trend towards publishers printing the yellow color will ease or complicate the recycling of yellow directories remains to be determined.

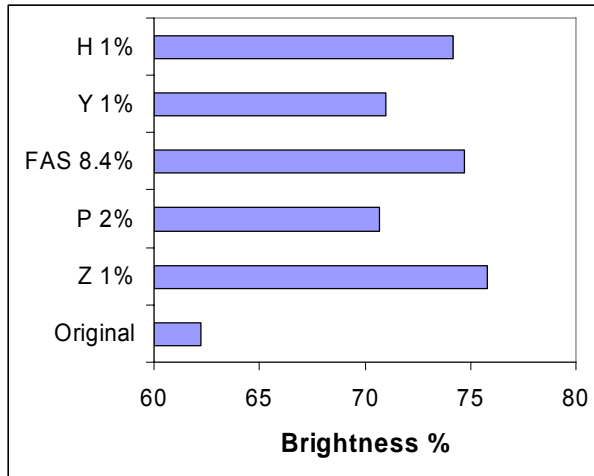
Table 3-9 Comparison of Published Yellow Directory Bleachability Studies

Author	Reducing Agent, pH	Temperature, °C, °F	Retention Time	Pulp Consistency	Change in b*
Sutman	Hydrosulfite, 1 wt%, pH 5	60°C, 140°F	60 min	1%	14.8
Dumont Mill 1	Hydrosulfite, 1 wt%, pH 4.6 (Basazol) pH 4.9 (Calcozine)	70°C, 158°F 60°C, 140°F	120 min 30 min	2.5%	20 25
Dumont Mill 2	Hydrosulfite, pH 5, 1.2 wt%, (Astra)	60°C, 140°F	30 min	2.8%	20
Marchildon	FAS, 0.25-0.75%, NaOH, 0.15-1.05%	25-75°C, 77-167°F	20 min	8%	Up to 8
Cao	Hydrosulfite, 1 wt%, pH 6.6 start, 6.9 end	60°C, 140°F	45 min	12%	< 1
Cao	FAS, 1 and 2 wt% pH 10 start, 7 end	80-86°C, 177-188°F	30 min	15%	Increase ≈ 1

3.3.4 RECYCLING OF MIXED OFFICE WASTE

Recycling colored ledger or mixed office waste containing color continues to be difficult. Figure 2 compares the effectiveness of different bleaching agents used as single stages on a mixed office waste pack. In Figure 2 high brightness values were obtained but the furnishes contained only 8.5% colored ledger paper.

Table 3-10 shows that the final pulp brightness is much lower when 'pure' unmixed colored furnishes are used.

Figure 2 Single Stage Bleaching Results According to Ferguson⁴

Furnish = 91.5 % Office Waste + 8.5 % Colored Ledger
H=hypochlorite
Y=hydrosulfite (dithionite)
FAS = formamidine sulfinic acid
P=peroxide
Z=ozone

Table 3-10 Bleaching of Unmixed Office Wastes Results According to Ferguson⁴

Raw Material Type	% Brightness After Treatment			
	Original Deinked	FAS 0.5%	P 1%	H 2%
Mixed Office Waste	62.1	81.5	66.7	81.8
Colored Ledger	65.5	80.7	69.0	79.7
File Stock	54.3	72.3	61.5	71.0
Goldenrod	15.0	40.5	22.8	20.2

Bleaching goldenrod wastepaper with FAS did produce significantly brighter pulp than did bleaching with hypochlorite or peroxide. However, even after bleaching, the pulp from goldenrod paper was still darker than unbleached pulp from mixed office waste. Goldenrod office paper is dyed with a stilbene yellow dye which is “one of the notorious dyes in office wastepaper recycling.”⁴ Although all dyes vary in their bleachability, yellow dyes are particularly troublesome. Stilbene dyes such as C.I. Direct Yellow 11 and Direct Orange 15 are considered “unbleachable under normal bleaching conditions”.⁵¹ To increase recycling of mixed office waste, a way to bleach these dyes must be developed.

In 1991, Michael Cheek⁵² tested common bleaching agents for the ability to removal color from a number of dissolved pure direct dyes. He bleached 24 dyes with 2 and 4% chlorine, at pH 4 and 8. Only two dyes out of 24 did not have 90% or greater color removal under at least one of the four conditions tested. He also bleached dyes with sodium hydrosulfite, FAS, and hydrogen peroxide. Hydrogen peroxide bleaching was not very effective. Color removal was less than 60% for 8 out of 12 dyes when hydrosulfite

was used and 4 out of 15 dyes when FAS was used.⁵² Table 3-11 summarizes his data. Only dyes with less than 90% color removal are listed.

Table 3-11 Results from Bleaching of Direct Dyes as Reported by Cheek⁵²

% Color Removal by Treatment (Dyes with removal >90% omitted)								
Direct Dye	H ₂ O ₂	FAS	Hydro sulfite	2% Avail. Cl pH 4	2% Avail. Cl pH 8	4% Avail. Cl pH 4	4% Avail. Cl pH 8	Ozone
Yellow 11	4	50(Red)	60(Red)	48	10	84	33	99
Yellow 147	16	NA	60	89	99	89	97	NA
Yellow 166	42% Inc.	20	10	95	25	95	35	NA
Red 236/239	6	98	80	98(Dull)	97	99(Dull)	99	NA
Violet 9	0	98(Dull)	80	96(Dull)	80	98(Dull)	88	NA
Blue 218	22	92(Dull)	50	97(Dull)	82(Redder)	99	96(Redder)	NA
Blue 86	0, Cons. Redder	30	37	73	55(Redder)	98	92(Redder)	NA
Blue 199	20% Inc., Redder	35	34	90	97	90	98	NA

To increase the utilization of wastepaper, the bleaching of dyed office and directory papers must be improved. Examination of Table 3-11 shows that the hardest direct dye to bleach is C.I. Direct Yellow 11. This project will focus on bleaching/decolorization of C.I. Direct Yellow 11 (used in goldenrod office paper) and Basazol Yellow 46L (used in telephone directories). Before examining possible decolorization methods, the properties and characteristics of color and of dyes will be discussed.

3.4 CHEMICAL REQUIREMENTS FOR DYES

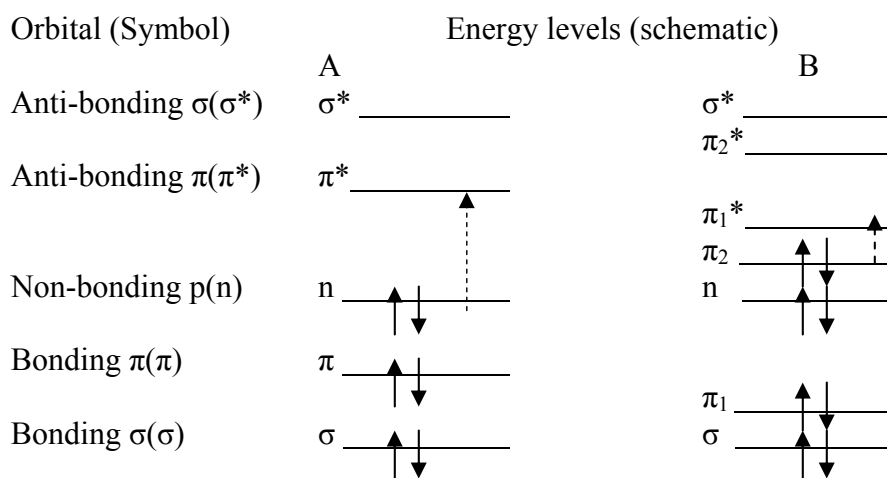
3.4.1 ELECTRONIC REQUIREMENTS FOR COLOR

This project focused on two specific dyes. Dyes are organic compounds. Like all organic compounds, their atomic orbitals combine to form molecular orbitals. When a group with π orbitals (e.g. NO₂ or COOH) is attached to an aromatic compound, overlapping of the two π orbitals normally occurs, leading to new bonding and anti-bonding states. Orbital overlapping and combination also occurs when aromatic carbons carry substituents that have lone pair of electrons (e.g. OH, NR₂, etc.)

Figure 3 shows the essential types of energy levels in two compounds. Compound A has a pair each of σ , π and n electrons and compound B has one pair of σ and n electrons, but two pairs of conjugated π electrons.

Color is produced when light is absorbed. The absorbed energy promotes one of the electrons inhabiting the molecular orbitals to a level of higher energy. The spacing between available bonding orbitals determines the amount of energy that can be accepted and thus the wavelength of light absorbed. In Figure 3, the transition from the non-bonding orbital n to the anti-bonding orbital π^* requires the least energy. The $n \rightarrow \pi$ transition results in an absorption band at a longer wavelength than the $\pi \rightarrow \pi^*$ transition. In compound B, the π orbitals are non-degenerate and the $\pi \rightarrow \pi^*$ transition corresponds to the longest wavelength absorption band.

Figure 3 Molecular Orbitals Schematic as per Marcus ⁴⁴



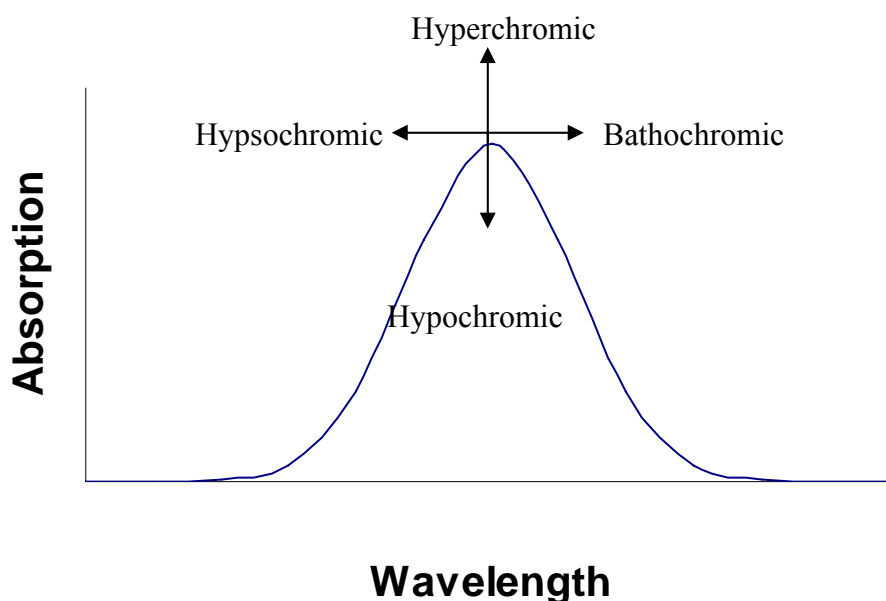
Schematic representation of energy levels. Molecule A has one σ , one π and one n electron pairs. Molecule B has one σ , one n and two conjugated π electron pairs. (Number of s electrons not specified)

The degree of conjugation (number of π orbitals and electrons) determines whether the electromagnetic radiation absorbed is within the range of visible wavelengths. Table 3-12 demonstrates how color changes with the amount of conjugation. The additional carbon-carbon double bonds increase the number of delocalized π electrons and decrease the energy required for excitation. The shifting of absorption from the ultraviolet range to the range of visible wavelengths is described as a bathochromic shift (Figure 4).

**Table 3-12 Relationship of Conjugated Double Bonds and Absorption
as Described by Rivlin⁵³**

Color of Diphenylpolyenes $C_6H_5(CH=CH)_N C_6H_5$ (N defines the number of conjugated double bonds)			
Value of N	Color	Value of N	Color
1	None	6	Brownish orange
2	None	7	Copper-bronze
3	Pale yellow	11	Violet-black
4	Greenish yellow	15	Greenish black
5	Orange		

Figure 4 Designations for Wavelength Shifts and Absorptivity Changes as per Marcus⁴⁴



3.4.2 CHEMICAL STRUCTURE OF DYE MOLECULES

All dyes are organic aromatic compounds with a conjugated double bond system, to which chromophores and auxochromes are attached. These functional groups reduce the number of conjugated double bonds needed for light absorption and result in molecules that are small enough to diffuse into fibers.

Chromophores (Table 3-13) are unsaturated functional groups (except for $-NR_3^+$). Alone, they absorb visible or near ultraviolet radiation. In a dye molecule, they function as electron acceptors. Auxochromes (Figure 14) are saturated functional groups.

Auxochromes act as electron donors because the atom attached to the conjugated system has nonbonding electrons.

Table 3-13 Examples of Dye Chromophores From Rivlin and Burstone^{54,53}

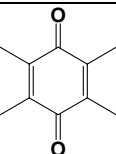
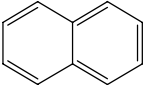
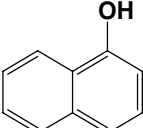
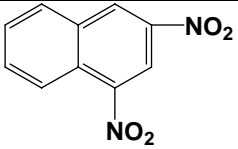
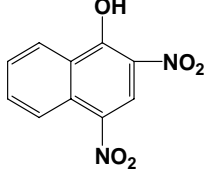
-NO ₂	Nitro group		Azo group
-C=O	Carbonyl group		Quinoid rings
-NR ₃ ⁺	Alkyl ammonium derivatives		
-C=C-	Methylene group		
-C=N-	Cyano group		

Table 3-14 Examples of Dye Auxochromes From Rivlin and Burstone^{53,54}

-NH ₂	Amino group
-NHR	Mono alkyl amino group
-NR ₂	Dialkyl amino group
-OH	Hydroxy group
-OR	Ether group
-NH ₃	Ammonium group
-COOH	Acetyl group
-HSO ₃	Sulfonic acid group

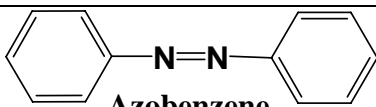
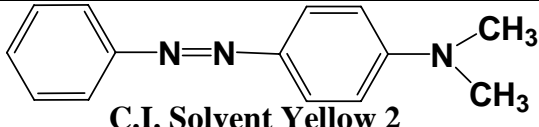
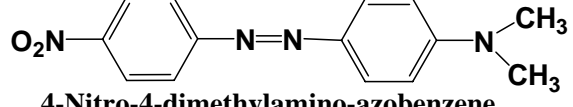
Dye chromogens can be described as electron acceptor(s) (chromophores) interacting with electron donor(s) (auxochromes) through a conjugated double bond system. The effect of adding an auxochrome and chromophore is qualitatively discussed in Table 3-15.

Table 3-15 Enhancing Color by Group Additions as per Fessenden⁵⁵

	To the left is naphthalene, a colorless compound
	The addition of a single hydroxyl group to naphthalene produces 1-naphthol which is also a colorless compound, but one which can ionize.
	If instead of a hydroxyl group we add the nitro group, which is a chromophore, we get the compound 2, 4-dinitronaphthalene. The addition of this chromophore has caused it to become pale yellow.
	If instead of a hydroxyl or nitro groups, both a hydroxyl and nitro groups are added, we get the deep yellow dye, Martius yellow
The addition of both an auxochrome and a chromophore results in a much stronger alteration of the absorption maximum of the compound. The hydroxyl group must have deepened the color, showing that auxochromes are also chromophores.	

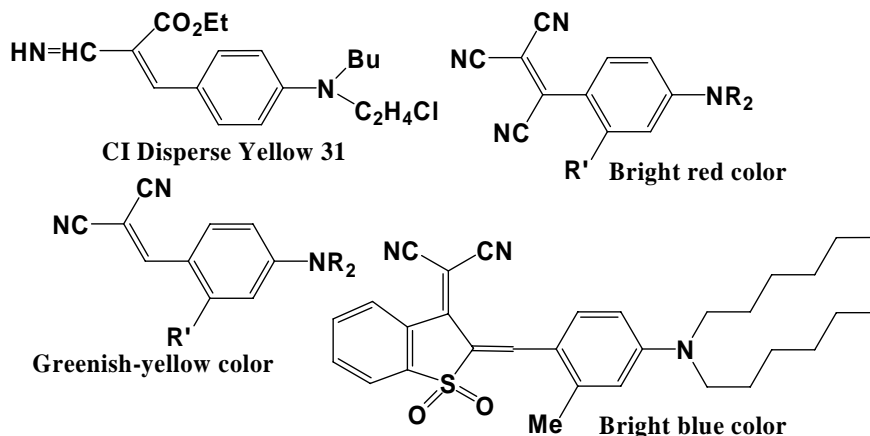
The quantitative effects of adding an auxochromes and chromophore are illustrated in Table 3-16. The addition of a dimethyl amino auxochrome to azobenzene shifts the absorption to longer wavelength (bathochromic shift) and increases the molar absorptivity (hyperchromic shift). The addition of a nitro chromophore causes both a bathochromic shift and a hyperchromic increase in absorption.

Table 3-16 Auxochrome and Chromophore Addition Effects as per Rivlin⁵³

Absorption Spectra of Azo Compounds		
	λ_{Max} nm	ϵ_{Max} $\text{L mol}^{-1} \text{cm}^{-1}$
 Azobenzene	330	17,000
 C.I. Solvent Yellow 2	408	27,500
 4-Nitro-4-dimethylamino-azobenzene	478	33,100

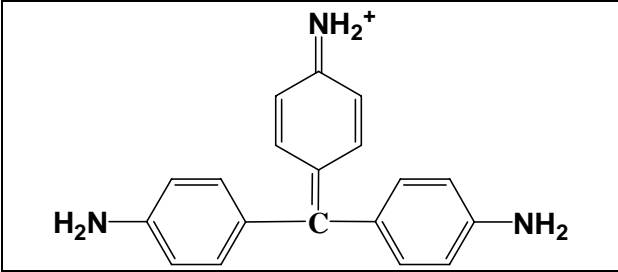
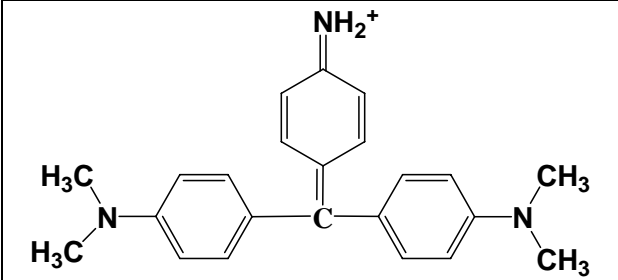
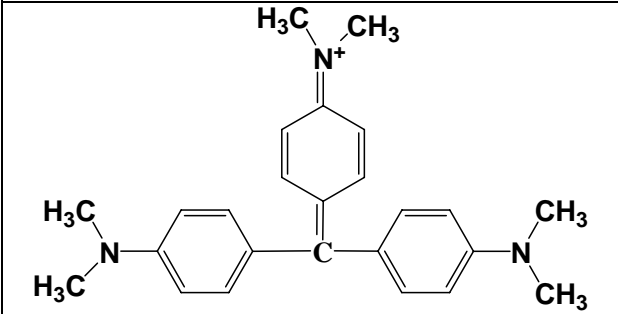
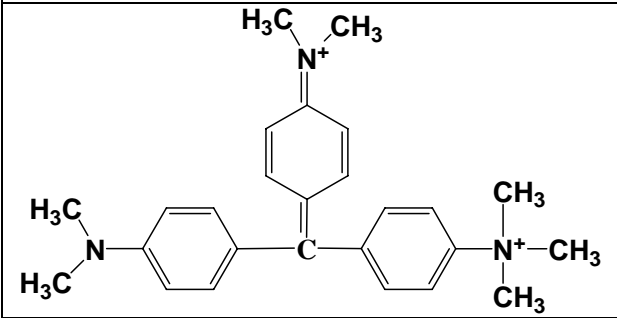
For any chromogen, changing the electron density can cause a bathochromic shift in absorption. In Figure 5 a typical yellow styryl dye is shown at the top. Addition of a second cyano group shifts the absorption to produce greenish-yellow color. The addition of a third cyano group shifts the absorption yet further to produce red color. At the bottom of Figure 5 is shown a bright blue dye that is created if an even more powerful electron-withdrawing group is used.

Figure 5 Bathochromic Shift with Electron-Withdrawing Groups as per Waring⁵⁶



This principle of altering dye color by adding modifying groups is shown more clearly in Table 3-17 where a series of differently colored homologues can be produced by the addition of methyl groups.

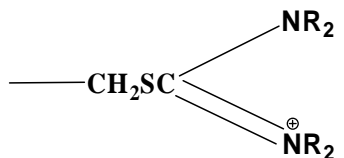
Table 3-17 Alteration of Color by Modifiers as Described by Burstone⁵⁴

	<p>Without any methyl groups the parent dye is called Pararosanilin and is red</p>
	<p>When four methyl groups are added the result is the reddish purple dye Methyl violet.</p>
	<p>As more methyl groups are added the result is the purple blue dye Crystal violet which has six such groups.</p>
	<p>If a seventh methyl group is added, the resulting dye is Methyl green.</p>

Dye molecules may also contain other groups in addition to chromophores and auxochromes. Reactive dyes contain groups that form covalent bonds with cellulose.

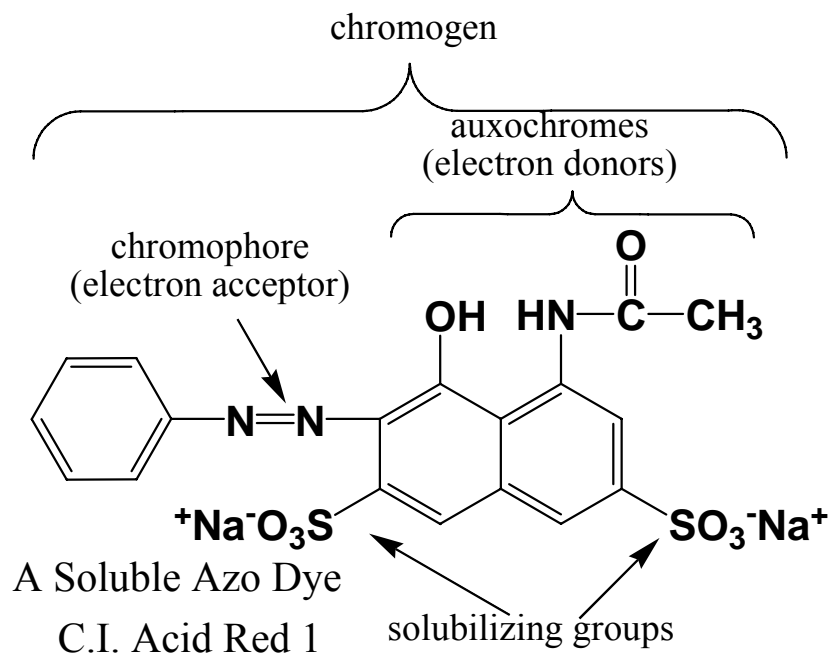
Saturated hydrocarbon chain(s) may be added to increase the hydrophobicity or molecular size. Soluble dyes will contain solubilizing groups, usually sulfonic acid groups ($-\text{SO}_3^- \text{Na}^+$). (Table 3-18)

Table 3-18 Solubilizing Groups From Johnson ⁵⁷

Permanent	Temporary
$-\text{SO}_3\text{Na}$	$-\text{O}-\text{Na}^+$
$-\text{COONa}$	$-\text{OSO}_3-\text{Na}^+$
$-\text{N}^+\text{H}_2\text{HCl}^-$	Onium groups e.g. 
$-\text{N}^+\text{R}_3\text{Cl}^-$	
$-\text{OH}$	
$-\text{NH}_2$	
$-\text{SO}_2\text{NH}_2$	

The important parts of a dye molecule are summarized in Figure 6.

Figure 6 Structure of Dye Molecule as Described by Rivlin ⁵³



3.4.3 FLUORESCENCE AND PHOSPHORESCENCE

An excited molecule or ion can lose the absorbed energy by 1) radiationless transitions, such as internal conversion or intersystem crossing (observable as heat formation) 2) emission of radiation (fluorescence and phosphorescence) and 3) photochemical reactions. Processes 1) and 2) are represented schematically in Figure 7

Most fluorescent organic compounds are characterized by stiffness in their structure. The stiffness prevents energy loss by torsional vibrations in the molecule. This principle is illustrated in Figure 8. Phenolphthalein shows no fluorescence because the excited state loses energy by internal conversion (vibrations of the benzene rings). The ether linkage in fluorescein prevents the benzene rings from twisting. Consequently, fluorescein is highly fluorescent.

Figure 7 Energy Absorption, Fluorescence and Phosphorescence
Schematic from Peters⁵⁸

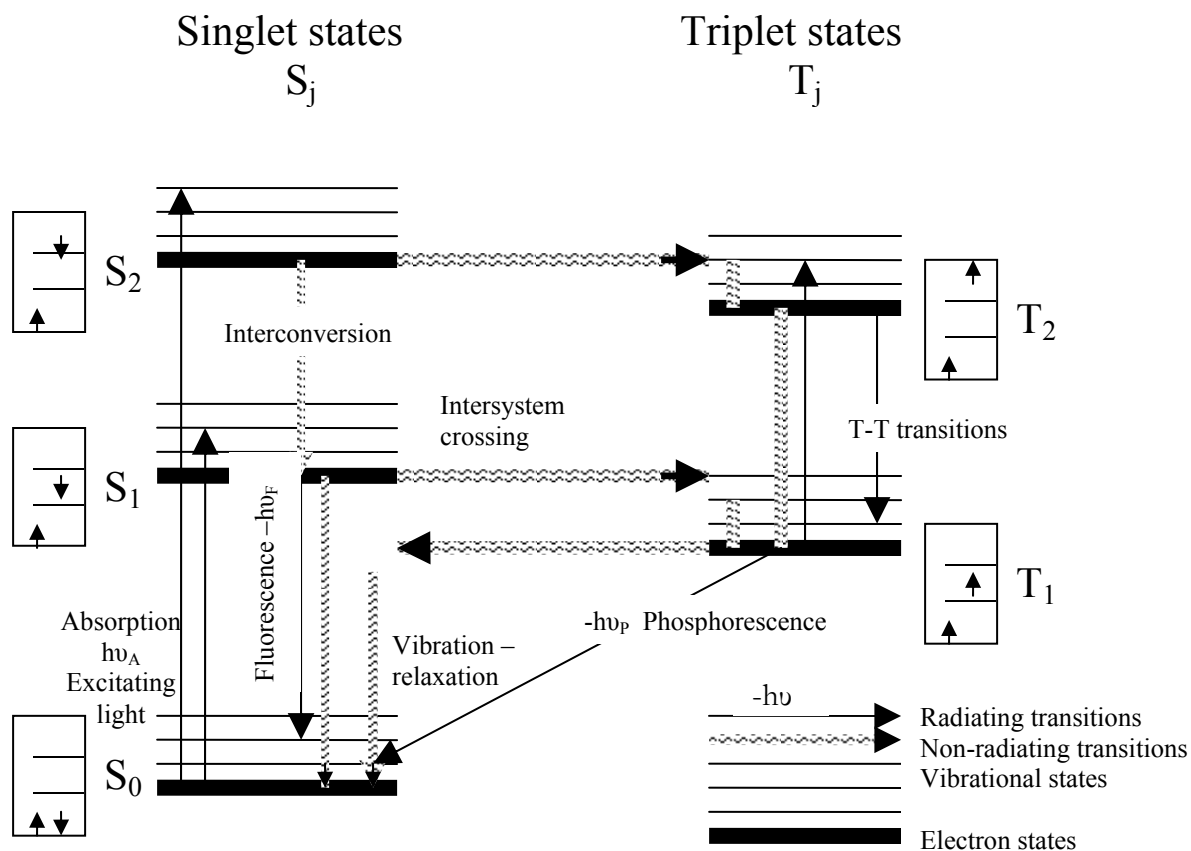
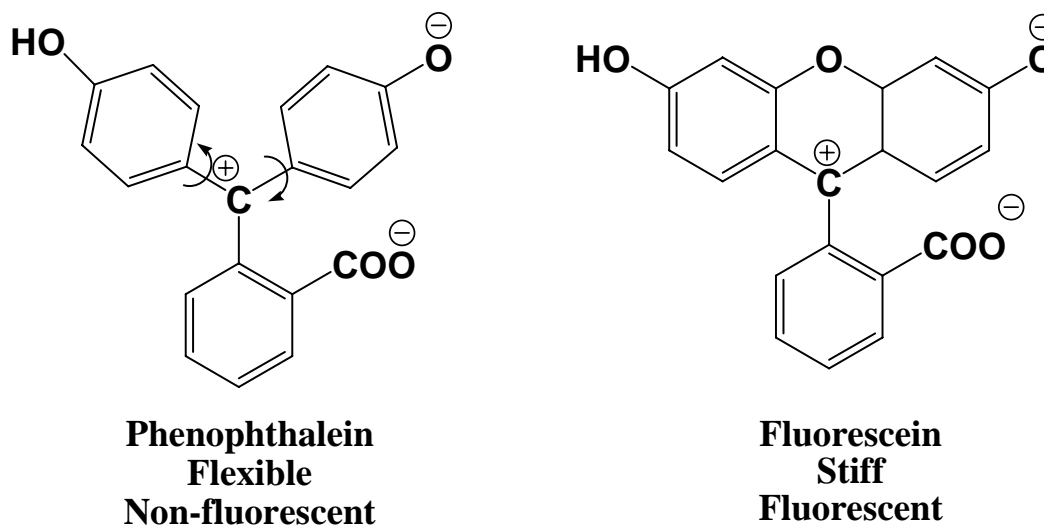


Figure 8 Example of Fluorescence Due to Structure Stiffness From Zollinger⁵⁹



3.4.4 CLASSIFICATION OF DYES

Dyes can be classified by solubility, application method or by chemical composition (Table 3-19). Only the relevant dye types will be discussed. These include acid/anionic dyes, basic/cationic dyes and direct dyes.

Table 3-19 Dye Classifications According to Rivlin⁵³

1. By Solubility	3. By Chemical Constitution
Soluble dyes (anionic or cationic)	Azo dyes and pigments (mono azo, diazo, etc.)
Disperse dyes (non-ionic, very slightly water soluble)	Anthraquinone Dyes
	Benzodifuranone Dyes
Pigments (insoluble)	Polycyclic Aromatic Carbonyl Dyes
2. By Method of Application	Indigoid Dyes
Acid dyes (anionic)	Polymethine and Related Dyes
Basic dyes (cationic)	Styryl Dyes
Direct dyes	Di- and Tri-Aryl Carbonium and Related Dyes
Azoic dyes	Phthalocyanines
Vat dyes	Quinophthalones
Sulfur dyes	Sulfur Dyes
Reactive dyes	Nitro and Nitroso Dyes
Disperse dyes	Miscellaneous Dyes (Stilbene and Formazan Dyes)

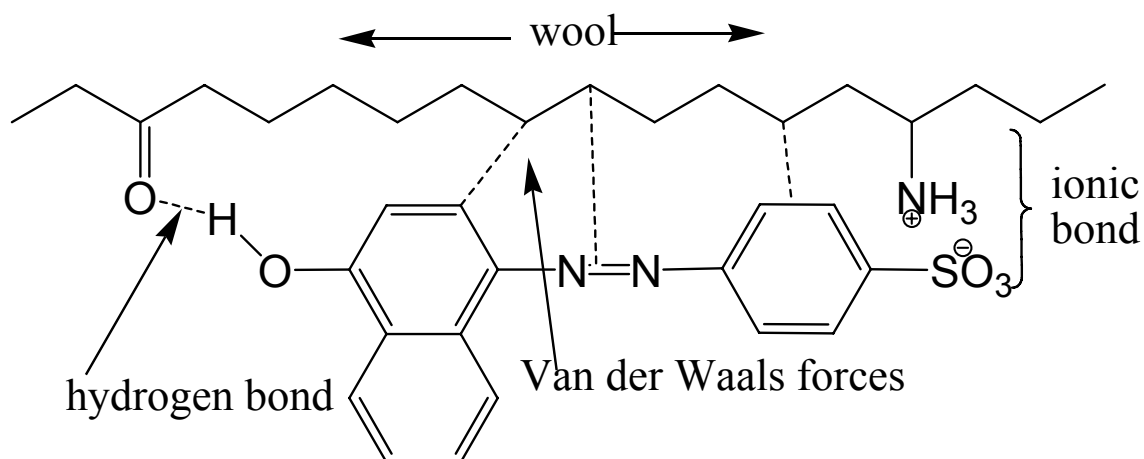
Of the major dye classifications, only basic and direct dyes are routinely applied to paper. Direct dyes are substantive to cellulose and are used to color fine papers with low-lignin-content chemical pulps. Basic dyes are cheap and have some affinity for

lignin. Consequently, basic dyes are preferred for directory and newsprint papers with high-lignin-content mechanical pulps.

3.4.5 ACID (ANIONIC) DYES

Acid dyes were the first synthetic dyes for wool. This type of dye is called 'acid' because the dye bath must be acidic for the dye to attach to the fiber. These dyes are also referred to as 'anionic' because they are negatively charged when dissolved. All acid dye molecules possess at least one group of atoms that impart water solubility, usually the sodium salt of a sulfonic acid group. The sulfonic or carboxylic acid group reacts with a basic group on the fiber to form a salt bridge. For wool, the basic groups are amines or amide groups present in the protein fiber. ()Since acid dyes require basic groups on the fiber, acid dyes are not used to color cellulose.

Figure 9 Acid Dye Binding to Wool Protein as Visualized by Rivlin⁵³



3.4.6 BASIC (CATIONIC) DYES

Like acid dyes, basic dyes are soluble ionic compounds. Unlike acid dyes, in basic dyes the color is contained within the cationic portion of the molecule. These dyes are often preferred over other dyes because most cationic dyes are characterized by intense and brilliant colors, and a complete range of colors is available.⁵³ Cationic dyes bound to acrylic fibers (Figure 10) are durable to washing and sunlight. When applied to cellulose, cationic dyes are not light fast and have limited wash-fastness. Consequently, they are

little used for cotton textiles. However, due to their intense color and low cost, basic dyes are often used for paper. Basic dyes are favored for mechanical pulps because the dye has affinity for both cellulose and for lignin.⁴⁷ Basazol 46L is a basic dye. Examples of typical basic dyes are shown in Figure 11.

Figure 10 Cationic Dye Attached to Anionic Fiber as Visualized by Rivlin⁵³

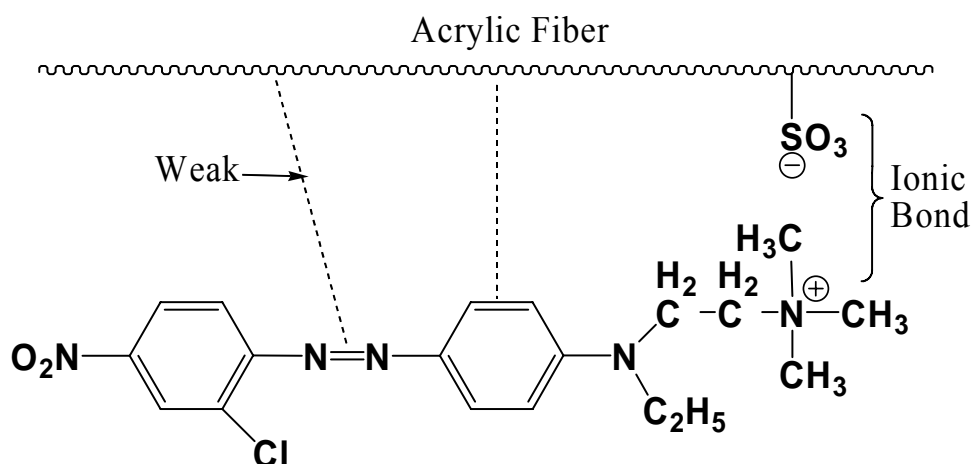
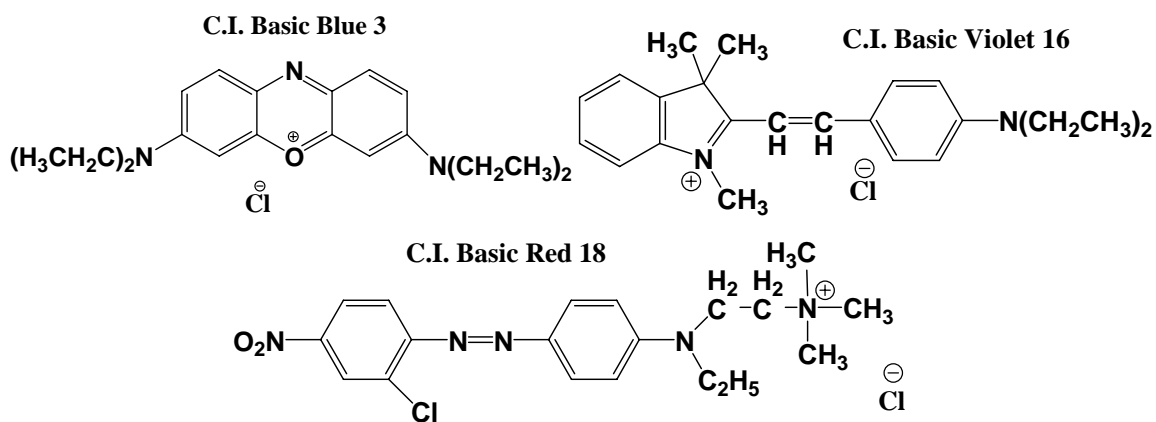


Figure 11 Examples of Cationic Dyes From Rivlin⁵³

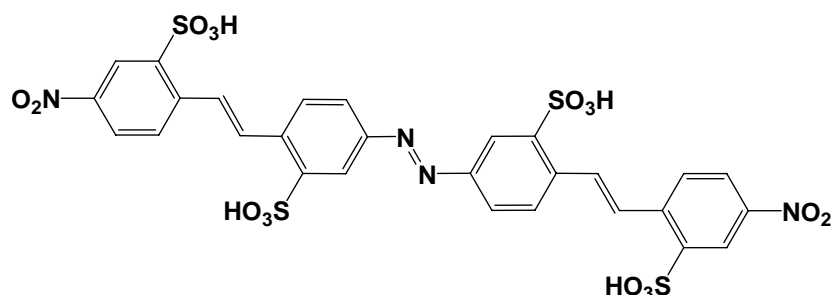


3.4.7 DIRECT DYES

These dyes are called 'direct' because of the directness and simplicity of their application to cellulosic fibers such as cotton, flax, and viscose rayon.⁶⁰ They are moderate in cost and easy to apply. During dye application, common salt (sodium chloride) or Glauber's salt (sodium sulfate)⁶¹ is added to overcome repulsion between anionic cellulose and the charged dye molecules. Their poor wet-fastness limits the use of direct dyes for coloring textiles.

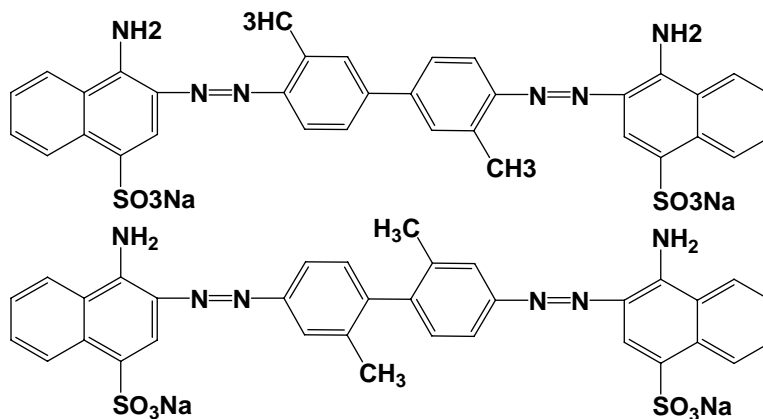
Direct dyes are generally long, linear, planar molecules such as C.I. Direct Yellow 11 (Figure 12). (This dye is the direct dye hardest to bleach by current wastepaper recycling methods, Table 3-11, page20). The sulfonate groups impart water solubility to the molecule.

Figure 12 C.I. Direct Yellow 11 as Published by Cheek⁵²



Although the substantivity of planar anionic polysulfonated dyes has been established empirically, a precise explanation of the phenomena has not yet been developed.⁶² Planarity is important. Two dyes are shown in Figure 13. The upper compound is planar and has high substantivity for cellulose. Steric hindrance from the methyl groups on the benzidine moiety in the lower structure prevents linearity, and the molecule has poor substantivity to cellulose.

Figure 13 Direct Dye Planarity Necessary for Substantivity as per Johnson⁵⁷



Relationship between Planarity and Substantivity

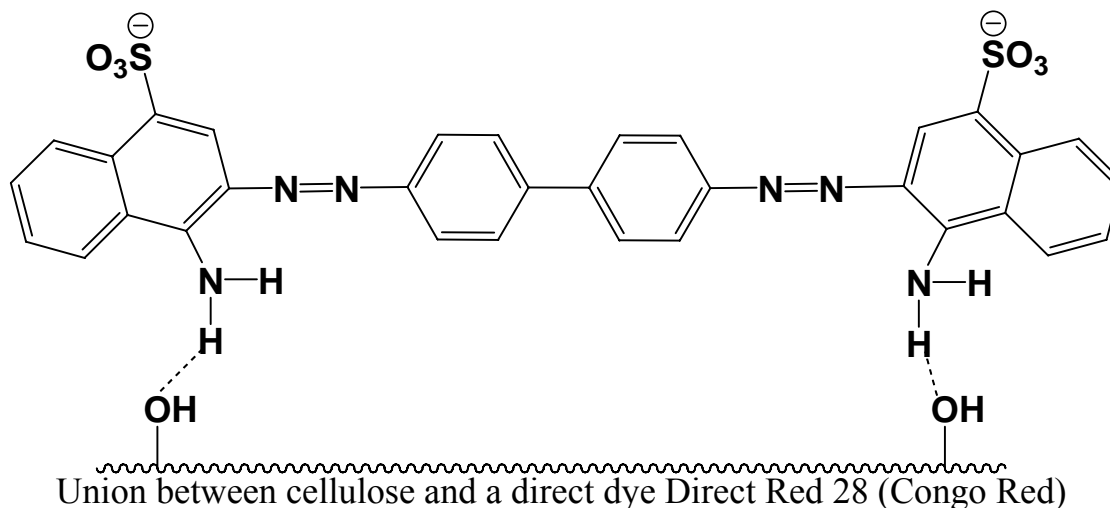
Top = Planar = High Substantivity

Bottom = Non-Planar = Low Substantivity

Planarity considerations led to the hypothesis that hydrogen bonds are formed between the dye and the fiber molecules, (Figure 14). The bonds are formed either

directly or after strong van der Waals forces cause the fiber and dye molecules to approach closely.⁶³

Figure 14 Proposed Model for Binding of Direct Dye to Cellulose as per Fessenden⁵⁵

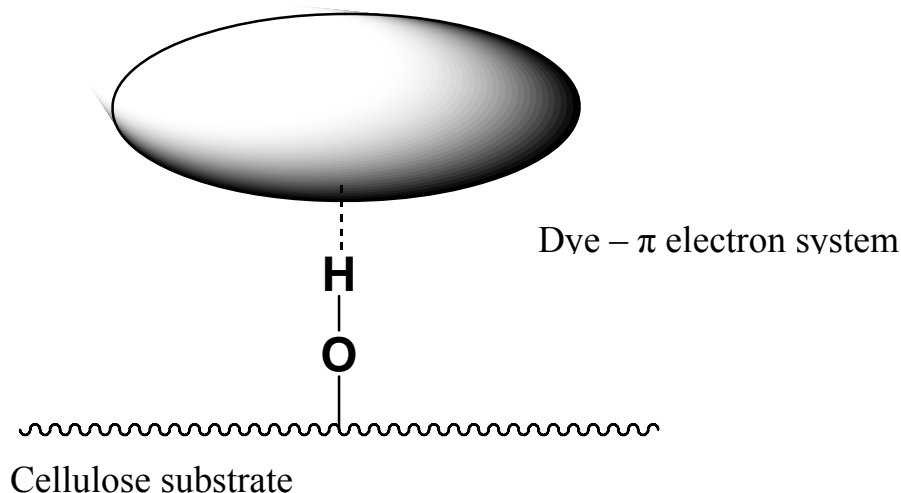


However, later evidence suggested that hydrogen bonds are not formed.⁵⁷ The cellulose hydroxyl groups are firmly attached to water and may not bind to polar groups in a dye. A monolayer experiment supported this suggestion. A surface-active benzidine diazo dye was spread as a monolayer on water containing cellobiose. The measured area per dye molecule matched the value expected for a single layer of water molecules between each dye and cellobiose molecule.⁵⁷ The water would prevent the formation of hydrogen bonds. When the cellobiose was replaced with mannitol, no such water layer was detected. Unlike cellobiose, mannitol in water does form hydrogen bonds with other solutes, including dyes.

Formation of hydrogen- π bonds has been suggested as a source of dye-fiber attraction. (Figure 15) Yoshida *et al.*⁶³ had found that sucrose, glucose, and cellobiose form 1:1 complexes in water with simple *aromatic* sulfonate anions, but not with an *aliphatic* anion. Yoshida inferred that the bond involves the aromatic group. However, hydrogen- π bonds can not be important in cellulose dyeing. Typical sulfonated azo direct dyes do not form any complexes with sucrose or with methyl- β -D-glycopyranoside (a water-soluble model of cellulose). It is not known why the presence of an azo group in an aromatic structure would prevent the formation of the π -bond complex. These models

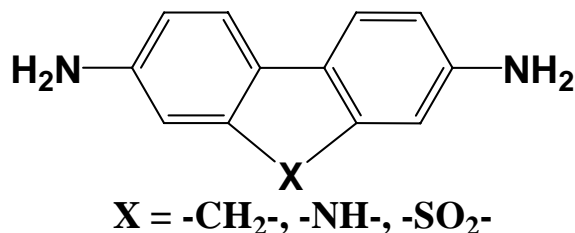
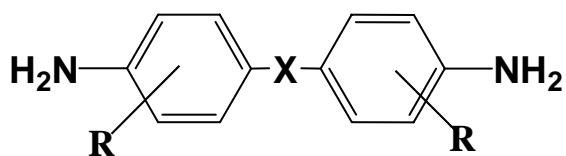
and theories had been advanced and ruled out by 1989. In 1998, the mechanism for substantivity of direct dyes for cellulose was still unknown.⁶²

Figure 15 Yoshida Bonds between Aromatic Dye and Cellulose as Described by Lewis⁶²



More recently, FT-IR spectroscopy was used to detect the places at which 8 experimental direct dyes interacted with cotton cellulose.⁶⁴ Pielesz *et al.* found that direct dye-cellulose interactions involved hydroxyl groups on the cellulose fibers. They also postulate that intermolecular interactions also occur between the dye molecules and glycosidic groups on the fibers. The concept that direct dyes bind at specific sites is compatible with other published studies. Data on the sorption of direct dyes onto cellulose substrates correlate better with the Langmuir equation than with the Freundlich equation.^{65,66} Since Langmuir postulates that absorption occurs on specific sites or surface area while Freundlich proposes diffuse binding, the sorption results collaborate the FT-IR results.

In Figure 16 are shown dyes based on benzidine, a carcinogen. The production of these dyes has been discontinued in the United States. However, once benzidine is incorporated into a direct dye it is no longer toxic. Therefore, some benzidine dyes are imported. The general structure of benzidine-based direct dyes is shown in Figure 17.

Figure 16 Direct Dyes Based on Benzidine From Cegarra⁶⁷*Figure 17 General Structure of Benzidine-based Direct Dyes*

X can be:

HN-NH- (aryldiamine)

simple link (benzidine)

CH=CH- (Stilbenes)

NH-

O-

S-

CONH-

NH-CO-NH-

R can be

-H

-SO₃Na

-Cl

-NO₂

-OH

-CH₃

-OCH₃

-OCH₂-COONa

3.5 DYES USED FOR COLORATION OF PAPER

3.5.1 DYE CHARACTERISTICS

The desired characteristics for dyes are summarized in Table 3-20. For paper, water fastness is not needed, but high substantivity is necessary. Papermachine headbox solutions are low consistency (0.3 to 0.6% fiber in water). To exhaust dyes from the solution, the dye must have high substantivity to cellulose and/or other materials, such as lignin, that are present in pulp fibers. High molar absorptivity reduces cost by requiring less dye to achieve the desired color intensity. However, when recycling dyed paper fibers, the high molar absorptivity of the dyes requires fiber decolorization to be very efficient.

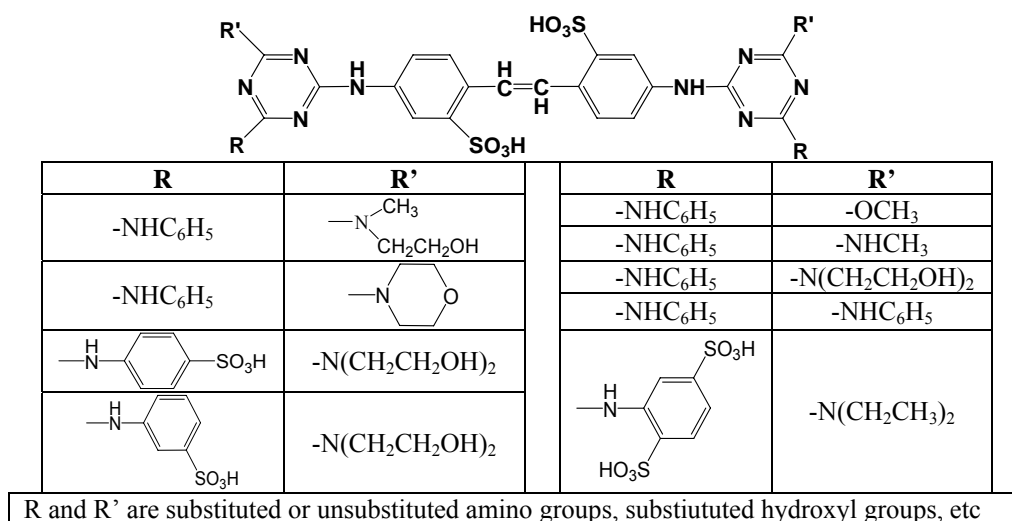
Table 3-20 Characteristics of Dyes

A. Intense color (molar absorptivity $\epsilon > 10,000$)
B. Solubility in water (fibers adsorb individual dye molecules from solution regardless whether bulk of dye is soluble aggregates or dispersed in micelles)
C. Substantivity to the fiber in question (dyeing is dynamic and reversible so dye must have greater affinity for fiber than for water)
D. Durability to wet treatments (may require chemical fixation of dye to fiber or altering dye chemical structure to become insoluble after dye has diffused into fiber)
E. Durability to further treatments in production and normal use (Normal use includes sunlight, bleaching, perspiration, dry-cleaning. For cellulosic fabrics, production includes low pH and high temperatures when adding durable press finish)
F. Safe, easy to handle and reasonably priced (Derivatives of carcinogenic benzidine are no longer produced in the US. Mordanting of acid dyes with chromium salts has become very limited because of disposal difficulties for chromium polluted effluents)

3.5.2 FLUORESCENT WHITENING AGENTS

Fluorescent whitening agents are often applied to fine papers. About 80% of all⁶⁸ commercial fluorescent whitening agents are stilbene derivatives (Figure 18). The generic formula is 4, 4'-diaminostilbene-2, 2'-disulfonic acid structures acylated by cyanuric acid derivatives.⁶⁸ As mentioned in section 3.4.3 on page 27, compounds need to have a rigid structure to exhibit fluorescence. The triazine rings increase the fluorescent efficiency of these compounds. Fluorescent whitening agents are significantly different from stilbene dyes because of the triazine rings.

Figure 18 Stilbene Fluorescent Whitening Agents as per Coulston⁶⁸



3.5.3 RECYCLABILITY

Concern about recyclability has become more important. Pressure sensitive adhesives were recently redesigned to be repulpable.^{69,70,71} The successful^{72,73,74,75,76} redesign occurred because the United States Post Office had decided that postage stamps should be repulpable. The Post Office convinced adhesive manufacturers and paper recyclers to work together on creating repulpable pressure sensitive adhesives.^{77,78}

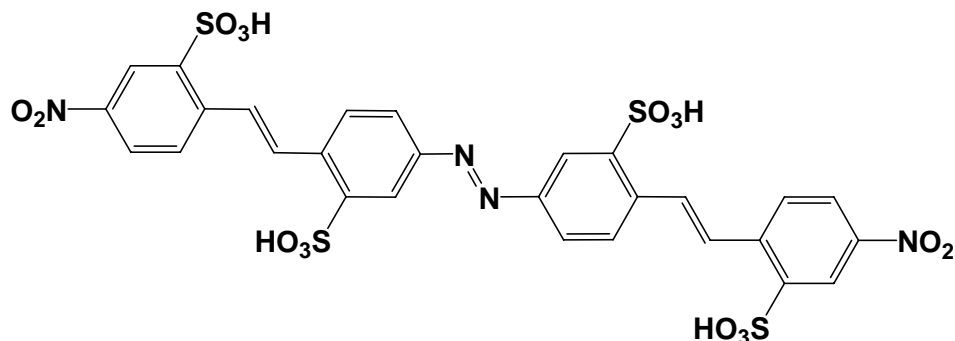
In Europe, ink manufacturers are cooperating with paper researchers to develop more recycling friendly inks for telephone book directories.⁷⁹ Dye manufacturers are giving some consideration to recyclability. In 1998⁸⁰ and 1999,⁸¹ paper dyes were patented that are readily bleached when the paper is recycled. In 2003⁸² another dye was patented that claims to yield a colorless wastewater that avoids effluent treatment problems. However, other paper dyes were also recently patented^{83,84,85,86,87} that do not claim to be bleachable. Dye manufacturers have other concerns such as improving the water- and light- fastness of textile dyes. In addition, the manufacturing process must be safe and the finished dye product must meet regulatory requirements. As long as regulations allow the profitable production and application of existing dyes, little motive exists to replace current paper dyes with bleachable alternatives.

3.5.4 PAPER DYES SELECTED FOR STUDY

Most dyes for bleached chemical pulp fine papers are direct dyes such as C.I. Direct Yellow 11. Basic dyes such as Basazol Yellow 46L are applied to high-yield pulps that contain a significant amount of lignin. Dyes were selected for this research project because they are known to be recalcitrant to existing chemical bleaches.

The structure for Direct Yellow 11 has been published and is shown below.

Figure 19 Stilbene Dye Direct Yellow 11 as Published by Cheek⁵²



Basazol Yellow 46L, used for yellow directory paper, is classified as a methine dye. Unlike C.I. Direct Yellow 11, Basazol Yellow 46L (C.I. Basic Yellow 96), is manufactured by only one company. According to Dwight Pakan of BASF, the exact structure of Basazol Yellow 46L is proprietary. However, the general structure of Basazol Yellow 46L is covered in US Patent #4,256,458 (March 17, 1981) Basazol Yellow 46L is a cyanine-type dye. The patent information for this dye is summarized in Figure 20.

Figure 20 Patent Information Relevant to Basazol Yellow 46L Basic Yellow Dye

United States Patent

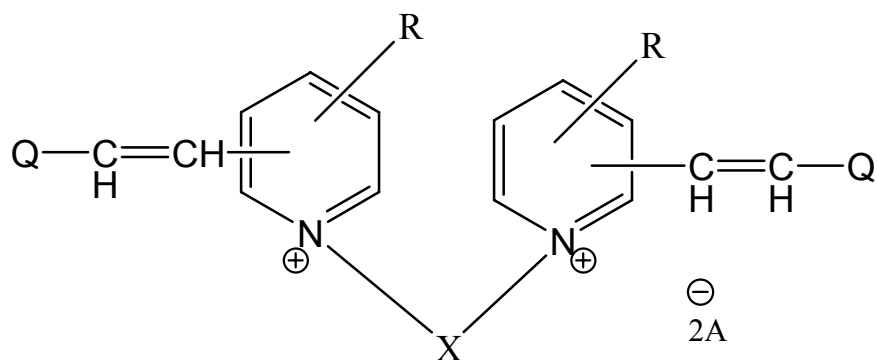
Number 4,256,458

March 17, 1981

Methine Dyes for Paper and Amonically-Modified Fibers

Inventors: Hans-Juergen Degen, Franz Feichtmayr, and Klaus Grychtol
 Assignee: BASF Aktiengellschaft, Ludwigshafen, Fed. Rep. of Germany

Patent applies to methine dyes of the formula

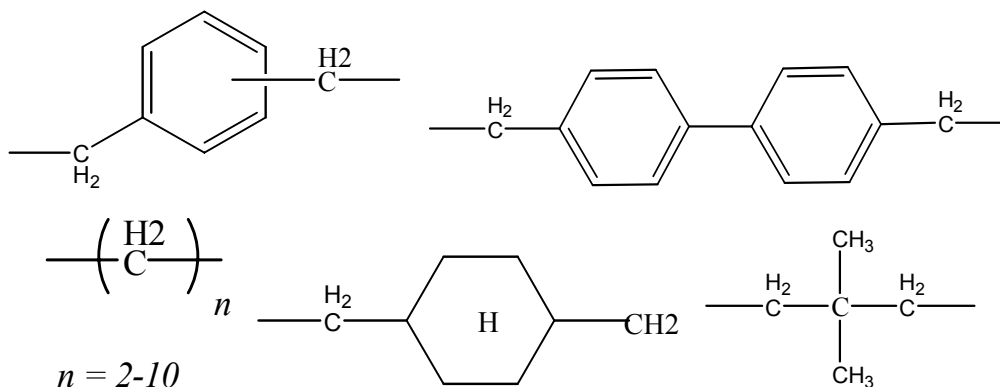


A is an organic or inorganic ion

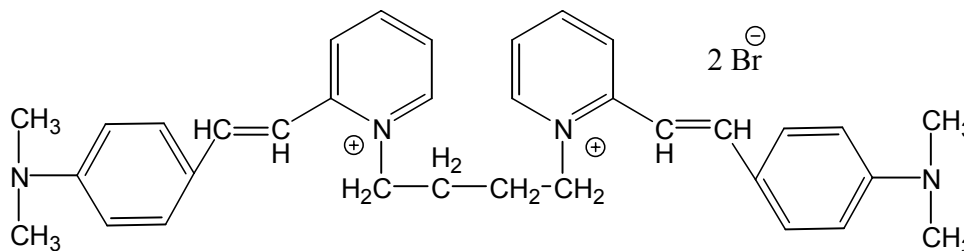
R is hydrogen, methyl or ethyl

Q groups are the same or different substituted or unsubstituted aryl or hetaryl radicals or selected from the group consisting of N, N-disubstituted aminophenyl, indolyl and carbazolyl radicals which are substituted or unsubstituted by chlorine, methoxy, ethoxy, methyl or ethyl groups.

X represents a bridge member selected from the group consisting of aliphatic, aromatic and heteroaromatic radicals such as the group below.



The synthesis of numerous dyes is described, and the resultant colors include yellow, orange and red. An example structure of an orange dye is shown below.



3.5.5 SUMMARY OF DYE CHEMISTRY

The possibility of organic compounds to absorb light and produce color is created by the overlap of π -orbitals. Dye chromogens consist of electron-donor(s) and electron-acceptor(s) interacting through a conjugated double bond system. Molar absorptivity and wavelength(s) of absorption are controlled by the electronic structure of the molecule and the amount of energy required for electron excitation. The molar absorptivities of major commercial dye chromogens are listed in Table 3-21.

Table 3-21 Molar Absorptivity of Chromogens as Described by Rivlin⁵³

Dye Type	Molar Absorptivity (ϵ)
Anthraquinone	5,00-15,000
Azo	20,000-40,000
Basic cyanine	40,00-80,000
Basic triarylmethane	40,000-160,000

3.6 DECOLORIZATION OF DYES

3.6.1 DEFINITION OF DECOLORIZATION AND RELATED TERMS

A number of different terms are used when discussing dyes. *Color-stripping* refers to the removal of dye molecules from the fiber. Reduction in absorbance or visible color is referred to as *bleaching* or as *decolorization*. Decolorization implies degradation or destruction of the dye chromogen, but decolorization can result from destruction of only a small portion of a large dye molecule. *Mineralization* refers to degradation of dye molecule to carbon dioxide and water. Biological systems may be able to decolorize dyes without mineralizing the dyes. If this occurs, intermediate byproducts will accumulate. In a process called biosorption, biomass may also remove dyes from liquid solutions by physical adsorption of the dyes to the cell membranes. Dead cells often exhibit greater biosorption than living material.⁸⁸

3.6.2 CHEMICAL DECOLORIZATION

The basic 'components' of dye molecules are listed in Table 3-22. Decolorization and/or color-stripping methods can be classified by how the method interacts with the dye components. Since light absorption is characteristic of the electronic structure of dye molecules, all of these approaches involve redox reactions that alter the available electron density.

3.6.2.1 Decolorization agents applied during paper recycling

Because of chemical differences, it is understood that no single chemical treatment is capable of handling all the various dyes present in wastepaper.⁸⁹ The treatments listed in the TAPPI database of dye-breaking agents⁹⁰ include chlorine,

hypochlorite, chlorine dioxide, hydrosulfite (dithionite), formamidine sulfinic acid (FAS), hydrogen peroxide, oxygen, and ozone at various concentrations, pHs, and temperatures.

Chlorine, chlorine dioxide, sodium hypochlorite, oxygen and ozone can be considered as degrading agents because they adversely affect pulping yield.³¹ They all destroy phenolic groups and carbon-carbon double bonds. With the exception of hypochlorite, these degrading agents do not have any affect on carbonyl bonds.

Hydrogen peroxide, sodium hydrosulfite, and formamidine sulfinic acid are nondegrading reagents. Their action is limited to the destruction of carbonyl groups, and thus pulp yield is not affected. FAS and hydrosulfite reduce just quinone structures, but peroxide can oxidize virtually all carbonyl groups. FAS is effective in treating many colored furnishes because FAS strips the dye from fiber in addition to reducing colored quinones and carbonyls to less colored phenols and hydroxyls.⁹¹

These bleaching agents can be compared to the decolorization approaches outlined in Table 3-22.

Table 3-22 Approaches to Decolorize Dyes or Color-Strip Dyed Fibers

Component	Examples	Approaches for Decolorization/Color-Strip
Electron-donating group (Auxochrome)	-NH ₂ , -NHR, -NR ₂ -OH -OR	Oxidize to lower electron density Split from molecule
Electron-accepting group (Chromophore)	-C=O -N=N- -NO ₂ -NR ₃ ⁺	Reduce to add electron density Split from molecule
Conjugated double bonds	-C=C- -N=N- -C=N-	Cleave double bonds by redox rxns Oxidize: C → C=O N → N=O Reduce: C → CH ₂ N → NH _s Add to bond(s): convert π bonds to σ bonds C=C → ClC-CCl or C-Cepoxide
Solubilizing groups	-SO ₃ Na, -COONa -N ⁺ H ₂ HCl ⁻ , -N ⁺ R ₃ Cl ⁻	Change pH to change solubility, fiber affinity
Reactive groups	Covalent bonds to fiber	Cleave dye-fiber bonds

The alkali that is added during wastepaper processing helps color-strip the dyed fibers. The cellulose fibers swell and dye molecules detach from the fibers. Chlorine, chlorine dioxide, and hypochlorite (oxidizing agents) are usually not very effective in

decolorizing dyes in general.⁵¹ Peroxide is an oxidizing agent that not very effective on the electron-rich direct dyes.⁵² FAS and hydrosulfite are reducing agents that are effective on most dyes, but not on C.I. Direct Yellow 11.⁵²

3.6.2.2 Decolorization of C.I. Direct Yellow 11

The structures of C.I. Direct Yellow 11 and possible cleavage products are shown in Figure 21. C.I. Direct Yellow 11 can be described as a symmetrical compound consisting of two, 4, 4'-dinitro, 2, 2'-disulfonated stilbene units that have been joined together by the conversion of a nitro group from each unit into an azo bond. This dye is applied to chemical pulps, which are commonly used in alkaline papermaking.

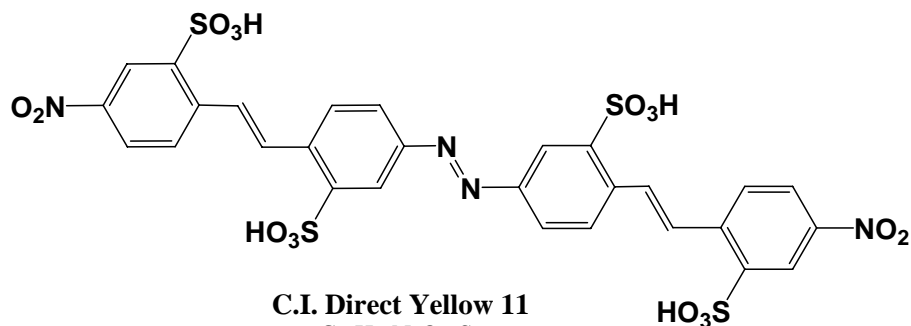
No electron-donating auxochromes are in the molecule. This structure lacks phenolic groups and cannot form a hydrazone. The nitro groups in the *para* position are strong chromophores, as is the azo bond. In addition, the electron-withdrawing *para* nitro groups reduce the already low π electron density of the stilbene units. The low electron density results in low reactivity and explains why C.I. Direct Yellow 11 does not react well with chlorine compounds, FAS, and hydrosulfite. The dye can be decolorized by ozone because ozone creates highly-reactive free radicals.

The yellow color of C.I. Direct Yellow 11 is created by absorbing blue light. Reduction of the terminal nitro groups would alter the absorption of the dye. However, creation of two electron-donating groups *para* to the electron-accepting azo group is unlikely to be helpful. The terminal amines could shift the absorption bathochromic, creating even more color.

The azo bond should be much more reactive than the stilbene because of the unpaired electrons on the azo nitrogens. Cleavage of the central azo bond would break conjugation between the stilbene units. Cleavage would shift absorption hypsochromic, to shorter wavelengths. The cleaved halves may absorb some ultraviolet light, but the visible absorption would disappear. Stilbene units are not rigid so the dye molecule 'halves' will not become fluorescent. Reduction of the azo bond or addition of oxygen across the azo bond would also be useful. Changing the azo bond interrupts the

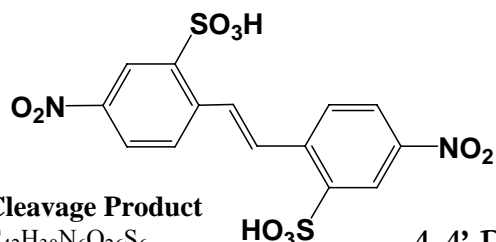
conjugated system, and would shift absorption from visible toward ultraviolet wavelengths.

Figure 21 C.I. Direct Yellow 11 and Possible Cleavage Products



C.I. Direct Yellow 11

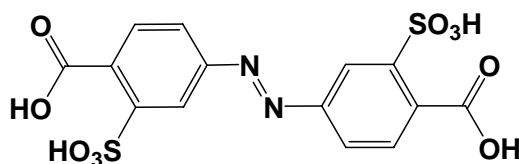
$C_{28}H_{20}N_4O_{16}S_4$
 Exact Mass: 795.98
 Mol. Wt.: 796.74
 C, 42.21; H, 2.53; N, 7.03; O, 32.13; S, 16.10



Azo Cleavage Product

$C_{42}H_{30}N_6O_{26}S_6$
 Exact Mass: 1225.95
 Mol. Wt.: 1227.1
 C, 41.11; H, 2.46; N, 6.85; O, 33.90; S, 15.68

4, 4'-Dinitrostilbene-2, 2'-disulfonic acid, CAS Registry # 128-42-7



4-[(4-carboxy-3-sulfophenyl) diazenyl]-2-sulfobenzoic acid

Stilbene Cleavage Product

$C_{56}H_{40}N_8O_{36}S_8$
 Exact Mass: 1655.93
 Mol. Wt.: 1657.47
 C, 40.58; H, 2.43; N, 6.76; O, 34.75; S, 15.48

3.6.2.3 Decolorization of Basazol Yellow 46L (C.I. Basic Yellow 96)

Without an exact structure, the response of Basazol Yellow 46L to chemical bleaches can not be predicted. However, this dye is considered unbleachable. Any system developed that decolorizes C.I. Direct Yellow 11 may also decolorize Basazol Yellow 46L.

3.7 ENZYMES

3.7.1 AN OVERVIEW OF ENZYMES

All living cells depend on enzymes. They produce enzymes to catalyze the reactions necessary to life. Although some catalytic RNAs have been found,⁹² the enzymes that regulate cellular metabolism are proteins. Each enzyme has a specific function, or biochemical reaction in which it participates. Enzymes vary greatly in the number of substrates. Some enzymes bind to only one specific molecule or a set of closely related molecules. Others will bind to a class of compounds such as all phenols small enough to enter the active site. In general, there are four kinds of enzyme affinities (Table 3-23).

Table 3-23 Four Types of Enzyme Affinities as per Ma and Jiang⁹³

Type of Affinity	Definition
Absolute	Enzyme has one and only one function
Group	Enzyme acts on molecules that have specific functional groups, such as amino group
Linkage	Enzyme acts on particular type of bond
Stereochemical	Enzyme acts on a particular stereo or optical isomer

The rates of enzymatically catalyzed reactions are much greater than the corresponding uncatalyzed reactions (by a factor of 10^6 - 10^{12}) (⁹⁴). Enzymatic reactions generally occur under mild conditions of pH (4-8), temperature (<100°C) and pressure (atmospheric). Not only are enzymatic reactions many times faster than uncatalyzed reactions, in many cases extreme conditions are required to chemically produce the same reactions. Some enzymes are so specific – a single enantiomer is recognized and a single product is produced – that the reaction can not be reproduced chemically.

Some enzymes require metal ions to function – most commonly, iron, zinc, copper and/or calcium. Enzymes may also require small molecules (coenzymes) that participate in the catalytic reaction. Cofactors range from dietary-based molecules such as pyridoxal-5-phosphate (from vitamin B6) to synthesized molecules like heme. Cofactors may be consumed/altered during the enzymatic reaction. Examples include the electron transport carriers NADPH (nicotinamide adenine dinucleotide phosphate) and NADH

(nicotinamide adenine dinucleotide) which cycle between oxidized and reduced forms as they participate in biosynthetic and energy generation reactions. The oxidation and reduction are generally performed by separate enzymes. Another example is the conversion of essential energy metabolism compounds ATP (adenosine triphosphate) to ADP (adenosine diphosphate) and. In the conversion of ATP to ADP, a high-energy phosphate bond is broken to provide energy the enzyme captures to drive an endothermic reaction.

3.7.2 NAMING OF ENZYMES

The International Union of Biochemistry and Molecular Biology (IUBMB) has adopted a systematic protocol for the classification and nomenclature of enzymes based on the nature of chemical reactions they catalyze. There are six main classes of reactions that enzymes catalyze. (Table 3-24). Within these major classes there are subclasses and sub-subclasses. Each enzyme is assigned two names and a four-digit classification.

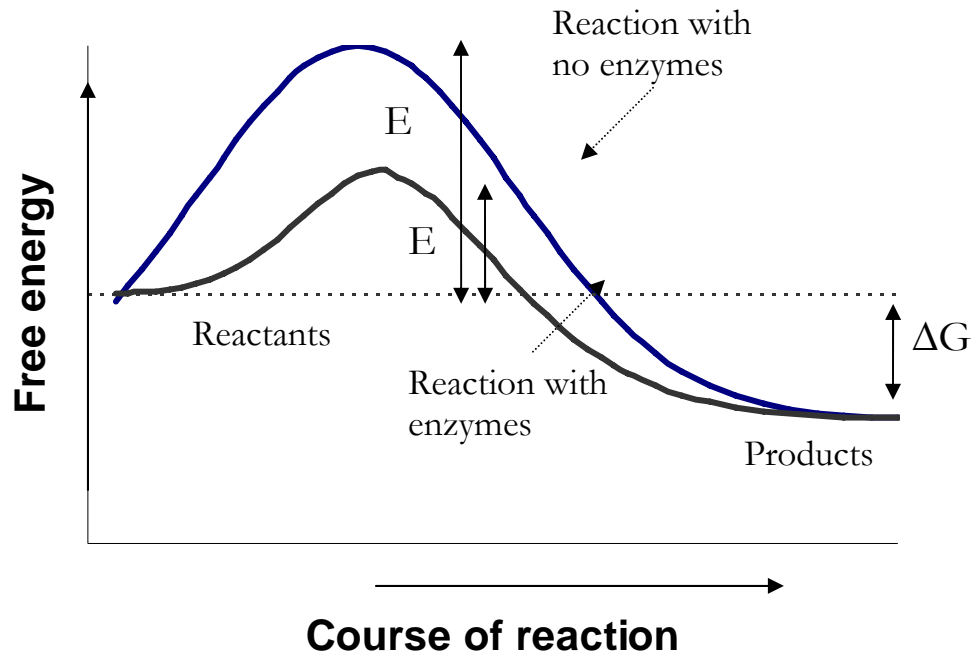
Table 3-24 Classification of Enzymes By Reaction Type as Described by Voet⁽⁹⁴⁾

Classification	Type of reaction catalyzed
1. Oxidoreductase	Oxidation-reduction reactions – adding or removing hydrogen atoms
2. Transferases	Transfer of functional groups between donor and acceptor molecules
3. Hydrolases	Hydrolysis reactions
4. Lyases	Group elimination to form double bonds or addition of water, ammonia or carbon dioxide across double bonds
5. Isomerases	Isomerization (such as L to D)
6. Ligases	Bond formation coupled with ATP hydrolysis

3.7.3 ENZYMATIC REACTIONS

As catalysts, enzymes greatly accelerate reactions without being consumed. Chemical reactions may not proceed, even if energy would be released, because a large activation energy is required to initiate the reaction. Enzymes lower the activation energy of reactions by many means including stabilizing transition states, controlling relative orientations of reactants in S_N2 reactions, and serving as electron transfer agents in multi-step redox reactions. The enzyme alters the reaction rate. The enzyme does *not* affect the change in free energy between reactants and products.

Figure 22 Enzyme Accelerates Reaction by Lowering Activation Energy
*Schematic as per Ma and Jiang*⁹³



A number of theories have been proposed to explain the catalytic reaction of enzymes. Arrhenius proposed that the substrate and the enzyme form an intermediate complex. The variation in binding affinities between an enzyme and related substrate compounds lead to the Lock-and-Key theory proposed by Emil Fischer in 1890. The binding affinity between enzyme and substrate depends on how well the substrate (“Key”) fits into the active site of the enzyme (“Lock”).

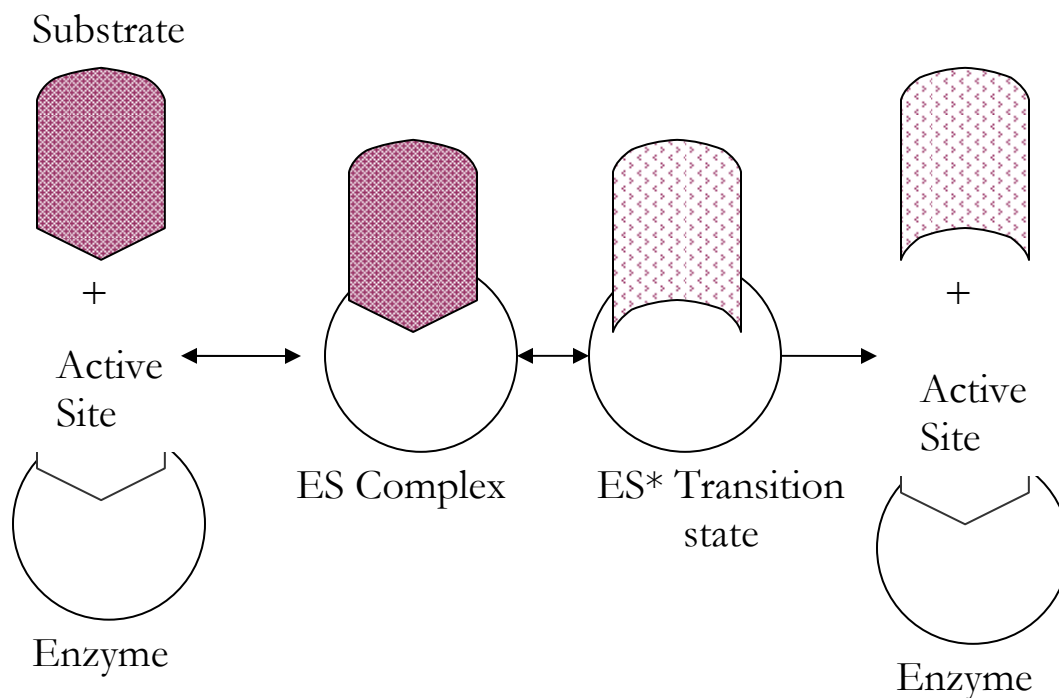
Figure 23 Enzyme Substrate Complex Theory as per Ma and Jiang⁹³



Where S is substrate, E is enzyme, ES is the enzyme-substrate complex, ES* is the transition state of the intermediate substance, EP is the enzyme product complex and P is product.

Although the theories have been refined in detail for many enzymes, they still serve as simplified yet adequate models of enzyme catalysis. They explain why the same enzyme from two related fungi or plant species can exhibit differences in substrate specificity. Each enzyme is made from unique sequence of amino acids that is specified by the cell that produces it. Although portions of a sequence may be conserved during evolution, enzymes serving the same function for two different species will exhibit slight changes in the protein sequence. The changes in protein sequence result in changes in the active site. Changes in the active site result in changes in the binding affinity between the enzyme and its substrates. Because of structural variations, an enzyme's binding affinity depends on the specific substrate. Different substrates also vary in reactivity. It is possible for a substrate to exhibit both strong binding affinity and poor reactivity. Binding affinity varies with how well the substrate fits in the enzyme's active site, while reactivity is dependent on electronic and steric factors.

Figure 24 Lock and Key Model of Enzyme-Substrate Interaction as per Ma and Jiang⁹³



3.7.4 FACTORS AFFECTING ENZYMATIC REACTIONS

Enzymes can be applied over a wide range of pH, temperature and solution conditions. However, enzyme activity is dependent on reaction conditions such as pH, temperature, and solution conditions such as ionic strength, presence/absence of metal ions and presence of inhibitory compounds. These factors must be controlled during enzymatic reactions.

Temperature is an important factor – both when the enzyme is applied and during storage. Like all chemical reactions, the rate of an enzyme-catalyzed reaction increases when reaction temperature is raised. All enzymes have optimum working temperatures at which optimum activity is observed. Often, an enzyme is maximally active at high temperatures that also lead to quick denaturation (irreversible inactivation) of the enzyme. Optimal conditions may depend on the length of the desired reaction period. Storage conditions are also important. Storage at 4°C or below is generally safe while storage at room temperature usually leads to loss of enzyme activity.

Since proteins are comprised of amino acid groups, the pH of a solution will affect the net charge on the enzyme. Thus changes in pH (concentration of $[H^+]$) alter the shape of the enzyme active site. Each enzyme has an optimum pH and a working pH range; extremes of pH outside the working pH range can denature the enzymes.

Concentrations of reaction components are also important and interrelated. Increasing either the substrate concentration or the enzyme concentration will increase the reaction rate until the enzyme active site is saturated and a maximum reaction rate is reached. The products from an enzyme reaction inhibit the reaction. Inhibition of an enzyme can be reversible (such as two substrates competing for the same enzyme active site) or irreversible (such as removal of a metal ion from the active site). All these factors have to be controlled to utilize an enzyme effectively.

3.7.5 ENZYME ACTIVITY

Since totally pure enzymes are difficult to prepare from living cells, and some activity is lost every time a solution is thawed or frozen, enzymes are usually quantified in terms of their activity. Enzyme activity is determined by measuring the rate of product

released from an enzyme-catalyzed reaction. The International Union of Biochemistry and Molecular Biology define an international unit (IU) as the amount of enzyme that catalyzes the conversion of 1 micromole of substrate to a product in 1 minute under the conditions of the assay. The International System Unit for enzyme activity is Katal (symbol: kat) which is the amount of enzyme converting 1 mole of substrate to product in 1 second under the assay conditions.

Equation 3 Conversion Between IU and Kat

$$1 \text{ kat} = 1 \text{ mole/second} = 60 \text{ mole/minute}$$

$$1 \text{ IU} = 1/60 \mu \text{ kat} = 16.67 \times 10^{-9} \text{ kat}$$

Because binding affinity and reactivity are substrate dependent, when an amount of an enzyme is given, the assay (either the substrate or the product) is also specified. As long as the same substrate and assay are used, enzymatic activities are comparable. However, the same enzyme preparation can have different IU or kat values if the substrate or assay conditions are altered.

3.7.6 OXIDOREDUCTASES

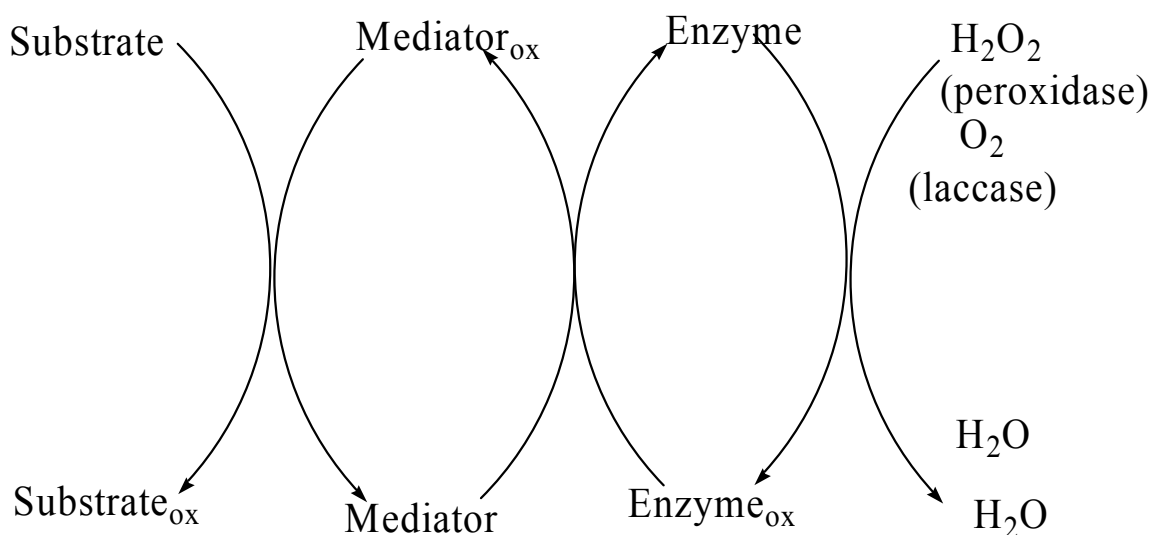
The ability of white rot fungi to digest trees and breakdown recalcitrant pollutants¹⁹⁷ has prompted research into the secreted enzymes of these fungi. Enzymes identified as participating in dye decolorization include laccase, manganese peroxidase and lignin peroxidase. These enzymes are oxidoreductases, which means they participate in redox reaction where one compound is reduced and another is oxidized. These enzymes contain metal ion(s). The metal center(s) and the enzyme itself undergo a redox cycle between at least one reduced and one oxidized form. Additional forms of the enzyme can exist for an enzyme like laccase that performs the four electron conversion of dioxygen to water using electrons collected one-at-a-time during the oxidation of four substrate molecules.

The addition of a mediator compound extends the range of compounds that can be oxidized by an oxidoreductase. The compounds that serve to mediate electron transfer reactions must meet a number of limiting criteria⁹⁵ outlined below (Table 3-25).

Table 3-25 Mediator Criteria for Oxidoreductase Reactions

1) Mediator functions as substrate of oxidoreductase enzyme
2) Both oxidized and reduced forms of the mediator are stable and do not inhibit enzyme activity
3) The mediator exhibits reversible electrochemical behavior

A general redox cycle is outlined below Figure 25.

Figure 25 Redox Cycle for Mediated Substrate Oxidation by an Oxidoreductase

3.7.7 LACCASE ENZYMES

Laccase belongs to a family of multi-copper oxidases that couple four one-electron substrate oxidations with the four-electron reduction dioxygen to water.⁹⁶ Spectroscopic studies, sequence alignments and crystal structures reveal that all multicopper oxidases contain at least one type 1 (T1), one type 2 (T2) and one type 3 (T3) Cu center.⁹⁶ Each type is defined by spectroscopic properties in the oxidized (Cu²⁺) state. The T1 site accepts electrons from the substrate. Type 3 is a binuclear site of two coupled Cu atoms. Type 2 and type 3 copper form a trinuclear copper cluster site that binds oxygen. The type 2/type 3 center distinguishes laccase from coupled binuclear copper proteins (such as tyrosinase) which are not able to produce water from dioxygen.⁹⁷

Laccase is the simplest of the multicopper oxidases, with one of each type of Cu center for a total of four Cu atoms. Other members of this family include plastocyanin,

human ceruloplasmin, ascorbate oxidase, and tree laccases. The type 2 and type 3 copper centers are very similar among these enzymes. However, the T1 Cu ligation for fungal laccase differs significantly. The change in protein structure alters the pH responsiveness and the redox potential. Redox potentials for some laccases are shown in Table 3-26.

Table 3-26 Comparison of Redox Potentials of the T1 Cu From Palmer⁹⁶

Enzyme	E° ^a
<i>Myceliophthora thermophila</i> laccase	450-480
<i>Rhizoctonia solani</i> laccase	630-680
<i>Polyporus pinsitus</i> (<i>Trametes villosa</i>) laccase	750-790
Spinach plastocyanin	370
<i>Coprinus cinereus</i> laccase ⁹⁸	550
<i>Trametes versicolor</i> laccase ⁹⁹	770

^a Units of mV vs NHE

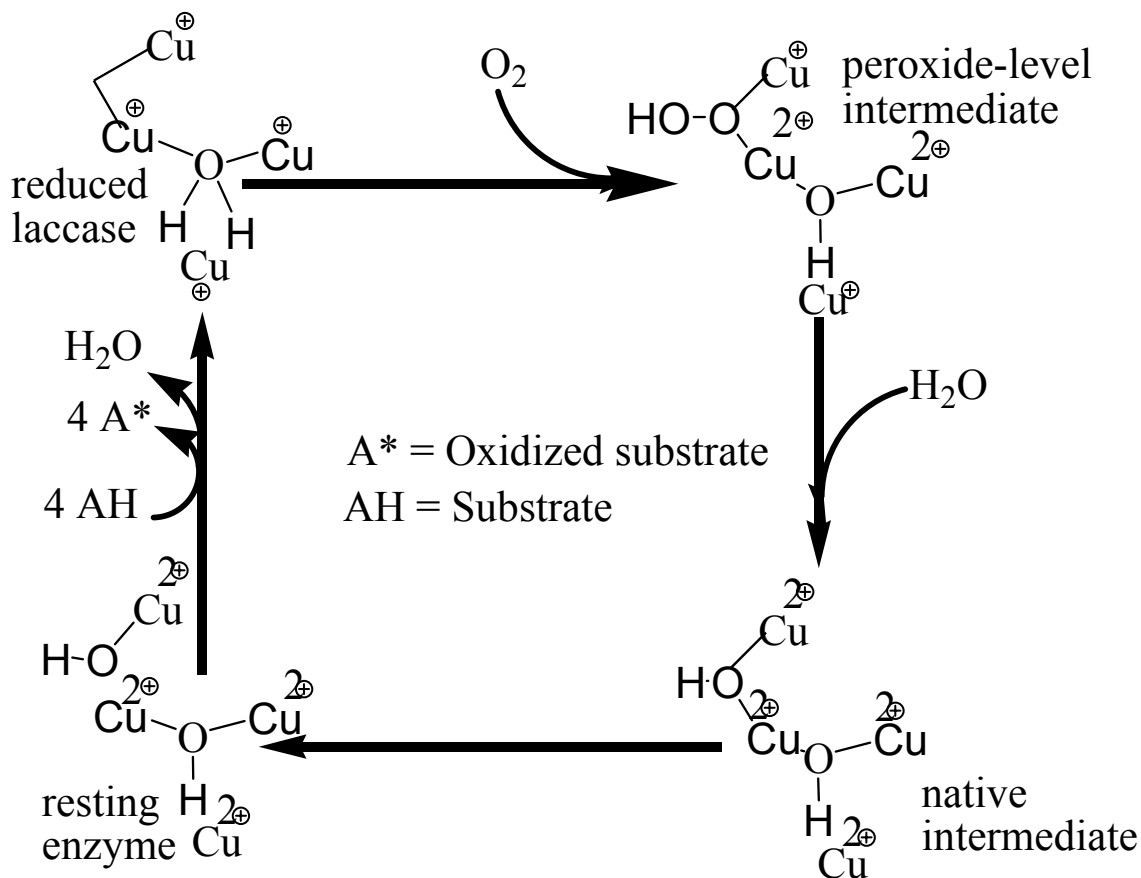
Laccase is classified as *p*-diphenol: dioxygen oxidoreductase, EC 1.10.3.2, where EC stands for the Enzyme Commission.¹⁰⁰ Laccases have a broad specificity that enables them to catalyze the one electron oxidation of a range of substrates. These substrates, usually phenols, provide electrons for the concomitant four electron reduction of atmospheric O₂ to water. This catalytic reaction depends on the four copper atoms¹⁰¹ found in the active sites. The molecular mass of the monomer¹⁰² ranges from 50 to 100 kDa. In trees, laccases participate in the biosynthesis of lignin. White-rot fungi secrete laccase and peroxidase enzymes to degrade lignin and enable digestion of cellulose from trees.

Enzymes such as poplar plastocyanin and zucchini ascorbate oxidase have redox potentials between 0.3 to 0.4 V. Laccases have higher redox potentials. They also fall into two groups. The 'low' redox potential laccases have E° values of 0.5-0.6 V. The 'high' potential laccases have E° values of 0.7-0.8 V.¹⁰³

Laccase can directly oxidize phenolic compounds. The reaction conditions would be as per McCarthy *et al.*¹⁰⁴ Since laccases have low redox potentials, to react with non-phenolic compounds, (like C.I. Direct Yellow 11); a low redox mediator is necessary. Also, since the enzyme is too large to enter cellulose fibers, mediator compounds are

added when biobleaching virgin pulp.⁸ The general redox cycle involving laccase and a mediator would be as in Figure 25 page49 with oxygen being converted to water. A schematic outline showing the oxidation state of the copper atoms during the reaction is shown below (Figure 26).

Figure 26 Laccase Catalytic Cycle as per Wesenberg¹⁰⁵



3.7.7.1 Laccase mediators

The production and the redox potential of laccases vary between fungal species. Likewise, the ability to bind to any particular mediator and to oxidize the mediator varies between laccases. Binding affinity is not directly correlated with speed of oxidation. Consequently, laccase effectiveness will change if the mediator compound is changed. In

addition, the optimum pH for dye decolorization depends on the enzyme, the dye, and the mediator.^{180,174} Different mediators are compared in Table 3-27.

Table 3-27 Structure and Properties of Laccase Mediators According to Paice¹⁰⁶

Mediator Name	Mediator Structure	Redox Potential vs SHE ^a V	Relative O ₂ uptake rate	% Pulp delignification ^b
HBT 1-Hydroxy benzotriazole		1.04*	1	45
NHA N-hydroxy acetanilide		0.83*; 1.01*	42	42
Violuric acid		0.91	14	42
ABTS 2,2'azinobis-3-ethyl-benzthiazoline-5-sulfonate		0.67; 1.09	100	37
HNNS 2-nitroso-1-naphthol-4-sulfonic acid		0.91*; 1.10*	46	34
NNDS 1-nitroso-2-naphthol-3,6-disulfonic acid		0.82	46	29
Promazine		0.77; 1.06*	59	18

*Electrochemically irreversible, redox potentials are lower than the values shown

^a SHE: Standard Hydrogen Electrode

^b Softwood oxygen delignified kraft pulp; initial Kappa = 15.

Mediator applied at 1% on pulp, followed by alkaline extraction.

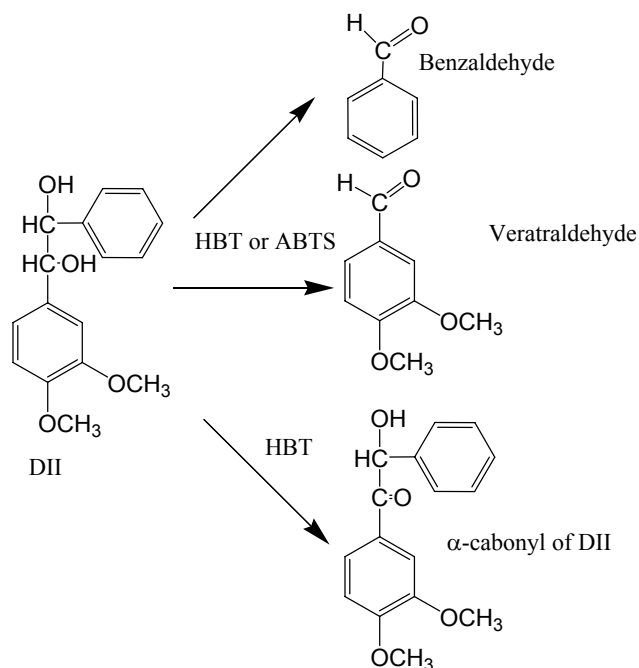
The first mediator introduced for biobleaching of virgin pulp with laccase was ABTS (2-2' azinobis-(3-ethyl benzthiazodine) in 1990.⁸ A new class of mediators containing the N-OH moiety was introduced in 1993.⁹ This class includes the mediators HBT (1-

hydroxybenzotriazole) and VA (violuric acid) used in this research as well as NHA, HNNS and NNDS as shown above in Table 3-27.

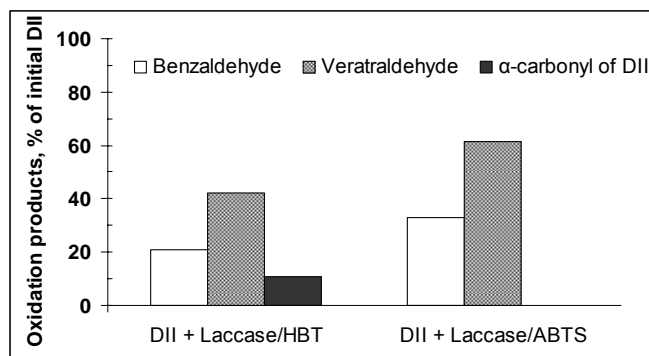
Changing from one mediator to another can change the products of the reaction. For example, when Bourbonnais *et al.*²⁰⁹ tested the reactivities of various mediators and laccase with lignin model compound they found that when β -1 dimer II (D II on left in Figure 27) was oxidized with *Trametes versicolor* laccase II, the products were veratraldehyde and benzaldehyde if ABTS was the mediator, but if HBT was the mediator, an α -carbonyl derivative of β -1 dimer II was also produced. The structures of the products are shown in Figure 27 and the relative amounts produced are shown in Figure 28.

Figure 27 Products From β -1 Dimer Produced by HBT and ABTS as Mediators

Schematic From Bourbonnais²⁰⁹



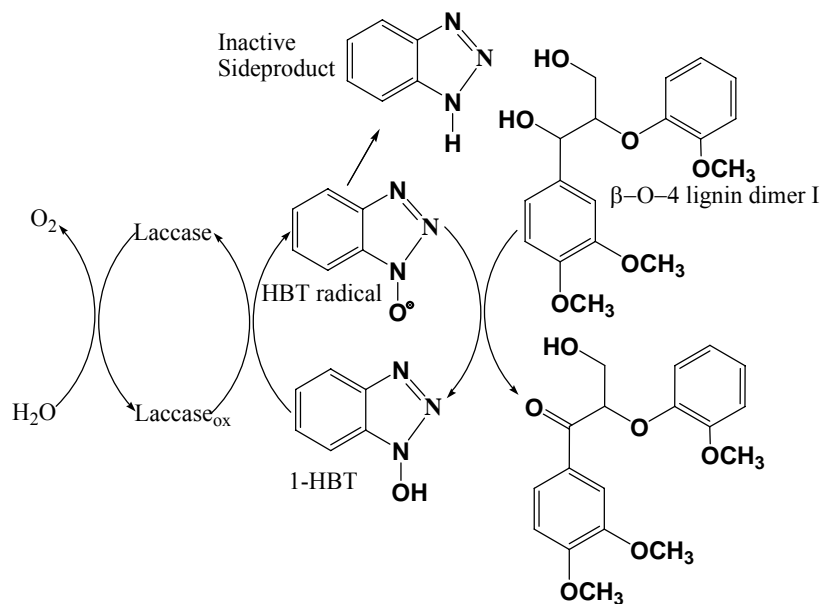
**Figure 28 Amounts of Products from Laccase Oxidation Depends on Mediator
Results from Bourbonnais²⁰⁹**



3.7.7.2 HBT - hydroxy benzotriazole as mediator

During the course of the reaction, the mediator abstracts an electron from the substrate which is then transferred to the enzyme. Figure 29 shows the HBT is reversibly reduced and oxidized by the formation of a nitroxyl radical. HBT, (1-hydroxy benzotriazole) is not fully catalytic because an inactive side product is occasionally formed.

