ENZYMATIC FORMATION OF COLORANTS

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Rosary Ann Stephen

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ENZYMATIC FORMATION OF COLORANTS

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A Thesis

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ENZYMATIC FORMATION OF COLORANTS

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Date of Graduation

VITA

Rosary Ann Stephen, daughter of Paul and Jane Stephen, was born November 2, 1975 in Coimbatore, India. She graduated from Patrick Henry High School, in San Diego, California, in June of 1993. She entered San Diego State University in the year of 1994, and graduated with a Bachelor of Science in Biology and Chemistry in August 2000. After graduation, she worked as a research associate for Diversa Corporation. In fall of 2005, she was enrolled by Auburn University in the Department of Polymer and Fiber Engineering for a Master's degree.

THESIS ABSTRACT

ENZYMATIC FORMATION OF COLORANTS

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The aim of this research is to understand if colorants can be synthesized enzymatically, and if the formed products can be used in various applications. Several phenolic compounds were chosen and reacted in the presence of laccase under moderate conditions, which resulted in colored products. Using a combination of techniques (DSC, IR, GC–MS, NMR), an attempt was made to characterize the newly synthesized colorants. In terms of application, the colorants were used to dye linen fabric and added to transparent films.

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INTRODUCTION

Enzymes are proteins that catalyze specific reactions under mild nontoxic conditions. They have been used in a wide range of fields, such as, biobleaching and scouring agents for pulp, paper, and textile processing, as detergents, as active compounds in food processing, and in the production of biofuels. Due to increased concerns over environmental pollution, better use of renewable resources, and an increasing need for ecologically sound processes, the interest in nontraditional applications of enzymes has grown over the past decades. One such novel application is concerned with techniques involved in the in-vitro synthesis of organic compounds (such as polymers, oligomers, or colorants).

Utilizing enzymes to produce organic compounds has great advantages. The reaction can occur under mild conditions, which results in an added benefit of energy efficiency. In addition, enzymatic reactions are highly regio-, stereo-, and enantiospecific, making the process more efficient and less wasteful.

The objective of this research is to find an environmentally sound process in the synthesis of colored compounds and investigate probable applications. While numerous dyes are produced by the reaction of dye fragments through a synthetic route, the coupling of dyes by green chemistry via enzymatic means has yet to be approached.

In this research work, colorants (dyes or pigments) were synthesized by introducing laccases to phenolic compounds in an aqueous medium. Phenolic

compounds were chosen based on its similarity in structure to that of dye fragments and to parts of the lignin molecule, since laccases are known to be involved in the synthesis of lignin.

CHAPTER ONE

LITERATURE REVIEW

1.1 Coloration Process

Commercial dyeing of textile fibers can be approached by either batch or continuous methods. Generally, the most widely used commercial route is of the former one, exhaust dyeing. Along with a particular process, the choice of dye is dependent upon the color needed and the material chosen to be dyed (e.g. cotton, linen, wool, nylon). Other factors must also be taken into account, such as cost, quantity of material to be dyed, logistics, etc. Pollution control, especially in regard to dye house effluents as well as worker safety, are aspects more and more strictly regulated, thus adding to the cost of the coloration process.

Typically, to prepare for dyeing the fibrous materials are scoured to remove any hydrophobic impurities that could hinder the dyeing process and then bleached in order to remove any color already present. This is particularly important for natural fibers which have a slight yellowish hue to them. A dye-bath is prepared by mixing dye in the form of powder, granules, or in liquid form with water. Also, any auxillaries (salts, surfactants, pH- adjusting acids or bases, etc.) are added that are required as dyeing aids to assist in the proper absorption of the dye to the fibers (1).

During the dyeing process the bath is agitated to create efficient circulation and assure maximum contact between fiber and dye. The temperature is slowly raised to the optimum temperature, often near the boiling point of water, and kept there for a period of time (0.5-2hr). After absorption of the dye, the fibers can be further treated with fixatives to improve the fastness properties of the fiber. Finally, extensive rinsing removes any unfixed dye and all auxiliaries.

1.1.1 Dyeing

In order to add color to a material, the dye molecule must first adsorb onto the fiber then penetrate its accessible areas. This process is quite complex. It can be described by four stages: diffusion of dye through the aqueous dye-bath to the surface of the fibers; adsorption of dye on the surface of the fiber; absorption/diffusion of dye into the fiber from the surface toward the interior; and finally the sorption of the dye at dye-sites. The major driving force in the dyeing process, besides the concentration gradient of dye in bath versus dye in fiber, is ultimately the potential interaction between colorant and fiber. In the case of cellulosic materials direct dyes can only form weak interactions with the fiber and are held inside by physical entrapment. In some cases, a fixation after treatment might be necessary. Reactive dyes, on the other hand, form covalent bonds with the hydroxyl functional groups of cellulose under alkaline conditions. Vat and sulfur dyes are held inside the fiber as insoluble pigments and their fiber/dye interaction is minimal.

1.2 Enzymes

All enzymes are proteins which catalyze chemical reactions, with the exception of a few RNA enzymes. They assist in the conversion of substrates into products by increasing the reaction rate. This is accomplished by the enzymes' active site, which is a small pocket of the entire structure of the enzyme. Initially, the substrate loosely binds/interacts with amino acids within the active site. This sets it up for a series of reactions to occur; and during these processes, the active site allows the substrate to resemble more closely that of the structure of the transition state. Through a sequence of reactions, the substrate is finally converted into the product.

Enzymes are categorized into six groups by the International Union of Pure and Applied Sciences (IUPAC): Oxidoreductases, Transferases, Hydrolases, Ligases, Lyases, and Isomerases. These classifications are based upon the reactions which they catalyze (e.g., transferases move chemical groups from one molecule to another). Within these groupings, they are further divided into subclasses, and categories. This is to better identify the specific reactions in which they provide assistance. An enzyme is given an Enzyme Commission number (EC.#.#.#.#), which denotes its specific function. Each subsequent number indicates a subset within a group, with the final number making it distinct from others.