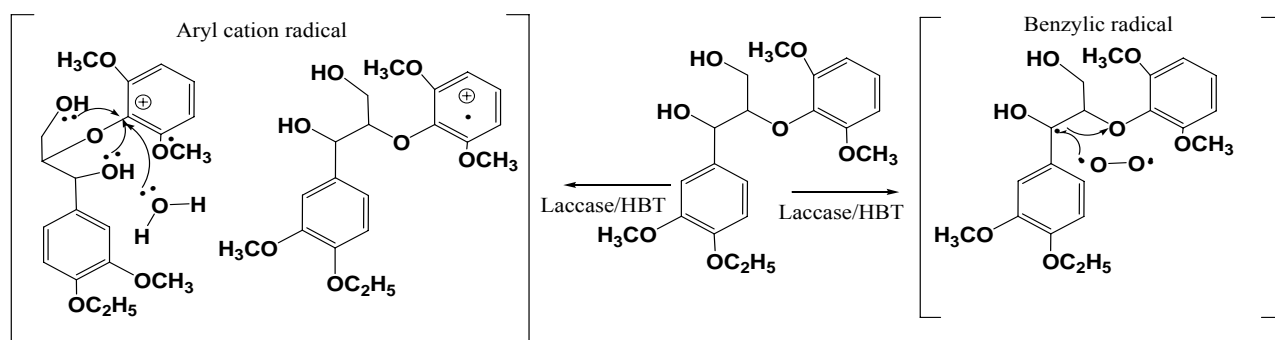
**Figure 29 Laccase/HBT Oxidation of β -O-4 Lignin Dimer
As Described by Bourbonnais²⁰⁹**



When treated with the laccase-mediator system, the substrate forms radicals which can lead to the formation of many different products. A thorough study of the

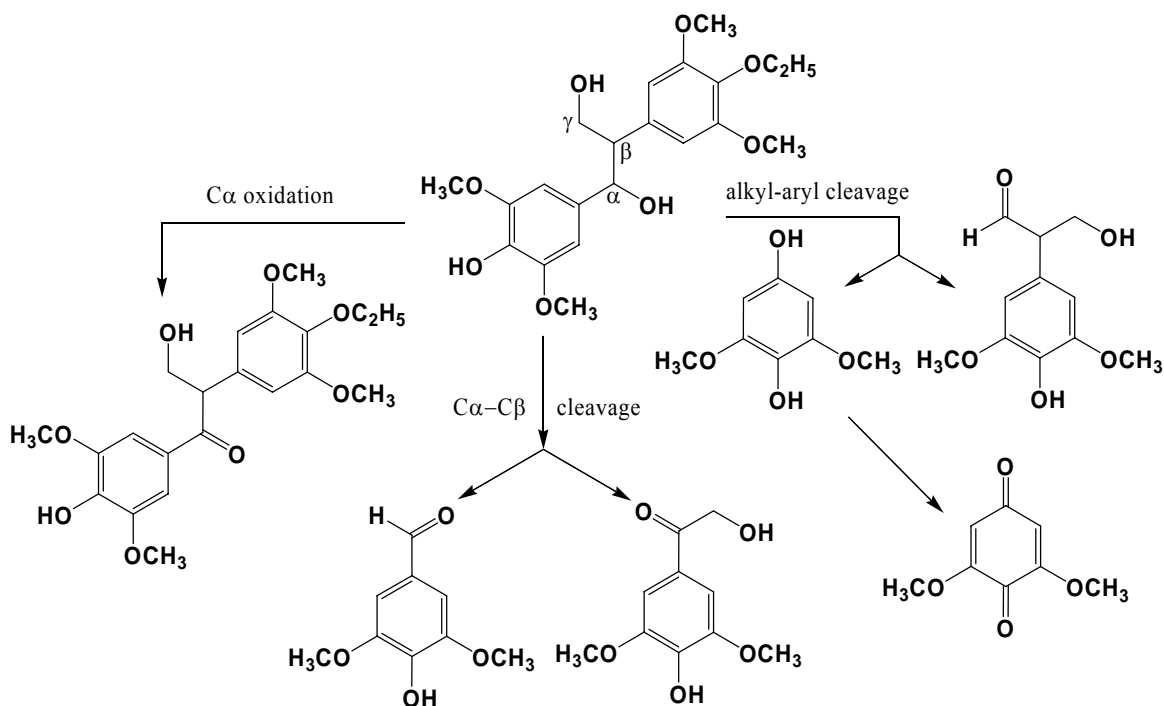
substrate degradation mechanisms has been made by Kawai *et al.*¹⁰⁷ They examined the incorporation of ^{18}O from H_2^{18}O and $^{18}\text{O}_2$. Treatment of a nonphenolic β -O-4 lignin dimer with *Trametes versicolor* laccase plus HBT has been reported to lead to $\text{C}\alpha$ - $\text{C}\beta$ cleavage, $\text{C}\alpha$ -oxidation, β -ether cleavage, and aromatic ring cleavage products.¹⁰⁷ The proposed initial radical formation mechanisms are shown in Figure 30. Most of the products they observed were formed from the aryl cation radical. They did find a novel $\text{C}\alpha$ - $\text{C}\beta$ cleavage that proceeded from the benzylic radical.

Figure 30 Formation of Benzylic and Aryl Cation Radicals from β -O-4 Lignin Dimer As Described by Kawai¹⁰⁷



The kinds of products that result from laccase-mediated oxidation of lignin are exemplified from the products shown in Figure 31 for the degradation of β -1 lignin model compounds. The degradation products shown were generated through three types of cleavages via phenoxy radicals. These were: $\text{C}\alpha$ - $\text{C}\beta$ cleavage, alkyl-aryl cleavage, and $\text{C}\alpha$ oxidation.

**Figure 31 Products of Laccase Degradation of Lignin through Phenoxy Radicals
As Per Higuchi¹⁰⁸**



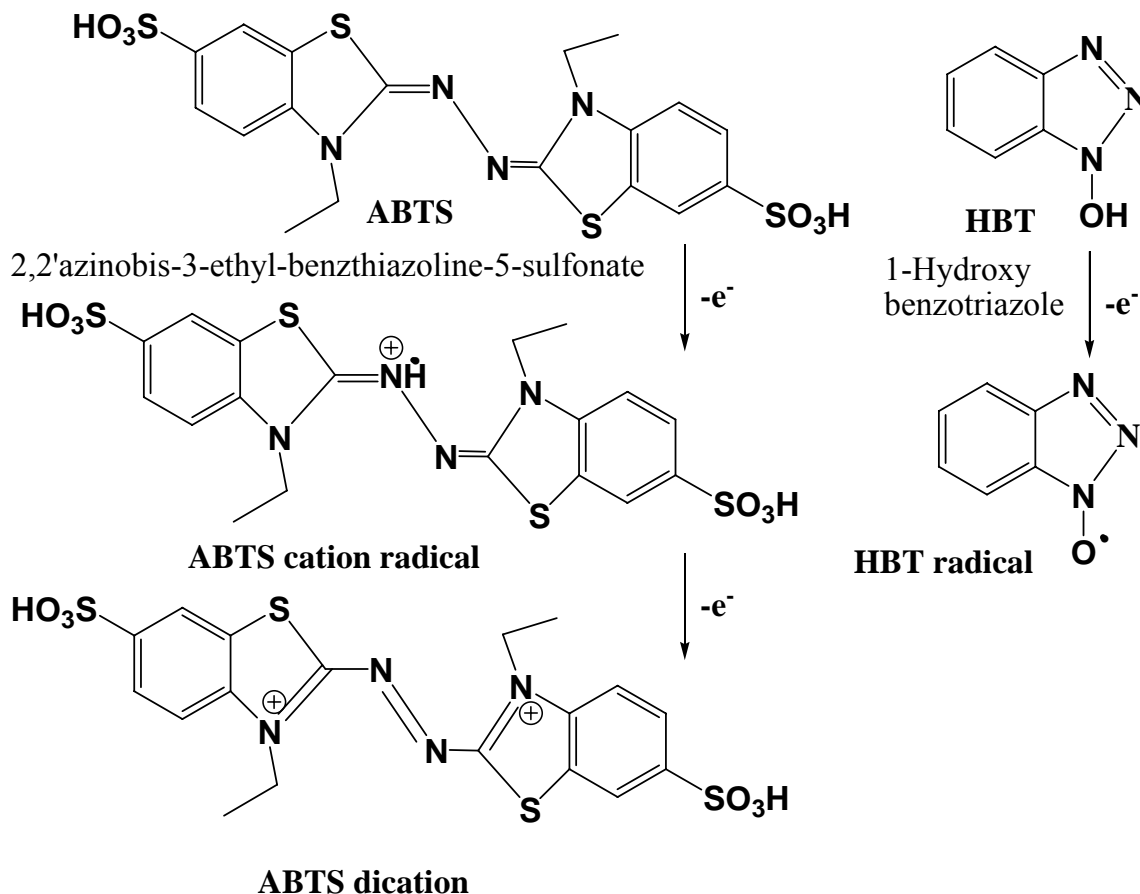
3.7.7.3 ABTS - 2,2'azinobis-3-ethyl benzthiazoline-5-sulfonate as mediator

The mechanism and kinetics of the reaction of ABTS with laccase are different than for HBT is able to transport one electron. ABTS however can abstract two electrons. The structures and intermediates formed are shown in Figure 32. Potthast *et al.*¹⁰⁹ explored the kinetics of the laccase-mediator oxidation of 2,4-dimethoxybenzyl alcohol to 2,4-dimethoxybenzaldehyde. Two distinguishable stages were observed in the consumption of dioxygen if ABTS was the mediator. In the first phase, a rapid decrease in oxygen occurred and no reaction product could be detected. In the second stage, oxygen consumption was slower and followed second-order kinetics while the benzaldehyde product was produced according to a zero-order rate law. In the first stage, ABTS was being converted to the oxidized species. In the second stage, the oxidized ABTS acts as the oxidant for the substrate. The formation of the ABTS radical cation is fast; the slower conversion of ABTS radical cation to the dication is the rate-limiting step in the oxidation of substrate. Increasing the ABTS/substrate ratio lead to less oxygen

consumption in the second stage indicating that the oxidized ABTS formed in the first stage acts as an oxidant reservoir.

The LMS reaction with HBT as mediator was different. No distinguishable phases could be observed. The reaction was characterized by comparatively slow uptake with zero-order kinetics throughout. The enzymatic oxidation of HBT to the HBT radical was the rate limiting step. Product formation also followed a zero-order rate law. Increasing the HBT/substrate ratio lowered the amount of oxygen consumed per equivalent of benzaldehyde formed, indicating that a different reaction pathway occurs at higher mediator concentrations. At low HBT/substrate concentrations, the HBT radical serves as a one-electron oxidant and is reduced to HBT in a reversible process. At higher HBT/substrate levels, the HBT radical acts as a three-electron oxidant and is irreversibly reduced to benzotriazole (the inactive side product shown in Figure 29). Potthast *et al.*¹⁰⁹ note that both mechanisms occur simultaneously when HBT is added at commonly applied concentrations.

The differences in mechanism between HBT and ABTS suggest that ABTS should be tested as well as the commonly applied nitroxyl mediators such as HBT. A poor result obtained with laccase/HBT system does not mean the laccase/ABTS system would also produce poor results.

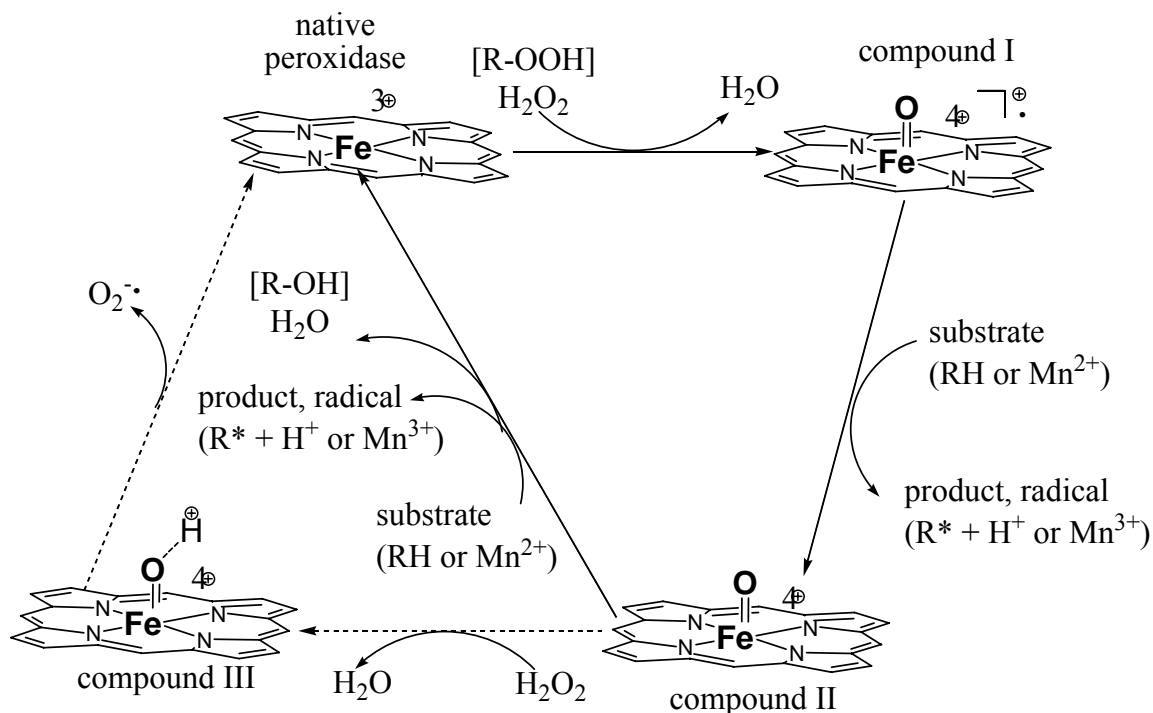
Figure 32 ABTS and HBT and Their Intermediates

3.7.8 PEROXIDASE ENZYMES

Peroxidases¹¹⁰ are heme (haem) containing enzymes that use hydrogen peroxide (H_2O_2) as the electron acceptor to catalyze a number of oxidative reactions.¹¹¹ In the resting state, the iron ion in the heme is in the +3 oxidation state. The catalytic process is a multistep reaction as outlined in Figure 33. The first step is the reaction of the active site with hydrogen peroxide. The peroxide is reduced to water. The protein has been oxidized by two electrons. This form of the enzyme is called Compound I and contains an oxyferryl ($Fe [IV] =O$) center and an organic cation radical.¹¹² Next, Compound I oxidizes one substrate molecule (R) to give a substrate radical ($R\bullet$) and Compound II, where the organic cation radical is reduced to its resting state. Finally, Compound II is reduced by a second substrate molecule to the resting $Fe [III]$ state. In the absence of an

electron donor, Compound II will react with hydrogen peroxide to form the inactive intermediate Compound III.¹¹³

Figure 33 The Peroxidase Catalytic Cycle as Visualized by Barr and Aust¹⁹⁷



Heme peroxidases include fungal lignin peroxidase, fungal manganese peroxidase, soybean peroxidase and horseradish peroxidase. Peroxidases are more reactive than copper oxidases like laccase. The relative reactivities of laccase (Lac), soybean peroxidase (SBP), horseradish peroxidase (HRP) and fungal lignin peroxidase (LiP) are illustrated in Table 3-28. The $E_{1/2}$ values are oxidation potentials for substrate molecules. Laccase is obviously much less reactive than the peroxidases. Although dye decolorization studies have focused on lignin and manganese peroxidases, the similarities between the fungal peroxidases, which have to be isolated from fungi, and the commercially available soybean and horseradish peroxidases suggest that soybean and horseradish peroxidases may also be able to decolorize dyes.

Table 3-28 Oxidation of Methoxybenzenes by SBP, HRP, and LiP as per McEldoon^{a,114}

Compound	$E_{1/2}$ V ^c	SBP/ H_2O_2	HRP/ H_2O_2	LiP/ H_2O_2	Lac/ O_2 ¹¹⁵
----------	--------------------------	---------------	---------------	---------------	---------------------------

Methoxybenzene	1.76	-	-	-	-
1,3,5-Trimethoxybenzene	1.49	-	-	±	-
1,2-Dimethoxybenzene	1.45	-	-	+	-
1,2,3-Trimethoxybenzene	1.42	±	-	+	-
1,4-Dimethoxybenzene	1.34	+	+ ^b	+	-
Hexamethoxybenzene	1.24	+	+ ^b	+	-
1,2,4-Trimethoxybenzene	1.12	+	+	+	-
1,2,3,5-Tetramethoxybenzene	1.09	+	+	+	-
Pentamethoxybenzene	1.07	+	+	+	-
1,2,4,5-Tetramethoxybenzene	0.81	+	+	+	+

+ strongly reactive ± slightly reactive - unreactive Reactions at pH 2.4

^a SBP=soybean peroxidase HRP=horseradish peroxidase

LiP=*Phanerochaete chrysosporium* lignin peroxidase

^b HRP was active with these compounds at pH 2.4 only if initiated by enzyme addition. Initiation by peroxide addition produced no reactivity

^c E_{1/2} relative to saturated calomel electrode

Peroxidases (donor: H₂O₂ oxidoreductase) are classified as EC 1.11.1.X - where X is the digit that represents the particular type of peroxidase in question.

The overall reaction is as follows: donor + H₂O₂ <=> oxidized donor + 2 H₂O

Peroxidases are found in bacteria, fungi, plants and animals. Fourteen subclasses of peroxidases have been defined. On the basis of sequence similarity, fungal, plant and bacterial peroxidases can be viewed as members of a superfamily consisting of three major classes.¹¹⁰

Class I, the intracellular peroxidases, include yeast cytochrome c peroxidase, ascorbate peroxidase and bacterial catalaseperoxidases, which exhibit both peroxidase and catalase activities.

Class II consists of secretory fungal peroxidases: ligninases, or lignin peroxidases (LiPs), and manganese dependent peroxidases (MnPs). These are monomeric glycoproteins involved in the degradation of lignin. The peroxidases most commonly studied for dye decolorization are fungal lignin peroxidase and manganese peroxidase.

Class III consists of the secretory plant peroxidases, which have multiple tissue specific functions: e.g., removal of hydrogen peroxide from chloroplasts and cytosol; oxidation of toxic compounds; biosynthesis of the cell wall; defense responses towards wounding; indole3acetic acid (IAA) catabolism; ethylene biosynthesis; and so on.¹¹⁶

Class III peroxidases are also monomeric glycoproteins, containing four conserved disulphide bridges and two calcium ions. The peroxidases used in this project are

horseradish peroxidase and soybean seed coat peroxidase. Both are classified as peroxidase E.C.1.11.1.7 and are included in the class III superfamily.

3.7.8.1 Lignin peroxidase

Lignin peroxidase has an unusually high redox potential. A particular question has been how Lip could catalyze the oxidation of compounds having a higher redox potential than those estimated for LiPI (Compound I) and LiPII (Compound II). It has been inferred¹¹⁷ from kinetic studies that LiPI and veratryl alcohol (VA) react to form a LiPII-VA^{•+} that has a higher redox potential than uncomplexed LiPI and LiPII. The redox potentials for the Fe³⁺/Fe²⁺ couple in some peroxidases are shown in Table 3-29.

The crystal structure for lignin peroxidase has been defined to 1.70 Å.¹¹⁸ Lignin peroxidase shows the "typical peroxidase fold" and the heme has a close environment as found in other peroxidases.¹¹⁸ The bond between the heme iron and the N atom of the proximal histidine residue is longer than in cytochrome c peroxidase.¹¹⁸ The weaker Fe-N bond would make the heme more electron-deficient and may explain the higher redox potential. In addition, a surface residue tryptophan is stereospecifically hydroxylated by an autocatalytic process. A transient radical may form during the catalytic cycle that is different from the type of radical expected for this type of peroxidase.¹¹⁸

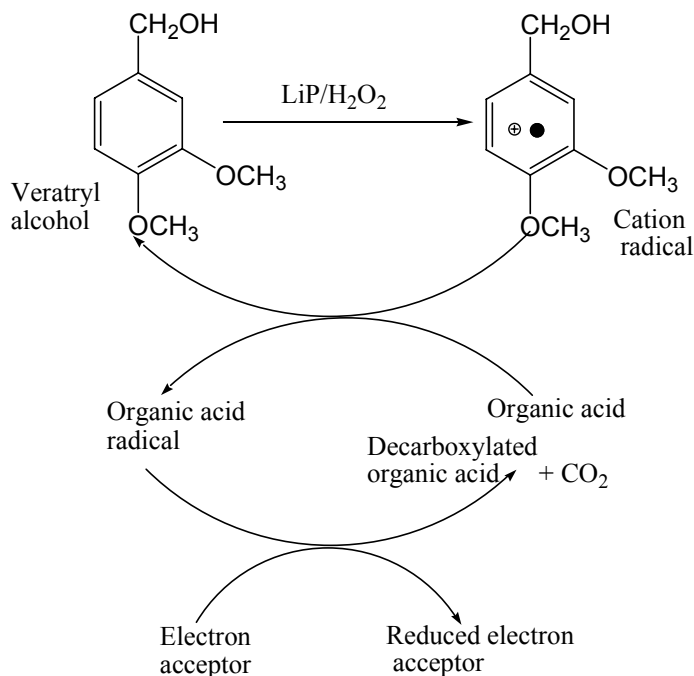
Table 3-29 Redox potential of Several Heme Proteins as per Millis and Cai^{119,120}

LiP Lignin peroxidase	-130 mV
MnP Manganese peroxidase	-90 mV
HRP Horseradish peroxidase	-278 mV
CcP Cytochrome c peroxidase	-194 mV
Turnip peroxidase (P ₁)	-223 mV
Turnip peroxidase (P ₇)	-100 mV
Chloroperoxidase	-140 mV
Lignin peroxidase H1	-142 mV
Lignin peroxidase H8	-137 mV
Lignin peroxidase H2	-135 mV
Lignin peroxidase H10	-127 mV
Mn-dependent peroxidase H4	-93 mV
Mn-dependent peroxidase H3	-88 mV

It is not clear what roles laccase, lignin peroxidase and manganese peroxidase have during delignification. Fungi have been found that produce only one of these three

enzymes yet are still capable of delignification. Many ligninolytic fungi produce veratryl alcohol (VA, 3,4-dimethoxybenzyl alcohol) that serves as cofactor in lignin peroxidase degradation of lignin¹²¹ Veratryl alcohol may function as a redox mediator in the indirect oxidation of compounds by LiP¹²¹ (Figure 34), protect lignin peroxidase from inactivation by excess hydrogen peroxide¹²² or serve to complete the catalytic cycle.

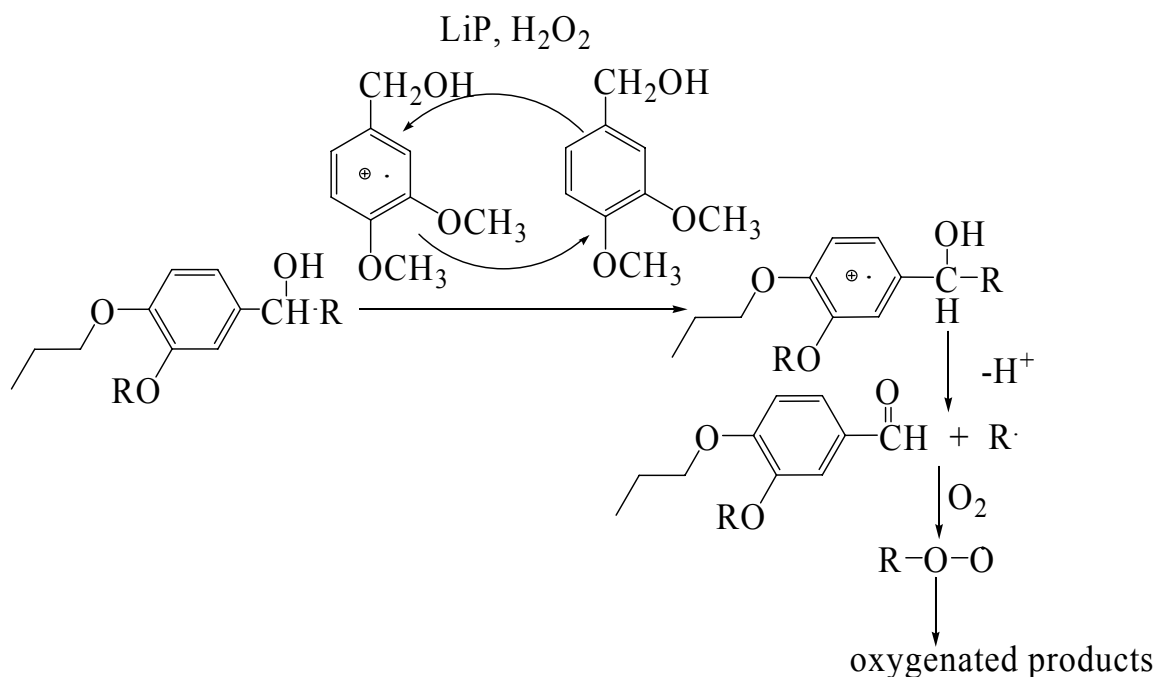
Figure 34 Mechanism of Mediated Lignin Peroxidase Oxidation



Organic acids shown to be electron donors include EDTA and oxalate (which is secreted by the fungus)
 Electron acceptors studied include cytochrome c, nitroblue tetrazolium, tetranitromethane, molecular oxygen, carbon tetrachloride and ferric iron.

Lignin peroxidase (LiP) catalyzes the H₂O₂-dependent oxidation of veratryl alcohol (VA) to veratraldehyde, with the enzyme-bound veratryl alcohol cation radical (VA^{•+}) as an intermediate.¹¹⁷ Subsequently VA^{•+} undergoes C α -H protonation¹²³ to form a carbon centered radical which is eventually converted to veratryl aldehyde.¹²⁴ (Figure 35) From kinetic studies of the deuterium isotope effect, it appears that the veratryl alcohol radical cation, once generated by the lignin peroxidase/hydrogen peroxide system, is released from the enzyme and is deprotonated by the medium.¹²⁴

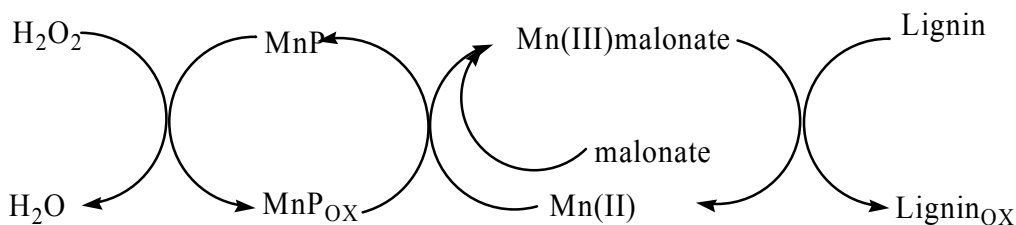
Figure 35 Veratryl Alcohol as Mediator for Peroxidase Reactions
As Published by Basiocchi¹²⁵



3.7.8.2 Manganese peroxidase

Both lignin peroxidase and manganese peroxidase can oxidize substrates by mediated reactions. For manganese peroxidase, the mediator is Mn²⁺/Mn³⁺ chelated by an organic acid like malonate or oxalate. The mechanism for mediated oxidation of lignin by manganese peroxidase is shown below. Chelated Mn²⁺ binds to MnP and is oxidized to Mn³⁺. The Mn³⁺-complex diffuses to the media where it oxidizes the final substrate.

Figure 36 Manganese Peroxidase Mechanism



The chelation of Mn²⁺ appears to be necessary. In the presence of succinate, a weak chelator, most of the Mn²⁺ is present as free Mn²⁺ and the reduction of MnP-II is

slow.¹²² If oxalate or malonate are present, most of the Mn^{2+} is complexed and the rate of MnP-II reduction is increased.

3.7.8.3 Horseradish peroxidase

Horseradish peroxidase (HRP) is an important enzyme that has been studied for more than a century.¹²⁶ Fifteen peroxidase isozymes have been isolated from horseradish root. Of these, horseradish peroxidase C (HRP C) is the most studied. Most reactions catalyzed by HRP C can be expressed as



Where AH_2 and AH^* represent a reducing substrate and its radical product, respectively.

Typical reducing substrates include aromatic phenols, phenolic acids, indoles, amines and sulfonates. The mechanism of catalysis has been investigated extensively (for reviews see Veitch 2001,¹²⁷ Dunford 1999¹²⁸) HRP has many applications and has been used as a reagent for organic synthesis and biotransformation. It is used in coupled enzyme assays, chemiluminescent assays and immunoassays (for reviews of applications see Veitch 2001,¹²⁷ Kreig 2003¹²⁹ and Ryan 1994¹³⁰). HRP has also been investigated for the treatment of waste waters and color removal from effluent from paper mill bleach plant.¹³¹

3.7.8.4 Soybean peroxidase

Soybean peroxidase (SBP) is noteworthy for its stability. It exhibits high heat stability with 66% of the enzymatic activity remaining after 90 minutes at 80°C and pH 5.0¹³² It is also stable over a wide pH range^{250,251} (pH 2.4-8.0). Inclusion of polyethylene glycol (PEG) has been reported to stabilize the enzyme.²⁵² One major application for soybean peroxidase is the polymerization of phenols, biphenols, anthraquinones and anilines^{133,134} SBP has also been used for enantioselective sulfoxidations¹³⁵ and for brominations.¹³⁶ SBP can be used to catalyze reactions in ionic liquids¹³⁷ and in organic solvents¹³⁸ Other applications include bioremediation of phenolic wastes¹³⁹ and of soils contaminated with chlorophenols.¹⁴⁰ Dye decolorization with soybean peroxidase has also been reported.¹⁴¹ The reaction mechanism for soybean peroxidase is similar to other

the peroxidases. Like the other peroxidases, inclusion of a mediator such as veratryl alcohol extends the range of compounds that the enzyme can oxidize.

3.8 BIOLOGICAL DEGRADATION AND DECOLORIZATION

Removal of color from textile plant effluents is currently a major problem.^{142,143} Overall decolorization of dyes is being heavily researched and a number of reviews articles^{144,145,6,146,147,148,149} about biological effluent treatments have been published. After a brief discussion of the current status, some papers involving azo dyes will be discussed to illustrate the chemistry involved. The discussion of biological degradation will focus on azo dyes since C.I. Direct Yellow 11 contains an azo bond and azo dyes are commonly studied. However, many of these studies did also include other dyes.

3.8.1 SUMMARY OF EFFLUENT DYE DECOLORIZATION RESEARCH

Effluents containing textile dyes can be subjected to chemical, physical or biological treatments. Chemical treatments are usually oxidative processes and include ozonation, photochemical treatment, addition of sodium hypochloride (NaOCl), electrochemical destruction and addition of Fenton's reagent (H_2O_2 -Fe (II) salts).¹⁴⁸ Physical methods are based on adsorption of dyes to solid materials. Although activated carbon is most commonly used, cost considerations have promoted application many other cheap adsorbents such as peat, wood chips, fly ash corn cobs, rice hulls, etc. Dead biomass (fungal or microbial) may also be utilized in this fashion.

Concerns about cost and possible toxicity of dyes continue to prompt research into biological treatments. Standard municipal aerobic treatments that rely on biological activity are inefficient in dye removal.¹⁴⁸ The biological treatments consist of four basic types: physical adsorption to dead biomass; treatment by anaerobic bacteria; treatment with aerobic fungi; and treatment with enzymes from white-rot fungi. Research into biological degradation of dyes also includes the reductive degradation to which ingested dyes are subjected.

3.8.2 AZO REDUCTASES IN MAMMALS AND INTESTINAL MICROFLORA

At least 3000 azo dyes are used commercially.¹⁵⁰ Five azo dyes are used as food colorants: Citrus Red No. 2, Allura Red, Tartrazine, Sunset Yellow and Orange B. More than 2 million kilograms of these dyes are consumed yearly. Consequently, the metabolism of these dyes has been well studied.

Many of the early azo dyes were benzidine derivatives. Aromatic amines, such as benzidine, induce urinary bladder cancer in man and tumors in some experimental animals.^{151,152,153} Many times intact dyes, like Trypan Blue, are not mutagenic but an intestinal anaerobe reduces the dye to products that are mutagenic. The many different intestinal microflora capable of reducing azo dyes. are anaerobes or facultative anaerobes. Hepatic enzymes can also metabolize a variety of azo dyes. While the reduction of azo dyes by intestinal microflora and hepatic enzymes is important to the toxicity of the dye, neither method is applicable the decolorizing colored fibers.

3.8.3 MICROBIAL TREATMENT OF TEXTILE EFFLUENTS

Conventional wastewater-treatment methods are biological oxidation (by bacteria) and chemical precipitation.^{154,155} However, in the case of textile dyes, biomass often appears to remove color by absorption,^{156,157,158} not metabolism. Even dead mushrooms have been used to remove color,^{142,159} and biosorption is generally more effective with dead microbes than live ones.^{88,160,156} Some azo dyes have been identified as the most problematic compounds in textile effluents as they are difficult to remove due to their high water solubility and low exhaustion.¹⁶¹

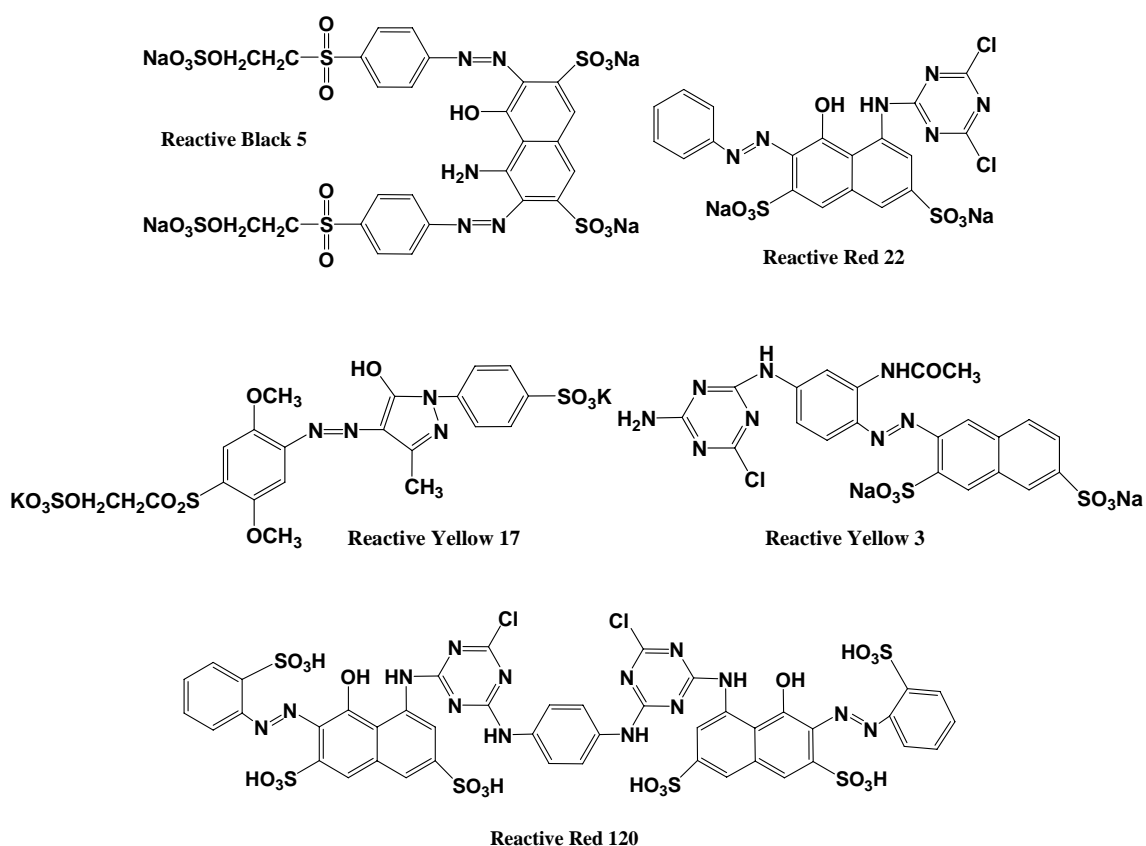
3.8.3.1 Dye decolorization by inoculated soil microorganisms

In an attempt to find bacteria that could degrade textile dyes, Nigam¹⁶² isolated bacteria from columns of soil samples inoculated with textile effluent over a year earlier. He found five aerobic organisms that could grow on media containing textile dyes. However, no decolorization was observed. From an anaerobic system, he isolated two bacterial strains and one fungus. The fungus grew well but produced no visual decolorization. The bacteria could grow aerobically, but decolored the test dyes only when grown anaerobically.

3.8.3.2 Decolorization of reactive azo dyes by methanogenic bacteria

Beydilli *et al.*¹⁶³ have been able to decolorize reactive azo dyes (Figure 37) with bacteria under methanogenic (low redox potential) conditions. They are trying to develop a fixed-film anaerobic reactor to treat textile dyebaths. Supplementing the cultures with an additional carbon source (dextrin and peptone) increased the production of methane and the extent of color removal.

**Figure 37 Reactive Azo Dye Substrates Decolorized by Methanogenic Bacteria
As Published by Beydilli¹⁶³**

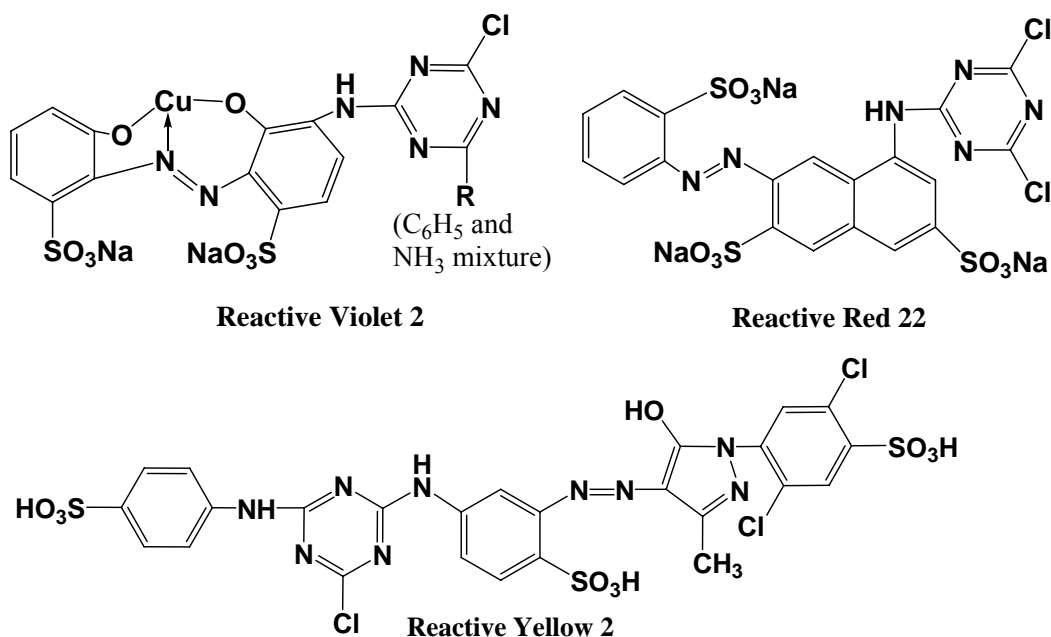


3.8.3.3 Decolorization of 3 azo dyes by *Pseudomonas luteola*

Hu¹⁶⁴ reported that the facultative anaerobe *Pseudomonas luteola* could decolor three azo dyes when grown 5 days through shaking-static incubation. Of the three dyes tested (Figure 38), all were chlorinated and one did not have any oxygen atoms attached to an aromatic ring. *P. luteola* produced an inducible azoreductase. HPLC analysis of the culture supernatant did detect 2-aminobenzene sulfonic acid (or orthonilic acid), the

metabolite expected from azo bond cleavage. However, the amount of reduction metabolite detected was lower (30%) than the amount of dye decolorized. Hu did not determine the cause. The low amount of orthanilic acid detected may have been due to difficulty in extracting the metabolite from the culture supernatant. The low amount of orthanilic acid could also indicate that the main degradation mechanism is the reduction of the azo double bond to a single bond (with no cleavage).

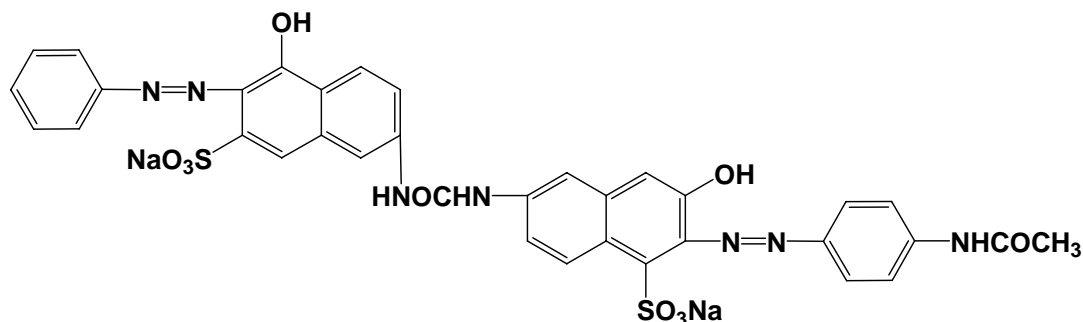
Figure 38 Reactive Azo Dye Substrates Decolorized by *Pseudomonas luteola*
As Published by Hu¹⁶⁴



3.8.3.4 Dye decolorization by microbial consortium

Using samples from the wastewater treatment facilities of a local dyeing house, He *et al.*¹⁶⁵ isolated four fungi and four bacterial strains that were highly effective at decolorizing Direct Fast Scarlet 4BS (4BS), a model azo dye substrate. An optimal microbial consortium was determined through combination decolorization experiments.

Figure 39 Direct Fast Scarlet 4BS as per He¹⁶⁵



He *et al.* performed decolorization experiments of individual strains and the microbial consortium under static and shaking culture conditions. In their experiments, when gel beads were included, the fungus 8-4* grew in the peripheral, high oxygen regions while *Pseudomonas 1-10* colonized the interior. They measured the rate of decolorization. By spectrophotometer, they monitored the chemical alterations in the dye molecule as it was mineralized. The key step to decolorization of azo dyes is usually reduction and cleavage of the azo bonds by azo reductase under anaerobic conditions to form the corresponding amines.^{166,167} Based on their results, He *et al.* propose that the decolorization mechanisms and degrading pathway as follows: azo bonds were firstly cleaved by the azoreductase of *Pseudomonas 1-10*. The white-rot fungus 8-4* was stimulated to increase its production of extracellular enzymes. Synergism between *Pseudomonas 1-10* and the extracellular enzymes of white-rot fungus 8-4* increased decolorization of 4BS, leading to complete mineralization.

3.8.3.5 Summary of Microbial Treatments of Textile Effluents

Efforts are underway to apply microbial treatment to effluents from textile dyeing. Different reactor designs are currently being developed.^{168,169,170} The faster growth of bacteria versus fungi suggests that microbial treatment may be more effective than systems based on white rot fungi. The dependence of microbial decolorization on azo reductase production by anaerobic microbes is not a significant limitation in the treatment of wastewater. However, the requirement for anaerobic conditions indicates that this method of dye decolorization is not suitable for treatment of colored pulp fibers.

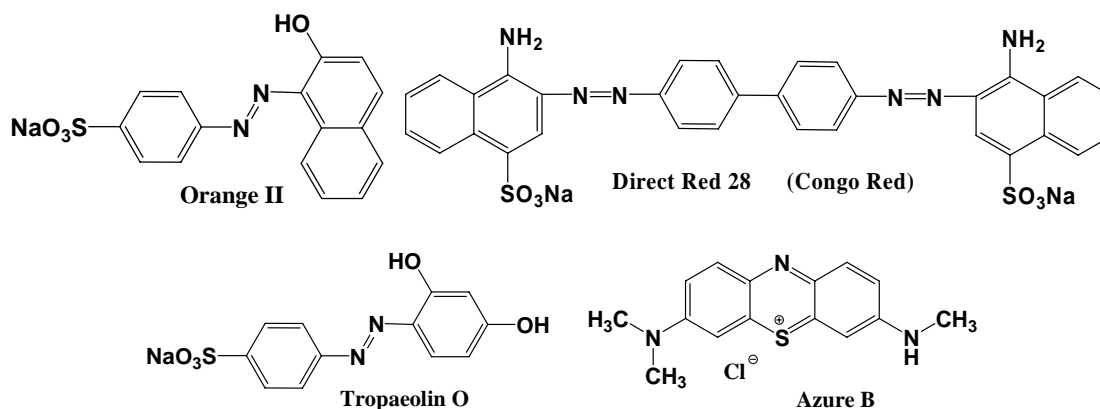
3.8.4 DECOLORIZATION OF DYES BY INTACT FUNGI

A number of white-rot fungi have been reported to degrade textile dyes. The anthraquinone dyes are structurally similar to the phenolic units in lignin. To develop an easier assay for lignolytic activity, in 1983 Glenn and Gold¹⁷¹ tested *Phanerochaete chrysosporium* for the ability to degrade three polycyclic aromatic carbonyl dyes. The fungus decolorized the dyes, but only during secondary metabolism, when lignolytic activity appears in this fungus. Chemical inhibitors of lignin degradation also inhibited dye degradation. Likewise, a mutant lacking lignolytic activity was unable to decolorize the polymeric dyes.

3.8.4.1 Decolorization of azo dyes by *Phanerochaete chrysosporium*

Cripps *et al.*¹⁷² grew *Phanerochaete chrysosporium* cultures for six days, then added azo dyes to the media. (The dyes are shown in Figure 40) To determine the extent of decolorization, the absorbance of the culture media was measured at the λ_{Max} for each dye. Extensive loss of color occurred during five days of culture, with most color loss occurring within the first six hours. To determine whether the dye was degraded or absorbed to the mycelium, Cripps extracted the mycelial mat with methanol. After five days, Azure B, Tropaeolin O and Orange II contained no dye. For Congo Red, 6% of the applied amount could be extracted after five days of growth and allowing the cultures to grow 12 days still did not result in complete decolorization of Congo Red.

Figure 40 Azo Dyes Degraded by *Phanerochaete chrysosporium* as per Cripps¹⁷²



Lignolytic activity is induced by nitrogen-limiting conditions. In nitrogen sufficient cultures, substantial amounts of dye (11 to 49%) were bound to the mycelia and not degraded. Using crude lignin peroxidase from nitrogen-limited cultures of *P. chrysosporium*, three of the four dyes were partially decolorized within 20 minutes. The absorbance of Congo Red did not decrease. It should be noted that orange II, tropaeolin O and Congo Red are all azo dyes, but only Congo Red lacks phenolic groups.

The enzyme reaction was initiated by the addition of hydrogen peroxide. The crude lignin peroxidase was dialyzed during preparation. Cripps did not add veratryl alcohol. Cripps's crude lignin peroxidase may have decolorized if veratryl alcohol had been present to serve as an electron shuttle. Cripps did demonstrate that *Phanerochaete chrysosporium* could decolorize azo dyes when grown under lignolytic conditions.

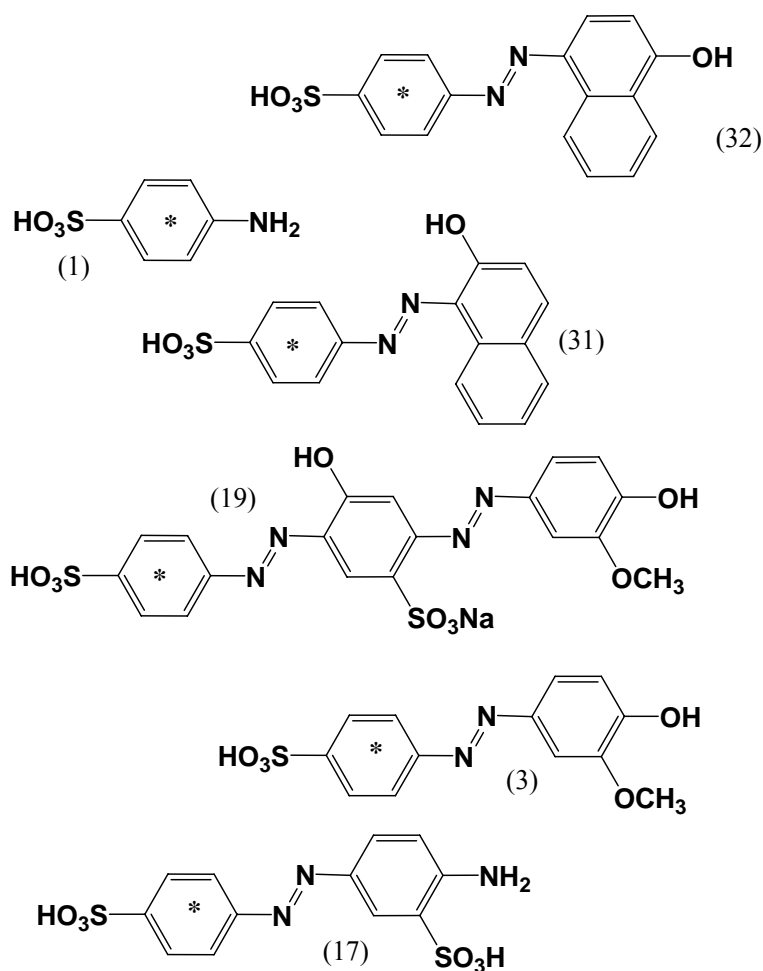
3.8.4.2 Dye decolorization by *P. chrysosporium* and *S. chromofuscus*

Paszczynski *et al.*^{173,176} examined the ability of both *Phanerochaete chrysosporium* and *Streptomyces chromofuscus* to mineralize sulfonated azo dyes and sulfanilic acid. Structures of the radiolabeled substrates used are shown in Figure 41, and the amount of radioactivity recovered is shown in Table 3-30. *Phanerochaete chrysosporium* was tested because it is most-studied white-rot fungus. *Streptomyces chromofuscus*, a ligninocellulolytic microorganism, belongs to an actinomycete group abundant in soil. When tested with xenobiotic compounds, actinomycetes can catalyze hydroxylations; O, N, and S oxidations; and O- and N-dealkylations. Paszczynski radiolabeled the dyes in the most recalcitrant portion of the molecule, the benzene ring attached directly to the azo bond and containing a sulfonate group in the *para* position.

S. chromofuscus showed mineralization only when the lignin-like guaiacol structure was attached to the sulfonated aromatic ring. *P. chrysosporium* mineralized all the dyes tested. Paszczynski measured the rate of dye mineralization during the 21-day incubation period. The mineralization of dye 3 (with hydroxy and methoxy substituents) was twice the rate of degradation of dyes 17 and 19 for the first 10 days. By the end of the incubation, dyes 3, 17, and 19 had been mineralized to similar extents.

Hydroxynaphthol dyes 31 and 32 differ in the position of the hydroxyl group. Paszczynski *et al.* report that the degradation rate of dye 31 was 2½ times greater than dye 32. The degradation of dye 31 also started two days earlier. Paszczynski did not speculate why the degradation rates for dyes 31 and 32 were so different. The position of the hydroxyl group may have some effect. However, dye 32 is in the sulfonic acid form while dye 31 is the sodium salt. Differences in water solubility could account for the slower mineralization of dye 31.

Figure 41 ¹⁴C-Labeled Azo Dyes Degraded by *P. chrysosporium* and *S. chromofuscus*
As Published by Paszczynski¹⁷³



Structures of radiolabeled compounds used.
* aromatic rings uniformly labeled with ¹⁴C

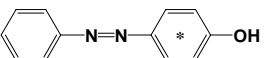
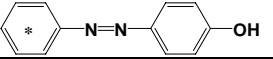
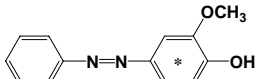
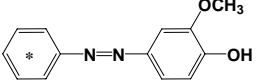
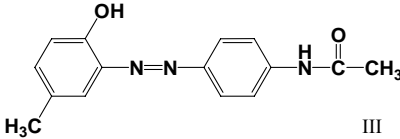
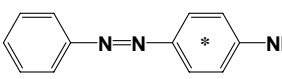
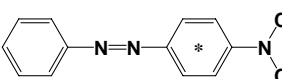
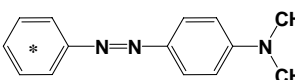
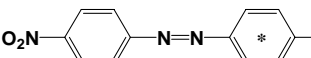
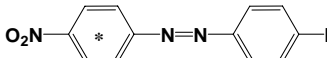
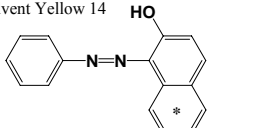
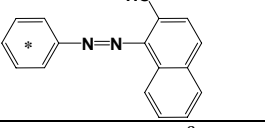
Table 3-30 Radioactivity Recovered from ¹⁴C Labeled Dyes as per Paszczynski¹⁷³

Percentage of radioactivity incorporated into CO ₂ and biomass and Removed from medium after 21 days of growth by <i>Phanerochaete chrysosporium</i> and <i>Streptomyces chromofucus</i>						
Azo dye	% Radioactivity in:					
	<i>P. chrysosporium</i>			<i>S. chromofucus</i>		
	Medium ^a	CO ₂ ^b	Cells	Medium	CO ₂	Cells
32 (Orange I)	29.6	19.7	4.0	22.0	1.1	7.0
1 Sulfanilic acid	25.0	17.2	1.3	0.0	0.0	0.0
31 (Orange II)	41.6	34.8	2.3	0.0	0.0	0.0
19	28.0	23.2	3.6	17.9	1.3	3.9
3	31.7	25.7	5.2	19.0	3.6	4.6
17 (Acid Yellow 9)	33.0	26.9	2.3	0.0	0.0	0.0
^a ¹⁴ C not accounted for as CO ₂ or biomass was lost by absorption to cell material and could be removed by washing with distilled water ^b In addition to CO ₂ , a small amount (0.1 to 0.5%) of organic volatiles was detected						

3.8.4.3 Decolorization of ¹⁴C-ring-labeled azo dyes by *P. chrysosporium*

Spadro *et al.*¹⁷⁴ examined the ability of *Phanerochaete chrysosporium* to mineralize specifically ¹⁴C-ring-labeled azo dyes. (Table 3-31) *P. chrysosporium* had been reported to decolorize a number of azo dyes,^{172,175,176} but decolorization of the chromophore is not the same as mineralization of the compound. All of the dyes tested were degraded much more rapidly under nitrogen limiting (lignolytic) conditions than under nitrogen-sufficient conditions. Phenolic ring-labeled compounds Ia and IIa were also significantly degraded under nitrogen-sufficient conditions suggesting these phenolic compounds are susceptible to enzymes other than lignin-degrading enzymes. Comparison of the degradation of compounds Ia and IIa suggests that the addition of a methoxy group to the phenolic ring enhanced degradation. For Disperse Orange 3, (compound VI), the amino-substituted and the nitro-substituted ring were about equally degraded. Although the nitro group would be expected to retard degradation, the degradation of 2, 4-dinitrotoulene by *P. chrysosporium* has been shown to be initiated with reduction of a nitro group to an amine.¹⁷⁷ Addition of a phenolic, amino or acetamide group to the aromatic ring increased the degradation. Spadro demonstrated that *P. chrysosporium* mineralizes as well as decolorizes azo dyes and that the degradation rate was affected by the substitution pattern.

Table 3-31 ¹⁴C- Labeled Azo Dyes Degraded by *P. chrysosporium* as per Spadro¹⁷⁴

¹⁴ C-Substrate ^a	Mineralization (%) 12 days after substrate addition	
	Low nitrogen culture	High nitrogen culture
Side chain ¹⁴ C-labeled DHP	21.9 ± 1.2	2.9 ± 0.05
4-Phenylazophenol		
 Ia	38.3 ± 1.6	21.9 ± 2.7
 Ib	28.4 ± 1.7	6.9 ± 1.2
4-Phenylazo-2-methoxyphenol		
 IIa	48.1 ± 3.4	15.1 ± 1.2
 IIb	20.7 ± 1.2	0.8 ± 0.3
Disperse Yellow 3		
 III	42.7 ± 0.5	5.7 ± 0.38
4-Phenylazoaniline		
 IV	25.8 ± 0.2	4.7 ± 0.06
N,N-Dimethyl-4-phenylazoaniline		
 Va	46.0 ± 0.4	6.7 ± 0.2
 Vb	29.9 ± 0.2	2.2 ± 0.1
Disperse Orange 3		
 VIa	40.1 ± 0.5	9.5 ± 0.1
 VIb	42.5 ± 0.68	2.5 ± 0.59
Solvent Yellow 14		
 VIIa	4.5 ± 0.05	0.0 ± 0.04
 VIIb	23.1 ± 0.68	3.4 ± 0.6
^a * indicates ¹⁴ C label. DHP, dehydropolymerizate		

3.8.4.4 Dye decolorization of 3 varied dyes by *Trametes versicolor*

Wang and Yu¹⁷⁸ investigated the ability of *Trametes versicolor* to adsorb and degrade three dyes: an indigoid, an anthraquinone and an azo dye. (The azo dye used has a phenolic group on the aromatic ring attached to the azo bond. Figure 42)

To ascertain the importance of physical adsorption and enzymatic degradation, dye adsorption was determined under four conditions. (Figure 43) 1.) Dead mycelium + autoclaved spent broth. This control had minimal adsorption of the three dyes. 2.) Dead mycelium + intracellular enzymes. A solution of intracellular enzymes prepared by homogenization of mycelium in broth followed by removal of debris. 3.) Dead mycelium + extracellular enzymes. A solution of extracellular enzymes was prepared by removing the mycelium from a 10-day old culture broth. The solution contained 78 U/L of peroxidase activity and 16,000 U/L of laccase activity. 4.) Living mycelium + extracellular enzymes. In general, the adsorbed dyes were removed and degraded fastest by the living mycelium plus extra cellular enzymes. The anthraquinone dye was degraded faster by the extracellular enzymes. The indigo dye was degraded faster by the intracellular enzymes. The azo dye was degraded at the same rate with the enzymes. For these dyes, degradation by live fungal cells was more important than biosorption by dead cells.

Figure 42 Three Synthetic Dye Substrates Decolorized by *Trametes versicolor* As Published by Wang and Yu¹⁷⁹

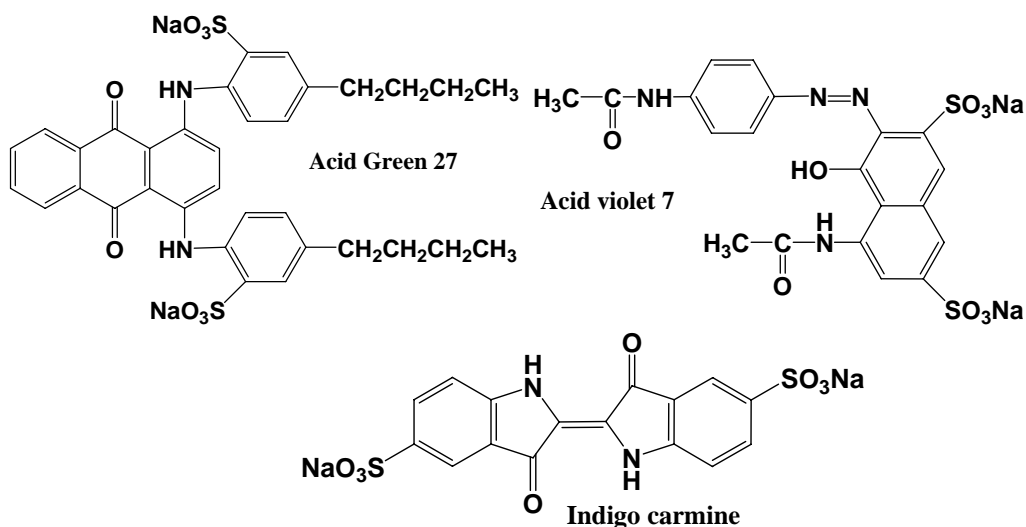
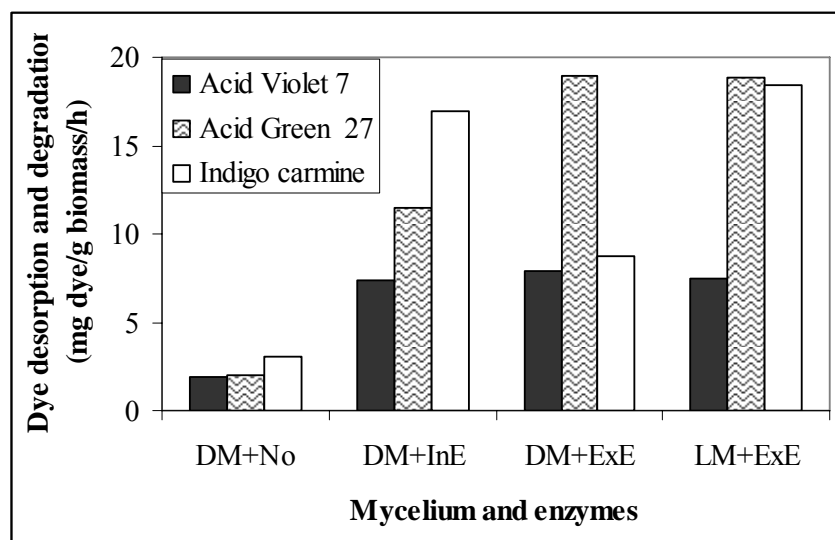


Figure 43 Dye Degradation by Mycelium Plus Enzymes as Measured by Wang¹⁷⁸

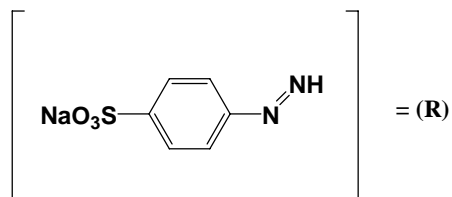


Physical desorption and enzymatic degradation of adsorbed dyes with four types of fungal biomass and enzymes. DM+No: dye-saturated dead mycelium in an autoclaved spent broth without enzyme; DM+InE: dye-saturated dead mycelium in a supernatant of intracellular enzymes; DM+ExE: dye-saturated dead mycelium in a broth containing ligninolytic extracellular enzymes; LM+ExE: dye-saturated living mycelium in a broth with the extracellular enzymes

3.8.4.5 Effect of aromatic substitution pattern on decolorization

Pasti-Grigsby, Paszynski, *et al*¹⁸⁰. continued research with *Phanerochaete chrysosporium* and *Streptomyces chromofuscus* and examined the effect of different aromatic substitution patterns on azo dye degradability.¹⁸⁰ The substrate dye structures are shown in Figure 44. *Streptomyces chromofuscus* A11 decolorized azo dyes that had a hydroxyl group in the *para* position and at least one methoxy and/or alkyl group *ortho* to the hydroxyl group. The rate of dye decolorization by intact *Streptomyces chromofuscus* correlated with the decolorization rate by isolated *Phanerochaete chrysosporium* manganese peroxidase and commercial horseradish peroxidase. Isolated *Phanerochaete chrysosporium* lignin peroxidase showed a different dye specificity. LiP was the only enzyme tested that could decolorize compound [11], an azo compound with two strongly electron-withdrawing fluorine groups attached to the aromatic ring. Their work demonstrates that the aromatic substitution pattern of the substrate is important and that manganese peroxidase and lignin peroxidase differ in specificity.

Figure 44 Monosulfonated Azo Dyes Degraded by Intact Fungi
From Publication by Pasti-Grigsby¹⁸⁰



(1)	(9)	(17)
(2)	(10)	(18)
(3)	(11)	
(4)	(12)	(19)
(5)	(13)	
(6)	(14)	(30)
(7)	(15)	(31)
(8)	(16)	(32)

3.8.4.6 Summary of treatments of textile effluents with intact fungi

Research efforts continue in this area ranging from screening new fungal isolates for the ability to decolorize dyes,^{181,182,183} examining the effect of culture conditions on decolorizing effectiveness^{184,185,186} and exploring potential methods for industrial application.^{187,188} Treatment with intact fungi may be appropriate for wastewater treatment. However, days of treatment are usually needed. Decolorization with intact fungi is not appropriate for removing color from cellulose fibers.

3.9 DEGRADATIVE REDOX SYSTEMS OF WHITE-ROT FUNGI

White-rot fungi have been studied for dye decolorization because they are able to degrade a large variety of substrates. White-rot fungi break down both hydrophilic cellulose and hydrophobic lignin. Fungi can degrade a greater variety of substrates than bacteria because fungi excrete enzymes that can attack compounds too large or insoluble to enter a cell. The redox mechanisms of white-rot fungi are listed in Table 3-32. These same pathways have also been studied for biobleaching^{189,190,191,192,193} and for treatment of papermill and bleach plant effluents.^{194,195,196}

Table 3-32 Redox Systems of White-Rot Fungi

System	Mediator	Electron Source
Lignin Peroxidase	Veratryl Alcohol	H ₂ O ₂
Manganese Peroxidase + Chelator	Mn ^{+2/+3}	H ₂ O ₂
Laccase	Various Small Molecules	O ₂
Organic Chelators	Fe ^{+2/+3} , Mn ^{+2/+3}	H ₂ O ₂

3.9.1 DEGRADATION OF RECALCITRANT POLLUTANTS

White-rot fungi are able to degrade pollutants like polychlorinated phenols and dinitrotoulene that persist in the environment.¹⁹⁷ The degradation mechanisms used by white-rot fungi are outlined in an excellent review by Barr and Aust.¹⁹⁷ These compounds

are already highly oxidized. The initial step in mineralization is a reduction. Published reports of compound degradation should be evaluated carefully. In some cases, a fungus can perform the initial reduction, but the breakdown products accumulate. Fungi have been incorrectly assumed to mineralize compounds when only the disappearance of the initial compound was monitored.

3.9.2 DECOLORIZATION OF DYES BY FUNGAL ENZYMES

White-rot fungi contain at least three redox systems: lignin peroxidase (LiP or ligninase), manganese peroxidase (MnP) and laccase (Lac). Each of these systems has been evaluated for ability to decolorize synthetic dyes.

3.9.2.1 Lignin peroxidase treatment of 10 varied dyes

Ollikka *et al.*¹⁹⁸ isolated lignin peroxidase isoenzymes from carbon-limited cultures of *Phanerochaete chrysosporium*. The fungus produces a number of veratryl alcohol-oxidizing proteins that could be expected to vary in specificities and/or efficiencies. Ten different dyes were treated with a crude enzyme preparation. These dyes included azo, triphenyl methane, heterocyclic and polymeric dyes.

The color removal was greater than 75% for seven of the dyes. Poly R-478 and Poly T-128, (polycyclic aromatic carbonyl dyes), were decolorized 46% and 48%, respectively. Congo Red (an azo dye, structure given in Figure 40), was decolorized 54%.

Three lignin peroxidase isoenzymes were isolated by preparative isoelectric focusing, followed by chromatofocusing with an exchange column. The purified isoenzymes were used to decolorize five dyes. When 2-mM veratryl alcohol was added, the decolorization activity of the purified isoenzymes was similar to the activity of the crude enzyme preparation. In the absence of veratryl alcohol, decolorization was greatly reduced.

Ollikka determined the kinetic constants, k_{CAT} , K_m , for the purified isoenzymes. The three isoenzymes did not exhibit significant kinetic differences. For some dyes, the

pH range for effective decolorization was very narrow. The enzymes function in the pH range of 3-5.

The addition of veratryl alcohol to the crude enzyme preparation did not affect decolorization. Veratryl alcohol is added to the fungal culture media to activate enzyme production. To determine if veratryl alcohol had carried over into the crude enzyme preparation, Ollikka removed low-molecular weight compounds from the crude enzyme preparation by diafiltration. After diafiltration, the activity of the crude LiP preparation decreased to the level of the purified isoenzymes. The presence of veratryl alcohol in the crude enzyme preparation was verified and its concentration was determined by two independent methods. The crude enzyme preparation was found to contain 150 μM veratryl alcohol by capillary electropherograms and by measurement of veratraldehyde production at 310 nm.

Cripps¹⁷² had not observed decolorization of Congo Red by crude lignin peroxidase from nitrogen-limited *Phanerochaete chrysosporium*. Ollikka¹⁹⁸ found significant decolorization of crude lignin peroxidase from carbon-limited *Phanerochaete chrysosporium*. The discrepancy could be due to the difference in the fungal culture conditions. However, the lack of veratryl alcohol in Cripps's dialyzed enzyme preparation is the most likely cause for the difference.

3.9.2.2 Lignin and manganese peroxidase treatment of 8 varied dyes

Young and Yu¹⁹⁹ selected 8 dyes of very diverse structures as substrates. (Figure 45) They obtained partially purified ligninase (lignin peroxidase) and Mn-dependent peroxidase (manganese peroxidase) from Tienzyme. Fungal cultures of *P. chrysosporium* and *T. versicolor* were also tested. The results are shown in Table 3-33. No results are shown for manganese peroxidase because MnP had negligible ability to decolorize the dyes. Both the manganese and hydrogen peroxide concentrations were varied.

Table 3-33 Decolorization (%) of Dyes by Fungi and Lignin Peroxidase as per Young¹⁹⁹

Dye	Lignin peroxidase	<i>P. chrysosporium</i>	<i>T. versicolor</i>
Indigo caramine (IC)	98.1	29.8	92.8
Reactive blue-15 (RB-15)	94.1	75.6	91.4
Acid violet-7 (AV-7)	92.9	26.9	12.4
Reactive black-5 (RB-5)	91.4	11.3	15.6
Acid green-27 (AG-27)	85.3	0.2	18.6
Acid blue-25 (AB-25)	81.6	55.6	69.0
Acid orange-74 (AO-74)	79.1	6.6	7.3
Acid black-24 (AB-24)	25.4 (0.6 mM H ₂ O ₂) 83 (0.2 mM H ₂ O ₂)	98	96.6

Effectiveness of the decolorization treatment depended on the dye structures. The fungi were cultured for nine days. Decolorization data include dye adsorption and degradation. Altering the pH affected the decolorization of individual dyes to different extents. All of the dyes did require acidic conditions, (pH 3.7-4.9), for better decolorization.

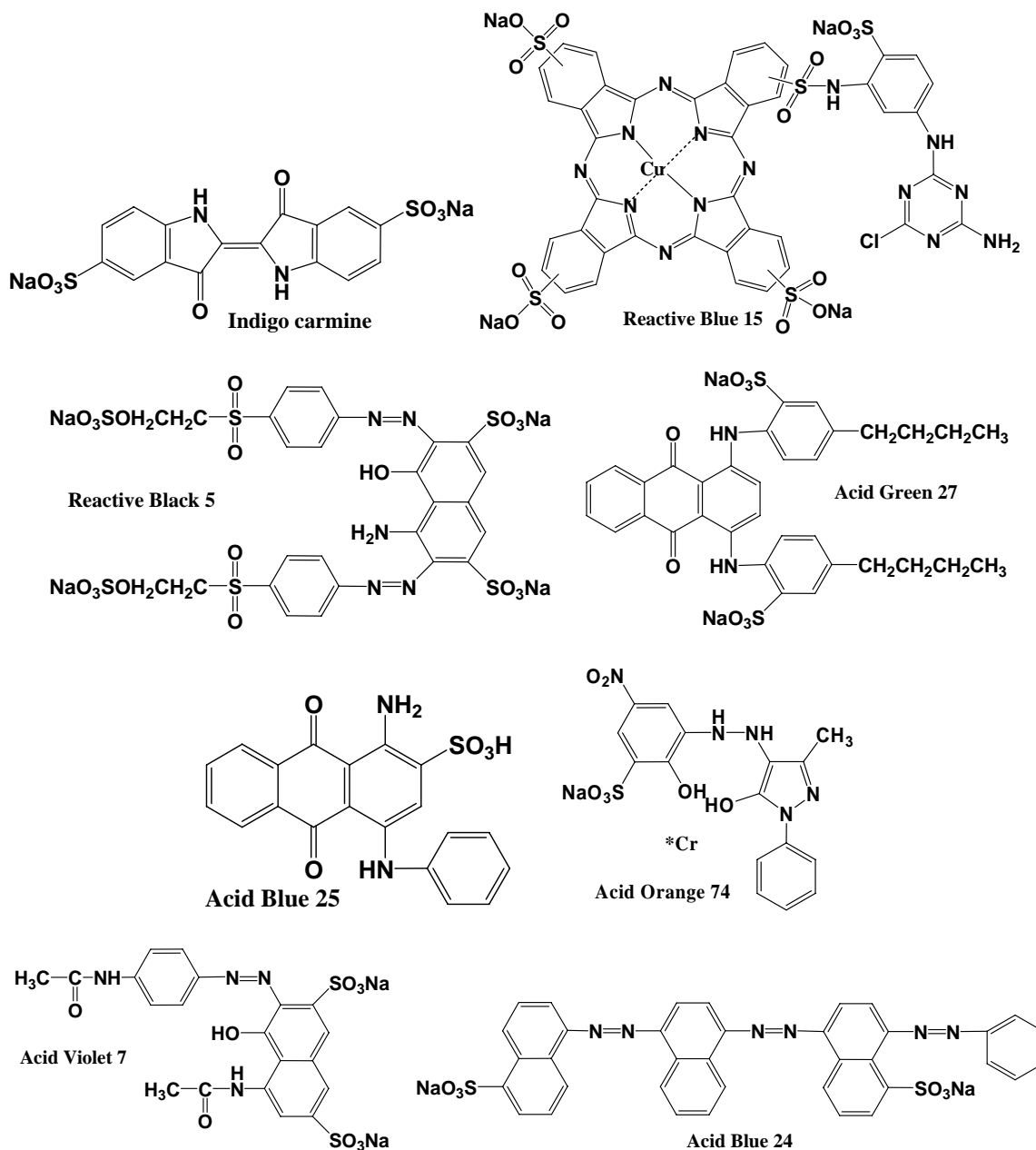
Two types of peroxide effects were observed. The dye decolorization of some dyes was enhanced by high peroxide concentrations (up to 0.8 mM). For other dyes, an optimum peroxide concentration was observed (about 0.18 mM). Overdose with hydrogen peroxide caused a rapid and significant decline in dye decolorization. Decolorization of all eight dyes required the addition of veratryl alcohol. The response to veratryl alcohol addition varied between dyes. Increasing veratryl alcohol concentration above 1 mM brought no further enhancement.

Some inhibition of decolorization was observed at high dye concentrations. The reaction was dependent on LiP and the decolorization rates increased linearly with LiP doses. The amount of rate response to increased LiP dosage depended on the dye structure.

Of the dyes tested, only acid black-24 (AB-24) contained a non-phenolic azo group. Lignin peroxidase was able to decolorize acid black-24. The reaction also had an optimum peroxide concentration. This redox system of lignin peroxidase, veratryl alcohol

and 'low dosage' hydrogen peroxide might be effective in decolorizing non-phenolic C.I. Direct Yellow 11.

**Figure 45 Diverse Synthetic Dye Substrates Decolorized by Lignin Peroxidase
As Published by Young and Yu¹⁹⁹**



3.9.2.3 Manganese peroxidase treatment of 4 azo dyes

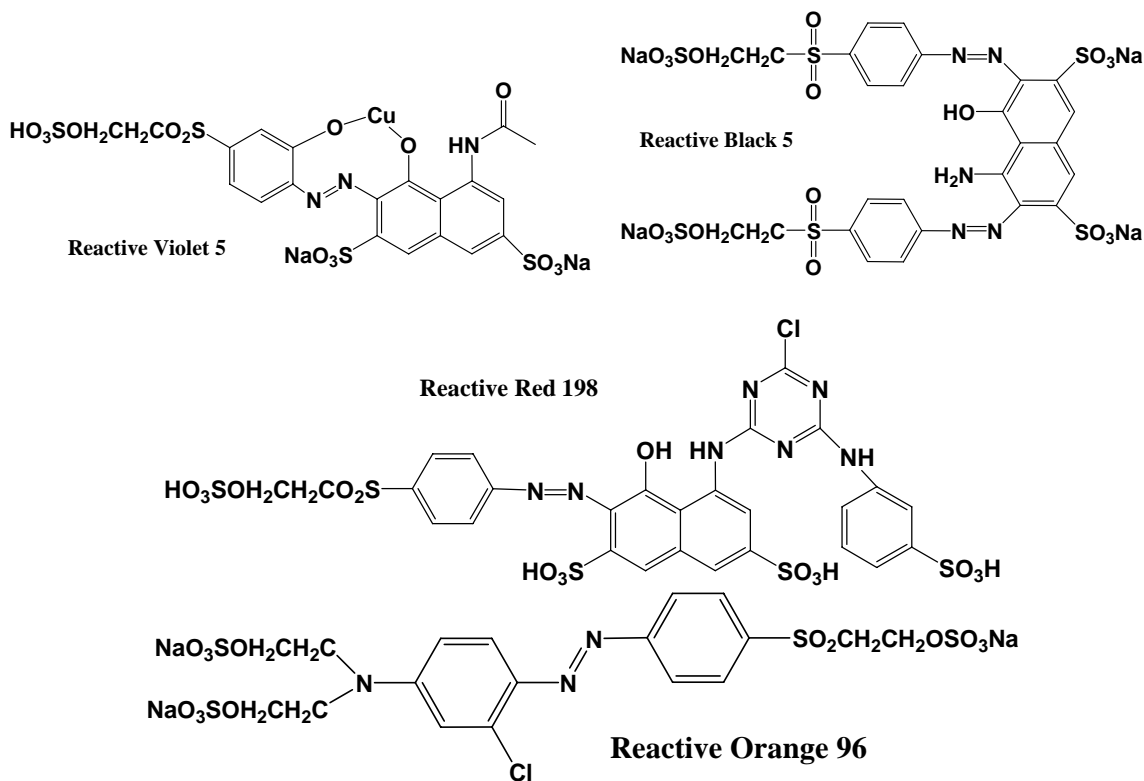
Heinfling *et al.*²⁰⁰ selected one phthalocyanine dye and four azo dyes for substrates (Figure 46) One of the dyes has a non-phenolic azo linkage. The selected

enzymes were manganese peroxidases from *Phanerochaete chrysosporium*, *Pleurotus eryngii* and *Bjerkandera adusta*. MnP from *P. chrysosporium* did not decolorize the dyes. The MnPs from *P. eryngii* and *B. adusta* did decolor the dyes, but the reaction was Mn^{2+} independent. The reaction rates with the dyes could not be increased by adding 1 mM Mn^{2+} .

Manganese peroxidase degrades xenobiotic compounds by converting Mn^{2+} to Mn^{3+} . The Mn^{3+} then reacts with the substrate. To determine if Mn^{3+} was involved, Heinfling also treated the dyes with a Mn^{3+} -lactate complex. The Mn^{3+} complex did not decolorize the dyes. Lignin peroxidase from *B. adusta* was able to decolor the dyes when veratryl alcohol was added. The addition of veratryl alcohol to MnP did not stimulate dye decolorization. So the dyes were decolorized by lignin peroxidase plus veratryl alcohol and by manganese peroxidases without additional manganese.

Figure 46 Azo Dye Substrates Decolorized by Manganese Peroxidase

As Published by Heinling²⁰⁰



3.9.2.4 Laccase treatment of 3 varied dyes

Wong and Yu¹⁷⁹ isolated the decolorizing enzyme from *Trametes versicolor*. The same substrates were used as in previous work with the intact fungus. (Figure 42, page 75) These compounds include an anthraquinone dye, an indigo dye and an azo dye with phenol group attached to the azo bond. Lignin peroxidase and manganese peroxidase activities were not detected in carbon-limited cultures of *T. versicolor*. Laccase activity was present in these cultures.

The initial decolorization rates were determined for each dye by adding an aliquot of culture supernatant to a dye solution. For the anthraquinone dye, the initial decolorization rate increased in proportion to the laccase activity. The linear response indicates that the anthraquinone dye was a substrate for laccase. The azo and indigo dyes were decolorized, but the rate was not proportional to the laccase activity. Decolorization of these dyes may involve a low molecular weight mediator.

The crude media preparation was checked for the presence of a mediator by membrane filtration. The filtered high molecular weight fraction could decolor the anthraquinone dye, but not the azo or indigo dyes. In a designed experiment, the crude laccase solution was enclosed in a dialysis membrane that allowed free diffusion of small molecules but prohibited the release of the enzyme. When the known laccase mediator ABTS (2, 2'-azino-bis (3-ethylthiazoline-6-sulfonate) was added to a dye solution with the confined laccase, the azo and indigo dyes were decolorized. Subsequent experiments determined that the anthraquinone dye could serve as a mediator for the decolorization of the azo and indigo dyes. This work indicates that a laccase-mediator redox system may be able to decolorize C.I. Direct Yellow 11.

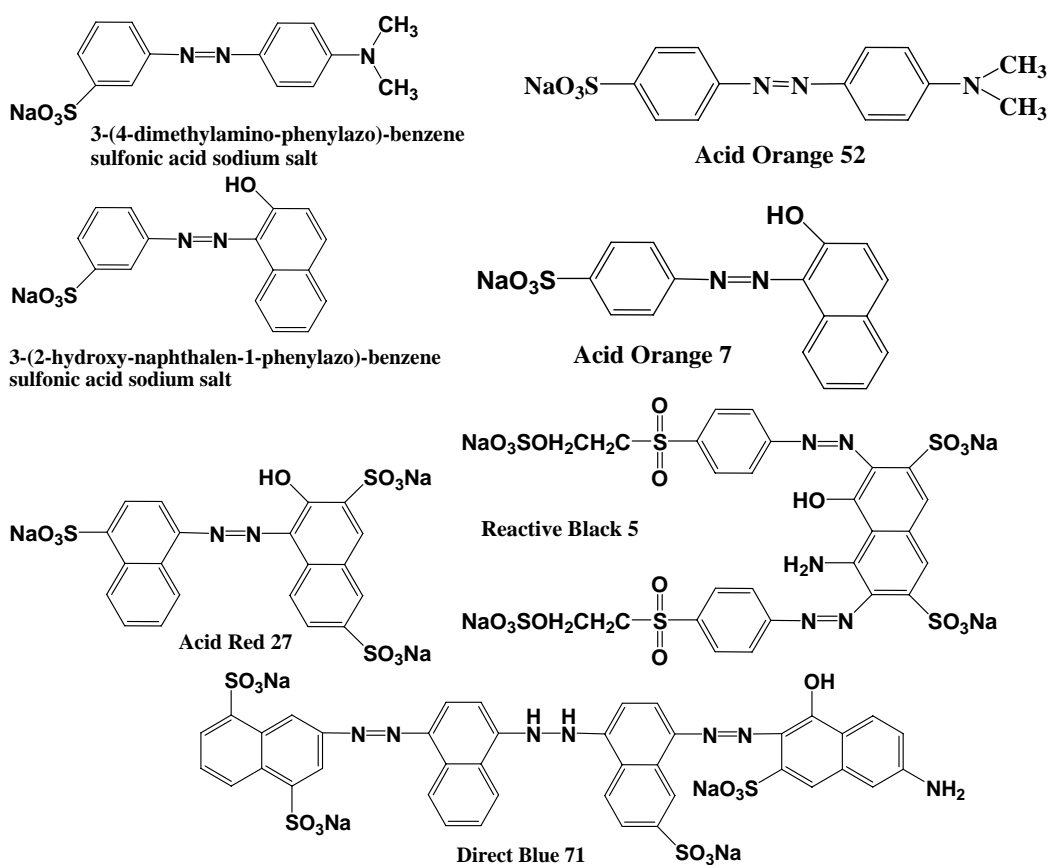
3.9.2.5 Relationship between redox potential and decolorization by laccase

The ability of microbes, fungi and enzymes to degrade azo dyes depends on the structural characteristics of the dye, the temperature and pH of treatment, the presence of intermediates, and the difference between redox potentials of the bioagent and the dye. Zille *et al.*²⁰¹ explored the biodegradation of some azo dyes under aerobic conditions by the ascomycete yeast *Issatchenkia occidentalis* and by laccase with or without mediator.

The dyes and mediator are shown in Figure 47. They compared these two approaches on the basis of the electrochemical properties of the dyes and bioagents.

Zille found a linear relationship during the initial period of decolorization with laccase and a laccase/mediator system between the percentage decolorization of each dye and the respective anodic peak potential. Contrarily to the laccase system, *I. occidentalis* decolorizes azo dyes through a reductive mechanism, but also in this system a linear relationship between the cathodic peak potentials and the time of maximum decolorization of the azo compounds was observed.

Figure 47 Dye Structures with Different Redox Potentials Decolorized by Laccase
From Publication by Zille²⁰¹



3.9.2.6 Summary of dye decolorization by fungal enzymes

Decolorization with fungal enzymes offers a number of advantages over using intact fungi. To produce the necessary enzymes, fungi may require very precise culture conditions. The reaction is slower and the amount of waste material (sludge) increases

due to fungal growth. Consequently, dye decolorization with fungal enzymes offers great potential. In addition to screening new sources, research efforts include creating enzyme membrane bioreactors²⁰² and enzyme production under solid state fermentation conditions.²⁰³

Table 3-34 Redox Potentials for Delignification Enzymes and Chemicals as per Call²⁰⁴

Compound	Redox potential		Literature
	E ₀ (mV) ^a	E _{1/2} (mV) ^b	
Laccase (fungal; type 1/type 3) pH 5.5	+780/+785		Reinhammar 1984
Laccase (plant; type 1/type 3) pH 7.5	+394/+434		Reinhammar 1984
Ceruloplasmin pH 5.5	+490/+580		Reinhammar 1984
Stellamycin pH 7.1	+184		Reinhammar 1984
Mavicyanin pH 7.0	+285		Reinhammar 1984
Rusticyanin pH 2.0	+680		Reinhammar 1984
Plastocyanin pH 7.0	+370		Reinhammar 1984
Azurin pH 7.0	+300		Reinhammar 1984
HRP-I/HRP-II	+920		Hayashi and Yamazaki 1979
LiP-I/LiP-II	+1000-+1100		Shoemaker et al. 1994
O ₂ + e ⁻ → O ₂ ⁻	-330		Lippard and Berg 1994
O ₂ + H ⁺ + e ⁻ → HO ₂	-130		Lippard and Berg 1994
O ₂ + 2H ⁺ + 2e ⁻ → H ₂ O ₂ (aq)	+1500		Lippard and Berg 1994
O ₂ + 4H ⁺ + 4e ⁻ → 2H ₂ O	+815		Ishihara and Nishida 1983; Lippard and Berg 1994
O ₂ [*] + 2H ⁺ + e ⁻ → H ₂ O ₂	+890		Lippard and Berg 1994
O ₂ [*] (reductant/oxidant)	-330/+950		Henry et al. 1981
OH + e ⁻ → OH ⁻	+1400		CRC 1982
OH ₂ + H ⁺ + e ⁻ → H ₂ O ₂	+1500		CRC 1982
OH [*] + H ⁺ + e ⁻ → H ₂ O	+2180/+2310		Wood 1994; Lippard and Berg 1994
CO ₂ [*]	+1100		Henry et al. 1981
Cu ²⁺ + e ⁻ → Cu ⁺	+153		Lippard and Berg 1994
Fe ³⁺ + e ⁻ → Fe ²⁺	+771		Lippard and Berg 1994
Mn ³⁺ (chelated)	+900 - +1200		Demmer et al. 1980; Cui and Dolphin 1990
Mn ³⁺ + e ⁻ → Mn ²⁺	+1510		Lippard and Berg 1994
(Fe(CN) ₆) ³⁻ /(Fe(CN) ₆) ⁴⁻	+420		Morpurgo et al. 1993
Dehydroascorbate + 2H ⁺ + 2e ⁻ → ascorbate	+0.06		Dawson et al. 1986
Veratryl alcohol (3,4-dimethoxybenzyl alcohol)	+1450 (pH 3, water)	+1220	Fawer et al. 1991
Several tetra-, tri-, di- and methoxybenzene(s)		Increasing from +810 - +1760	De Jong et al.; Popp and Kirk 1991
Benzotriazole (4N HCl)		-1000 ^d	Lund and Kwee 1968
N-hydroxybenzotriazole (pH 0)		≈ 800 ^d	Lund and Kwee 1968
N-hydroxybenzotriazole (pH 7)		+760	Aurich et al. 1977

^aE₀ is the potential; vs. normal hydrogen electrode (NHE, pH 7, 25°C)
^bE_{1/2} (half-wave potential-polarography vs. saturated calomel electrode (SCE) is the potential where the ratio of oxidized/reduced state is valid, i.e. in this case E₀=E_{1/2} according to Nernst equation (for correction E_{0,SCE} = +240 mV)

^cSolvent acetonitrile

^dControlled polarographical reductions of benzotriazole to *o*-aminophenylhydrazine can be obtained at -1.0 V (SCE) with a consumption of 4 electrons per molecule under acidic conditions (up to pH 3)

3.10 SUMMARY OF LITERATURE REVIEW

To increase the value of recycled fiber, dyes present in wastepaper have to be bleached/decolorized. For fine papers from chemical pulp, the hardest dye to bleach is a stilbene yellow dye. This dye, C.I. Direct Yellow 11, responds poorly to current chemical bleaching agents. Ozone can destroy the dye, but ozone also damages cellulose fibers and requires investment in an expensive ozone generator. In directory paper from high-yield (mechanical) pulps, a major problem is the dye in telephone directory yellow pages. The most commonly applied dye for directory paper is the methine dye, Basazol Yellow 46L (C.I. Basic Yellow 96).

Dyes produce color by absorbing different wavelengths of light. Human perception responds to all wavelengths of visible light received. Visually identically material may not absorb light identically. Spectrophotometric analysis of absorbed or reflected light is necessary to distinguish whether visually identical materials contain the same dye.

Visible and ultraviolet electromagnetic radiation is absorbed by a compound if the energy of the radiation matches the energy required to promote an electron in the compound to a higher energy level. Many compounds absorb only high-energy ultraviolet light. Dyes consist of planar conjugated systems. Many dyes also contain nitrogen or sulfur. Because $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions require less energy in conjugated systems, dyes absorb lower-energy visible light. If reduction, oxidation, or cleavage interrupts the conjugated double bonds in a dye, the absorption will be shifted toward higher-energy shorter wavelengths. For yellow dyes (which absorb blue light), this hypsochromic shift can result in the disappearance of visible color.

C.I. Direct Yellow 11 contains an azo bond. Azo dyes are the most-commonly used textile dyes. Environmental considerations have prompted studies of biological degradation of azo and other dyes. Bacteria are not very effective in degrading these large molecules. However, treatments with white-rot fungi or isolated fungal enzymes have

been successful in degrading many dyes, including azo dyes. Non-phenolic azo dyes have been harder to degrade than phenolic azo dyes. However, degradation of non-phenolic azo dyes has been reported.

There are some published reports that are of particular relevance to this project. Beginning with yellow pulp produced from yellow colored recycled paper sorted out from mixed office paper at a recycling mill, Li *et al.*²⁰⁵ compared treatments with oxygen, alkaline hydrogen peroxide, sodium dithionite in the presence of detergent, and laccase with violuric acid as mediator. Bleaching effectiveness was evaluated by measuring TAPPI brightness²⁰⁶ which is based on absolute reflectance of blue light of 457 nm. The laccase/violuric acid treatment was for 18 hours and yielded pulp with lower brightness than pulp treated for one hour with alkaline hydrogen peroxide. Sodium dithionite treatment, nearly equal to alkaline hydrogen peroxide treatment in bleaching effectiveness, provided higher brightness than alkaline hydrogen peroxide when a detergent was added during treatment. Their pulp contained a mixture of unidentified dyes and the laccase reaction time was long (18 hours). This report suggests that results from laboratory experiments with one dye may be difficult to apply to the mixed dyes in commercial operations.

One German recycle mill investigated whether laccase mediator system would be suitable for deinking processes (such as ink detachment and dirt speck reduction) that are run in neutral conditions under atmospheric pressure.²⁰⁷ They tested two laccases (from *Trametes villosa* and *Myceliophthora thermophila*) and three mediators: ABTS, HBT and violuric acid. Treatment with laccase or laccase with mediator lowered the brightness of artificially aged prints, deinked pulp, and unprinted recycling papers. Subsequent tests indicated that laccase treatment resulted in yellowing of lignin in groundwood and thermomechanical pulp. This yellowing could not be reversed by subsequent alkaline extraction, peroxide bleaching or FAS bleaching. These results suggest that laccase-mediator bleaching would not benefit a mill recycling lightly colored papers containing a high percentage of groundwood or thermomechanical pulp. A recycle mill treating highly colored papers with a low percentage of groundwood and thermomechanical pulp may

still benefit. Also, the neutral pH and/or atmospheric oxygen conditions may have affected laccase selectivity.

Finally, according to a Japanese report,²⁰⁸ the white rot fungus *Coriolus versicolor* was effective at decolorizing 5 of 7 different groups of dye tested, including stilbene. They obtained a maximum decolorization of >80% after 7 days. They examined cell-free extracts and found laccase, peroxidase, and catalase activities. Cell-free decolorizing of stilbene dye is not mentioned. This fungus may be useful for effluent treatment, but short reaction times with cell-free extracts are necessary for bleaching cellulose fibers dyed with stilbene dyes.

4 DISSERTATION OBJECTIVES

The goal of this research proposal was to determine whether treatment with the laccase-mediator system and/or with peroxidase-hydrogen peroxide could effectively decolorize recalcitrant dyes Direct Yellow 11 and Basazol 46L. This research was novel in two aspects. First, at the inception of this research program, there were no reported investigations of the ability of lignolytic fungal enzymes to decolorize stilbene dyes. Second, the reported decolorization studies applied fungal enzymes to dissolved dyes. It was unknown whether the enzymes could decolorize dyes while the dye was attached to cellulose fibers. On this basis, the findings reported in this thesis were important contributions to the field of biobleaching of cellulose fibers and to the potential applications of lignolytic enzymes.

The main objectives of this research proposal were to:

1. Determine the effectiveness of laccase-mediator system on decolorizing Direct Yellow 11 and Basazol 46L.
2. Develop an efficient LMS to decolorize pulp dyed with Direct Yellow 11
3. Propose potential mechanism for LMS decolorization
4. Compare effectiveness of developed LMS system to conventional treatments.
5. Investigate whether peroxidase enzymes are capable of decolorizing Direct Yellow 11 and Basazol 46L
6. Examine the reaction rates of LMS and peroxidase treatments
7. Investigate potential mechanisms for peroxidase decolorization

5 THESIS FORMAT

The author has elected to present the research results in the format of publications, conference proceedings and manuscripts submitted for publication. Because of this format, there will be some duplication of the introduction and experimental sections. The results and discussion section of this thesis is divided into four chapters. The four sections comprising the core of this thesis are titled:

1. An Auspicious Application of Laccase and Hydrogen Peroxidases for Biobleaching of Recalcitrant Paper Dyes, *Proceedings International Society of Wood Pulping and Chemistry*, Madison, Wisconsin, June 2003.
2. Laccase-Mediator Biobleaching Applied to a Direct Yellow Dyed Paper, *Biotechnology Progress* (in press)
3. Enzymatic biobleaching of two recalcitrant paper dyes with horseradish peroxidase, soybean peroxidase and laccase-mediator system (submitted to *Biotechnology Letters*)
4. Decolorization of Colored Paper by Laccase-Mediator System and Conventional Treatments (in preparation)

Publication 1 describes a preliminary study on the ability of the laccase-mediator system to decolorize Direct Yellow 11. This report demonstrates that LMS can decolorize pulp dyed with Direct Yellow 11.

In Publication 2, ABTS is shown to be more effective as laccase-mediator than either HBT or violuric acid. In addition, the dye removal of 60% can be maintained as the ABTS concentration is lowered from 5 mM to 0.01 mM.

For Publication 3, the laccase/ABTS system, soybean peroxidase and horseradish peroxidase were applied to Direct Yellow 11 and Basazol 46L. All three treatments were able to reduce the signal intensity of Direct Yellow 11 and Basazol 46L. Direct Yellow 11 responded slowly to both soybean peroxidase and laccase/ABTS. Basazol 46L responded quickly, with 74% reduction in signal within 5 minutes.

The last section describes the results from a comparative study between the laccase/ABTS system and conventional bleaching treatments. The best results were obtained for bleaching regimes that included reductive bleaching with sodium dithionite. For pulp dyed with Direct Yellow 11, laccase/ABTS followed by dithionite removed as much color as when the pulp was treated with oxygen or hydrogen peroxide followed by dithionite.

6 EXPERIMENTAL PROCEDURES

6.1 MATERIALS AND REACTION EQUIPMENT

6.1.1 CHEMICALS

A sample of C.I. Direct Yellow 11 (Pergasol) has been provided by Ciba-Geigy. Samples of Direct Yellow 11 (Fastusol) and Basazol Yellow 46L were donated by Dwight Paeon of BASF. Bleached hardwood kraft dry lap pulp was obtained from an IPST member company. Laccase, NOVO 51002, from *Trametes villosa*, was donated by Novozyme.

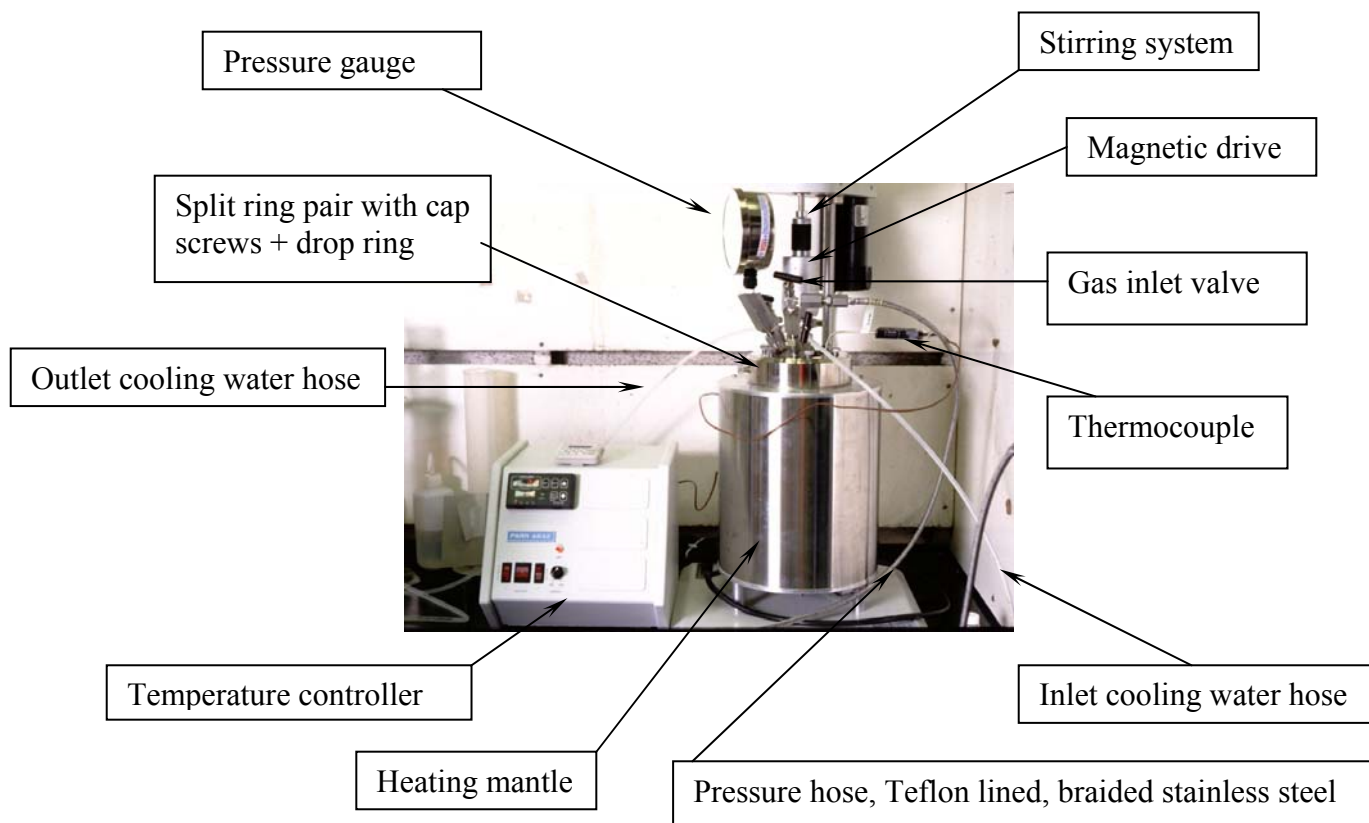
Laccase mediators 1-Hydroxy benzotriazole (HBT), violuric acid, and 2, 2'-azinobis-3-ethyl-benzthiazoline-5-sulfonate (ABTS) were purchased from Sigma. Polyethylene glycol of 10,000 and 35,000 molecular weight were purchased from Fluka. Soybean peroxidase, veratryl alcohol and 4-aminoantipyrine (4-AAP) were purchased from Sigma.

6.1.2 REACTION EQUIPMENT

A water-jacketed Parr High-Pressure system (Figure 48) was used for heated pressurized reactions such as laccase-mediator reactions (45 °C, 10 bar oxygen) and oxygen pulp bleaching (90 °C, 90 psi (6 bar) oxygen). A thermostat-controlled water bath was used for heated ambient pressure reactions such as soybean peroxidase (45 °C), alkaline hydrogen peroxide pulp bleaching (75 °C), sodium dithionite pulp bleaching (75 °C), and alkaline extraction of bleached pulp (60 °C). For water batch reactions involving pulp, the pulp and bleaching solutions were sealed in heat-sealable Kapak bags 2.5 mils thick.

Figure 48 Photograph of a 1000-mL-Capacity Parr Reactor with Components

Safety rupture disc, gas release valve, bomb cylinder, and other internal bomb fittings are not visible in this photograph.



6.1.3 ANALYSIS EQUIPMENT

Spectra were obtained on a Perkin-Elmer Lambda 900 UV/Vis/NIR Spectrophotometer. The color of the handsheets were measured following TAPPI Standard Method T422 om-88, "Spectral reflectance factor transmittance, and color of paper and pulp polychromatic illumination" CIELAB values for handsheets were obtained on a Technibrite Micro TB-1C machine (Technidyne Corp., New Albany, Indiana, USA) which measures light reflected at 0° from the surface. For the comparison among laccase/ABTS, hydrogen peroxide and oxygen bleaching treatments, CIELAB

values for handsheets were obtained on a Brightmeter Micro S-5 (Technidyne Corp., New Albany, Indiana, USA) which measures light reflected at 45° from the surface.

6.2 ENZYME ACTIVITY ASSAYS

6.2.1 LACCASE ACTIVITY ASSAY

Laccase activity was measured by monitoring the rate of oxidation of ABTS²⁰⁹ in a 100.0 mM pH 5.0 sodium acetate buffer. . The reaction was followed at 420 nm ($\epsilon_{420} = 36,000$) One International Unit (IU) of activity is defined as the conversion of 1 μ mole of substrate/minute. One Katal (kat) of activity is defined as 1 mole/second of substrate converted. For laccase-mediator treatment of dyed pulp, 10.3 U or 50 μ kat was added for each mL of reaction.

Equation 4 Conversion Between IU and Kat

$$1 \text{ kat} = 1 \text{ mole/second} = 60 \text{ mole/minute}$$

$$1 \text{ IU} = 1/60 \mu \text{ kat} = 16.67 \times 10^{-9} \text{ kat}$$

6.2.2 SOYBEAN PEROXIDASE ACTIVITY ASSAY

Soybean peroxidase (SBP) activity was measured by monitoring the production of a red quinoneimine dye at 510 nm. In the presence of hydrogen peroxide, phenol and 4-aminoantipyrine (4-AAP), SBP catalyzes the formation of phenol free radicals which then react with 4-AAP to form the quinoneimine dye. ($\epsilon_{510} = 97,210 \text{ a.u. /M/cm}$)²¹⁰ The standard reaction conditions were 10.0 mM phenol, 2.40 mM 4-AAP and 0.20 mM H₂O₂ at 25 °C, pH 7.0.

6.3 PEROXIDE CONCENTRATION ASSAY

For reactions involving hydrogen peroxide, the concentration of the stored stock solution (nominally 30%) must be determined. The assay procedure²¹¹ involves titration of an acidic solution of hydrogen peroxide with sodium dithionite in the presence of an indicator. Two (2.0) mL of peroxide solution is mixed with 100 mL distilled water, 10-15 mL of 25% sulfuric acid, 5.0 mL of 10% potassium iodide (KI) solution and 3 drops of

saturated ammonium molybdate solution. This mixture is titrated with 0.10 N sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) until a pale yellow color appears. Then 10.0 mL of soluble starch solution is added and the thiosulfate titration continues until the blue color disappears. The calculation of hydrogen peroxide concentration is shown in Equation 5.

Equation 5 Calculation of Hydrogen Peroxide Concentration

$$\text{Grams/Liter} = \frac{0.1(\text{norm. thio.}) \times 34 (\text{equiv. H}_2\text{O}_2 - 50\%) \times (\text{mL. thio used})}{2 \text{ mL (sample size)}}$$

6.4 SELECTION OF DYE CONCENTRATION

6.4.1 DYE MANUFACTURERS' SAMPLE AND STOCK CONCENTRATIONS

The donated dye samples are commercial preparations that, in addition to the dye chromophore, include fillers, salts and other materials. For instance, according to the MSDS sheet, the Basazol 46L sample contains 40% acetic acid. The first step was to obtain a solution concentration of g/mL of non-volatile solids.

One mL of each dye was dried down using a rotary evaporator. In Table 6-1 below are listed the concentration of non-volatile solids/mL for each dye and the final solution volumes when 1.0 mL of dye was diluted to prepare a 10.0 mg/mL solution. For the basic dye Basazol 46L, it was necessary to include 10% acetic acid when preparing a 10 mg/mL solution

Table 6-1 Concentrations of Dye Samples and Stock Solutions

Commercial Dye	g/mL non-volatile solids	Final volume for stock solutions from 1mL of dye	Stock solution concentration g/L
Pergasol (Direct Yellow 11)	0.613	61.0	10.05
Fastusol (Direct Yellow 11)	0.266	26.5	10.03
Basazol 46L	0.555	55.0	10.09

6.4.2 WORKING CONCENTRATION FOR DYE SOLUTIONS

The dye concentration used for the enzyme reactions was a concentration whose absorbance is at least 0.5 AU and less than 1 AU. For both Direct Yellow 11 and Basazol

46L, that concentration was 10 ppm (10 mg/L). For Direct Yellow 11, the absorption peak is broad and λ_{Max} is 405-411 nm. For Basazol 46L, λ_{Max} is 436-438 nm.

6.5 PULP RELATED PROCEDURES

6.5.1 PULP PREPARATION

Dry lap pulp sheets were used to prepare virgin fully bleached kraft hardwood pulp for dyeing with Direct Yellow 11. Sheets of dry pulp were ripped into small pieces and allowed to soak in distilled water for 4-16 hours. The soaked pulp was then transferred to a laboratory-scale pulper (Catep by Kādant Lamort, Vitry Le Francois) and pulped for at least five minutes. The resultant pulp was then drained to thicken it and transferred to a 5-gallon pail for storage at 4°C until used.

Commercial dyed office papers were used for some bleaching experiments. Reams of RepliCopyColors™ paper designed for high-speed copying, laser and offset printing were purchased. The colors selected for use were blue, cherry (pink) and goldenrod (orange). 80 sheets (8 ½ x 11", about 4.2 g each) of each color were ripped into small pieces and soaked in 3 L of distilled water. The paper was processed in batches in a Waring blender (with 4 additional L of distilled water) to produce pulp. The pulp was thickened and stored at 4°C until used.

6.5.2 DYEING PROCEDURE

Analysis of spectra containing Direct Yellow 11, laccase and mediators was complicated because absorbance by laccase mediators overlaps with absorption by Direct Yellow 11. Therefore most experiments were performed on pulp dyed with Direct Yellow 11. The applied dye dosage was based on the commercial dosage recommended by BASF. In a large Hobart mixer, 1200 g of fully bleached hardwood chemical pulp was mixed with 3.0 g of liquid dye concentrate and 24 g alum. This 5% slurry, (24 kg total volume), was mixed for one hour at room temperature. The dyed pulp was then subjected to extensive washing until a mixture of yellow pulp in clear water was obtained.

6.5.3 DETERMINATION OF CONCENTRATION OF DYE IN DYED PULP

Determining the amount of dye on the fiber is difficult; however, it is possible to determine how much remains in the liquid phase after the dyeing process. After dyeing, a sample of dyed pulp was filtered to obtain effluent from the dyeing reaction. A spectrum was then obtained of the effluent sample. To prepare a standard curve, dye concentrate and alum were mixed at the concentrations used for pulp dyeing. This "100%" solution was measured by spectrophotometer then used to prepare a series of dilutions ("50%", "25%", "10%", etc) that were also measured by spectrophotometer. Comparison between the effluent sample and the standard curve allows determination of the amount of dye associated with the fiber. Spectrophotometric measurement of dye remaining in pulp filtrate indicated that more than 90% of the applied dose of Direct Yellow 11 was attached to the pulp.

6.5.4 HANDSHEET PREPARATION

Handsheets were prepared following TAPPI Method T218 (the Büchner Funnel Method).²¹² For this process, 4.0 o.d.g. of pulp in about 12000mL solution were subjected to 15,000 revolutions on a disintegrator. This slurry is then pH adjusted to be $\text{pH } 6.5 \pm 0.5$. The slurry is then continuously poured into a 15.0 cm Büchner funnel. After inversion onto a metal plate, the filter paper is removed. The metal plate and formed handsheet are then stacked between sheets of blotter paper on a press, and pressed at 50 psi for 90 seconds. Each metal plate and handsheets are then placed on a restrained drying ring and dried under conditions as defined in TAPPI Standard Method 402 om-93, "Standard conditioning and testing atmosphere for paper, board, pulp handsheets and related products". After drying for 12-20 hours, the CIELAB color values were then measured following TAPPI Standard Method T442-om88.²¹³

6.6 SOLUTION SAMPLE TREATMENTS

6.6.1 LACCASE/ABTS TREATMENT OF DIRECT YELLOW 11 IN SOLUTION

For kinetic studies of laccase/ABTS treatment of Direct Yellow 11, 20.0 mg/L Direct Yellow 11 was treated with 1.0 mM ABTS and 10.3 Units laccase/mL in 100 mM

sodium acetate buffer pH 4.5. The reaction was allowed to continue for 24 hours under 10 bar oxygen. During the course of the reaction, aliquots of at least 10 mL were removed for analysis.

The reaction solution with Direct Yellow 11, buffer, mediator and water were mixed and preheated to 45°C. Laccase was added. After purging with oxygen for 60 seconds, the Parr reactor was sealed and the reaction timing began. At each time point, the sampling valve was opened and the sample collected. The first 3-5 mL of sample was discarded since the volume of the sampling tube was about 3 mL.

Since absorbance by ABTS would interfere with quantifying the dye peak, a control reaction was performed in the same way, containing laccase and ABTS but no dye. For each time point, the spectrum from the control was subtracted from the spectrum of the dye reaction. To calculate the absorbance area, a baseline was first drawn from the lowest point between 450 and 550 nm. The area under the curve was then summed from 285 – 550 nm.

Similar experiments were performed with 1-hydroxybenzotriazole and violuric acid. However, after subtracting the matching control, the difference spectra obtained showed both a peak with maxima around 410 nm (the λ_{Max} for Direct Yellow 11) and an overlapping peak with maxima around 305 nm. Although efforts were made to model the Direct Yellow 11 and other components of these complex spectra using Unscrambler Chemometric software, Principal Component Analysis software and Multiple Linear Regression software, consistent models of the Direct Yellow 11 absorbance as it changed over time were not found.

6.6.2 LACCASE/ABTS TREATMENT OF BASAZOL 46L IN SOLUTION

The concentration of Basazol 46L was set at 10.0 mg/L of non-volatile dye solids. The reaction mixture included 100 mM sodium acetate buffer, pH 4.5 and 1.0 mM ABTS. After preheating the reaction mixture to 45°C, laccase was added (10.3 units/mL reaction solution) and a time zero sample was taken. The system was flushed with oxygen for 60 seconds, sealed, pressurized to 10 bar and reaction timing started. After

two hours the pressure was released and the treated sample was collected. A control of laccase plus mediator was also run.

6.6.3 HORSERADISH PEROXIDASE TREATMENTS

The ability of horseradish peroxidase (HRP) to decolorize Basazol 46L and Direct Yellow 11 was examined by treating the dyes with HRP for two hours at 45°C. The dye concentration was set at 10.0 mg/L of non-volatile dye solids. The reaction mixture included 100 mM sodium acetate buffer, pH 3.8 and 1.0 mM veratryl alcohol. Five units (5.0) of horseradish peroxidase (activity 116 PPU/mg, assay based on the production of purpurogallin from pyrogallol) was added per mL reaction volume. After preheating this mixture to 45°C, the reaction was initiated by adding hydrogen peroxide (0.10 mM final concentration).

6.6.4 SOYBEAN PEROXIDASE TREATMENTS

Treatment conditions for soybean peroxidase (SBP) were virtually the same as for HRP treatment. The dye concentrations were the same, 10.0 mg/L, and the reaction proceeded for two hours at 45°C. In this case, the reaction mixture included 0.10 M calcium chloride as well as 100 mM sodium acetate buffer, pH 3.8 and 1.0 mM veratryl alcohol. 1.0 unit of SBP was added for every mL of reaction mix. After preheating the reaction mixture to 45°C, the reaction was initiated by adding hydrogen peroxide (0.10 mM final concentration). In studies where the pH was varied, the buffers (100 mM) used included glycine-HCl (pH 2.4), sodium acetate (pH 2.8 and pH 4.5), sodium citrate, phosphate (pH 6.5) and glycine-NaOH (pH 8.5 and pH 10.5). To obtain an estimate of experimental error, 8 identical samples at pH 4.5 were treated with SBP. The standard deviation of the measured absorbance area divided by the average absorbance area yields a variation of 6%. For tests to determine if a free radical mediator was involved, pH 6.5 samples were prepared with and without 5.0 mM dimethyl sulfoxide.

6.7 PULP SAMPLE TREATMENTS

6.7.1 LACCASE-MEDIATOR TREATMENT OF COLORED PULPS

The laccase-mediator treatments were carried out in a 1000-mL-capacity temperature-controlled stirred pressure reactor. Mediator type and amount varied in experiments, but was added as an aqueous solution. The selected reaction conditions, based on optimal LMS biobleaching conditions for biodelignification of chemical pulps²³⁸ were as follows: 45°C, pH 4.5, 10 bar oxygen, stirred for 2-hour reaction followed by extensive washing. The basic procedure was as follows: The pulp (3% final consistency), mediator, sodium acetate buffer, pH 4.5, (final concentration 20 mM) and water were preheated to 45°C. Laccase (4.365 kats for 27 g o.d. pulp) was added and stirred. The reactor was closed and pressurized with oxygen to 10 bar. After mixing two hours, the pulp was filtered and washed thoroughly with distilled water until the filtrate contained no visible color. Reaction conditions such as pH, temperature and time were not altered since previous work²¹⁴ had established that these conditions were optimum for this laccase.

6.7.2 ALKALINE EXTRACTION OF LACCASE-TREATED PULP

A major effect of xylanase treatment is to improve the efficacy of subsequent deinking treatments.²¹⁵ To determine if laccase/ABTS treatment would also improve subsequent steps, colored pulps were subjected to laccase-1.0 mM ABTS treatment, followed by alkaline extraction. Colored pulp (25g, o.d.) in a heat-sealable bag was placed in a 60°C water bath and preheated for at least five minutes. Sodium hydroxide (0.10 g, or 0.4%) and water (final volume 250 mL, consistency 10%) were added. The bag was then heat sealed, mixed thoroughly, and placed in the 60°C water bath for 60 minutes. The bag was then opened; the pulp was suction-filtered and washed with 1 L of distilled water.

6.7.3 OXYGEN BLEACHING OF COLORED PULPS

Alkaline oxygen bleaching of colored pulps was performed in the Parr pressure reactor. The reaction conditions²¹⁶ include 90°C, 90 psi (6 bar) oxygen pressure and 0.8%

charge of sodium hydroxide (0.216 g NaOH for 27 g o.d. pulp). Water, sodium hydroxide and the reaction vessel were preheated to 90°C. The pulp was rapidly heated to 90°C in a microwave and added to the pressure reactor. For the time course studies, the pulp consistency was 10%. For the bleaching comparison studies, a consistency of 5% was used.

6.7.4 PEROXIDE BLEACHING OF COLORED PULPS

Bleaching of colored pulp with alkaline hydrogen peroxide was performed following the procedure of Li *et al.*²¹⁷ Colored pulp (53 g, o.d.) was mixed with water (final volume 1060 mL, 5% consistency) and DTPA (diethylene triamine pentaacetate, 0.11 g or 0.2%) in a heat-sealable bag and preheated in 75°C water bath for at least 10 minutes. (The DTPA is a metal chelator that stabilizes hydrogen peroxide.) Sodium hydroxide (0.53 g or 1%) and hydrogen peroxide (0.53 g or 1%) were added. After heat-sealing, the pulp slurry was mixed thoroughly and placed in 75°C water bath for 60 minutes. The bag was then opened; the pulp was suction-filtered and washed with 1 L of distilled water.

6.7.5 DITHIONITE TREATMENT OF COLORED PULPS

Reductive bleaching of colored pulp was performed following the procedure of Li *et al.*²¹⁷ Sodium dithionite (0.50 g, 2% on o.d. pulp) was dissolved in 50 mL of distilled water that had been boiled and then cooled while a stream of nitrogen gas bubbled through it. The colored pulp (25 g, o.d.) was mixed with water (final reaction volume 750 mL, consistency 3.33%) and placed in a heat-sealable Kapak bag. The pulp slurry was then placed in a 75°C water bath and allowed to preheat for 10 minutes as nitrogen gas was bubbled through the pulp slurry. Nitrogen bubbling continued as the pulp slurry was removed from the water bath, the dithionite solution was added and the contents were mixed for 60 seconds. After measuring pH, the bag was then heat sealed and the pulp slurry was placed in the water bath. After reacting for 60 minutes, the bag was opened and the pH was measured. The pulp was suction filtered and washed with 1 L of distilled water.

6.8 ANALYSIS OF RESULTS

6.8.1 MEASUREMENT OF COLOR (TRISTIMULUS VALUES)

The TAPPI brightness measurement (commonly used to test virgin fibers) does not measure color because it depends on the blue portion of the visible spectrum. To accurately characterize colored fibers and sheets, a three number colorimetric measurement must be used. The color of the handsheets were measured following TAPPI Standard Method T422 om-88, "Spectral reflectance factor transmittance, and color of paper and pulp (polychromatic illumination)" A Technidyne Corporation Technibrite Micro TB-k located in the Paper Testing Laboratory of IPST was used to perform this measurement. This machine illuminates the sample with diffuse light, measures the reflected light, and calculates the CIE L^* , a^* , b^* values. For the comparison among laccase/ABTS, hydrogen peroxide and oxygen bleaching treatments, CIELAB values for handsheets were obtained on a Brightmeter Micro S-5 (Technidyne Corp., New Albany, Indiana, USA) which measures light reflected at 45° from the surface.

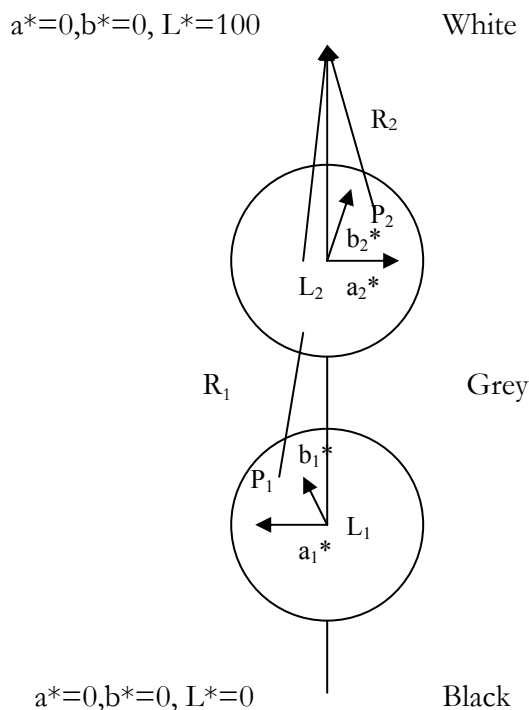
6.8.2 DETERMINATION OF DYE REMOVAL INDEX

For any one sheet, the 'difference' from a white sheet can be reduced to a single number that represent the distance in color space from an ideal bleach. (Figure 49) The ideal bleach is pure white. The CIE $L^*a^*b^*$ coordinates for the ideal bleach point are $a^*=b^*=0$, $L^*=100$. The first step is to determine the tristimulus values for the sheet. Then the geometric distance (R) from the measured location in L^* , a^* , b^* color space to pure white is calculated. The distance in color space from a point P_1 with coordinates a_1 , b_1 , L_1 , to the ideal bleach point is calculated from the formula $R^2 = a^2 + b^2 + (100-L)^2$.

After bleaching, the material is now at point P_2 in color space with coordinates a_2 , b_2 , L_2 . The reduction in distance from the ideal bleach point in moving from point P_1 to P_2 is $-\Delta R^2$. The formulas for calculating ΔR^2 are given in Equation 6. The dye removal index (DRI) for a bleaching process is based on the change in R that occurs during that process. The dye removal index is expressed as a percentage of the original distance from

the ideal bleach point. The DRI does not give the direction of the color change, but rather measures how much color was removed.

Figure 49 Distance in Color Space from an Ideal Bleach⁴⁵



Equation 6 Dye Removal Index Calculation⁴⁵

$$\text{Distance from Ideal Bleach Point} = R^2$$

$$R^2 = a^2 + b^2 + (100-L)^2$$

$$\text{Amount of Color Removal} = -\Delta R^2$$

$$\Delta R^2 = R_2^2 - R_1^2$$

$$\Delta R^2 = (a_2^2 - a_1^2) + (b_2^2 - b_1^2) + [(100-L_2)^2 - (100-L_1)^2]$$

$$\text{Dye Removal Index (DRI)} = -100 [\Delta R^2 / R_1^2]$$

$$\text{DRI} = \text{Percent Color Removal by Bleaching Process}$$

6.8.3 ANALYSIS OF SPECTRAL DATA

To determine the amount of color removed during treatment of dissolved dyes, the areas under the peak were determined. Because the absorption peaks from laccase/ABTS overlap with the absorption peaks of the dyes, the spectra of the

appropriate laccase/ABTS control was subtracted from the spectra of the dye/laccase/ABTS spectra before quantifying. For laccase-mediator treatments, a straight baseline was drawn from the lowest point between 380 and 550 nm. Examination of the absorbance curves from HRP and SBP treatments outside the area of the dye peak showed that a sloping baseline was needed. To this purpose, a small Excel Macro program was written to take the absorbance data, draw the sloping baseline and sum the area between the baseline and the observed absorbance. The wavelengths used for summation were 285-550 nm for Direct Yellow 11 and 335-498 nm for Basazol 46L. Extent of decolorization was determined by calculating the percentage decrease in signal intensity (i.e., 100% would equal no dye peak remaining).

7 RESULTS AND DISCUSSION

7.1 PUBLICATION 1

An Auspicious Application of Laccase and Hydrogen Peroxidases for Biobleaching of Recalcitrant Paper Dyes.

Kristina Knutson and Arthur Ragauskas

Proceedings International Society of Wood Pulping and Chemistry, Madison WI, June 2003.

7.1.1 ABSTRACT

Two commonly applied paper dyes, Direct Yellow 11 and Basazol 46L, are known to cause color problems in recycling paper. Biobleaching of these dyes was explored by treating the dyes with hydrogen peroxidases and laccase-mediator systems (LMS). Treatment with soybean peroxidase achieved a 90% reduction in the absorption of Basazol 46L. For Direct Yellow 11-dyed pulp, 60% of the color could be removed by LMS treatment.

7.1.2 INTRODUCTION

Mixed office waste and colored directory paper are underutilized for generation of recycled fibers. A major difficulty in using these furnishes is the problems associated with decolorizing the dyes present in the paper.^{3,2} Of the commonly used paper dyes, the stilbene dye Direct Yellow 11 (goldenrod yellow office paper)³ and methine dye Basazol 46L (yellow directory paper) are notorious⁴ for poor bleachability. When the bleachability of a number of common paper dyes was examined,⁵² Direct Yellow 11 was found to respond poorly to treatment with hydrogen peroxide, FAS, hydrosulfite, chlorine, and hypochlorite. Only ozone treatment could remove more than 90% of the color. Removing dye from yellow directory paper is also problematical.⁴³

Laccase-mediator systems (LMS) have been extensively studied for the biobleaching of wood pulps (for reviews, see Paice *et al.*²¹⁸ Call and Mücke⁹⁷) Laccases (EC 1.10.3.2) are oxidoreductases that combine single-electron oxidization of many aromatic substrates with the reduction of oxygen to water.²⁵⁴ Bourbonnais and Paice demonstrated that the substrate range of laccase can be extended to nonphenolic compounds by the addition of mediator compounds such as 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS)²¹⁹ Peroxidases also oxidize many aromatic substrates but obtain electrons from hydrogen peroxide rather than oxygen. Horseradish peroxidase (HRP) is the most widely used peroxidase, but soybean peroxidase (SBP) is more stable.

Since these dyes respond poorly to chemical bleaching, peroxidases and the LMS biobleaching system were tested for the ability to decolorize the dyes.

7.1.3 METHODS AND MATERIALS

7.1.3.1 Enzyme activity assays

Laccase activity was measured by the oxidation of ABTS²⁰⁹ at 420 nm, pH 5.0 ($\epsilon_{420} = 36,000$). For these experiments, 50 μ kats of laccase (NOVO 51002, from *Trametes villosa*) were added per mL of liquid reaction volume. Soybean peroxidase activity was determined by the oxidation of phenol in the presence of 4-aminoantipyrine at 510 nm ($\epsilon_{420} = 36,000$).²¹⁰ One unit of SBP was used per mL of solution.

7.1.3.2 Dyeing pulp with Direct Yellow 11

To obtain a reproducible defined substrate for biobleaching, pulp dyed with Direct Yellow 11 was prepared. Fully bleached hardwood kraft pulp (1200 o.d.g) was mixed at room temperature for one hour (5% cs) with the equivalent of the common commercial dosage: 3 g of liquid dye concentrate and 24 g alum (to attach the dye to the pulp). After extensive washing, a mixture of yellow pulp in clear water was obtained.

7.1.3.3 LMS treatment of dyed pulp

Laccase-mediator treatments were carried out in a 1000-mL-capacity temperature-controlled stirred pressure reactor. Direct Yellow 11-dyed pulp (27 o.d.g), sodium acetate buffer, pH 4.5, (final concentration 20 mM), and distilled water (to dilute pulp slurry to 7.5% final consistency) were preheated to 45°C. Three mediators were tested (5.0 mM each): ABTS, 1-hydroxybenzotriazole (HBT), and violuric acid (VA). After mixing mediator with the pulp, 50 μ kats of laccase per mL of liquid reaction volume was added and mixed again. The reactor was closed and pressurized with oxygen to 10 bar. After two hours, the pulp was filtered and washed thoroughly with distilled water until the filtrate contained no visible color.

7.1.3.4 Calculation of Dye Removal Index (DRI)

The LMS decolorization of pulp dyed Direct Yellow 11 was quantified by measuring the CIELAB color values for handsheets prepared following TAPPI Standard Method T218.²¹² The DRI measures the percent change in geometric distance from the ideal bleach point. It is calculated following the equations shown below.⁴⁵

Equation 7 Distance from Ideal Bleach Point

$$R^2 = a^{*2} + b^{*2} + (100-L^*)^2$$

Equation 8 Amount of Color Removal

$$-\Delta R^2 = R_2^2 - R_1^2$$

$$\Delta R^2 = (a_2^{*2} - a_1^{*2}) + (b_2^{*2} - b_1^{*2}) +$$

Equation 9 Dye Removal Index (DRI)

$$\text{Dye Removal Index (DRI)} = -100 [\Delta R^2 / R_1^2]$$

7.1.3.5 Soybean peroxidase treatment

Both the stilbene dye Direct Yellow 11 and the methine dye Basazol 46L were treated with SBP. To obtain accurate spectra, 10 ppm of nonvolatile dye solids was used. The 40-mL reaction solution contained 2 mM veratryl alcohol and 0.1 M CaCl₂ in 0.1 M

glycine-HCl buffer, pH 2.4.²¹⁰ One unit of SBP was added per mL solution. Reaction was initiated by the rapid addition of 0.5 μL of 27% hydrogen peroxide (final concentration 0.1 mM). After inversion, reaction solutions were placed for 2 hours in a 45°C water bath.

The effectiveness of SBP was evaluated by recording spectra, measuring the major peak area, and calculating the percent reduction in peak area from untreated dye.

7.1.3.6 Horseradish peroxidase treatment

Treatment with HRP was conducted in the same way as the SBP treatment. The dye (10 ppm) and H_2O_2 (0.1 mM) concentrations were the same. The buffer system was 0.1 M sodium acetate, pH 3.8 containing 1 mM veratryl alcohol (no CaCl_2). Five units of horseradish peroxidase (stated activity 116 PPU/mg based on a purpurogallin assay) was added per mL reaction volume.

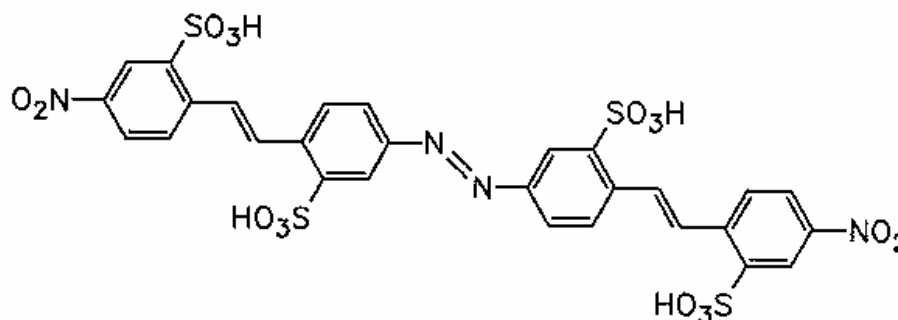
7.1.3.7 Evaluation of pH and PEG effects on SBP

The effectiveness of SBP treatment of Basazol 46L was evaluated at several different pH values by changing the composition of the 0.1-M buffer system. The buffers used included glycine-HCl (pH 2.4), sodium acetate (pH 3.8), potassium acetate (pH 4.5), and glycine-NaOH (pH 8.5). SBP stabilization by polyethylene glycol (PEG) was also examined by including none or 500 mg/mL of either 10,000 or 35,000 molecular weight PEG.

7.1.4 RESULTS AND DISCUSSION

The major chromophore in Direct Yellow 11 is shown in Figure 50.⁵² This stilbene dye is more unreactive than most dyes with azo bonds because the lack of phenolic groups eliminates the formation of azo/hydrazone tautomers.

Figure 50 Chromophore in Direct Yellow 11



7.1.4.1 LMS treatment of Direct Yellow 11

Initial work focused on testing the effectiveness of LMS to bleach Direct Yellow 11. Since laccase alone did not decolorize the dye, three mediators were tested: ABTS, HBT, and VA. In solution, dye decolorization is difficult to quantify because laccase and the mediators also absorb near the λ_{Max} for Direct Yellow 11 (411nm).

To avoid the spectral interference, LMS was applied to pulp that had been dyed with Direct Yellow 11. The biphasic system allows laccase and mediator compounds to be washed away. In this system, ABTS was much more effective than HBT or VA in bleaching pulp dyed with Direct Yellow 11 (Figure 51).

LMS-ABTS retained effectiveness even when the concentration of ABTS was cut 500-fold from 5 mM to 0.01 mM (Figure 52). These results were completely unexpected. For delignifying virgin pulp, both violuric acid and 1-hydroxy benzotriazole (HBT) are more effective than ABTS.^{209,220}

Figure 51 Bleaching Effectiveness of Different Mediators at 5 mM in the LMS Treatment of Pulp Dyed with Direct Yellow 11.

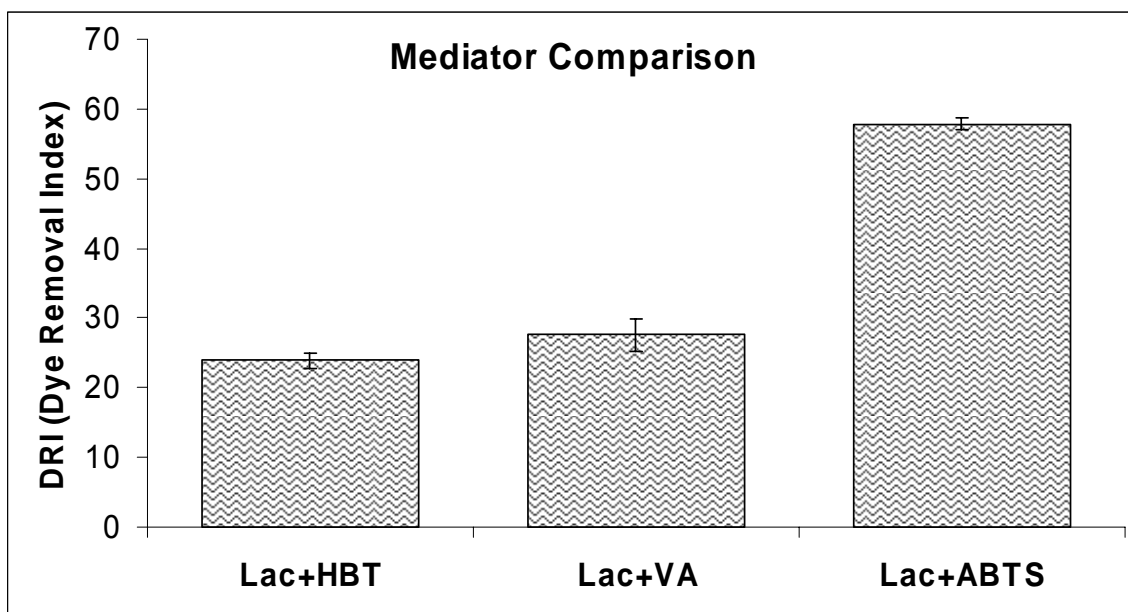
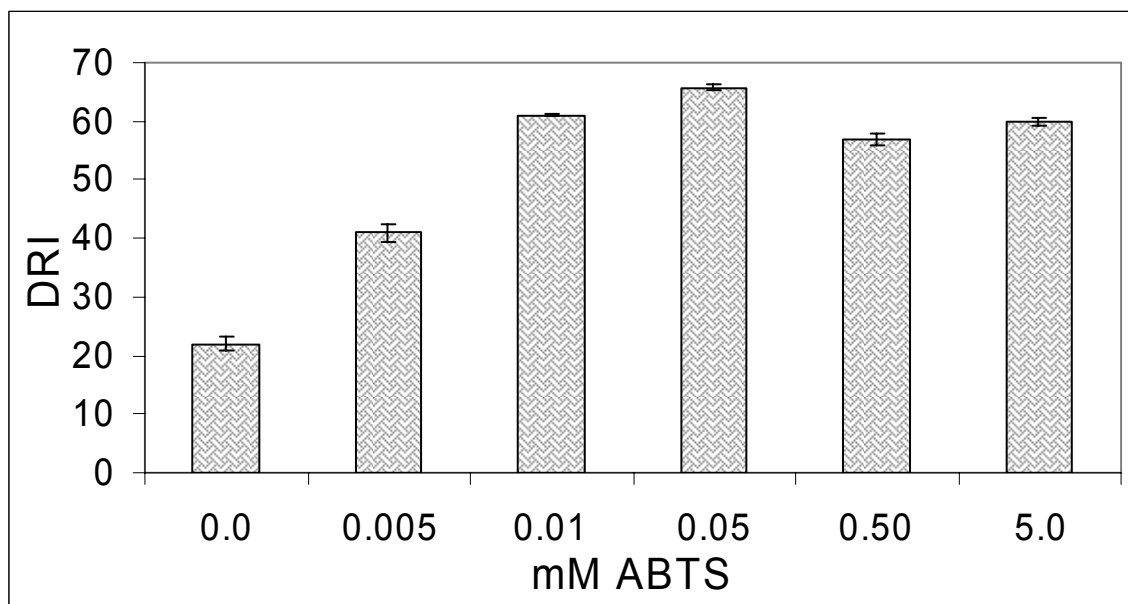


Figure 52 Dye Removal From Direct Yellow 11-Dyed Pulp as ABTS Dose is Altered



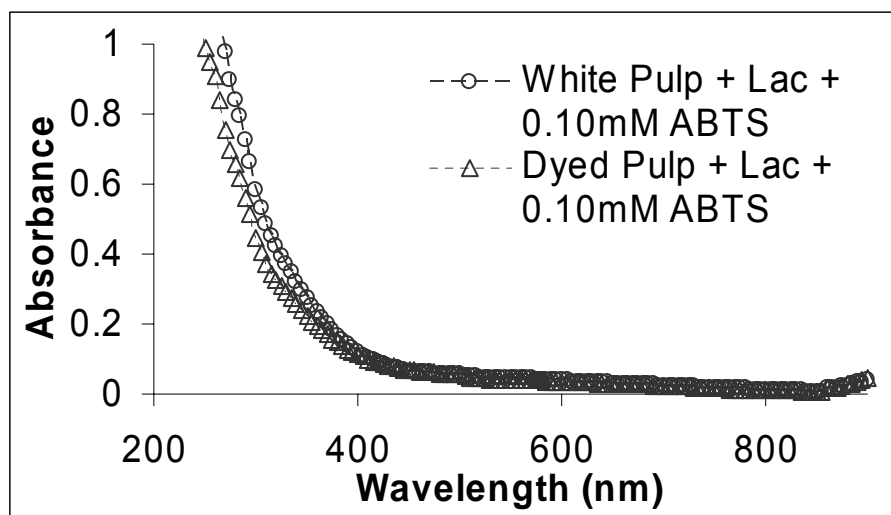
Although LMS treatment does reduce the color of dyed pulp, it is unclear whether Direct Yellow 11 is being stripped from the pulp or chemically altered while remaining attached to the pulp. Since significant electron delocalization is required for the

absorption of light in the visible range, interruption of conjugated double bonds would reduce visible color. If absorption shifts to the ultraviolet range, the pulp color would decrease even though the dye is still attached to the fiber.

When the effluent from the treated dyed pulp was compared with the effluent from the treatment of the starting (undyed) white pulp, little difference can be noticed (Figure 53). No dye peak at 411nm is observed which indicates that simple stripping of the intact dye is not occurring. The observed spectra can be attributed to absorption by laccase and the ABTS mediator. If any breakdown products from the dye are present in the effluent, these putative products do not absorb visible light.

Figure 53 Effluents (Post-Reaction Solutions)

From the Treatment of Bleached and Dyed Pulps with Laccase and 0.1mM ABTS



7.1.4.2 Peroxidase treatments

Stilbene dye Direct Yellow 11 responds poorly to treatment with peroxidases (Table 7-1). In fact, HRP slightly increased the area of absorbance for Direct Yellow 11 (Figure 54).

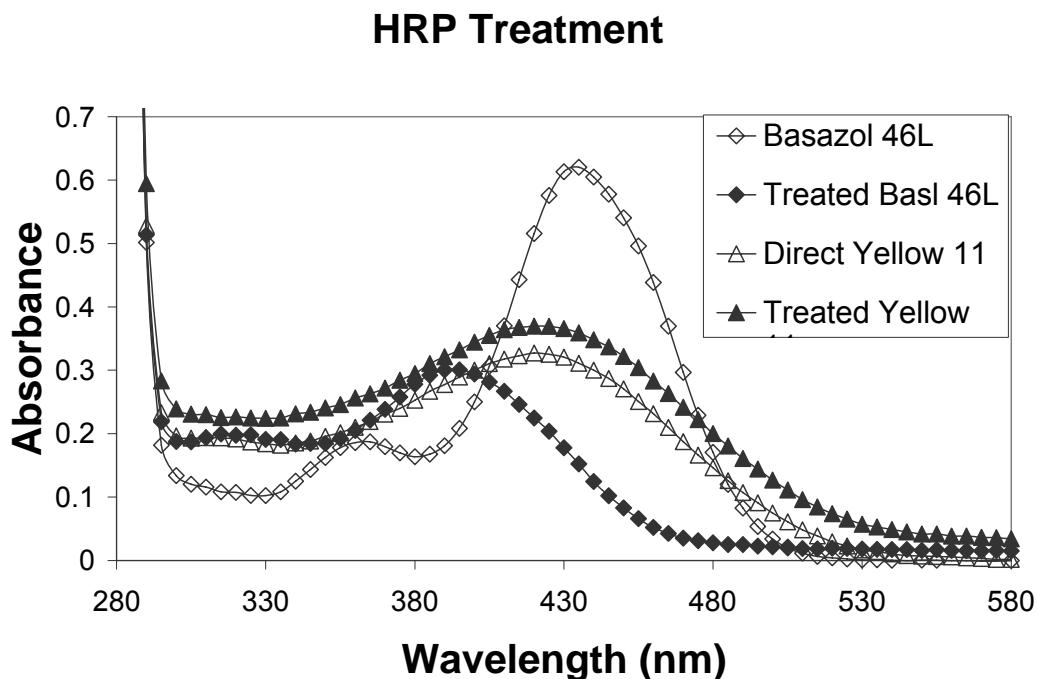
Table 7-1 and Figure 54 also show that the methine dye Basazol 46L (U.S. Patent # 4,256,458 1981) responds well to treatment with peroxidases. Given that SBP is less expensive, more effective, and stable through a broader pH range than HRP, soybean peroxidase treatment was explored further.

Table 7-1 Comparison of Peroxidases

% Reduction in Absorbance Area		
Dye	Direct Yellow 11	Basazol 46L
SBP	19.5	88.9
HRP	-14.6	73.5

7.1.4.3 Effects of pH and PEG on SBP treatment

Because polyethylene glycol (PEG) has been reported to stabilize SBP,²¹⁰ 500 mg/mL of different PEGs (Table 2) were included while examining the effect of pH on SBP bleaching. Table 7-2 shows that SBP biobleaching is most effective between pH 3.8 and 8.5. The inclusion of 10,000 or 35,000 molecular weight PEG has little effect on decolorization.

Figure 54 Results from Treating Dyes with Horseradish Peroxidase

*Table 7-2 % Decrease in Absorbance Area at Various pHs
in Presence of Different PEGs*

SBP Treat	pH	PEG	% Dec.	PEG	% Dec.	PEG	% Dec.
Basazol 46L	2.4	None	69.2	10,000 MW	60.2	35,000 MW	67.3
	3.8		85.1		85.3		86.9
	4.5		89.7		87.4		90.6
	8.5		89.0		89.7		89.4

7.1.5 CONCLUSIONS

This research reports one of the first successful applications of laccase and peroxidase to bleach these recalcitrant paper dyes. For the methine dye Basazol 46L, soybean peroxidase showed the most promise particularly for near neutral pH systems. The stilbene dye Direct Yellow 11 did not respond to peroxidases but could be bleached by LMS. Of the mediators tested, ABTS provided the most decolorization.

7.1.6 ACKNOWLEDGMENTS

We would like to thank Novozyme for the donation of the laccase and BASF for the donation of Direct Yellow 11. This work was supported by the member companies of the Institute of Paper Science and Technology.

7.2 PUBLICATION 2

Laccase-Mediator Biobleaching Applied to a Direct Yellow Dyed Paper

Kristina Knutson and Arthur Ragauskas

Biotechnology Progress (2004) **20(6)**: 1893-1896

7.2.1 ABSTRACT:

Laccase-Mediator System (LMS) for biobleaching was applied to a bleached chemical pulp dyed with stilbene dye Direct Yellow 11. Of mediators tested, 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) was found to be more effective than either violuric acid (VA) and N-hydroxybenzotriazole (HBT), which had been shown to be superior to ABTS when bleaching virgin chemical pulp. The laccase-ABTS system removed more than 60% of the color.

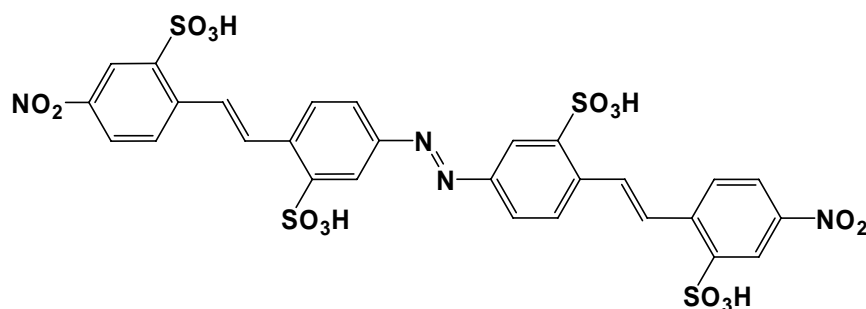
7.2.2 INTRODUCTION

The ability to use enzymatic systems to convert waste materials into value added products in an environmentally friendly manner and cost-effective manner is a hallmark of modern biotechnology. The use of biotechnology in the pulp and paper industry, especially for recycling applications is now a well established process. The use of cellulases to deink newspaper^{221,222,223} is a commercially accepted practice and the use of amylase for deinking xerographic office waste paper is nearing routine commercial applications.^{224,225}

Mixed office waste paper (MOW) is defined as wastepapers collected from business offices, consisting of various coated and uncoated printing and writing papers.²²⁶ It contains both chemical and mechanical pulp fibers, various types of printing inks, and a wide assortment of mineral fillers and sizing materials. This source of waste paper is frequently underutilized because it includes dyed copy papers and dyed file folders that are difficult to bleach.^{19,227} Many different bleaching approaches have been

studied including ozone,²²⁸ oxygen,²²⁷ hydrogen peroxide^{229,230} and various combinations^{216,231,227} of commonly used chemical treatments including chlorine dioxide, hypochlorite, hydrosulfite, formamidine sulfinic acid (FAS), as well as ozone, oxygen, and hydrogen peroxide. When dealing with colored papers, some dyes are particularly difficult to bleach. Goldenrod yellow copy paper is one of the most difficult papers to bleach¹⁹ and detrimentally impacts the value of the wastepaper resource.⁸⁹ This paper is dyed with a yellow stilbene dye that is notorious⁴ for poor bleachability and has been referred to as “unbleachable under normal bleaching conditions.”⁵¹ When the bleachability of a number of common paper dyes was examined,⁵² Direct Yellow 11 (see Figure 55) was found to respond poorly to treatment with hydrogen peroxide, FAS, hydrosulfite, chlorine, and hypochlorite.

Figure 55 Major Chromophore in Direct Yellow 11



In principal, laccase-mediator systems are a promising biotreatment to oxidatively decolorize MOW since it has been already established that they can bleach several textile dyes including: triarylmethane, indigoid, azo, and anthraquinone dyes.^{179,232} In addition, the application of LMS for biobleaching chemical pulps has been extensively studied (see Paice *et al.*²¹⁸; Call and Mücke⁹⁷; Kleen *et al.*²³³; Chandra *et al.*²³⁴; and Crestini, *et al.*²³⁵). These biobleaching studies are dependent upon the application of the chemical mediator which is attributed to the fact that the enzyme is too large to enter a fiber and oxidize the lignin.⁷ Bourbonnais and Paice were the first to demonstrate that substantial oxidative removal of lignin from a fiber could be accomplished in the presence of ABTS.²¹⁹ Extensive screening efforts^{236,9,209} have found several suitable laccase mediators including 1-hydroxybenzotriazole (HBT) and violuric acid (VA) which can significantly outperform ABTS with respect to lignin removal.²³⁷ Although the use of laccase-

mediated bleaching has been mentioned²⁰⁵ as a possible method to remove dyes from recycled paper, little²⁰⁷ has been published. Perhaps the most notable was a recent report by Li *et al.*²⁰⁵ indicating that a LMS treatment, employing violuric acid as a mediator, was able to increase the brightness of an unspecified yellowed dyed recycled paper from TAPPI Brightness 43.5 to 55.0 after biobleaching. This paper examines the viability of biobleaching the problematical stilbene dye, Direct Yellow 11 with LMS employing ABTS, HBT and VA as mediators.

7.2.3 METHODS AND MATERIALS

Violuric acid and N-hydroxybenzotriazole were obtained from Aldrich. ABTS, (2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate)) was purchased from Sigma. Direct Yellow 11 was acquired from BASF. Laccase (NOVO 51002, from *Trametes villosa*) was a gift from Novozymes. A commercial hardwood fully bleached chemical pulp was employed for all test sheet studies.

7.2.3.1 Enzyme activity assays

Laccase activity was measured by the oxidation of ABTS²⁰⁹ at 420 nm, pH 5.0 ($\epsilon_{420} = 36,000$). For decolorization experiments, 48.5 μ kats of laccase were added per mL of reaction solution.

7.2.3.2 Dyeing pulp with Direct Yellow 11

To obtain a reproducible defined substrate for biobleaching, pulp dyed with Direct Yellow 11 was prepared. Fully bleached hardwood chemical pulp 5% slurry (24 kg containing 1200 g fiber) was mixed at room temperature for one hour with the equivalent of the common commercial dosage: 3 g of liquid dye concentrate and 24 g alum. After extensive washing, a mixture of yellow pulp in clear water was obtained. Spectrophotometric measurement of dye remaining in pulp filtrate indicated that more than 90% of the applied dose of Direct Yellow 11 was attached to the pulp. All UV/Vis

measurements were accomplished using a Perkin Elmer UV/Vis/NIR Lambda 900 Spectrometer.

7.2.3.3 LMS treatment of dyed pulp

The selected reaction conditions, based on optimal LMS biobleaching conditions for biodelignification of chemical pulps²³⁸ were as follows: 45°C, pH 4.5, 10 bar oxygen, stirred for 2-hour reaction followed by extensive washing. The laccase-mediator treatments were carried out in a 1000-mL-capacity temperature-controlled stirred pressure reactor. Direct Yellow 11-dyed pulp (27 g fiber), sodium acetate buffer, pH 4.5, (final concentration 20 mM), and distilled water (873mL) were preheated to 45°C. Three mediators were tested (5 mM each): ABTS, HBT, and VA. After mixing mediator with the pulp, 4.365 kats of laccase was added and stirred. The reactor was closed and pressurized with oxygen to 10 bar. After mixing two hours, the pulp was filtered and washed thoroughly with distilled water until the filtrate contained no visible color.

7.2.3.4 Measurement of color and calculation of Dye Removal Index (DRI)

Following TAPPI Standard Method T218 (the Büchner Funnel Method),²¹² five to seven 4 g fiber testsheets were prepared from the washed pulps. CIELAB color values (TAPPI Standard Method T442-om88²¹³) were then measured with a Technibrite Micro TB-1C machine (Technidyne Corp., New Albany, Indiana, USA). Five measurements were made on each testsheet and the values were statistically treated to obtain average CIELAB color values with error bars for each treatment. These values were then used to calculate the dye removal index (DRI) which measures the percent of color removed by each treatment.⁴⁵ The Lac+ABTS experiment was repeated to obtain an estimate of experimental error. Variation due to experimental error (included in error bar calculation) was significantly less ($p < .001$) than variation due to choice of mediator.

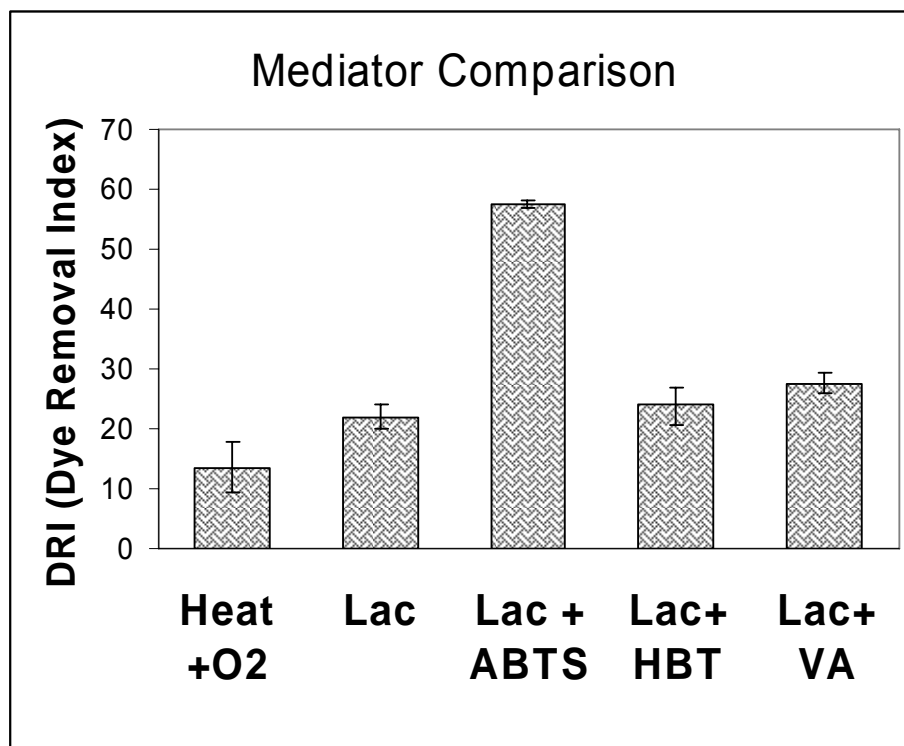
7.2.4 RESULTS AND DISCUSSION

7.2.4.1 Comparison of ABTS, HBT, and VA as laccase mediators.

To evaluate the biobleaching potential for MOW, a synthetic recycled furnish of Direct Yellow 11-dyed hardwood chemical pulp was treated with laccase-mediator systems. In the absence of enzyme and mediators, 13.6% of the color was removed as determined by dye removal index. Laccase treatment, without mediators, resulted in 22.0% color removal.

Subsequently, the laccase biobleaching studies were performed in the presence of a chemical mediator. Three mediators HBT, VA, and ABTS were employed with laccase to biobleach the dyed paper. The treated pulp was washed, filtered and converted into testsheets. The testsheets were subsequently evaluated employing standard CIELAB optical methods. DRI values were calculated based on optical measurements of treated and untreated pulps. The results of this analysis are summarized in Figure 56.

Figure 56 Bleaching Effectiveness of Different Mediators at 5 mM in the Laccase-Mediator Treatment of Pulp Dyed with Direct Yellow11

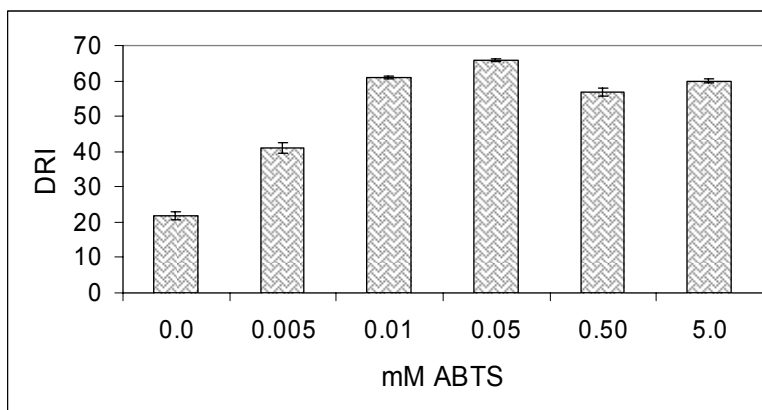


Whereas the DRI values for pulps treated solely with laccase were 22.0% the values of the pulps treated with the laccase mediator system varied between 22.0-59.6%. These results indicated that ABTS was the most effect laccase mediator for removal of Dye 11 under the conditions employed. This was unexpected in-light of the previously reported efficiency of VA and HBT at lignin removal from chemical pulps over ABTS.^{237,220,238} In this system, ABTS was much more effective than HBT in decolorizing pulp dyed with Direct Yellow 11 (Figure 56). To determine that this biobleaching effect was not due the mediator and oxygen, a control experiment with ABTS alone was preformed. CIELAB analysis of the treated sheets indicated dye removal of 22.2%. In summary, these initial results demonstrate the potential for an LMS treatment to remove dye Direct Yellow 11 from paper.

7.2.4.2 Determination of optimal ABTS concentration.

To determine the effectiveness of LMS biobleaching with ABTS a second series of experiments were performed in which the amount of dyed pulp and laccase were held constant while the concentration of ABTS was varied. The treated pulps were washed and subsequently converted into testsheets and analyzed by CIELAB. The results of this analysis are summarized in Figure 57. The optimal ABTS concentration was determined to be 0.01 mM for this system where 27 g of dyed pulp is treated with 4.365 kats of laccase. Even with the five-hundredfold reduction of ABTS concentration, the DRI value for 0.01 mM ABTS is still twice the values for treatment with 5 mM VA.

Figure 57 Bleaching Effectiveness with Varying ABTS Concentrations in the Laccase-Mediator Treatment of Pulp Dyed with Direct Yellow11



Although laccase-mediator systems have been used to bleach chemical pulp and to decolorize dyes in synthetic textile effluents, this study demonstrates the dependency of the LMS system on mediator employed for efficient oxidative removal of direct dye Yellow 11 from paper. In this study, the most effective mediator was ABTS and the least effective was VA. This was unexpected since VA has been shown to substantially outperform ABTS as a mediator for LMS bleaching of virgin chemical pulps.

This unexpected result must be related to the fact that oxidations with ABTS and with N-hydroxyl mediators (HBT and VA) occur by different mechanisms.^{239,240} In the laccase-mediator system, HBT forms benzotriazolyl-1-oxide radical²⁴¹ while ABTS forms both the radical cation and a dication.²⁴² Further experimental work is under way to determine the nature of the reaction between the dyed pulp and the ABTS-laccase system. The ability to enzymatically bleach a difficult stilbene dye has significant practical application during the recycling of mixed office waste and these applications are currently under review.

7.2.5 ACKNOWLEDGMENTS

We would like to thank Novozymes for the donation of the laccase and BASF for the donation of Direct Yellow 11. This work was supported by the member companies of the Institute of Paper Science and Technology.

7.3 PUBLICATION 3

Enzymatic biobleaching of two recalcitrant paper dyes with horseradish and soybean peroxidase

Authors: Kristina Knutson, Sylva Kirzan and Arthur Ragauskas

Accepted for publication by *Biotechnology Letters*

7.3.1 ABSTRACT

A stilbene and a methine dye known to be recalcitrant to common chemical bleaches were treated with horseradish and soybean peroxidases and both oxoreductive enzymes were shown to be effective at chromophore removal. On a comparative basis, soybean peroxidase was found to be more effective at bleaching these dyes. When compared to laccase in combination with ABTS as mediator, soybean peroxidase was found to be more effective at oxidative dye removal, especially for the methine dye Basazol 46L.

7.3.2 INTRODUCTION

Efficient utilization and recycling of natural resources is a hallmark of modern industrial manufacturing processes. Over the past decade paper recycling in America has progressed to about 45% in 1999.^{2,43} Indeed, for certain select grades, (newspaper and old packaging containers) greater than 68% is currently being recycled. In contrast, mixed office waste and colored directory papers are often underutilized (38% for printing/writing paper). A major difficulty in recycling these grades of paper is the problems associated with decolorizing the dyes present in the paper.² Of the commonly used paper dyes, the stilbene dye Direct Yellow 11 and methine dye Basazol 46L are notorious⁴ for poor bleachability with the commonly used chemical bleaching agents including chlorine dioxide, oxygen, hydrogen peroxide and sodium dithionite.

A number of enzymes from lignolytic fungi have been isolated and tested for the ability to bleach wood pulps including manganese peroxidase^{244,245} lignin peroxidase²⁴⁶ and laccase.²³⁸ Fungi producing these enzymes are capable of decolorizing a number of dyes including Malachite Green, Azure B, Poly R-478, Anthraquinone Blue, Congo Red and Xylidine.¹⁸¹ Dye decolorization by other peroxidases has also been described^{247,248}

The purpose of this study was to determine the ability of peroxidases to decolorize Direct Yellow 11 and Basazol 46L and to compare peroxidase bleaching to laccase-mediator bleaching. The development of a laccase mediator system utilizing 2, 2'-azinobis- (3-ethylbenzthiazoline-6-sulfonate), known as ABTS, as mediator to decolorize pulp dyed with Direct Yellow 11 has been recently reported.²⁴⁹ This study examines the relative reaction rates for laccase/ABTS and soybean peroxidase biobleaching.

7.3.3 MATERIALS AND METHODS

7.3.3.1 Dyes and enzymes

Basazol 46L (B46) and Direct Yellow 11 dyes were donated by BASF. Soybean peroxidase, horseradish peroxidase and laccase mediator 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) were purchased from Aldrich. Polyethylene glycol 10,000 MW and 35,000 MW were purchased from Fluka. Laccase (NOVO 51002, from *Trametes villosa*) was provided by Novozyme.

7.3.3.2 Enzyme assays

Soybean peroxidase activity was measured by the oxidation of phenol in the presence of 4-aminoantipyrine at 510 nm ($\epsilon_{420} = 36,000$)²¹⁰ (Kinsley and Nicell 2000). One unit was defined as μ mole peroxide consumed per min. One unit of SBP was used per mL of solution. Laccase activity was measured by the oxidation of ABTS²⁰⁹ at 420 nm ($\epsilon_{420} = 36,000$) in pH 5.0 acetate buffer. One unit of laccase was defined as μ mol ABTS oxidized per min. 10.3 units of laccase were added per mL of reaction.

7.3.3.3 Horseradish peroxidase treatments

The ability of horseradish peroxidase (HRP) to decolorize Basazol 46L and Direct Yellow 11 was examined by treating the dyes with HRP for two hours at 45°C. The dye concentration was set at 10.0 mg/L of non-volatile dye solids. The reaction mixture included 100 mM sodium acetate buffer, pH 3.8 and 1.0 mM veratryl alcohol. Five units (5.0) of horseradish peroxidase (activity 116 PPU/mg based on a purpurogallin assay) was added per mL reaction volume. After preheating this mixture to 45°C, the reaction was initiated by adding hydrogen peroxide (0.10 mM final concentration).

7.3.3.4 Soybean peroxidase treatments

Treatment conditions for soybean peroxidase (SBP) were virtually the same as for HRP treatment. The dye concentrations were the same, 10 mg/L, and the reaction proceeded for two hours at 45°C. In this case, the reaction mixture included 0.10 M calcium chloride as well as 100 mM sodium acetate buffer, pH 3.8 and 1.0 mM veratryl alcohol. 1.0 unit of SBP was added for every mL of reaction mix. After preheating the reaction mixture to 45°C, the reaction was initiated by adding hydrogen peroxide (0.10 mM final concentration). In studies where the pH was varied, the buffers (100 mM) used included glycine-HCl (pH 2.4), sodium acetate (pH 2.8 and pH 4.5), sodium citrate, phosphate (pH 6.5) and glycine-NaOH (pH 8.5 and pH 10.5). To obtain an estimate of experimental error, 8 identical samples at pH 4.5 were treated with SBP to yield average signal intensity of 7.95 with standard deviation of 0.49 (6% variation)

For tests to determine if a free radical mediator was involved, pH 6.5 samples of Basazol 46L (10.0 mg/L) were prepared with and without 5.0 mM dimethyl sulfoxide. These samples were then reacted with soybean peroxidase (1.0 U/mL) as described above.

7.3.3.5 Laccase-mediator system treatment of Basazol 46L dye

The laccase/ABTS system developed for treatment of Direct Yellow 11²⁴⁹ was tested for its ability to decolorize Basazol 46L dye. Since laccase uses oxygen as an electron source, the solution concentration of oxygen was increased by performing the reaction with stirring under 1000 kPa of oxygen pressure. As in the peroxidase reactions, the dye concentration was set at 10 mg/L of non-volatile dye solids. The reaction mixture included 100 mM sodium acetate buffer, pH 4.5 and 1.0 mM ABTS. After preheating the reaction mixture to 45°C, laccase was added (10.3 units/mL reaction solution) and a time zero sample was taken. The system was flushed with oxygen for 60 seconds, sealed, pressurized to 1000 kPa and stirred. After stirring for two hours, the pressure was released and the treated sample was collected. A control of laccase plus ABTS (no dye) was also performed.

7.3.3.6 Reaction rate study of laccase/ABTS decolorization of Direct Yellow 11

For reaction rate studies of the laccase/ABTS treatment of Direct Yellow 11, a reaction mixture of 20.0 mg/L Direct Yellow 11, 1.0 mM ABTS and 10.3 Units laccase/mL was prepared in 100 mM sodium acetate buffer pH 4.5 as described above. The reaction was allowed to continue for 24 hours under 10 bar oxygen. During the course of the reaction, 10 mL aliquots were removed for analysis. Samples were also obtained for the same time points from a pressurized control reaction containing laccase and ABTS, performed under analogous reaction conditions.

7.3.3.7 Reaction rate study of SBP/H₂O₂ decolorization of dyes

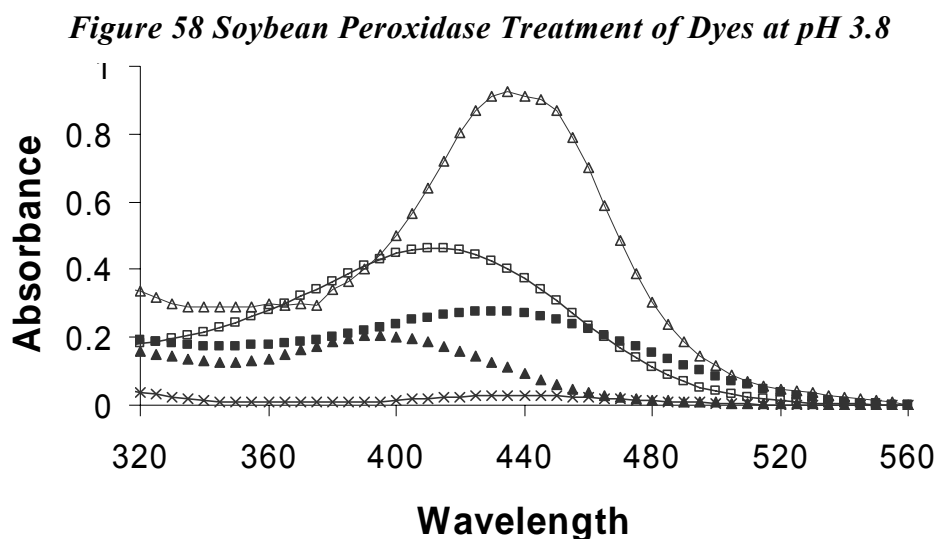
SBP treatment was performed as described above using individual aliquots of a large master solution of 10.0 mg/L of dye, 100 mM acetate buffer, pH 4.5, 1.0 mM veratryl alcohol and 1.0 unit of SBP/mL. Reaction was halted by removing from 45°C water bath. After chilling for one minute in an ice bath, the spectra were obtained.

7.3.3.8 Data collection and analysis

Spectra were obtained from all the samples prepared as described above using a Perkin Elmer Lambda 900 UV/Vis/NIR spectrophotometer. The areas under the peak curves (λ : 285-550 nm for Direct Yellow 11, 335-498 nm for Basazol 46L) were determined. Because the absorption peaks from laccase/ABTS overlap with the absorption peaks of the dyes, the spectra of the appropriate laccase/ABTS control was subtracted from the spectra of the dye/laccase/ABTS spectra before quantifying. Extent of decolorization was determined calculating the percentage decrease in signal intensity (i.e., 100% would equal no dye peak remaining).

7.3.4 RESULTS AND DISCUSSION

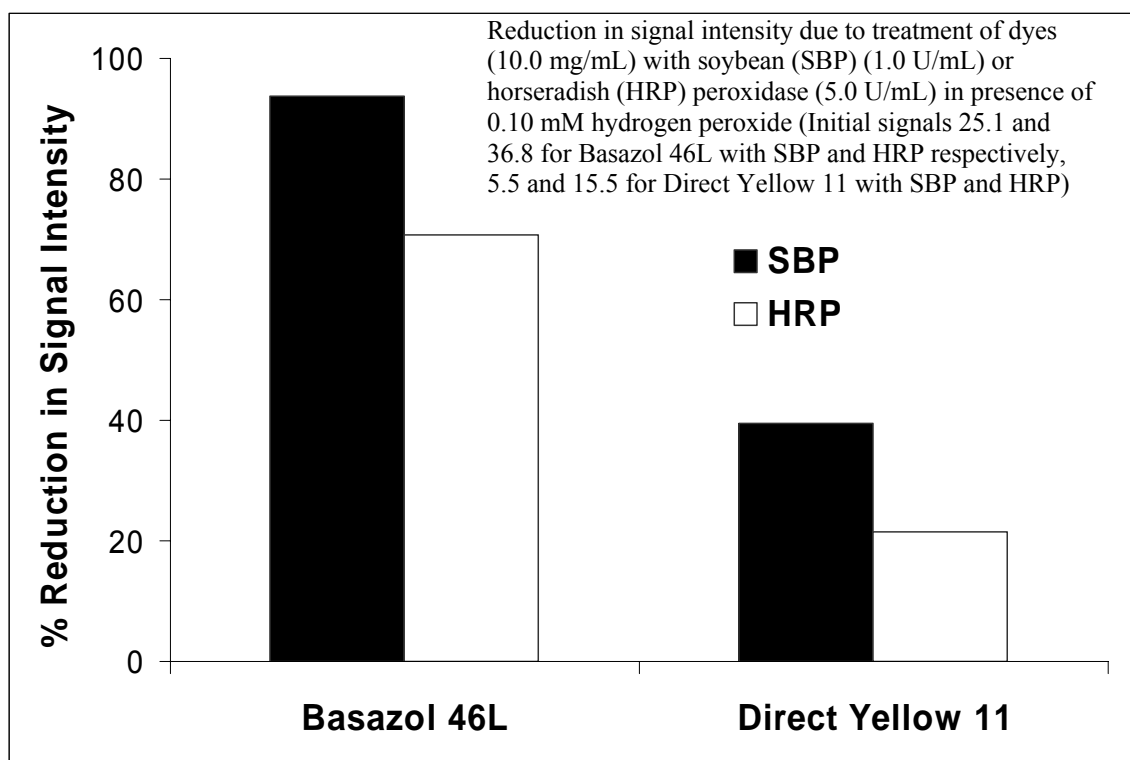
Spectra of stilbene dye Direct Yellow 11 and methine dye Basazol 46L before and after treatment with soybean peroxidase are shown in Figure 58. SBP treatment of Basazol 46L resulted in a substantial decrease in the signal intensity and in a shifting of the dye maxima suggesting a chemical alteration in the dye.



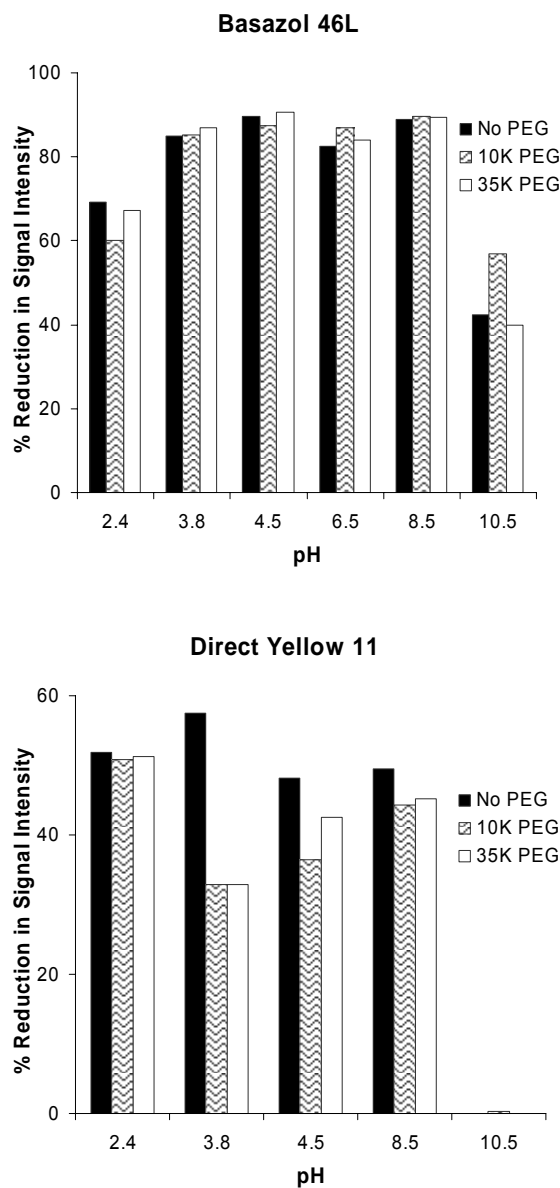
Treatment of 10 mg/L Basazol 46L (Δ) and 10 mg/L Direct Yellow 11 (\square) with soybean peroxidase (1.0 U/mL) and hydrogen peroxide (0.10 mM) for two hours at 45 °C. Corresponding solid symbols are the untreated dyes. No dye control (\times) is included for comparison.

When both dyes were subjected to treatment by HRP and SBP, measurement of the dye UV/Vis absorption intensity showed that both SBP and HRP significantly reduce signal intensity of methine dye Basazol 46L, as summarized in Figure 59. The signal intensity of Direct Yellow 11 UV/Vis spectrum also decreased with peroxidase treatment but the effect was not as great. The difference in effectiveness between SBP and HRP for biobleaching these dyes is greater for the stilbene dye Direct Yellow 11 than for the methine dye Basazol 46L.

Figure 59 Comparison Between Horseradish and Soybean Peroxidases



Previous published reports had indicated that soybean peroxidase is stable over a wide pH range^{250,251} and that inclusion of polyethylene glycol (PEG) could further stabilize the enzyme.²⁵² Thus a study was undertaken treating both dyes at a variety of pHs, in the absence and presence PEG. Based on the work by Kinsley and Nicell,²¹⁰ two high molecular weight PEGs (10,000 MW and 35,000 MW), were chosen with an application concentration of 500 mg/L. The results of these studies are shown in Figure 60.

Figure 60 Effect of pH and PEG on SBP Decolorization

Reduction in signal intensity due to treatment of dyes (10 mg/mL) with soybean (SBP) (1.0 U/mL) at various pHs in presence of 0.10 mM hydrogen peroxide (plus 0.10 M calcium chloride and 1.0 mM veratryl alcohol) for 2 h at 45 °C with and without 10,000 (10K) and 35,000 (35K) MW PEG. (Average signal before treatment: 49.1 Basazol 46L, 31.0 Direct Yellow 11)

The inclusion of PEG appeared to inhibit decolorization of Direct Yellow 11 at pH 3.8. At pH 10.5, inclusion of 10,000 MW PEG appeared to enhance the decolorization of Basazol 46L. Otherwise, the presence of PEG had relatively little effect. SBP appears to be effective at biobleaching from pH 4 to pH 8.

To determine if a free radical reaction was responsible for the biobleaching effect, peroxidase activity was measured in the presence and absence of 5.0 mM dimethyl sulfoxide (DMSO), a hydroxyl radical quencher.²⁵³ The amount of dye decolorization was unaffected by the inclusion of DMSO, suggesting that the mechanism of dye decolorization by soybean peroxidase does not involve a free radical. This was true for reactions that included PEG as well as in the absence of PEG

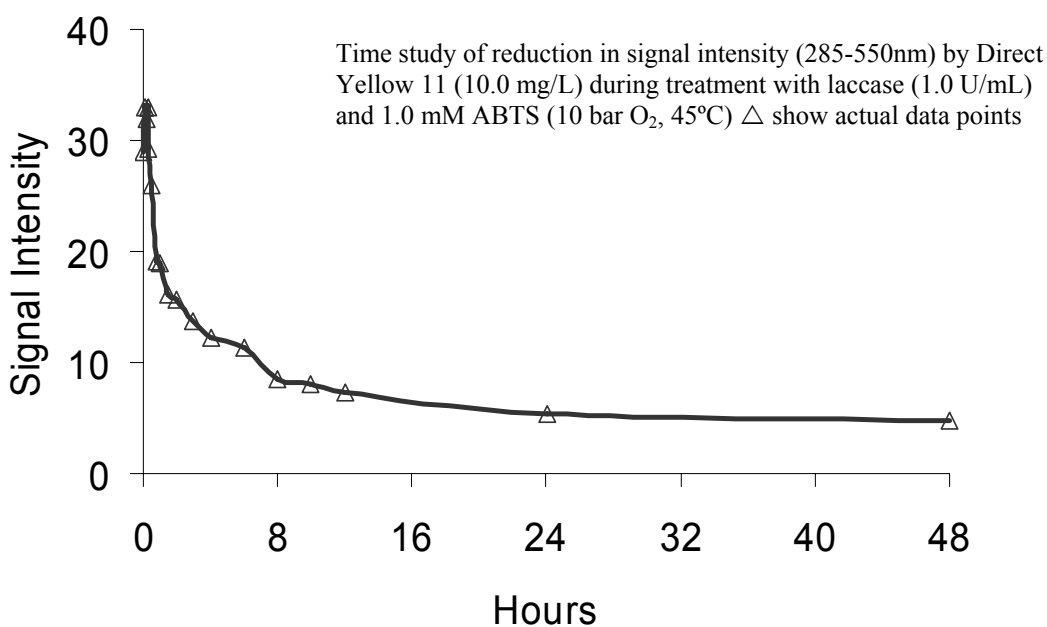
The relative effectiveness of enzymatic bleaching by SBP and by laccase/ABTS can be seen in Table 7-3. Soybean peroxidase appears to be more effective than laccase/ABTS, especially for decolorization of Basazol 46L. Reaction rate study of the decolorization of Direct Yellow 11 by laccase/ABTS is shown in Figure 61.

Table 7-3 Reduction in Signal Intensity Following Treatment with Lac/ABTS or SBP

	Lac/ABTS	SBP
Direct Yellow 11	46%	49%
Basazol 46L	34%	87%

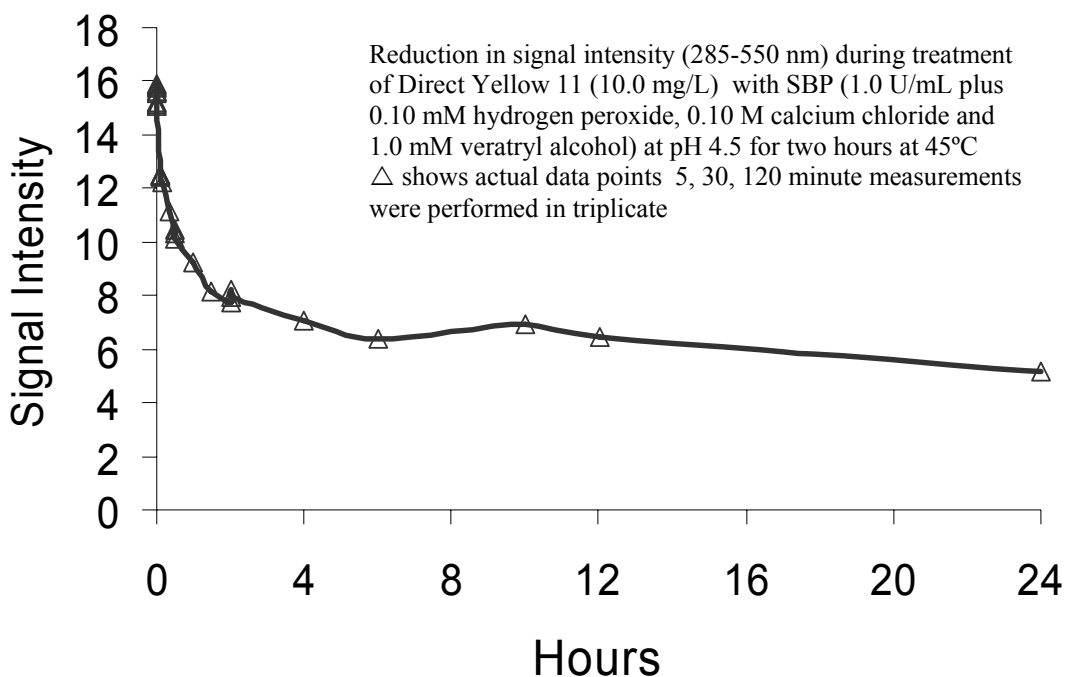
Reduction in signal intensity at 285-550nm (Direct Yellow 11, initial signal 29) and 334-508nm (Basazol 46L, initial signal 49) due to treatment of dyes (10 mg/L) with laccase (10.3 U/mL plus 1.0 mM ABTS and 10 bar O₂) and with soybean peroxidase (1.0 U/mL plus 0.10 mM hydrogen peroxide, 0.10 M calcium chloride and 1.0 mM veratryl alcohol) at pH 4.50 for two hours at 45 °C

Figure 61 Decolorization of Direct Yellow 11 by Laccase/1 mM ABTS



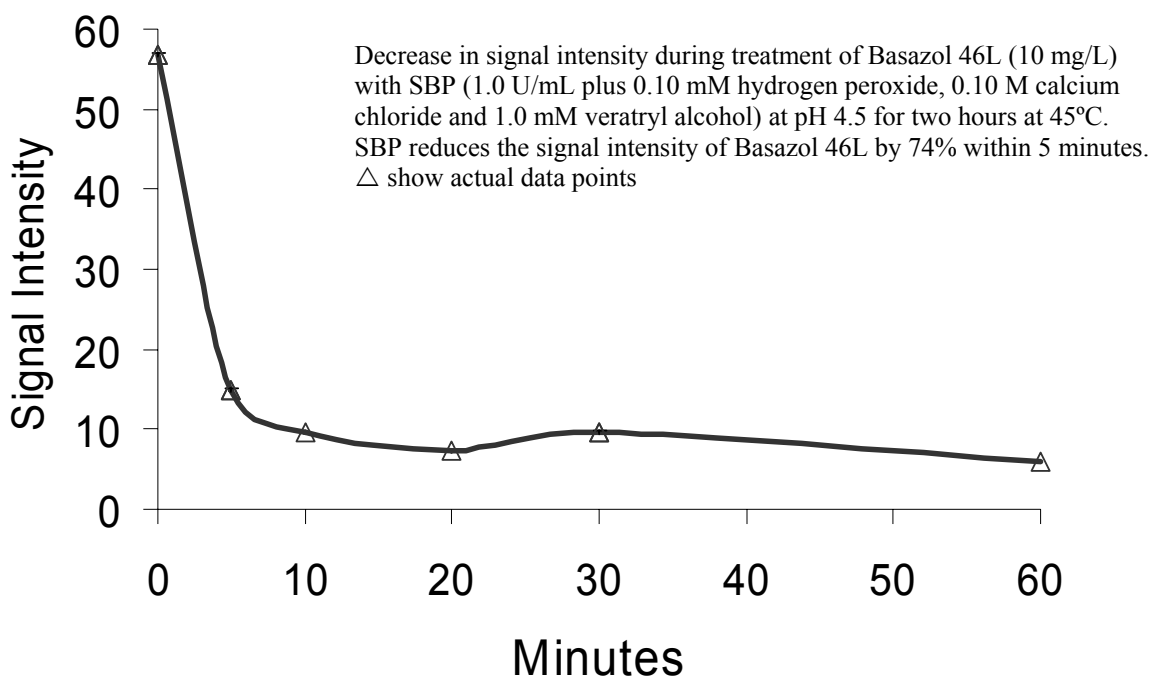
After 90 minutes of biobleaching with the laccase system, 51% of the dye signal has disappeared. As shown in Figure 62, decolorization of Direct Yellow 11 by SBP follows similar kinetics with 48% dye decolorization in 90 minutes. Stilbene dyes, such as Direct Yellow 11, are considered to be relatively unreactive to bleaching chemicals. This is consistent with the relatively slow decolorization of Direct Yellow 11.

Figure 62 Decolorization of Direct Yellow 11 by SBP/Veratryl Alcohol



The methine dye Basazol 46L responds more rapidly to soybean peroxidase. Within five minutes, the measured signal intensity decreases 74% (Figure 63). Longer incubation times result in a slight additional decrease in signal intensity (data not shown).

Figure 63 Decolorization of Basazol 46L by SBP/Veratryl Alcohol



These results demonstrate that these two recalcitrant dyes can be effectively decolorized by enzymatic treatments by horseradish peroxidase, soybean peroxidase, and laccase with ABTS as mediator. SBP is effective from pH 4.5 to 8.5. The stilbene dye Direct Yellow 11 responds to both SBP and laccase/ABTS. For the methine dye Basazol 46L, SBP is more effective and the reaction is relatively fast.

7.3.5 ACKNOWLEDGEMENTS

We would like to thank Novozymes for the donation of the laccase and BASF for the donation of Basazol 46L and Direct Yellow 11. This work was supported by the member companies of the Institute of Paper Science and Technology.

7.4 PUBLICATION 4

Decolorization of Colored Paper by Laccase-Mediator System and Conventional Treatments

Kristina Knutson and Arthur Ragauskas

Manuscript in preparation

7.4.1 ABSTRACT

A laccase-mediator has been developed for the decolorization of pulp dyed with recalcitrant stilbene dye Direct Yellow 11 using 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the mediator. To compare the bleaching effectiveness of this system to existing bleaching methods, three commercial colored pulps and pulp dyed with Direct Yellow 11 were subjected to bleaching with the laccase-ABTS system, alkaline oxygen and alkaline hydrogen peroxide. Pulp subjected to these treatments was also further bleached with sodium dithionite. For pulp dyed with Direct Yellow 11, treatment with laccase/ABTS followed by sodium dithionite was as effective as alkaline hydrogen peroxide followed by sodium dithionite. For the commercial colored pulps, hydrogen peroxide followed by sodium dithionite was the most effective bleaching method.

7.4.2 INTRODUCTION

Mixed office waste and colored directory paper are underutilized for generation of recycled fibers. A major difficulty in using these furnishes is the problems associated with decolorizing the dyes present in the paper.^{3,2} Of the commonly used paper dyes, the stilbene dye Direct Yellow 11 is notorious⁴ for poor bleachability. When the bleachability of a number of common paper dyes was examined,⁵² Direct Yellow 11 was found to respond poorly to treatment with hydrogen peroxide, FAS, hydrosulfite, chlorine, and hypochlorite.

Laccase-mediator systems (LMS) have been extensively studied for the biobleaching of wood pulps (for reviews, see Paice *et al.*,²¹⁸ Call and Mücke⁹⁷). Laccases (EC 1.10.3.2) are oxidoreductases that combine single-electron oxidization of many aromatic substrates with the reduction of oxygen to water.²⁵⁴ Bourbonnais and Paice demonstrated that the substrate range of laccase can be extended to nonphenolic compounds by the addition of mediator compounds such as 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate), as known as ABTS.²¹⁹

A laccase-mediator system²⁴⁹ has been recently developed for bleaching pulp dyed with Direct Yellow 11 using ABTS as the mediator. The purpose of this study was to compare the effectiveness of laccase/ABTS treatment with existing chemical bleaching treatments.

7.4.3 METHODS AND MATERIALS

7.4.3.1 Materials

Direct Yellow 11 dye was donated by BASF. Laccase (NOVO 51002, from *Trametes villosa*) was provided by Novozyme. Laccase mediator 2, 2'-azinobis-3-ethylbenzthiazoline-5-sulfonate (ABTS) was purchased from Sigma.

7.4.3.2 Enzyme activity assays

Laccase activity was measured by monitoring the rate of oxidation of ABTS²⁰⁹ in a 100mM pH 5 sodium acetate buffer. One International Unit (IU) of activity is defined as the conversion of 1 μ mole of substrate/ minute. One Katal (kat) of activity is defined as 1 mole/second of substrate converted. The reaction was followed at 420nm ($\epsilon_{420} = 36,000$)

7.4.3.3 Peroxide concentration assay

For reactions involving hydrogen peroxide, the concentration of the stored stock solution (nominally 30%) must be determined. The assay procedure²¹¹ involves titration of an acidic solution of hydrogen peroxide with sodium dithionite in the presence of starch indicator. Two (2) mL of peroxide solution is mixed with 100 mL distilled water,

10-15 mL of 25% sulfuric acid, 5 mL of 10% potassium iodide (KI) solution and 3 drops of saturated ammonium molybdate solution. This mixture is titrated with 0.1N sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) until a pale yellow color appears. Then 10mL of soluble starch solution is added and the thiosulfate titration continues until the blue color disappears. The calculation of hydrogen peroxide concentration is shown below.

Equation 10 Calculation of Hydrogen Peroxide Concentration

$$\text{Grams/Liter} = \frac{0.1(\text{norm. thio.}) \times 34 (\text{equiv. H}_2\text{O}_2 - 50\%) \times (\text{mL. thio used})}{2 \text{ mL (sample size)}}$$

7.4.3.4 Dyeing pulp with Direct Yellow 11

To obtain a reproducible defined substrate for biobleaching, pulp dyed with Direct Yellow 11 was prepared. Fully bleached hardwood kraft pulp (1200 o.d.g) was mixed at room temperature for one hour (5% cs) with the equivalent of the common commercial dosage: 3 g of liquid dye concentrate and 24 g alum (to attach the dye to the pulp). After extensive washing, a mixture of yellow pulp in clear water was obtained.

7.4.3.5 Preparation of commercial dyed pulp

Reams of RepliCopyColors™ paper designed for high-speed copying, laser and offset printing were purchased. The colors selected for use were blue, cherry (pink) and goldenrod (orange). 80 sheets (8 ½ x 11", about 4.2 g each) of each color were ripped into small pieces and soaked in 3 L of distilled water. The paper was processed in batches in a Waring blender (total of 7 L of distilled water used) to produce pulp. The pulp was thickened, and stored at 4°C until used.

7.4.3.6 LMS treatment of dyed pulp

The selected reaction conditions, based on optimal LMS biobleaching conditions for biodelignification of chemical pulps²³⁸ were as follows: 45°C, pH 4.5, 10 bar oxygen, stirred for 2-hour reaction followed by extensive washing. The laccase-mediator treatments were carried out in a 1000-mL-capacity temperature-controlled stirred pressure reactor. Colored pulp (27 g, o.d. final consistency 3%), sodium acetate buffer, pH 4.5, (final concentration 20 mM), ABTS (10mM), and distilled water (873mL) were mixed and preheated to 45°C. Laccase (4.365 kats) was then added. The reactor was

closed and pressurized with oxygen to 10 bar. After mixing two hours, the pulp was filtered and washed with a liter of distilled water.