1.2.1 Oxidoreductases

Oxidoreductases are a group of enzymes which conduct oxidation reduction reactions by the transfer of electrons or hydride ions from one molecule to another. They have been designated an EC number of one. Their reaction can be described as follows:

$$A^- + B \rightarrow A + B^-$$

Some oxidoreductases have metals within their active site. Metals which are tightly bound to the enzyme are referred to as a prosthetic group, without which the enzyme would be functionless.

1.2.1.1 Laccase

Laccases, 1,4-benzenediol oxidase (EC 1.10.3.2), are multi-copper containing enzyme which have broad substrate specificity; they carry out one-electron oxidations of phenolic and related compounds, and reduce molecular oxygen to water, according to the schematic in Figure 1.1.

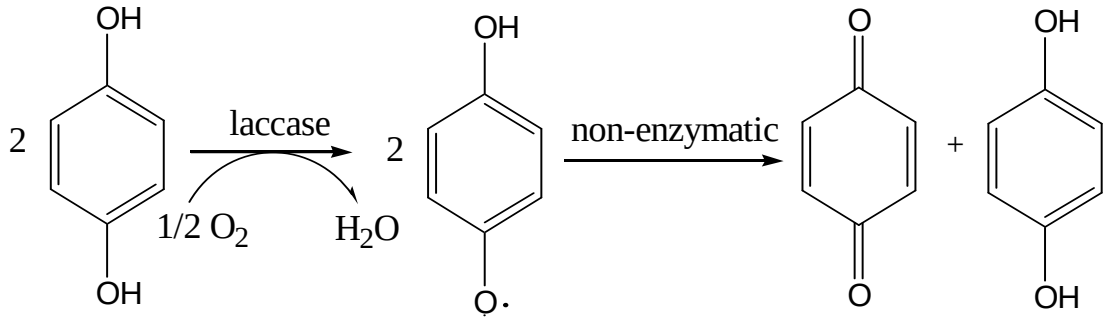


Figure 1.1: General schematic of a laccase reaction.

Laccases generally contain a type 1, type 2, and type 3 copper center per subunit. They are found in higher plants and microorganisms and are capable of oxidizing various aromatic compounds using molecular oxygen as a terminal electron acceptor. The incorporation of copper atoms in the protein structure, allows the proteins to perform electron transfer reactions, because copper atoms are able to switch their oxidation states between Cu^{I} and Cu^{II} (2).

In the presence of phenolic groups, laccases can initiate oxidative coupling of phenolic molecules, such as dye fragments via one-electron transfer reactions. These one-electron transfers produce radicals which could possibly further react with other radicals present, and hence, possible coupling can occur.

Fungal laccases often occur as isozymes, which differ in their quaternary structure. A carbohydrate moiety is commonly found as part of its structure, which is known to play an important role in its stability. For the catalytic activity, a minimum of four copper atoms per active protein unit is needed: Type 1: (one Cu atom), Type 2: (one Cu atom), and Type 3 (two Cu atoms), see Figure 1.2.

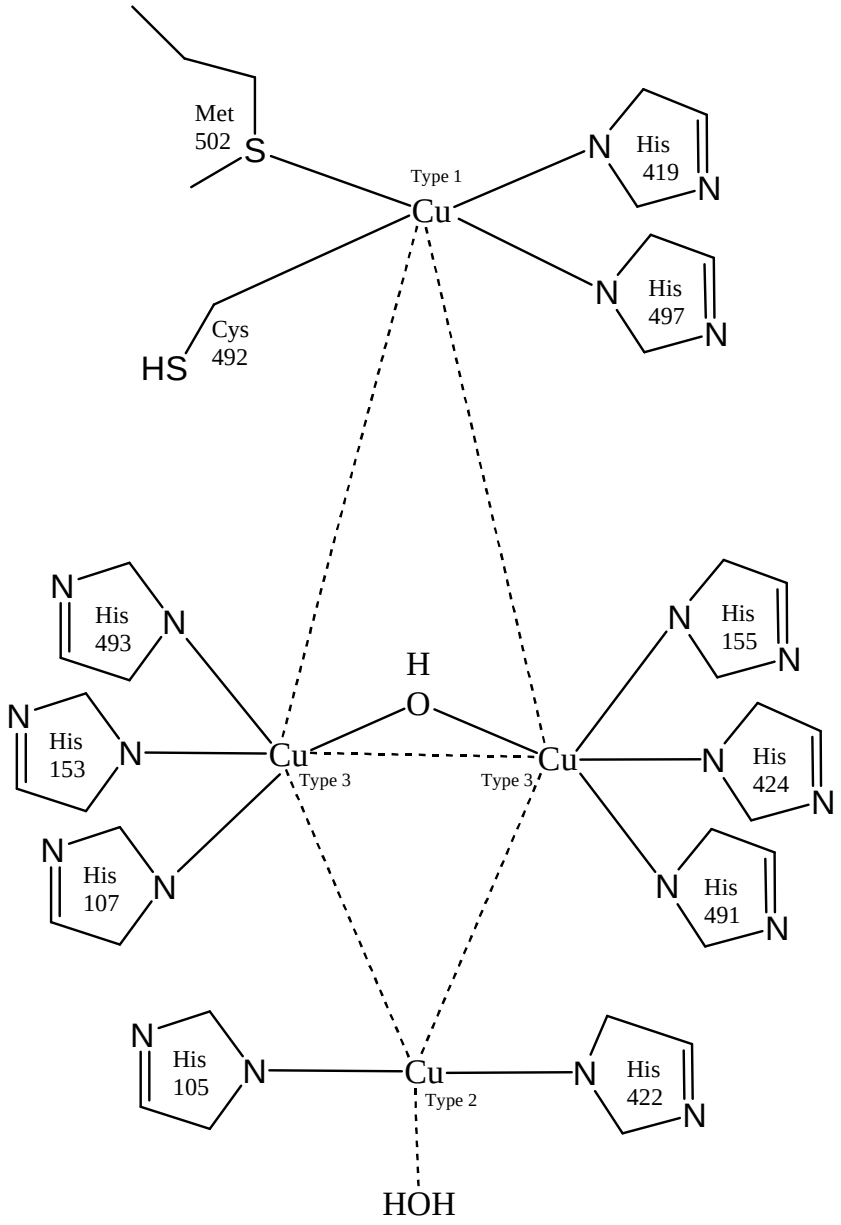


Figure 1.2: Copper centers of laccase (CotA) from B. subtilis (3).