7.4.3.7 Alkaline extraction of laccase-treated pulp

Xylanase treatment improves the efficacy of subsequent deinking treatment.²¹⁵ To determine if laccase/ABTS treatment would also improve subsequent steps, colored pulps were subjected to laccase-1mM ABTS treatment, followed by alkaline extraction.

Colored pulp (25g, o.d.) in a heat-sealable bag was placed in a 60°C water bath and preheated for at least five minutes. Sodium hydroxide (0.1g, or 0.4%) and water (final volume 250 mL, consistency 10%) were added. The bag was then heat sealed, mixed thoroughly, and placed in the 60°C water bath for 60 minutes. The bag was then opened; the pulp was suction-filtered and washed with 1 L of distilled water.

7.4.3.8 Oxygen bleaching of colored pulps

Alkaline oxygen bleaching of colored pulps was performed in the Parr pressure reactor. The reaction conditions²¹⁶ include 90°C, 90 psi (6 bar) oxygen pressure and 0.8% charge of sodium hydroxide (0.216g NaOH for 27g o.d. pulp). Water, sodium hydroxide and the reaction vessel were preheated to 90°C. The pulp was rapidly heated to 90°C in a microwave and added to the pressure reactor. For the time course studies, the pulp consistency was 10%. For the bleaching comparison studies, the pulp consistency was 5%. As a control, the time course studies included pulp samples that were also mixed with alkali but which were allowed to react for 2 hours at room temperature under ambient pressure.

7.4.3.9 Peroxide bleaching of colored pulps

Bleaching of colored pulp with alkaline hydrogen peroxide was performed following the procedure of Li *et al.*²¹⁷ Colored pulp (53g, o.d.) was mixed with water (final volume 1060 mL, 5% consistency) and DTPA (diethylene triamine pentaacetate, 0.11g or 0.2%) in a heat-sealable bag and preheated in 75°C water bath for at least 10 minutes. (DTPA is a metal chelator that stabilizes hydrogen peroxide.) Sodium hydroxide (0.53g or 1%) and hydrogen peroxide (0.53g or 1%) were added. After heat-sealing, the

pulp slurry was mixed thoroughly and placed in 75°C water bath for 60 minutes. The bag was then opened; the pulp was suction-filtered and washed with 1 L of distilled water.

7.4.3.10 Dithionite treatment of colored pulps

Reductive bleaching of colored pulp was performed following the procedure of Li *et al.*²¹⁷ Sodium dithionite (0.5g, 2% on o.d. pulp) was dissolved in 50 mL of distilled water that had been boiled and then cooled while a stream of nitrogen gas bubbled through it. The colored pulp (25g, o.d.) was mixed with water (final reaction volume 750 mL, consistency 3.33%) and placed in a heat-sealable Kapak bag. The pulp slurry was then placed in a 75°C water bath and allowed to preheat for 10 minutes as nitrogen gas was bubbled through the pulp slurry. Nitrogen bubbling continued as the pulp slurry was removed from the water bath, the dithionite solution was added and the contents were mixed for 60 seconds. After measuring pH, the bag was then heat sealed and the pulp slurry was placed in the water bath. After reacting for 60 minutes, the bag was opened and the pH was measured. The pulp was suction filtered and washed with 1 L of distilled water.

7.4.3.11 Calculation of Dye Removal Index (DRI)

The LMS decolorization of pulp dyed Direct Yellow 11 was quantified by measuring the CIELAB color values for handsheets prepared following TAPPI Standard Method T218. The DRI measures the percent change in geometric distance from the ideal bleach point. It is calculated following the equations shown below.⁴⁵

Equation 11 Dye Removal Index Calculation⁴⁵

$$\text{Distance from Ideal Bleach Point} = R^2$$

$$R^2 = a^2 + b^2 + (100-L)^2$$

$$\text{Amount of Color Removal} = -\Delta R^2$$

$$\Delta R^2 = R_2^2 - R_1^2$$

$$\Delta R^2 = (a_2^2 - a_1^2) + (b_2^2 - b_1^2) + [(100-L_2)^2 - (100-L_1)^2]$$

$$\text{Dye Removal Index (DRI)} = -100 [\Delta R^2 / R_1^2]$$

$$\text{DRI} = \text{Percent Color Removal by Bleaching Process}$$

7.4.4 RESULTS AND DISCUSSION

7.4.4.1 Reaction rate study for oxygen bleaching

To determine how quickly oxygen bleaching can remove color, samples of each of the three commercial colored pulps were subjected to oxygen bleaching for different amounts of time. Brightness and $L^*a^*b^*$ values were then obtained for handsheets made from the treated pulps. The brightness values are shown in Figure 64 below. Base values (shown in Figure 64 as open circles on vertical axis) are measurements from handsheets made directly from the colored pulps. Zero time brightness values are higher because the pulp was diluted and washed in the same manner as the pulps exposed to oxygen pressure. Controls (RT, points at 2 hour in graphs below) were also prepared: pulp mixed with caustic and reacted for 2 hours at room temperature and ambient pressure. The brightness of the blue and cherry pink pulp increases quickly (within 10 minutes) and further incubation does not yield further improvement. The brightness of the goldenrod orange paper was low and extending bleaching time to 120 minutes did not improve brightness. Room temperature treatment with sodium hydroxide for an extended time (2 hours) lead to brightness improvements achievable with 10 minutes of oxygen bleaching.

Figure 65 shows why evaluation of $L^*a^*b^*$ values are necessary when evaluating bleaching treatments for colored materials. Little change in brightness was observed for pink and blue pulps between 10 minutes and 60 minutes of oxygen bleaching. Also, little difference in brightness was observed between pulps bleached for 60 minutes with oxygen at 90° and pulps bleached with caustic for 120 minutes at room temperature and pressure. However, when the dye removal index values are compared (calculations based on color in time zero handsheets), it can be seen that extending bleaching time from 10 minutes to 60 minutes did lead to a distinct improvement in the amount of color removed. Also, the dye removal index showed more color was removed by oxygen bleaching at 90°C for 60 minutes than was removed by 120 minutes at room temperature and pressure. Despite the nearly unchanged brightness values, even the color in goldenrod orange decreased when the $L^*a^*b^*$ values are measured.

Figure 64 Improvements in Brightness during Oxygen Bleaching

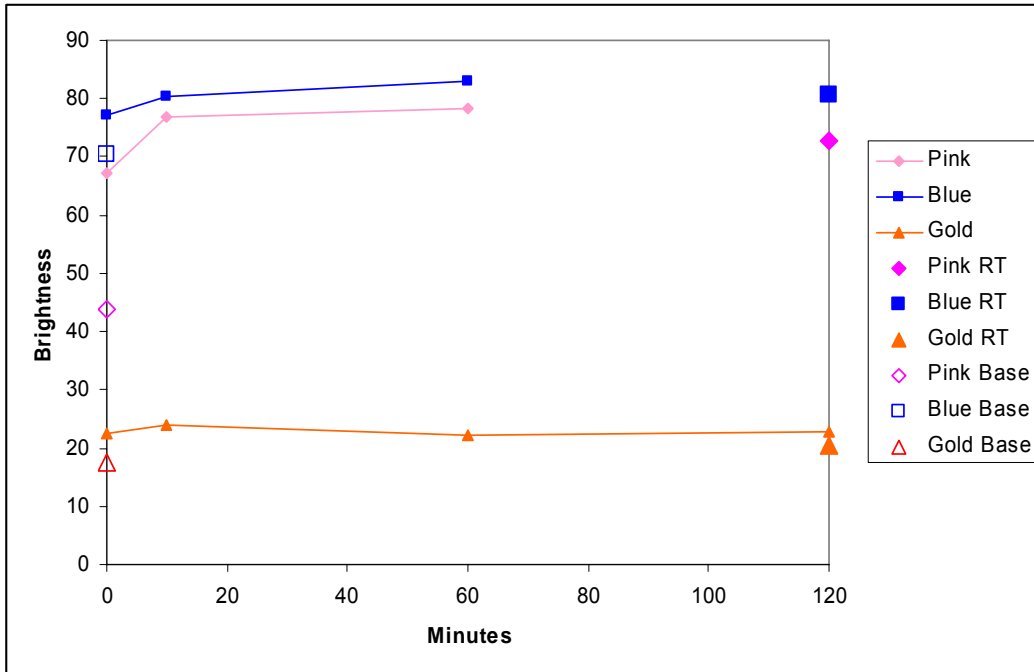
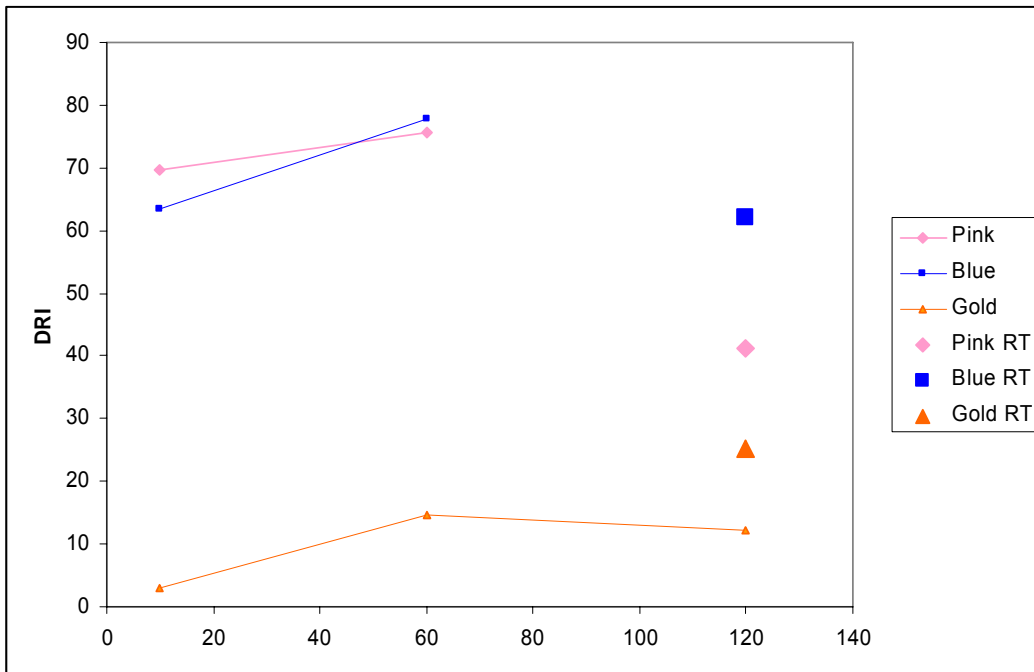


Figure 65 Improvements in Dye Removal Index Values during Oxygen Bleaching



7.4.4.2 Comparison between bleaching treatments

To evaluate bleaching effectiveness of the laccase/ABTS system, four colored pulps were subjected to a series of bleaching treatments as outlined below in Table 7-4. The bleaching treatments are defined in the right column of Table 40. The left column lists associated abbreviation applied to the results of that treatment as shown in the succeeding figures.

Table 7-4 Bleaching Treatments Applied to Colored Pulps

L	1. Laccase/ABTS bleaching: 2hrs, 10bar O ₂ , 45°C, pH 4.5, 0.01mM ABTS, 3% cs*
LAE	2. Laccase/ABTS bleaching as above; followed by Alkaline Extraction: 1 hr, 60°C, 10% cs, 0.4% NaOH or 0.1g NaOH/ 25g o.d. pulp
LDT	3. Laccase/ABTS bleaching as above; followed by Dithionite Bleaching: 1 hr, 75°C, cs 3.33%, 2% Na ₂ S ₂ O ₄ or 0.5 g Na ₂ S ₂ O ₄ / 25g o.d. pulp
O	4. Oxygen bleaching: 2 hr, 90°C, cs 5%, 0.8% NaOH or 0.2g NaOH/ 25g o.d. pulp
ODT	5. Oxygen bleaching as above, followed by Dithionite Bleaching: 1 hr, 75°C, cs 3.33%, 2% Na ₂ S ₂ O ₄ or 0.5 g Na ₂ S ₂ O ₄ / 25g o.d. pulp
P	6. Alkaline hydrogen peroxide bleaching: 1 hr, 75°C, cs 5%, 1.0 % NaOH or 0.5g NaOH/ 50g o.d. pulp, 0.2% DTPA or 0.1g DTPA/ 50g o.d. pulp
PDT	7. Alkaline hydrogen peroxide as above followed by Dithionite Bleaching: 1 hr, 75°C, cs 3.33%, 2% Na ₂ S ₂ O ₄ or 0.5 g Na ₂ S ₂ O ₄ / 25g o.d. pulp
*cs = consistency = wt% pulp in slurry	

The brightness values are plotted in Figure 66 through Figure 69. Bleaching treatments increased the already high brightness values for the pink and blue pulps. The largest brightness gain was 10 brightness points for the blue pulp and 22 points for the pink pulp. For both goldenrod and Direct Yellow 11 pulps, treatments including dithionite bleaching yielded the best results. The Direct Yellow 11-dyed pulp did respond to bleaching treatments with the largest gain being 37 points in brightness. The goldenrod pulp has a low brightness of 23 and the maximum brightness gain observed was 19 points.

Figure 66 Brightness Values for Bleached Blue Commercial Pulp

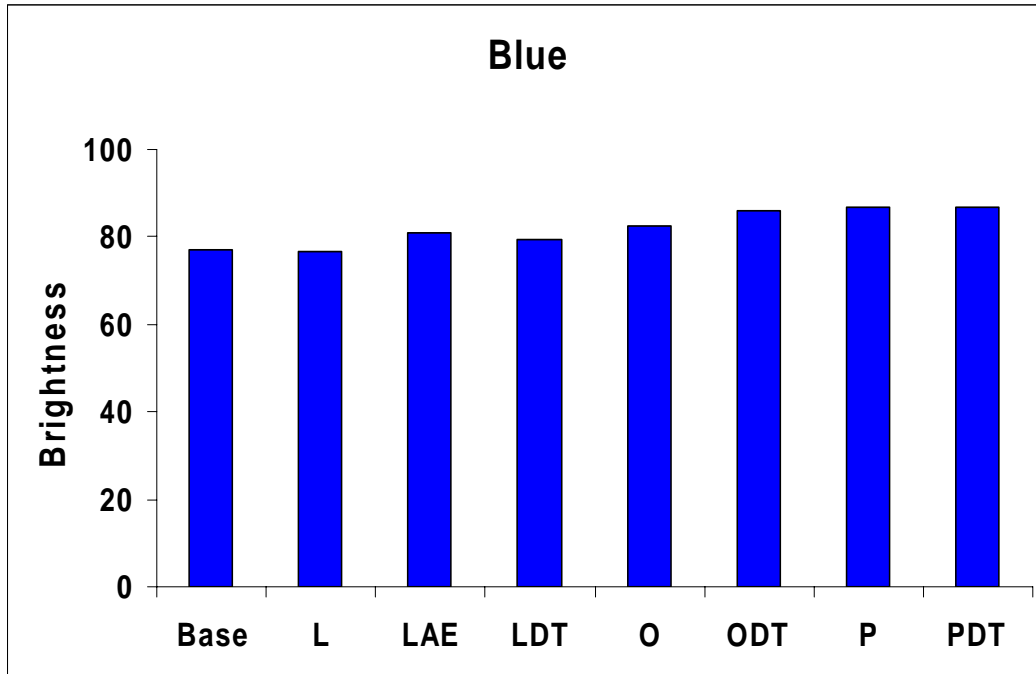


Figure 67 Brightness Values for Bleached Cherry Pink Commercial Pulp

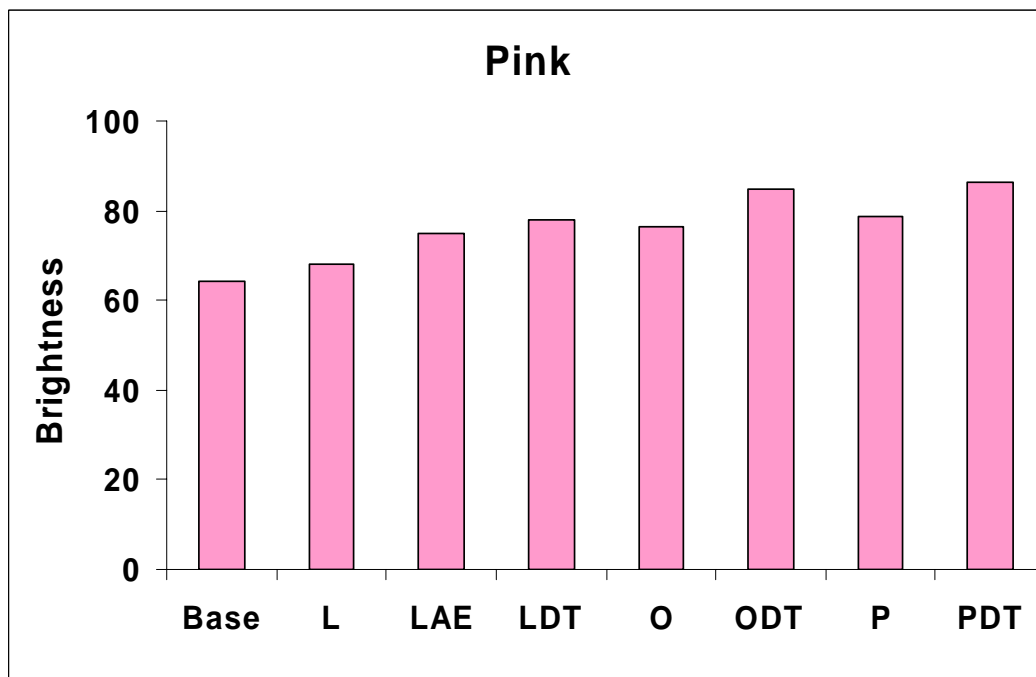


Figure 68 Brightness Values for Bleached Goldenrod Commercial Pulp

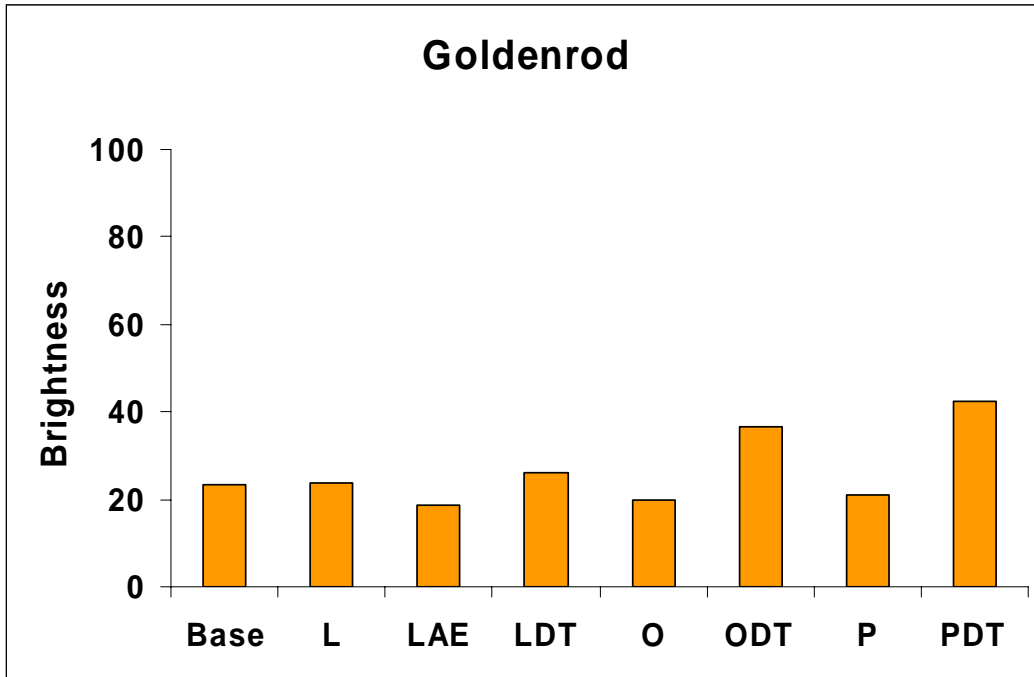
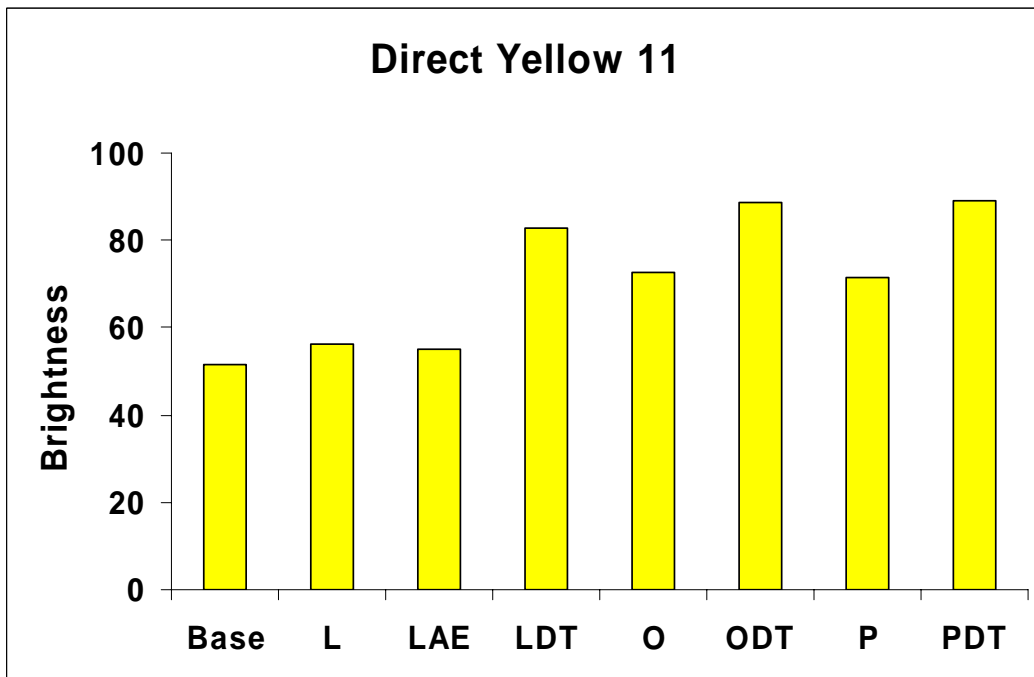


Figure 69 Brightness Values for Bleached Direct Yellow 11-Dyed Pulp



Examination of the dye removal index values (Figure 70 through Figure 73) shows that dithionite bleaching yields the best result. LDT treatment results are comparable to PDT treatment results for the pink pulp, but not for the blue pulp.

Figure 70 Dye Removal Index Values for Bleached Blue Commercial Pulp

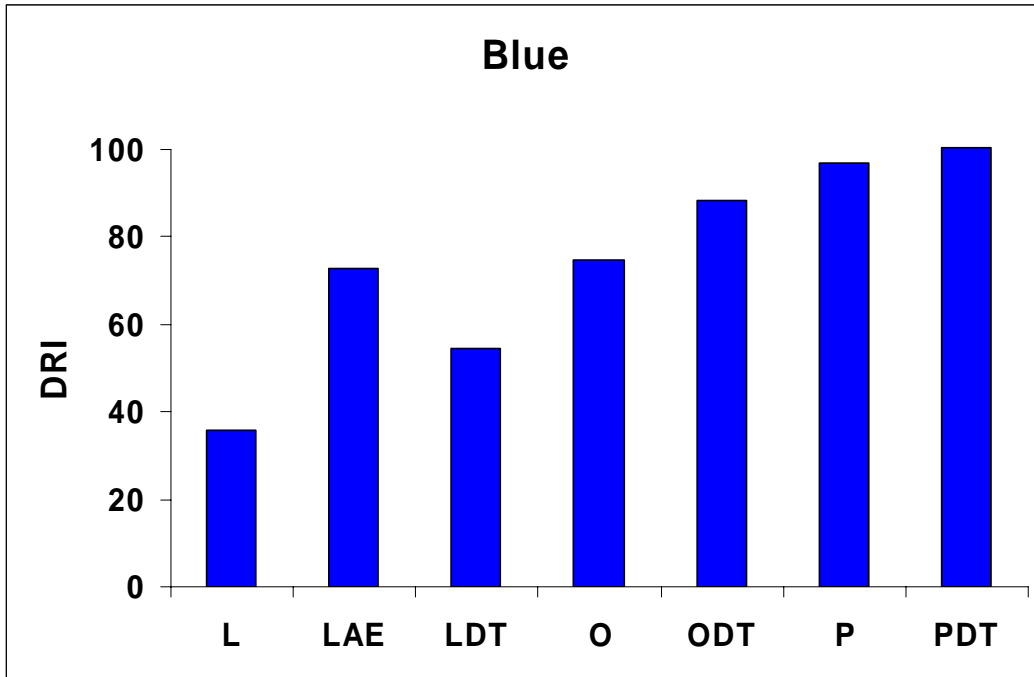


Figure 71 Dye Removal Index Values for Bleached Cherry Pink Commercial Pulp

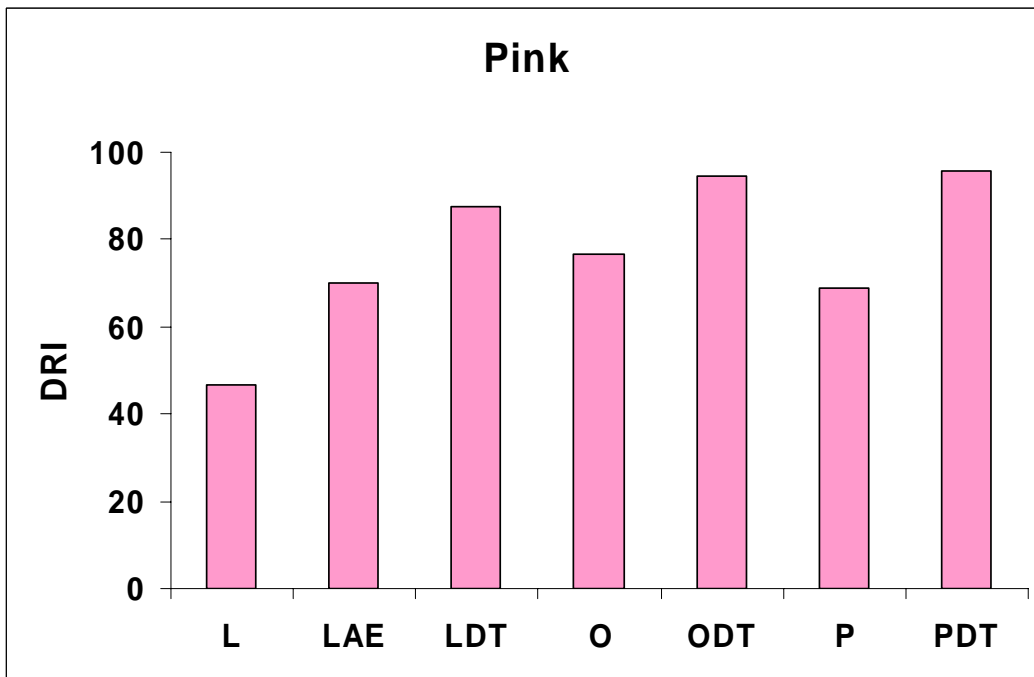


Figure 72 Dye Removal Index Values for Bleached Goldenrod Commercial Pulp

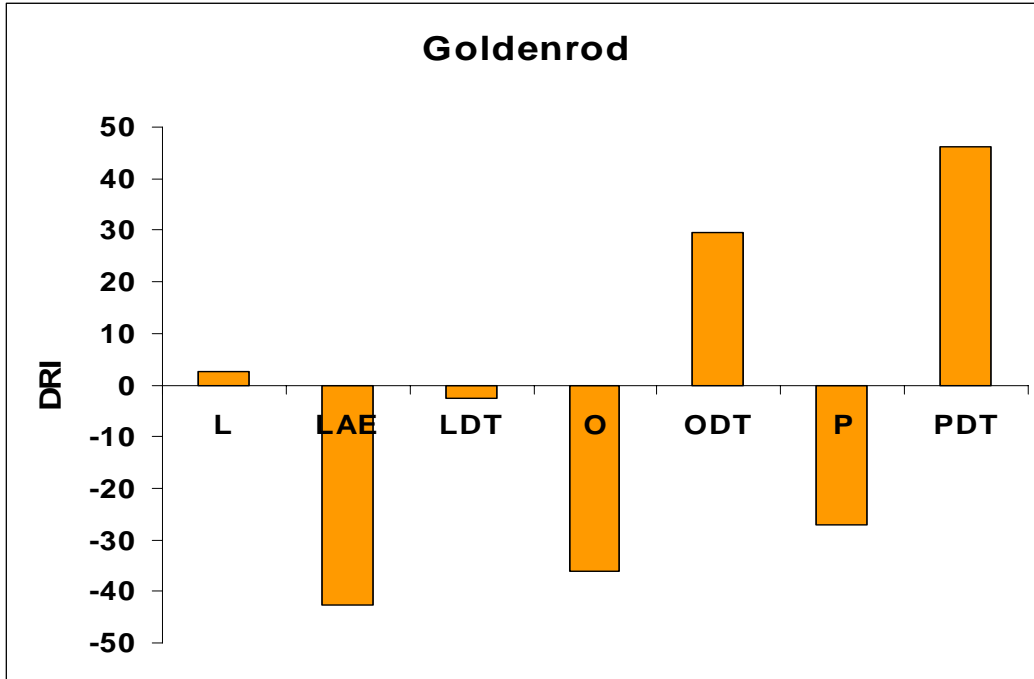
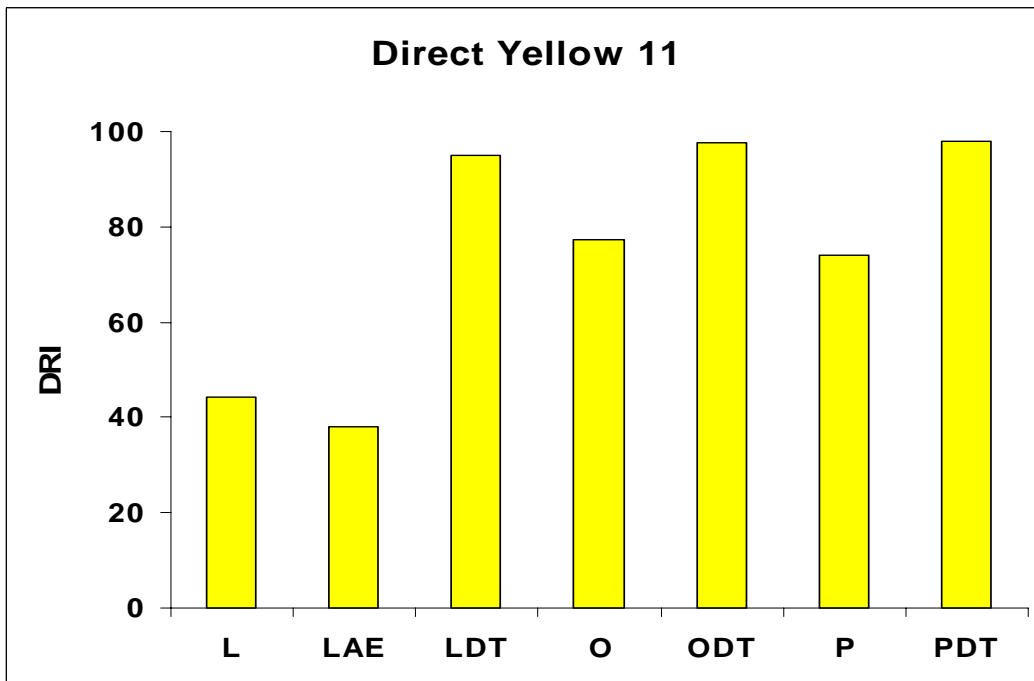


Figure 73 Dye Removal Index Values for Bleached Direct Yellow 11 Dyed-Pulp



For Direct Yellow 11, the dye removal index values of laccase/ABTS treatment followed by dithionite bleaching are comparable to the results obtained with oxygen or peroxide bleaching followed by dithionite treatment. When brightness values are

compared, the final brightness for LDT treatment of Direct Yellow 11 pulp is not quite as high as for ODT or PDT. This may be related to the observation that Direct Yellow 11 pulp treated with oxygen or peroxide remains yellow, while Direct Yellow 11 pulp treated with laccase/ABTS changes hue to cream/yellow-brown. The change in hue suggests a chemical alteration occurred.

The results obtained for treatment of goldenrod pulp were unexpected. The large negative dye removal index values were obtained for treatments that involve heating the pulp at elevated temperatures (60-75°C) in *strongly* alkaline treatments. It is unlikely to be due to residual alkalinity as all pulps were washed at the end of the treatment. In addition, during the preparation of handsheets, the pH of the pulp slurry is adjusted to 6.5 ± 0.5 . Whatever the cause for the increase in color during alkaline treatment, the color increase was reversed by reduction with sodium dithionite.

7.4.4.3 Summary

Examination of oxygen bleaching of colored pulps show that $L^*a^*b^*$ color values are better than brightness values for evaluating the effectiveness of a bleaching treatment. When followed by sodium dithionite treatment, the results of the laccase/ABTS system designed for bleaching Direct Yellow 11 dyed pulp is comparable to the results from oxygen or hydrogen peroxide bleaching followed by sodium dithionite. For the commercial colored pulps containing dyes of unknown composition, the laccase/ABTS treatment had some effect, but was not as effective as alkaline hydrogen peroxide followed by sodium dithionite.

7.4.5 ACKNOWLEDGEMENTS

We would like to thank Novozymes for the donation of the laccase and BASF for the donation of Basazol 46L and Direct Yellow 11. This work was supported by the member companies of the Institute of Paper Science and Technology.

8 SUMMARY OF RESULTS

The first issue this research project addressed was whether laccase-mediator system could decolorize Direct Yellow 11. An affirmative answer could not be assumed. As of 2004, the only reports of cell-free decolorization of a stilbene dye are the publications from this research project. As mentioned in Section 3.8.4.1, page 70, when 4 different azo dyes were added to cultures of *Phanerochaete chrysosporium*,¹⁷² the one dye the fungi did not completely decolorize was Congo Red - a direct dye that contains azo bonds between two phenyl groups that do *not* have hydroxyl groups. Like Direct Yellow 11, Congo Red is unable to form azo/hydrazone tautomers. When Cripps *et al.* prepared a crude extract of lignin peroxidase from *P. chrysosporium*, the three other dyes were partially decolorized, but not Congo Red.

The addition of a mediator compound could be necessary for decolorization of Direct Yellow 11. Laccase can degrade phenolic units of lignin, but a mediator is necessary to degrade nonphenolic lignin.⁸ During degradation of wood, white-rot fungi secrete low molecular weight compounds that function as mediators.²⁵⁵ Decolorization of a phenolic azo dye with the laccase from *Trametes versicolor*¹⁷⁹ (discussed in section 3.9.2.4 on page 84), appeared to involve a small molecular weight mediator. In the experiments reported here, it was found in control experiments under standard conditions of 45°C for 2 hours at pH 4.5 under 10 bar oxygen pressure followed by suction filtration and washing, the amount of color removed was similar (~22% DRI) whether mediator alone, laccase alone or nothing was added to the Direct Yellow 11-dyed pulp. Laccase alone does *not* decolorize Direct Yellow 11.

Another possibility was that laccase with mediator could decolorize dissolved Direct Yellow 11 in solution, but not when the dye was attached to fiber. A slurry of fiber in water is a biphasic solution. The presence of solid material inhibits diffusion and can result in uneven distribution of dissolved material within the solution.

In addition, cellulose fibers are negatively charged. At the interface between a charged solid and a liquid, a separation of electrical charges occurs. Positive ions would be attracted to the surface and attach, forming the rigid zone known as the Stern layer.

Additional positive ions are attracted by the negative fiber but also repelled by the other positive ions in the Stern layer. These additional positive ions form a diffuse layer. The diffuse layer and the Stern layer together form the double layer that exists surrounding cellulose fibers in water. The thickness of the double layer depends on pH and ionic strength of the solution. For some dyes, salt must be added during the dyeing process to shrink the size of this double layer to enable the dye molecules to reach and attach to the fibers. Not only are the cellulose fibers charged, the enzyme is also charged. The charge on an enzyme varies with the pH since the amino acids of proteins are amphoteric. Proteins contain both carboxylic acid groups that can ionize and amino groups that can be protonated. Since a double layer would exist around cellulose fibers and around enzymes in solution, it is probable that the ability of the enzyme to directly approach the fiber is limited. These limitations would be much less for low molecular weight mediators. The mediators have very little charge or surface area compared an enzyme (about 60,000 in molecular weight) or the micron-sized cellulose fibers.

This research determined that laccase with mediator could decolorize both Direct Yellow 11 in solution and pulp dyed with Direct Yellow 11.

One explanation for the decolorization of Direct Yellow 11 dyed pulp is that laccase with mediator could be stripping the intact dye from the fiber. However spectrophotometric measurement of the post-reaction effluent from laccase/ABTS treatment (Figure 53, page 112) shows no dye peak around 400 nm and little difference is discernable between post-reaction solutions of laccase/ABTS treatment of white and of Direct Yellow 11-dyed pulp. The pulp color changes from yellow to cream/yellowish brown, suggesting that an alteration in the dye chromophore has occurred. The decolorization observed when Direct Yellow 11 in solution (no pulp, Figure 61, page 129) was treated with laccase with mediator proves that laccase with mediator is reacting with the dye chromophore.

Another issue was which of the available mediators would be the most effective. Although the first mediator introduced for biobleaching of virgin pulp with laccase was ABTS (2-2' azinobis-(3-ethyl benzthiazodine),⁸ most research has focused on the class of mediators containing the N-OH moiety.⁹ This class includes the mediators HBT (1-

hydroxybenzotriazole) and VA (violuric acid) used in this research as well as NHA (N-hydroxy acetanilide), HNNS (2-nitroso-1-naphthol-4-sulfonic acid) and (NNDS) 1-nitroso-2-naphthol-3,6-disulfonic acid) (see Table 3-27, page 52). Previous research had found that for biobleaching virgin pulp HBT was more effective than ABTS⁹. Later work showed that mediators NHA and VA outperform HBT.²⁵⁶

For the decolorization of Direct Yellow 11 however, ABTS was found to a better mediator than either HBT or violuric acid (Figure 56 page 119). Laccase/ABTS remained effective even when the ABTS concentration was decreased to 10 nM (Figure 57 page 120). The differences in mediator effectiveness could be due to a difference in redox potentials (redox potentials are 1.04 for HBT; 0.91 for violuric acid and 0.67 and 1.09 for the two electron transfers to ABTS). It is also possible that some other mechanism is occurring. There is some structure similarity between Direct Yellow 11, with an azo bond between phenyl groups bearing sulfonic acid groups (Figure 55 page 116), and the ABTS dication (Figure 32 page 58) which has an azo bond between heterocycles attached to phenyl groups bearing sulfonic acid groups. The greater similarity between Direct Yellow 11 and ABTS intermediates than between Direct Yellow 11 and HBT and VA intermediates may facilitate closer association and/or better stereochemical orientation during the electron transfer from activated mediator to dye chromophore. For example, the positive charges on the heterocyclic nitrogens of the ABTS dication (Figure 32 page 58) may draw the ABTS dication closer to Direct Yellow 11 which is negatively charged from four ionized sulfonic acid groups.

Experiments with dissolved dye suggest that Direct Yellow 11 reacts slowly. About one hour was needed for maximal decolorization to occur regardless of whether the decolorization agent was the laccase/ABTS system (Figure 61 page 129) or soybean peroxidase/veratryl alcohol (Figure 62 page 130). For laccase/ABTS, previous kinetic studies had indicated that the radical cation formed quickly while the dication formation was slow,¹⁰⁹ and limited the reaction rate. It could be that the slow formation of ABTS dication limited the rate of Direct Yellow 11 decolorization. However, the reaction of soybean peroxidase/veratryl alcohol with Basazol 46L was rapid (Figure 63 page 131) while the reaction of soybean peroxidase/veratryl alcohol with Direct Yellow 11 was

slow, even though both reactions were performed at same concentrations of nonvolatile dye solids, enzyme, mediator and hydrogen peroxide. The slow rate of reaction of Direct Yellow 11 with soybean peroxidase/veratryl alcohol together with the slow reaction with laccase/ABTS suggests that the observed slow decolorization reaction is due to the dye, not the mediator transferring the electron or the enzyme activating the mediator. The reaction rate studies were performed on dissolved Direct Yellow 11 so the factors that could limit the reaction rate of a dye attached to a solid fiber are not relevant.

Possible explanations for the observed slow decolorization include a) difficulties in electron transfer between mediator and dye chromophore and b) slow transformation of the activated dye chromophore to a stable chemical form with less molar absorptivity.

Direct Yellow 11 is a long planar molecule with four ionized sulfonic acid groups. Being long and planar reduces the likelihood of random collisions with the necessary stereochemical orientation between the dye chromophore and the activated mediator. The four charged sulfonic acid groups would tend to repel the close approach of other molecules. Since ABTS dication is positively charged (Figure 32 page 58) direct repulsion may not be a problem. However, the charged sulfonic acid groups are bulky and would further attract sodium ions (from the buffer solution), and hydronium ions (solution pH 4.5 for laccase and pH 3.8 for reaction rate study of soybean peroxidase with Direct Yellow 11). Even though the sulfonic acid groups are in the plane of the aromatic ring, the bulk of the associated ions around these charged groups would reduce close collisions between mediator and the dye chromophore. It is also possible that electron transfer occurs only when the dye chromophore enters a rare resonance state or conformation and the rate of decolorization is limited by the rare appearance of that resonance state or conformation.

Stilbene compounds are very stable. The oxygens in Direct Yellow 11 (Figure 55 page 116) are in nitro and sulfonic acid groups and are already fully oxidized. Direct Yellow 11 could form a radical that is moderately stable and continues to absorb light. Eventually the dye radical could decay into a more stable form that has a shifted λ_{Max} (resulting in the hue change from yellow to cream) and a lower molar absorptivity (resulting in lower absorbance in solution and decolorization of dyed pulp). This

prolonged decay is similar to the process of phosphorescence in that time passes between the initial activation and formation of the final stable structure. In this case, the reason for the slow decolorization of Direct Yellow 11 would be difficulty in formation of the oxidized dye products. The difficulty could be related to necessary intramolecular rearrangements and/or to a requirement for a second intermolecular reaction such as extraction of a hydrogen atom from the medium or even a second electron transfer with the activated mediator. The slow decolorization of Direct Yellow 11 observed in these enzymatic redox systems collaborates the reputation of Direct Yellow 11 as a stable dye that is recalcitrant to chemical bleaching.

When Direct Yellow 11-dyed-pulp was subjected to a series of bleaching treatments, it was found that single stage oxidative bleaching was not as effective as two-stage oxidative bleaching followed by reductive treatment. Single stage laccase/ABTS treatment was also not as effective as single stage oxygen or alkaline hydrogen peroxide. That result was not expected. However, it should be noted that after single stage treatments, the color of laccase/ABTS pulp has changed from yellow to cream while oxygen and hydrogen peroxide treated pulps are still yellow. Oxygen and hydrogen peroxide may be color stripping the dye off the fiber rather than altering the dye chromophore.

Since the Direct Yellow 11-dyed pulp did respond to chemical bleaches, it may appear that the dye's reputation as recalcitrant is unwarranted. However, it must be noted that in commercial production, the dye fixative applied would be a compound designed for that purpose. The dye fixative applied to the pulp in this research (alum) is a readily available chemical that has many uses in papermaking. It was suggested by JC Cantrell at BASF because he knew our laboratory would already have this chemical on hand. If the color reduction by oxygen and by hydrogen peroxide bleaching is by color stripping, then the choice of dye fixative could be important. Oxygen and hydrogen peroxide did reduce the color of Direct Yellow 11-dye pulp. However, unlike treatment with laccase/ABTS, in treatment with oxygen or with alkaline hydrogen peroxide there is no evidence indicative of chemical alteration of the dye chromophore.

The two stage treatment including laccase/ABTS was as effective as two stage treatment with oxygen or alkaline hydrogen peroxide, whether judged by brightness values (Figure 69 page 141, maximum gain 37 points) or by dye removal index values (Figure 73 page 143). Although the two stage treated pulps vary slightly in their a^* and b^* values, the handsheets for all of the two stage treatments are visibly white. Whatever the chemical alteration is that occurs in the Direct Yellow 11 dye chromophore during laccase/ABTS treatment, that alteration does not prevent reductive bleaching by sodium dithionite.

The results of bleaching the commercial colored pulps demonstrate why brightness values are not conclusive when evaluating bleaching treatments of colored pulps. For the blue pulp, the initial brightness is high and bleaching treatments did not alter the brightness greatly (Figure 66 page 140, maximum gain 10 points). Brightness value changes for the pink pulp were similar (Figure 67 page 140, maximum gain 22 points). The brightness values show little distinction among the bleaching treatments. The dye removal index values were more informative. The blue pulp responded well to bleaching with alkaline hydrogen peroxide, experiencing only a slight improvement by secondary bleaching with sodium dithionite. The blue pulp did not respond well to laccase/ABTS treatment, even if followed by alkaline extraction or dithionite bleaching. For the pink pulp, a two stage treatment including laccase/ABTS achieved results comparable to two stage bleaching with oxygen or alkaline hydrogen peroxide.

As for the Direct Yellow 11-dyed pulp, the most effective bleaching treatments for commercial goldenrod pulp were treatments that included reductive bleaching by sodium dithionite. The goldenrod pulp has a low brightness of 23 and the maximum brightness gain observed was 19 points (Figure 68 page 141) For the goldenrod pulp, very different information is conveyed by the dye removal index values (Figure 72 page 143) when compared with the brightness values. Treatments that result in only slight differences in brightness values, exhibited major differences in dye removal index values. An increase in color was observed for bleaching treatments that include heating strongly alkaline solutions (alkaline extraction, oxygen bleaching, and hydrogen peroxide

bleaching). The poor results obtained for the goldenrod pulp demonstrate how the bleaching response can vary greatly among dyes.

The methine dye Basazol 46L responded to laccase/ABTS, horseradish peroxidase/veratryl alcohol and soybean peroxidase/veratryl alcohol treatments. Decolorization results were 71% for treatment with horseradish peroxidase/veratryl alcohol (Figure 59 page 127) and 33% for treatment with laccase/ABTS (Table 7-3 page 129). However the rapid rate of decolorization of Basazol 46L when treated with soybean peroxidase/veratryl alcohol (74% reduction within 5 minutes, Figure 63 page 131) suggests that decolorization by soybean peroxidase/veratryl alcohol may be the best route. Soybean peroxidase is inexpensive and readily available. The acceptable range of solution pH is also very broad including both acidic (pH 3.8) and mildly alkaline (pH 8) conditions (Figure 60 page 128). Although polyethylene glycol (PEG) has been reported to stabilize soybean peroxidase,²¹⁰ the inclusion of 500 mg/L of PEG with 10,000 or 35,000 molecular weight had little effect on the decolorization of Basazol 46L.

Addition of 5 mM dimethyl sulfoxide (a free radical scavenger) had negligible effect on the decolorization of the dye. Since inclusion of a radical scavenger had no effect, it appears that the reaction mechanism may not include a free radical. However, the dye decolorization is nearly instantaneous. The reaction solution with soybean peroxidase, veratryl alcohol and Basazol 46L dye was prepared and allowed to equilibrate. Dye decolorization occurs immediately upon addition of the hydrogen peroxide. There was not much time for the radical scavenger to affect the reaction.

The experiments with Basazol 46L were performed in solution. It is possible that soybean peroxidase/veratryl alcohol may not decolorize the dye when it is attached to cellulose fibers. However, the veratryl alcohol in the solution functions a mediator, in the same manner that ABTS mediates in laccase reactions. Since ABTS is able to overcome mass transfer limitations in the laccase-mediated decolorization of Direct Yellow 11, it is probable that the mediated soybean peroxidase reaction could decolorize Basazol 46L attached to fibers. One complication is relevant. Direct Yellow 11 is applied to bleached kraft fibers. Basic dye Basazol 46L is designed for application to lignin-containing pulps.

The lignin present could consume the mediator and greatly reduce the ability of the soybean peroxidase to decolorize Basazol 46L.

In summary, the results reported in this research project demonstrated the potential benefits of biotechnology for solving existing problems. This research program has also shown that a recalcitrant stilbene dye can be decolorized by the lignolytic enzyme laccase combined with a mediator. This dye and a recalcitrant methine dye can also be decolorized by soybean peroxidase, suggesting that the extensive existing research on dye decolorization by lignin and manganese peroxidases may also apply to this inexpensive commercially available enzyme.

9 THESIS CONCLUSIONS

Laccase-mediator treatment reduced the color of pulp dyed with Direct Yellow 11. For this purpose, ABTS was a better mediator than either HBT or violuric acid. Laccase/ABTS remained effective even when the ABTS concentration was decreased to 10 nM. The pulp color changes from yellow to cream/yellowish brown, suggesting that the dye molecule has been chemically altered.

Experiments with dissolved dye suggest that Direct Yellow 11 reacts slowly. About one hour was needed for maximal decolorization to occur regardless of whether the decolorization agent was the laccase/ABTS system or soybean peroxidase/hydrogen peroxide. When compared with conventional bleaching treatments, laccase/ABTS treatment followed by dithionite bleaching removed as much color as oxygen or hydrogen peroxide treatment followed by dithionite treatment.

Both laccase/ABTS and horseradish peroxidase/hydrogen peroxide treatments could reduce the signal intensity of Basazol 46L. However, soybean peroxidase achieved 74% reduction within 5 minutes. Soybean peroxidase was able to reduce the signal from Basazol 46L under both acidic (pH 3.8) and mildly alkaline (pH 8) conditions.

In summary, the results reported in this research project demonstrated the potential benefits of biotechnology for solving existing problems. This research program has also shown that a recalcitrant stilbene dye can be decolorized by the lignolytic enzyme laccase combined with a mediator. This dye and a recalcitrant methane dye can be decolorized by soybean peroxidase, suggesting that the extensive existing research on dye decolorization by lignin and manganese peroxidases may also apply to this commercially available enzyme.

10 RECOMMENDATION FOR FUTURE WORK

The research described in this thesis could be continued in a number of ways.

First, the chemistry of the reaction between laccase/ABTS and Direct Yellow 11 remains to be elucidated. The data suggest that laccase/ABTS causes a chemical change in the dye molecule. The laccase/ABTS treatment of pulp dyed with Direct Yellow 11 changes the pulp color from yellow to cream/yellow-brown.

Another question is whether the laccase/ABTS system can be used to break down other dyes? In addition, testing laccase with other mediators against a variety of dyes could yield some useful information. Data suggests a correlation between the redox potential of a dye and the mediator used

Finally, possible industrial application of this system raises issues to be explored. It is possible that when treating mixed office waste that includes a variety of colored paper, dyes from some colored papers could act as laccase mediators to allow laccase to attack the recalcitrant dyes. Another issue concerns where in the recycling process should laccase or laccase and mediator be added? For a recycle mill that is already adding cellulases and/or xylanses before deinking, would there be any value to also adding laccase or laccase and mediator? Or alternatively, must the treatment with the laccase-mediator system come as part of the bleach cycles after deinking?

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Georgia Tech Mass Spectrometry Laboratory

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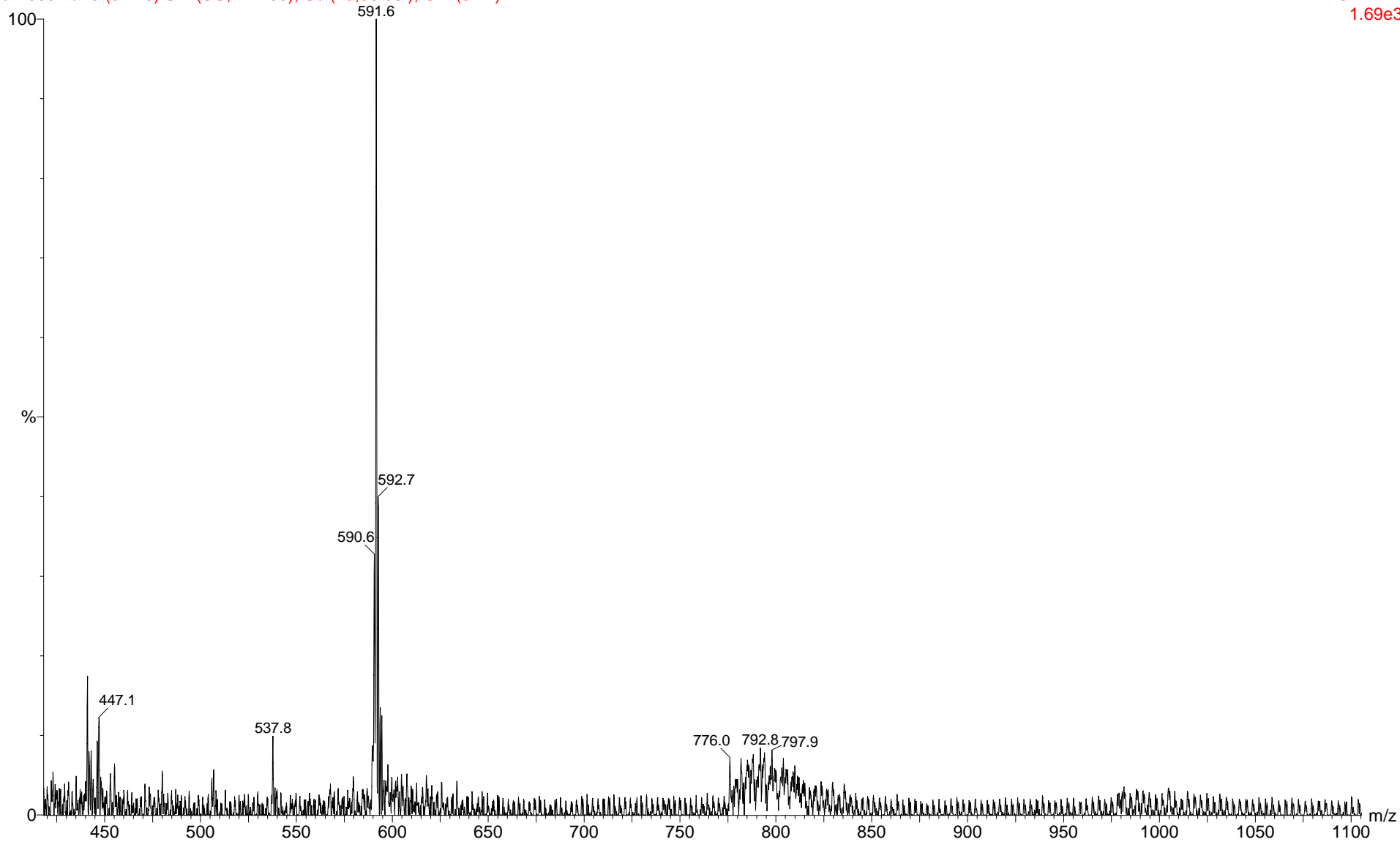
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Laser Power: 20/80

TOF LD+

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Georgia Tech Mass Spectrometry Laboratory

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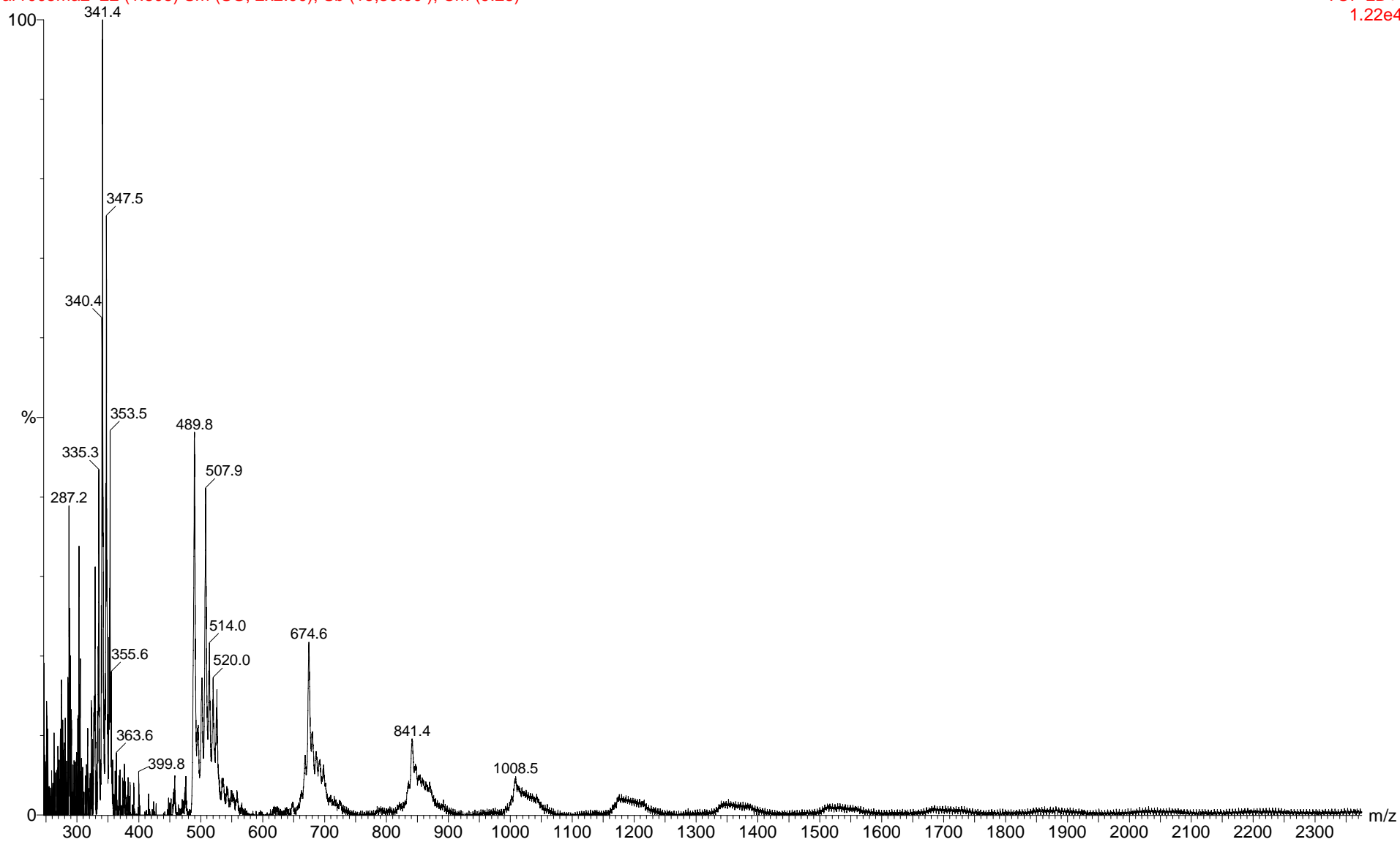
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Georgia Tech Mass Spectrometry Laboratory

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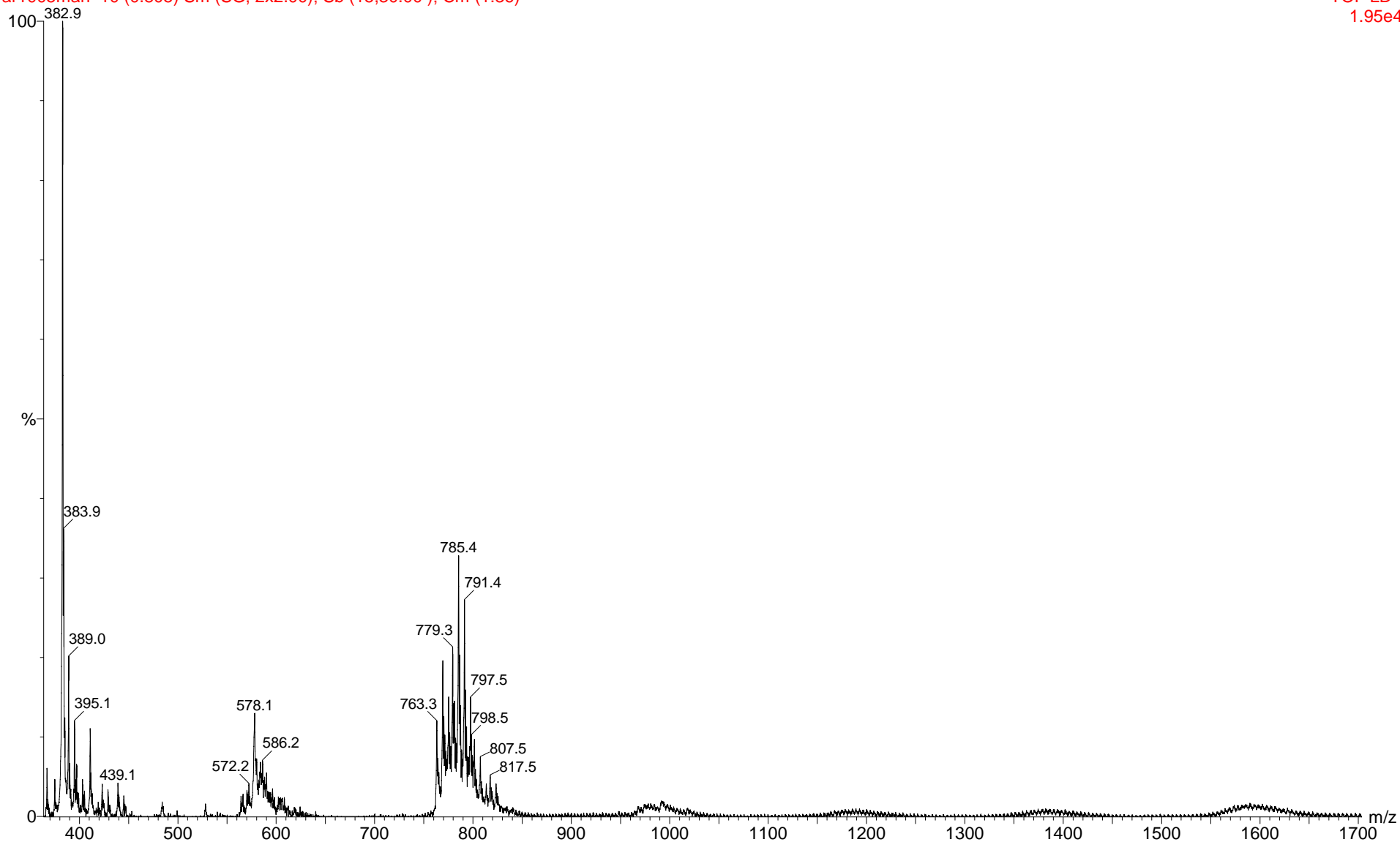
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TOF LD-
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Georgia Tech Mass Spectrometry Laboratory

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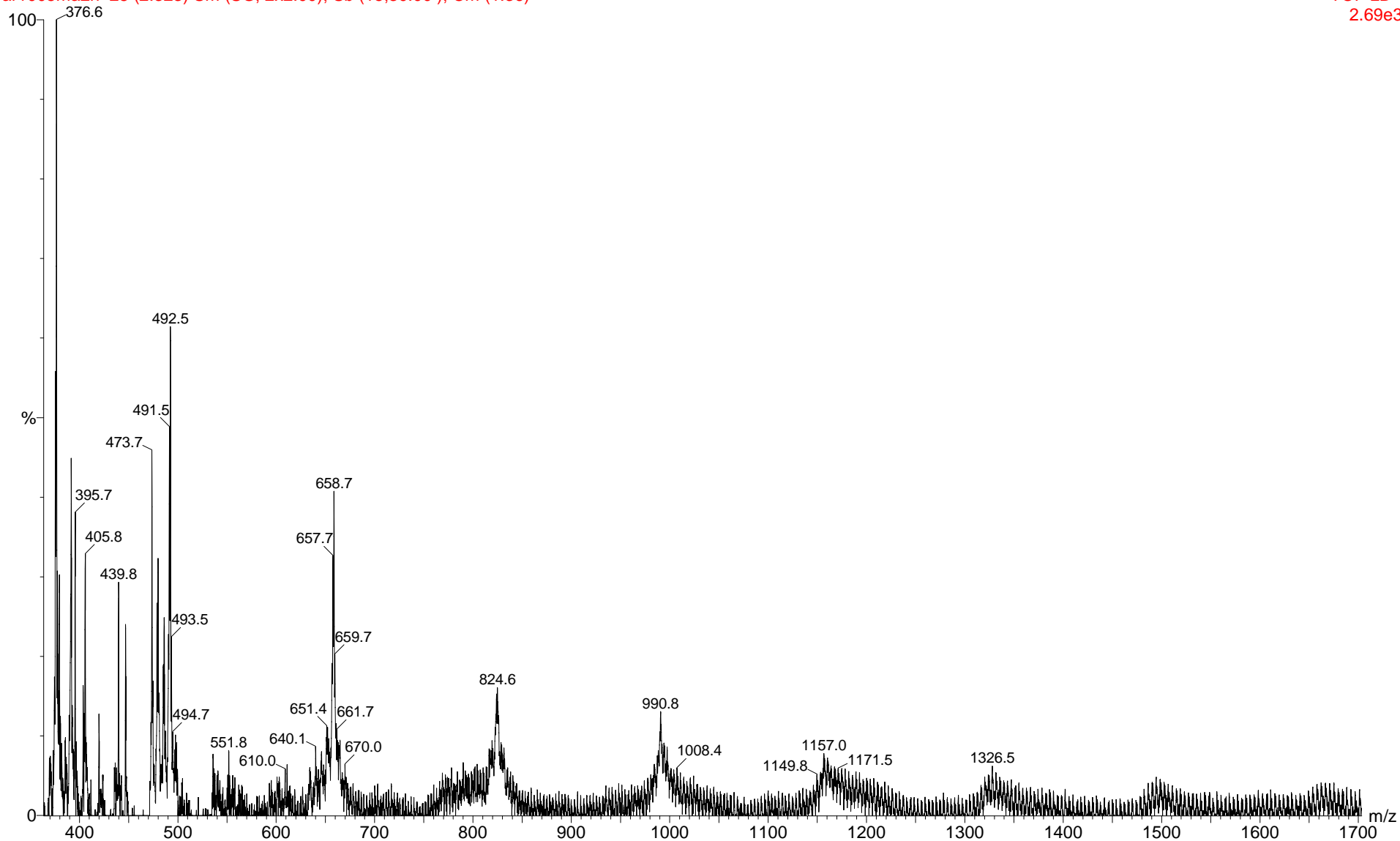
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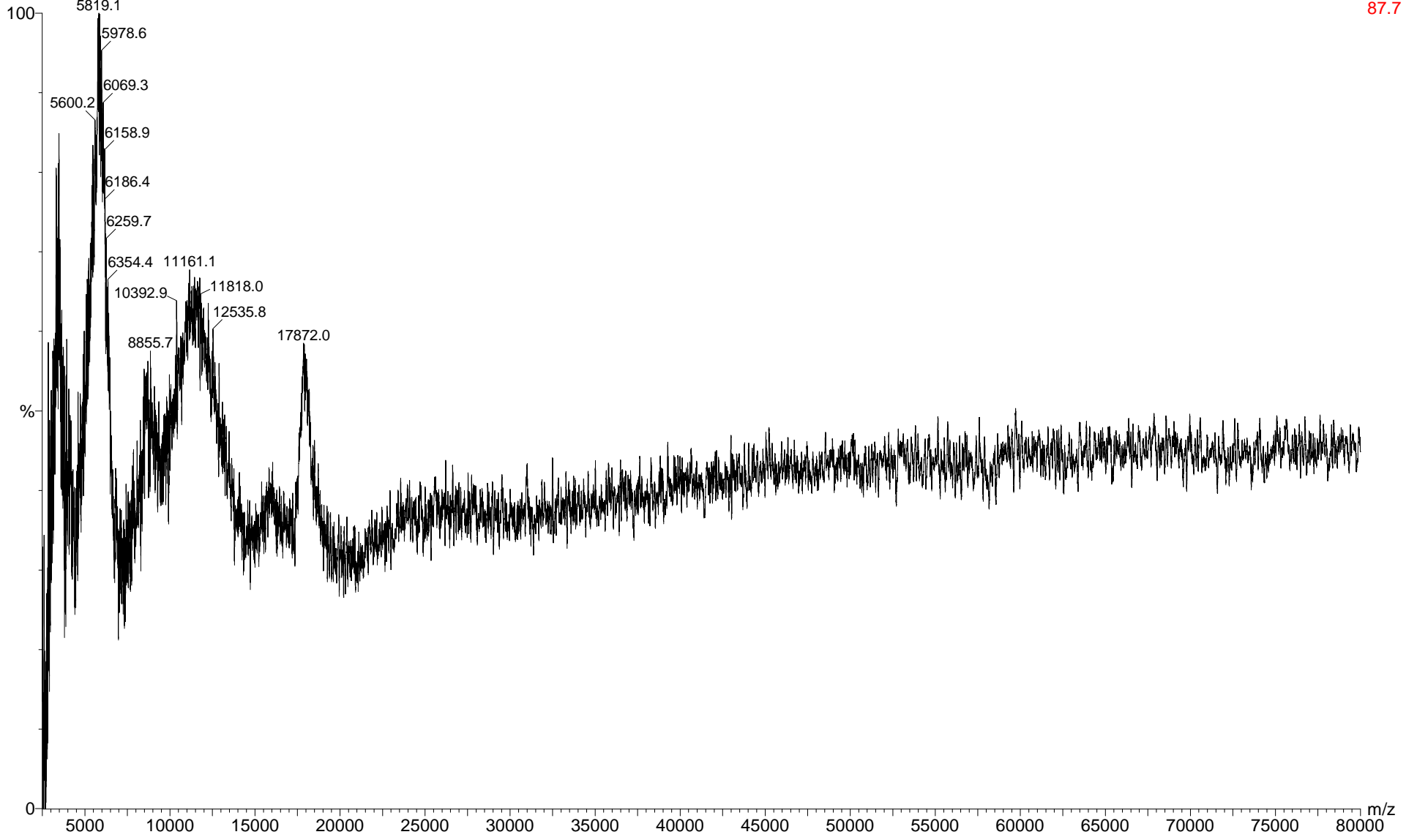
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TOF LD+
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Georgia Tech Mass Spectrometry Laboratory

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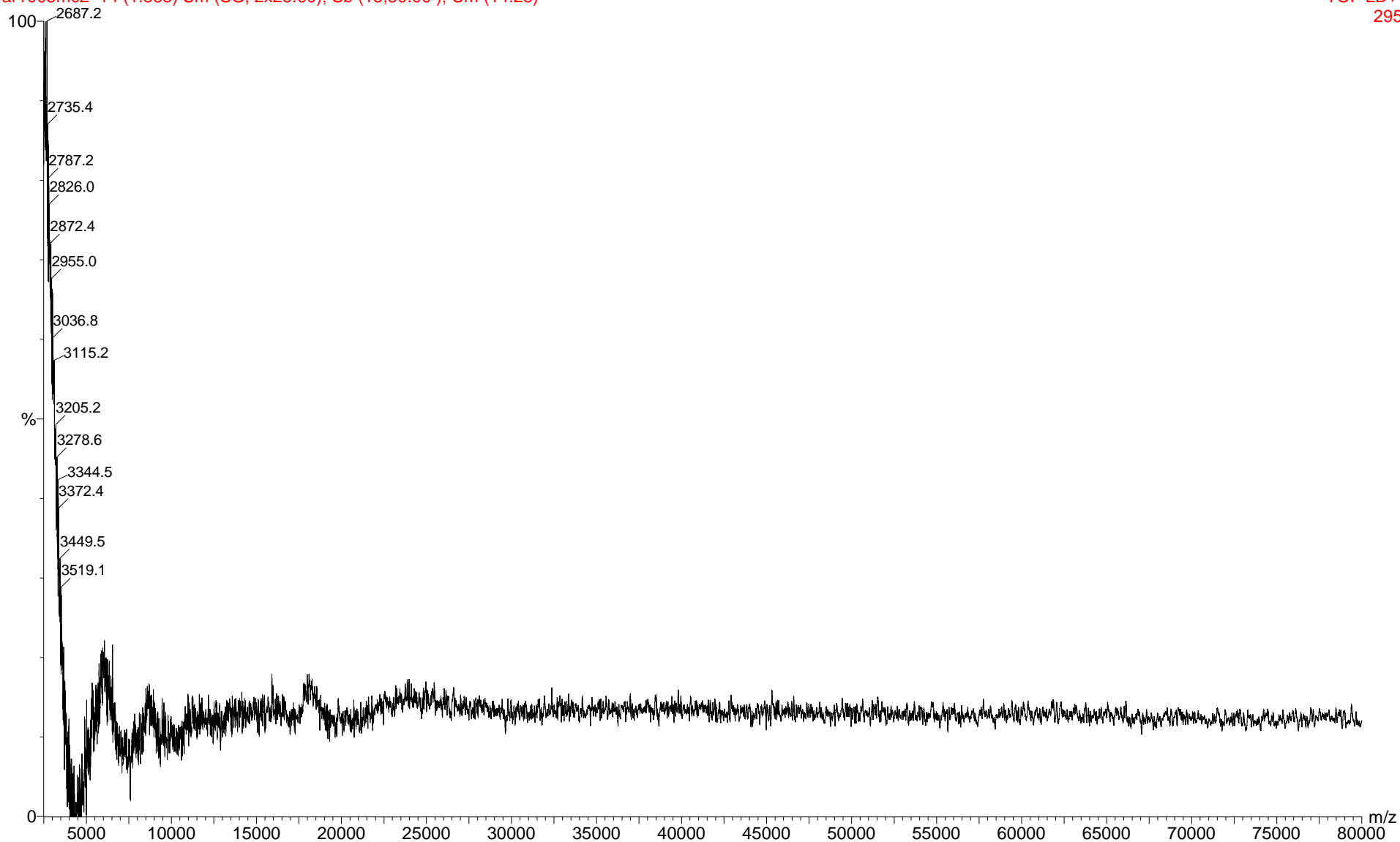
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Laser Power: 50/80

TOF LD+

295



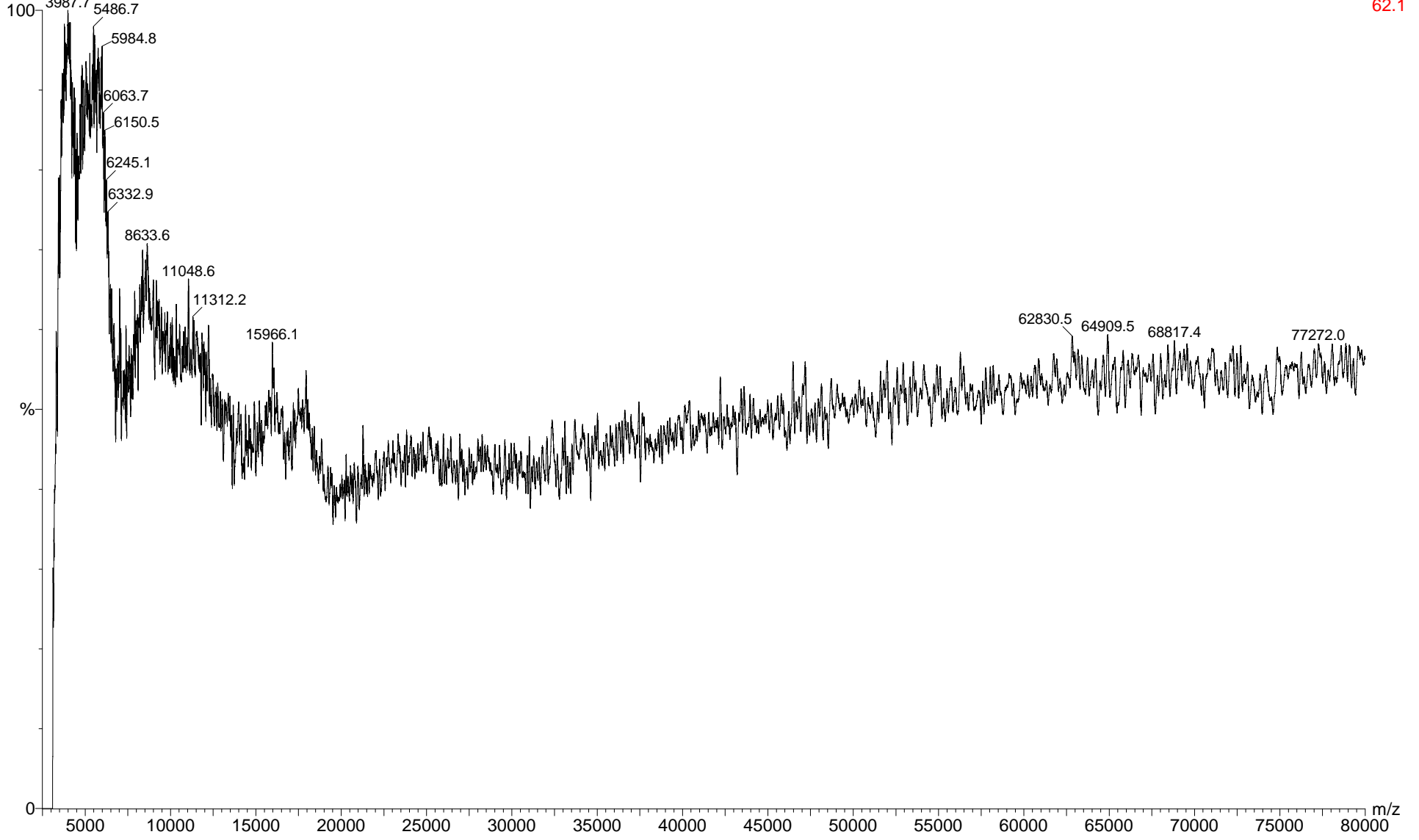
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Knutson laccase (CHCA)

Pulse Voltage: 2200
Laser Power: 50/80

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TOF LD-
62.1



Georgia Tech Mass Spectrometry Laboratory

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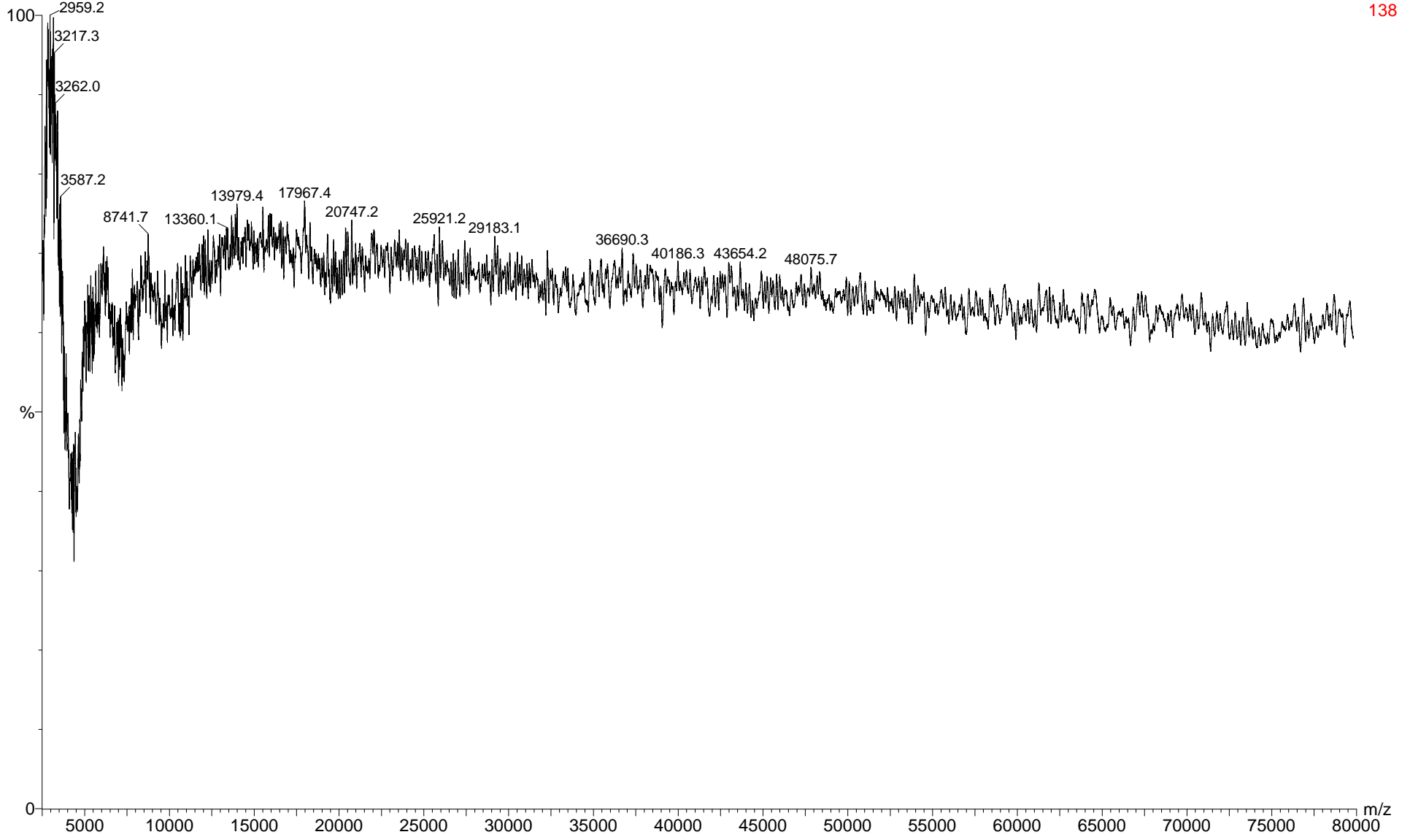
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Pulse Voltage: 2200

Laser Power: 50/80

TOF LD-

138



Georgia Tech Mass Spectrometry Laboratory

5-OCT-2004 15:25:38

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Knutson ABTS (CHCA)

Pulse Voltage: 1050

Laser Power: 20/80

TOF LD+

3.75e3



Georgia Tech Mass Spectrometry Laboratory

5-OCT-2004 17:20:04

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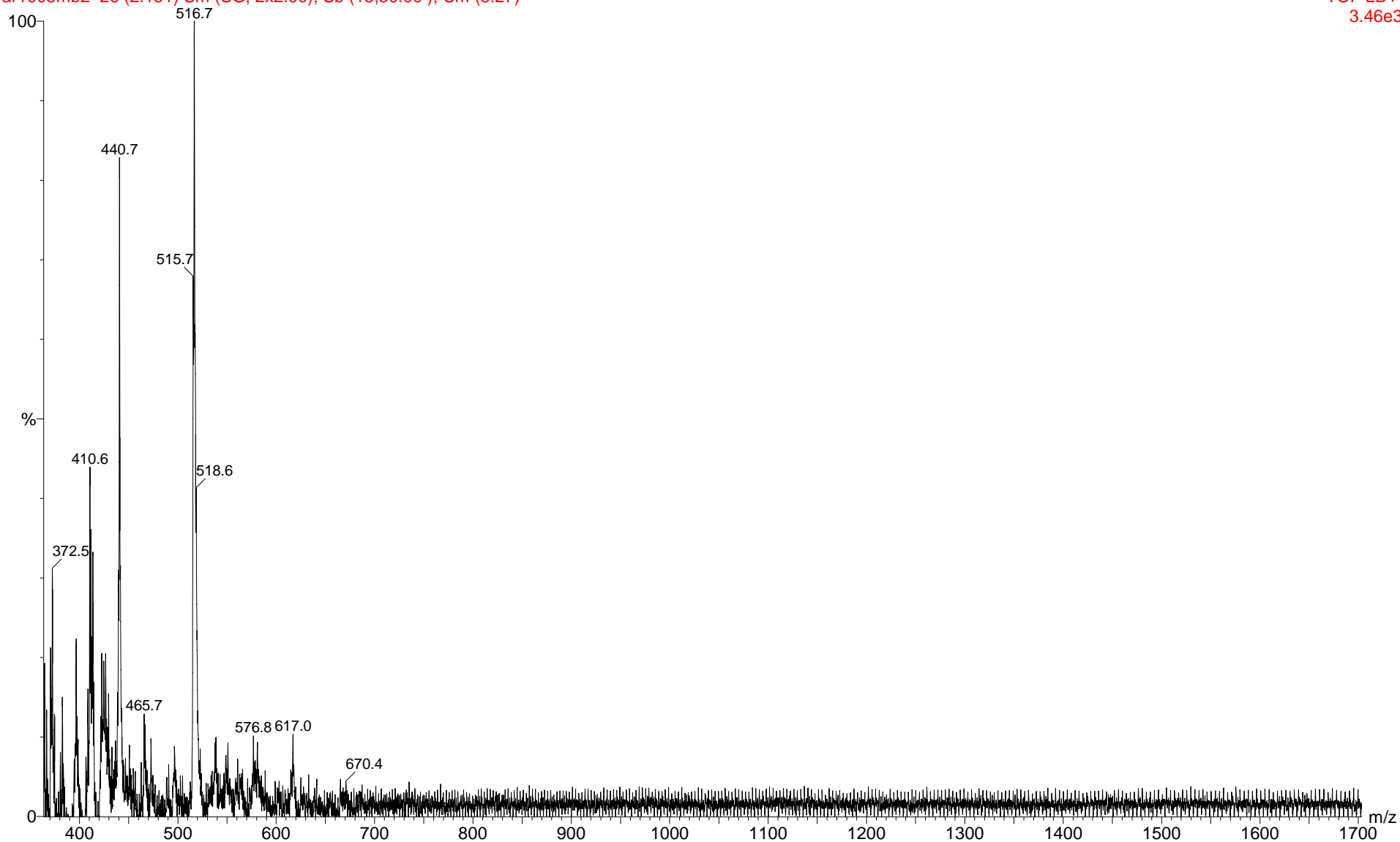
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Pulse Voltage: 1050

Laser Power: 50/80

TOF LD+

3.46e3



Georgia Tech Mass Spectrometry Laboratory

4-OCT-2004 18:42:46

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Knutson ABTS (CHCA)

Pulse Voltage: 1100

Laser Power: 20/80

TOF LD-
2.50e4



Georgia Tech Mass Spectrometry Laboratory

4-OCT-2004 18:16:56

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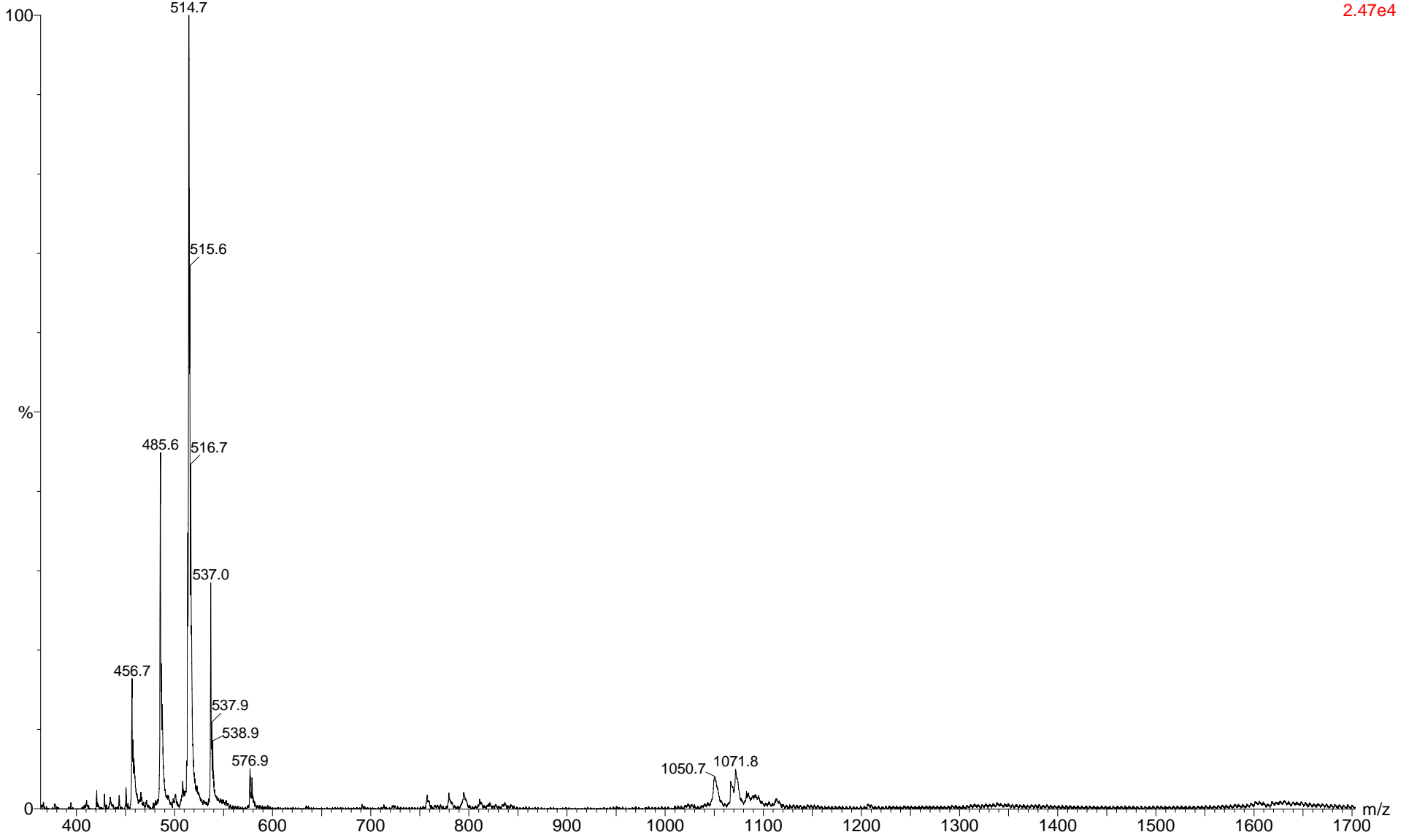
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Pulse Voltage: 1100

Laser Power: 50/80

TOF LD-

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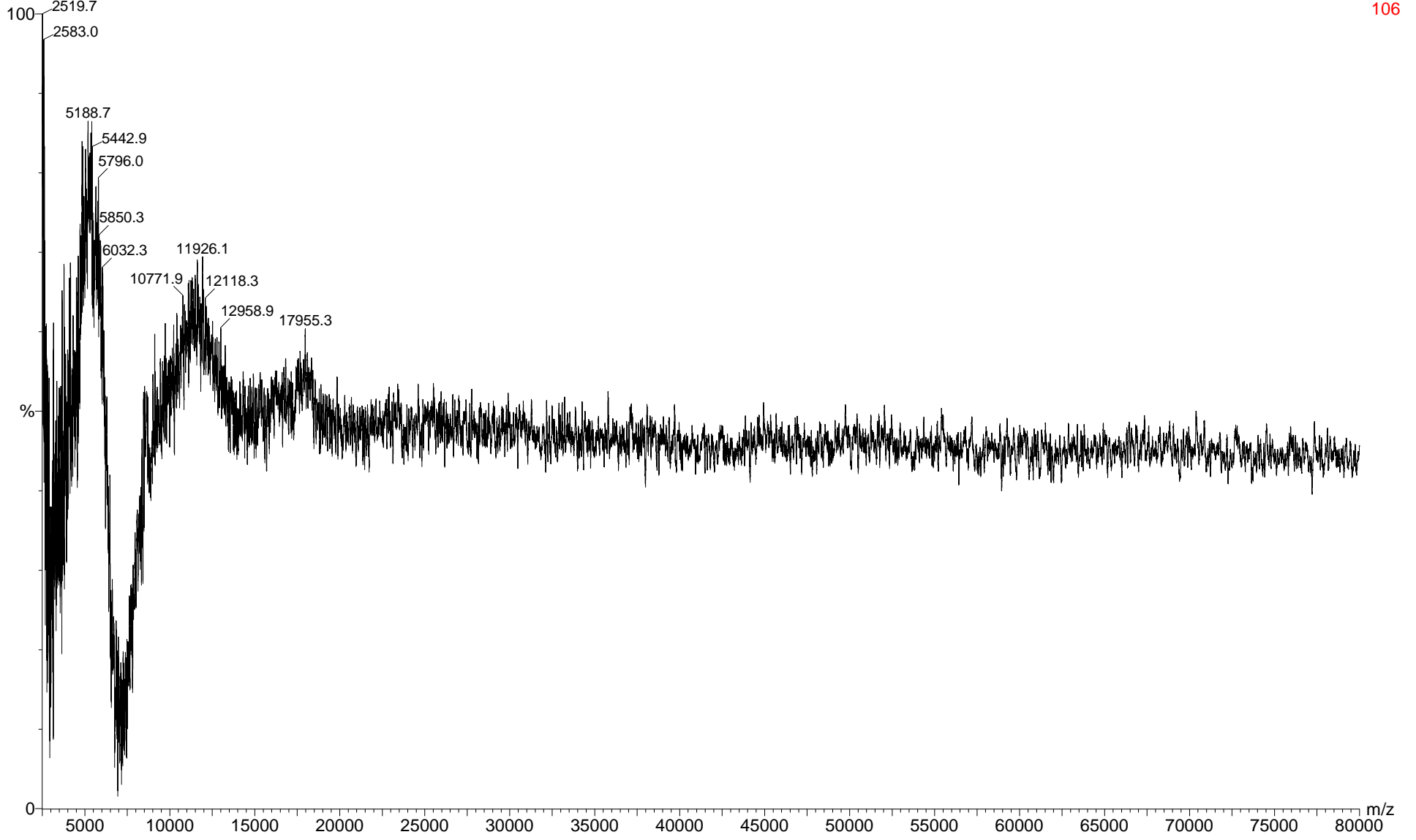
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Knutson laccase + ABTS (CHCA)

Pulse Voltage: 1700
Laser Power: 50/80

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TOF LD+
106



Georgia Tech Mass Spectrometry Laboratory

5-OCT-2004 17:50:05

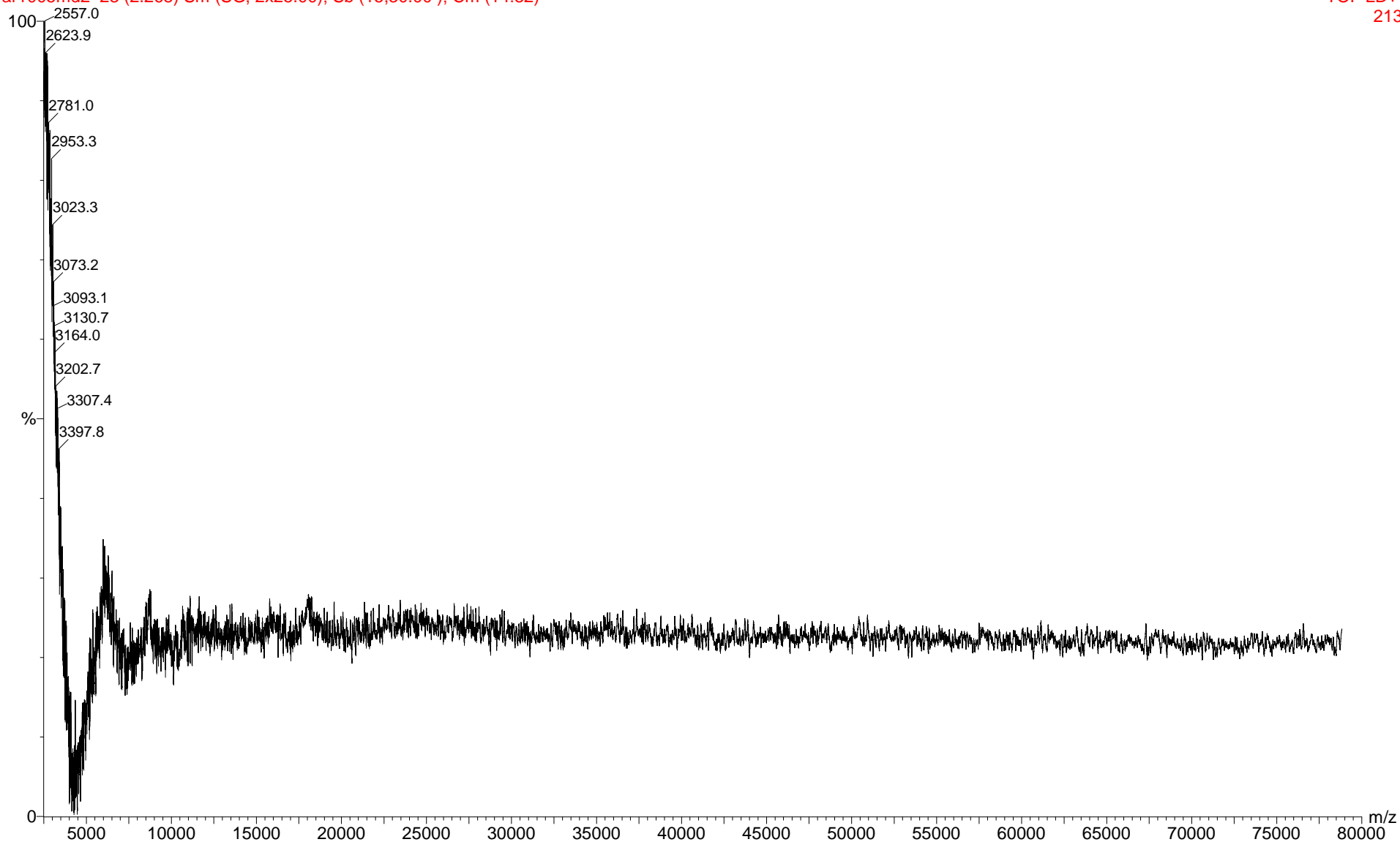
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Pulse Voltage: 1700

Laser Power: 50/80

TOF LD+
213



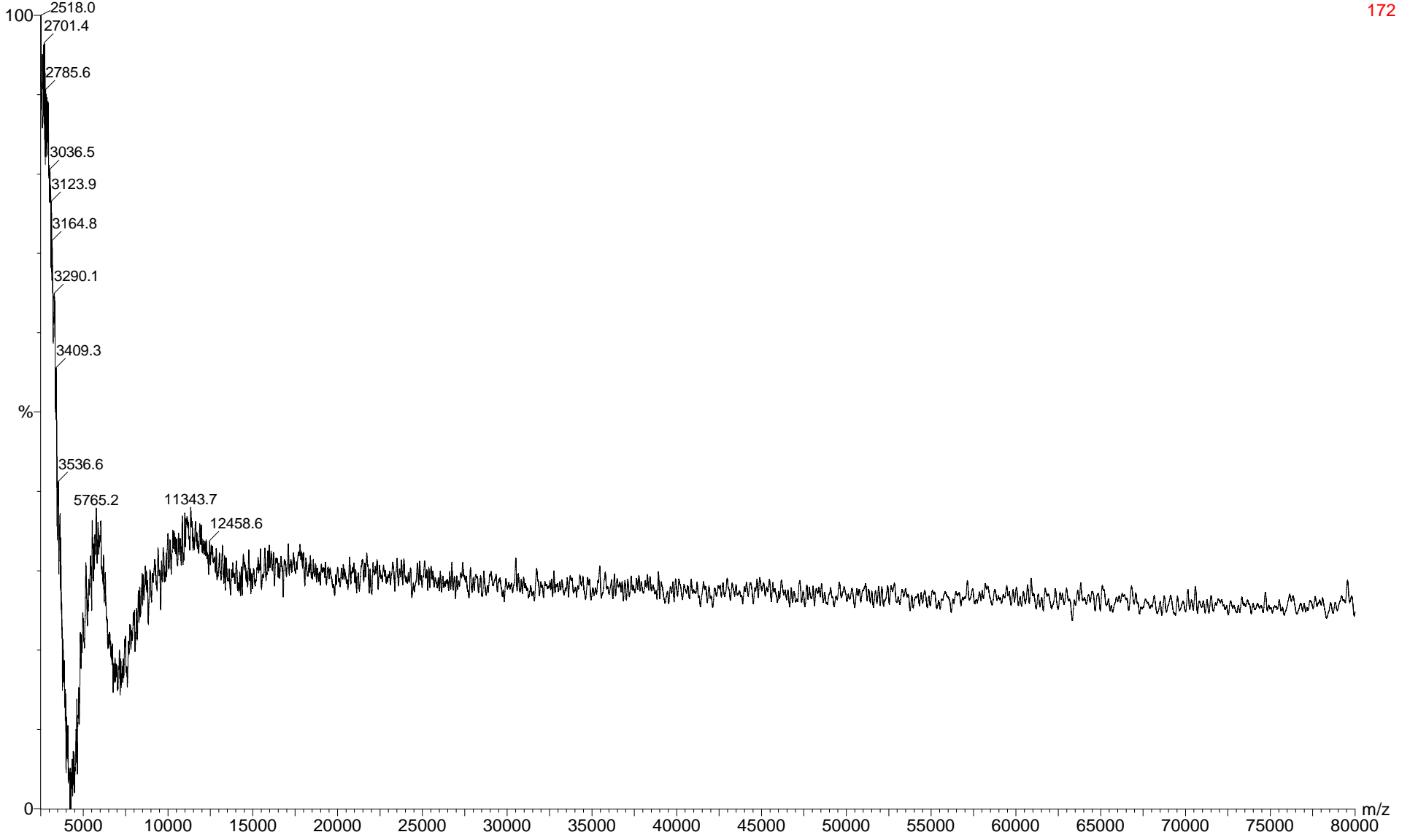
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4-OCT-2004 20:02:43

Knutson laccase + ABTS (CHCA)

Pulse Voltage: 2200
Laser Power: 50/80

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TOF LD-
172



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Knutson laccase + ABTS (DHB)

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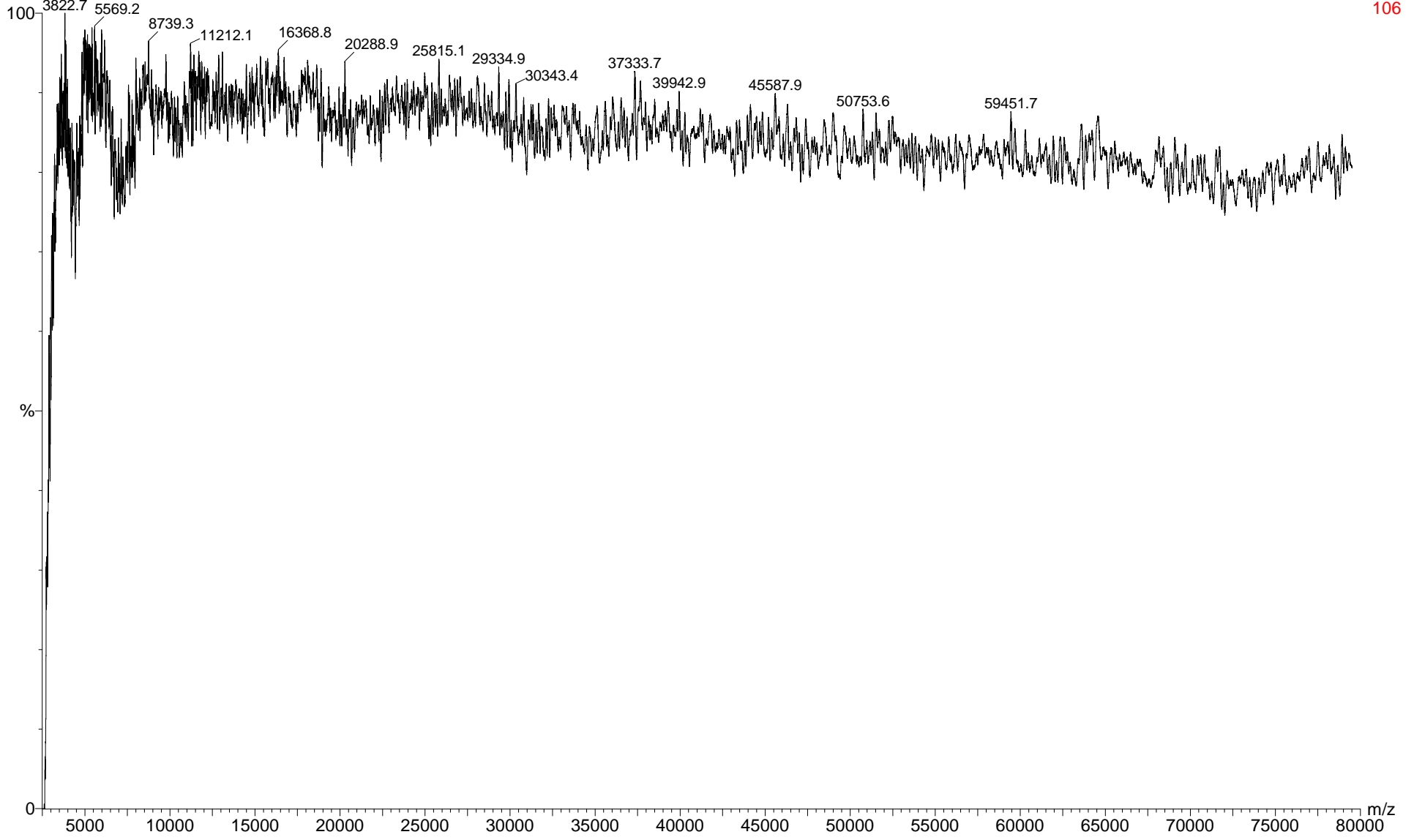
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TOF LD-

106



Georgia Tech Mass Spectrometry Laboratory

5-OCT-2004 18:39:44

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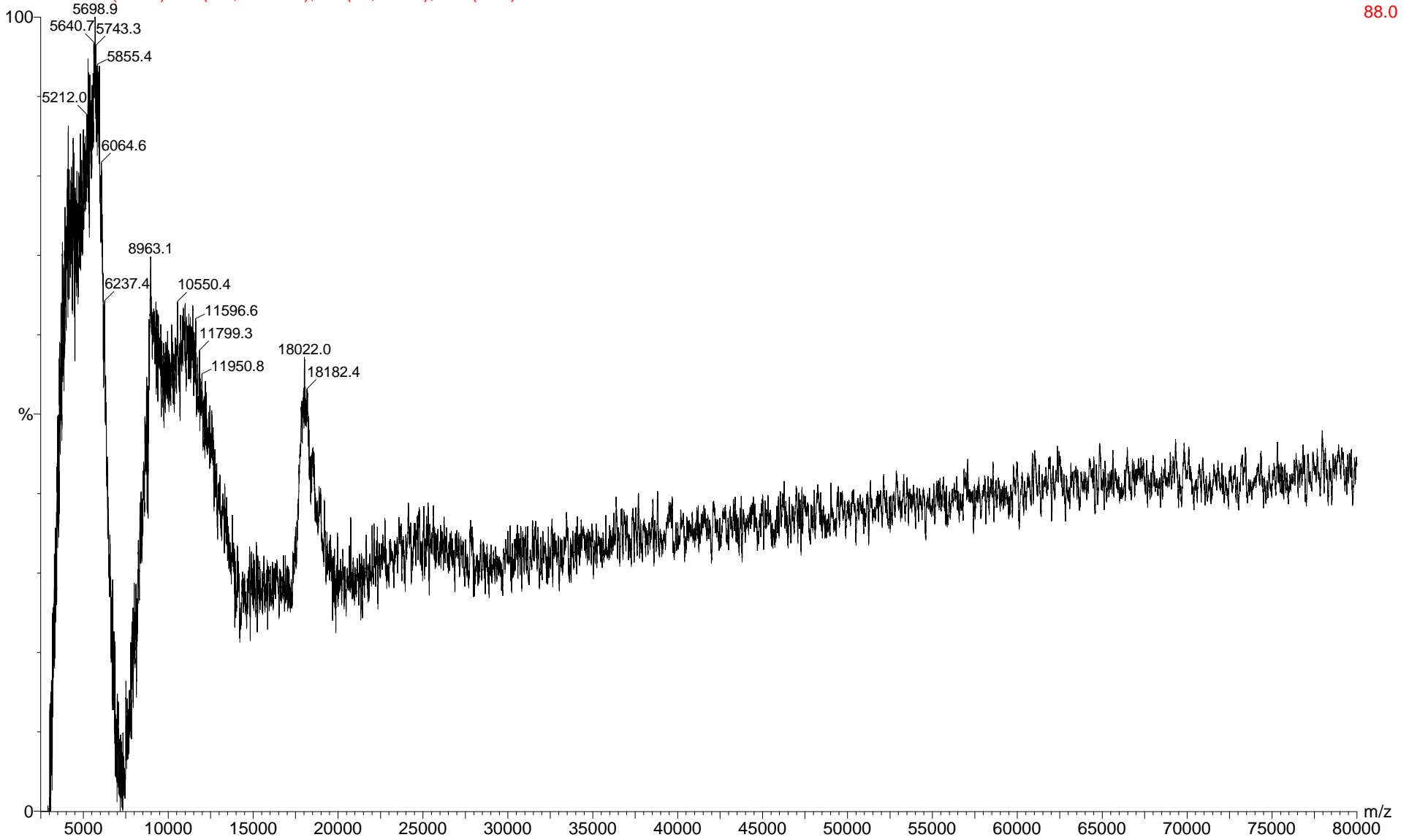
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Pulse Voltage: 1700

Laser Power: 50/80

TOF LD+

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Georgia Tech Mass Spectrometry Laboratory

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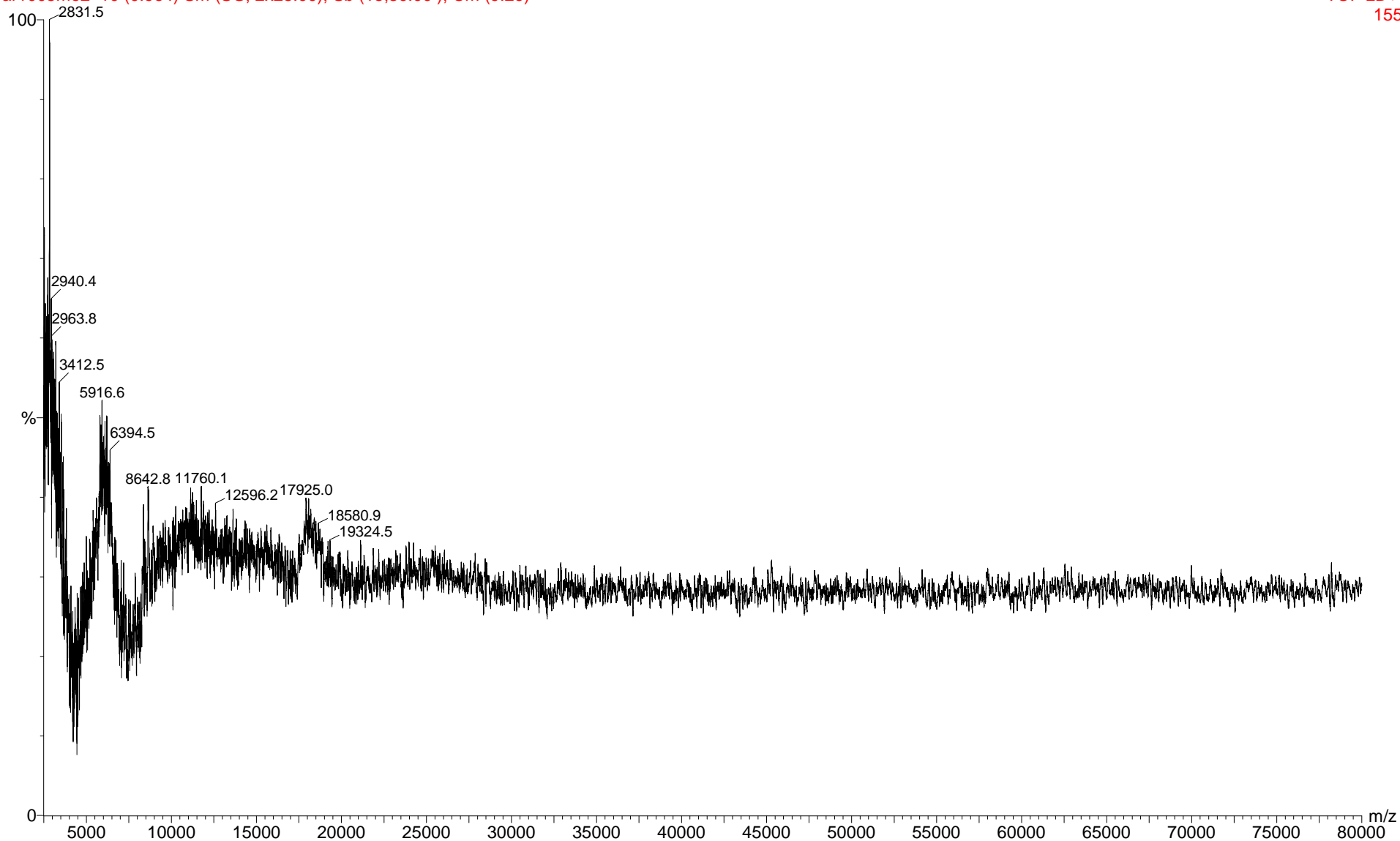
Knutson laccase + ABTS + DY11 (DHB)

Pulse Voltage: 1700

Laser Power: 50/80

TOF LD+

155



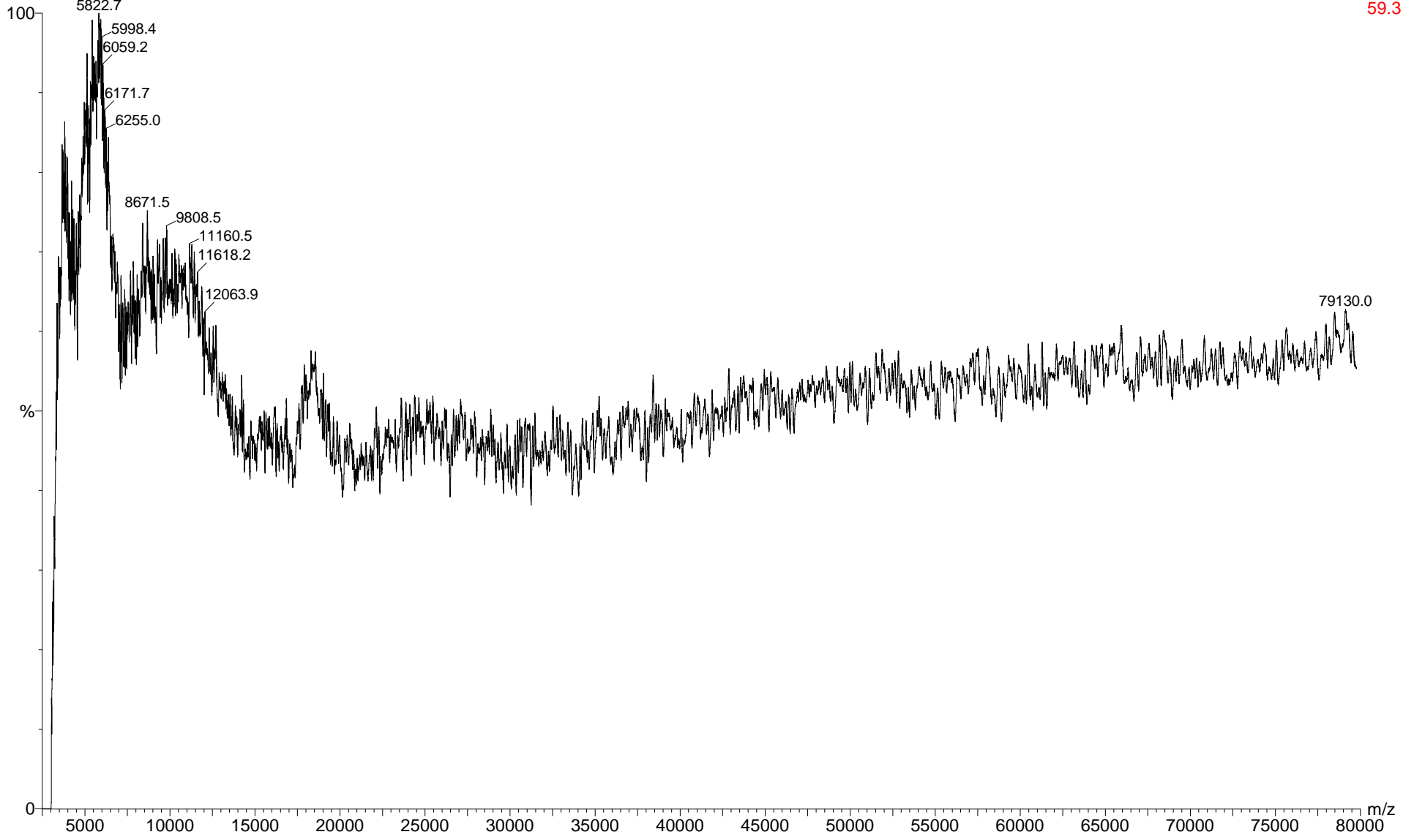
Georgia Tech Mass Spectrometry Laboratory
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Knutson laccase + ABTS + DY11 (CHCA)

Pulse Voltage: 2200
Laser Power: 50/80

ar1005men 14 (1.311) Sm (SG, 2x50.00); Sb (15,30.00); Cm (9:28)

TOF LD-
59.3



Georgia Tech Mass Spectrometry Laboratory

4-OCT-2004 19:32:57

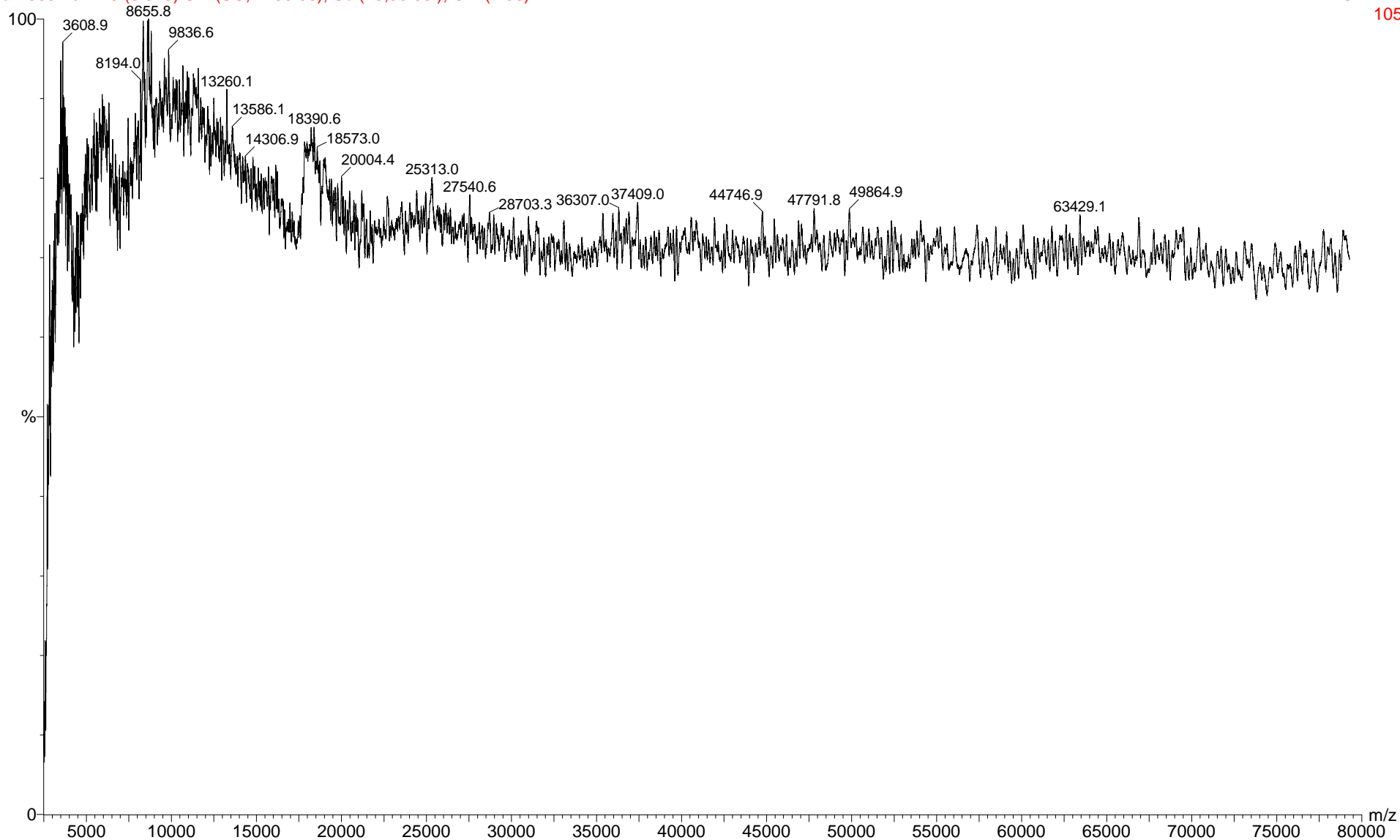
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Knutson laccase + ABTS + DY11 (DHB)

Pulse Voltage: 2200

Laser Power: 50/80

TOF LD-
105



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LIST OF ABBREVIATIONS

ABTS	2-2' azinobis (3-ethylbenzthiazoline-6-sulfonate)
cs	Consistency (wt% pulp in slurry)
CHCA	α -Cyano-4-hydroxycinnamic acid
DHB	2,5-dihydroxybenzoic acid
FAS	Formamidine sulfinic acid
HBT	1-Hydroxybenzotriazole
HRP	Horseradish Peroxidase
o.d.	Oven-Dried
VA	Violuric acid
SBP	Soybean Peroxidase

A APPENDIX

A.1 PURIFICATION AND CHARACTERIZATION

Since the samples of Direct Yellow 11 supplied were from commercial preparations, they contained salts, fillers and other compounds in addition to the dye chromophore(s). Attempts were made to purify the dye chromophore.

A.1.1 PURIFICATION BY SOLID PHASE EXTRACTION (SPE)

In solid phase extraction, a sample is applied to a bed of modified silica beads. Ideally, the dye chromophore would initially adhere to the silica, (forming a visible yellow band) while other ingredients would pass through. The column could then be washed to remove any other ingredients that had bound to the beads. Finally, the dye chromophore would be eluted from modified silica with a stronger solvent.

Table A-1 Samples Subjected to SPE Extraction

Available Samples (* marks samples selected)
*Filtrate from treated Direct Yellow 11 dyed pulp
*Filtrate from treated white pulp
ABTS
Laccase
*Laccase + ABTS
*Direct Yellow 11
*Direct Yellow 11 + Alum
Solution of Direct Yellow 11 + laccase + ABTS
Solution from treating Direct Yellow 11 with laccase/ABTS for 2 hrs at 45°C, 10 bar O ₂

A method development kit with 5 each of 10 different types of solid phase extraction cartridges was obtained. Five samples were selected from available solutions. (Table A-1). The concentrations of Direct Yellow 11 and alum were those applied to pulp during the dyeing process. It should be noted that Fastusol is the brand name for the Direct Yellow 11 used for most experiments.

Application conditions were as follows:

- 1) Room temperature
- 2) 3 mL of each sample
- 3) Applied to column; collected and reapplied two times for total of applying each 3 mL sample three times to maximize removal of dye from sample
- 4) Obtained spectra of each filtrate to examine decrease in absorbance

The results obtained using 3 mL of 75 µg/mL Direct Yellow 11 are shown below. All columns were white before sample was applied. SPE Column refers to the group attached to the silica beads to modify it. SAX stands for strong anion exchange; SCX for strong cation exchange.

Table A-2 Results of SPE Extraction of 75 µg/mL Direct Yellow 11

SPE Column	Filtrate Color	Column Color
NH ₂	Colorless	Yellow band concentrated at very top
SAX	Colorless	Dark yellow throughout with darker yellow at top
C ₈	Slightly yellow	Faint yellow with darker band at top
C ₁₈	Slightly yellow	Light yellow throughout
CH	Slightly yellow	Light yellow throughout
Diol	Faint yellow	Light yellow throughout
CN	Faint yellow	Light yellow throughout
Silica	Faint yellow	Light yellow throughout
C ₂	Yellow	White
SCX	Yellow	White

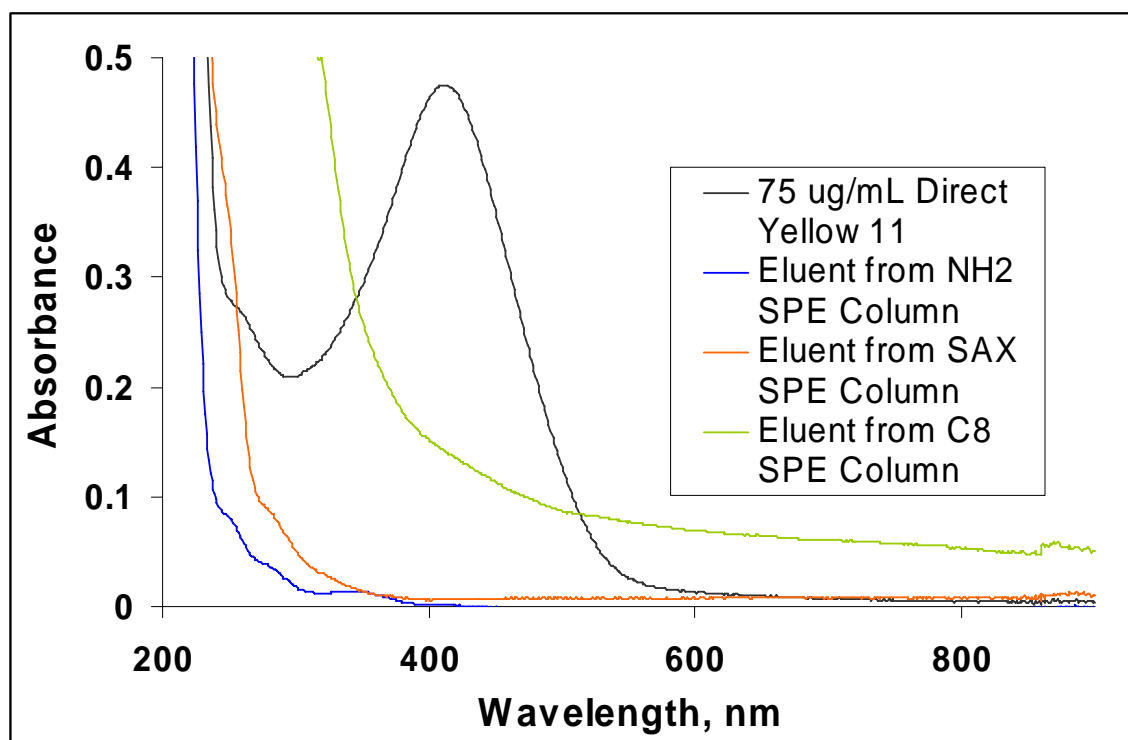
Conclusion: the NH₂ modified silica column gave the best results in binding. However, SAX and C₈ also appeared potentially useful. These three columns, NH₂, SAX and C₈ were applied to the remaining samples: A) 75 µg/mL Direct Yellow 11 + 600 µg/mL alum B) filtrate from laccase-ABTS treatment of Direct Yellow 11-dyed pulp C) filtrate from laccase-ABTS treatment of white pulp The results are shown below in Table A-3.

Table A-3 Results of SPE Extractions of Reaction Filtrates

Sample	SPE Column	Filtrate Color	Column Color
Direct Yellow 11 75 µg/mL + Alum 600 µg/mL	NH ₂	Colorless	Thin dark yellow band concentrated at top
	SAX	Colorless	Yellow throughout
	C ₈	Slightly yellow	Dark yellow at top and yellow throughout
Filtrate from dyed pulp treated with laccase-ABTS	NH ₂	Colorless	Thin brown band concentrated at top
	SAX	Colorless	Slight yellow color throughout
	C ₈	Faint yellow tinge	Faint cream color throughout
Filtrate from white pulp treated with laccase-ABTS	NH ₂	Colorless	Concentrated brown band at top
	SAX	Colorless	Light tan throughout
	C ₈	Faint cream tinge	Faint cream color throughout

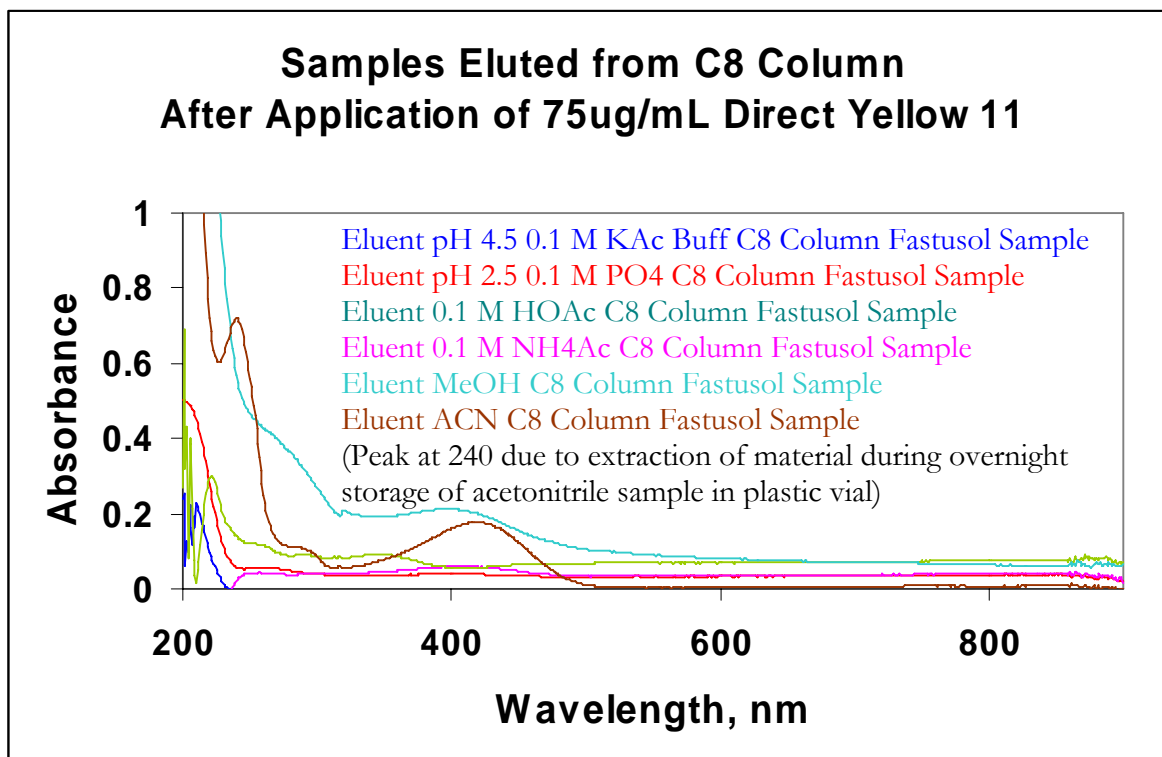
The results indicate that the NH₂-modified SPE column was able to effectively extract Direct Yellow 11 from solution. The SAX column was also very good at removing Direct Yellow 11 from solution. All 3 SPE column types did appear to be able to remove from solution and concentrate Direct Yellow 11.

Figure A-1 Removal of Dye from Solution by NH₂, SAX and C8 SPE Columns



However, elution of absorbed dye was problematical. Changes in solution pH or ionic strength were unable to elute dye. As shown in Figure A-2, some dye could be removed from the C8 column by applying methanol (MeOH) or acetonitrile (ACN) as eluent. However, visible dye color remained on column after elution. The absorption of Direct Yellow 11 by the C8 column appears to non-specific. The binding of Direct Yellow 11 to NH₂ and SAX (strong anion exchange) was more specific and also stronger. Although a number of solvents were tested as eluents, the binding between Direct Yellow 11 and NH₂ and SAX SPE columns was too strong to be disrupted. SPE can not be easily used to separate Direct Yellow 11 and products from laccase-mediator solutions.

Figure A-2 Elution of Adsorbed Dye from C8 SPE Column



A.1.2 PURIFICATION BY HPLC

Considerable effort was expended trying to develop a high-pressure liquid chromatography (HPLC) system to separate Direct Yellow 11 from laccase, ABTS and breakdown products. These efforts were guided by Practical HPLC Method Development, 2nd Ed, by Snyder, Kirkland and Glajch.¹

A.1.2.1 Overview of HPLC Theory and Definitions

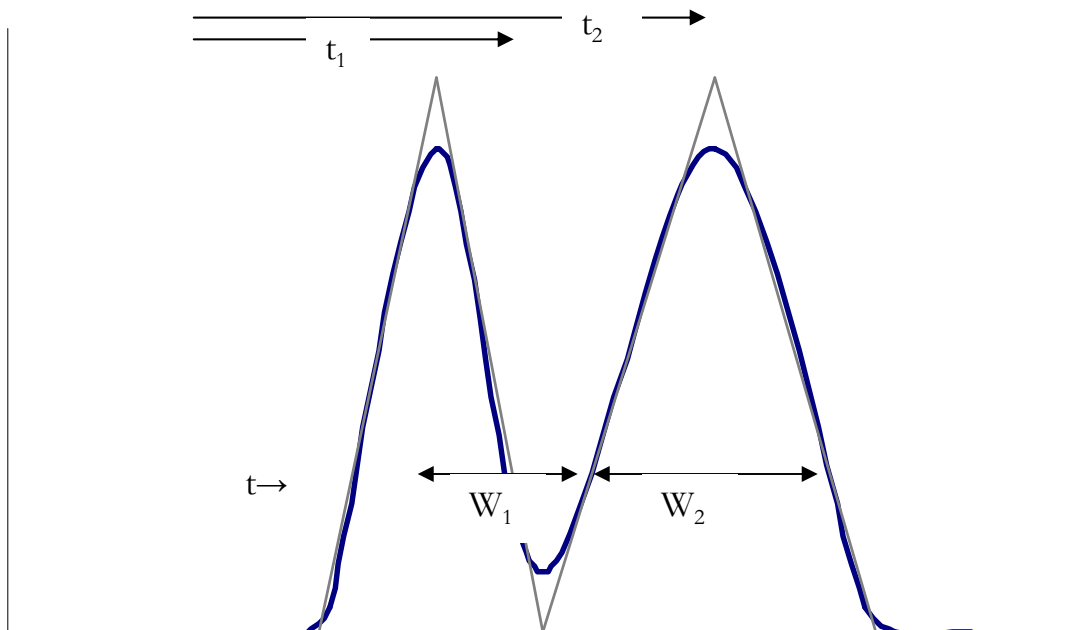
HPLC is used to separate components in a mixture so that the individual components can be analyzed and/or quantitated. For analytical HPLC, the separation achieved depends on solution components having some affinity for the column. Component partitioning between the mobile phase and the column retards elution of the components. Differences in component affinity and partitioning create the component separation.

The quality of a separation is often determined by the resolution between the component peaks. Resolution is based on peak widths and retention times. (Equation A-1).

Equation A-1 Definition of Resolution between Two HPLC Peaks

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

Where W_1 and W_2 are the widths at the peak base of peaks 1 and 2, t_1 and t_2 are the retention times for peaks 1 and 2. (Figure A-3).

Figure A-3 Example of HPLC Peak Resolution

The separation between any two bands in a chromatogram can be altered systemically by changing the experimental conditions. The resolution R_s can be described in terms of three parameters (k , α , and N) which are directly related to experimental conditions.

Equation A-2 Resolution Depends on Selectivity, Efficiency and Retention

$$R_s = \frac{1}{4} (\alpha - 1) N^{1/2} \frac{k}{1 + k}$$

(selectivity) (efficiency) (retention)

Here k is the average retention factor for the two bands, N is the column plate number and α is the separation factor: $\alpha = k_1/k_2$, where k_1 and k_2 are values of k for adjacent bands 1 and 2. Equation A-2 is useful in method development because it

classifies the many experimental variables into three categories: retention (k), column efficiency (N) and selectivity (α). This classification simplifies the systematic variation of conditions to improve resolution between bands.

If conditions are changed so k becomes smaller (earlier elution), resolution usually becomes worse. If α is increased, the two peaks will move apart, increasing R_s . When column efficiency N is increased, the bands become narrower and better separated, but their relative positions do not change.

The parameters k and α are determined by conditions that affect retention or component partitioning between the mobile phase and the column packing. The column plate number N primarily depends on column quality and is affected by changing column conditions.

Table A-4 Parameters Affecting HPLC Peak Resolution

Conditions that Affect k (retention factor) and α (selectivity)
1. Composition of the mobile phase
2. Column composition (stationary phase)
3. Temperature

Conditions the Affect N (column efficiency)
1. Flow rate
2. Column length
3. Particle size

The retention factor k is based on retention times.

Equation A-3 Definition of Retention Factor k

$$k = \frac{t_R - t_0}{t_0}$$

Where t_R is the band retention time and t_0 is the column dead time. The column dead time is related to the column dead volume V_m (volume of mobile phase inside the column) and the flow rate F .

Equation A-4 Definition of Column Dead Time

$$t_0 = \frac{V_m}{F}$$

An estimate of the value of t_0 can be obtained by the retention time of the first significant baseline disturbance, use of a very strong solvent as the mobile phase,

injection of an unretained sample or calculation from column dimensions. Equation A-4 allows the calculation of t_0 from estimated V_m .

Equation A-5 Calculation of Dead Volume V_m

$$V_m \approx 0.5 L d_c^2$$

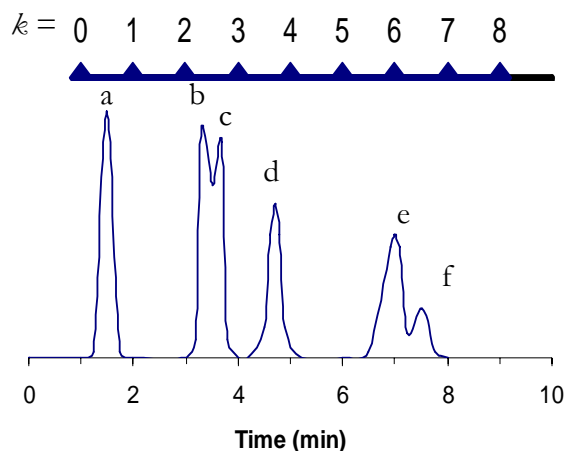
Where V_m (dead volume, in mL), L (cm) is column length and d_c (cm) is internal diameter of the column. For the commonly used 0.46 cm ID columns, Equation A-5 reduces to Equation A-6.

Equation A-6 Calculation of V_m for 0.46cm ID Column

$$V_m \approx 0.1 L$$

A simulated chromatogram is shown in Figure A-4. The resolution is poor between peaks b and c and between e and f. For this system, t_0 is 1. The values of retention factor k are shown at the top.

Figure A-4 Simulated HPLC Chromatogram



A.1.2.2 Experimental HPLC Systems

Two different hardware systems were used. One was a Hewlett Packard (Agilent) 1090 LC system that included 3 mobile phase reservoirs, an autosampler and an 8-channel photodiode array detector based on a xenon lamp, range 200-600 nm. This integrated system was computer controlled, using Chem Station software, version 5. The second hardware system consisted of Gilson equipment: Model 305 (master,

programmable) pump, Model 306 (slave pump) Model 805 Manometric Module (measures system pressure), Model 811B Dynamic Mixer, column oven, Model 116 UV Detector (range 200-350 nm) and Model FC 204 Fraction Collector. A second detector, SpectroPhotometric Model 8450 UV/Vis detector was also included after the Gilson UV detector. Injections were made through a Rheodyne manual injector. Analog data from the two detectors were collected and analyzed utilizing Pyramid Chromatography Manager Software by Axxiom Chromatography.

A.1.2.3 HPLC Results

Two different reversed-phase silica-based columns were tested: Discovery C-18 and Discovery C-16 Amide, both from Supleco Company. A number of combinations of water, acetonitrile and methanol were tested. Although laccase, ABTS and reaction mixtures were also subjected to HPLC, the method development focused on the major chromophore for Direct Yellow 11

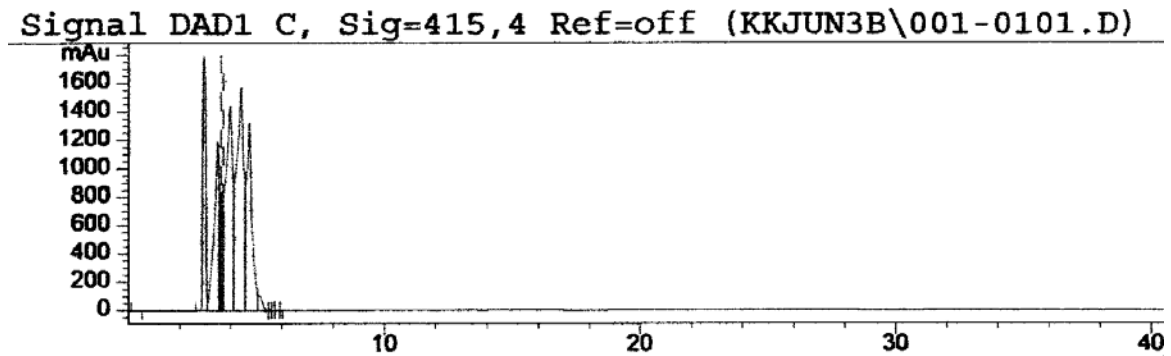
Peaks broaden as components move through the column so that late eluting peaks are much broader and shallower than early eluting peaks. At the same time, some retention is necessary to separate desired component peaks from the initial baseline disturbance and from early-eluting/nonretained impurity bands. For a good separation, the HPLC method should result in retention factor $0.5 < k < 20$. To lower high values of k (late-eluting peaks), the solvent strength of the mobile phase can be increased or the method can be changed from isocratic to a gradient system.

. The major problem observed with Direct Yellow 11 was a low value for k . Since the pK_a for sulfonic acid groups is very low (~ 1.0 pH), changing mobile phase pH had little effect. High pressure problems experienced with the columns prevented the addition of ion-pair reagents since these reagents significantly increase column pressures in normal separations.

All the various combinations tested, (10, 20, 40, 60% acetonitrile in water; 20, 40, 60 % methanol in water; gradients from 5-100% acetonitrile in water or buffers; gradient 5-100% methanol in water) did not improve retention of Direct Yellow 11 on the C-18 or Amide columns. Figure A-5 illustrates a typical HPLC separation of the manufacturer's sample of Direct Yellow 11. The chromatogram was obtained at 415 nm, near the dye

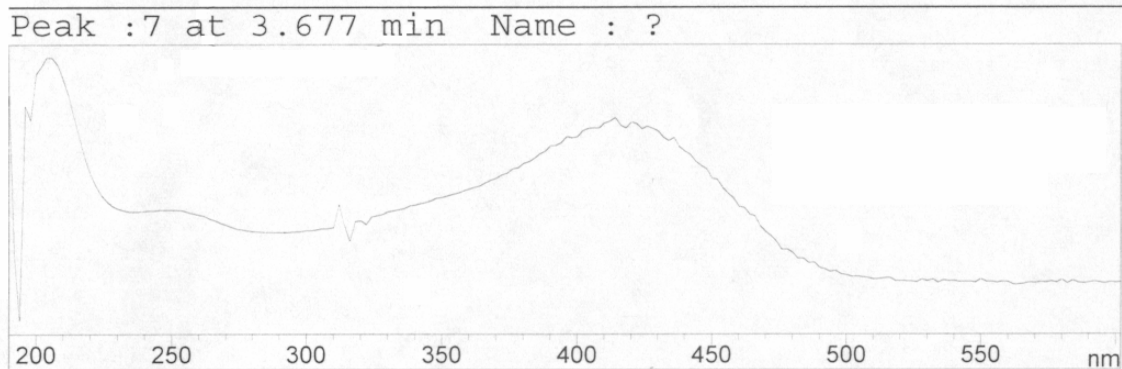
maxima observed on spectra of the Direct Yellow 11 manufacturer's sample. Fillers and buffer salts added to the manufacturer's sample should not be observed at this wavelength. A number of peaks can be observed in Figure A-5. These various peaks correspond to different isomers of the major chromophore.

Figure A-5 Example of HPLC Separation of Direct Yellow 11



This chromatograph was obtained on the Agilent HPLC system. The photodiode array detector in the system obtains spectra for each observed peak. One such spectrum is shown below (peak corresponds to the dotted line in Figure A-5). This spectrum for the 3.677 min peak matches the spectrum obtained for the unfractionated manufacturer's sample. Similar spectra were also obtained for peaks eluting at 2.927, 3.470, 3.958, 4.386, and 4.713 minutes. The HPLC method did sufficiently separate the different chromophore isomers to produce the distinct peaks in Figure A-5, but baseline resolution was not obtained for any method tested.

Figure A-6 Spectra of Direct Yellow 11 Peak from HPLC Chromatogram

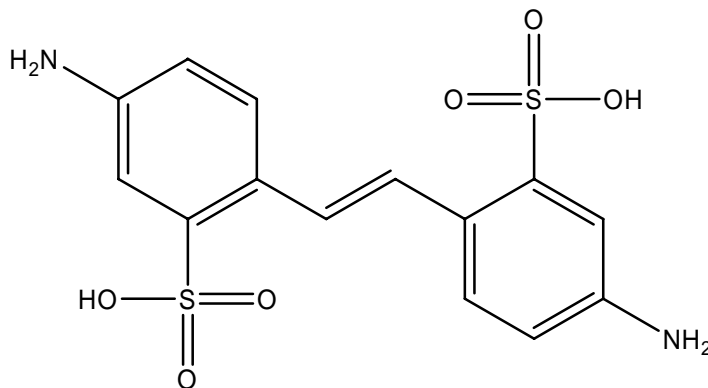


The earliest peak with spectrum corresponding to Direct Yellow 11 eluted at 2.927 minutes. For this system that included a 25 cm x 0.46 id. reverse phase C-18 column, the observed t_0 was 2.6 min. The retention factor $k = (2.9-2.6)/2.6 = 0.11$. (Equation A-3). A k of at least 0.5 (3.9 min for this separation) is considered necessary to separate desired peaks from unretained components and contaminants. The combination of poor peak retention and multiple isomers of the major chromophore limited application of HPLC in this research project.

A.1.2.4 Model Compound

A simpler, single chromophore model compound was obtained to test for decolorization by the laccase/mediator system. However the compound, 4,4'-diamino-2,2'-stilbenedisulfonic acid (Figure A-7) was not water soluble. Treating the insoluble dye with laccase in water would require the addition of alcohol, which in turn would decrease the ability of laccase to function and could alter reaction mechanisms. Consequently, experiments with the model compound were not pursued.

Figure A-7 Model Compound 4,4'-diamino-2,2'-stilbenedisulfonic acid



$C_{14}H_{14}N_2O_6S_2$
Exact Mass: 370.03
Mol. Wt.: 370.40

C, 45.40; H, 3.81; N, 7.56; O, 25.92; S, 17.31

A.1.3 PURIFICATION BY TLC

Attempts were made to purify Direct Yellow 11 by thin-layer chromatography (TLC). With four sulfonic acid groups on the major chromophore, Direct Yellow 11 is very hydrophilic. Direct Yellow 11 (as supplied) was applied to Whatman

chromatography paper and to silica TLC plates. When low polarity solvents such as ethyl acetate or acetonitrile are applied, no color is eluted. Color is eluted however in paper chromatography when 10% methanol is added to acetonitrile. Acetone also elutes color.

Dr. Giesla Buschle-Diller suggested using a mobile phase of (% by volume) of 16% *n*-butanol, 12% ammonium hydroxide, 43% pyridine and 4% methanol. Total for this recipe is 75%. Mobile phases tested included either adding the remaining 25% as water or keeping the component ratios the same and adjusting the total to equal 100%. The samples were applied as listed in Table A-5; with top sample in table on the far left of TLC plate proceeding to the last sample listed being on the far right of the TLC plate. DASS represents a model compound 4,4'-diamino-2,2'-stilbene disulfonic acid.

Table A-5 Samples Subjected to TLC Analysis

Abbreviation	Chromophore	Notes	Purpose
F	Direct Yellow 11	As supplied by BASF	Dye Samples
P	Direct Yellow 11	As supplied by Ciba	
B40	Basazol 40	As supplied by BASF	
B46	Basazol 46L	As supplied by BASF	
ABTS		Laccase Mediator	Separating Direct Yellow 11 from ABTS and checking purity
F ABTS	Direct Yellow 11 + ABTS		
TLC Pure	Direct Yellow 11	After purification by TLC	
NMR SPE	Direct Yellow 11	Purified by SPE; retrieved from NMR sample	
SPE 1	Direct Yellow 11	Purified by SPE	
SPE 2	Direct Yellow 11 + ABTS	Purified by SPE	
Fresh DASS	4,4'-Diamino-2,2'-stilbene disulfonic acid	Freshly dissolved solution; Light or air sensitive	Model Compound
NMR DASS	4,4'-Diamino-2,2'-stilbene disulfonic acid	Retrieved from NMR sample	
NMR Rept'd DASS	4,4'-Diamino-2,2'-stilbene disulfonic acid	Retrieved from NMR sample	
Fresh Rept'd DASS	4,4'-Diamino-2,2'-stilbene disulfonic acid	Freshly dissolved solution; Light or air sensitive	

Following Dr. Bushle-Diller's recommendations, 16 mL of *n*-butanol, 12 mL of concentrated ammonium hydroxide, 43 mL of pyridine and 4 mL methanol was mixed with 0, 10, 15 or 25 mL of distilled water to produce mobile phases for elution of

analytical TLC plates. The volumes and percentages of each component in the mobile phases tested are shown in Table A-6.

For the dyes, 10 µL of 10 mg/L stock solutions were applied to the plate by adding 2.5 µL four times in same spot, allowing drying between applications. The ABTS samples also contained 10 µL from 1 mg/mL solution. The results are shown in Figure A-8 through Figure A-12. The areas outlined in pencil exhibited fluorescence under ultraviolet illumination.

Table A-6 Mobile Phases Tested for Analytical TLC

Chemical	mL	%	mL	%	mL	%	mL	%	mL	%
n-Butanol	16	21.3%	16	18.8%	16	17.8%	16	16.0%	40	40.0%
NH ₄ OH	12	16.0%	12	14.1%	12	13.3%	12	12.0%	30	30.0%
Pyridine	43	57.3%	43	50.6%	43	47.8%	43	43.0%	20	20.0%
MeOH	4	5.3%	4	4.7%	4	4.4%	4	4.0%	10	10.0%
Water	0	0.0%	10	11.8%	15	16.7%	25	25.0%	0	0.0%
Total	75		85		90		100		100	

Figure A-8 Analytical TLC (No Water) Applied to Samples in Table A-5

Elution for 60 Minutes Mobile Phase by Volume: n-Butanol 16 mL: Ammonium Hydroxide 12 mL: Pyridine 43 mL: Methanol 4.0 mL

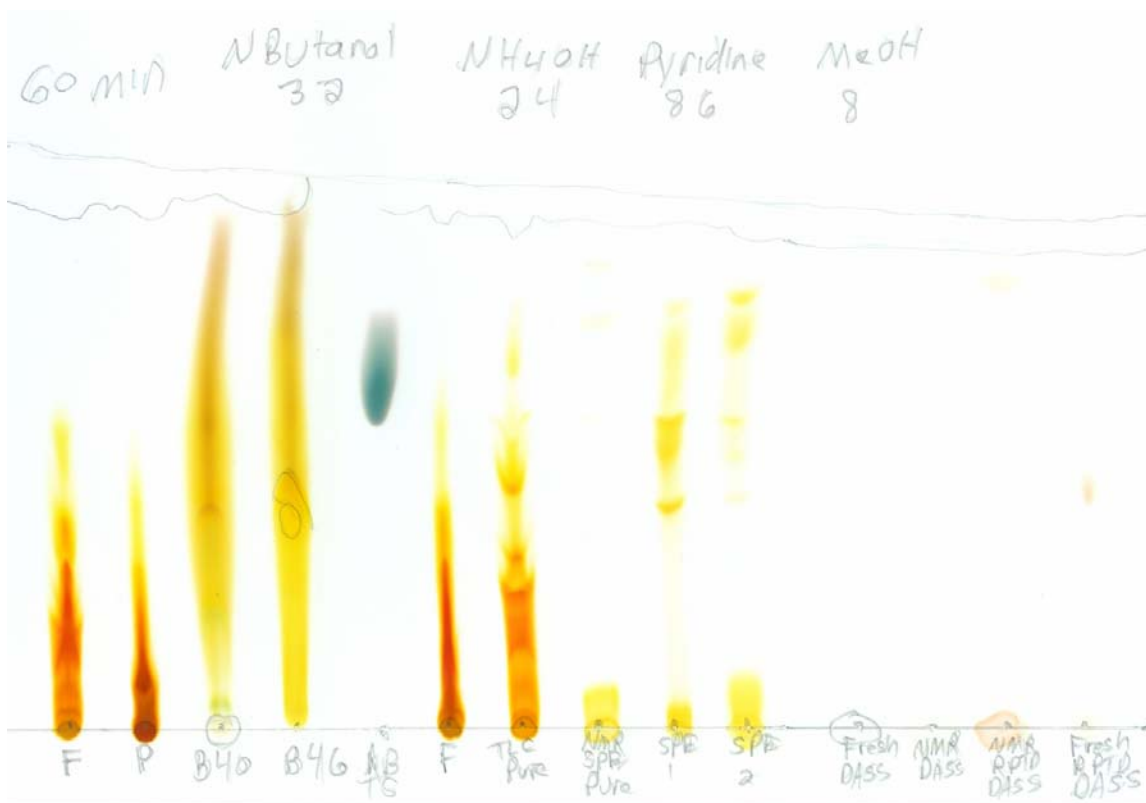


Figure A-11 Analytical TLC (25 mL Water) Applied to Samples in Table A-5
Elution for 60 Minutes Mobile Phase by Volume: n-Butanol 16 mL: Ammonium Hydroxide 12 mL: Pyridine 43 mL: Methanol 4 mL: Water 25 mL

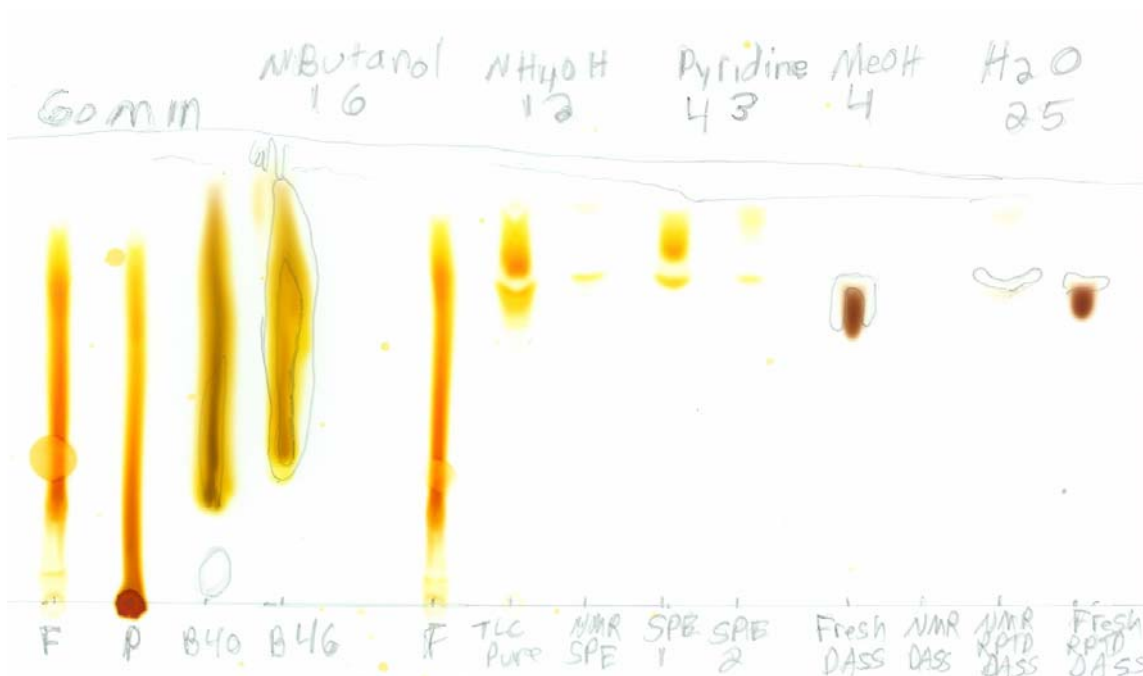
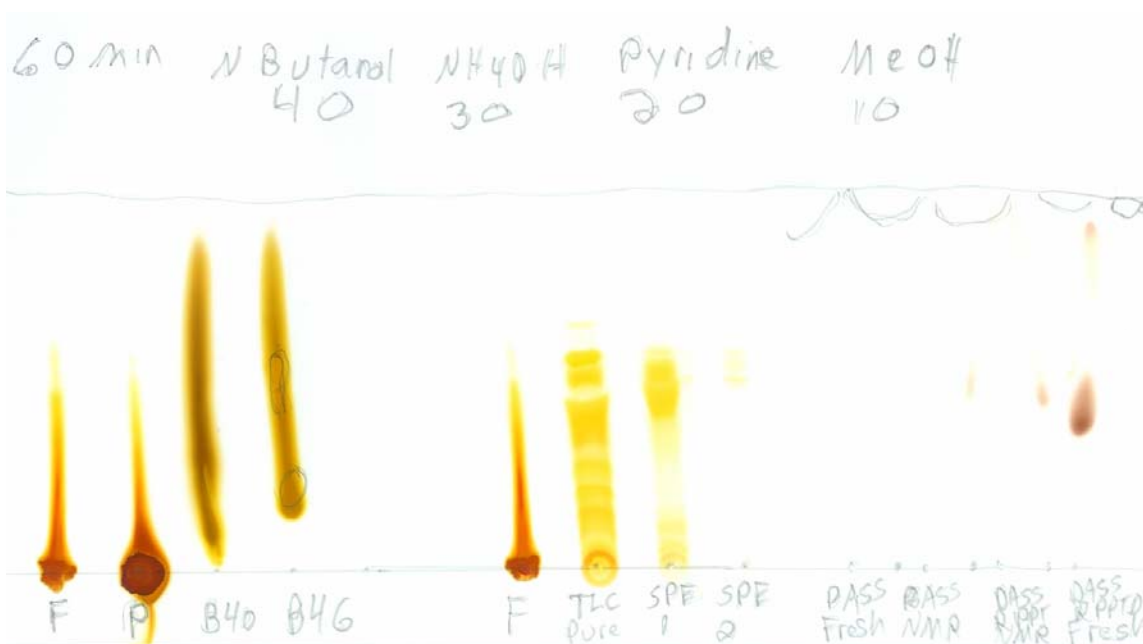


Figure A-12 Analytical TLC (No Water, Altered) Applied to Samples in Table A-5
Elution for 60 Minutes Mobile Phase by Volume: n-Butanol 40 mL: Ammonium Hydroxide 30 mL: Pyridine 20 mL: Methanol 10 mL:



The best separation appears to be with the inclusion of 25 mL of water in the mobile phase (Figure A-11). Preparative TLC plates were obtained and used to obtain “TLC Pure” Direct Yellow 11. To improve separation of the dye chromophore(s) from uncolored fillers and salts in manufacturer’s sample, two additional “wash” steps were added. After application of Direct Yellow 11, the plates were subjected to elution first with methylene chloride, then with ethyl acetate. The dye chromophore does not elute with either solvent. These “wash” steps were included to move eluting uncolored components to the top of the TLC plates, away from the desired chromophore(s). A scan on one preparative TLC plate after elution is shown in Figure A-13. It can be seen that the manufacturer’s sample consists of multiple chromophores. The chromophores in the upper bands may be closely related, while the lower band may be different.

It is known that commercial dye preparations can contain multiple components such as a major dye chromophore, contaminants from the manufacturing processes, acids or buffer salts (to control pH and solubility), fillers, viscosity modifiers, and even additional unrelated dye chromophores. For Direct Yellow 11 manufacturer’s sample, in addition to the published structure that contains four sulfonic acid groups, manufacturer’s sample may include chromophores with only 2 or 3 sulfonic acid groups. Also, stilbene structures readily undergo *cis-trans* isomerizations in the presence of light. The wide upper bands are probably geometric isomers. If chromophores are present that have less than 4 sulfonic acid groups, the molecule would not be as soluble and would move more slowly, possibly creating the observed lower band.

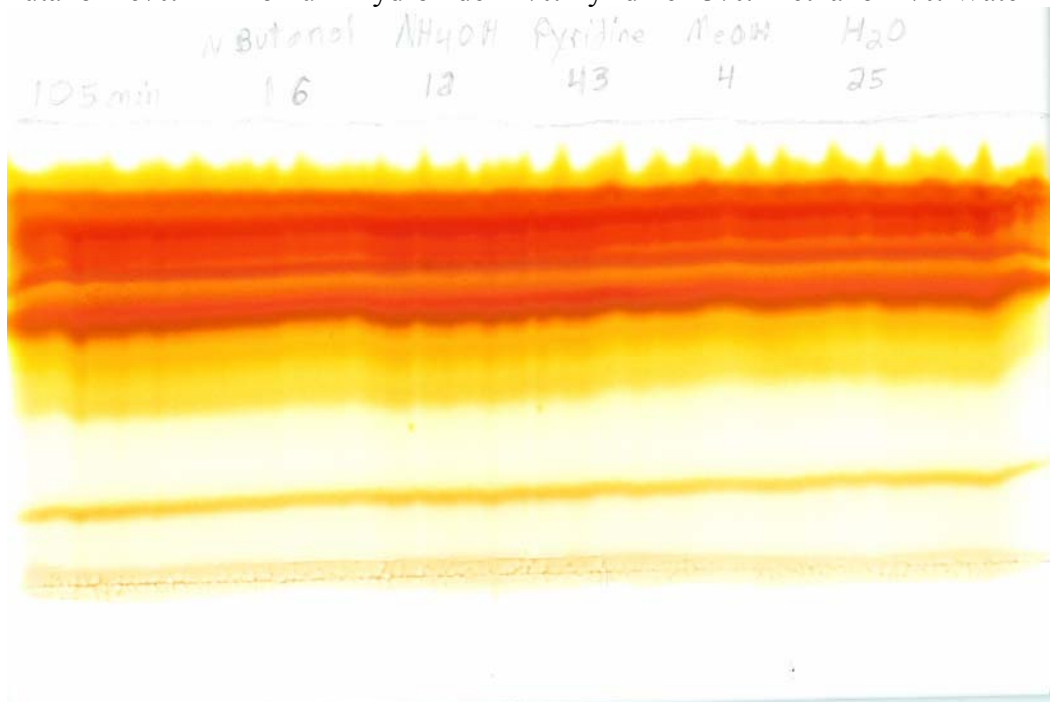
The dark “orange” regions of the upper bands were collected and eluted with water. Enough material was obtained that a NMR scan was run of the sample. However, too little “TLC pure” dye was obtained to be used for further experiments.

Figure A-13 Scan of Preparative TLC Applied to Direct Yellow 11

Step 1: Wash with Methylene Chloride Step 2: Wash with Ethyl Acetate

Step 3: Elution for 90 Minutes Mobile Phase by Volume %:

n-Butanol 16%: Ammonium Hydroxide 12%: Pyridine 43%: Methanol 4%: Water 25%

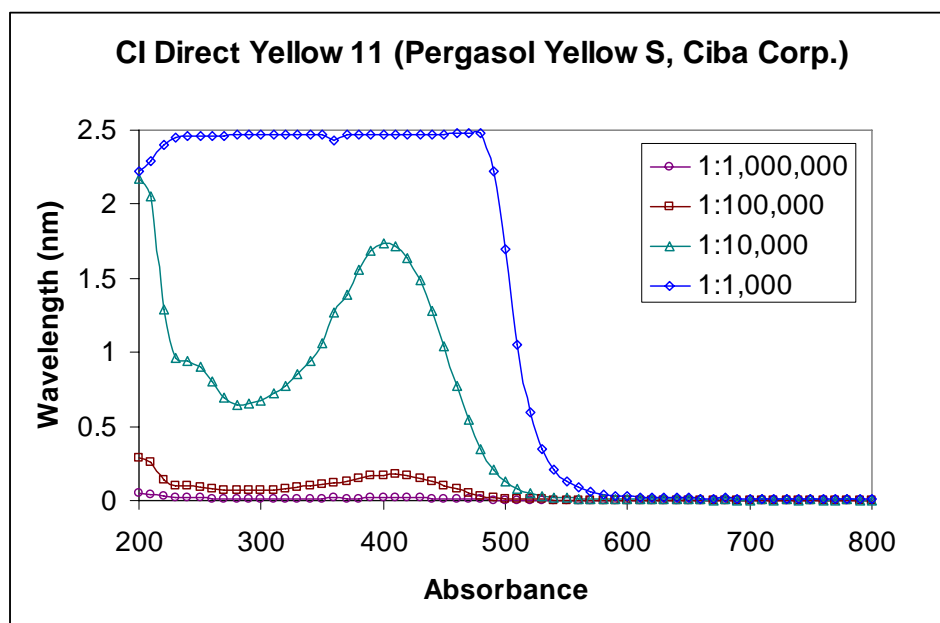


A.1.4 CHARACTERIZATION OF DYES BY UV/VIS SPECTROPHOTOMETRY

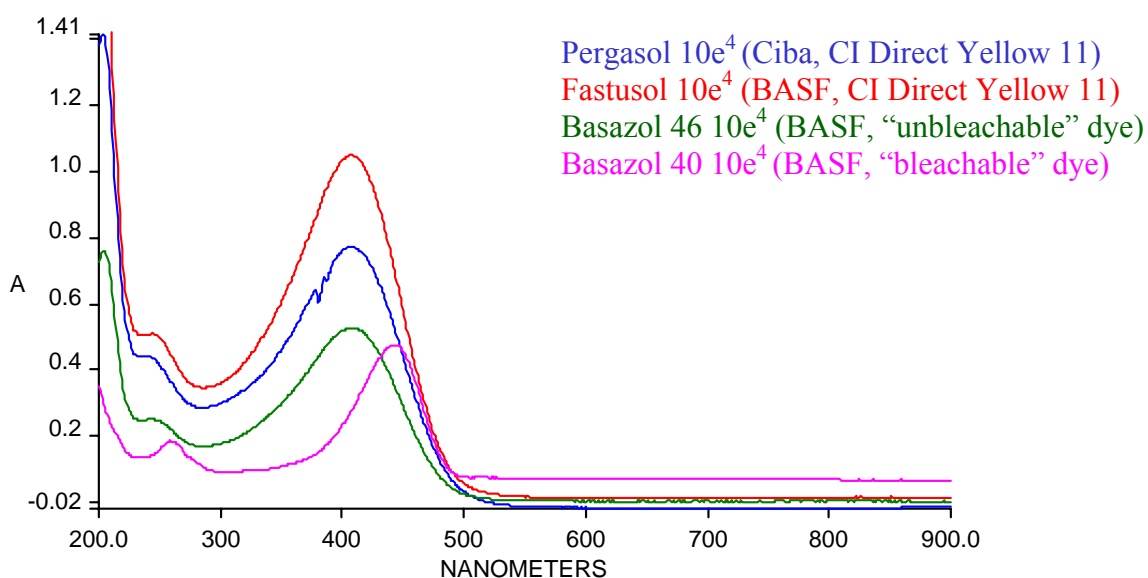
Concentration curves were prepared to enable determination of amount of non-volatile solids/mL necessary to produce absorbance between 0.2 and 0.8 AU. The dilutions selected were $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$. The dilutions were prepared as described in Table A-7 below. The spectra for four dilutions made for Direct Yellow 11 dye sample from Ciba Corporation is shown in Figure A-14. Similar results were obtained for the other dye samples (Direct Yellow 11 Fastusol, Basazol 46L and Basazol 40, all from BASF).

Table A-7 Dye Dilutions for Relating Absorbance to Concentration

Dilution	Concentrate	Amount of Concentrate	Final Volume
$1:10^3$	Dye Sample	0.10 mL	100 mL
$1:10^4$	$1:10^3$	5.0 mL	50 mL
$1:10^5$	$1:10^3$	0.5	50 mL
$1:10^6$	$1:10^3$	0.10 mL	100 mL

Figure A-14 Absorbance of Four Dilutions of Direct Yellow 11

Four different dye samples were received from CIBA and BASF, including a sample of C.I. Direct Yellow 11 from both Ciba and BASF. Fastusol, the Direct Yellow 11 sample from BASF, was used for virtually all the research in this project. The spectra of these dyes are shown in Figure A-15. The absorbance spectra of both Direct Yellow 11 samples are very similar with the Fastusol sample having a higher concentration of dye chromophore.

Figure A-15 Characteristic Spectra of Dye Samples

A.1.4.1 Effect of Changes in Solution pH on Dye Spectra

Since pH can affect dye spectra, each dye sample was dissolved in pH 2.5; pH 4.5 and pH 9.0 buffers in addition to water and the spectra were obtained for all samples. The effect of pH on dye absorbance is shown below, beginning with spectra of the buffers used to dissolve the dyes. Full spectrums (200-900 nm) were obtained. The graphs below show only 200-600 nm because only baseline absorbance was observed in the 600-900 nm range. It can be seen that 25 mM sodium phosphate buffer pH 2.5 did not exhibit significant absorbance. However the more concentrated 100 mM sodium acetate buffer pH 4.5 and the 100 mM sodium phosphate pH 9 buffer did show significant absorbance below 220 nm (pH 9 buffer) and 240 nm (pH 4.5 buffer)

Figure A-16 Absorbance of Different pH Buffers

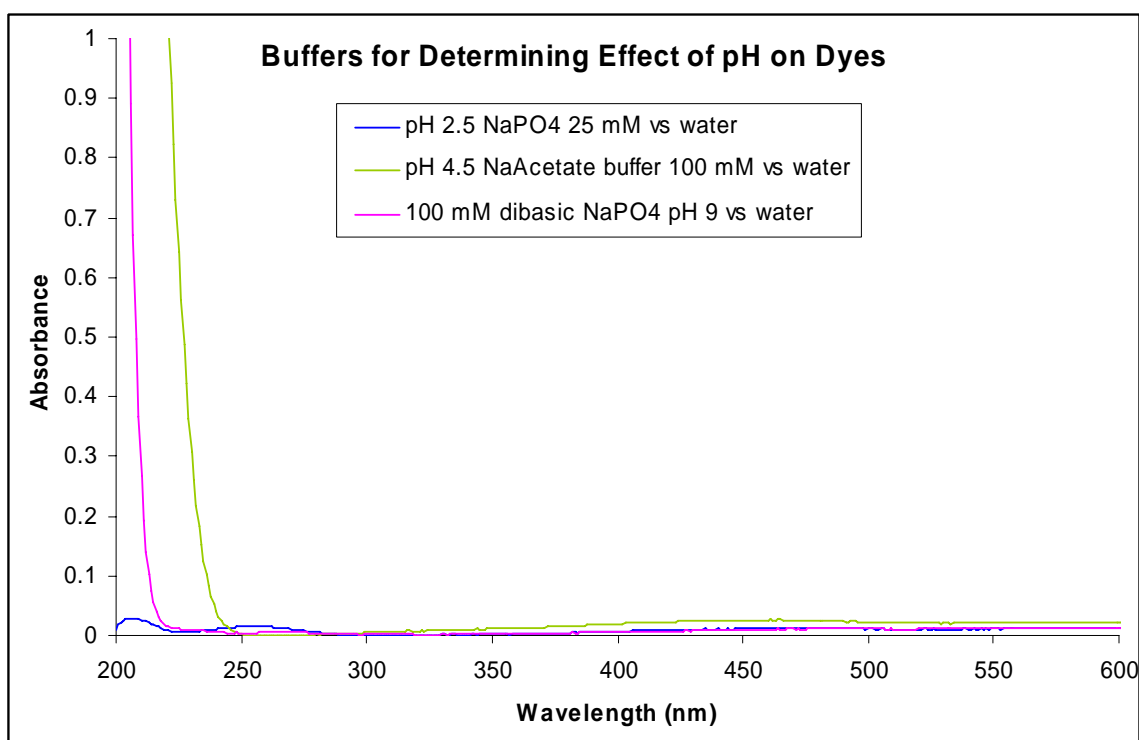


Figure A-17 Effect of pH on Light Absorbance of Fastusol

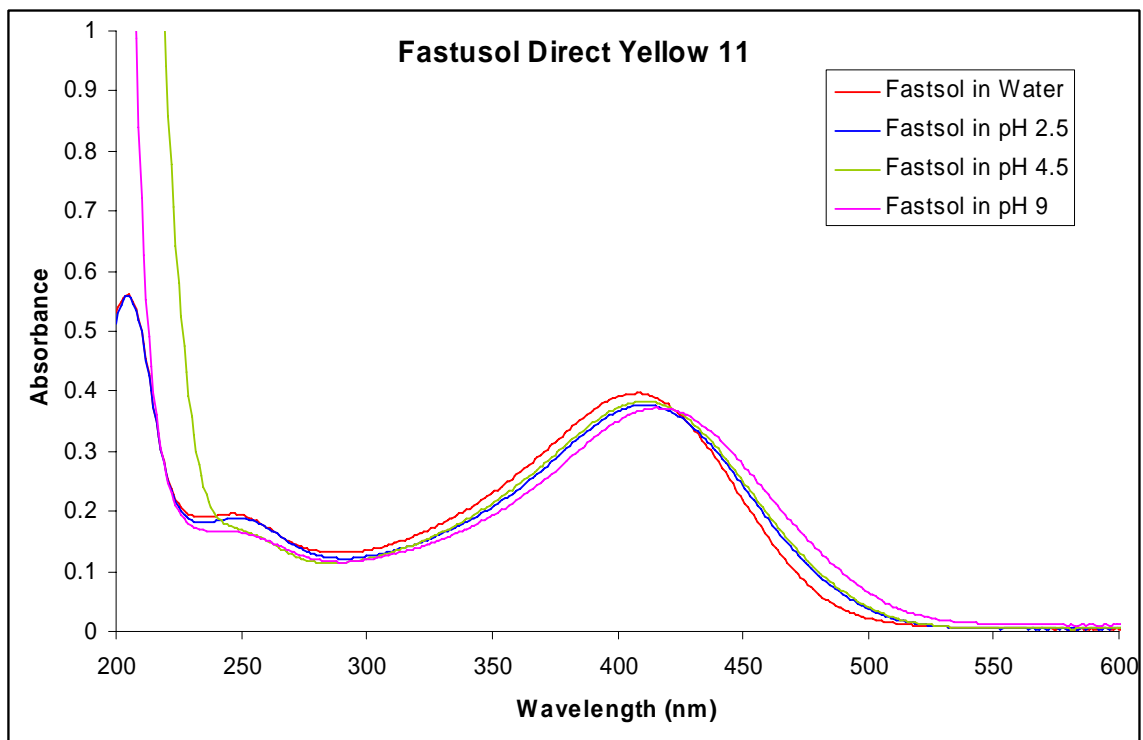


Figure A-18 Effect of pH on Light Absorbance of Pergasol

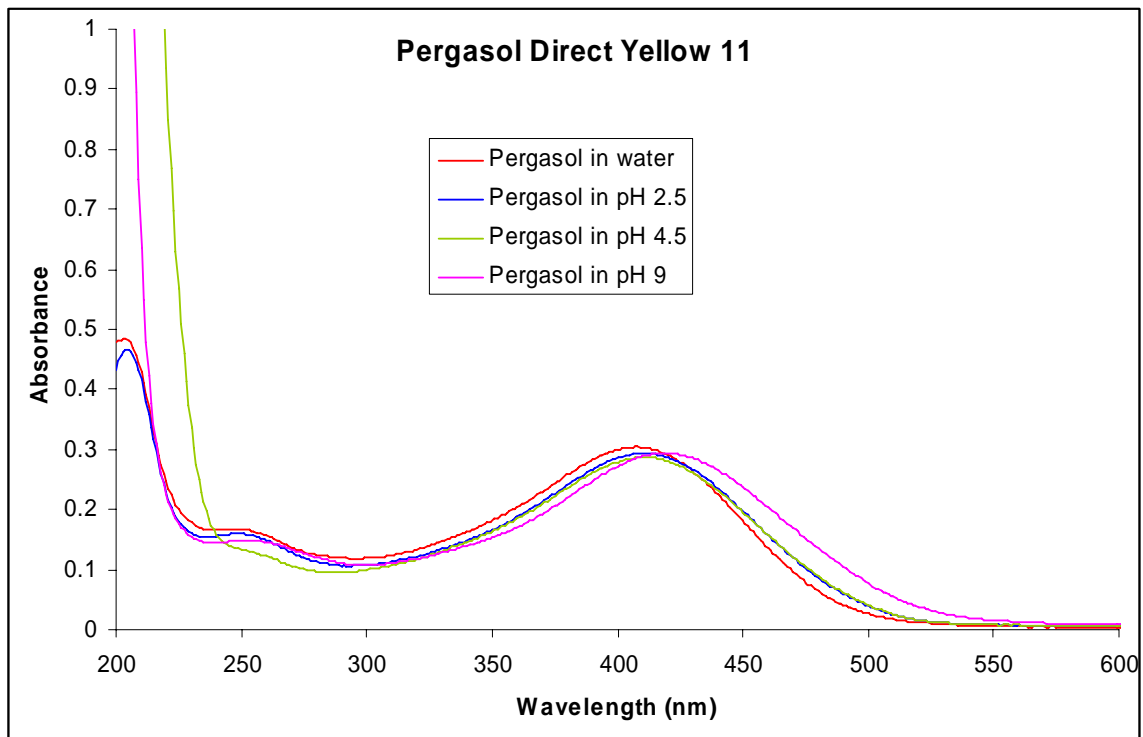


Figure A-19 Effect of pH on Light Absorbance of Basazol 46l

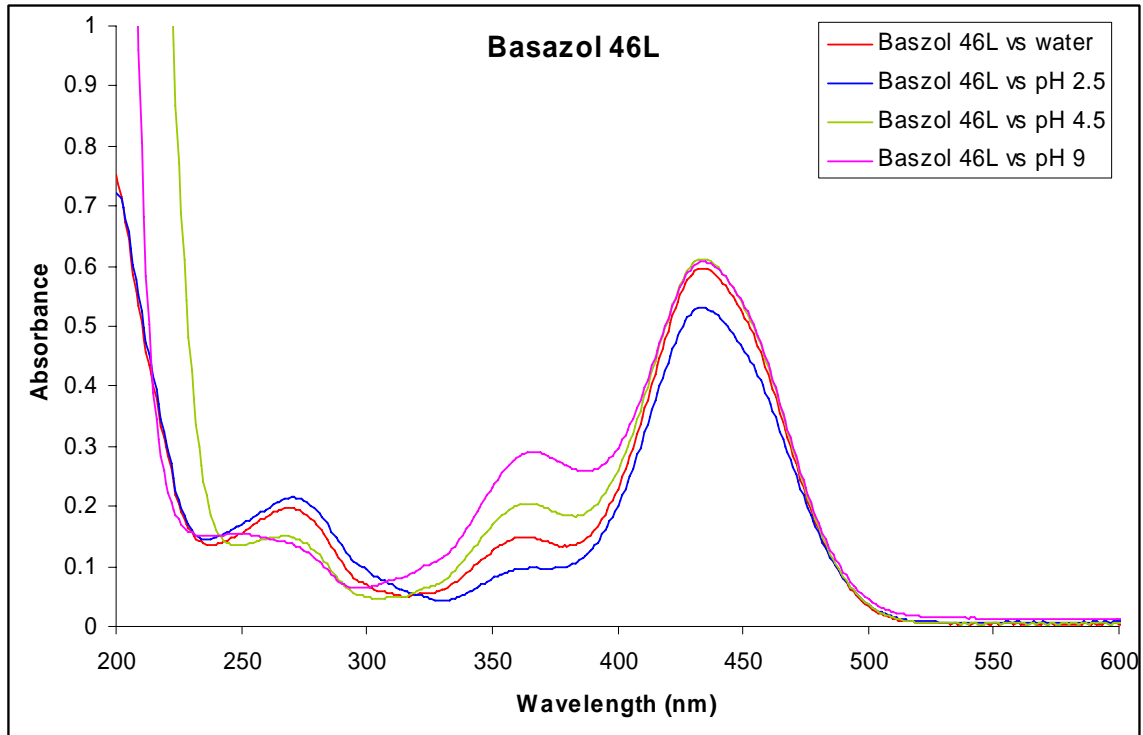
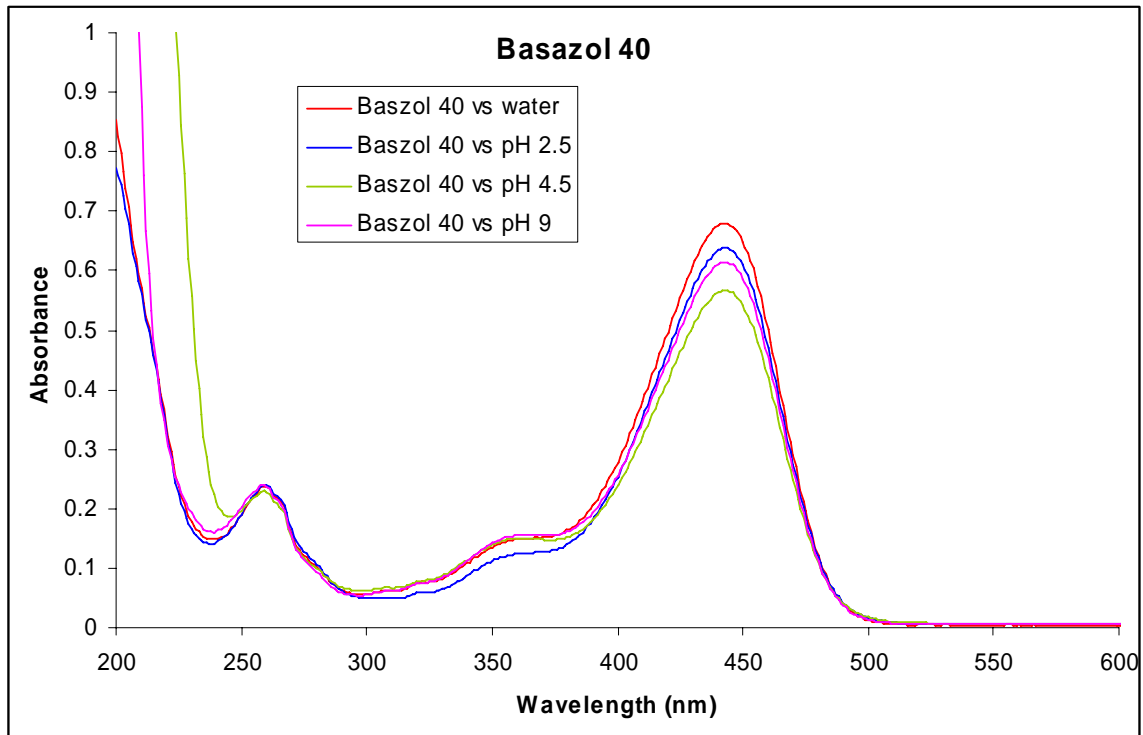


Figure A-20 Effect of pH on Light Absorbance of Basazol 40



Basic pH 9 solution did shift the absorbance maxima to longer wavelengths for Direct Yellow 11 samples. The absorbance spectra of basic dyes Basazol 40 and Basazol 46L were not altered by changing solution pH.

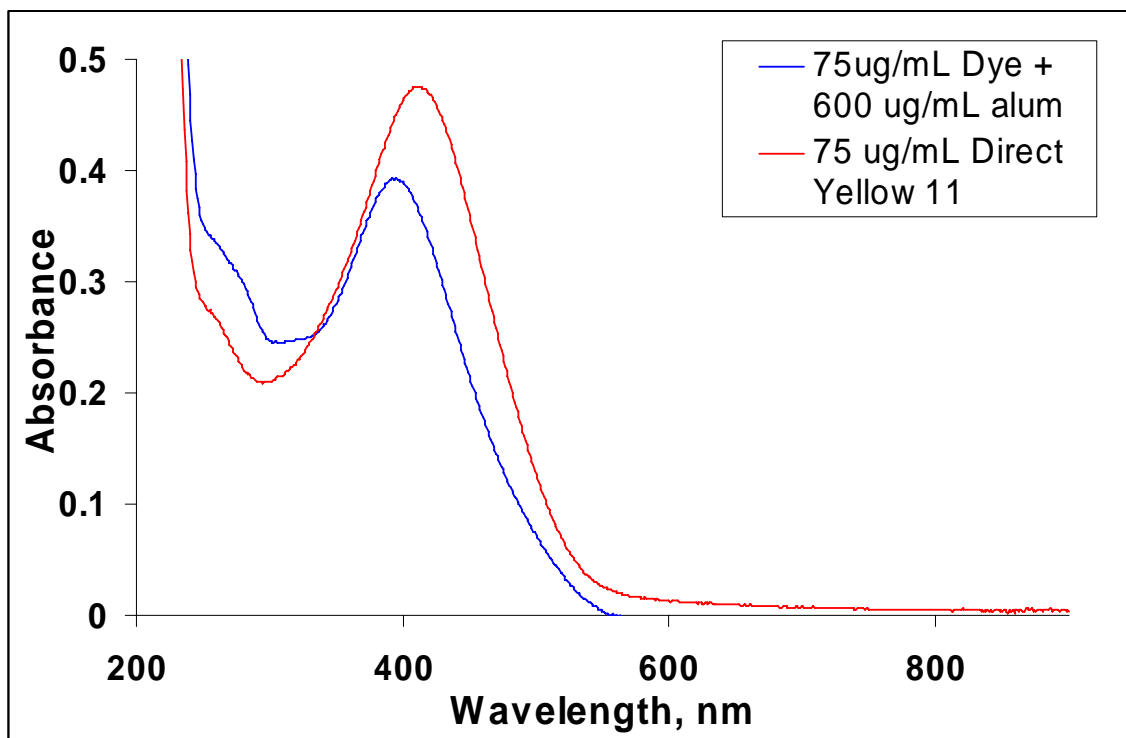
Table A-8 Dye λ_{Max} (nm) in Different pH Solutions

	Pergasol	Fastusol	B46L	B40
Water	408	408	435	443
pH 2.5	410	410	434	443
pH 4.5	410	411	435	443
pH 9.0	419	416	435	443

A.1.4.2 Effect of Alum on Dye Spectra

The alum (aluminum sulfate 18-hydrate) applied as dye fixative may form complexes with Direct Yellow 11 that alter its absorption spectra. When tested, alum did slightly shift the λ_{Max} as can be seen in Figure A-21.

Figure A-21 Alum Shifts λ_{Max} of Direct Yellow 11

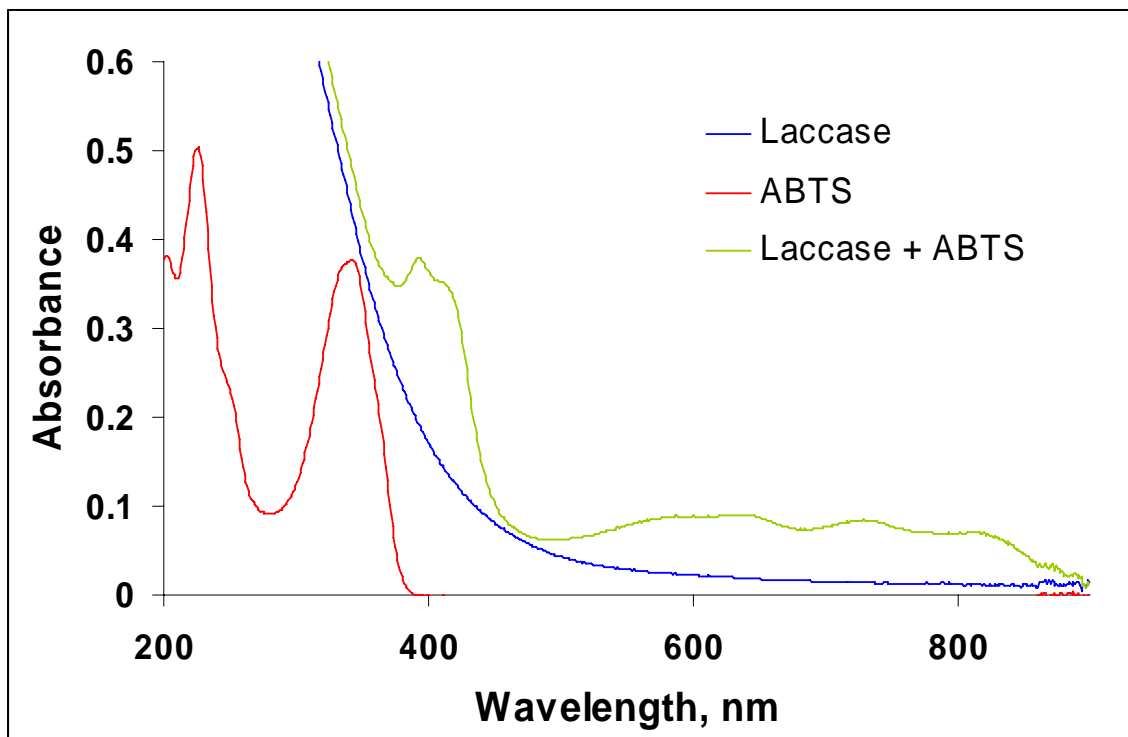


A.1.4.3 Effect of Mixing Laccase with ABTS

When ABTS is dissolved in solution, the blue powder forms a blue solution. When laccase is added, the solution immediately becomes much darker. Laccase reacts

with ABTS and converts it to the dication. This change can also be observed by spectrophotometer as in Figure A-22.

Figure A-22 Formation of ABTS Dication by Addition of Laccase



A.1.5 CHARACTERIZATION OF REACTION MIXTURES BY UV/VIS

The initial experiments with Direct Yellow 11 in solution showed that laccase/HBT did react with the dye but quantization would be difficult. Complications from overlapping absorbances forced the research focus to turn to dyed pulp. What are shown below are spectra showing how the mediator absorbance overlaps the Direct Yellow 11 (FS for Fastusol) spectra for ABTS, HBT and VA.

The first spectra (Figure A-23) show the reaction control of Direct Yellow 11 (Fs) heated at 45°C for 2 hours under 10 bar oxygen pressure. No change in dye absorbance can be observed. The succeeding spectra (Figure A-24) are for Direct Yellow 11 treated with laccase and oxygen, but in the absence of any mediator. No reaction can be observed which is expected since laccase generally reacts with phenols and Direct Yellow 11 lacks phenolic groups.