Type 1 copper has a trigonal coordination, with two histidines and one cysteine. The fourth amino acid position is found to be varied among species. Phenylalanine is usually the ligand for fungal laccases (3). This position is believed to strongly influence the oxidation potential of laccases. A mutation from phenylalanine to methionine

significantly lowered the oxidation potential of a fungal laccase from *Trametes villosa* (4).

Type I copper gives the blue color to the multi-copper proteins, due to the absorption of the copper–cysteine bond at 600 nm. Type 1 copper has a high redox potential, and thus, substrate oxidation takes place at this site.

Type 2 copper is tactically positioned close to the type 3 copper to form a cluster, which is referred to as the trinuclear center; this is due to the contribution of one copper from type 2 and two copper atoms from type 3. It is at this site where molecular oxygen is reduced and water is released. Type 2 copper is bound by two histidines and is in near proximity of two type 3 copper atoms, which are surrounded by a total of six histidines. The strong anti-ferromagnetical coupling between the two type 3 copper atoms is maintained by a hydroxyl bridge (Figure 1.2) (3). Type 2 and type 3 copper have a tetrahedral geometry.

The different copper centers of laccases direct electrons from the reducing substrate to molecular oxygen without releasing toxic peroxide intermediates. This is achieved by four single electronic oxidations of the substrate at the mononuclear site. The electrons are then transported to the trinuclear cluster, where molecular oxygen is reduced to water.

The oxidation of substrates creates reactive radicals. Oxidation of phenolic compounds and anilines by laccases produce radicals which can react with each other to form dimers, oligomers or polymers covalently coupled by C–C, C–O, and C–N bonds. Also, these radicals can further undergo non-enzymatic reactions, resulting in cross-linking.

1.3 Enzymatic Polymerization

Organic synthesis of polymers or oligmers (which can possibly form colorants) via enzymatic means has several advantages. Firstly, the reaction can occur under mild conditions, such as moderate temperature and pH; due to this, the overall process becomes more energy efficient. Table 1.1 shows a summary of enzymatic polymerizations and their products, according to enzyme class.

Table 1.1: Polymer products according to enzyme class

Oxidoreductases	polyphenols, polyanilines, vinyl polymers
Transferases	polysaccharides, cyclic oligosaccharides, polyesters
Hydrolases	polysaccharides, polyesters, polycarbonates, poly(amino acids)

1.3.1 Polymerization via Laccase

Laccases from various species have been used with starting phenolic compounds to yield polymers with high molecular weight (5). The polymerization behavior is dependent on the origin of the enzyme, reaction conditions, and nature of substrate. Few reports are found in the literature regarding the enzymatic synthesis of colored products (or polymers).

Laccase derived from *Pycnoporus coccineus* was used to synthesize polymers with phenol, m-cresol, bisphenol-A (6). The reaction was carried out in equal amounts of

methanol and acetate buffer. It was found that formation of polyphenols occurred, resulting in equal units of phenylene and oxyphenylene, which was comfirmed by IR spectral analysis; the ratio of these units could be controlled by the amount of organic solvent used.

Polymers of various molecular weights were synthesized using laccase derived from *Trametes hirsute* (7). The initial starting materials used were guaiacol and erol, in the presence of ABTS (2,2´-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) as a mediator. It was found that higher molecular weight polymers were obtained with ABTS than without. The results indicate that the mediator functioned both as an electron carrier and as a coupling product.

Polymers were formed using 8-hydroxyquinoline in the presence of laccase from *Trametes pubescens* (8). The reaction was carried out in an aqueous organic solution. Structural characterization of the polymer was done via FT-IR, and antioxidant properties were investigated.

It was found that by using 1-naphthol and catechol in the presence of laccase from *Trametes versicolor* yielded a polymerized product (9 & 10). The reaction was carried out in an 10% acetone/acetate buffer under mild conditions and initial reaction rates were investigated.

1.4 Characterization of Enzymatically Formed Polymers

Polymerization of catechol in a batch system has been achieved by the use of laccase to yield polycatechol. The reaction occurred in an acetone/50 mM acetate buffer

(10:90) solution at pH 5. Comparisons between catechol and polycatechol products obtained by laccase polymerization were done by IR analysis (10).

Laccase from *Ustilago maydis* polymerized kaempferol and quercetin has also been achieved. The ratios of monomer and polymers were assessed by measurement of antioxidant activity (11). It was observed that products obtained by addition of the laccase with the monomer increased the antioxidant activity, thus, indicating an increase in molecular weight of the flavanoid. IR spectra were taken of the initial compound versus the polymer product; spectral data indicated differences between the two, again suggesting an increase in molecular weight. Greater molecular weights were reached with kaempferol than with quercetin.