Figure A-23 Heating Direct Yellow 11 under Oxygen has no Effect

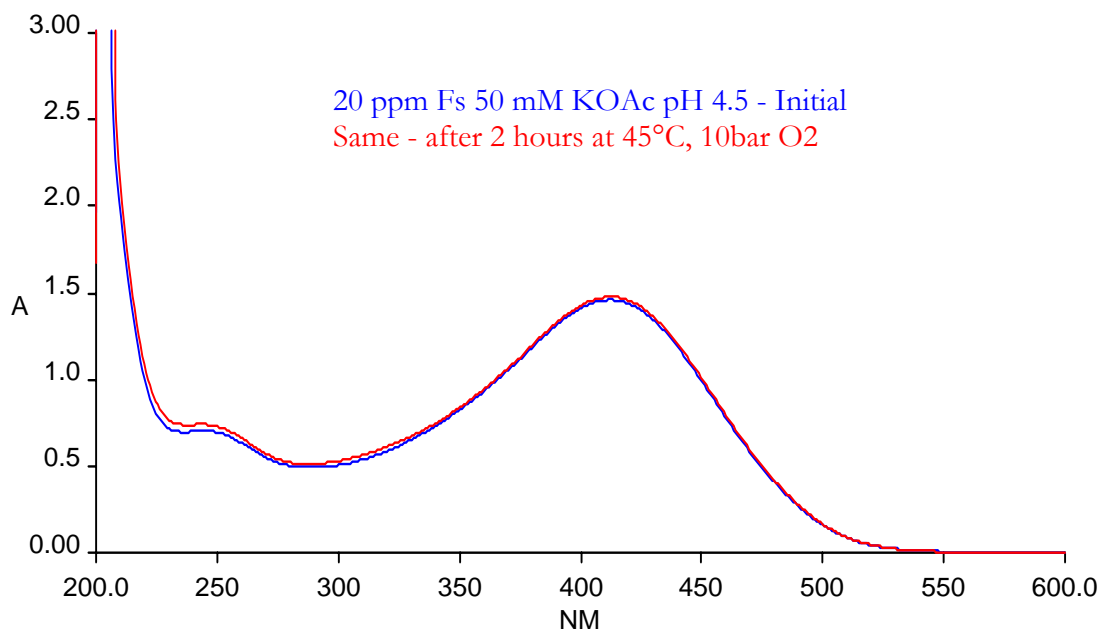
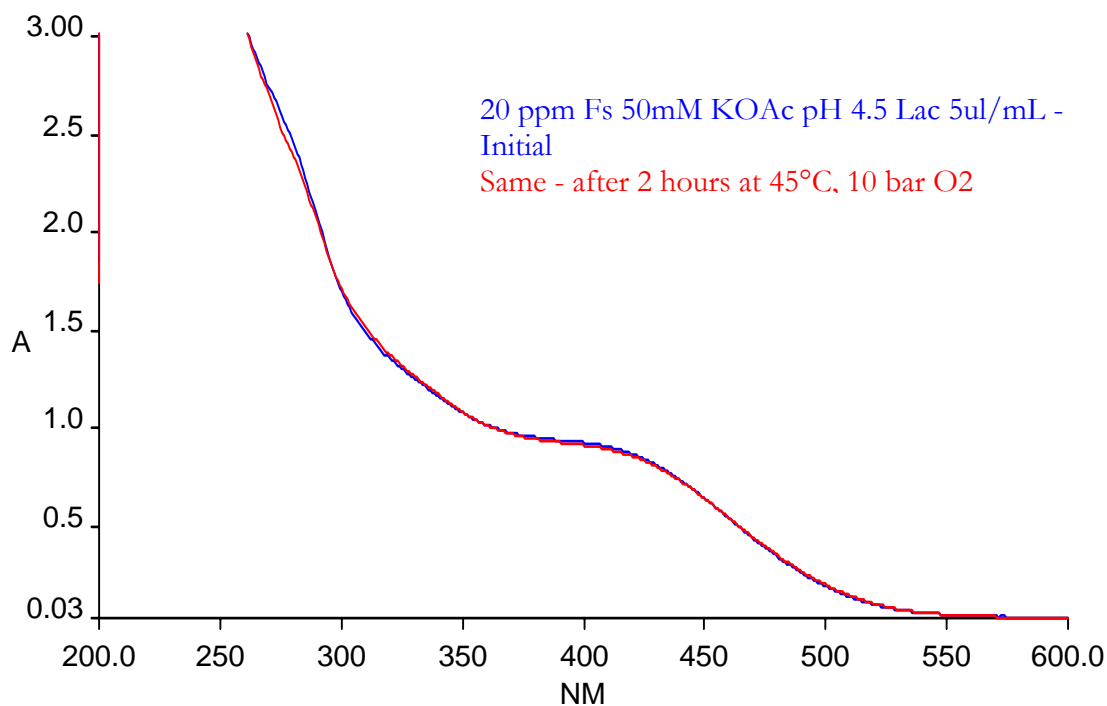
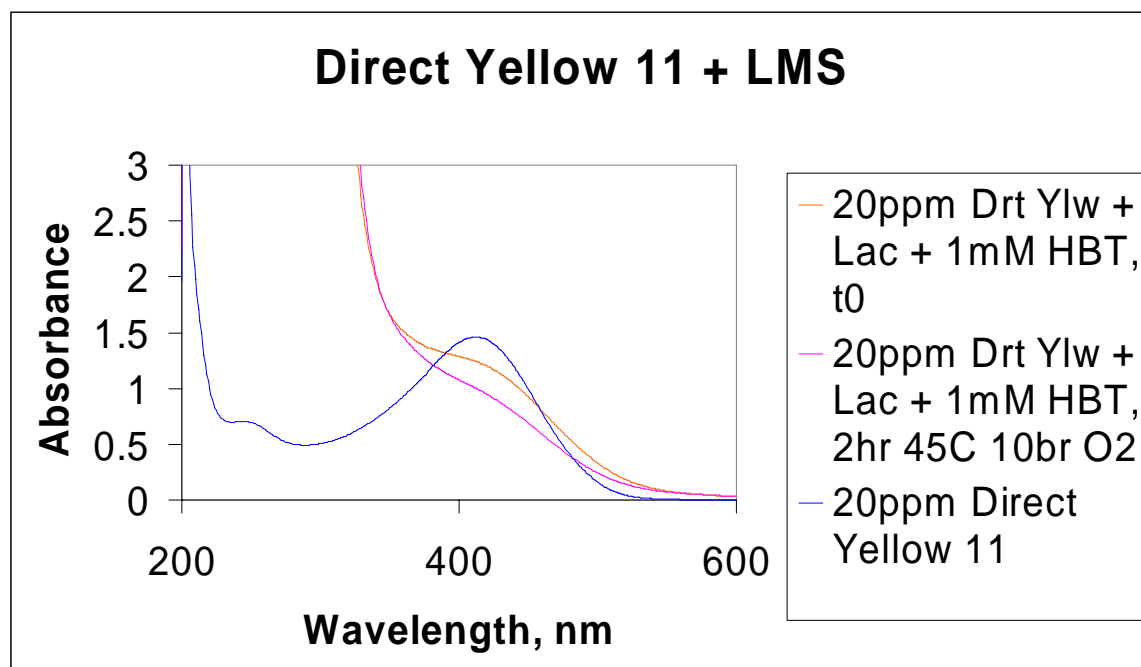


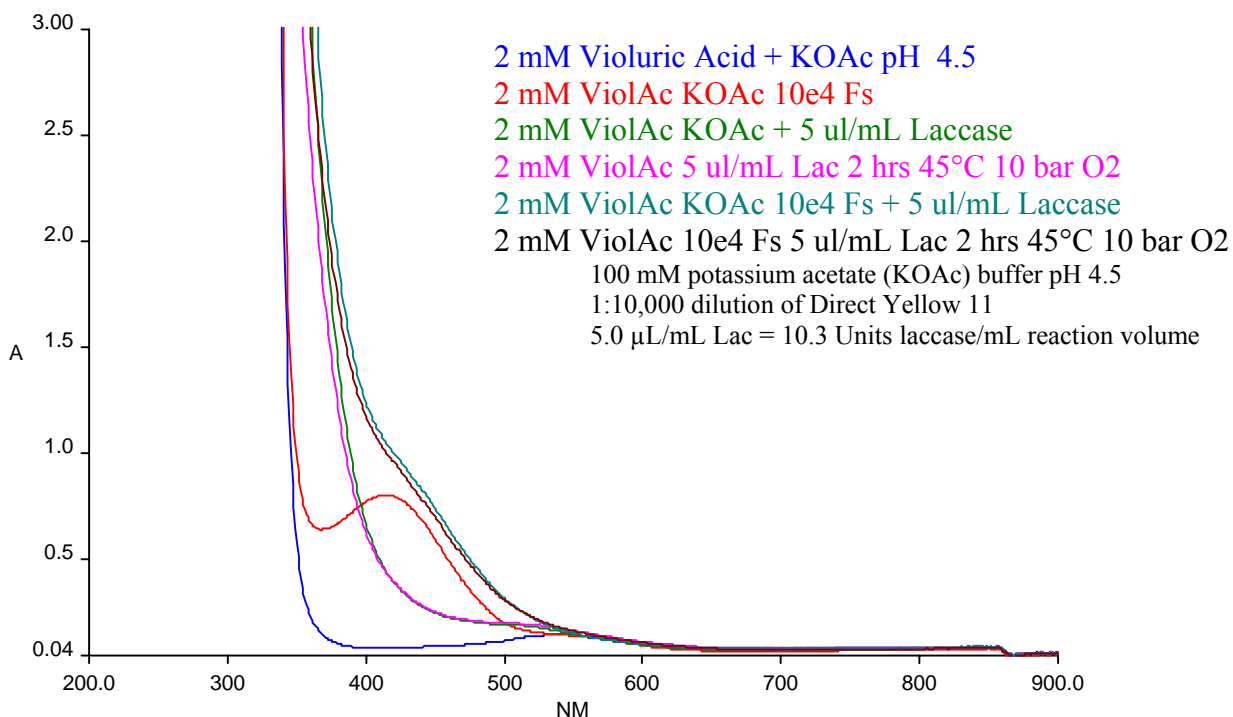
Figure A-24 Laccase Treatment of Direct Yellow 11 has no Effect



Treatment with laccase and mediator for two hours at 45°C 10 bar oxygen pressure could decrease dye absorbance but quantization would be difficult due to overlap between dye absorbance and enzyme/mediator absorbances. (Figure A-25)

Figure A-25 Direct Yellow Treated with HBT

When Direct Yellow 11 (1:10,000 dilution) is treated with laccase in combination with 2.0 mM violuric acid, no decrease in dye absorbance can be discerned in the presence of the large absorbances of enzyme and mediator.

Figure A-26 Direct Yellow 11 + Laccase + 2.0 mM Violuric Acid

At low ABTS concentration, a slight decrease in absorbance near dye maxima (394-415 nm) can be observed when reaction solution with Direct Yellow 11, laccase and ABTS after two hours at 45°C 10 bar oxygen (Figure A-27, green line) is compared with the same solution before reaction (Figure A-27, blue line). The decrease after treatment is more noticeable at higher ABTS concentration.

Figure A-27 Direct Yellow 11 + Laccase + 0.01 mM ABTS

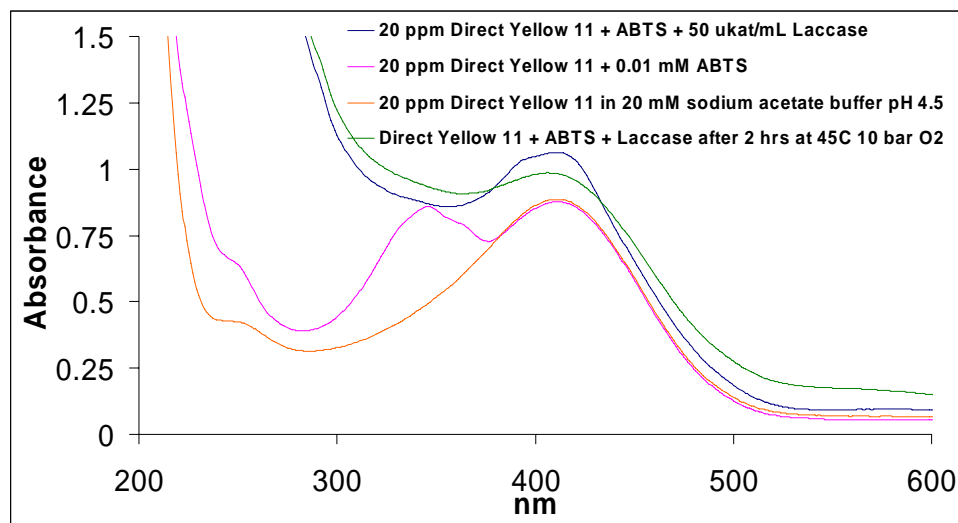
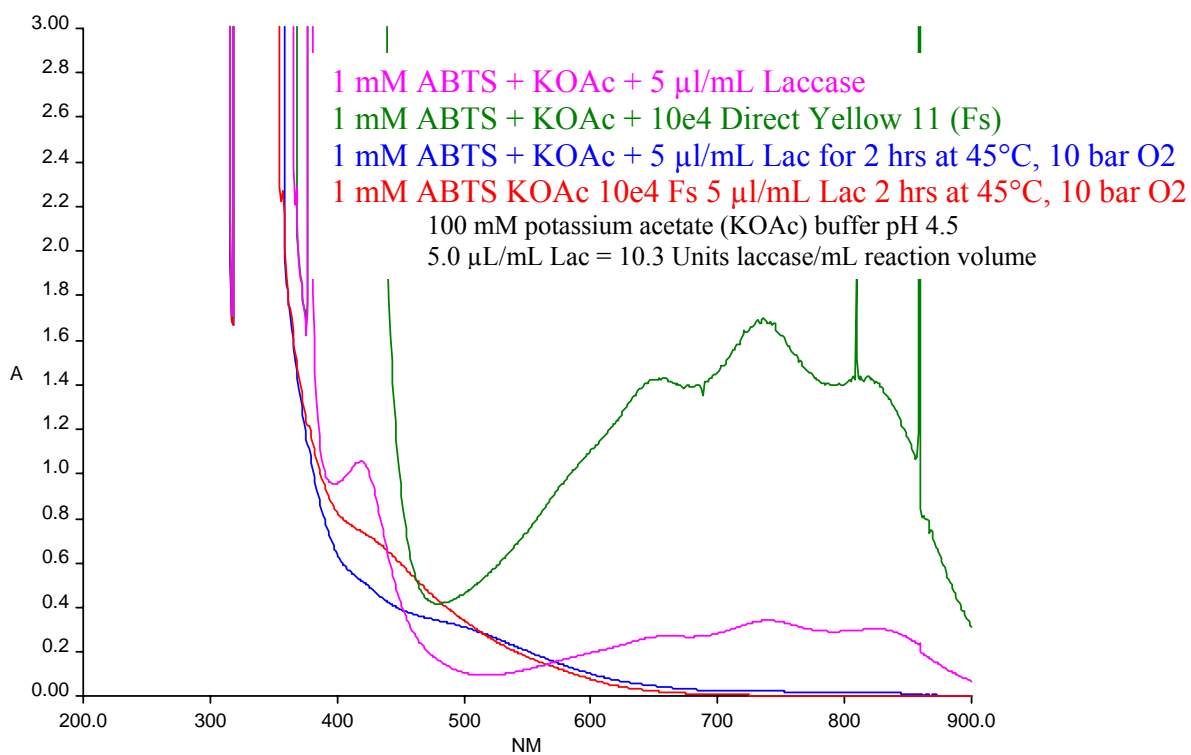


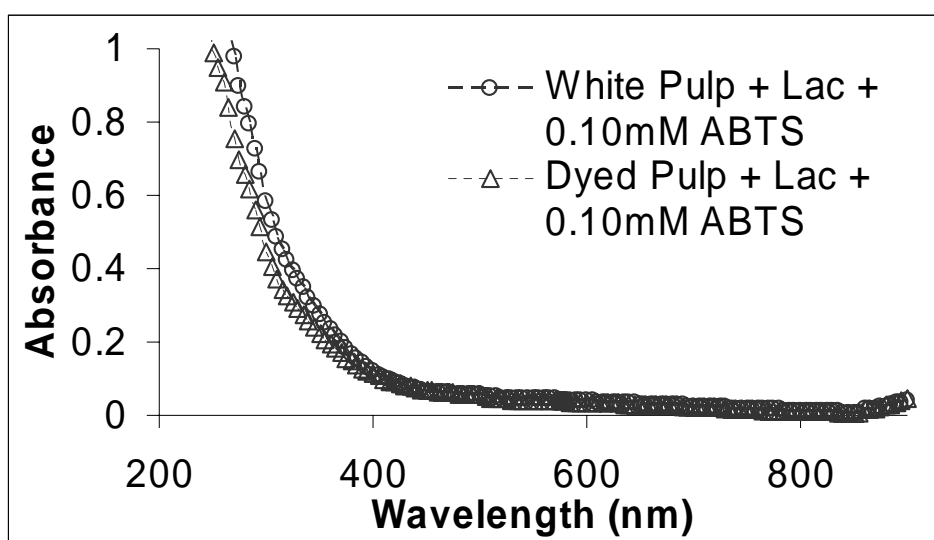
Figure A-28 Direct Yellow 11 + Laccase + 1.0 mM ABTS



A.1.6 CHARACTERIZATION OF PULP REACTION EFFLUENTS BY UV/VIS

Switching from dye in solution to dyed pulp allows washing of pulp and removal of laccase and mediator. One question is whether laccase treatment is stripping intact Direct Yellow 11 dye chromophores off the fiber. Consequently post-reaction effluents were obtained by filtration of dyed and white pulp samples treated with laccase and 0.10 mM ABTS. Spectrophotometric examination of these post reaction effluents showed little difference resulting from treatment of dyed pulp versus treatment of white pulp.

Figure A-29 Spectra from Effluents (Post-Reaction) for Laccase/ABTS Treated Pulps



A.1.7 CHARACTERIZATION BY NMR

A number of NMR spectra were obtained. Commercial dyes are mixtures that include fillers in addition to one – or more- chromophores. Attempts were made to purify Direct Yellow 11. Direct Yellow 11, as supplied, purified Direct Yellow 11 and ABTS/Laccase combinations were all subjected to NMR. The spectra are complex and difficult to interpret.

Figure A-30 NMR Spectrum of Direct Yellow 11 as Supplied

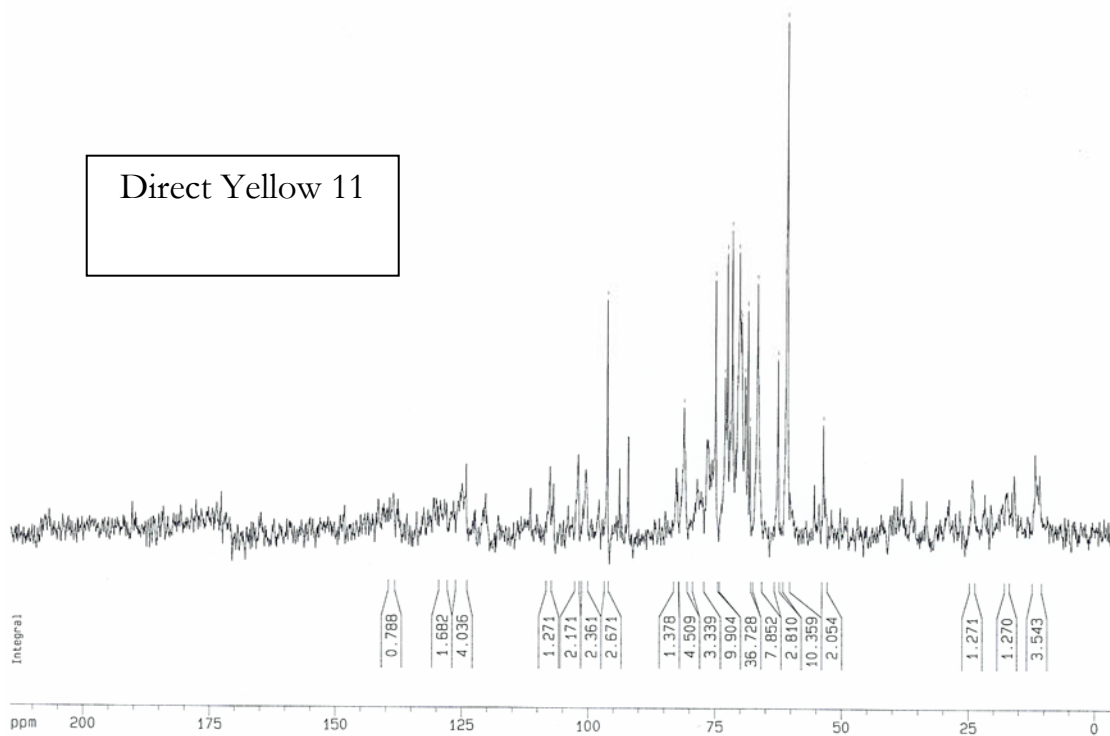


Figure A-31 NMR Spectrum of Direct Yellow 11 Purified by TLC

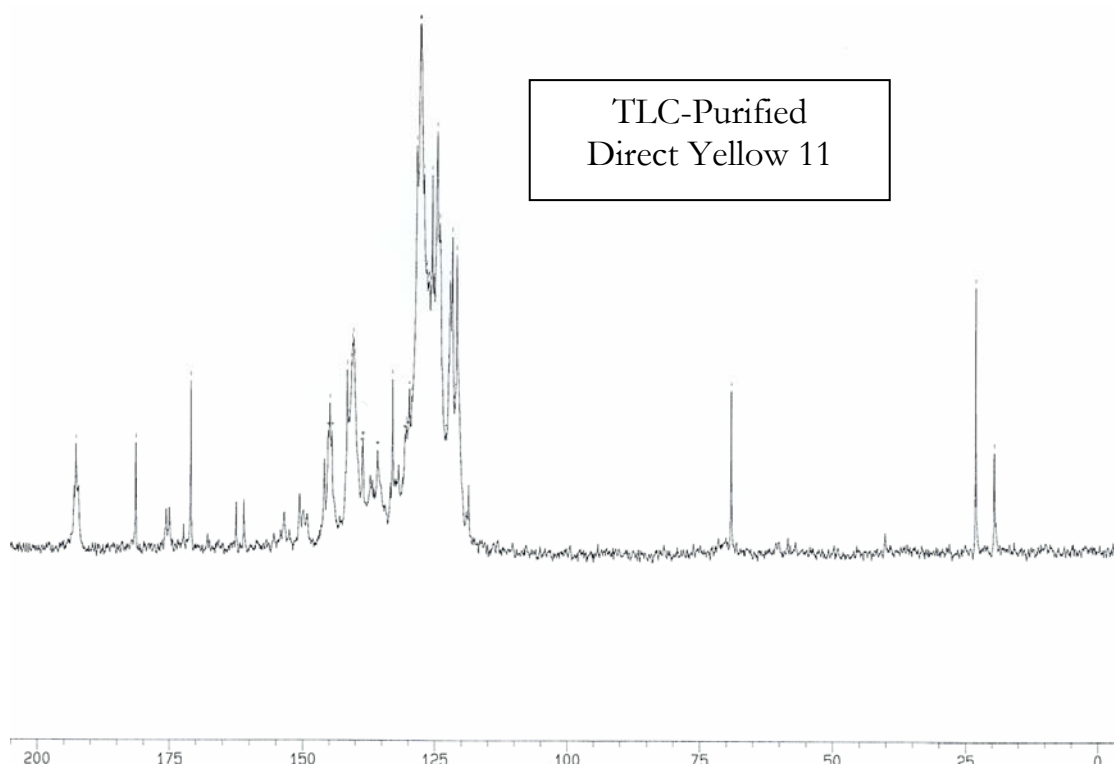


Figure A-32 NMR Spectrum of Direct Yellow 11 Purified by Paper Chromatography

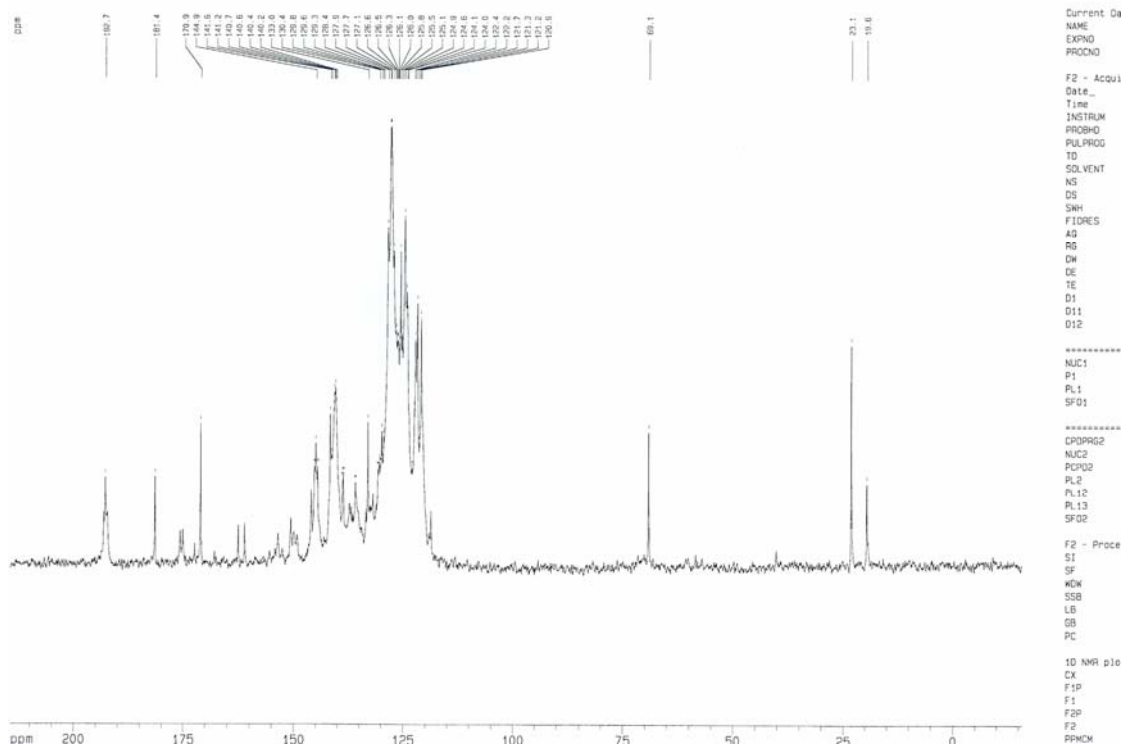


Figure A-33 NMR Spectrum of Direct Yellow 11 Purified by NH₂-SPE

8/19/03, pure dye in D2O

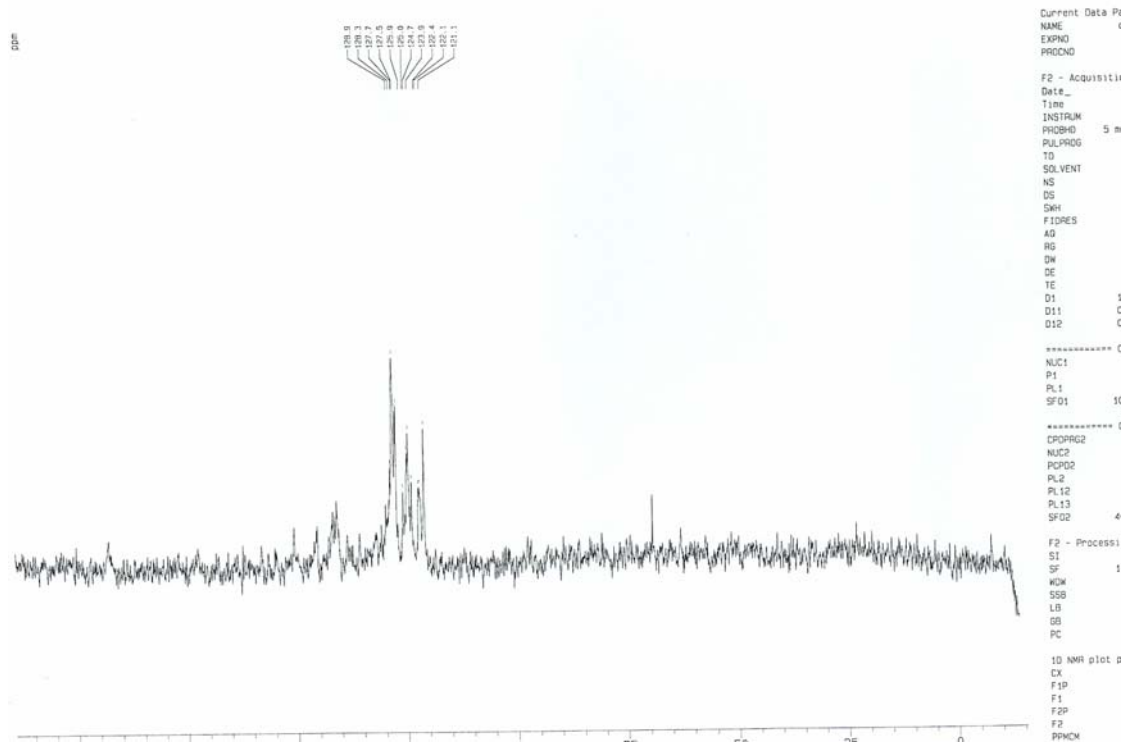


Figure A-34 NMR Spectrum of 10.3 $\mu\text{L}/\text{mL}$ Laccase + 5.0 mM ABTS

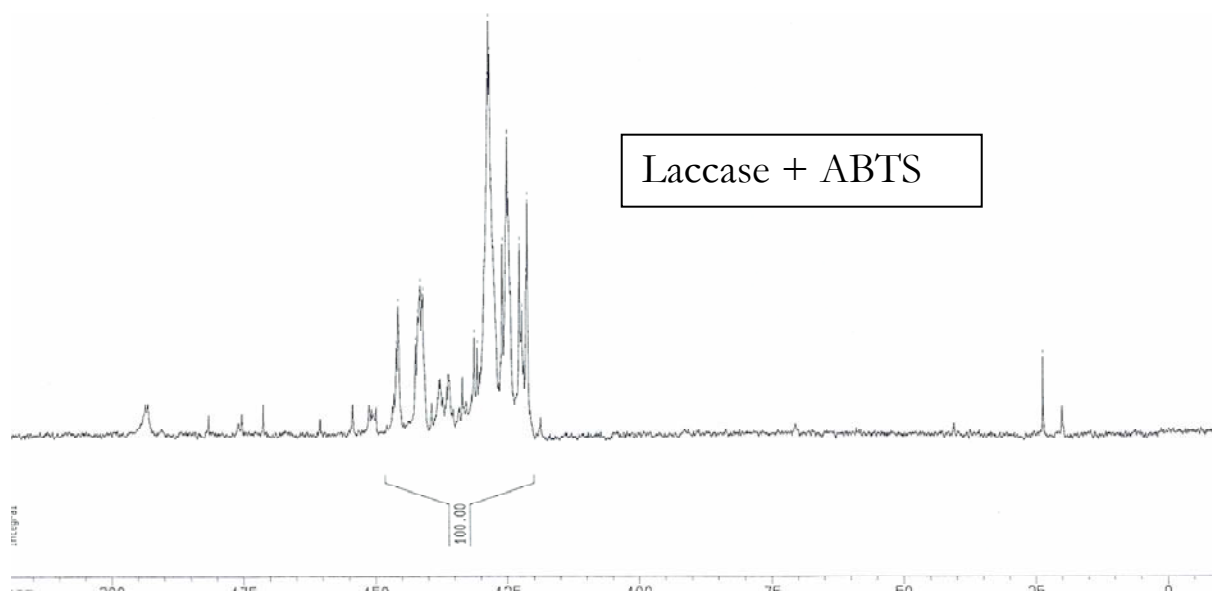


Figure A-35 NMR Spectrum of Direct Yellow 11 Treated with Laccase + ABTS After 2hr at 10 bar O₂, 45 °C

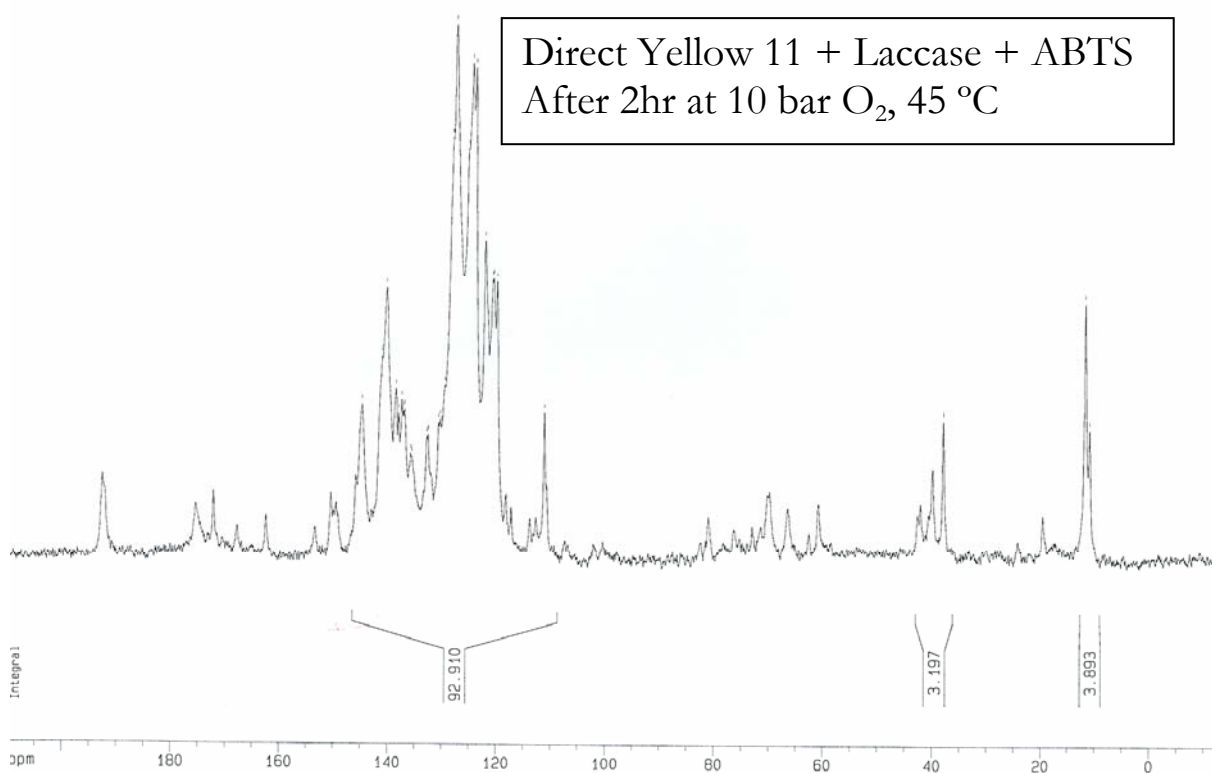


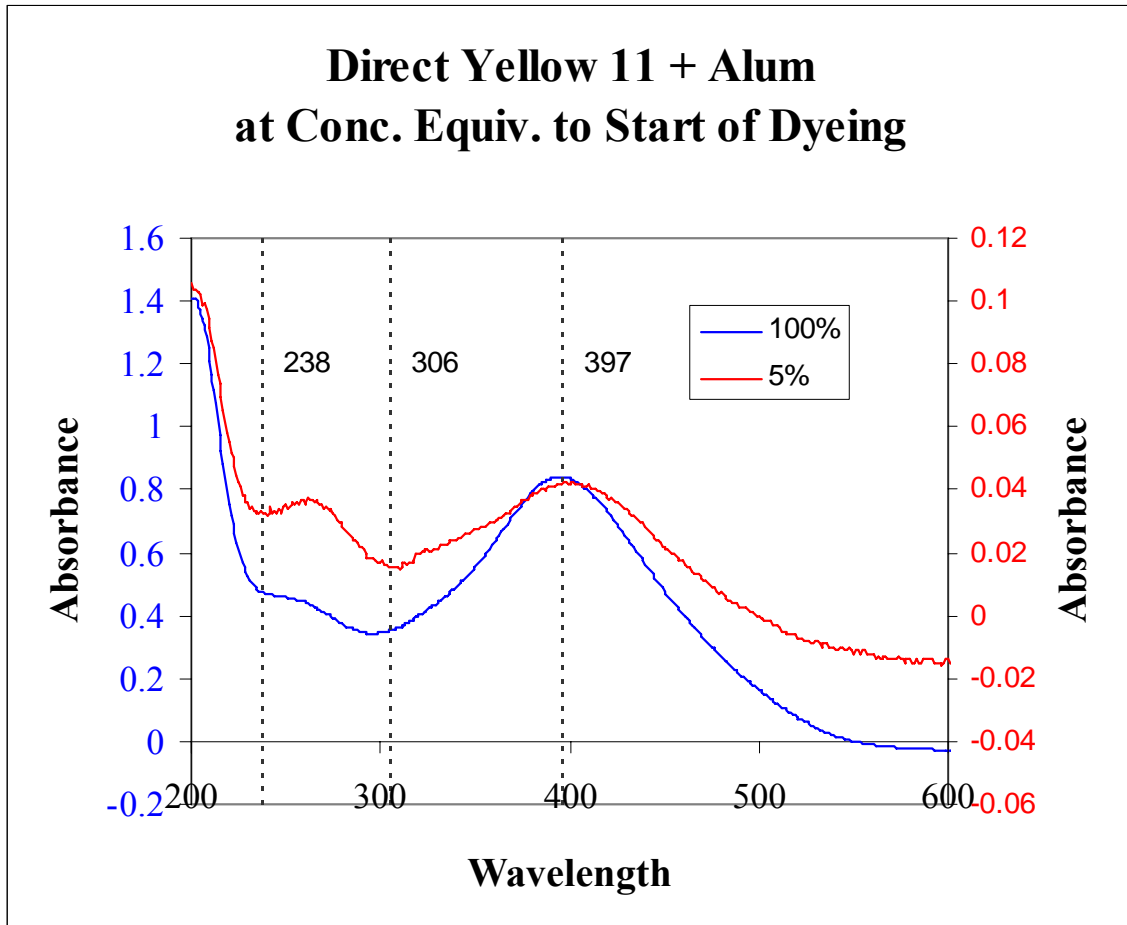
Table A-9 NMR Peaks

Sample	Peak ppm
Direct Yellow 11	96.24
	81.53
Lac + ABTS	146-121
	23.6
Dye Rxn w/ Lac + ABTS	144-111
	37.9
	11-10

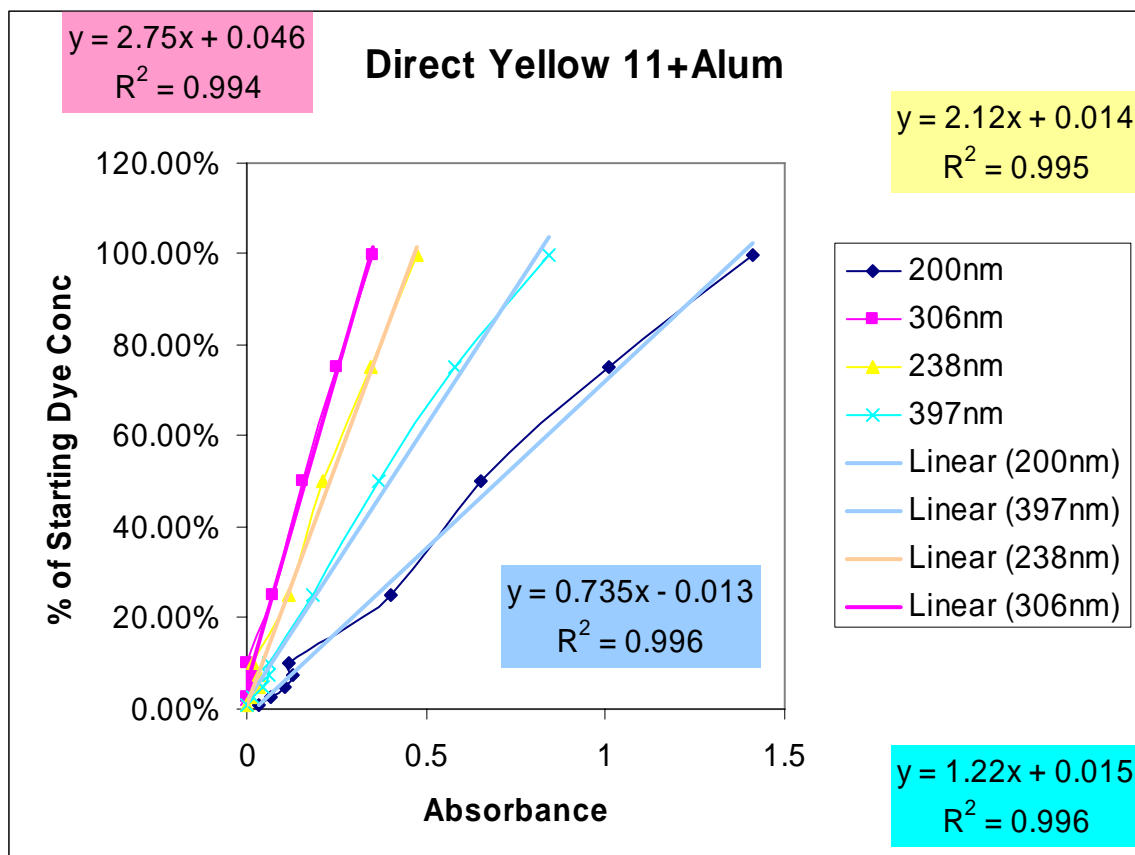
A.2 NECESSARY SUPPORTING PROCEDURES

A.2.1 DETERMINATION OF EXTENT OF PULP DYEING

As mentioned in the Experimental Procedures section of the Thesis, the amount of Direct Yellow 11 attached to dyed pulp was determined by spectrophotometric examination of the filtrate of the dyeing reaction. The filtrate spectrum was compared to spectra from serial dilutions of a mixture of Direct Yellow 11 and alum prepared at the concentrations present at the beginning of the dyeing reaction. Figure A-36 shows the spectrum of the “100%” solution of Direct Yellow 11 + alum. It also shows the spectrum of the “5%” diluted dye+alum mixture plotted on a separate axis. The spectra show that the λ_{Max} of Direct Yellow 11 remains around 400 nm but the secondary peak around 270 nm becomes more pronounced as the dye/alum solution is diluted.

Figure A-36 Spectra of Direct Yellow 11 + Alum

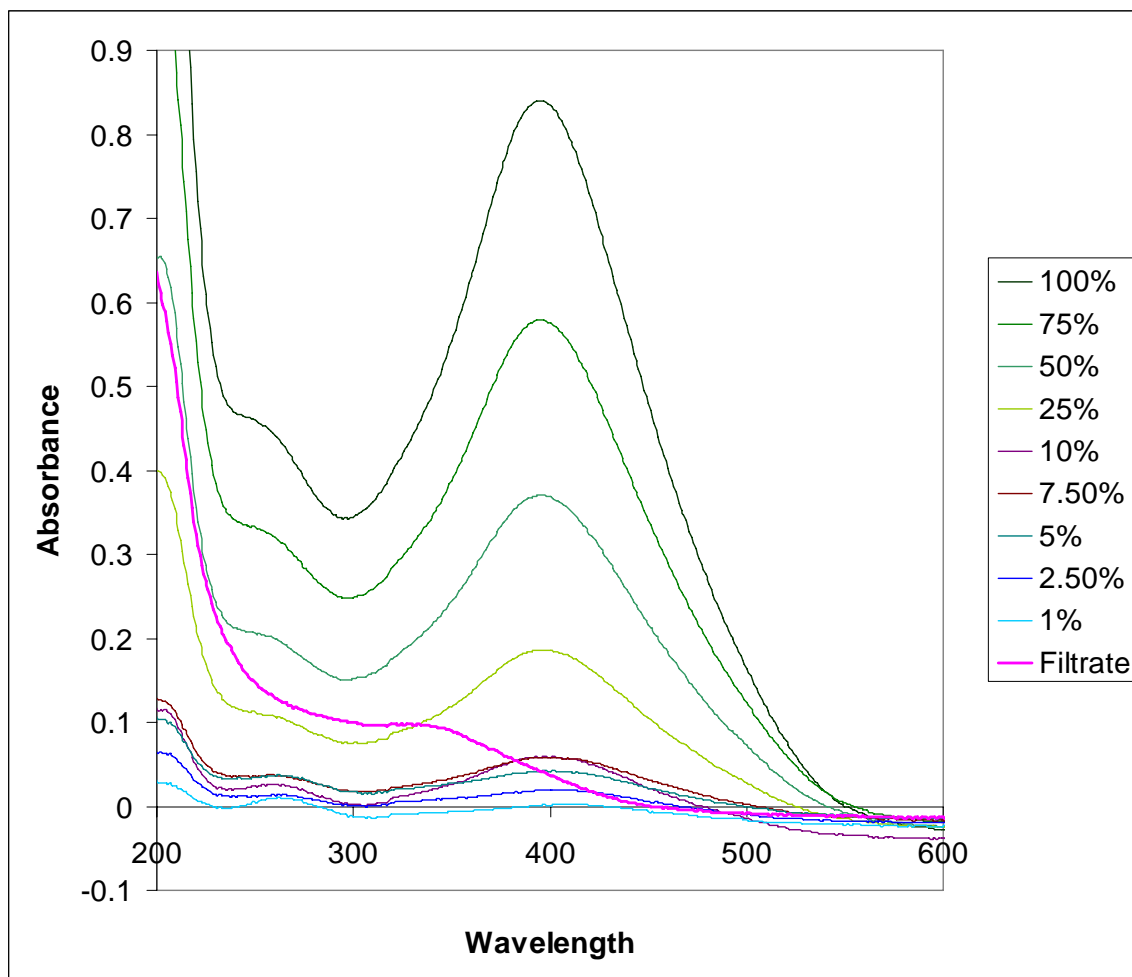
The serial dilutions measured include 100%; 75%; 50%; 25%; 10%; 7.5%; 5%; 2.5% and 1%. To see if a linear relationship existed between dye concentration and absorbance, the signal (baseline to spectral line) was measured for each dilution at the wavelengths of inflection (shown as dotted lines in Figure A-36: 397 nm; 306 nm; 238 nm; and 200 nm). These signal intensity values were then plotted and fitted with linear trend lines. The results are shown in Figure A-37. Although the rate of change in signal depends on the wavelength being measured, the measured signal decreases linearly as the dye + alum mixture is diluted.

Figure A-37 Linear Models of Signals of Different Dilutions of Dye + Alum

Comparison of the spectrum of the filtrate from the completed dyeing reaction with the spectra of the diluted Direct Yellow 11 + alum standards revealed that a change occurred during the dyeing reaction. As can be seen in Figure A-38, the spectrum of the dyeing reaction filtrate is *not* the same shape as the diluted Direct Yellow 11 + alum mixture. Since the uptake of Direct Yellow 11 and of alum are dependent of their affinity for cellulose fiber, it is likely that the uptake of dye and of alum would differ, resulting in a changed ratio of dye to alum in the filtrate from the completed dyeing reaction. In addition, alum (aluminum sulfate) demonstrates complex behavior in water such as changing the number of water molecules bound depending on solution pH². Examination of the spectrum at λ_{Max} around 400 nm does show that the filtrate signal at 400 nm is less than the signal from dye + alum diluted to 5%. The shape difference between filtrate spectrum and diluted dye + alum mixtures complicates analysis. However, it does appear that less than 10% of the applied amount of Direct Yellow 11 (0.25% or 3.0 g dye

concentrate/1200 g. o.d. pulp) remains in the filtrate. During the dyeing process, greater than 90% of the dye was attached to the pulp.

Figure A-38 Spectra of Diluted Dye + Alum and Dyeing Reaction Filtrate



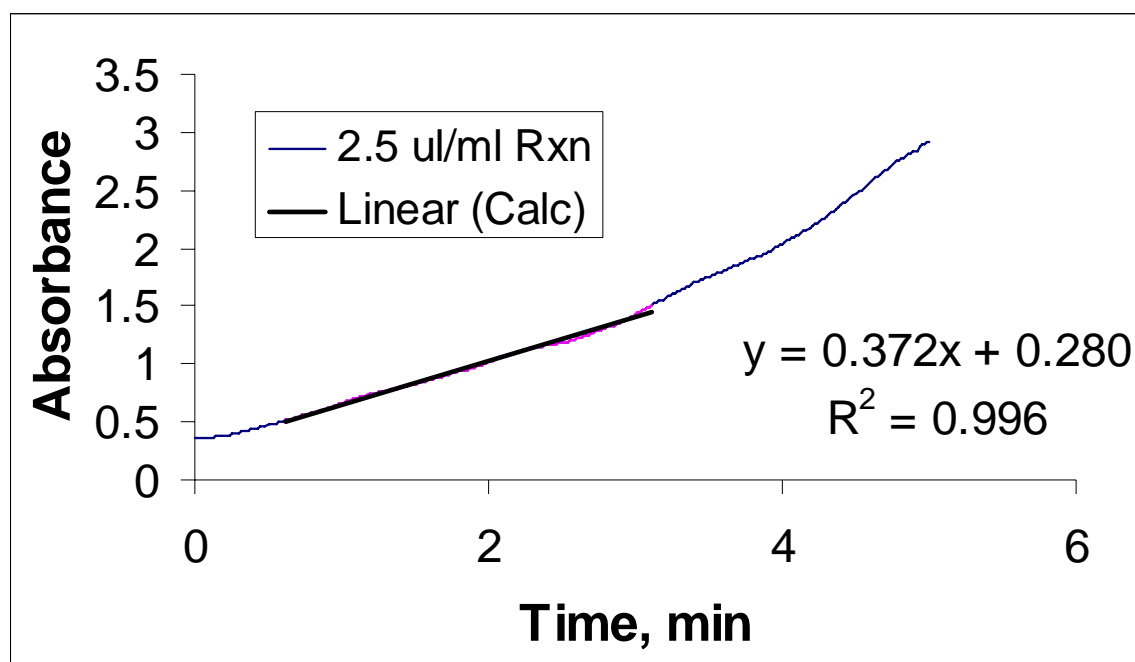
A.2.2 LACCASE ACTIVITY ASSAYS

Laccase activity was measured by monitoring the rate of oxidation of ABTS³ in a 100 mM pH 5.0 sodium acetate buffer. The course of the reaction was followed by using a Perkin-Elmer Lambda 900 UV/Vis/NIR Spectrophotometer set to continuously collect absorbance values at 420 nm.

One mL solutions with ABTS and buffer were prepared. The reaction was started by the addition of laccase, followed by immediate placement in spectrophotometer and start of data collection. The reaction of an enzyme with substrate is linear as long as substrate is in excess. As the reaction nears completion, it slows down and is no longer

linear. Enzyme activity values are calculated from a linear portion of the time course spectra. To obtain suitable spectra, the concentrations of ABTS and of laccase were adjusted until the completion of the reaction took at least one minute. Figure A-39 shows a laccase assay spectrum and the linear fit obtained from it.

Figure A-39 Laccase Activity Assay



For the product of the laccase-ABTS reaction, $\epsilon_{420} = 36,000$. The slope of the fitted line is 0.372. During the course of one minute, the accumulated absorbance is 0.186 which corresponds to 0.00517 mmol (5.17 μmol) of product ($0.186/36,000 \text{ M}^{-1}\text{cm}^{-1}$) created by 1.0 mL of reaction solution. The amount of laccase added to the solution was 2.5 μL . Laccase activity = $5.17 \mu\text{mol} / 2.5 \mu\text{L} = 2.07 \mu\text{mol product} / 1.0 \mu\text{L laccase} \cdot \text{minute}$. One International Unit (IU) of activity is defined as the conversion of 1 μmol of substrate/minute. Therefore the laccase activity is 2.07 U/ μL of laccase solution. For laccase-mediator treatments of dyed pulp, 10.3 U or 5.0 μL laccase is added per mL of reaction.

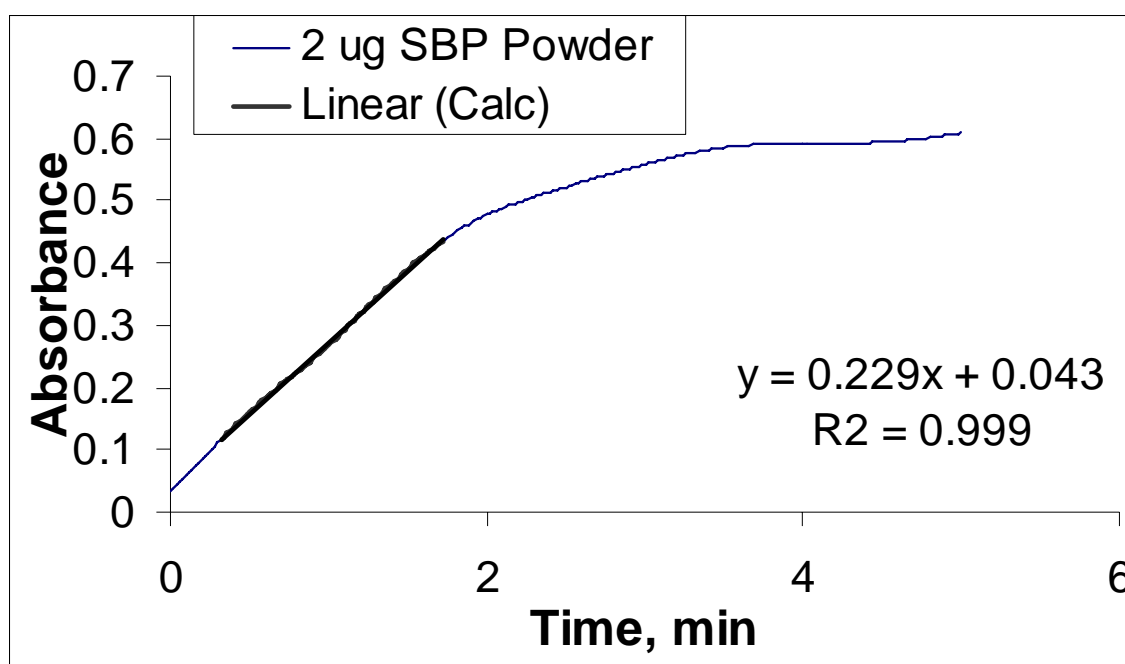
A.2.3 SOYBEAN PEROXIDASE ACTIVITY ASSAYS

Soybean peroxidase activity was measured by monitoring the production of a red quinoneimine dye at 510 nm. In the presence of hydrogen peroxide, phenol and 4-aminoantipyrine (4-AAP), SBP catalyzes the formation of phenol free radicals which

then react with 4-AAP to form the quinoneimine dye. ($\epsilon_{510} = 7,210 \text{ a.u./M/cm}$)⁴ The standard reaction conditions are 10 mM phenol, 2.4 mM 4-AAP and 0.2 mM H₂O₂ at 25°C, pH 7.0.

The reaction mixture was prepared and absorption at 510 nm was monitored. For this enzyme, the reaction is started by the addition of hydrogen peroxide. As with the laccase activity assay, determination of enzyme activity is done by fitting a trend line to the linear portion of the time course spectra.

Figure A-40 Soybean Peroxidase (SBP) Activity Assay



For the original SBP activity assay performed in the summer of 2001, the calculated activity was 7.95 Units/mg SBP powder. When additional soybean peroxidase experiments were run in the summer of 2004, the same frozen powder preparation of SBP was used, but the activity was reassayed. The same assay reaction conditions were used, but the amount of enzyme was varied. A solution of 1 mg SBP powder/mL was prepared. Activities for 1, 2, 5 and 10 µL aliquots were determined and linear trend lines were obtained for each concentration. The results from enzyme activity assays can vary with concentration of the reaction solution. When concentration-corrected enzyme activity values were calculated, the activity calculated from the 1 µL aliquot was low (4 U/mL), and the activity calculated from the 10 µL aliquot was high (8 U/mL). Consequently, the

similar activities (~6U/mL) obtained from the 2 and the 5 μ L aliquots were averaged. The activity value calculated in August 2004 was 6.3 Units/mL. This slight decrease in enzyme activity is reasonable for powdered enzyme stored at -4°C.

A.2.4 ASSAY OF HYDROGEN PEROXIDE “30%” STOCK SOLUTION

For accurate concentration, hydrogen peroxide stock must be titrated before application. The method was supplied by Interlox America. A mixture of 100 mL distilled water, 10-15 mL 4N sulfuric acid, 5.0 mL 1N potassium iodide and 3 drops of saturated ammonium molybdate is prepared and 2.0 mL of “30%” hydrogen peroxide stock is added. This mixture is then titrated with a 0.2 N sodium thiosulfate solution. Near the end, as the yellow color becomes pale, 10 mL of starch indicator solution is added. Titration endpoint is then readily visible as the dark blue color disappears.

An example calculation is outlined below.

(thio = sodium thiosulfate)

176.0 mL of 0.2 N thiosulfate was used in the first titration (173.0 in second)

Weight of 2 mL H₂O₂ sample was 2.226 g

$$0.2 \text{ N thio} \times 17\text{g/mole (equiv thio/H}_2\text{O}_2) \times 176.0 \text{ mL (from titration)} / 2 \text{ mL sample} = 299.2 \text{ g/l}$$

$$2.226 \text{ g} / 2 \text{ mL H}_2\text{O}_2 \text{ solution (density)} * 1000 \text{ mL} = 1113 \text{ g (for 1 L volume of H}_2\text{O}_2 \text{ sln)}$$

$$299.2 \text{ g/l} / 1113 \text{ g} = 26.88\% \text{ wgt/wgt H}_2\text{O}_2 \text{ (from first titration)}$$

For second titration, 173.0 mL thio yields 294.1 g/L result: 26.42%

Average of 26.88 & 26.42 (from first and second titrations) = 26.65% wgt/wgt

A.3 DATA FOR PUBLICATION TABLES AND BAR GRAPHS

A.3.1 COMPARISON BETWEEN LACCASE MEDIATORS

These experiments were performed by treating Direct Yellow 11 dyed pulp with laccase in the presence of 5.0 mM of violuric acid (VA), 1-hydroxybenzotriazole (HBT), or 2-2' azinobis (3-ethyl benzthiazoline-6-sulfonate) (ABTS). After the laccase-mediator treatment, the treated pulps were washed and made into handsheets. The CIELAB values were then measured. For dye removal index value (DRI) calculations, CIELAB values for

handsheets made from untreated Direct Yellow 11 dyed pulps were used for the reference starting point.

Error bars for these treatments were calculated. For each treated pulp, 5 to 7 handsheets were prepared and 5 CIELAB were obtained per handsheet. The resultant 25-35 CIELAB readings were used to calculate average CIELAB values for the treated pulp. Standard deviations were also calculated. DRI values are a single number derived from calculations based on L*, a* and b* for the reference pulp and the treated pulp. The calculated standard deviation for L*, a* and b* were added and subtracted from the average L*a*b* values of the treated pulp. DRI values were calculated for the average L*a*b* values, the “low” and the “high” L*a*b* values. The DRI values calculated from the “low” and “high” L*a*b* were used to produce the error bars for the DRI value calculated from the average L*a*b* values.

Table A-10 DRI Values for Pulps Treated with Different ABTS Concentrations

Mediator	Brightness	L*	a*	b*	DRI (avg)	DRI (hi)	DRI (low)	L* SD	a* SD	b* SD
Lac	56.3	94.1	-3.41	24.8	22.0	23.1	20.9	0.09	0.14	0.23
Lac + ABTS	54.6	87.4	-0.57	15.0	57.9	61.8	54.9	0.18	0.08	0.18
Lac + HBT	56.6	94.0	-3.76	24.4	23.9	26.1	21.6	0.21	0.08	0.46
Lac + VA	55.9	93.2	-3.04	23.7	27.6	28.4	26.7	0.12	0.06	0.20

A.3.2 BLEACHING RESPONSE TO ABTS CONCENTRATIONS

Pulp dyed with Direct Yellow 11 was treated with laccase in the presence of different amounts of ABTS. As mentioned above, standard deviations in L*, a* and b* values were used to calculate “low” and “high” DRI error bars.

Table A-11 DRI Values for Pulps Treated with Different ABTS Concentrations

mM ABTS	Brightness	L*	a*	b*	DRI (avg)	DRI (hi)	DRI (low)	L* SD	a* SD	b* SD
0	56.3	94.1	-3.41	24.8	22.0	23.1	20.9	0.09	0.14	0.23
0.005	58.5	93.3	-3.24	21.1	41.0	42.5	39.4	0.16	0.11	0.38
0.01	60.4	91.2	-1.85	15.8	61.1	61.3	60.8	0.14	0.10	0.16
0.05	57.5	86.3	0.97	10.1	65.8	65.3	66.2	0.28	0.13	0.19
0.50	53.5	82.6	2.37	7.7	56.8	55.8	57.8	0.37	0.13	0.27
5.0	54.8	83.5	2.31	7.9	59.9	59.3	60.6	0.23	0.11	0.12

A.3.3 COMPARISON BETWEEN ENZYMATIC TREATMENTS

Both dyes, Direct Yellow 11 and Basazol 46L, were treated with soybean peroxidase, horseradish peroxidase and laccase with 1.0 mM ABTS. The results from soybean peroxidase treatment of Direct Yellow 11 varied with dye concentration. When the absorption area was low, as for 10 ppm (mg/L) Direct Yellow 11, the calculated reduction was lower (39%) than when the area was higher (49%) due to higher (20 ppm) dye concentration.

Table A-12 Comparisons between Enzymatic Treatments

	Basazol 46L			Direct Yellow 11			Dye Conc.
	Area Before	Area After	% Reduction	Area Before	Area After	% Reduction	
Soybean Peroxidase (pH 3.8)	25.1	1.55	93.8%	5.47	3.31	39.4%	10 ppm
Horseradish Peroxidase (pH 3.8)	36.8	10.7	70.8%	15.5	12.2	21.4%	20 ppm
Laccase + 1.0 mM ABTS (pH 4.5)	41.8	27.8	33.6%	29.0	15.7	45.8%	20 ppm
Soybean Peroxidase (pH 3.8)	62.0	7.95	87.2%	15.5	7.99	48.5%	20 ppm

A.3.4 EFFECT OF pH ON SOYBEAN PEROXIDASE DECOLORIZATION

The data for the published bar graphs are listed in Table A-13 and Figure A-14. In these tables, the molecular weight (10,000 or 35,000) of the poly ethylene glycol (PEG) included in the reaction is listed in the PEG column. The published bar graphs for SBP treatment of Direct Yellow 11 did not include pH 6.5, but that data is included here. Soybean peroxidase is inhibited by the presence of phosphate, which was present since sodium phosphate buffer was used for the pH 6.5 reactions. The inhibition was significant for the decolorization of Direct Yellow 11, but had only minor effect on SBP decolorization of Basazol 46L. The decolorization reaction was attempted using potassium citrate buffer for pH 6.5. However, the reaction mixture also includes calcium chloride and the calcium citrate precipitated.

Table A-13 Effect of pH on SBP Decolorization of Basazol 46L

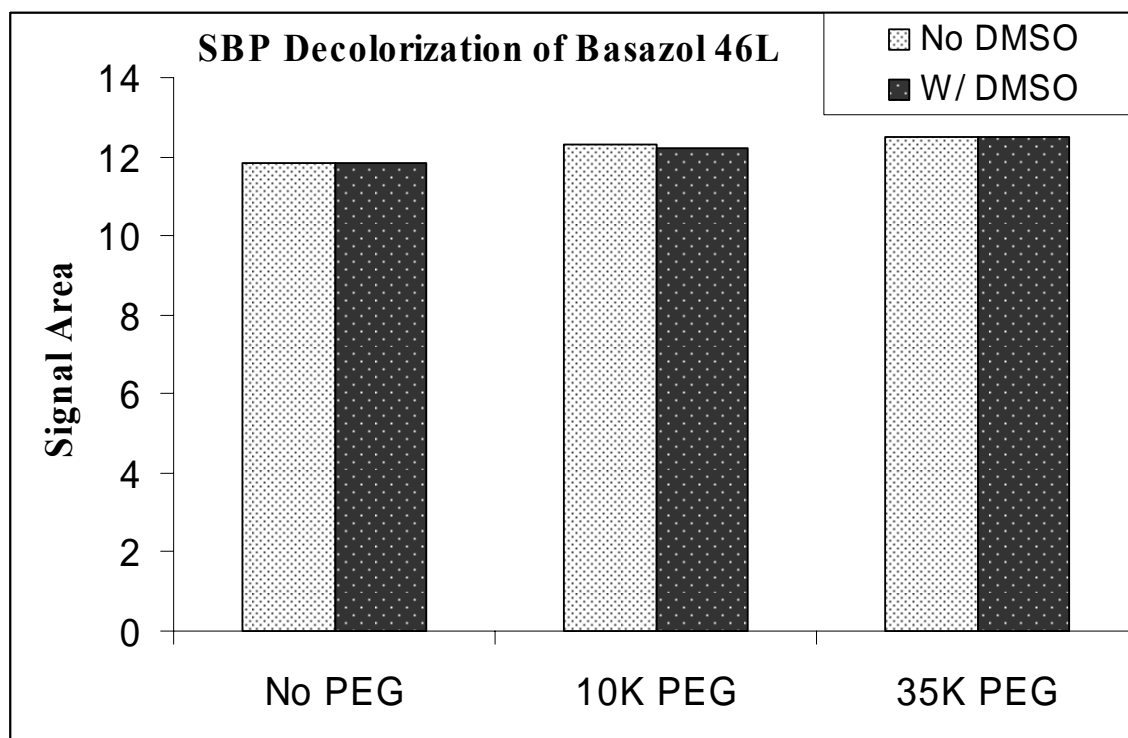
		Peak Area			
Dye	PEG	pH	Before	After	% Reduction
Basazol 46L	None	2.4	55.6	17.1	69.2%
		3.8	48.6	7.3	85.1%
		4.5	41.4	4.3	89.7%
		6.5	44.8	7.9	82.4%
		8.5	53.6	5.9	89.0%
		10.5	36.0	20.7	42.4%
Basazol 46L	10K	2.4	45.8	18.2	60.2%
		3.8	49.2	7.3	85.3%
		4.5	49.0	6.2	87.4%
		6.5	48.4	6.3	87.0%
		8.5	63.2	6.5	89.7%
		10.5	45.4	19.6	56.9%
Basazol 46L	35K	2.4	56.0	18.3	67.3%
		3.8	50.4	6.6	86.9%
		4.5	51.9	4.9	90.6%
		6.5	50.6	8.1	83.9%
		8.5	61.4	6.5	89.4%
		10.5	34.0	20.4	39.9%

Table A-14 Effect of pH on SBP Decolorization of Direct Yellow 11

Dye	PEG	pH	Peak Area		% Reduction
			Before	After	
Direct Yellow 11	None	2.4	43.1	20.8	51.8%
		3.8	28.7	12.2	57.5%
		4.5	24.1	12.5	48.1%
		6.5	25.4	22.9	9.6%
		8.5	34.2	17.3	49.5%
		10.5	23.5	24.7	-5.3%
Direct Yellow 11	10K	2.4	41.5	20.4	50.8%
		3.8	28.6	19.2	32.9%
		4.5	28.2	17.9	36.5%
		6.5	26.0	19.9	23.7%
		8.5	32.5	18.1	44.3%
		10.5	24.7	24.7	0.2%
Direct Yellow 11	35K	2.4	41.8	20.3	51.3%
		3.8	28.9	19.4	32.9%
		4.5	29.4	16.9	42.5%
		6.5	25.3	21.7	14.2%
		8.5	32.3	17.7	45.1%
		10.5	23.9	24.0	-0.5%

A.3.5 EFFECT OF DMSO ON SBP DECOLORIZATION OF BASAZOL 46L

Peroxidase enzymes can create hydroxyl radicals. To examine whether free radicals were involved in the decolorization of Basazol 46L by soybean peroxidase, a set of experiments were performed at pH 6.5, with and without 5.0 mM dimethyl sulfoxide (DMSO). DMSO is a free radical quencher⁵. If the soybean peroxidase enzyme releases into the solution free radicals that are necessary for the decolorization reaction, the inclusion of DMSO would significantly inhibit the decolorization reaction. However, inclusion of 5.0 mM DMSO had little effect on the dye decolorization.

Figure A-41 Effect of DMSO on SBP Decolorization of Basazol 46L*Table A-15 Signal Intensity for pH 6.5*

	Before	After	% Reduction
No PEG	40.2	11.8	70.5%
10K PEG	40.8	12.3	69.8%
35K PEG	39.3	12.5	68.3%
No PEG w/ DMSO	40.5	11.8	70.8%
10K PEG w/ DMSO	39.8	12.2	69.3%
35K PEG w/ DMSO	41.0	12.5	69.5%

A.3.6 REACTION RATE DATA

In the dissertation, it is noted that when Basazol 46L is decolorized with soybean peroxidase, 74% of the color is removed within 5 minutes. To clarify, the numerical data for the reaction rate graphs are given below.

Table A-16 Reaction Rate Study of SBP Decolorization of Basazol 46L

Time, min	Area	Time, min	Time, Hour	Area	% Reduced	Time, Hour	Area	% Reduced
0	56.1	5	0.08	15.02	73.6%	2	5.99	89.5%
0	56.8	5	0.08	14.84	73.9%	2	7.76	86.3%
0	56.9	5	0.08	14.77	74.0%	2	9.52	83.2%
0	57.0	10	0.17	9.79	82.8%	4	10.24	82.0%
0	57.0	20	0.33	6.65	88.3%	6	6.93	87.8%
0	56.8	30	0.5	9.77	82.8%	8	8.11	85.7%
0	56.9	30	0.5	9.38	83.5%	10	5.55	90.2%
0	56.8	30	0.5	9.54	83.2%	12	8.99	84.2%
0	57.0	60	1	7.34	87.1%	24	7.05	87.6%
0	56.9	90	1.5	6.35	88.8%			
Avg, 0	56.8							

Table A-17 Reaction Rate Study of SBP Decolorization of Direct Yellow 11

Time, min	Area	Time, min	Time, Hour	Area	% Reduced	Time, Hour	Area	% Reduced
0	15.8	5	0.08	12.53	19.3%	2	7.78	49.9%
0	15.5	5	0.08	12.47	19.7%	2	8.22	47.0%
0	15.9	5	0.08	12.42	20.0%	2	7.96	48.7%
0	15.6	10	0.17	12.25	21.1%	4	7.08	54.4%
0	15.8	20	0.33	11.13	28.3%	6	6.37	59.0%
0	15.6	30	0.5	10.31	33.6%	8	8.19	47.3%
0	15.2	30	0.5	10.46	32.6%	10	6.93	55.3%
0	15.2	30	0.5	10.14	34.7%	12	6.44	58.5%
0	15.1	60	1	9.22	40.6%	24	5.18	66.6%
Avg, 0	15.5	90	1.5	8.12	47.7%			

Table A-18 Reaction Rate Study of Direct Yellow 11 with Laccase+1.0 mM ABTS

Time, Minutes	Time, Hour	Area	Time, Hour	Area
0	0	29.0	2	15.7
5	0.08	33.0	3	13.7
10	0.17	31.9	4	12.2
15	0.25	32.9	6	11.4
20	0.33	29.2	8	8.6
30	0.5	26.0	10	8.0
45	0.75	19.2	12	7.3
60	1	19.0	24	5.4
75	1.25	14.9	48	4.7
90	1.5	16.2		

A.3.7 COMPARISON AMONG BLEACHING TREATMENTS

Sample	Treatment	Averages:		Change in			R ²	dR2 (Ref)	DRI (Ref)
		Brightness	Brightness	L*	a*	b*			
2Blue Base (Peroxy)	Base 2	76.83494		86.12714	-6.8727	-6.38169	280.4161		
Blue Base	Base 1	77.0955		86.675	-6.63713	-5.60575	253.0315		
Blue	1 Lac/ABTS	76.49214	76.49214	88.35179	-4.58857	-2.35107	162.2634	-90.7681	35.87224
	2 Lac + Alk Ext	80.84208	80.84208	92.2825	-2.79938	1.023958	68.4448	-184.587	72.95009
	3 Lac + DiThio	79.50417	79.50417	90.57417	-5.04271	-0.70521	114.7726	-138.259	54.641
	4 O2	82.64479	82.64479	92.92354	-3.65979	0.777083	64.0742	-188.957	74.67738
	5 O2 + DiThio	86.02045	86.02045	95.46682	-1.30386	2.644545	29.24342	-223.788	88.44277
	6 Peroxide	86.59268	86.59268	94.75036	-2.60607	0.916071	35.18955	-245.227	96.91544
	7 P + DiThio	86.70021	86.70021	95.85042	-1.27771	2.764583	26.4945	-253.922	100.3518
Cherry Pink	Base	64.43236		83.74778	18.34639	-0.60806	601.0944		
Cherry Pink	1 Lac/ABTS	67.92732	67.92732	86.40125	11.55214	1.257321	319.9589	-281.136	46.77062
	2 Lac + Alk Ext	74.93313	74.93313	89.47958	8.330625	0.70625	180.5773	-420.517	69.95859
	3 Lac + DiThio	77.78688	77.78688	92.51396	2.089375	3.776042	74.6648	-526.43	87.57852
	4 O2	76.25964	76.25964	90.94357	7.383929	2.105536	140.9746	-460.12	76.54702
	5 O2 + DiThio	84.65042	84.65042	95.31354	0.2075	3.400208	33.56736	-567.527	94.41563
	6 Peroxide	78.54909	78.54909	90.412	9.728182	-0.61345	186.9436	-414.151	68.89946
	7 P + DiThio	86.22839	86.22839	95.65839	0.290179	2.761786	26.56122	-574.533	95.58119
Gold Base	Base	23.54075		86.72463	1.81775	53.42525	3033.797		
Goldenrod	1 Lac/ABTS	23.66688	23.66688	86.42354	-2.07396	52.58563	2953.869	-79.9277	2.634575
	2 Lac + Alk Ext	18.50854	18.50854	87.97333	-2.71875	64.63417	4329.608	1295.811	-42.7125
	3 Lac + DiThio	25.99833	25.99833	89.80875	-5.47938	54.57146	3111.929	78.13206	-2.57539
	4 O2	19.75175	19.75175	88.5185	-1.9005	63.16525	4125.286	1091.488	-35.9776
	5 O2 + DiThio	36.48255	36.48255	93.03255	-8.67809	44.84021	2134.499	-899.298	29.64265
	6 Peroxide	21.07563	21.07563	88.71688	-1.15333	61.03583	3854.012	820.2149	-27.0359
	7 P + DiThio	42.3675	42.3675	93.78917	-8.38729	39.01604	1631.173	-1402.62	46.2333
Direct Yellow	Base	51.56963		93.585	-2.88	28.8275	880.4714		
Direct Yellow	1 Lac/ABTS	56.2055	56.2055	91.138	-1.82125	20.228	491.024	-389.447	44.23169
	2 Lac + Alk Ext	55.241	55.241	91.8055	2.439	21.73125	545.3458	-335.126	38.06207
	3 Lac + DiThio	82.84104	82.84104	95.57167	1.081042	4.96375	45.4176	-835.054	94.84167
	4 O2	72.65688	72.65688	95.87146	-2.26833	13.35583	200.5685	-679.903	77.22033
	5 O2 + DiThio	88.47667	88.47667	96.81625	-0.22896	3.158125	20.16244	-860.309	97.71004
	6 Peroxide B	71.46438	71.46438	95.90344	-2.52969	14.355	229.2472	-651.224	73.96313
	7 P + DiThio	89.00982	89.00982	96.80589	-0.21429	2.72625	17.68068	-862.791	97.99191

A.4 ALTERNATIVES/VARIATIONS OF LACCASE-MEDIATOR SYSTEM

A.4.1 GRAFTING

For the first attempt to dye bleached hardwood kraft pulp with Direct Yellow 11, the pulp was simply mixed with the dye. After filtration, a yellow solution and nearly white pulp was obtained. A dye fixative is necessary to attach Direct Yellow 11 to pulp. Dyed pulp was later prepared using alum (aluminum sulfate 18-hydrate) as fixative. The need for a fixative led to a set of experiments to determine if laccase could graft Direct Yellow 11 onto pulp. Direct Yellow 11 (75 mg) was mixed with (30 g) of white bleached hardwood kraft pulp then subjected to one of three treatments: 1) [“laccase”] treatment with 10.3 units of laccase in 100 mM sodium acetate buffer pH 4.5, at 45°C for 2 hours under 10 bar oxygen pressure 2) [“oxygen”] treatment with 10 bar oxygen pressure in 100 mM sodium acetate buffer pH 4.5, at 45°C for 2 hours and 3) [“alum”] treatment by adding (0.60 g) alum to Direct Yellow 11/pulp mixture and stirring for one hour at room temperature. The results are shown below in Table A-19. “Wh Pulp” refers to the starting white pulp. D^2 is the calculated distance for the measured Lab* values from the ideal bleach point (where $a=b=0$, $L=100$). The difference in D^2 between the treated and starting white pulps is shown as ΔD^2 . These results are shown graphically in Figure A-42 and Figure A-43. Figure A-42 shows the TAPPI brightness values for handsheets made from the white and each treated pulp. The calculated distance from the ideal bleach point, D^2 , measures the amount of color present. As can be seen in Figure A-43, laccase treatment under oxygen pressure was less effective in attaching Direct Yellow 11 to the pulp than was oxygen treatment alone. The addition of alum greatly increased the attachment of Direct Yellow 11 to the bleached pulp. Conclusion: laccase is not effective in grafting Direct Yellow 11 to pulp. Laccase, which is known to react with phenols, may not directly interact with the dye Direct Yellow 11 since it lacks phenolic groups.

Anecdotally, laccase does appear to be able to graft ABTS to pulp. In later experiments, a set of sheets were prepared for TOF-SIMS experiments. For a control, white pulp was treated with laccase plus 1.0 mM ABTS. Handsheets made from the treated white pulp were faintly blue/purple. Since ABTS does bind to laccase and forms

the dication, in the absence of reactive compounds to complete the redox cycle, some of the ABTS (a blue compound) may attach to the pulp and impact some blue color.

Table A-19 Results of Grafting Direct Yellow 11 onto Pulp

Treatment	Brightness	L*	a*	b*	D ²	ΔD ²
Oxygen	66.11	95.71	-3.37	18.37	367.0	344.1
Laccase	68.88	95.79	-2.83	16.13	285.9	262.9
Alum	53.08	94.30	-3.39	28.4	850.6	827.6
Wh Pulp	87.39	96.85	-0.43	3.59	23.0	0

Figure A-42 Brightness after Grafting Treatments

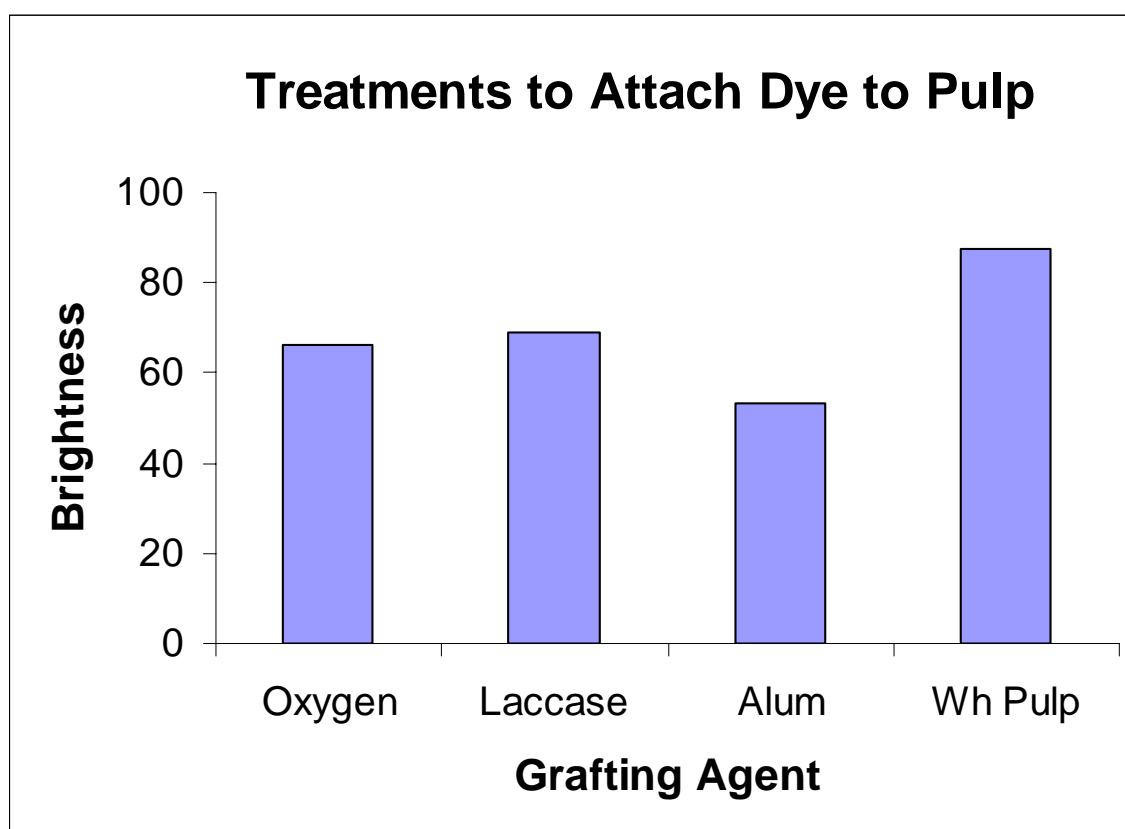
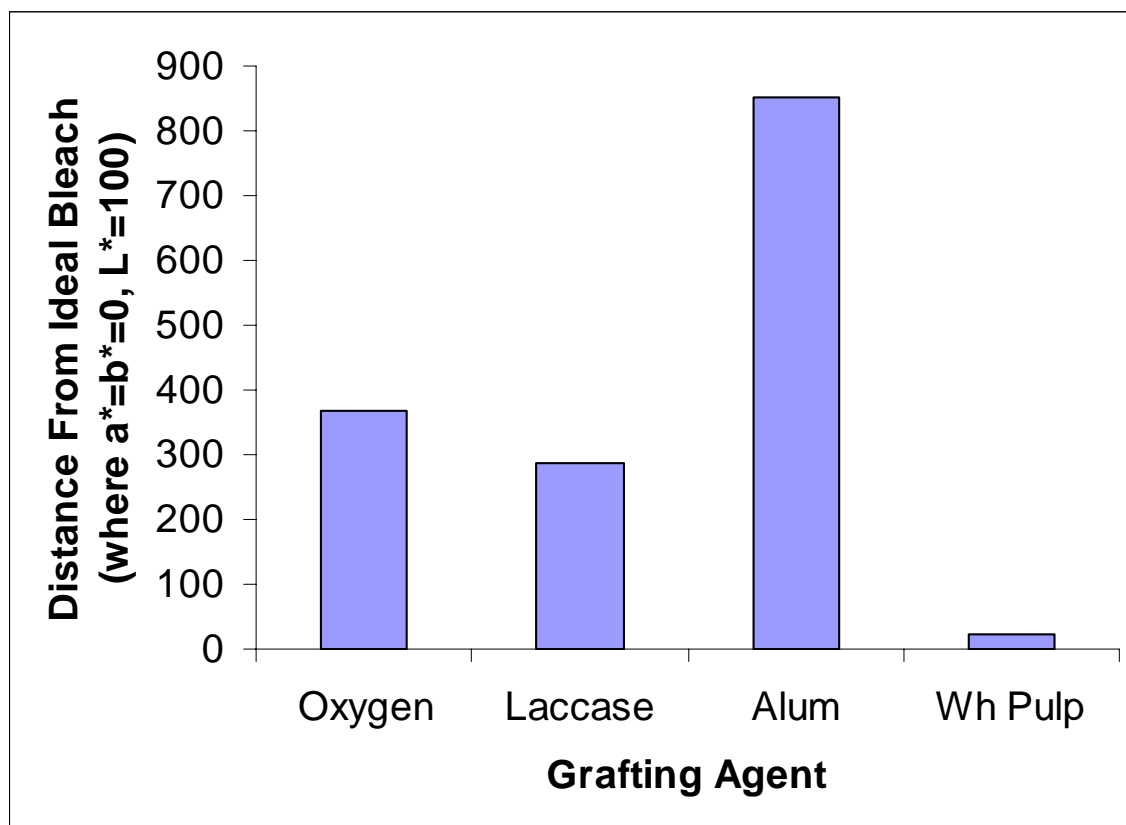
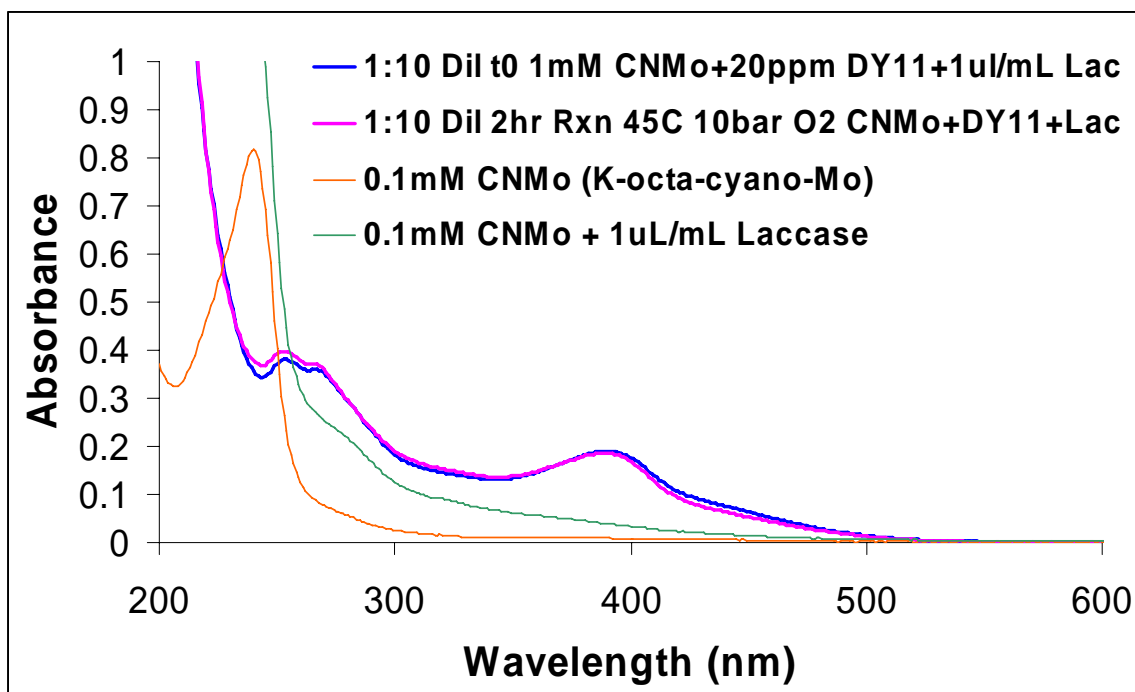
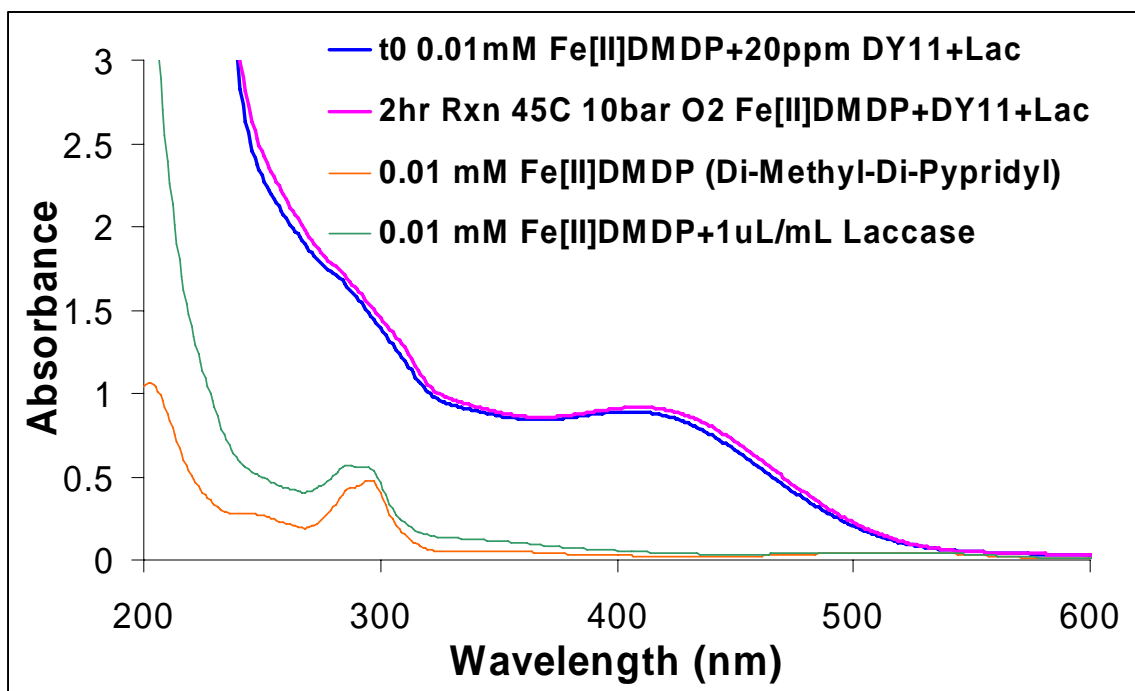


Figure A-43 Color from Grafted Direct Yellow 11

A.4.2 LACCASE TREATMENT USING INORGANIC LACCASE MEDIATORS

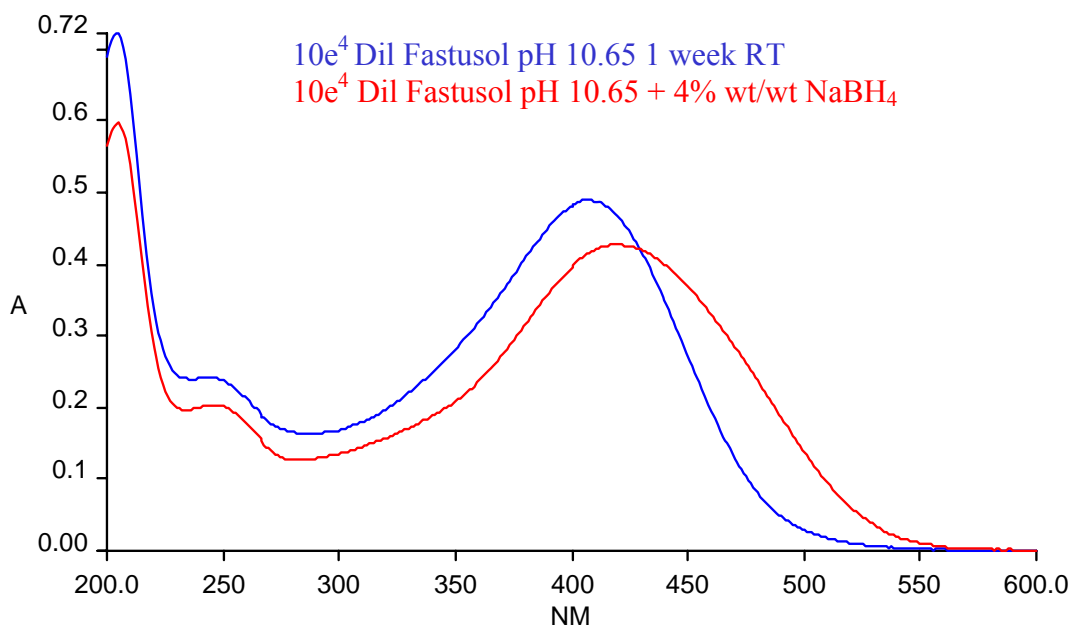
Inorganic mediators have been discovered for laccase.^{6,7} Two commercially available inorganic laccase mediators were obtained for testing. Potassium octacyanomolybdate (IV) hydrate (CNMo) was purchased from Sigma. The second was produced by mixing 3 moles of 4,4'-dimethyl-2,2'-dipyridyl with 1 mole of ferrous chloride, 4-hydrate to produce Fe[II]DMDP. Spectra were obtained for both compounds and both mediators were applied to the decolorization of 20 ppm Direct Yellow 11. However, strong spectral absorbance by the mediators limited application. For Fe[II]DMDP, the mediator concentration was reduced to 0.01 mM to allow spectral measurement. For decolorization with CNMo, 1 mM concentration was applied, but the t₀ and 2 hour reaction samples were diluted 1:10 to improve spectral measurement. Neither mediator appeared to be effective at the concentrations tested.

Figure A-44 Ineffective Decolorization with Potassium Octacyanomolybdate*Figure A-45 Ineffective Decolorization by Laccase with Fe[II]DMDP*

A.4.3 BOROHYDRIDE REDUCTION OF DIRECT YELLOW 11-DYED PULP

Because of the electronic requirements for light absorption and the creation of color, Direct Yellow 11 could be decolorized by *either* oxidation or reduction. Laccase-mediated reactions oxidize substrates. It was relevant to determine whether Direct Yellow 11 could be decolorized by reduction. To do this, Direct Yellow 11 (Fastusol) was reacted with sodium borohydride for a week at room temperature (RT). Dye solution was diluted 1:10,000 and the pH was adjusted to pH 10.65. Some of the diluted dye was transferred to a flask and stored in the dark for one week. To the remaining portion sodium borohydride was added and mixed. The borohydride-Direct Yellow 11 solution was then also placed in the dark for one week at room temperature. Spectra were obtained from both samples after one week.

Figure A-46 Reduction with Sodium Borohydride Shifts λ_{Max} slightly



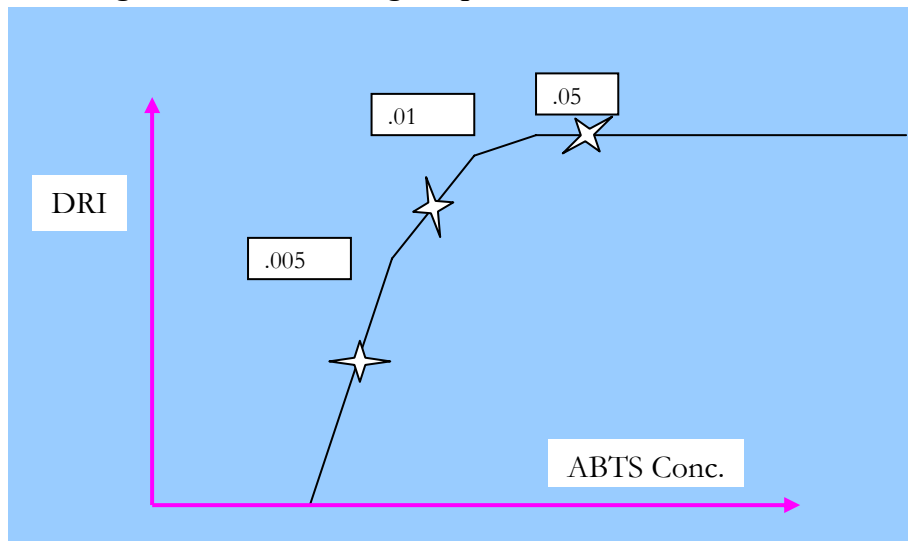
A.5 OPTIMIZATION OF LACCASE/ABTS DECOLORIZATION

A statistical design experiment was performed to determine the optimum combination of laccase, ABTS, and oxygen pressure. The selected format was a central composite design with additional axial points. The designed structure was a three variable version of the two dimension design shown below. Each level of an experimental variable was assigned a coded value between -2 and +2. In a central composite experiment, the

A.5.2 CALCULATING VARIABLE VALUES FOR EACH CODED LEVEL

From what is known about the reaction mechanism, increasing the concentrations of oxygen, (consumed during the reaction), ABTS and laccase would each increase the reaction rate until a constraint is reached and no further improvement occurs. The bleaching response for different ABTS concentrations at constant laccase and oxygen concentration shows this logarithmic behavior. (Figure A-48) Based on this, Dr. McDonough suggested that rather than plotting the variable concentrations on a linear scale and then selecting fixed intervals between values, the variable concentrations should be plotted on a logarithmic scale and a fixed “logarithmic” interval selected. Based on the sketch shown in Figure A-48, Dr. McDonough suggested that for the concentrations of ABTS, the “0” level should be between 0.005 and 0.01 mM ABTS, the “-1” value below 0.005, and the “+1” value above 0.05 mM.

Figure A-48 Bleaching Response to ABTS Concentrations



Following Montrogenometry,⁸ the alpha value between levels was kept at 1.6818 and a was adjusted to limit variables to achievable concentrations. As per Dr. McDonough’s recommendation, the “0” value for ABTS was set at 0.075 mM. The “0” values selected for laccase and oxygen pressure were the amounts applied in the “standard” decolorization reaction 10.3 Units or 5 μ L of laccase/mL reaction and 10 bar oxygen pressure.

The calculations of variable values are shown in Table A-21. The calculations were performed as follows:

The “0” variable values were set (5, 0.0075 and 10).

The log was obtained for those values (0.699, -2.125 and 1.0).

For each level, the coded value was multiplied by the a value and added to the “0” log value to obtain the level log value. For example, for the -2 laccase log value, -3.3636 multiplied by 0.3 (a) and added to “0” log value (0.699) yielded the log value -0.310.

The inverse logarithm is then calculated to obtain the actual values of variables that were applied during the experiment. For example, the inverse logarithm of -0.310 yields 0.490 as the value for the μL of laccase to add/mL reaction solution for the -2,0,0 axial run. If necessary, the value of a was adjusted to yield achievable values for the variables. The variable values used in the experiment were equally spaced on the logarithmic scale. The actual values and calculations are shown in Table A-21. From the assay of laccase activity, 5 μL laccase stock/mL of solution equals 5 Units laccase/mL.

Table A-21 Calculation of Variable Values for Statistical Designed Experiment

Variables:	Laccase, $\mu\text{L}/\text{mL}$	ABTS, mM	Oxygen Pressure	Laccase, Units	
Actual Values					
Level -2	0.49	0.0011	5	1.0094	
Level -1	1.57	0.0028	7	3.2342	
Level 0	5	0.0075	10	10.3	
Level 1	16.0	0.020	14	32.96	
Level 2	51.1	0.052	20	105.266	
Calculation					
Factor a	0.3	0.25	0.09	alpha =	1.6818
Variable Values				Level	Coded
Level -2	0.490	0.0011	4.981	-2	-3.3636
Level -1	1.565	0.0028	7.057	-1	-1.6818
Level 0	5	0.0075	10	0	0
Level 1	15.978	0.0197	14.170	1	1.6818
Level 2	51.056	0.0520	20.078	2	3.3636
Log Variable Values					
Level -2	-0.310	-2.966	0.697		
Level -1	0.194	-2.545	0.849		
Level 0	0.699	-2.125	1.000		
Level 1	1.204	-1.704	1.151		
Level 2	1.708	-1.284	1.303		

A.5.3 EXPERIMENTAL RESULTS

Since these experimental points took several weeks to perform, to prevent a blocking effect, points from the various levels were interspersed. Every week, one of the center points (0,0,0) was run as well as one of the axial points. The numerical results are shown in Table A-22. Maximum and minimum DRI values are in bold.

Table A-22 Results from Statistical Design Experiment for Lac/ABTS Decolorization

Sample	Date	Brightness	L*	a*	b*	DRI	DRI Error Values		Std Dev			Lac	ABTS	O2
							Hi	Low	L*	a*	b*			
Yellow Base Sheet		52.3	93.8	-2.74	28.4				0.17	0.16	0.55			
0,0,0	27-Jan	54.7	89.8	-1.48	19.1	44.6	44.0	45.2	0.34	0.07	0.32	0	0	0
0,0,0	7-Feb	56.3	91.2	-1.73	19.9	43.9	42.3	45.4	0.12	0.06	0.39	0	0	0
0,0,0	17-Apr	56.1	90.7	-1.79	19.3	45.6	44.7	46.5	0.14	0.06	0.27	0	0	0
0,0,0	23-Apr	56.7	90.7	-1.58	18.7	48.4	47.6	49.1	0.22	0.07	0.28	0	0	0
0,0,0	1-May	56.5	91.1	-1.69	19.5	45.6	44.8	46.4	0.12	0.07	0.24	0	0	0
0,0,0	7-May	56.0	90.6	-1.63	19.3	45.8	44.9	46.8	0.14	0.05	0.28	0	0	0
Avg 0,0,0		56.1	90.7	-1.65	19.3	45.7	44.7	46.6						
+2,0,0 Hi Lac	6-May	62.9	93.0	-1.92	16.8	60.5	59.8	61.1	0.23	0.09	0.28	2	0	0
-2,0,0 Low Lac	2-May	54.6	90.4	-1.69	20.2	40.9	40.2	41.6	0.20	0.21	0.27	-2	0	0
0,+2,0 Hi ABTS	24-Apr	54.3	84.7	1.26	10.6	58.8	59.1	58.5	0.18	0.08	0.12	0	2	0
0,-2,0 Low ABTS	16-Apr	56.5	93.0	-2.28	22.8	32.6	30.1	35.0	0.24	0.13	0.54	0	-2	0
0,0,+2 Hi O2	28-Jan	58.7	91.9	-1.83	18.8	50.2	49.1	51.2	0.22	0.20	0.35	0	0	2
0,0,-2 Low O2	6-Feb	56.9	91.5	-1.75	19.8	44.9	43.9	45.9	0.13	0.05	0.27	0	0	-2
+1,+1,+1	5-May	58.8	91.0	-1.40	16.9	56.5	54.8	58.0	0.35	0.07	0.59	1	1	1
+1,+1,-1	10-Feb	56.0	89.6	-1.11	17.5	51.1	50.5	51.8	0.17	0.06	0.26	1	1	-1
+1,-1,+1	18-Apr	60.1	93.2	-1.92	19.8	48.2	47.5	49.0	0.15	0.09	0.22	1	-1	1
+1,-1,-1	11-Feb	59.4	92.9	-1.82	20.0	46.9	45.9	47.9	0.15	0.09	0.28	1	-1	-1
-1,+1,+1	29-Apr	57.8	89.3	-1.09	15.2	59.2	58.9	59.5	0.18	0.07	0.21	-1	1	1
-1,+1,-1	21-Apr	55.3	88.5	-0.84	16.3	53.3	53.1	53.5	0.19	0.08	0.19	-1	1	-1
-1,-1,+1	30-Apr	56.2	92.2	-2.22	21.7	37.0	35.9	38.1	0.18	0.08	0.28	-1	-1	1
-1,-1,-1	29-Jan	57.0	92.5	-2.21	21.4	38.9	37.9	39.8	0.15	0.07	0.24	-1	-1	-1

The results obtained showed small differences. To magnify the differences, the brightness values are plotted for the range of 45-65. Of the six center point values, the brightness values were 56.0 to 56.7 for five points; 54.7 for the remaining point.

Figure A-49 Statistical Experiment: Brightness Values for 0,0,0 Center Point Runs

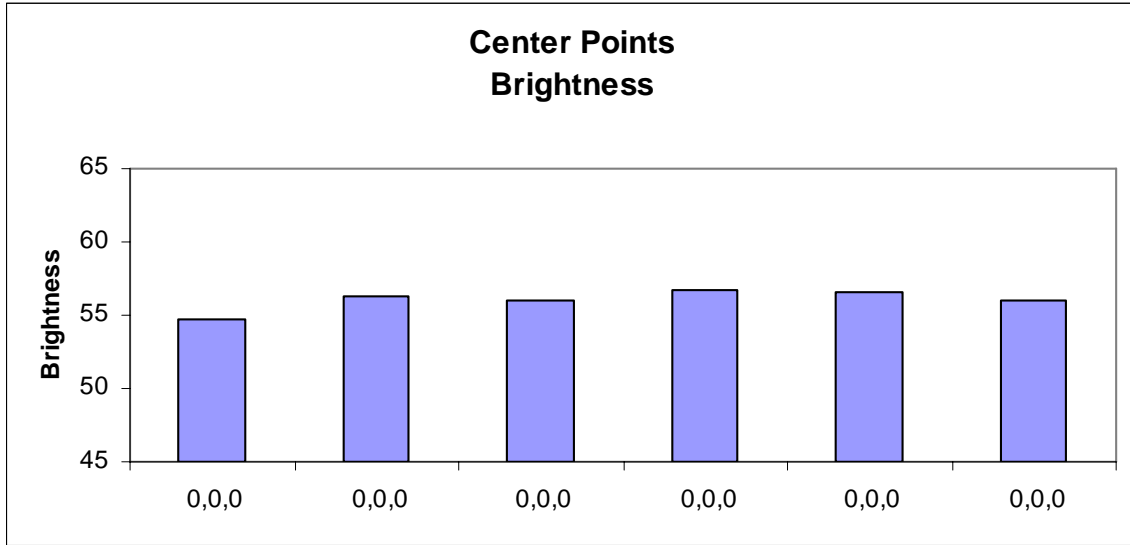


Figure A-50 Statistical Experiment: Brightness Values for Axial Runs

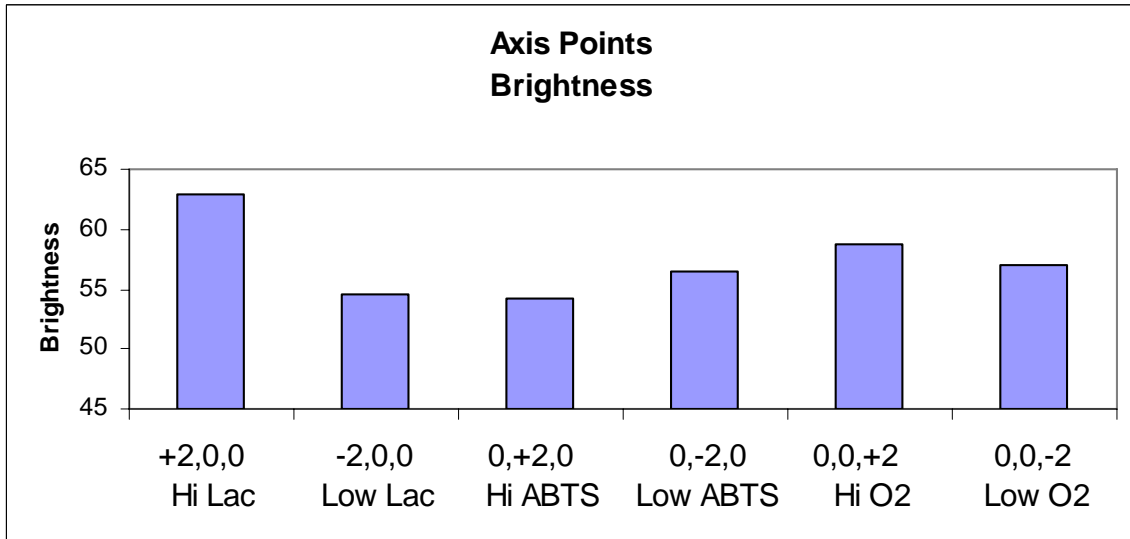
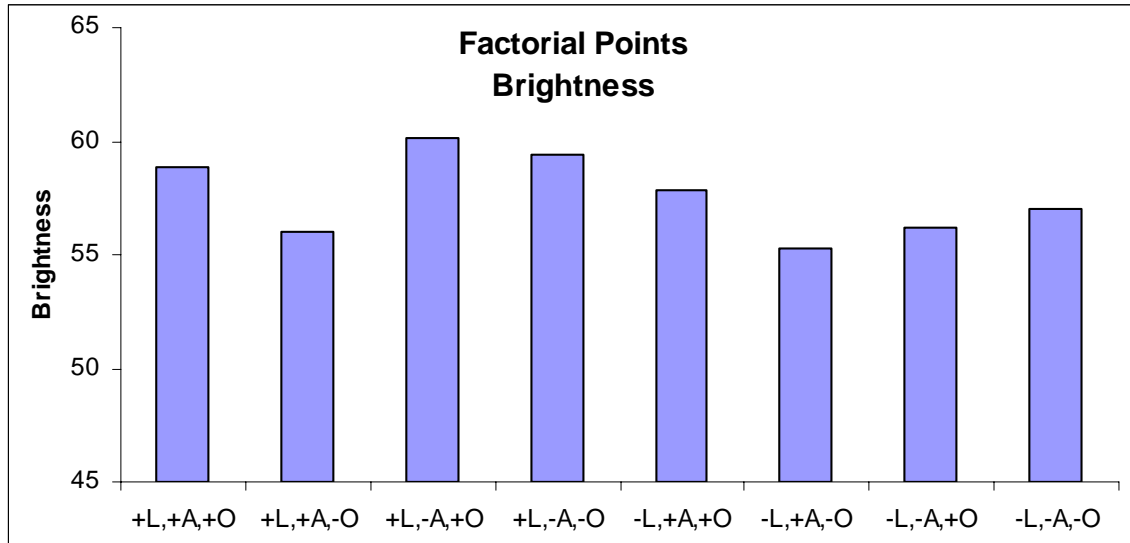


Figure A-51 Statistical Experiment: Brightness Values for Factorial Runs



One way to examine the data is to examine by comparison to the center point data. The dye removal index values all appeared high – above 90 % when initially calculated. However, that was due to miscalculating the distance from ideal bleach point (“color content”) of the initial base sheets. The final correct dye removal index values are 32 to 60%. All the DRI values are plotted below.

Figure A-52 Statistical Experiment: DRI Values for 0,0,0 Center Point Runs

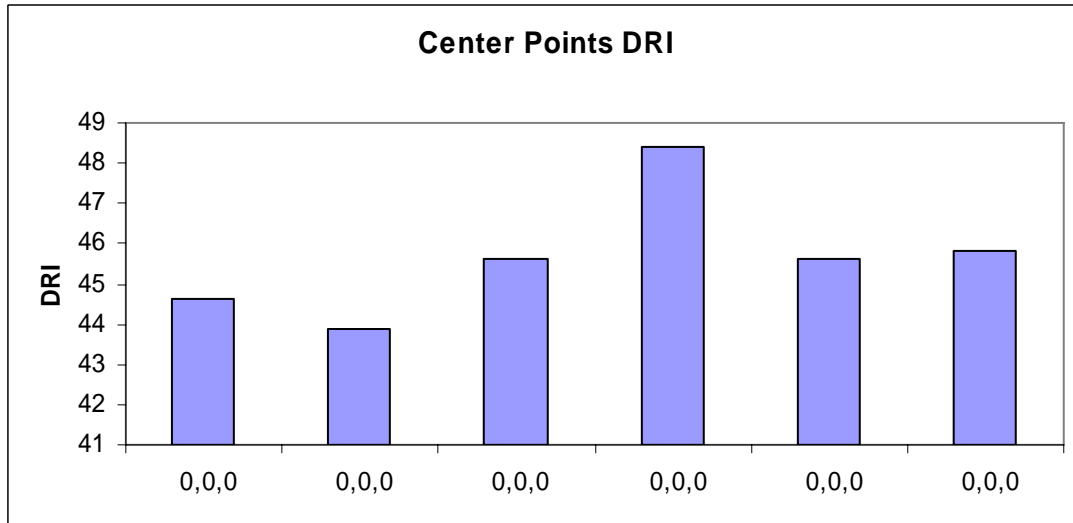


Figure A-53 Statistical Experiment: DRI Values for Axial Runs

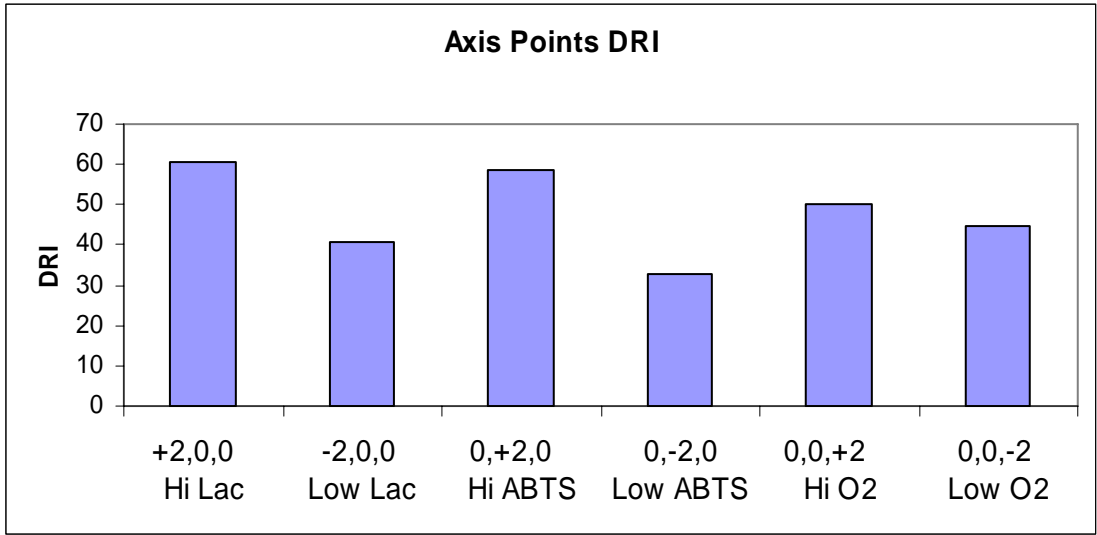
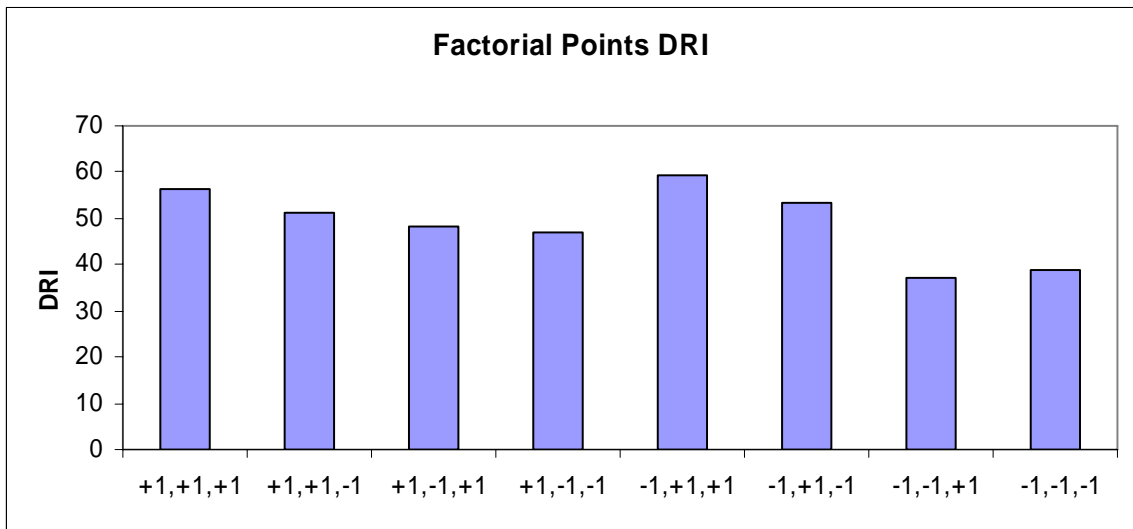


Figure A-54 Statistical Experiment: DRI Values for Factorial Runs



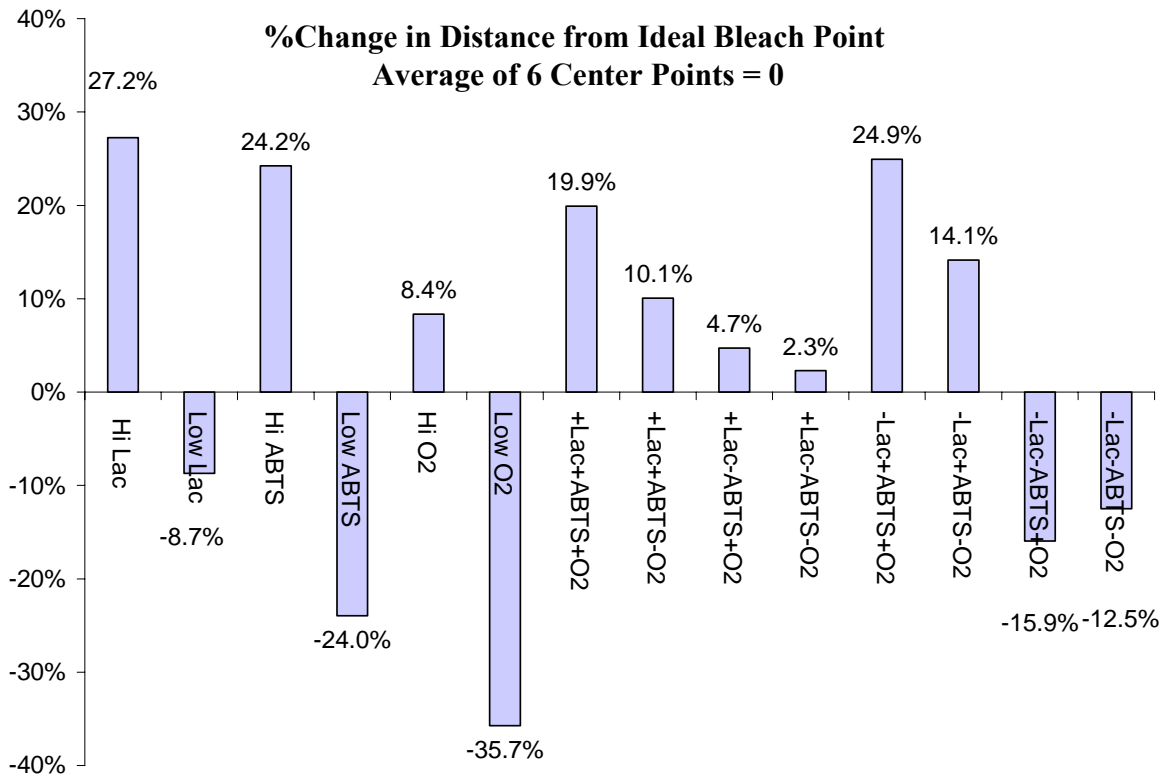
There are many ways to examine this data. One method is to set the amount of color (distance from ideal bleach point) of the center point data as zero and look at the percent change between in color content between pulp subjected to the (0,0,0) treatment and the other treatments. The results are shown in Figure A-55. As expected for the axial experiments (one variable ± 2 while others stay at 0), increasing just one variable increased the removal of color while decreasing it decreased color removal. The variable levels were actually spaced logarithmically equidistant. The relatively large increase in

oxygen pressure from 10 bars to 20 only increased color removal 8 %. Decreasing oxygen level from 10 bars to 5 bars caused a 36% decrease in the amount of color removed during standard (0,0,0) treatment. For laccase mediator ABTS, equal size logarithmic changes in concentration (0.0075 down to 0.0011 or up to 0.052 mM) resulted in equal size changes in amount of color removed (± 24). Decreasing from 5 $\mu\text{L}/\text{mL}$ of laccase to 0.49 $\mu\text{L}/\text{mL}$ (10 fold decrease) decreased color removal by 9%. Increasing laccase from 5 $\mu\text{L}/\text{mL}$ to 51.1 (10 fold increase) increased color removal by 27%.

The factorial points (all variables either +1 or -1 level) also yield interesting results. In the presence of higher concentration of ABTS (0.02 mM), dropping the laccase concentration from 16.0 $\mu\text{L}/\text{mL}$ to 1.6 $\mu\text{L}/\text{mL}$, actually *increased* the amount of color removal (25% vs. 20% if $\text{O}_2 = +1$, 14% vs. 10% if $\text{O}_2 = -1$). In the presence of the lower concentration (0.003 mM) of ABTS, dropping the laccase concentration decreased the amount of color removal (-16% vs. 5% if $\text{O}_2 = +1$, 13% vs. 2% if $\text{O}_2 = -1$). If both the laccase and ABTS concentrations are dropped, the amount of color removal is less than for standard conditions. For the same combinations of laccase and ABTS, decreasing the oxygen pressure (+1 = 14 bar, -1 = 7 bar) does lower the amount of color removed. As expected, decreasing the amount of ABTS (+1 = 0.02 mM, -1 = 0.003 mM) decreases the amount of color removed.

The difference in response to laccase concentration at higher ABTS concentrations does suggest some interaction may be occurring between the enzyme and the mediator that does not involve decolorization of the dye. At the higher enzyme concentration, the putative interaction occurs more often, and maybe reducing the availability of mediator to participate in the dye decolorization reaction.

Figure A-55 Change in Color Content Using 0,0,0 Treated Pulp as Zero Values



A.5.4 STATISTICAL ANALYSIS OF OPTIMIZATION EXPERIMENT RESULTS

To analyze the results, NCSS statistical analysis software was applied to the data. The types of analysis applied include response surface regression analysis, multiple regression, robust regression, multivariate ratio of polynomials, and all possible regression. The complete report results from the software application are included, following the discussion of significance.

The goal is a statistical model of the experimental data. As explained above, the coded variable levels (-2 to +2) are derived from the logarithms of the actual values. For the statistical analysis, the coded variable values were used. To apply any derived statistical model, conversion functions are needed. The conversion equations are given below. Coded variables were numbers -2 to +2. The actual values are given in Table A-21, page 51 and range from 5-20 bar oxygen pressure, 0.0011 to 0.052 mM ABTS, 0.49 to 51.1 μL Laccase/mL solution or 1.009 to 105.3 Units of laccase/mL solution. For this experiment, the amount of laccase added was calculated as μL of enzyme concentrate

/ mL of reaction solution. Assay of the enzyme concentrate shows that 5 $\mu\text{L}/\text{mL}$ is the equivalent of 10.3 Units/mL

Equation A-7 Conversion of Coded Variables to Actual Values

$$\begin{aligned}\text{Oxygen: bar} &= 10\exp[0.3485 * (\text{Coded Value})] \\ \text{ABTS: mM} &= 0.0075\exp[0.9663 * (\text{Coded Value})] \\ \text{Laccase: } \mu\text{L}/\text{mL} &= 5.0009\exp [1.1616 * (\text{Coded Value})] \\ \text{Laccase: Units} &= 10.302\exp[1.1616 * (\text{Coded Value})]\end{aligned}$$

Equation A-8 Conversion of Coded Variable to Log (Actual Value)

$$\begin{aligned}\text{Oxygen: Log[bar]} &= 0.1514 * (\text{Coded Value}) + 1 \\ \text{ABTS: Log[mM]} &= 0.4205 * (\text{Coded Value}) - 2.1248 \\ \text{Laccase: Log}[\mu\text{L}/\text{mL}] &= 0.5046 * (\text{Coded Value}) + 0.699 \\ \text{Laccase: Log[Units]} &= 0.5045 * (\text{Coded Value}) + 1.0134\end{aligned}$$

Equation A-9 Conversion of Actual Values to Coded Variables

$$\begin{aligned}\text{Oxygen: (Coded Value)} &= 2.8694\text{Ln}(\text{bar}) - 6.607 \\ \text{ABTS: Coded Value} &= 1.0348\text{Ln}(\text{mM}) + 5.0639 \\ \text{Laccase: Coded Value} &= 0.8609\text{Ln}(\mu\text{L}/\text{mL}) - 1.3857 \\ \text{Laccase: Coded Value} &= 0.8609\text{Ln}(\text{Units}) - 2.0079\end{aligned}$$

Equation A-10 Conversion of Log (Actual Value) to Coded Variable

$$\begin{aligned}\text{Oxygen: Coded Value} &= 6.605 * [\text{Ln}(\text{bar})] - 6.605 \\ \text{ABTS: Coded Value} &= 2.3781 * [\text{Ln}(\text{mM})] + 5.053 \\ \text{Laccase: Coded Value} &= 1.9818 * [\text{Log}((\mu\text{L}/\text{mL})] - 1.3853 \\ \text{Laccase: Coded Value} &= 1.9823 * [\text{Log}(\text{Units})] - 2.0088\end{aligned}$$

A.5.4.1 Discussion of Statistical Models

Response surface regression yielded a model with R^2 value of 0.941, which is a fairly good fit. This analysis includes both ANOVA and ANOVA using pure error. The estimated regression coefficients are the same: what differs are the calculated Probability levels, F Ratios for factors and T Ratios for regression coefficients. The full results are given in Section A.5.4.2, page 61. The relative importance of the three factors is shown below. The ABTS level is clearly very important. The laccase level is also important. The oxygen level however is only marginally significant.

Table A-23 Importance of Factors in Response Surface Regression

Factor	Standard ANOVA			ANOVA Using Pure Error	
	df	F-Ratio	Prob Level	F-Ratio	Prob Level
Lac	4	12.16	0.000743	32.09	0.000927
ABTS	4	29.68	0.000016	78.36	0.000107
O2	4	2.25	0.136064	5.94	0.038634
Total Error	10				
Lack of Fit	5	4.28	0.068238	4.28	0.068238
Pure Error	5				

The model determined by response surface regression is described below. The residual differences between model and data are plotted below in Section A.5.4.2 and show no significant trends. The probability levels for each regression coefficient are also given in Section A.5.4.2. If probability level of 0.1 is used to determine which regression terms are significant, based on the standard ANOVA, the terms $ABTS(\text{Level})^2$, $O2(\text{Level})^2$, $Lac(\text{Level}) * O2(\text{Level})$ and $ABTS(\text{Level}) * O2(\text{Level})$ can be omitted from the model.

Equation A-11 Model From Response Surface Regression (R^2 0.941)

$$\begin{aligned}
 DRI = & 45.92814 + Lac(\text{Level}) * 3.338322 + ABTS(\text{Level}) * 6.347024 + O2(\text{Level}) * 1.328575 \\
 & + Lac(\text{Level})^2 * 1.3943 + ABTS(\text{Level})^2 * 0.1527651 + O2(\text{Level})^2 * 0.6083103 + \\
 & Lac(\text{Level}) * ABTS(\text{Level}) * 3.025076 + Lac(\text{Level}) * O2(\text{Level}) * 0.3339178 + \\
 & ABTS(\text{Level}) * O2(\text{Level}) * 1.471562
 \end{aligned}$$

The results from Multiple Regression Analysis (Section A.5.4.3, page 65) were similar. In correlation matrix, the dependent variable DRI strongly correlated with ABTS levels, significantly correlated with laccase levels but only marginally correlated with oxygen levels. Based on the T Value and the probability level, the multiple regression rejected oxygen levels as not being significant. A model was obtained but its R^2 value was only 0.805. The low R^2 value is probably due to limiting regression to linear terms. However, the predicted values in the report were based on all three terms, including oxygen. The data did pass normality tests such as skewness, kurtosis and omnibus.

Table A-24 Correlation of Factors with DRI in Multiple Regression Analysis

Correlation Matrix	
	DRI
Lac	0.410726
ABTS	0.780897
O2	0.163459

Equation A-12 Regression Equation from Multiple Regression (R^2 0.805)

$$DRI = 47.65244 + Lac(Level) * 3.338322 + ABTS(Level) * 6.347024 + O2(Level) * 1.328575$$

Table A-25 Oxygen Level NOT Significant in Multiple Regression

Independent Variable	Regression Coefficient	Standard Error	T-Value (Ho: B=0)	Prob Level	Decision (5%)	Power (5%)
Intercept	47.65244	0.8021212	59.4080	0.000000	Reject Ho	1.000000
Lac	3.338322	0.8967987	3.7225	0.001852	Reject Ho	0.937140
ABTS	6.347024	0.8967987	7.0774	0.000003	Reject Ho	0.999998
O2	1.328575	0.8967987	1.4815	0.157908	Accept Ho	0.285829
R-Squared	0.805214					

The Multivariate Ratio of Polynomials Analysis was run to see how well the model would fit. An R^2 value of 0.989 was obtained, indicating a good fit. The model consists of the ratio of two polynomial equations. Although the fit is good, the complicated equation limits the model to the range of variable levels tested.

Equation A-13 Format for Multivariate Ratio of Polynomials Model

Symbolic Model

$$Y = P1 (U, V, W) / P2 (U, V, W)$$

$$P1 (U, V, W) = B0 + B1 * U + B2 * U^2 + B3 * V + B4 * UV + B5 * V^2 + B6 * W + B7 * UW + B8 * VW + B9 * W^2$$

$$P2 (U, V, W) = 1 + B10 * U + B11 * U^2 + B12 * V + B13 * UV + B14 * V^2 + B15 * W + B16 * UW + B17 * VW + B18 * W^2$$

where

$$Y = DRI$$

$$U = Lac$$

$$V = ABTS$$

$$W = O2$$

Equation A-14 Ratio of Polynomials Model (R^2 0.989)

$$DRI = \{45.65816 - 16.05398 * Lac + 15.72261 * Lac^2 + 2.330253 * ABTS + 47.34002 * Lac * ABTS + 9.275566 * ABTS^2 + 1.32302 * O2 + 1.453849 * Lac * O2 + 4.770414 * ABTS * O2 + 0.473319 * O2^2\} / [1 - 0.5431627 * Lac + 0.3376349 * Lac^2 - 0.2267051 * ABTS + 1.141829 * Lac * ABTS + 0.2349017 * ABTS^2 + 0 * O2 + 0 * Lac * O2 + 4.349544E-02 * ABTS * O2 + 0 * O2^2]$$

The results from applying the NCSS robust regression to the data were very similar to the multiple regression results. A moderate R^2 value of 0.886402 was obtained.

The probability levels indicate that the oxygen pressure term could be omitted. However, the predicted values produced by the program do include the oxygen term in the calculation

Equation A-15 Regression Equation from Robust Regression (R^2 0.886)

$$DRI = 46.81763 + Lac(Level)*3.054206 + ABTS(Level)*6.066785 + O2(Level)*1.137815$$

Table A-26 Oxygen Level NOT Significant in Robust Regression

Independent Variable	Regression Coefficient	Standard Error	T-Value (Ho: B=0)	Prob Level	Decision (5%)	Power (5%)
Intercept	46.81763	0.5791607	80.8370	0.000000	Reject Ho	1.000000
Lac	3.054206	0.7395132	4.1300	0.000786	Reject Ho	0.972030
ABTS	6.066785	0.6475289	9.3691	0.000000	Reject Ho	1.000000
O2	1.137815	0.6292678	1.8082	0.089415	Accept Ho	0.397471
R-Squared	0.886402					

Analysis by All Possible Regressions Function was also performed but no additional insight was gained. The data indicate that ABTS level is most important, followed by the laccase level. Oxygen level is barely significant, but does improve fit when included. The models and fits are summarized below. The best fit was obtained with the ratio of polynomials. Both models with good fits (ratio of polynomials and response surface regression) include quadratic terms (squared values and products of two terms)

Table A-27 Summary of Statistical Models

Equation	Page	Model Type	R ²
A-11	59	Response Surface Regression	0.941
A-12	60	Multiple Regression	0.805
A-14	60	Ratio of Polynominals	0.989
A-15	61	Robust Regression	0.886

A.5.4.2 Response-Surface Regression Report

Page/Date/Time 1 04-13-2005 15:49:06
 Database P:\Public Files\Kristina Knutson\DRI Data.S0
 Response DRI

Descriptive Statistics Section

Variable	Count	Mean	Minimum	Maximum
Lac	20	0	-2	2
ABTS	20	0	-2	2
O2	20	0	-2	2
DRI	20	47.65244	32.63659	60.46495

Hierarchical Model Summary Section

Number of Terms Removed 0
 Number of Terms Remaining 9
 R-Squared Cutoff Value 0.010000
 R-Squared of Final Model 0.941378

Coded Hierarchical Model

	A	B	C	D	E	F
A Lac	2	1	1	0	0	0
B ABTS		2	1	0	0	0
C O2			2	0	0	0
D				0	0	0
E					0	0
F						0

Notes:

For off-diagonal entries:

1=u1w1, 2=u1w2, 3=u2w1, 4=u2w2, 5=u1w3,
 6=u3w1, 7=u2w3, 8=u3w2, 9=u3w3.

For diagonal entries:

1=u1, 2=u2, 3=u3.

Where u1=u, u2=u^2=u*u, and u3=u^3=u*u*u.

Sequential ANOVA Section

Source	df	Sequential Sum-Squares	Mean Square	F-Ratio	Prob Level	Incremental R-Squared
Regression	9	995.0316	110.5591	17.84	0.000050	0.941378
Linear	3	851.1075	283.7025	45.79	0.000004	0.805214
Quadratic	3	52.49934	17.49978	2.82	0.093026	0.049668
Lin x Lin	3	91.42462	30.47487	4.92	0.023698	0.086495
Total Error	10	61.9635	6.19635			0.058622
Lack of Fit	5	50.22861	10.04572	4.28	0.068238	0.047520
Pure Error	5	11.73489	2.346979			0.011102

Sequential ANOVA Section Using Pure Error

Source	df	Sequential Sum-Squares	Mean Square	F-Ratio	Prob Level	Incremental R-Squared
Regression	9	995.0316	110.5591	47.11	0.000265	0.941378
Linear	3	851.1075	283.7025	120.88	0.000044	0.805214
Quadratic	3	52.49934	17.49978	7.46	0.027092	0.049668
Lin x Lin	3	91.42462	30.47487	12.98	0.008521	0.086495
Total Error	10	61.9635	6.19635			0.058622
Lack of Fit	5	50.22861	10.04572	4.28	0.068238	0.047520
Pure Error	5	11.73489	2.346979			0.011102

ANOVA Section

Factor	df	Last Sum-Squares	Mean Square	F-Ratio	Prob Level	Term R-Squared
Lac	4	301.2905	75.32262	12.16	0.000743	0.285044
ABTS	4	735.6749	183.9187	29.68	0.000016	0.696006
O2	4	55.76162	13.9404	2.25	0.136064	0.052755
Total Error	10	61.9635	6.19635			0.058622
Lack of Fit	5	50.22861	10.04572	4.28	0.068238	0.047520
Pure Error	5	11.73489	2.346979			0.011102

ANOVA Section Using Pure Error

Factor	df	Last Sum-Squares	Mean Square	F-Ratio	Prob Level	Term R-Squared
Lac	4	301.2905	75.32262	32.09	0.000927	0.285044
ABTS	4	735.6749	183.9187	78.36	0.000107	0.696006
O2	4	55.76162	13.9404	5.94	0.038634	0.052755
Total Error	10	61.9635	6.19635			0.058622
Lack of Fit	5	50.22861	10.04572	4.28	0.068238	0.047520
Pure Error	5	11.73489	2.346979			0.011102

Estimation Section

Parameter	df	Regression Coefficient	Standard Error	T-Ratio	Prob Level	Last R-Squared
Intercept	1	45.92814				
Lac	1	3.338322	0.6223117	5.36	0.000317	0.168695
ABTS	1	6.347024	0.6223117	10.20	0.000001	0.609800
O2	1	1.328575	0.6223117	2.13	0.058536	0.026719
Lac^2	1	1.3943	0.496433	2.81	0.018518	0.046244
ABTS^2	1	0.1527651	0.496433	0.31	0.764605	0.000555
O2^2	1	0.6083103	0.496433	1.23	0.248516	0.008802
Lac*ABTS	1	-3.025076	0.8800817	-3.44	0.006360	0.069261
Lac*O2	1	0.3339178	0.8800817	0.38	0.712311	0.000844
ABTS*O2	1	1.471562	0.8800817	1.67	0.125459	0.016390

Estimation Section Using Pure Error

Parameter	df	Regression Coefficient	Standard Error	T-Ratio	Prob Level	Last R-Squared
Intercept	1	45.92814				
Lac	1	3.338322	0.3829963	8.72	0.000329	0.168695
ABTS	1	6.347024	0.3829963	16.57	0.000015	0.609800
O2	1	1.328575	0.3829963	3.47	0.017871	0.026719
Lac^2	1	1.3943	0.3055254	4.56	0.006037	0.046244
ABTS^2	1	0.1527651	0.3055254	0.50	0.638294	0.000555
O2^2	1	0.6083103	0.3055254	1.99	0.103114	0.008802
Lac*ABTS	1	-3.025076	0.5416386	-5.59	0.002538	0.069261
Lac*O2	1	0.3339178	0.5416386	0.62	0.564551	0.000844
ABTS*O2	1	1.471562	0.5416386	2.72	0.041931	0.016390

Optimum Solution Section

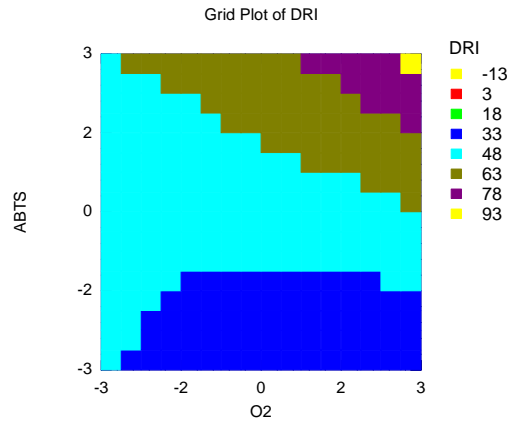
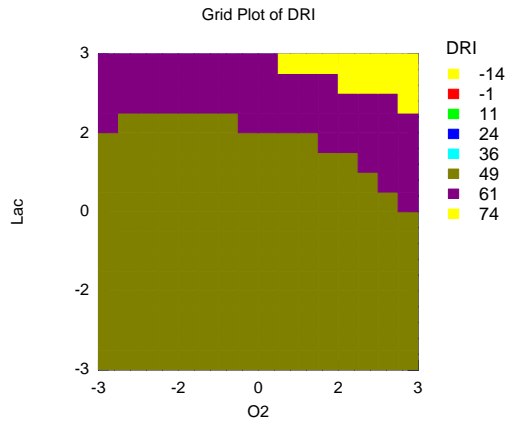
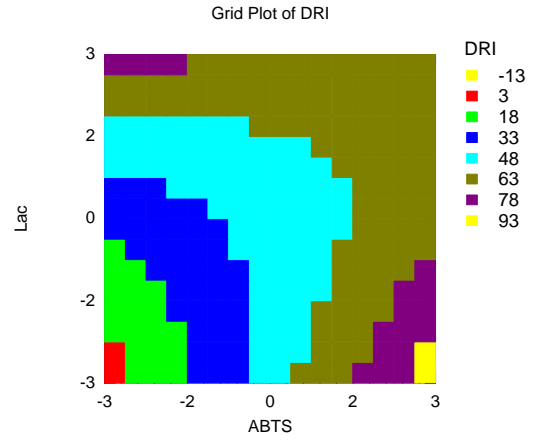
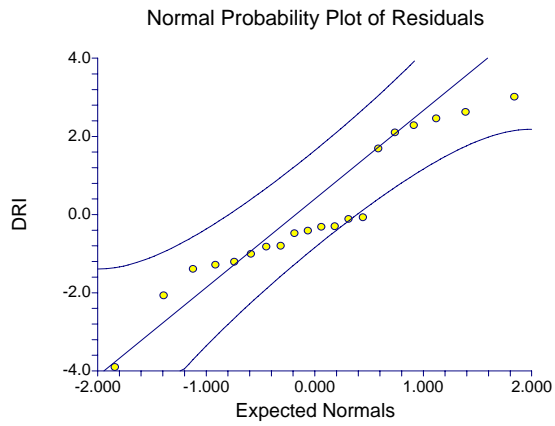
Parameter	Maximum Exponent	Optimum Value
Lac	2	1253
ABTS	2	-6669
O2	2	6554

Function at optimum -1215539
 Number of Function Evaluations 504
 Maximum Functions Evaluations 500

Residual Section

Row	DRI	Predicted	Residual
1	44.6439	45.92814	-1.284145
2	43.8597	45.92814	-2.068444
3	45.6145	45.92814	-0.313633
4	48.3866	45.92814	2.458475
5	45.6289	45.92814	-0.2992045
6	45.8151	45.92814	-0.1129449
7	60.4649	58.18198	2.282966
8	40.9258	44.82869	-3.902863
9	58.8219	59.23325	-0.411335
10	32.6365	33.84515	-1.208561
11	50.1974	51.01853	-0.8210567
12	44.9053	45.70423	-0.7988397
13	56.4857	57.87784	-1.392118
14	51.1302	51.60973	-0.4795131
15	48.2210	48.29082	-6.979143E-02
16	46.9043	47.90895	-1.004614
17	59.2080	56.58351	2.62451
18	53.3407	51.65107	1.689688
19	36.9955	34.89619	2.09941
20	38.8620	35.84999	3.012015

Plots Section



A.5.4.3 Multiple Regression Report

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 Database P:\Public Files\Kristina Knutson\DRI Data.S0
 Dependent DRI

Descriptive Statistics Section

Variable	Count	Mean	Standard Deviation	Minimum	Maximum
Lac	20	-1.387779E-17	0.9176629	-2	2
ABTS	20	0	0.9176629	-2	2
O2	20	0	0.9176629	-2	2
DRI	20	47.65244	7.458641	32.63659	60.46495

Correlation Matrix Section

	Lac	ABTS	O2	DRI
Lac	1.000000	0.000000	0.000000	0.410726
ABTS	0.000000	1.000000	0.000000	0.780897
O2	0.000000	0.000000	1.000000	0.163459
DRI	0.410726	0.780897	0.163459	1.000000

Regression Equation Section

Independent Variable	Regression Coefficient	Standard Error	T-Value (Ho: B=0)	Prob Level	Decision (5%)	Power (5%)
Intercept	47.65244	0.8021212	59.4080	0.000000	Reject Ho	1.000000
Lac	3.338322	0.8967987	3.7225	0.001852	Reject Ho	0.937140
ABTS	6.347024	0.8967987	7.0774	0.000003	Reject Ho	0.999998
O2	1.328575	0.8967987	1.4815	0.157908	Accept Ho	0.285829
R-Squared	0.805214					

Regression Coefficient Section

Independent Variable	Regression Coefficient	Standard Error	Lower 95% C.L.	Upper 95% C.L.	Standardized Coefficient
Intercept	47.65244	0.8021212	45.95202	49.35286	0.0000
Lac	3.338322	0.8967987	1.437194	5.23945	0.4107
ABTS	6.347024	0.8967987	4.445896	8.248153	0.7809
O2	1.328575	0.8967987	-0.5725538	3.229703	0.1635
T-Critical	2.119905				

Analysis of Variance Section

Source	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (5%)
Intercept	1	45415.1	45415.1			
Model	3	851.1075	283.7025	22.0472	0.000006	0.949548
Error	16	205.8875	12.86797			
Total(Adjusted)	19	1056.995	55.63132			

Root Mean Square Error	3.587195	R-Squared	0.8052
Mean of Dependent	47.65244	Adj R-Squared	0.7687
Coefficient of Variation	0.0752783	Press Value	346.8986
Sum Press Residuals	66.33203	Press R-Squared	0.6718

Normality Tests Section

Assumption	Value	Probability	Decision (5%)
Skewness	1.5600	0.118760	Accepted
Kurtosis	0.1222	0.902761	Accepted
Omnibus	2.4485	0.293974	Accepted

Serial-Correlation Section

Lag	Correlation	Lag	Correlation	Lag	Correlation
1	0.294452	9	0.157251	17	
2	-0.067348	10	0.159457	18	
3	-0.088960	11	0.101654	19	
4	-0.054537	12	-0.063515	20	
5	-0.027456	13	0.067477	21	
6	-0.234963	14	-0.153368	22	
7	-0.206917	15	-0.255146	23	
8	0.098583	16		24	

Above serial correlations significant if their absolute values are greater than 0.447214
 Durbin-Watson Value 1.3431

R-Squared Section

Independent Variable	Cumulative Sequential	Incremental Sequential	Incremental Last	Simple	Partial (Adj. for Rest)
Lac	0.168695	0.168695	0.168695	0.168695	0.464111
ABTS	0.778495	0.609800	0.609800	0.609800	0.757906
O2	0.805214	0.026719	0.026719	0.026719	0.120625

Variable Omission Section

Independent Variable	R-Squared When Omitted Vs Other X's	MSE	Mallow's Cp	Prob When Omitted	R-Squared When Omitted	Level
Full Model	0.805214	12.86797				
Lac	0.636519	22.59987	15.856914	0.001852	0.000000	
ABTS	0.195414	50.02606	52.089928	0.000003	0.000000	
O2	0.778495	13.77231	4.194734	0.157908	0.000000	

Sum of Squares and Correlation Section

Independent Variable	Sequential Sum Squares	Incremental Sum Squares	Last Sum Squares	Simple Correlation	Partial Correlation
Lac	178.3103	178.3103	178.3103	0.410726	0.681257
ABTS	822.8658	644.5555	644.5555	0.780897	0.870578
O2	851.1075	28.24176	28.24176	0.163459	0.347311

Sequential Models Section

Independent Variable	Included R-Squared	Omitted R-Squared	Included F-Ratio	Included Prob>F	Omitted F-Ratio	Omitted
Lac	0.168695	0.636519	3.65	0.072035	26.14	0.000009
ABTS	0.778495	0.026719	29.87	0.000003	2.19	0.157908
O2	0.805214	0.000000	22.05	0.000006		

Notes

1. INCLUDED variables are those listed from current row up (includes current row).
2. OMITTED variables are those listed below (but not including) this row.

Predicted Values with Confidence Limits of Means

Row	Actual	Predicted	Std Error of Predicted	95% LCL of Mean	95% UCL of Mean
1	44.64399	47.65244	0.8021212	45.95202	49.35286
2	43.8597	47.65244	0.8021212	45.95202	49.35286
3	45.61451	47.65244	0.8021212	45.95202	49.35286
4	48.38661	47.65244	0.8021212	45.95202	49.35286
5	45.62893	47.65244	0.8021212	45.95202	49.35286
6	45.81519	47.65244	0.8021212	45.95202	49.35286
7	60.46495	54.32908	1.964787	50.16392	58.49425
8	40.92583	40.9758	1.964787	36.81063	45.14096
9	58.82191	60.34649	1.964787	56.18132	64.51165
10	32.63659	34.95839	1.964787	30.79323	39.12355
11	50.19747	50.30959	1.964787	46.14442	54.47475
12	44.90539	44.99529	1.964787	40.83013	49.16045
13	56.48572	58.66636	1.748183	54.96038	62.37234
14	51.13022	56.00921	1.748183	52.30323	59.71519
15	48.22103	45.97231	1.748183	42.26633	49.67829
16	46.90434	43.31516	1.748183	39.60918	47.02114
17	59.20802	51.98972	1.748183	48.28373	55.6957
18	53.34076	49.33257	1.748183	45.62659	53.03855
19	36.99559	39.29567	1.748183	35.58968	43.00165
20	38.86201	36.63852	1.748183	32.93254	40.3445

Predicted Values with Confidence Limits of Individuals

Row	Actual	Predicted	Std Error of Predicted	95% LCL of Individual	95% UCL of Individual
1	44.64399	47.65244	3.675781	39.86013	55.44474
2	43.8597	47.65244	3.675781	39.86013	55.44474
3	45.61451	47.65244	3.675781	39.86013	55.44474
4	48.38661	47.65244	3.675781	39.86013	55.44474
5	45.62893	47.65244	3.675781	39.86013	55.44474
6	45.81519	47.65244	3.675781	39.86013	55.44474
7	60.46495	54.32908	4.090031	45.6586	62.99956
8	40.92583	40.9758	4.090031	32.30532	49.64627
9	58.82191	60.34649	4.090031	51.67601	69.01697
10	32.63659	34.95839	4.090031	26.28791	43.62887
11	50.19747	50.30959	4.090031	41.63911	58.98007
12	44.90539	44.99529	4.090031	36.32481	53.66577
13	56.48572	58.66636	3.990502	50.20687	67.12585
14	51.13022	56.00921	3.990502	47.54972	64.4687
15	48.22103	45.97231	3.990502	37.51283	54.4318
16	46.90434	43.31516	3.990502	34.85567	51.77465
17	59.20802	51.98972	3.990502	43.53023	60.4492
18	53.34076	49.33257	3.990502	40.87308	57.79205
19	36.99559	39.29567	3.990502	30.83618	47.75515
20	38.86201	36.63852	3.990502	28.17903	45.098

Residual Report

Row	Actual	Predicted	Residual	Percent Error	MSEi
1	44.64399	47.65244	-3.008446	6.74	13.09069
2	43.8597	47.65244	-3.792744	8.65	12.71636
3	45.61451	47.65244	-2.037933	4.47	13.43438
4	48.38661	47.65244	0.7341753	1.52	13.68801
5	45.62893	47.65244	-2.023505	4.43	13.43849
6	45.81519	47.65244	-1.837245	4.01	13.48896
7	60.46495	54.32908	6.135866	10.15	10.14023
8	40.92583	40.9758	-4.996345E-02	0.12	13.72559
9	58.82191	60.34649	-1.524575	2.59	13.50447
10	32.63659	34.95839	-2.321801	7.11	13.21243
11	50.19747	50.30959	-0.1121156	0.22	13.72463
12	44.90539	44.99529	-8.989861E-02	0.20	13.72506
13	56.48572	58.66636	-2.18064	3.86	13.31008
14	51.13022	56.00921	-4.878993	9.54	11.64456
15	48.22103	45.97231	2.248715	4.66	13.28371
16	46.90434	43.31516	3.589181	7.65	12.59952
17	59.20802	51.98972	7.218305	12.19	9.170297
18	53.34076	49.33257	4.008194	7.51	12.32119
19	36.99559	39.29567	-2.300071	6.22	13.26329
20	38.86201	36.63852	2.223493	5.72	13.29358

Regression Diagnostics Section

Row	Studentized Residual	Rstudent	Hat Diagonal	Cook's D	Dffits	Covratio
1	-0.860449	-0.853098	0.050000	0.009742	-0.195714	1.127423
2	-1.084768	-1.091215	0.050000	0.015483	-0.250342	1.003895
3	-0.582872	-0.570452	0.050000	0.004470	-0.130871	1.250568
4	0.209982	0.203595	0.050000	0.000580	0.046708	1.347713
5	-0.578745	-0.566327	0.050000	0.004407	-0.129924	1.252100
6	-0.525473	-0.513235	0.050000	0.003633	-0.117744	1.271013
7	2.044428	2.303048	0.300000	0.447824	1.507699	0.550875
8	-0.016647	-0.016119	0.300000	0.000030	-0.010552	1.849212
9	-0.507978	-0.495862	0.300000	0.027647	-0.324618	1.732894
10	-0.773608	-0.763457	0.300000	0.064122	-0.499800	1.587788
11	-0.037356	-0.036172	0.300000	0.000150	-0.023680	1.848695
12	-0.029954	-0.029003	0.300000	0.000096	-0.018987	1.848926
13	-0.696160	-0.684500	0.237500	0.037738	-0.382020	1.501214
14	-1.557598	-1.637377	0.237500	0.188918	-0.913820	0.879452
15	0.717893	0.706569	0.237500	0.040131	0.394336	1.489356
16	1.145831	1.157973	0.237500	0.102236	0.646265	1.205413
17	2.304413	2.729754	0.237500	0.413508	1.523475	0.338263
18	1.279599	1.307683	0.237500	0.127500	0.729818	1.102378
19	-0.734288	-0.723262	0.237500	0.041985	-0.403652	1.480217
20	0.709841	0.698385	0.237500	0.039236	0.389769	1.493784

Multicollinearity Section

Independent Variable	Variance Inflation	R-Squared Vs Other X's	Tolerance	Diagonal of X'X Inverse
Lac	1.000000	0.000000	1.000000	0.0625
ABTS	1.000000	0.000000	1.000000	0.0625
O2	1.000000	0.000000	1.000000	0.0625

Eigenvalues of Centered Correlations

No.	Eigenvalue	Incremental Percent		Cumulative Percent	Condition Number
1	1.000000	33.33	33.33	1.00	
2	1.000000	33.33	66.67	1.00	
3	1.000000		33.33	100.00	1.00

All Condition Numbers less than 100. Multicollinearity is NOT a problem.

Eigenvector Percent of Regression-Coefficient-Variance using Centered Correlations

No.	Eigenvalue	Lac	ABTS	O2
1	1.000000	0.00	0.00	100.00
2	1.000000	100.00	0.00	0.00
3	1.000000	0.00	100.00	0.00

Eigenvalues of Uncentered Correlations

No.	Eigenvalue	Incremental Percent	Cumulative Percent	Condition Number
1	1.000000	25.00	25.00	1.00
2	1.000000	25.00	50.00	1.00
3	1.000000	25.00	75.00	1.00
4	1.000000	25.00	100.00	1.00

All Condition Numbers less than 100. Multicollinearity is NOT a problem.

Eigenvector Percent of Regression-Coefficient-Variance using Uncentered Correlations

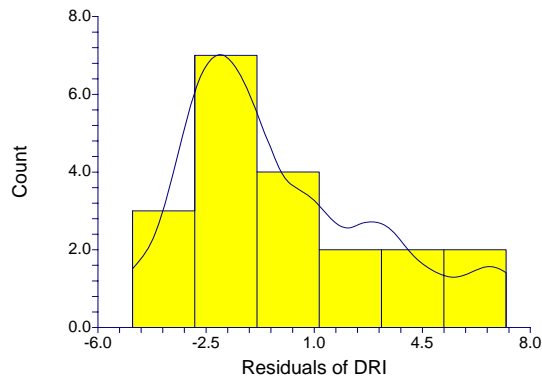
No.	Eigenvalue	Lac	ABTS	O2	Intercep
1	1.000000	0.00	0.00	0.00	100.00
2	1.000000	100.00	0.00	0.00	0.00
3	1.000000	0.00	100.00	0.00	0.00
4	1.000000	0.00	0.00	100.00	0.00

DFBETAS Section

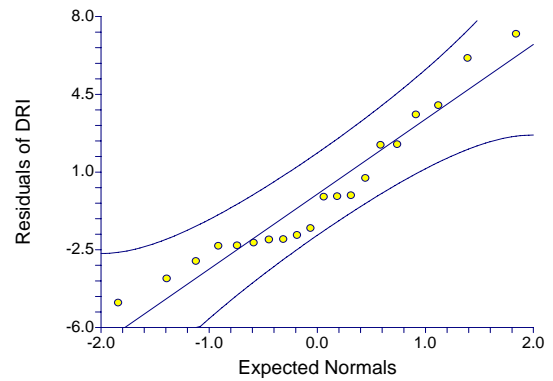
Row	Lac	ABTS	O2	Intercept
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2	0.0000	0.0000	0.0000	-0.2503
3	0.0000	0.0000	0.0000	-0.1309
4	0.0000	0.0000	0.0000	0.0467
5	0.0000	0.0000	0.0000	-0.1299
6	0.0000	0.0000	0.0000	-0.1177
7	1.3763	0.0000	0.0000	0.6155
8	0.0096	0.0000	0.0000	-0.0043
9	0.0000	-0.2963	0.0000	-0.1325
10	0.0000	0.4563	0.0000	-0.2040
11	0.0000	0.0000	-0.0216	-0.0097
12	0.0000	0.0000	0.0173	-0.0078
13	-0.1960	-0.1960	-0.1960	-0.1753
14	-0.4688	-0.4688	0.4688	-0.4193
15	0.2023	-0.2023	0.2023	0.1809
16	0.3315	-0.3315	-0.3315	0.2965
17	-0.7815	0.7815	0.7815	0.6990
18	-0.3744	0.3744	-0.3744	0.3349
19	0.2071	0.2071	-0.2071	-0.1852
20	-0.1999	-0.1999	-0.1999	0.1788

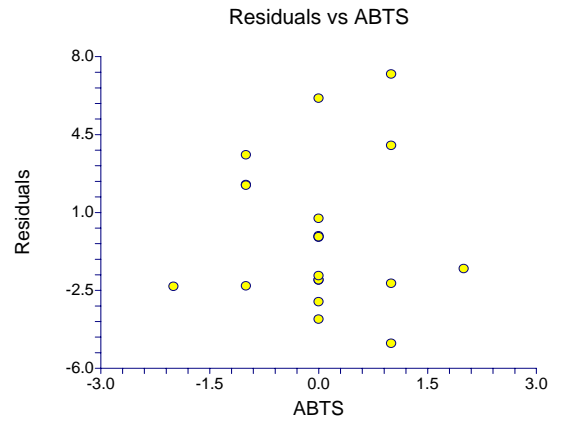
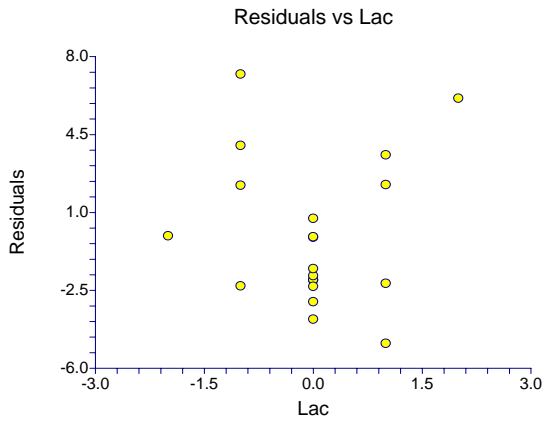
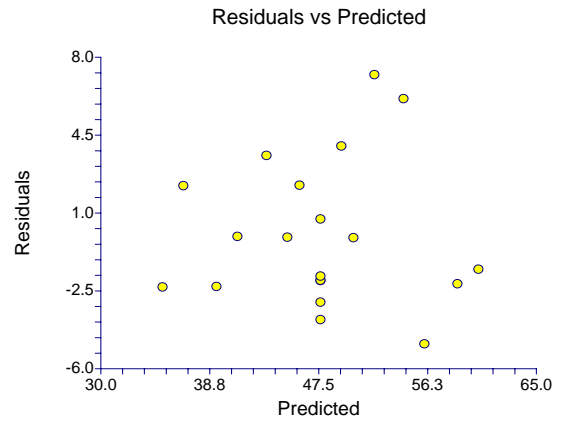
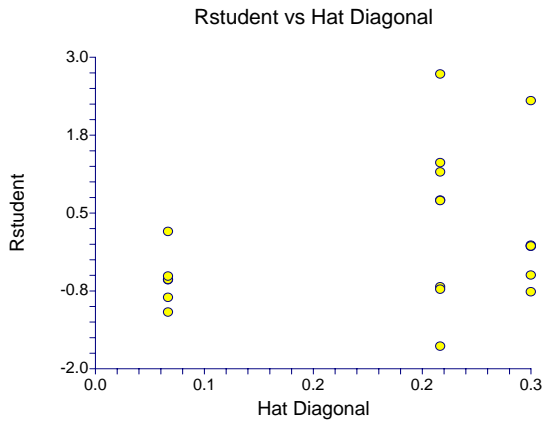
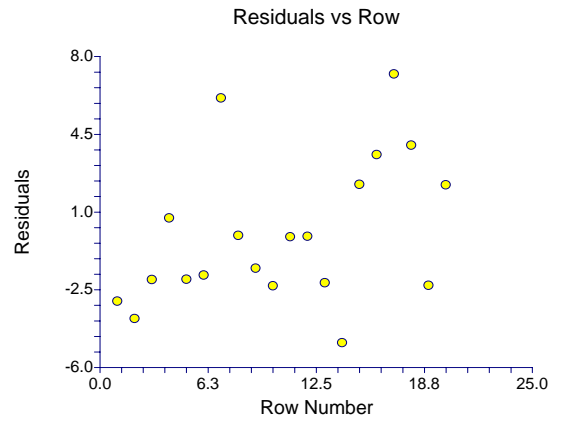
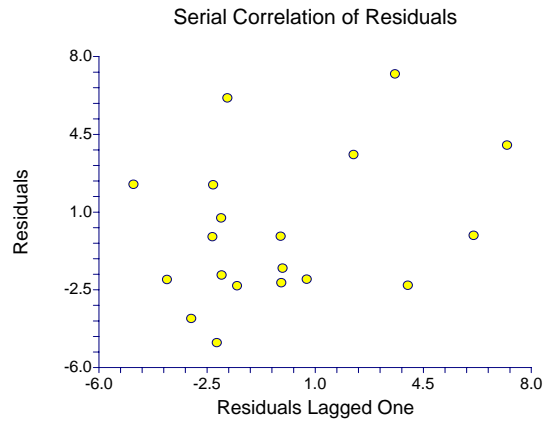
Plots Section

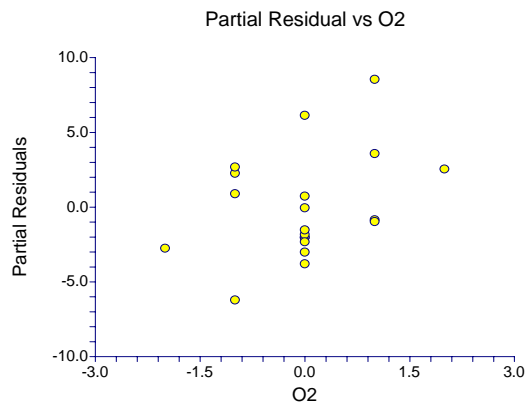
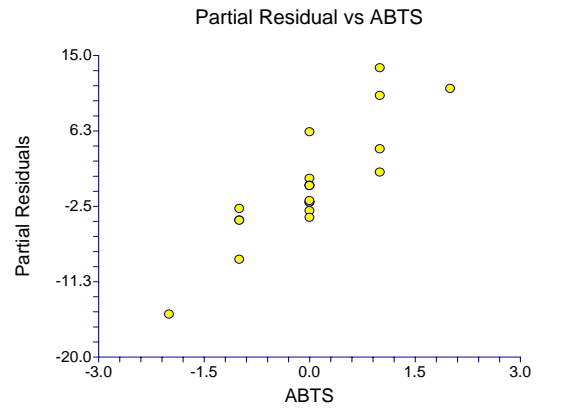
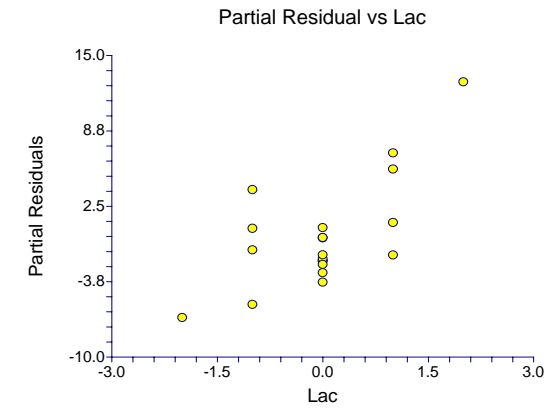
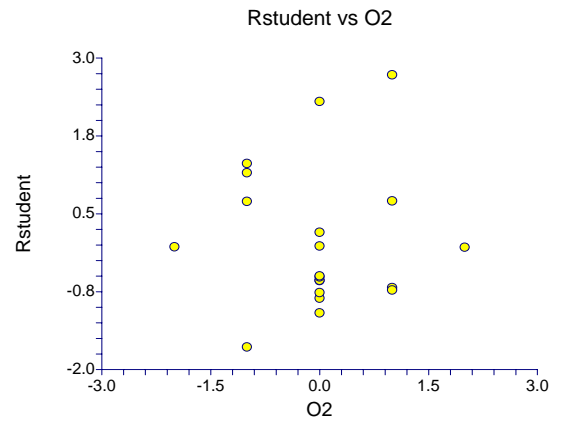
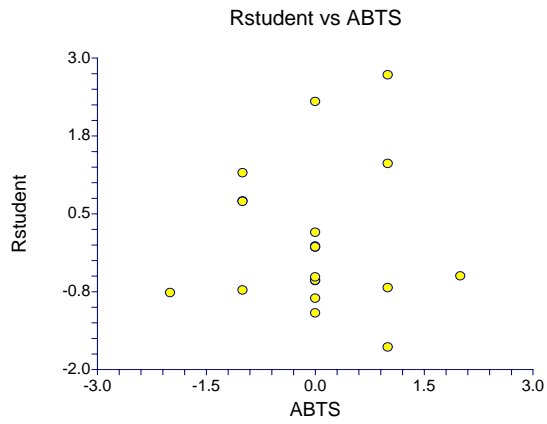
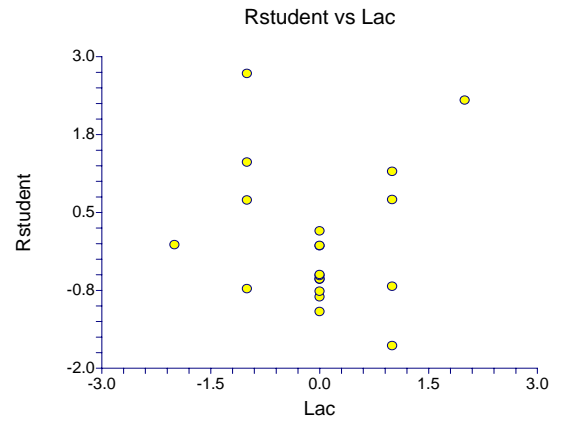
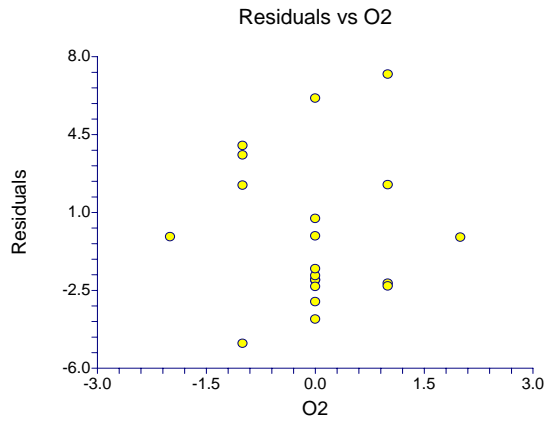
Histogram of Residuals of DRI



Normal Probability Plot of Residuals of DRI







A.5.4.4 Multivariate Ratio of Polynomials Report

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 Dependent DRI

Minimization Phase Section

Itn	Error Sum					
No.	Lambda	Lambda	B0	B1	B2	B3
0	11.73489	0.00004	45.65816	-16.05398	15.72261	2.330253

Convergence criterion met.

Model Estimation Section

Parameter Name	Term	Terms	Parameter Estimate	Asymptotic Standard Error	Lower 100% C.L.	Upper 100% C.L.
B0	Intercept		45.65816	0	45.65816	45.65816
B1	U		-16.05398	0	-16.05398	-16.05398
B2	U2	Lac	15.72261	0	15.72261	15.72261
B3	V	Lac^2	2.330253	0	2.330253	2.330253
B4	UV	ABTS	47.34002	0	47.34002	47.34002
B5	V2	Lac*ABTS	9.275566	0	9.275566	9.275566
B6	W	ABTS^2	1.32302	0	1.32302	1.32302
B7	UW	O2	1.453849	0	1.453849	1.453849
B8	VW	Lac*O2	4.770414	0	4.770414	4.770414
B9	W2	ABTS*O2	0.473319	0	0.473319	0.473319
B10	u	O2^2	-0.5431627	0	-0.5431627	-0.5431627
B11	u2	Lac	0.3376349	0	0.3376349	0.3376349
B12	v	Lac^2	-0.2267051	0	-0.2267051	-0.2267051
B13	uv	ABTS	1.141829	0	1.141829	1.141829
B14	v2	Lac*ABTS	0.2349017	0	0.2349017	0.2349017
B15	w	ABTS^2	0	0	0	0
B16	uw	O2	0	0	0	0
B17	vw	Lac*O2	4.349544E-0	0	4.349544E-	4.349544E-
B18	w2	ABTS*O2	0	0	0	0

R-Squared 0.988898

Iterations 0

Symbolic Model

$$Y = P1 (U, V, W) / P2 (U, V, W)$$

$$P1 (U, V, W) = B0+B1*U+B2*U2+B3*V+B4*UV+B5*V2+B6*W+B7*UW+B8*VW+B9*W2$$

$$P2 (U, V, W) = 1+B10*U+B11*U2+B12*V+B13*UV+B14*V2+B15*W+B16*UW+B17*VW+B18*W2$$

where

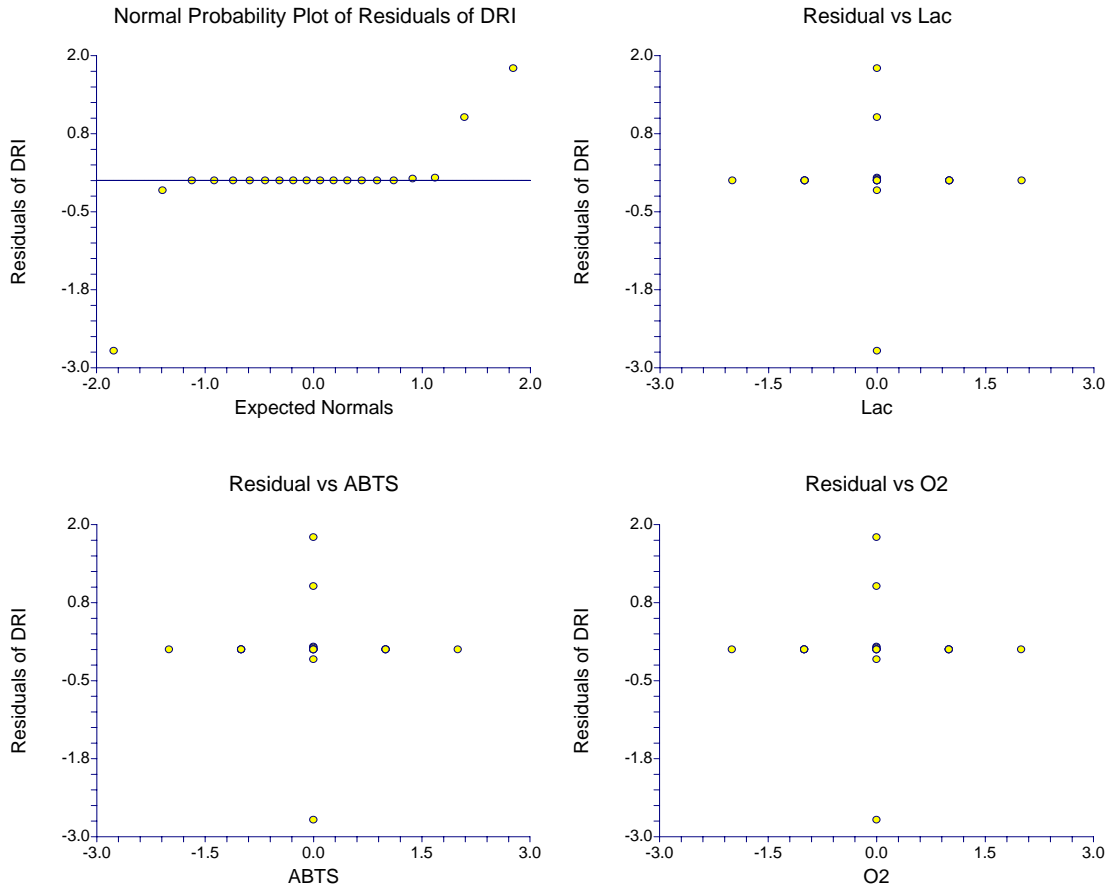
$$Y = \text{DRI}$$

$$V = \text{ABTS}$$

$$U = \text{Lac}$$

$$W = \text{O2}$$

Plot Section



A.5.4.5 Robust Regression Report

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 Dependent DRI

Regression Equation Section

Independent Variable	Regression Coefficient	Standard Error	T-Value (Ho: B=0)	Prob Level	Decision (5%)	Power (5%)
Intercept	46.81763	0.5791607	80.8370	0.000000	Reject Ho	1.000000
Lac	3.054206	0.7395132	4.1300	0.000786	Reject Ho	0.972030
ABTS	6.066785	0.6475289	9.3691	0.000000	Reject Ho	1.000000
O2	1.137815	0.6292678	1.8082	0.089415	Accept Ho	0.397471
R-Squared	0.886402					

Regression Coefficient Section

Independent Variable	Regression Coefficient	Standard Error	Lower 95% C.L.	Upper 95% C.L.	Standardized Coefficient
Intercept	46.81763	0.5791607	45.58986	48.04539	0.000000
Lac	3.054206	0.7395132	1.486508	4.621904	0.353256
ABTS	6.066785	0.6475289	4.694085	7.439485	0.799430
O2	1.137815	0.6292678	-0.1961731	2.471803	0.152736
T-Critical	2.119905				

Analysis of Variance Section

Source	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level	Power (5%)
Intercept	1	31797.01	31797.01			
Model	3	606.8008	202.267	41.6159	0.000000	0.999073
Error	16	77.76528	4.86033			
Total(Adjusted)		19	684.5662		36.0298	

Root Mean Square Error 2.204616 R-Squared 0.886402
 Mean of Dependent Variable 46.47531 Adj R-Squared 0.865102
 Coefficient of Variation 4.743628E-02

Residual-Percentile Section

Iter. No.	Max % Chang in any Beta	----- Percentiles of Absolute Residuals -----			
		25th	50th	75th	100th
1	0.0000	1.68091	2.236104	3.690963	7.218305
2	36.1484	1.28571	1.781523	3.227545	8.768864
3	18.8927	1.182553	1.941793	3.363799	8.550542
4	6.7566	1.114407	2.009939	3.469392	8.34147
5	2.1342	1.096623	2.027723	3.471458	8.283598
6	0.6262	1.091036	2.003117	3.478626	8.268036
7	0.9181	1.080936	1.9795	3.482112	8.255285
8	0.8055	1.071993	1.963812	3.48249	8.247221
9	0.5819	1.065481	1.954287	3.481377	8.243087
10	0.3976	1.061005	1.948794	3.479798	8.241152
11	0.2631	1.058032	1.945781	3.478276	8.240311
12	0.1689	1.056118	1.944221	3.477025	8.239984
13	0.1051	1.054925	1.943475	3.476089	8.239883
14	0.0633	1.054206	1.943161	3.475434	8.239877
15	0.0367	1.053788	1.943059	3.475	8.239902
16	0.0205	1.053555	1.943051	3.474725	8.239931
17	0.0110	1.05343	1.943078	3.474557	8.239957
18	0.0056	1.053366	1.943112	3.474459	8.239976
19	0.0026	1.053337	1.943141	3.474404	8.239989
20	0.0011	1.053324	1.943164	3.474374	8.239998

Robust Regression-Coefficient Section

Iter.	Intercept	Lac	ABTS	O2
1	47.65244	3.338322	6.347024	1.328575
2	47.02871	3.349287	5.911412	0.848316
3	46.97453	3.29421	5.968573	1.008586
4	46.89972	3.110624	6.00072	1.076732
5	46.85357	3.044237	6.020569	1.094516
6	46.83173	3.025175	6.033324	1.100103
7	46.82609	3.030473	6.046911	1.110203
8	46.82393	3.038511	6.056231	1.119146
9	46.82235	3.044704	6.061631	1.125659
10	46.82101	3.048809	6.064528	1.130134
11	46.81995	3.051328	6.065979	1.133107
12	46.81916	3.052783	6.066642	1.135022
13	46.8186	3.053571	6.066899	1.136215
14	46.81821	3.053969	6.066963	1.136933
15	46.81797	3.05415	6.066947	1.137351
16	46.81782	3.05422	6.066905	1.137585
17	46.81773	3.054236	6.066863	1.13771
18	46.81767	3.05423	6.066827	1.137773
19	46.81764	3.054218	6.066802	1.137803
20	46.81763	3.054206	6.066785	1.137815

Robust Residuals and Weights Section

Row	Actual Y	Predicted Value	Residual	Robust Weight
1	44.64399	46.81763	-2.173635	0.804706
2	43.8597	46.81763	-2.957934	0.656632
3	45.61451	46.81763	-1.203123	0.938010
4	48.38661	46.81763	1.568985	0.895489
5	45.62893	46.81763	-1.188695	0.939478
6	45.81519	46.81763	-1.002435	0.956936
7	60.46495	52.92604	7.538908	0.173571
8	40.92583	40.70922	0.2166146	0.998667
9	58.82191	58.9512	-0.1292854	1.000000
10	32.63659	34.68406	-2.047471	0.825573
11	50.19747	49.09326	1.104213	0.947741
12	44.90539	44.542	0.3633926	0.994917
13	56.48572	57.07644	-0.5907142	0.985394
14	51.13022	54.8008	-3.670587	0.503165
15	48.22103	44.94286	3.278161	0.589188
16	46.90434	42.66724	4.237108	0.376240
17	59.20802	50.96802	8.239998	0.210207
18	53.34076	48.69239	4.648369	0.284977
19	36.99559	38.83445	-1.838857	0.857922
20	38.86201	36.55882	2.303188	0.782333

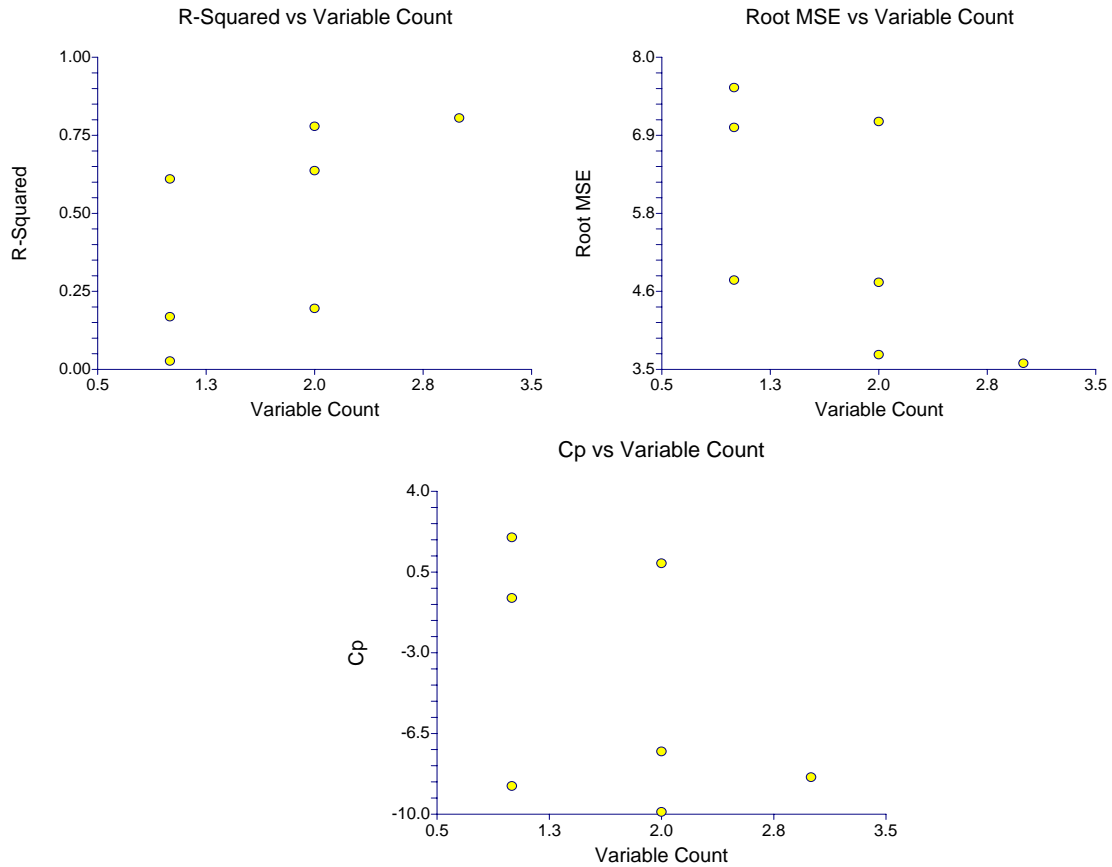
A.5.4.6 All Possible Regression Report

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 Dependent DRI

All Possible Results Section

Model Size	R-Squared	Root MSE	Cp	Model
1	0.609800	4.786785	16.051648	B (ABTS)
1	0.168695	6.986832	52.284662	A (Lac)
1	0.026719	7.559958	63.946842	C (O2)
2	0.778495	3.711106	4.194734	AB
2	0.636519	4.753932	15.856914	BC
2	0.195414	7.07291	52.089928	AC
3	0.805214	3.587195	4.000000	ABC

Plots Section



A.6 REACTION RATE STUDIES

A series of experiments were performed to examine the reaction rates of laccase-mediator decolorization of Direct Yellow 11 in solution. Initial experiments have indicated that results could be obtained by matching each dye decolorization reaction with a control with the same laccase-mediator concentrations but containing no dye. The Perkin-Elmer Lambda 900 UV/Vis/NIR spectrophotometer has the ability to subtract spectra yielding a difference spectrum corresponding to small differences between large absorbances.

A.6.1 REACTION CONDITIONS

Standard reaction conditions were used for these studies. The dye concentration was fixed at 20 ppm Direct Yellow 11. Laccase addition was constant at 10.3 U or 5.0 μL laccase is added per mL of reaction solution. Mediator concentrations did vary and the concentrations selected for study are shown in Table A-28. Samples from each reaction were collected at 18 time points as indicated in Table A-29. (Only one 48 hour time point was collected for each mediator: the remaining 18 samples were collected for each mediator concentration). In most cases, the absorbance of the reaction solution is too high to read directly. Samples were diluted to obtain solutions with absorbances less than 1 AU in the region around 410 nm (λ_{Max} for Direct Yellow 11). The dilutions made varied with the mediator concentration and ranged from 1:5 to 1:200. The same dilution was applied to all samples and controls for a given mediator concentration.

Table A-28 Laccase Mediator Concentrations for Decolorization Reaction Rates

Mediator Concentrations:					
Zero Mediator					
ABTS	1.0	0.10	0.010		mM
VA	4.0	8.0	16	32	mM
HBT	2.0	4.0	8.0	16	mM

Table A-29 Time Points for Sample Collection Reaction Rate Studies

Time Points:										
0	5	10	15	20	30	45	60	75	90	Minutes
2	3	4	6	8	10	12	24	48		Hours

A.6.2 CALCULATION OF DIFFERENCE SPECTRA

As mentioned, difference spectra were calculated to show small differences in large absorbances. For every spectrum obtained from laccase/mediator treatment of Direct Yellow 11, a matching spectrum was obtained for the same time period from a solution with the same amount of laccase and mediator, but no dye. Subtraction of the spectrum of the no dye control from the spectrum of the dye/laccase/mediator solution yields the difference spectrum for that time point for that mediator concentration.

For ABTS, difference spectra were obtained that showed a single dye peak that could be quantified. For low concentration, the peak was clear (Figure A-56). At higher concentrations the peak can be quantified, but the peak shape is distinctive in the difference spectrum (Figure A-57).

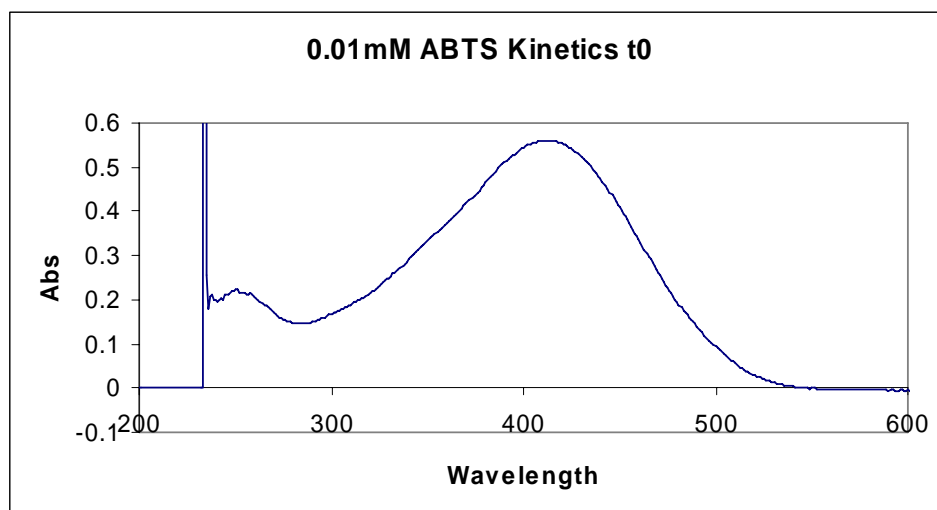
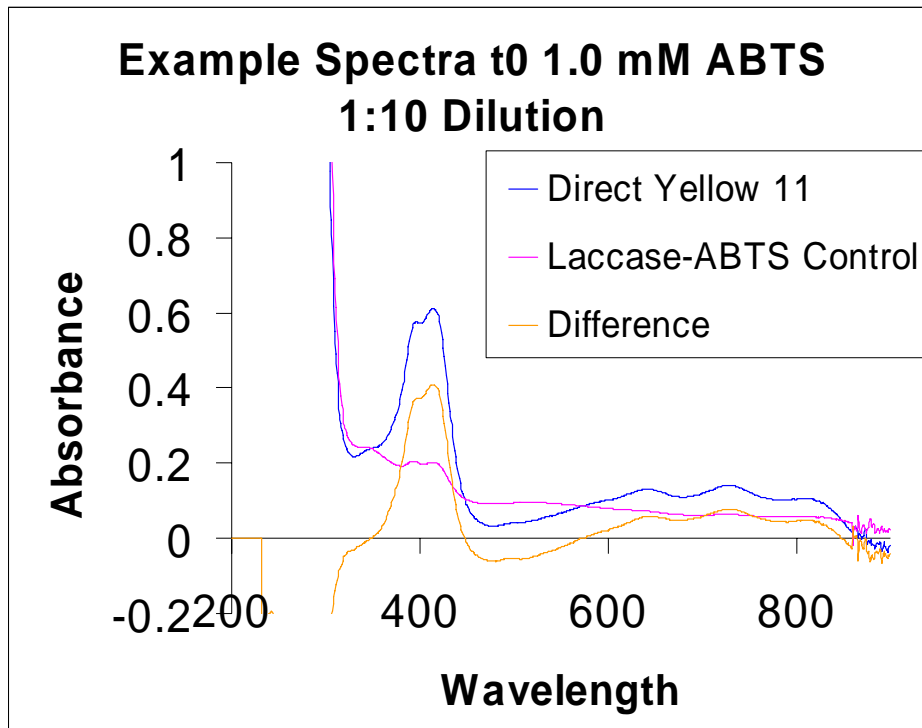
Figure A-56 Difference Spectra for Laccase Treatment with 0.01 mM ABTS

Figure A-57 Example of Original and Difference Spectra for 1.0 mM ABTS



The unusual peak shape in the difference spectra became more pronounced for the selected concentrations of hydroxyl benzotriazole (HBT) and violuric acid (VA).

Figure A-58 Example of Original and Difference Spectra for 2.0 mM HBT

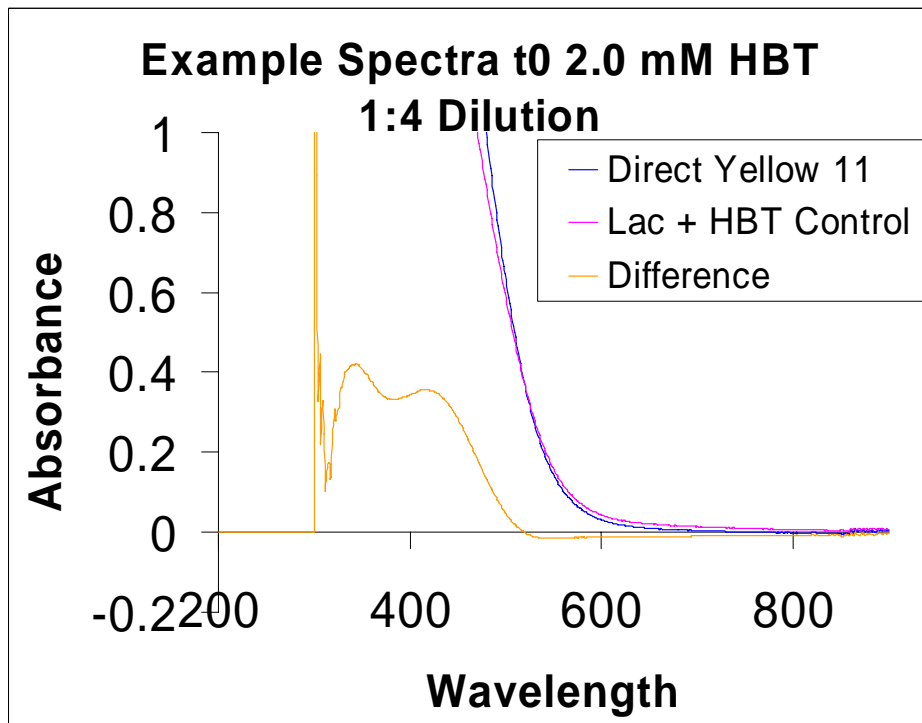
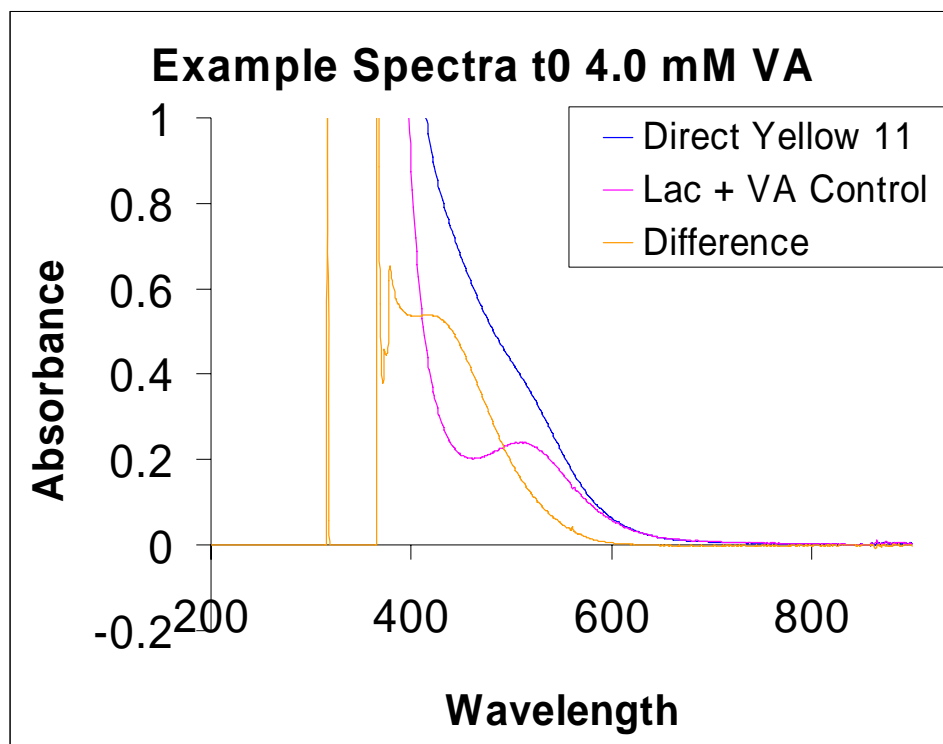


Figure A-59 Example of Original and Difference Spectra for 4.0 mM HBT



A.6.3 DIFFERENCE SPECTRA RESULTS FOR REACTION RATE STUDIES

The difference spectra obtained for each reaction rate study are shown in the following figures. The ABTS spectra suggested that the wavelength range of concern is 285-550 nm, so only that range is plotted. The data were zeroed by subtracting the lowest absorbance within that range.

For each mediator concentration, 18 time points were measured. For clarification purposes, the data for each concentration was divided into three sets of six time points for plotting. It can be observed that the time zero spectrum usually resembles the spectrum of Direct Yellow 11 (broad peak with maximum absorbance 400-412 nm), while spectra from the other time points are distinctly different with peaks in the 370-390 range.

The three graphs for a mediator concentration are followed by a graph showing either the peak area (for ABTS graphs) or the peak height for the peak corresponding to Direct Yellow 11. For ABTS, the area does appear to follow a recognizable pattern of decreasing with time. For VA and HBT, no such pattern is evident.