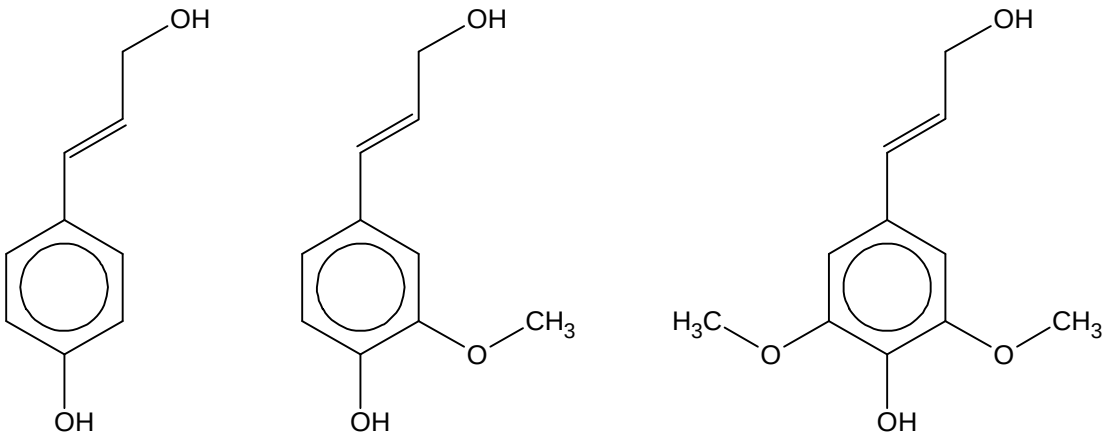
Polymerization of 8-hydroxyquinoline to poly (8-hydroxyquinoline) via laccase from *Trametes pubescens* (8) generating a polymeric product with a number average molecular weight of 789 m/z. The reaction occurred in a sodium acetate/acetone buffer solution under mild reaction conditions. The molecular weight of the final product was obtained by the use of MALDI-TOF. Formation of dimers, trimers, oligomers, and polymers were detected. The IR spectra of the product indicated a vibration from an OH group, thus, it was concluded that the polymer could be formed by either C-C or C-O-C bonds. The antioxidant activity of both the monomer and polymer were measured by quenching with DPPH (2,2´-diphenyl-1-pycrylhydrazyl); it was found that the product formed by 8-hydroxyquinoline was a much better antioxidant than the monomer, with a 53% difference in quenching activity between the two molecules. Antioxidant activity has been correlated with an increase in molecular weight and π-conjugated systems (8).

All the results combined indicate that a polymer was formed by the oxidation of 8-hydroxyquinoline via laccase.

1.5 Lignin

Plants are composed of three major components: cellulose, hemicellulose, and lignin. Lignin fills in the void between cellulose and hemicellulose. By doing so, it provides great mechanical strength to the plant and allows the plant to conduct water efficiently.

Lignin is a complex network polymer, which consists of phenylpropane units connected together in various ways. The chemical composition of lignin differs depending on its source and has not yet been fully elucidated.



p-coumaryl alcohol coniferyl alcohol syringal alcohol

Figure 1.3: Monomers involved in lignin synthesis.

Lignin, a hydrophobic polymer, is synthesized by the enzymatic dehydrogenation of the monomers shown in Figure 1.3. The first step of this reaction results in a phenoxy

radical; the radicals formed further react to a complex polymeric structure with various types of crosslinks and substituent groups. In Figure 1.4 the proposed structure of lignin from softwood (12) is shown.

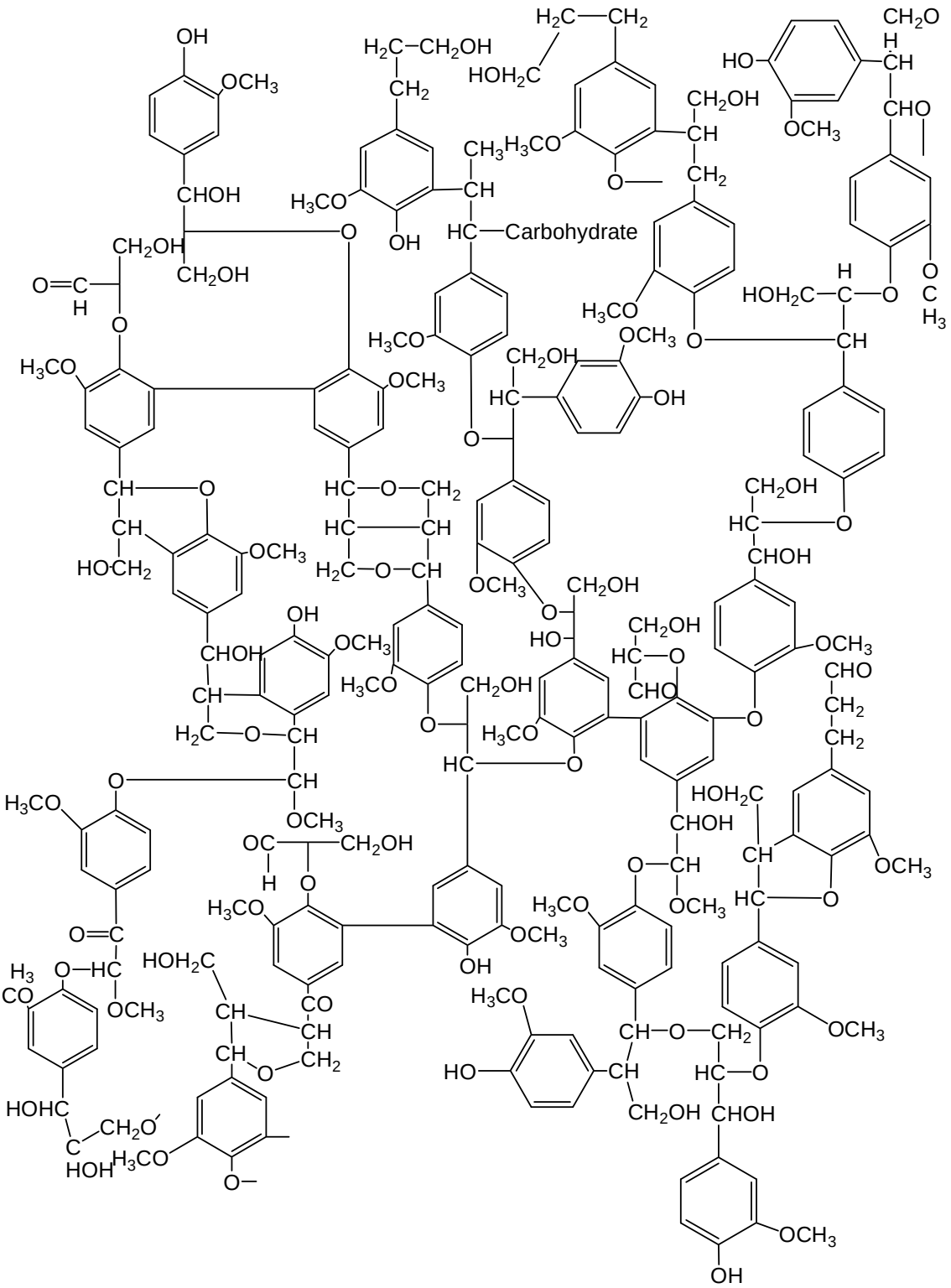
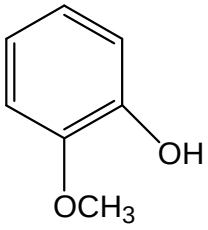