A.6.3.1 ABTS Data

Figure A-60 1 mM ABTS Reaction Rate Study Data

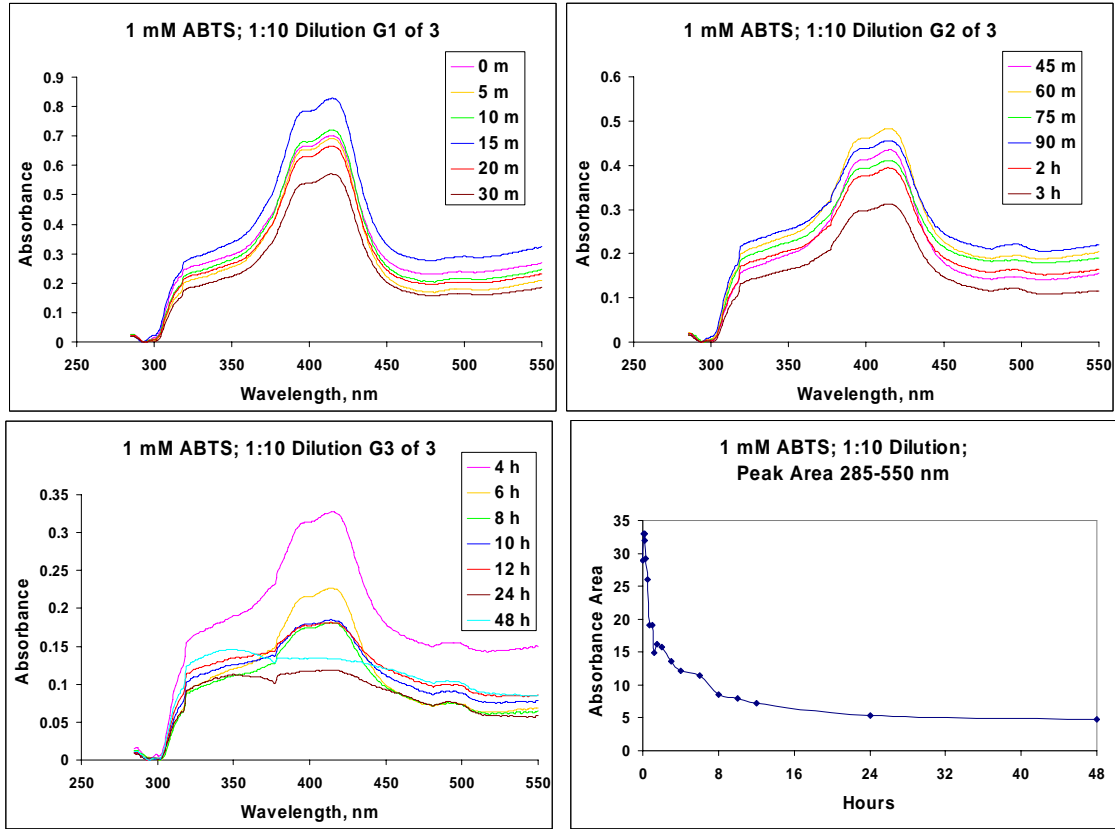
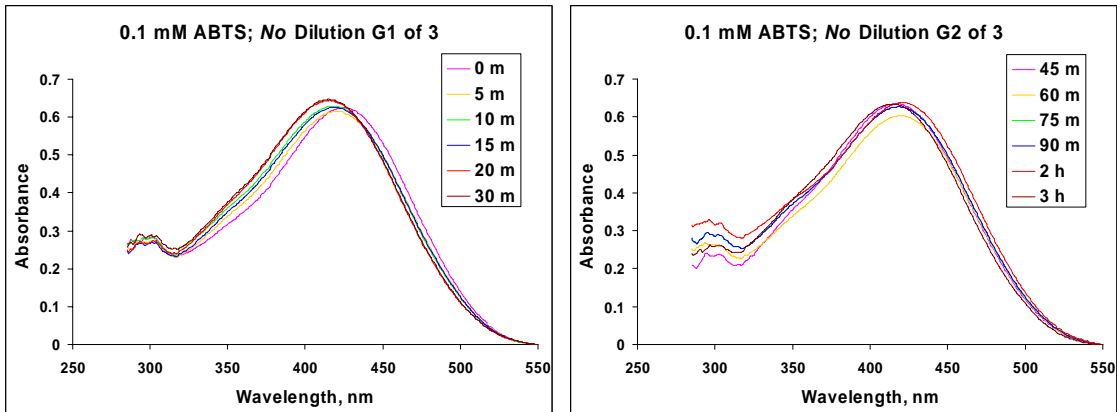


Figure A-61 0.1 mM ABTS Reaction Rate Study Data



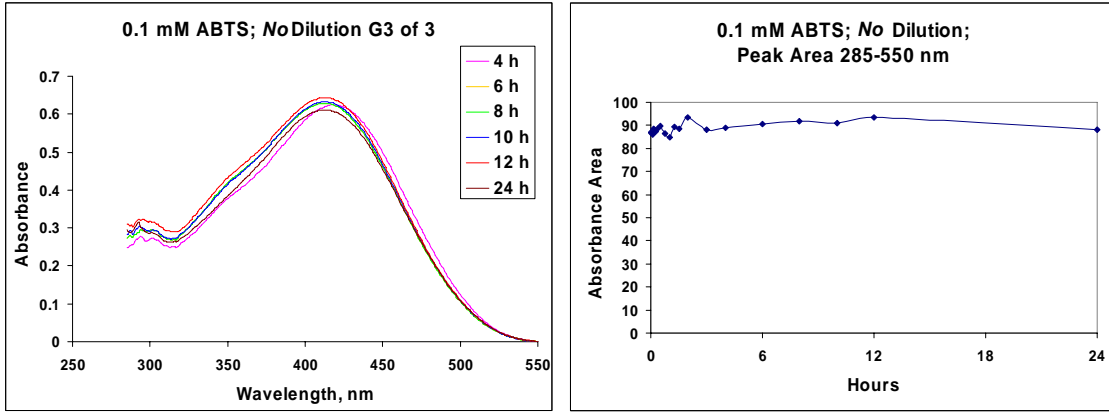
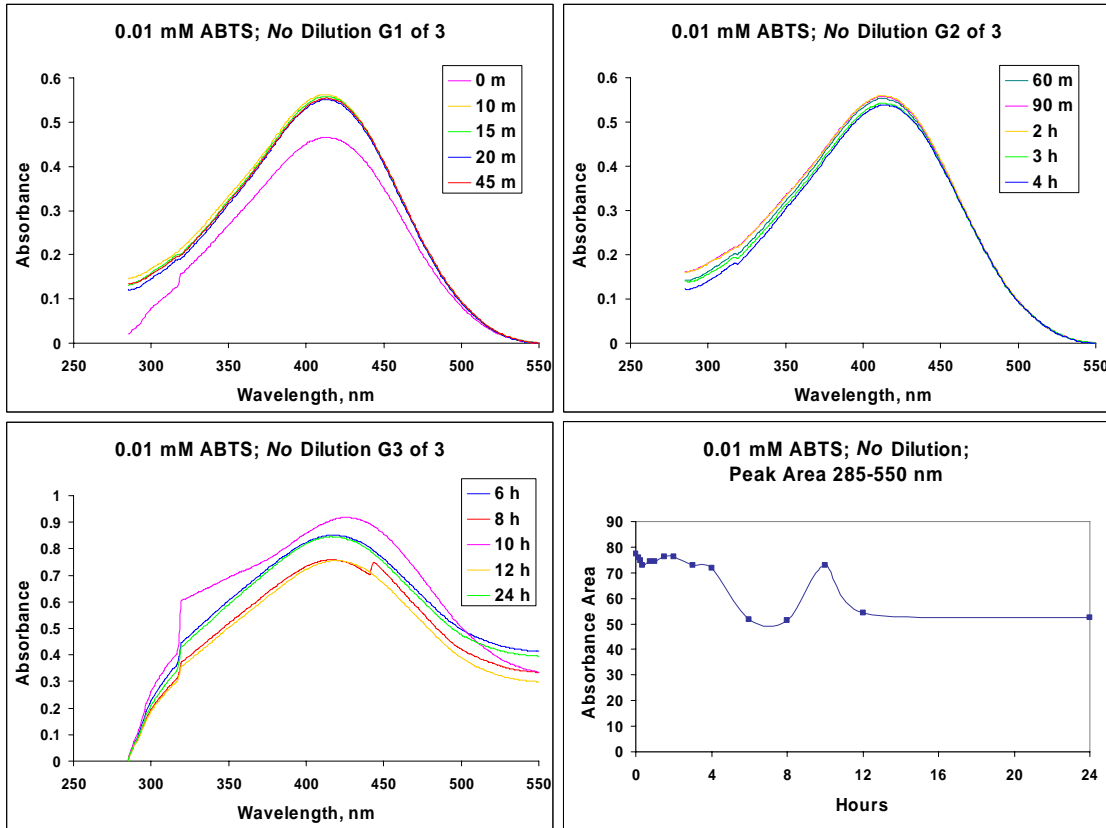


Figure A-62 0.01 mM ABTS Reaction Rate Study Data



A.6.3.2 *Violic Acid Data*

Figure A-63 4 mM VA Reaction Rate Study Data

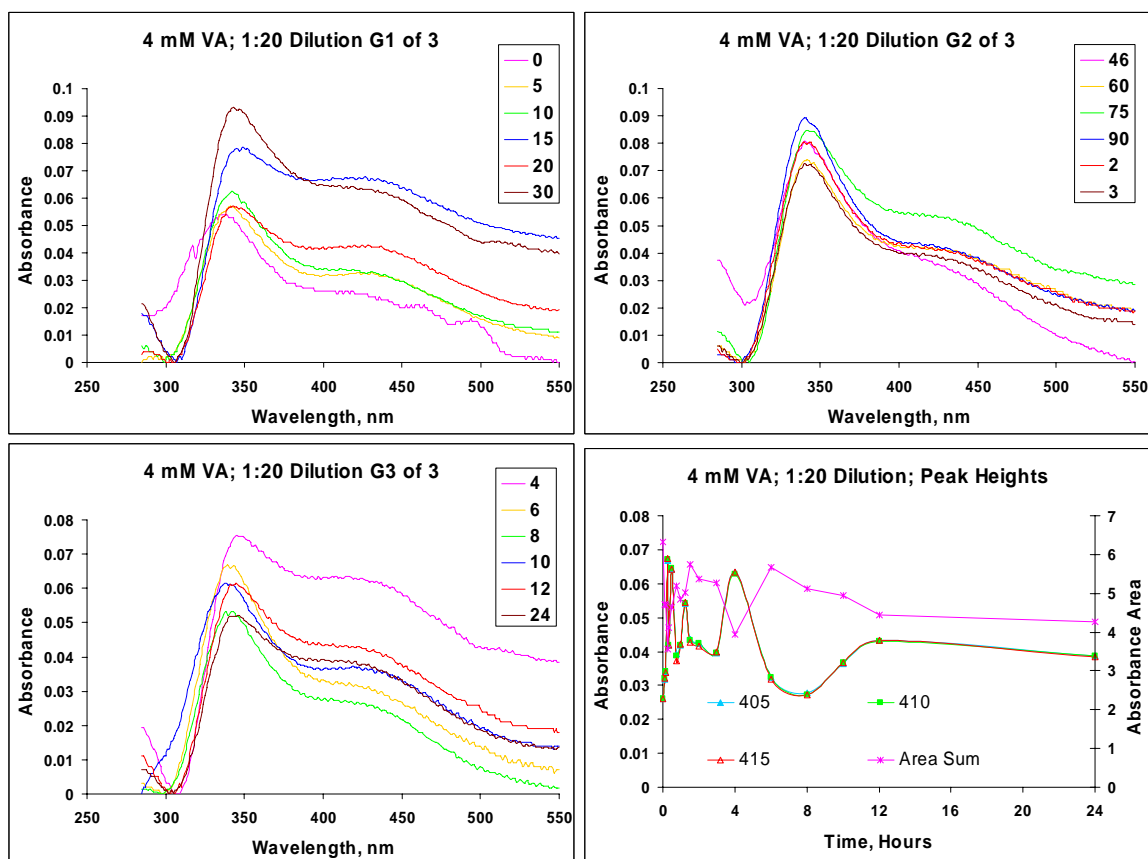
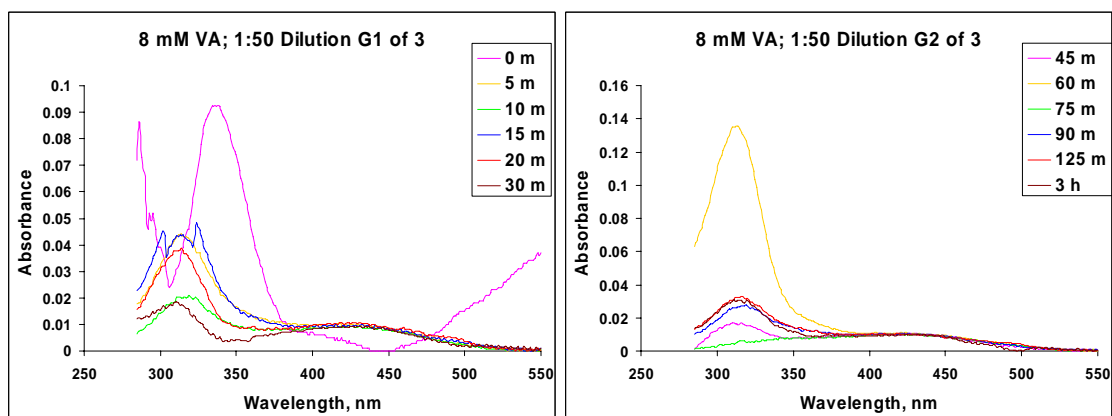


Figure A-64 8 mM VA Reaction Rate Study Data



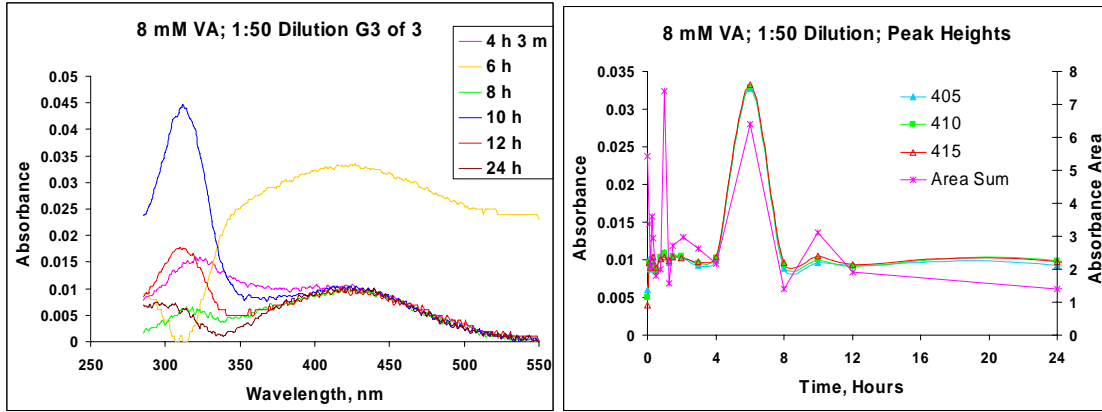


Figure A-65 16 mM VA Reaction Rate Study Data

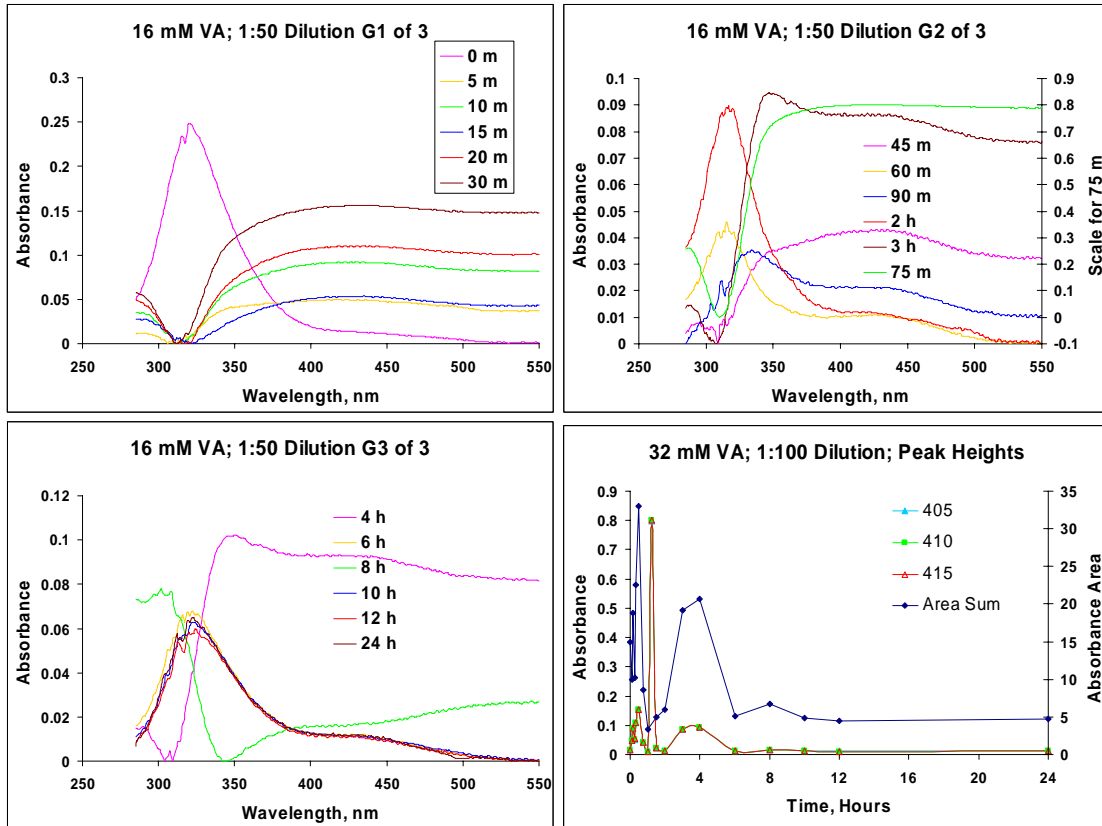
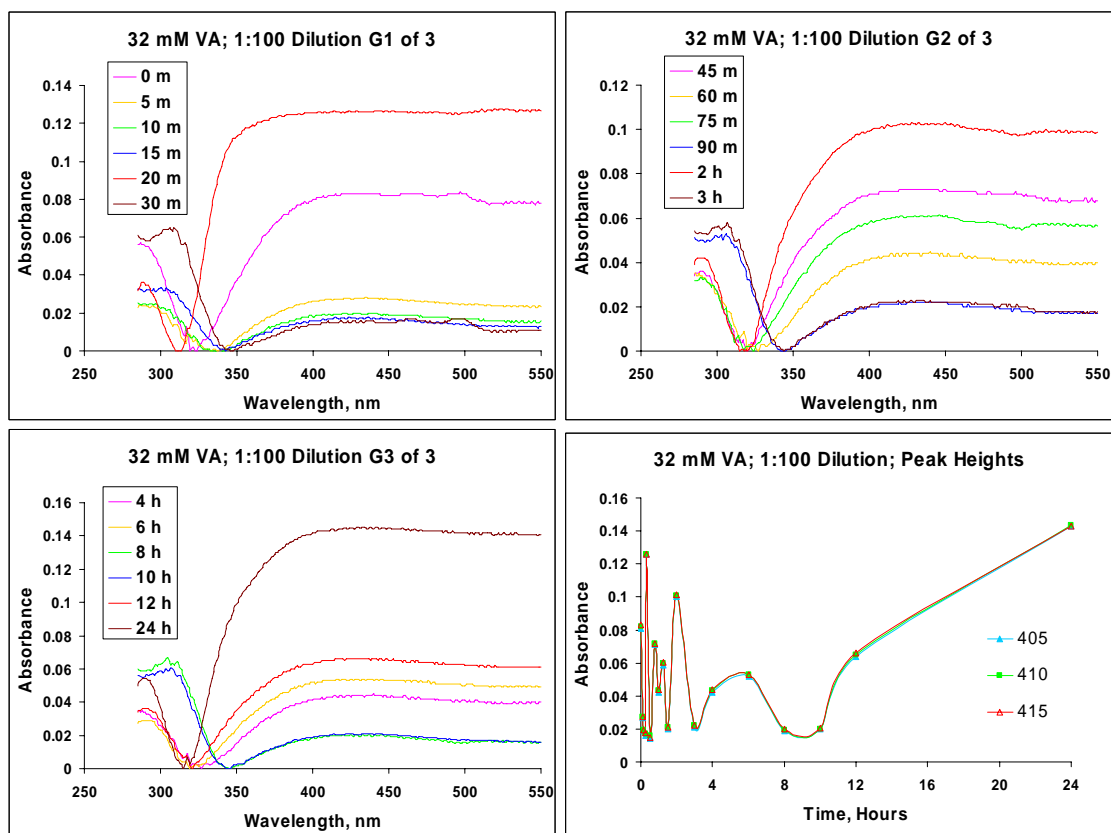
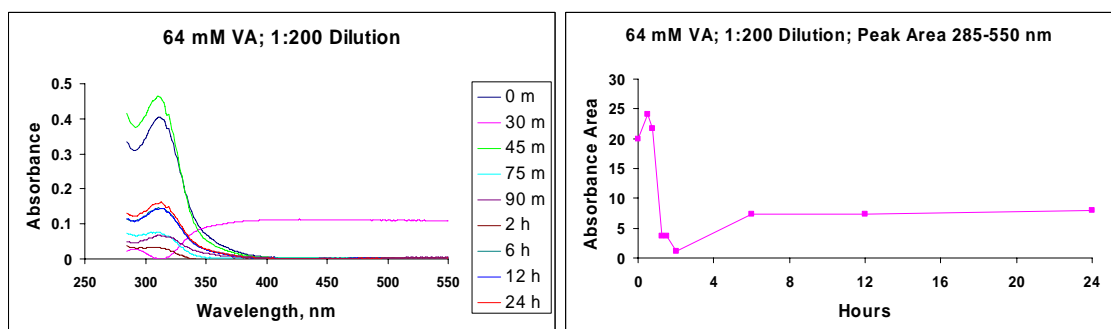


Figure A-66 32 mM VA Reaction Rate Study Data

An experiment utilizing 64 mM violuric acid was also performed. However, no peak around 400-415 nm due to Direct Yellow 11 can be distinguished in the difference spectra of these dark solution which had to be highly diluted to be measured.

Figure A-67 64 mM VA Reaction Rate Study Data

A.6.3.3 HBT Data

Figure A-68 2 mM HBT Reaction Rate Study Data

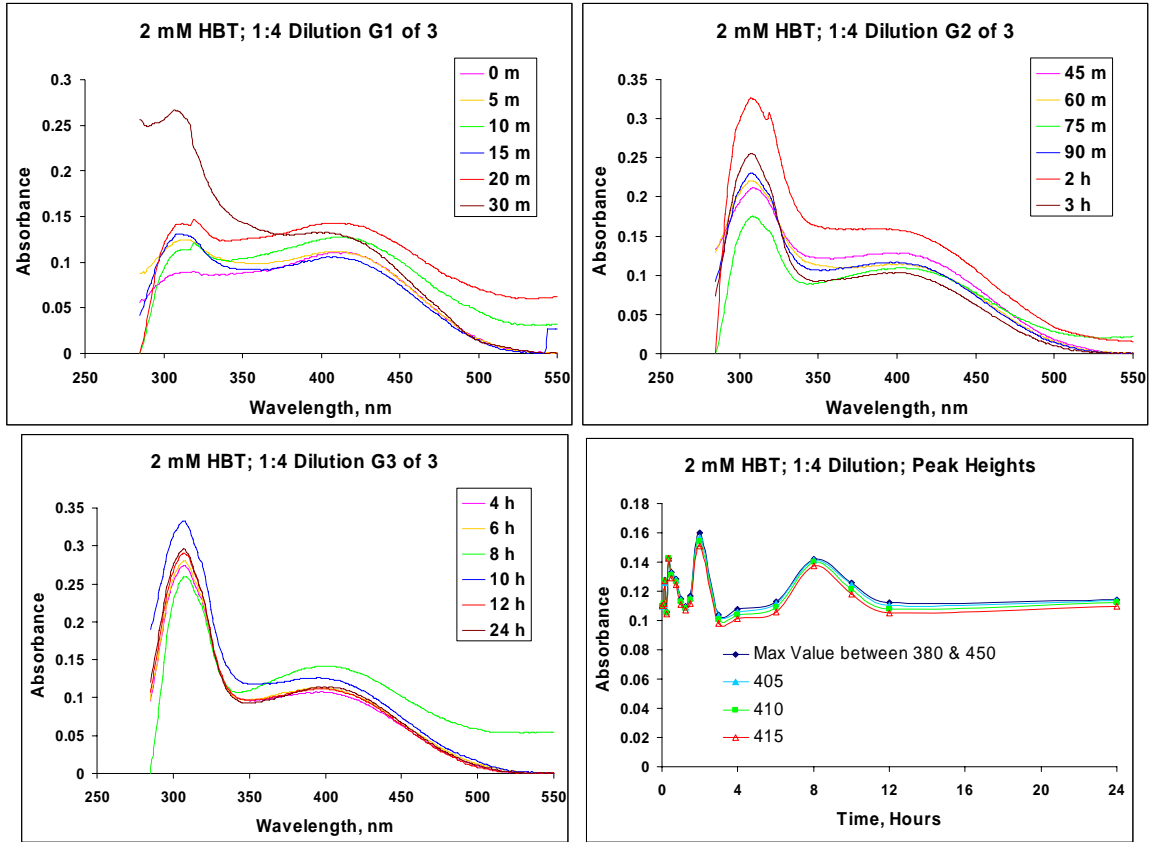
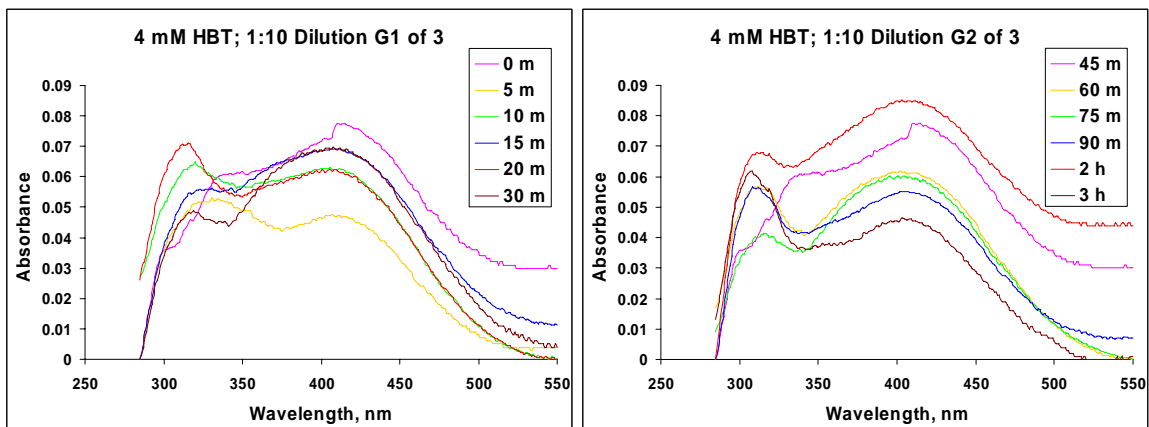


Figure A-69 4 mM HBT Reaction Rate Study Data



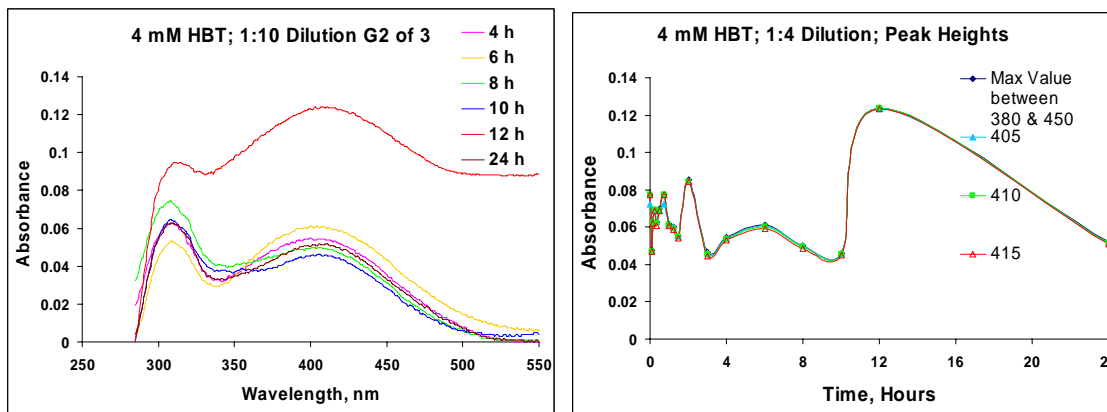


Figure A-70 8 mM HBT Reaction Rate Study Data

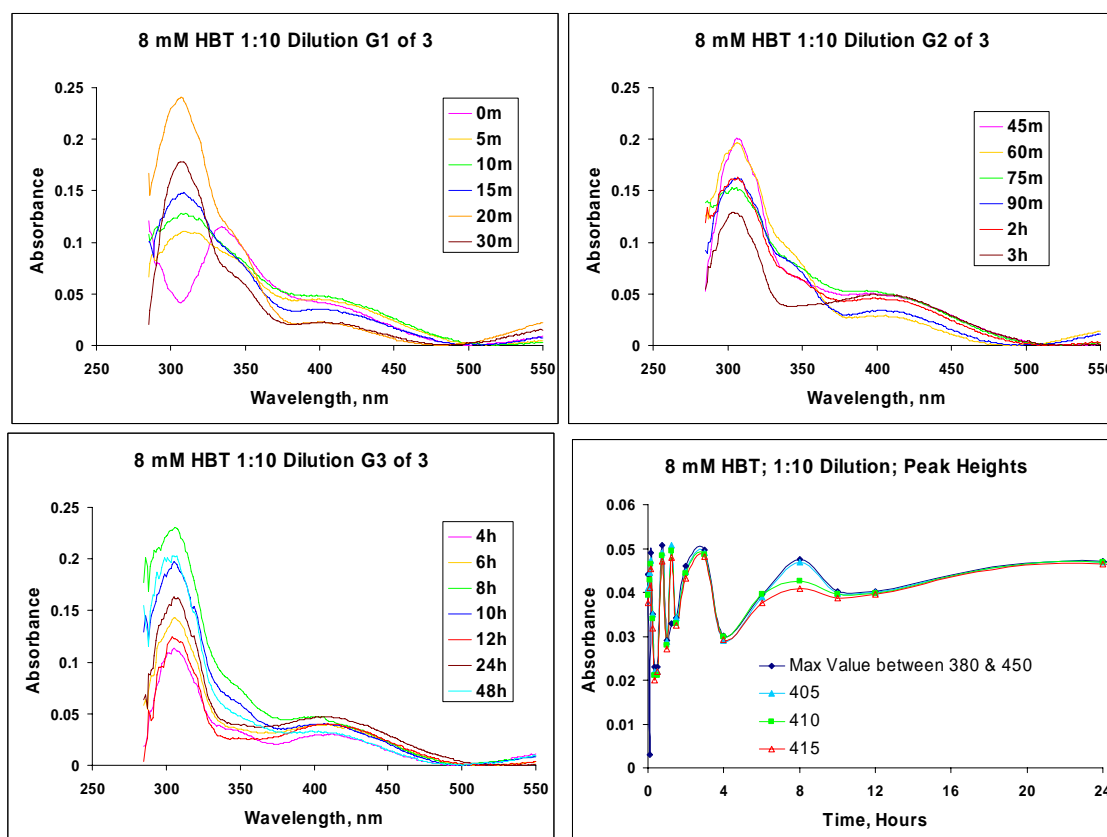
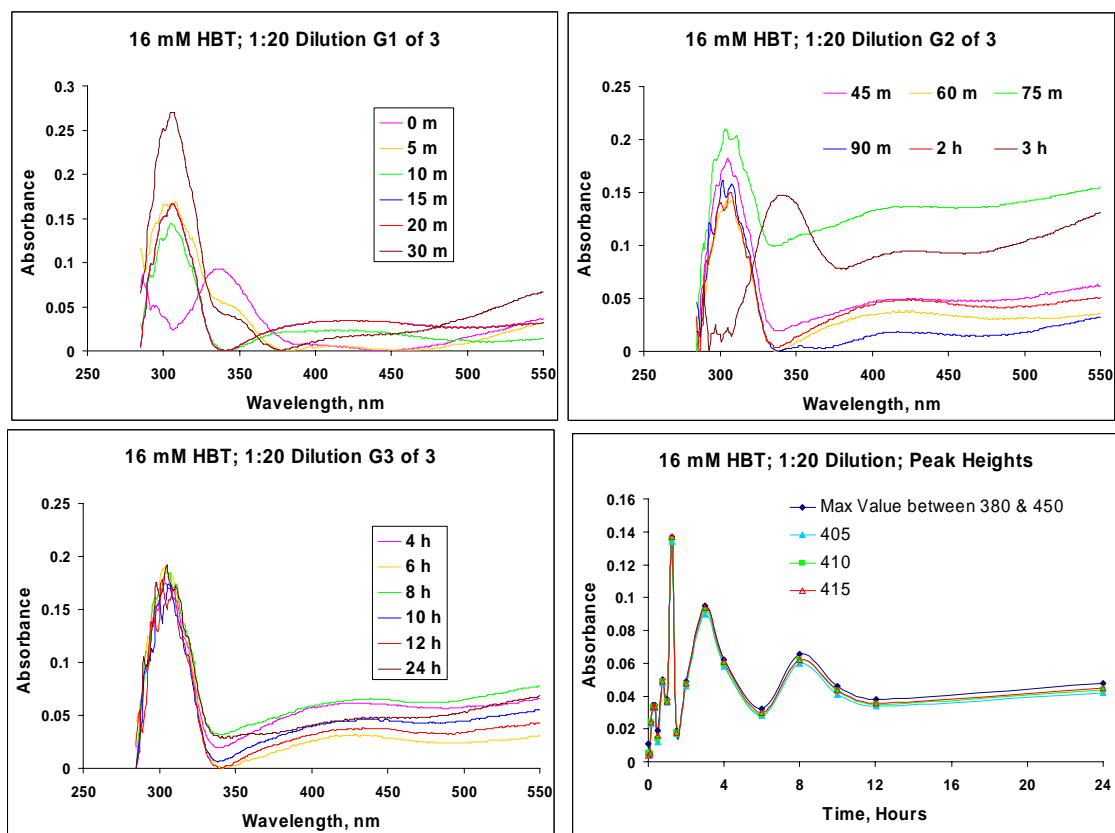
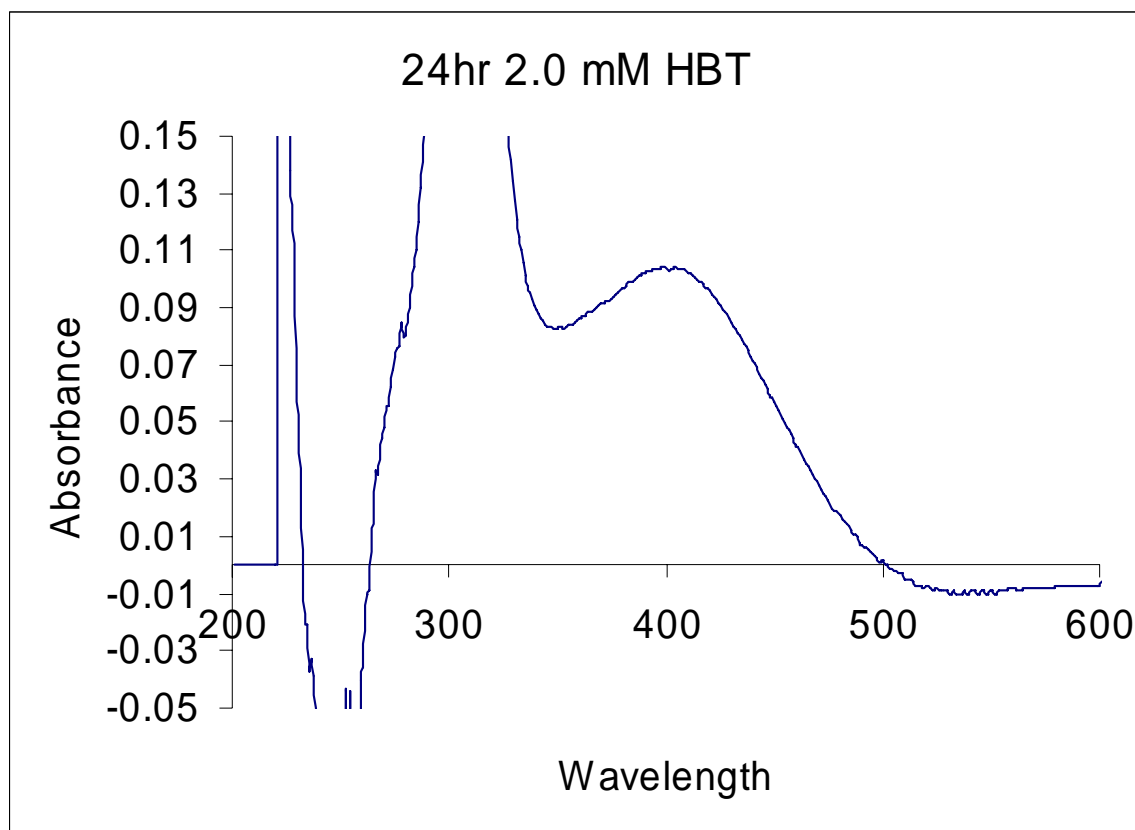


Figure A-71 16 mM HBT Reaction Rate Study Data



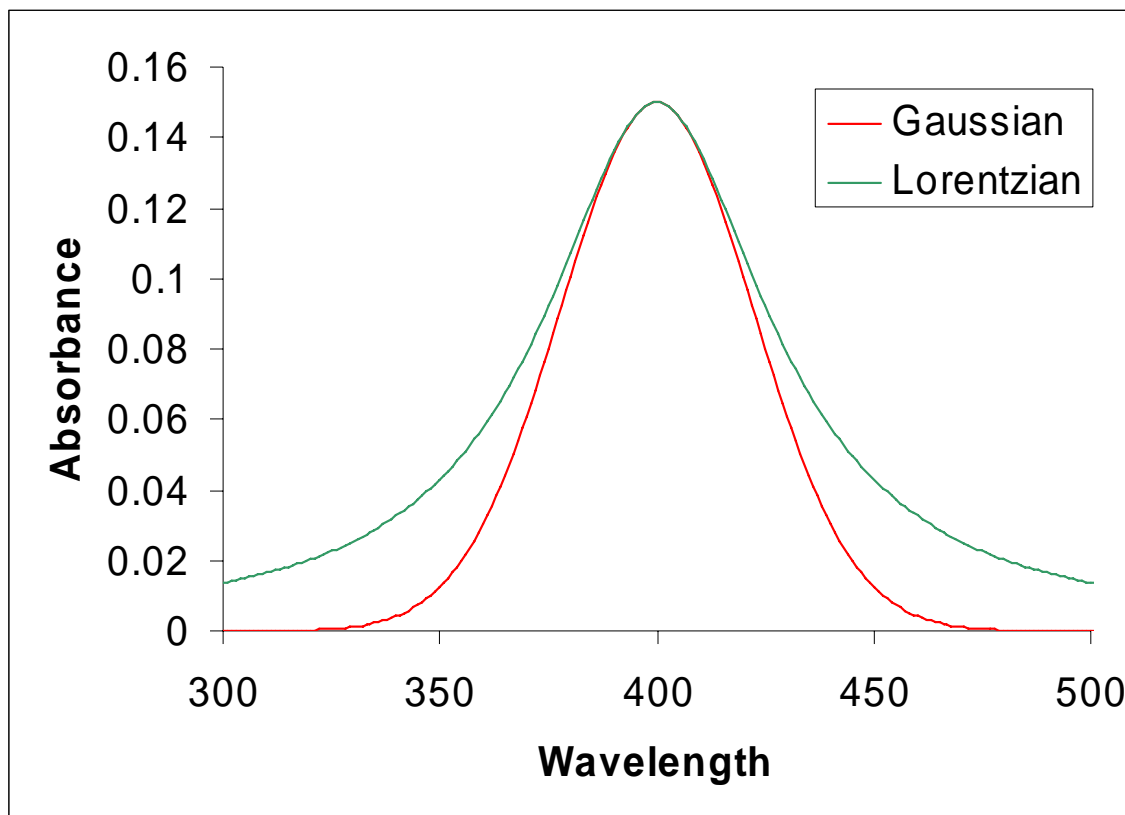
A.7 CHEMO METRIC ANALYSIS OF REACTION RATE SPECTRA

The unusual peak shapes of the reaction rate experiments made quantification difficult. Considerable time was spent on making mathematical models of these peaks. For example, consider Figure A-72. Direct Yellow 11 absorbance is centered on 405 nm. The peak around 300 nm does not need to be quantified. If a mathematical model could be fit to the spectrum, the portion centered on 405 nm could be quantized and compared to modeled areas from other time points from the same sample set.

Figure A-72 Example Difference Spectrum 2 mM HBT 24 Hour Reaction

A.7.1 MANUAL MODELING OF GAUSSIAN AND LORENTZIAN PEAKSHAPES

Modeling was guided by a textbook, Chemometrics (Data Analysis for the Laboratory and Chemical Plant) by Richard Brereton.⁹ The initial efforts were concentrated on using Gaussian and Lorentzian models of peaks. An example showing the difference between the models is shown in Figure A-73. Lorentzian model is based on the Cauchy distribution. While less common than the Gaussian (normal) distribution, Lorentzian peakshape often arises in NMR spectra. Three parameters are necessary for these models: x_0 , the position (wavelength) of the peak center, A the height at the center, and s , related to peak width. The equations for calculating these models are shown below.

Figure A-73 Example of Gaussian and Lorentzian Models of Spectral Data

Equation A-16 Equation to Calculate Gaussian Peakshape

$$x_i = A \exp[-(x_i - x_0)^2 / s^2]$$

Equation A-17 Equation to Calculate Lorentzian Peakshape

$$x_i = A / [1 + (x_i - x_0)^2 / s^2]$$

Equation A-18 Definitions for Peakshape Calculations

x_i = absorbance calculated for wavelength i

x_0 = position of peak center

A = height of peak at center

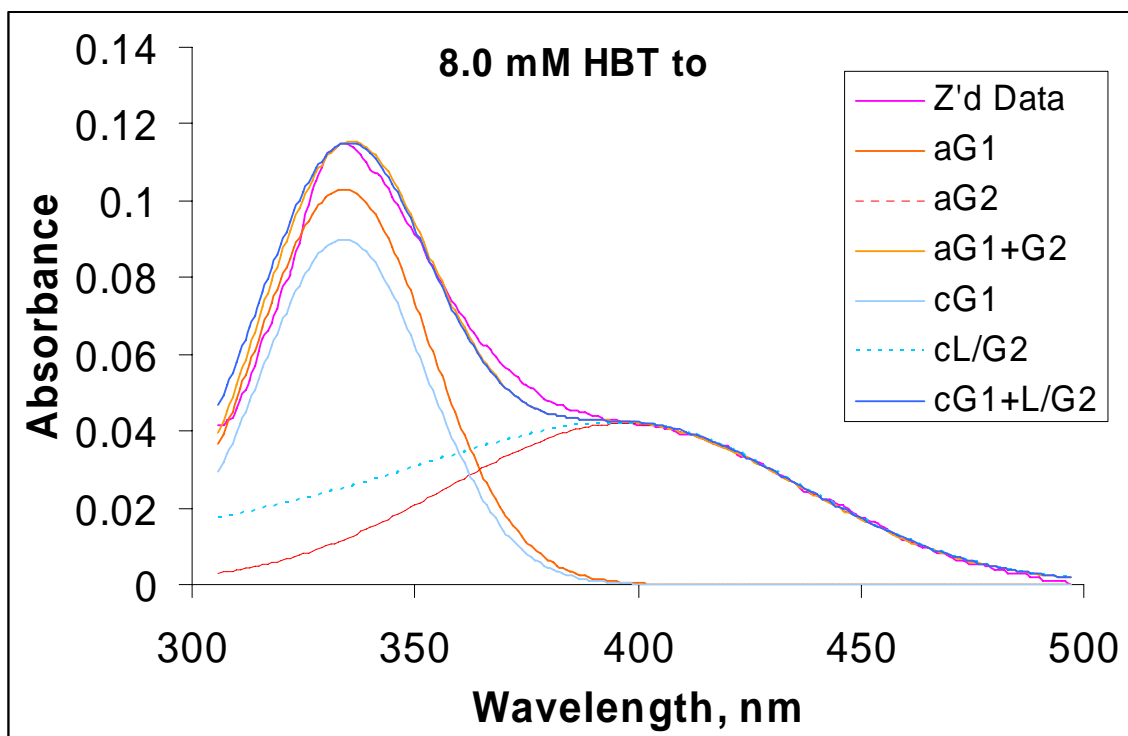
s relates to peak width

One issue to consider is that it is possible for the front and back portions of a peak to be of different kinds. For best fit of a peak, in addition to Gaussian and Lorentzian peakshapes, a Lorentzian front and Gaussian back should be considered as well as

Gaussian back and Lorentzian front. For modeling spectrums of two peaks, 16 combinations can be considered. For modeling, A and x_0 are set, but s would have to be adjusted as each of the 16 combinations are tried.

Examples of fitted models are shown in Figure A-74 where the two best combinations are shown. Close comparison of the raw data with model 1 (Gauss fit of peak 1 + Gauss fit of peak 2) and model 2 (Gauss fit of peak 1 + Lorentzian back and Gaussian front of peak 2) show only a slightly better fit for model 1. However, when the Gauss fit of peak 2 (dotted orange line, part of model 1) is compared with the combination fit of peak 2 in model 2 (dotted blue line) it appears that these two models that yield similar degrees of fit overall would yield significantly different areas for the dye peak of Direct Yellow 11 around 400 nm. This procedure can yield models with excellent fit to the data. However, it is tedious, and choosing between models is difficult.

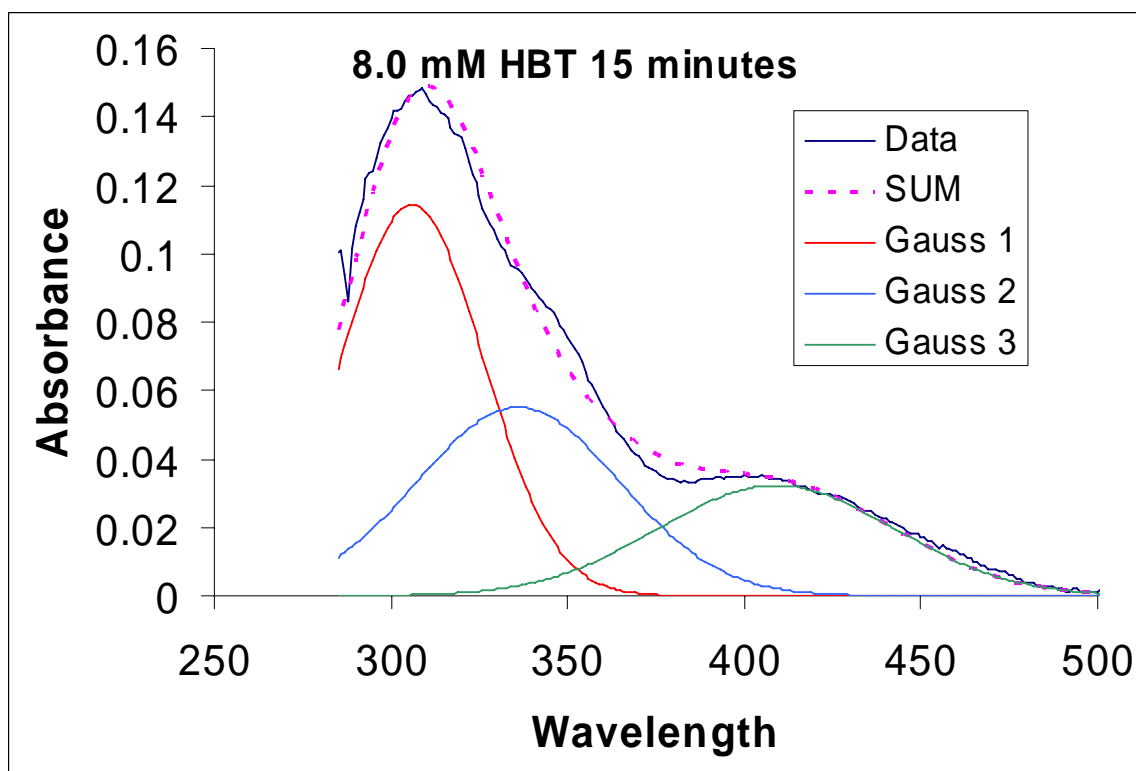
Figure A-74 Lorentzian/Gaussian Fits to 8.0 mM HBT Time Zero



To automate the model fitting, chemometric software was applied to the data. Both Unscrambler® software and software obtained from the Chemometric textbook

were tried. Visually, the data for each time point appear to consist of two components. To determine if this was correct, a matrix was compiled of the spectra for the complete set of time points for each concentration. Principal Component Analysis (PCA) was then applied to this matrix of data. PCA suggested that *three* components were represented in the spectral data. The PCA analysis is discussed in detail below. For 8.0 mM HBT data, the center of these three components appeared to be 306, 336, and 409 nm. Consequently, an effort was made to model the data using just Gaussian fits, but with three peaks, at 306, 336 and 409 nm. An example is shown in Figure A-75.

Figure A-75 Three Component Gaussian Model of 8.0 mM HBT



A.7.2 MODELING BY MULTIVARIATE TECHNIQUES

To understand the difficulties experienced in processing these data, the steps involved will be discussed. For easy reference, the different matrices discussed and their relationships are summarized in Table A-31, page 98. To simplify the discussion, factor analysis will be discussed as outlined by Brereton.⁹ The data set from HPLC-DAD (high

pressure liquid chromatography coupled to photodiode array detector) chromatography system consists of an elution profile (retention time for each individual compound) and a spectrum (wavelength versus time) plus noise and instrumental error. This data set can be written as a matrix that is the product of matrices C and S .

Equation A-19 Data Matrix = Component Concentration * Spectral Response

$$X = C.S + E$$

Where X is the original data matrix

C is a matrix of the elution profiles for each compound

S is a matrix consisting of spectra of each compound

E is an error matrix

For a chromatogram of two overlapping compounds recorded over 30 time points and 28 wavelengths, X is a matrix of 30 rows and 28 columns. C is matrix of 30 rows and 2 columns, each corresponding to elution profile of one compound. S is a matrix of 2 rows and 28 columns, each row corresponding to the spectrum of a single compound. E is a matrix the same size as X . Principal Component Analysis (PCA) performs an abstract mathematical transformation of the data to yield

Equation A-20 Data Matrix = Score x Loadings

$$X = T.P + E$$

Where T , called the scores, has the same number of rows as original data and P , the loadings, has as many columns as the original data. If the number of principal components found is denoted by A , then for the example $T = 30 \times A$ and $P = A \times 28$. In this example, to estimate the absorbance value for the 10th wavelength at the 8th time point, calculate the matrix sum for the values in the 8th row of the scores matrix (T) times the 10th column of the loadings matrix (P) (If two components are assumed, this number will be the sum of two multiplications).

The components found by PCA are mathematical constructs that can but does not necessarily correspond to physical factors (such as the number of compounds in a mixture). PCA is designed for determining the number of important components. To model the components, principal component regression can be used. Scores and loadings can be related to the concentration profile and spectra.

Equation A-21 Relationship between Data Matrices

$$X \approx C.S = T.R.R^{-1}.P$$

Where **C** corresponds to component concentration and **S** to spectral response of each component.

Equation A-22 Relationship between C and S Matrices and PCA Scores and Loadings

$$C = T.R \quad \text{and} \quad S = R^{-1}.P$$

If the number of components is known by PCA and pure spectra are available, the matrix R^{-1} can be found by regression.

Equation A-23 Definition of R^{-1} Matrix Relating C,S to T,P

$$R^{-1} = S.P'$$

The different types of matrices are summarized in Table A-30.

Table A-30 Types of Chemometric Matrices Discussed

Symbol	Name	Example for Reaction Data from Laccase-Mediator Treatment of Direct Yellow 11 in Solution
X	Raw Data	Absorbance Values for Wavelength x Time Points 266 x 19
C	Concentration/ Contribution for Components	266 x 3 Wavelength x Components
S	Spectral Response	3 x 19 Components x Time Points
T	PCA Scores	266 x 3
P	PCA Loadings	2 x 19
R	Regression Matrix	

Table A-31 Relationships between Chemometric Matrices

$X = C.S + E = T.P + E$
$X \approx C.S = T.R.R^{-1}.P$
$C = T.R$
$S = R^{-1}P$
$R^{-1} = S.P'$

A.7.3 APPLICATION OF PRINCIPAL COMPONENTS REGRESSION

The data set analyzed most intensively was 8.0 mM HBT. The data set consisted of 19 time points. For each time point a spectrum was available covering the range 200 to 900 nm. Omitting baseline portions of the spectra and the high absorbance near 200 resulted in useable spectra covering the range of 285 to 550 nm. Each spectrum analyzed consists of a 1x266 (wavelength versus absorbance value).

For this case, the data set X = 266 rows and 19 columns. PCA indicated that there were three principal components. T , the scores, was 266x3 and P , the loadings, was 3x19. Principal Component Regression uses regression to convert Principal Component scores into concentrations.

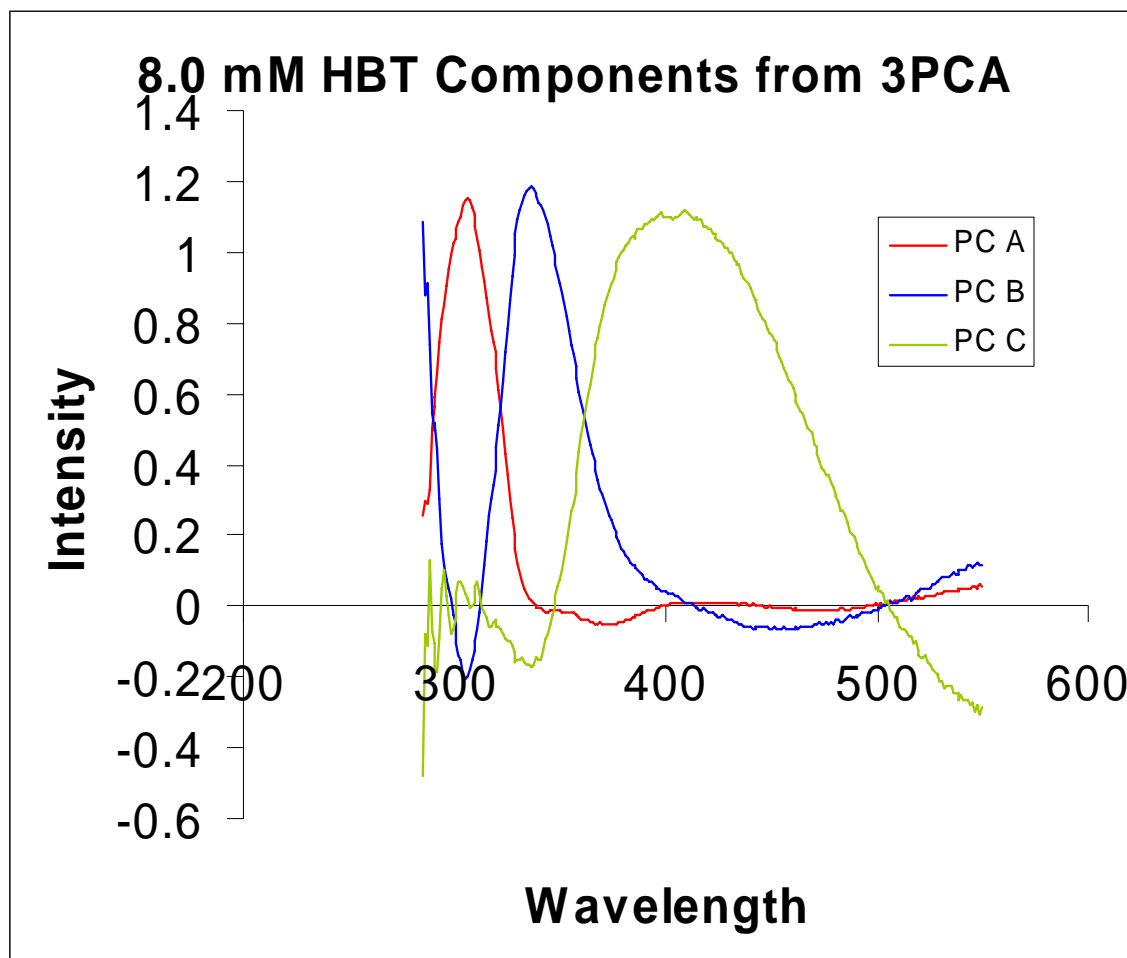
A.7.3.1 Calculation of C and S Matrices

The major problem with the laccase-mediator data is that pure spectra are *not* available. The spectrum for Direct Yellow 11 is available, but not whatever components absorb around 306 and 336. One way to handle this is to select a set of wavelengths where only one component is available. That data region can then be used to calculate R^{-1} . For the 8 mM HBT data, wavelengths 297-319 nm, 331-358 nm, and 382-455 nm were used to calculate S for Components 1, 2, and 3 respectively.

S , a 3x19 matrix, was transposed to create S' . The matrix $S*S'$ was calculated, then inverted to create $(S*S')^{-1}$. Next $S'*(S*S')^{-1}$ was calculated. C could then be obtained because $C = X*S'*(S*S')^{-1}$. X is the matrix of raw data (19 columns/timepoints x 266 rows/wavelengths).

A C matrix was obtained that represents the relative presence of each component. For 8.0 mM HBT, a C matrix of 266 columns and 3 columns was obtained. (Figure A-76) It should be noted that each component curve includes regions of *negative* contributions.

Figure A-76 Component "Contribution" Data for 8.0 mM HBT



Since the calculated model includes negative absorbance values, which cannot occur physically, it was not possible to obtain a chemometric model that would allow the absorbance area for Direct Yellow 11 to be isolated from the other peaks in the spectra.

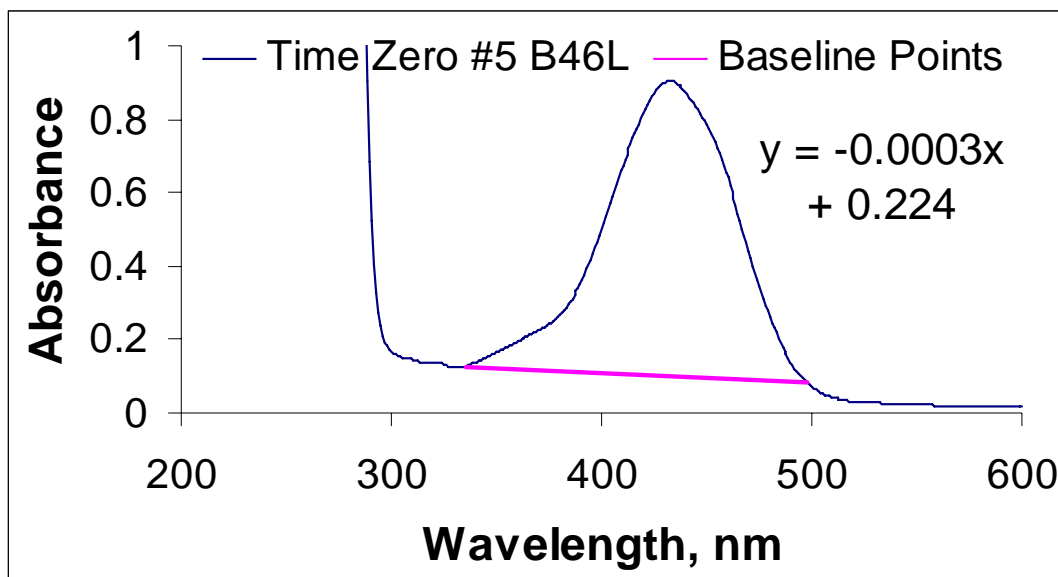
A.8 ANALYSIS OF SOYBEAN PEROXIDASE SPECTRA

The soybean peroxidase experiments were performed with the help of a Summer Undergraduate Research Experience (SURE) intern, Sylva Kirzan. Kristina Knutson designed the experiments, trained and supervised Sylva Kirzan. To quantify the decrease in dye absorbance, the absorbance area (area under the curve in the absorbance spectra) needed to be measured. A difficulty was encountered in that the baseline for these experiments was sloping. A simple approach for quantifying absorbance area is to correct the absorbance data by subtracting the lowest point (so all absorbance values are

positive), and sum the absorbance values for each wavelength within a specified peak area. This simple approach was applied to the quantization of the spectra for laccase treatments. However, because the baseline is sloping, for the soybean peroxidase experiments an additional step was added. Standard points at the beginning and ending of the dye absorbance peaks were selected. A sloping linear line was then drawn between these two points. The triangular area between the zero axis, and the corrected absorbance values for the selected beginning and ending points is then calculated and subtracted from the total summed peak area to yield the final dye absorbance area values. (Figure A-77) To facilitate these calculations, performed on each spectrum from each sample, Sylva Kirzan wrote two Visual Basic Excel Macro Programs which Kristina Knutson applied to analyze the data. These macro programs are listed in Table A-32 and Table A-33.

These macros are designed for processing spectral data from Perkin Elmer Lambda 900 UV/Vis/NIR machine which has been saved in ASCII format. The data files can then be opened by Excel, but the listing of absorbance and wavelength data start in row 89. To apply the macros, the spectral file is opened in Excel. Excel opens this file type as a one page read-only file. The data is then copied into a workbook containing the macro files. The data macro is run first, followed by the chart macro. The resultant Excel Chart sheet and data sheet can then be copied and saved before processing further spectral files.

Figure A-77 Example of Area Calculation for SBP Treatments



A.8.1 EXCEL MACRO PROGRAM FOR SUBTRACTING SLOPING BASELINE

Table A-32 Macro Program for Processing Spectral Data

Sub Data()
'
' Data Macro
' Macro recorded 8/1/2002 by IPST
'
' Keyboard Shortcut: Ctrl+q
'
Range("A1:F85").Select
Application.CutCopyMode = False
Selection.Cut
Range("L1").Select
ActiveSheet.Paste
Range("A20").Select
ActiveWindow.LargeScroll Down:=1
Range("A43").Select
ActiveWindow.LargeScroll Down:=1
Range("A66").Select
ActiveWindow.LargeScroll Down:=1
Range("A86:B868").Select
Selection.Cut
ActiveWindow.LargeScroll Down:=-1
Range("A63").Select
ActiveWindow.LargeScroll Down:=-1
Range("A40").Select
ActiveWindow.LargeScroll Down:=-1
Range("A17").Select
ActiveWindow.LargeScroll Down:=-1
Range("A1").Select
ActiveSheet.Paste
Range("A2:B2551").Select
Selection.Sort Key1:=Range("A2"), Order1:=xlAscending, Header:=xlGuess, OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom
Range("C1").Select
Sheets("Control 10KPEG+B40_DATA").Select
Range("C1:K1283").Select
Selection.Copy
ActiveWindow.ScrollWorkbookTabs Sheets:=1
Sheets("Test").Select
ActiveSheet.Paste
Range("A1").Select
Application.CutCopyMode = False
ActiveCell.FormulaR1C1 = "Wavelength"
Range("B1").Select
ActiveCell.FormulaR1C1 = "Absorbance"
Range("A1:B1").Select
Selection.Font.Bold = True
Range("B1").Select
End Sub

Table A-33 Macro for Generating Spectra from Translated Spectral Files

Sub Chart()
'
' Chart Macro
' Macro recorded 8/1/2002 by IPST
'
'
ActiveCell.Range("A1:B702").Select
Charts.Add
ActiveChart.ChartType = xlXYScatterSmoothNoMarkers
ActiveChart.SetSourceData Source:=Sheets("Test").Range("A1:B702"),
PlotBy:=xlColumns
ActiveChart.Location Where:=xlLocationAsNewSheet, Name:="Chart change"
With ActiveChart
.HasTitle = True
.ChartTitle.Characters.Text = "See Data"
.Axes(xlCategory, xlPrimary).HasTitle = True
.Axes(xlCategory, xlPrimary).AxisTitle.Characters.Text = "Absorbance"
.Axes(xlValue, xlPrimary).HasTitle = True
.Axes(xlValue, xlPrimary).AxisTitle.Characters.Text = "Wavelength"
End With
ActiveChart.PlotArea.Select
With Selection.Border
.ColorIndex = 16
.Weight = xlThin
.LineStyle = xlContinuous
End With
Selection.Interior.ColorIndex = xlNone
ActiveChart.Axes(xlCategory).Select
With ActiveChart.Axes(xlCategory)
.MinimumScale = 200
.MaximumScale = 600
.MinorUnitIsAuto = True
.MajorUnitIsAuto = True
.Crosses = xlAutomatic
.ReversePlotOrder = False
.ScaleType = xlLinear
.DisplayUnit = xlNone
End With
ActiveChart.Axes(xlValue).Select
With ActiveChart.Axes(xlValue)
.MinimumScale = -1
.MaximumScale = 1
.MinorUnitIsAuto = True
.MajorUnitIsAuto = True
.Crosses = xlAutomatic
.ReversePlotOrder = False
.ScaleType = xlLinear
.DisplayUnit = xlNone
End With
End Sub

A.9 TOF-SIMS EXPERIMENTS

In an attempt to determine what chemical transformation(s) were occurring during laccase/ABTS treatment, a set of handsheets were prepared from treated pulps and sent to Marjatta Kleen for ToF SIMS analysis. Four sheets were prepared as outlined in Table A-34. The laccase treatment conditions were the standard conditions of 10.3 Units of laccase /mL reaction solution, 2 hour reaction at 45°C, under 10 bar oxygen pressure. An initial set of treated pulp sheets was prepared using 0.5 mM ABTS. However, at this concentration the treated white pulp sheets exhibited a visible blue-purple tinge. Consequently a second set of treated handsheets was prepared using 0.1 mM ABTS. The sheets analyzed by ToF SIMS were treated with laccase and 0.1 mM ABTS. The analysis report from Marjatta Kleen is shown below.

A.9.1 REPORT OF TOF SIMS ANALYSIS RESULTS

KCL Science and Consulting/Marjatta Kleen and Heli Kangas 23.9.2003 Report
 Inked and deinked handsheets for ToF-SIMS testing
 A report to IPST/ Kristina Parks Knutson

Table A-34 Samples for TOF-SIMS Analysis

1. the initial virgin bleached hardwood pulp (Wh)
2. the pulp dyed with Direct Yellow 11 (DY)
3. the laccase-mediator treated dyed pulp (TR DY)
4. the laccase-mediator treated virgin pulp (Tr Wh)

A.9.1.1 Aim

Try to see if any differences can be found between the sample sheets, particularly any characteristics that are unique to the dyed pulp that was treated with laccase-ABTS (TR DY 0.01).

A.9.1.2 Experimental

The instrument used in ToF-SIMS analyses was a PHI TRIFT II at Top Analytica Oy. ToF SIMS spectra in positive and negative ion modes were acquired using Ga liquid

metal ion gun with 15 keV primary ions in bunched mode over the mass range of 2-2000 Da. The primary ion current was 600 pA, time per channel 0.138 ns, pulse width 19 ns, analysis area $200 \times 200 \mu\text{m}^2$ and acquisition time 5 minutes. Analytical charge compensation was used to neutralize charge in the insulating paper samples. The calculated ion dose was $2.7 \cdot 10^{11} / \text{cm}^2$ ensuring static conditions during the acquisition.

The marked side of each pulp sheet was analyzed. Three replicate runs were made from each sample.

A.9.1.3 Results and discussion

In general, the three ToF-SIMS spectra from each sample were visually similar, so the mass spectra in Figures and the numbers calculated and discussed are only based on one of the runs. This was done, since the calculations are very time-consuming and in this way we can get an idea how the results look like. Later, if needed, more exact calculations for certain interesting features can be done.

Positive SIMS spectra

Figure A-78 shows spectra for some of the metal ions present on the sample surfaces. It is evident that Wh and TR Wh samples contain some sodium (m/z 22.99) at their surface, which is hardly visible on the other samples. Samples DY and TR DY contain aluminum (m/z 26.98), the dyed sample more than the treated one. Samples DY and TR DY contain also silica (m/z 27.98), the dyed sample more than the treated one.

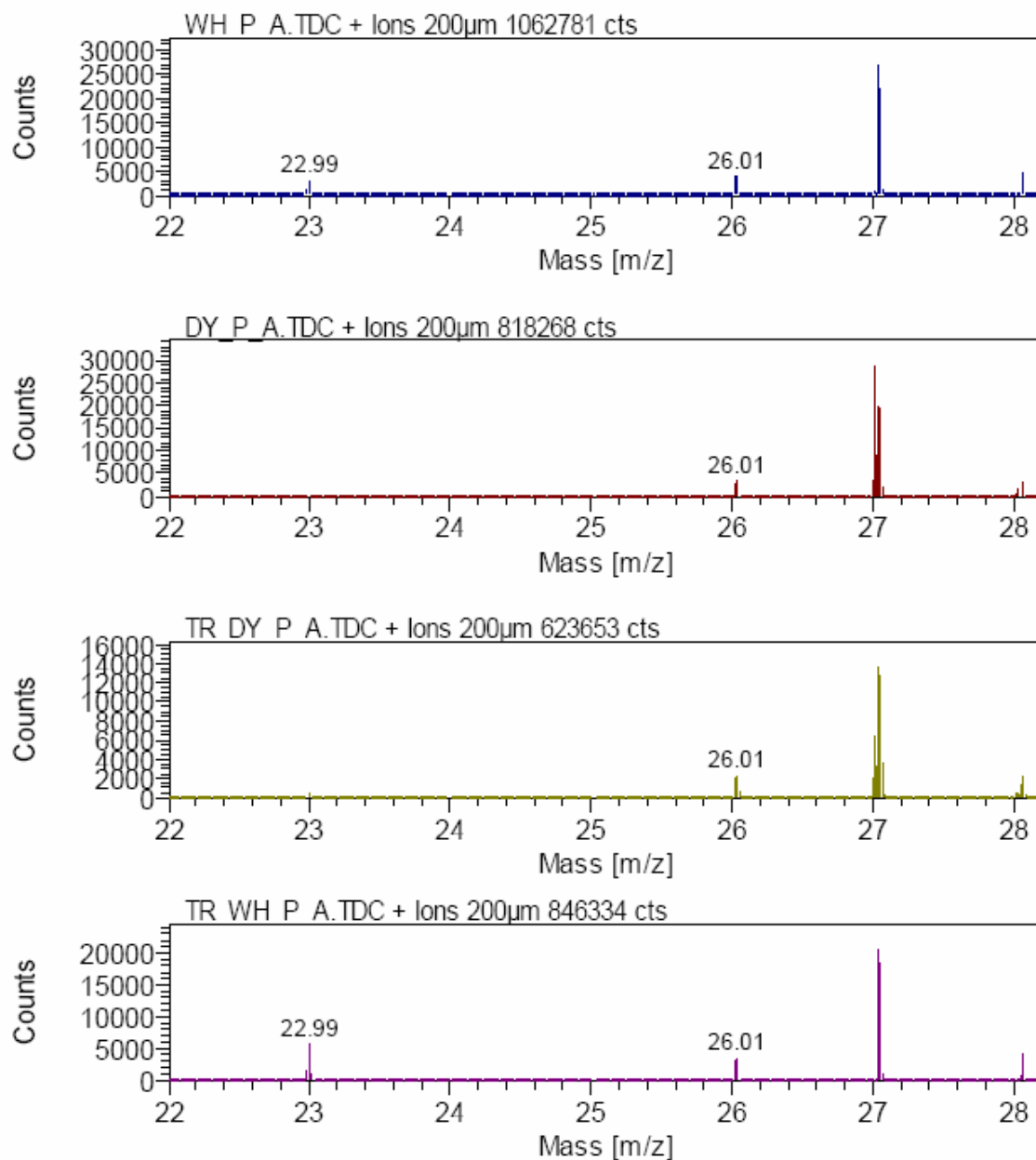
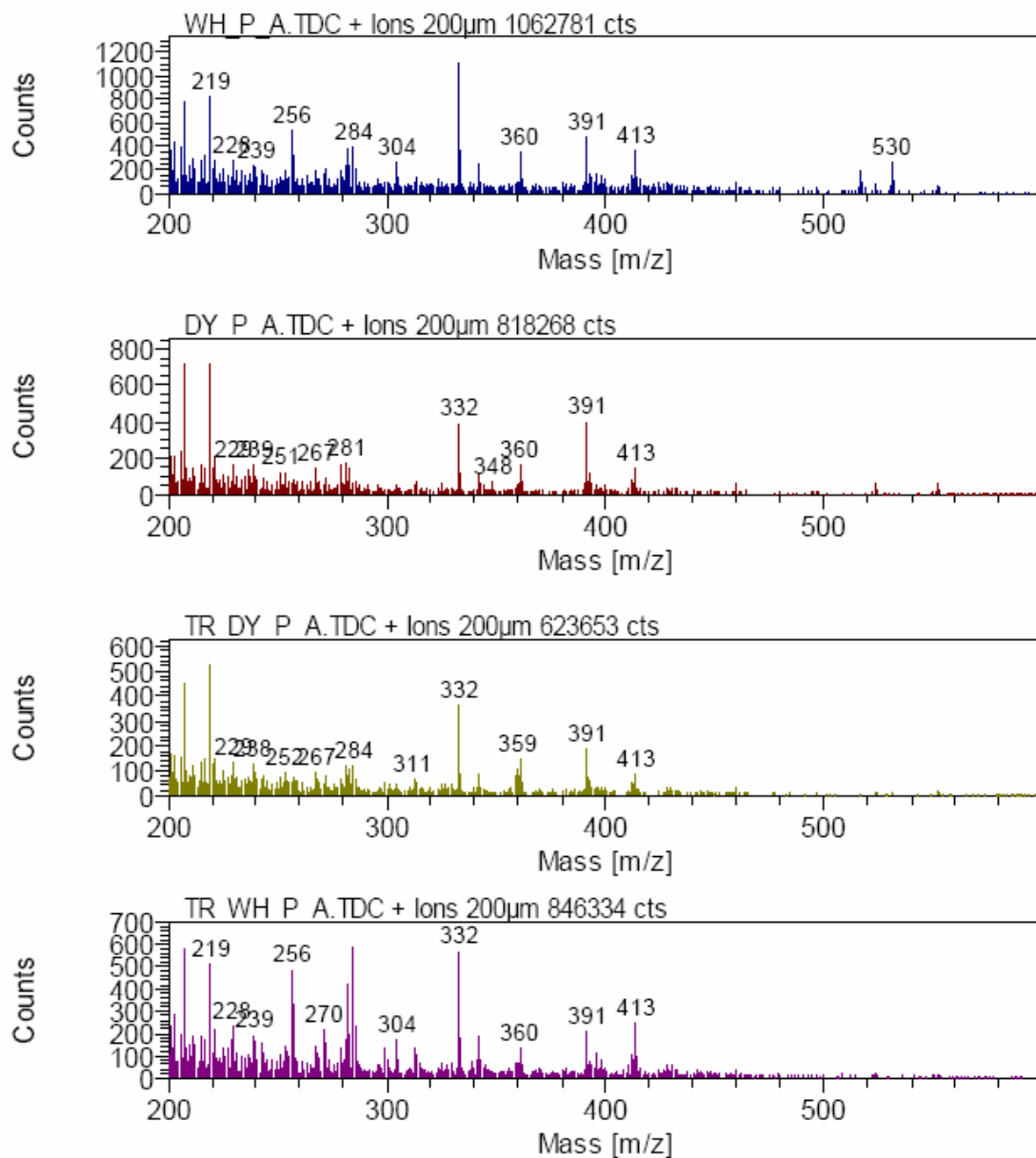
Figure A-78 Metal Ions on Surface of TOF SIMS Handsheet Samples

Figure A-79 shows the mass area from m/z 200 to 600. This area shows organic ion information. The largest differences were found here. However, no exciting new ions were observed for the DY sample compared to Wh sample. No clear differences were observed between the samples TR DY and DY either. On the other hand, the treatment changed slightly the relative abundance of some organic substances on pulp surface (comparison between TR WH and Wh).

Figure A-79 Positive Ions m/z 200 to 600**Negative SIMS spectra**

Differences were further traced in negative SIMS spectra. The DY sample showed relatively more of m/z 16 and 17, indicating more oxygen present on the dyed surface. The percentage of O (15.99) of the total ion count was about 35% (DY), 33% (Tr DY) and 31% for Wh and TR Wh.

Marjatta Kleen tried to trace differences in the amount of sulfur on the surface of samples. The positive SIMS spectra showed very low intensity for the sulfur ion (m/z 31.97), but the negative spectra showed some differences (Figure A-80).

Figure A-80 Sulfur Ion Spectra of Handsheet Surface

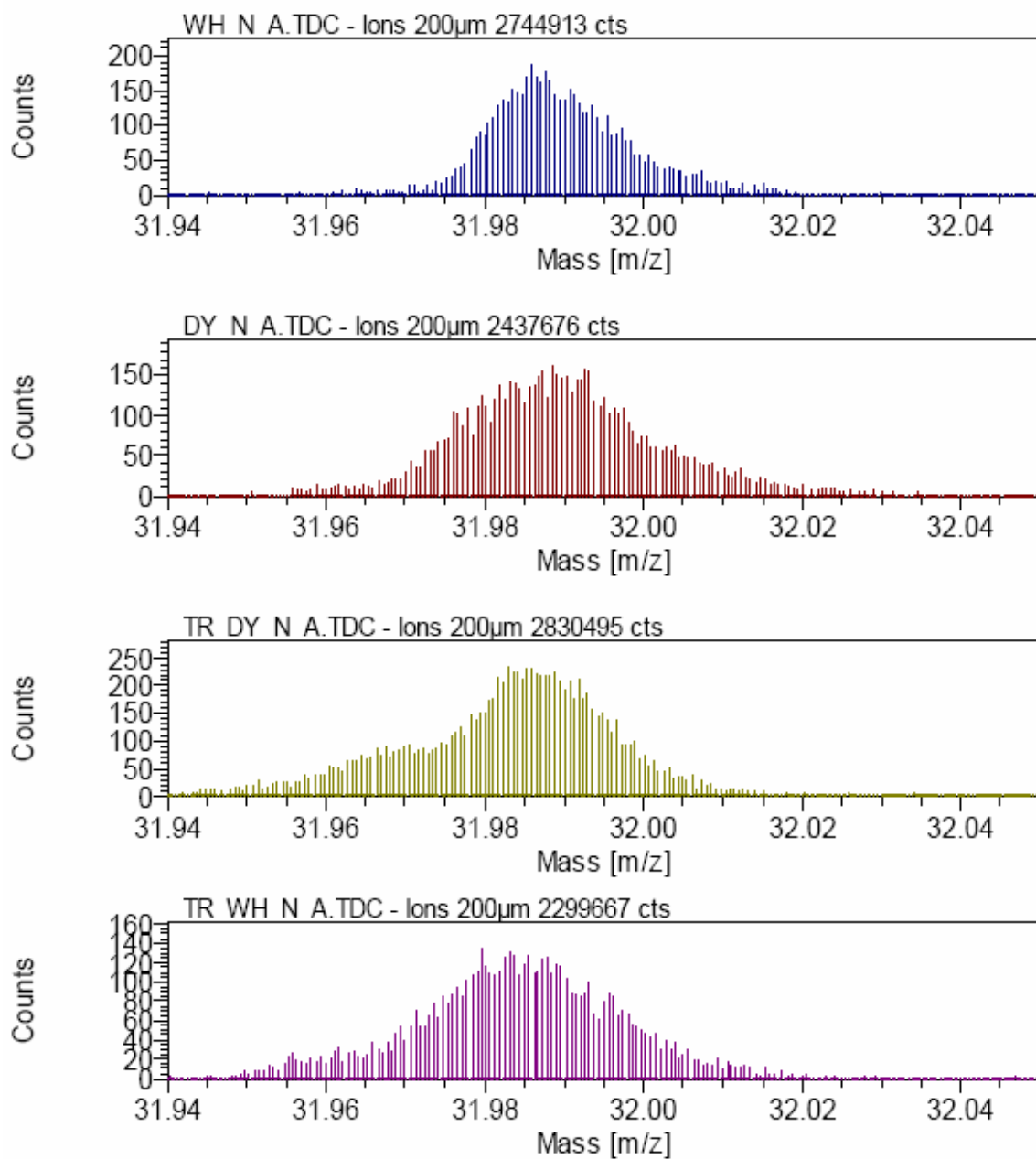


Fig. 3 shows that there is slightly more sulfur on the DY sample compared to Wh sample, but the largest difference is in fact between the TR DY and Wh. It seems that the dye leaves some more sulfur on the surface, but also the laccase treatment. In similar way SO at m/z 47.97 (Figure A-81) as well as SO₂ at m/z 63.96, SO₃ at m/z 79.96 and HSO₃ at m/z 80.96 was observed to have highest abundances for TR DY sample (Table A-35).

Figure A-81 SO and SO₂ Ion Spectra of Handsheet Surface

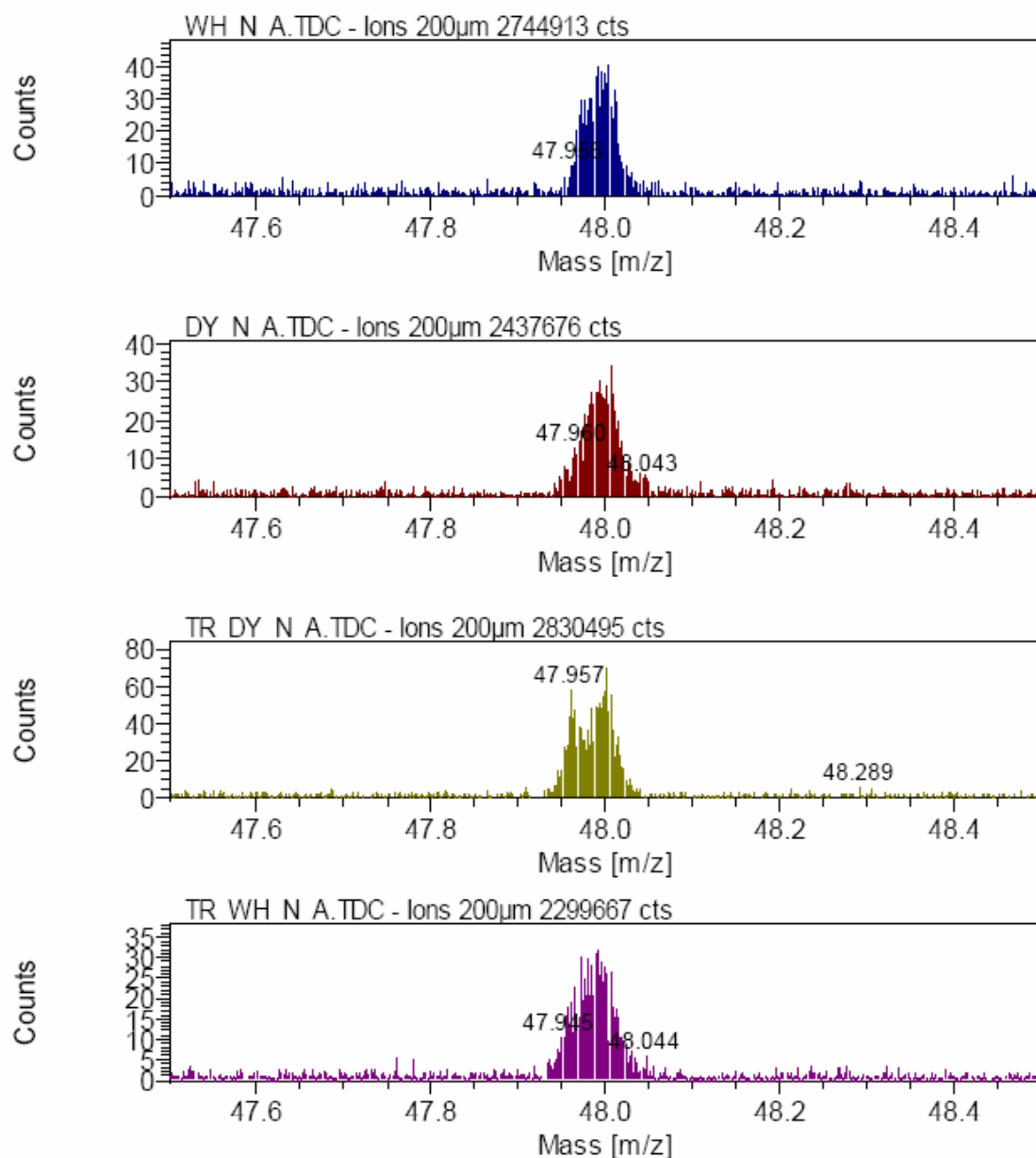


Table A-35 Ion Quantification Based on One Analyzed Spot/Sample

m/z	identity	Wh	DY	TR DY	TR Wh	
15.99	O	0.308	0.351	0.328	0.314	
26.00	CN	0.18	0.18	1.00	0.70	X 10-2
34.97	Cl	5.6	3.2	3.2	4.2	X 10-2
36.97	Cl	1.8	1.1	1.0	1.4	X 10-2
41.998	CNO	1.4	1.2	16.6	14.4	X 10-4
45.99	NO2	1.1	1.0	1.1	1.0	X 10-2
47.97	SO	1.2	1.0	2.2	1.6	X 10-4
50.94	V	+	+	+	+	
52.94	Cr	+	+	+	+	
62.96	PO2	1.2	3.9	2.6	1.6	X 10-5
63.96	SO2	3.0	3.9	8.7	4.6	X 10-5
69.93	Zn	+	+	+	+	
79.96	SO3	6.8	6.9	20.1	10.0	X 10-5
80.96	HSO3	3.4	4.8	7.9	5.0	X 10-5
121.02	C6H3NO2	1.9	2.3	2.0	0.7	X 10-4

+ = observed, not quantified

Table A-35 indicates slight differences in the relative amount of the fragment ion at m/z 121.02 (C6H3NO2), which could be formed from the ink. When normalized to the total ion count for each sample, the figures become very low, but at this point Dr. Kleen believes this is the best way for comparison. The ink fragment ion seems to be present already on the Wh surface, which seems strange (maybe some other organic compound gives the same fragment ion?), but there is about 20% more on the DY surface compared to Wh. Also surprisingly, the enzymatic treatment decreases clearly the amount of this ion on the Wh surface (about 60%), but much less on the TR DY surface (about 10%). This might indicate that this ion may originate from two different surface compounds.

Dr. Kleen also tried to trace differences in the amount of nitrogen on the surface of samples. Neither the positive nor the negative SIMS spectra showed any differences when looking on the area at m/z 14.00 (N) or at m/z 45.00 (NO2) (Table A-35). Table A-35 indicates that both treated samples (TR DY and TR Wh) have other nitrogen residues on their surface (see e.g. 26.00 CN and 41.998 CNO). This indicates that these fragment ions most probably originate from the enzyme.

Further it was noted that the sample Wh had relatively more chlorine (Cl at 34.97 and 36.97) on its surface compared to the other samples. In addition, zinc (Zn at 69.93) as

well as some less common metals like vanadium (V at 50.94) and chromium (Cr at 52.94) were observed on all samples.

Figure A-82 shows the mass spectral area where some differences in the organic substances can be noted.

Figure A-82 Mass Spectral Regions Showing Differences in Organic Substances

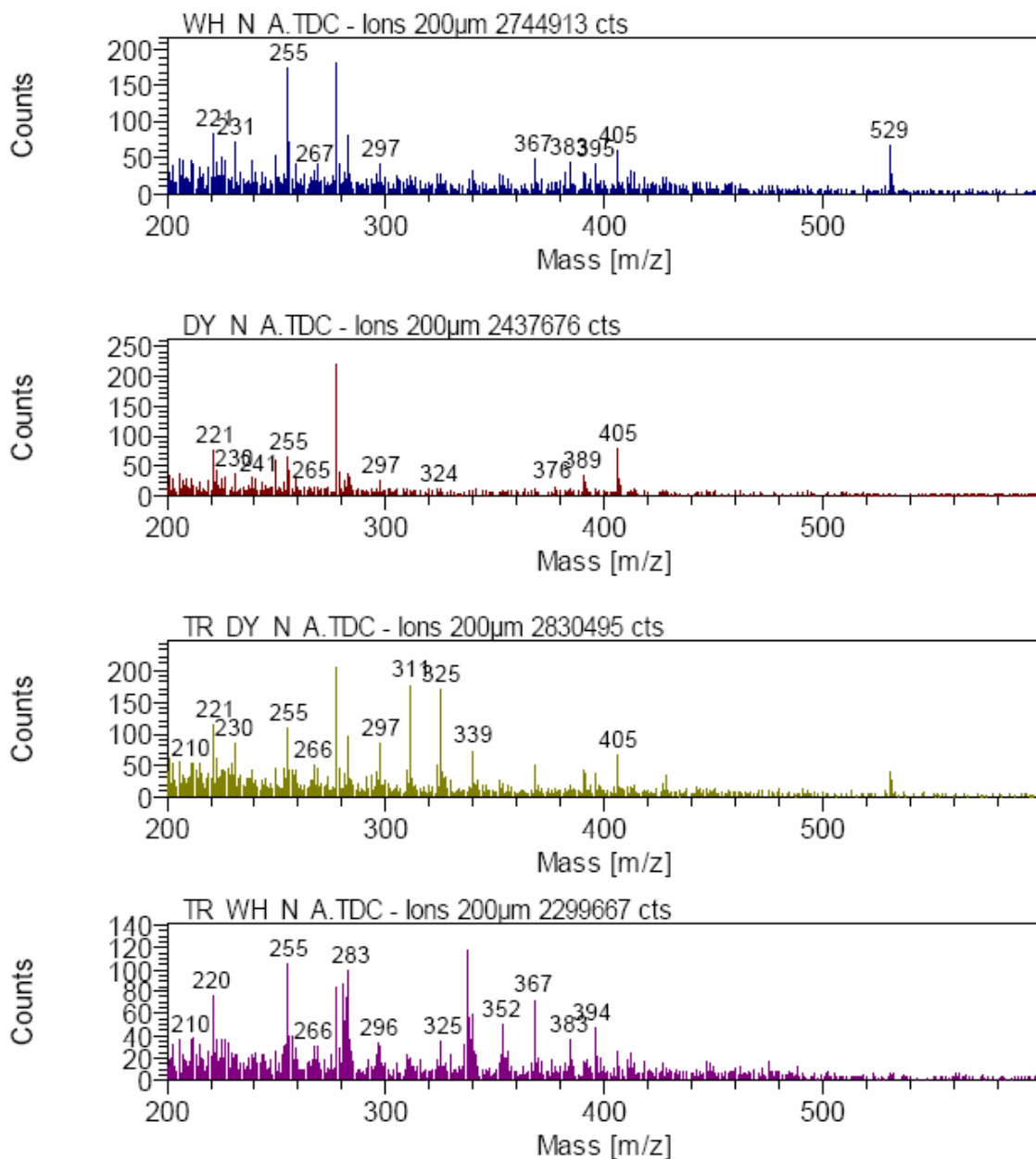


Figure A-82 shows that the ion distribution is different between the Wh and the DY samples, but same ions are found in both samples. No specific new ions were observed for the DY sample compared to Wh sample. Differently to the positive ion spectra, clear differences were observed between the samples TR DY and DY. The enzymatic treatment seems to partly modify the surface compounds or at least change the distribution pattern (compare TR Wh to Wh). However, the enzymatic treatment seems to change Wh and DY surfaces in different ways. The main ions seen in Figure A-82 originate from fatty acid structures (Table A-36).

Table A-36 ToF-SIMS Identified Fatty Acids and Their Characteristic Peaks

Extractive	Structure	Neg. peaks (m/z)
<i>Saturated fatty acids</i>		
Palmitic	C ₁₆ H ₃₂ O ₂	255
<i>Anteiso</i> heptadecanoic	C ₁₇ H ₃₄ O ₂	269
Stearic	C ₁₈ H ₃₆ O ₂	283
Arachidic	C ₂₀ H ₄₀ O ₂	311
Behenic	C ₂₂ H ₄₄ O ₂	339
Lignoceric	C ₂₄ H ₄₈ O ₂	367
<i>Unsaturated fatty acids</i>		
Oleic	C ₁₈ H ₃₄ O ₂ (9)	281
Linoleic	C ₁₈ H ₃₂ O ₂ (9,12)	279
Pinolenic	C ₁₈ H ₃₀ O ₂ (9,12,15)	277

A.10 MALDI EXPERIMENTS

Samples were submitted to Georgia Institute of Technology (GA Tech) Mass Spectrophotometry Laboratory to see if MALDI (matrix-assisted laser desorption/ionization mass spectroscopy) could detect any differences between liquid samples of Direct Yellow 11, laccase, ABTS, laccase+ABTS and laccase/ABTS treated Direct Yellow 11. It was hoped that the treated dye sample would exhibit new fragments due to the putative breakdown product(s) of treated Direct Yellow. The sample concentrations are outlined in Table A-37. Each sample was subjected to MALDI in two different matrices: α -Cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB). For each sample/matrix combination, spectra of both positive and negative m/z ions were obtained.

Table A-37 Samples Subjected to MALDI Analysis

Component	Concentration	Sample ID:	Molecular Weight
1. Dye Direct Yellow 11	10g/L stock	Dy11	796.7
2. Laccase	100% stock (semipure preparation)	Lac	~60,000
3. ABTS	10 mM stock	ABTS	548.7
4. Lac+ABTS	8.33 mM ABTS 16.7% Lac stock	ABTS+LAC	
5. Dye Treated with Lac/ABTS	very dilute Dye: 20 ppm Direct Yellow 11 Lac: 5 µl/mL ABTS: 0.01 mM	Dy11+Lac +ABTS	

A.10.1 RESULTS OF MALDI ANALYSIS

The spectra obtained are shown on the following pages. Sharp clearly defined spectra were obtained from moderately concentrated solutions of Direct Yellow 11 and laccase mediator ABTS. The MALDI spectra of the high molecular weight (~60,000) laccase enzyme did exhibit some peaks, but the signal-to-noise ratio is very small. The same low sensitivity/noisy baseline was observed for ABTS combined with laccase and the Direct Yellow 11, ABTS and laccase combination. No conclusions are easily drawn from these data.

Figure A-83 MALDI Spectrum Direct Yellow 11 Positive m/z from CHCA Matrix

Figure A-84 MALDI Spectrum Direct Yellow 11 Positive m/z Ions from DHB Matrix

Figure A-85 MALDI Spectrum Direct Yellow 11 Negative m/z from CHCA Matrix

Figure A-86 MALDI Spectrum Direct Yellow 11 Negative m/z Ions from DHB Matrix

Figure A-87 MALDI Spectrum Laccase Positive m/z Ions from CHCA Matrix

Figure A-88 MALDI Spectrum Laccase Positive m/z Ions from DHB Matrix

Figure A-89 MALDI Spectrum Laccase Negative m/z Ions from CHCA Matrix

Figure A-90 MALDI Spectrum Laccase Negative m/z Ions from DHB Matrix

Figure A-91 MALDI Spectrum ABTS Positive m/z Ions from CHCA Matrix

Figure A-92 MALDI Spectrum ABTS Positive m/z Ions from DHB Matrix

Figure A-93 MALDI Spectrum ABTS Negative m/z Ions from CHCA Matrix

Figure A-94 MALDI Spectrum ABTS Negative m/z Ions from DHB Matrix

Figure A-95 MALDI Spectrum Laccase+ABTS Positive m/z Ions from CHCA Matrix

Figure A-96 MALDI Spectrum Laccase+ABTS Positive m/z Ions from DHB Matrix

Figure A-97 MALDI Spectrum Laccase+ABTS Negative m/z Ions from CHCA Matrix

Figure A-98 MALDI Spectrum Laccase+ABTS Negative m/z Ions from DHB Matrix

Figure A-99 MALDI: Dye11 + Laccase+ABTS Positive m/z Ions from CHCA Matrix

Figure A-100 MALDI: Dye11+Laccase+ABTS Positive m/z Ions from DHB Matrix

Figure A-101 MALDI: Dye11+Laccase+ABTS Negative m/z Ions from CHCA Matrix

Figure A-102 MALDI: Dye11+Laccase+ABTS Negative m/z Ions from DHB Matrix

B APPENDIX REFERENCES

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⁹ Richard Brereton (2003) Chemometrics (Data Analysis for the Laboratory and Chemical Plant), Wiley & Sons, Pub. Information about Gaussian and Lorentzian curves to fit spectra on pages 122-125. Principal Component Analysis is in Chapter 4. {Principal Component Regression is in Chapter 5.