Figure 1.4: Proposed structure of lignin from softwood (12).

From Figures 1.3 and 1.4, it can be seen, that there are many possible combinations of various monomers. The types and proportions depend on the source, such as hardwood versus softwood, and between species within a category.

To isolate lignin from wood, the pulping industry uses two major chemical pulping reactions, alkaline pulping and sulfite pulping (13). The black liquor which is formed, as a result of the alkaline process, is precipitated with acid to yield kraft lignin. Kraft lignin is soluble in alkaline water and is hydrophobic below this condition. Under sulfite pulping, lignin is sulfonated to yield a water soluble compound.

Due to the possible variability of the composition of lignin, lignin model compounds were chosen for research (14). Examples of lignin model compounds are depicted in Figure 1.5. These compounds have been used to explore both enzymatic polymerization (9, 10) as well as enzyme delignification (15) reactions. For this research work, specific phenols were selected based upon both building blocks of colored compounds as well as are related to lignin chemistry. Guaiacol has been known to form a colored solution in the presence of lignin peroxidase (16).



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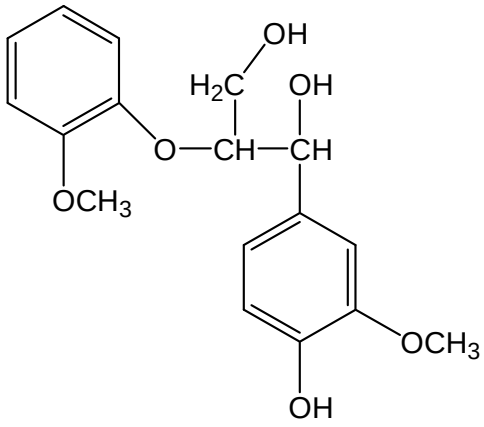

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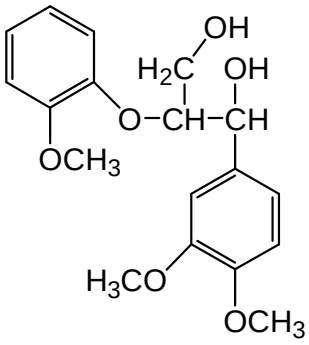

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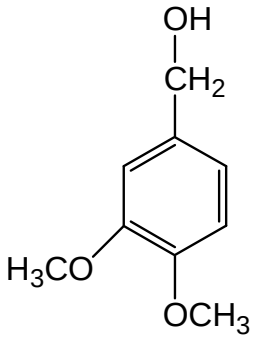

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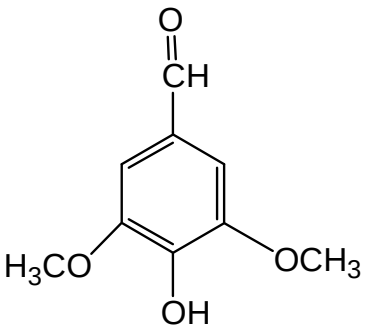

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Figure 1.5: A few examples of lignin model compounds: 1. guaiacol 2. erol 3. adlerol 4. veratrol 5. syringaldehyde.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

A light-weight 100% scoured unbleached linen fabric, with low lignin content of 4.4% (17), was contributed by Hefei Yayuan Dyeing & Finishing Company, China. The yarn count was 52/53 (warp/filling). The fabric weight per unit area was 131.46g/m^2.

Laccase 51002 and 51003 were supplied by Novozymes, Franklinton, NC. Laccase 51002 has an optimum pH at 5.0, which was provided with sodium acetate buffer; the optimum pH of laccase 51003 is 6.0, for which sodium phosphate buffer was used. All lignin model compounds (guaiacol, recorcinol, vanillic acid, vanillin, vanillyl alcohol, isovanillyl alcohol, catechol) were purchased from Sigma-Aldrich Chemical Company. Sodium acetate, glacial acetic acid, HPLC grade ethyl acetate, HPLC grade ethanol, monobasic sodium phosphate, dibasic sodium phosphate, tribasic sodium phosphate, carboxymethyl cellulose, polyvinyl alcohol, and silica beads were purchased from Fisher Chemical Company.

2.2 Methods

2.2.1 Preparation of the Laccase Catalyst

Laccase sample, in a 15 mL conical tube, was thawed, 400 µL pipetted out of the conical tube and placed in a new conical. Next, 4.6 mL of deionized water was added to the tube and mixed. The solution was kept at 4 ºC when not in use; any enzyme sample remaining after a week was not used for experiments.

2.2.2 Polymer Synthesis

50 mg of phenolic compound was placed in 25 mL of 0.05 M sodium acetate buffer, pH 5.0 (or 0.1 M phosphate buffer, pH 6.0). The solution was incubated to approximately to 50 ºC; next, 25 µL of diluted laccase was added to the solution. The reaction was allowed to proceed until the desired time length. The water was evaporated at 100 ºC. The solid sample was then transferred to a clean bottle and saved for further analysis or purification.

2.2.3 Coloration of Linen Fabric

Linen fabric was cut into squares to give an approximate weight of 1.0 g. Next, each piece was weighed and placed in a clean container with 100 mL of buffer solution (0.05 M sodium acetate, pH 5.0 or 0.1M phosphate buffer, pH 6.0). A lignin model compound (e.g. resorcinol) was selected and weighed to obtain the desired percentage to the weight of fabric (owf), and added into the bottle. The bottle was incubated in a shaker oven at 50 ºC and 125 rpm. Next, 0.1% (owf) laccase was added to the contents of the bottle and allowed to incubate for the desired time length. Once the reaction was

complete, the linen fabric was thoroughly washed with water and placed in a beaker with boiling deionized water for 10 min to deactivate the enzyme. The fabric was again rinsed with water, and then placed in the oven to dry overnight; the temperature was set to 60 ºC. Once dried, the fabric was conditioned at 21 ºC and 65% relative humidity for at least 24 hrs. The color coordinates of the treated fabric were measured with a CS-5 Chroma Sensor colorimeter.

2.2.4 Coloration of Polymer Films

A 10% (final volume) colorant solution was added to 18% polyvinyl alcohol (PVA) and 7.5% carboxymethyl cellulose (CMC) solutions to form films; the process required near boiling conditions, for complete dissolution of PVA and CMC. Once the reaction was complete, a few milliliters of solution were cast across a glass plate (10.4 cm x 12.8 cm) with a metal bar to spread the solution evenly across the plate. The plates were then allowed to dry overnight at room temperature. Once dried, the color coordinates of the films were measured with the CS-5 Chroma Sensor.

2.2.5 Purification using Gravitational Column Chromatography

Glass wool was placed at one end of the glass column (98 cm x 2 cm). Silica beads were then placed in a beaker with the mobile phase (ethyl acetate/ethanol/water); the mixture was slowly added to the column, and tapped with a vibrator to ensure tight packing. Once the column was prepared, the solid sample was diluted with the elutant and placed at the top of the column. Mobile phase was added to the top, and the spigot was opened to allow the elutant to flow at a pre-set rate. Fractions were collected and dried in an oven at a temperature between 60 – 70 ºC.

For the product obtained from incubation of laccase and resorcinol, it was found that a 33/33/33 mobile phase provided a separation of two phases (visible by two distinct yellow shades). However, there was a slight overlap between the two fractions. The testing of mobile phases was performed using TLC.

For the product obtained from incubation of laccase and guaiacol, various combinations of ethyl acetate/ethanol/water did not suffice. Thus, another approach was taken using the gravitational column method. Eluting began with a non-polar solvent and slowly progressed to a more polar solvent (hexane, methylene chloride, ethyl acetate, acetone, ethanol, water). Some separation with overlap occurred; however, results were not satisfactory.

2.2.6 Purification via Filtration

A vacuum filter flask was set up. Solid product was placed on top of the filter paper. The sample was then washed with 100 mL of deionized water, followed by rinsing with acetone. Once washed, the filter paper was placed in a 100 ºC oven. After drying, the solid particles were scraped of carefully, and placed in a clean glass bottle. This method was used for product obtained from guaiacol and catechol.

2.2.7 Purification via Separatory Funnel

Solid product obtained via enzymatic synthesis was diluted with 100 mL of water. Next, 100 mL of ethyl acetate was added to the solution and mixed very well. The solution was allowed to sit so that separation of the phases can occur. The ethyl acetate fraction was collected and the above series of steps repeated three times. All organic

fractions were combined and the solvent evaporated off at a temperature between 60 to 70 ºC. The solid was then used for characterization purposes.

2.2.8 Thin Layer Chromatography (TLC)

A 100 mL beaker was used as the developing container for TLC. The beaker was lined with filter paper and solvent added to a depth of 0.5 cm. Aluminum foil was used as a lid. The TLC plate was cut into 2.5 cm x 5 cm. A line was carefully drawn with a pencil approximately 0.8 cm from both ends of the plate. Using a pipette, 2 µL of sample was slowly spotted onto the bottom line until a total of 10 µL of sample was deposited. The TLC plate was placed in an oven set at 100 ºC for 2 min to remove any water which could have adhered to the silica gel and to dry out the sample. The plate was cooled and carefully placed into the developing chamber. The movement of the sample was monitored and stopped when the solvent reached the top line mark. Various combinations of ethyl acetate/ethanol/water mixtures were chosen for testing.

2.2.9 Ultraviolet – Visible Spectroscopy (UV/VIS)

For the UV/Vis measurements, a Thermo Spectronic Genesys-6 instrument was used. Liquid samples to be analyzed, were placed in a plastic cuvette, and absorbance measurements were read between 380 – 800 nm; readings were taken every 10 nm. Samples were diluted with buffer as necessary.

A time point assay for measurement of laccase activity was performed on various phenolic compounds using the UV-Vis spectrophotometer. A 2 mg/mL precursor solution in buffer was prepared in 500 mL total volume. A 25 mL aliquot of this solution was placed into 50 mL conical tubes. The tubes were shaken at 125 rpm and 50 ºC , and

25 µL of laccase was then added to each tube. Reaction was allowed to proceed until the desired time length (points between 1-30 min) and quenched with 300 µL of 5 M sodium hydroxide, so as to ensure deactivation of the enzyme. An aliquot of this liquid sample was taken and diluted with acetate buffer to obtain an absorbance reading between values of 0-1. The solution was diluted with the buffer to maintain the original pH, due to observed color change as alkalinity increased. Each time point was determined in triplicates, and a total of five time points were chosen for each precursor.

2.2.10 Infrared Spectroscopy (IR)

IR measurements were performed on the Thermo Scientific Nicolet 6700 FT-IR instrument. Samples to be characterized were initially cleaned using either the separatory funnel or the filtration purification method. Less than 1 mg of enzymatically formed colorant was placed on the ATR crystal surface and read using the FT-IR. The following settings were used: 50 scans, resolution of 4.0, % transmittance. Readings were taken in the mid-infrared range (4000 – 400 cm^{-1}).

2.2.11 Nuclear Magnetic Resonance (NMR)

For NMR analysis, laccase synthesized purified product was dissolved in deutrated methanol or dimethyl sulfoxide (DMSO). Samples were analyzed using a Bruker Advance NMR Spectrometer operating at 400.18 MHz (proton) or 100.64 MHz (carbon). Residual proton signals of DMSO-d_6 and CD_3OD were 2.50 and 3.31 respectively. ^{13}C chemical shift of standards are, 39.5 (DMSO-d_6) and 49.2 (CD_3OD).

2.2.12 Gas Chromatography – Mass Spectroscopy (GC/MS)

A Griffin 300 GC-MS was used for analysis of purified colored products and to run standard curves of the starting materials. The following concentrations were used for the standards: 0, 0.5, 2, and 4 mg/mL. From each standard or sample 2 µL were injected into the port and carried through the column by helium gas. The start temperature was set at 45 ºC, heating at 20 ºC/min until the temperature reached to 300 ºC; this was held for 5 min. The mass spec analysis was observed between 0 – 400 M/z. Area of the peaks, from the chromatogram, was calculated using the tools provided by the Griffin 300 GC-MS software. All purified products were dissolved in ethyl acetate. Triplicate measurements were performed for each sample.

2.2.13 Differential Scanning Calorimetry (DSC)

The thermal analysis of enzymatically formed products was performed on a DSC Q2000 from TA Instruments. Purified samples, approximately 5 – 10 mg, were weighed in DSC pans and tightly sealed. The change in heat flow (W/g) was monitored between 25 – 270 ºC. The heat flow was increased by 10ºC/min, both in heating and cooling mode.

2.2.14 Electro Kinetic Analysis (EKA)

The streaming potential measurements of the enzymatically treated linen fabric were carried out using a SurPASS Electrokinetic Analyzer (Anton Paar Instruments). Measurements were done using the cylindrical cell, with approximately 300 mg of cut pieces of fabric. The sample was pre-soaked in 1 mM potassium chloride for approximately 5 min, packed into the cell, and the electrodes were attached. Next, the

samples went through a rinsing procedure at a pressure of 400 mbar for 20 s, afterward, the cell was fully tightened. The flow check was then performed to ensure that a maximum pressure of 400 mbar was maintained, which ensures that the flow in the forward and reverse direction remains approximately the same. The pH was manually adjusted to 9.5 using 0.1M NaOH. Then 0.1 M HCl was added via the automatic titrator, so that the pH decreased stepwise by approximately 0.5 pH units. The zeta potential readings were taken until the pH reached to approximately 3.0.

2.2.15 Colorimeter

The CS-5 Chroma Sensor was used to take color measurements of enzymatically treated linen samples; CIE L*a*b* values were recorded for each sample and comparisons were made against the control. L* represents the lightness of a sample; a* signifies the value on the red-green coordinate; b* corresponds to the yellow-blue coordinate. Three random spots on the fabric were read, and the results averaged. Each condition was done in duplicate, resulting in an average from six points.

2.2.16 Washfastness to Laundering

Colored laccase treated linen fabrics were tested for colorfastness to laundering. Wash fastness tests were done using the Laundr-ometer (Atlas Instruments), with the AATCC test method 61-2001-Test 1A.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Synthesis and Characterization

3.1.1 Synthesis of Colorant in Solution

Laccases are known to play a major role in lignin chemistry. Various substituted phenols have been proposed in literature as model compounds for lignin synthesis (13). Some of these components were selected for this research for colorant synthesis since they also represent fragments of common dyes and pigments. Figure 3.1 shows the structure of guaiacol, resorcinol, vanillic acid (4-hydroxy-3-methoxy benzoic acid), vanillyl alcohol, isovanillyl alcohol, and vanillin.

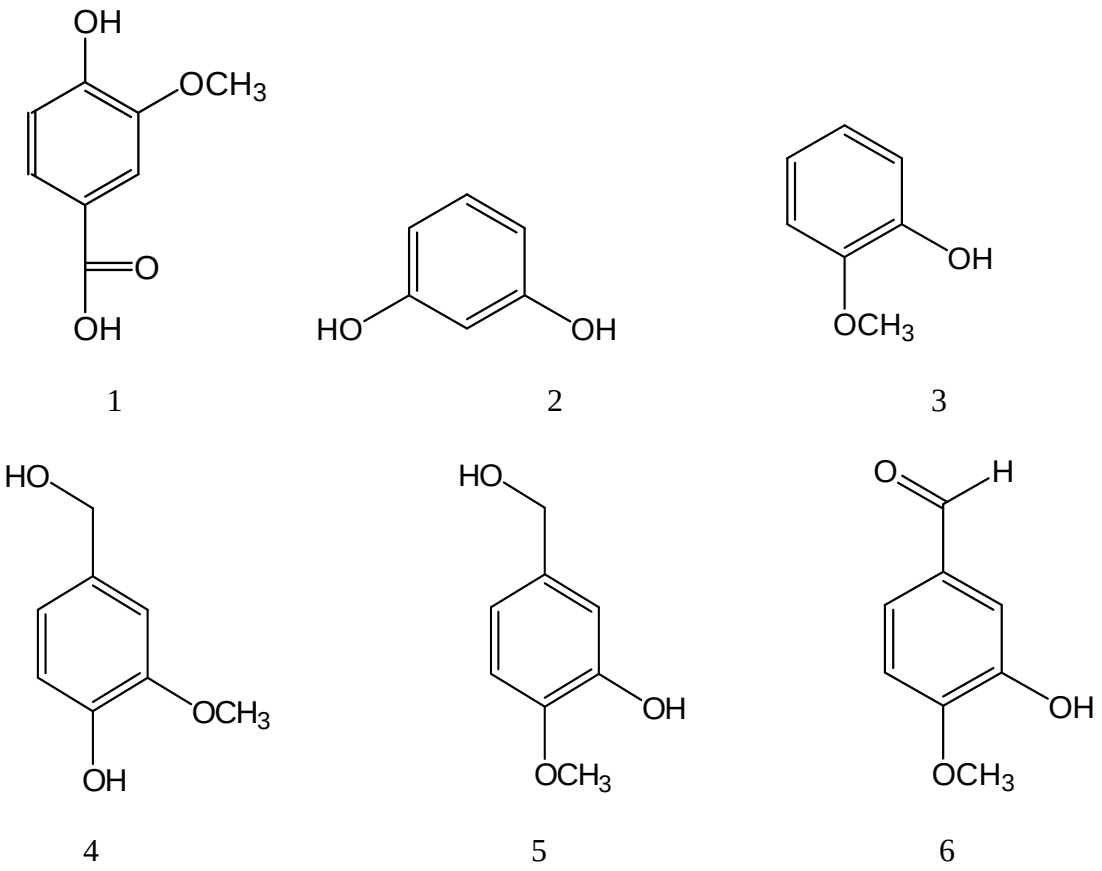


Figure 3.1: Common lignin model compounds: 1. vanillic acid, 2. resorcinol, 3. guaiacol, 4. vanillyl alcohol, 5. isovanillyl alcohol, 6. vanillin.

It was possible to form colored aqueous solutions with each of these phenols in the presence of a laccase under mild conditions. Resorcinol developed a clear bright orange color that did not seem to contain water-insoluble particles, while both guaiacol and vanillic acid resulted in opaque solutions of dark-purple and brownish-orange, respectively, with insoluble particular matter (see Figure 3.2).

Figure 3.2: Colored product obtained from vanillic acid (1), resorcinol (2), and guaiacol (3) in the presence of laccase.

Time point essays were performed for the enzymatic reaction with each phenolic compound and the color developing from the initially colorless solutions recorded. Figures 3.3 and 3.4 clearly demonstrate the time-dependent appearance of an intensifying color with reaction time. A maximum absorbance peak represents the hue of the sample. The peak did not shift significantly with the duration of the enzymatic reaction which means that the basic color shade did not change over time. The color only intensified; thus, more colorant could be produced.

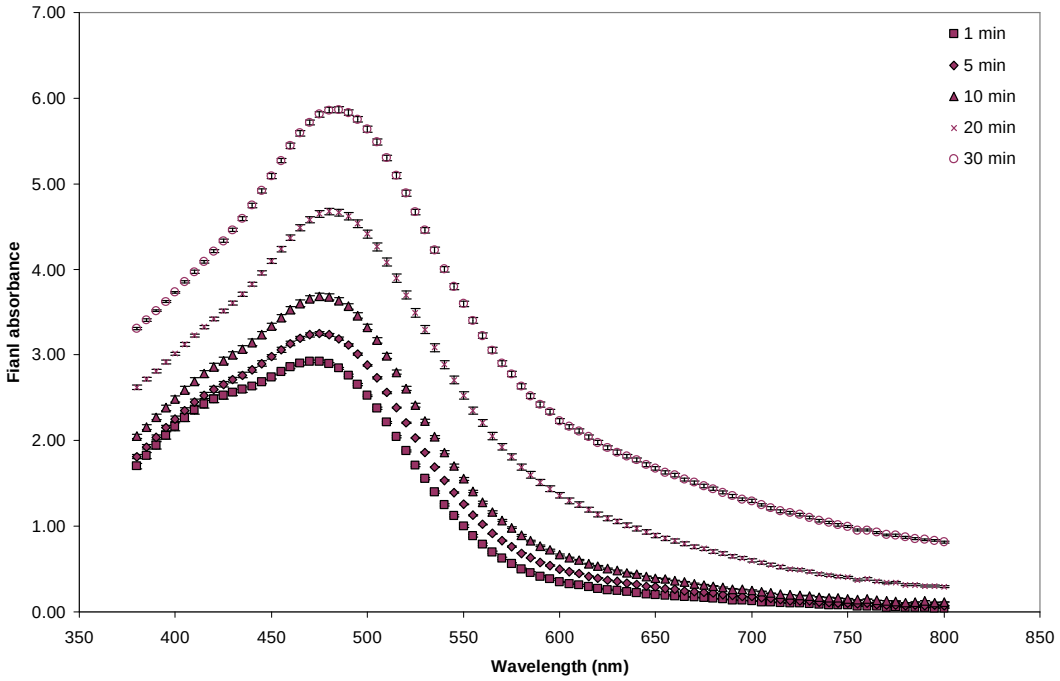


Figure 3.3: Absorbance spectrum of product formed from guaiacol at pH 6, using laccase 51003.

Colored guaiacol product (reddish/purple in hue) gives a bell shaped curve with a maximum at 480 nm (Figure 3.3), whereas, resorcinol product (orange/yellow in hue) resulted in a broad pointy peak, with a maximum at 490 nm (Figure 3.4). Comparisons between Figures 3.2, 3.3 and 3.4 indicate that differences in spectra relate to differences in color, which in turn, are due to the difference in structure.

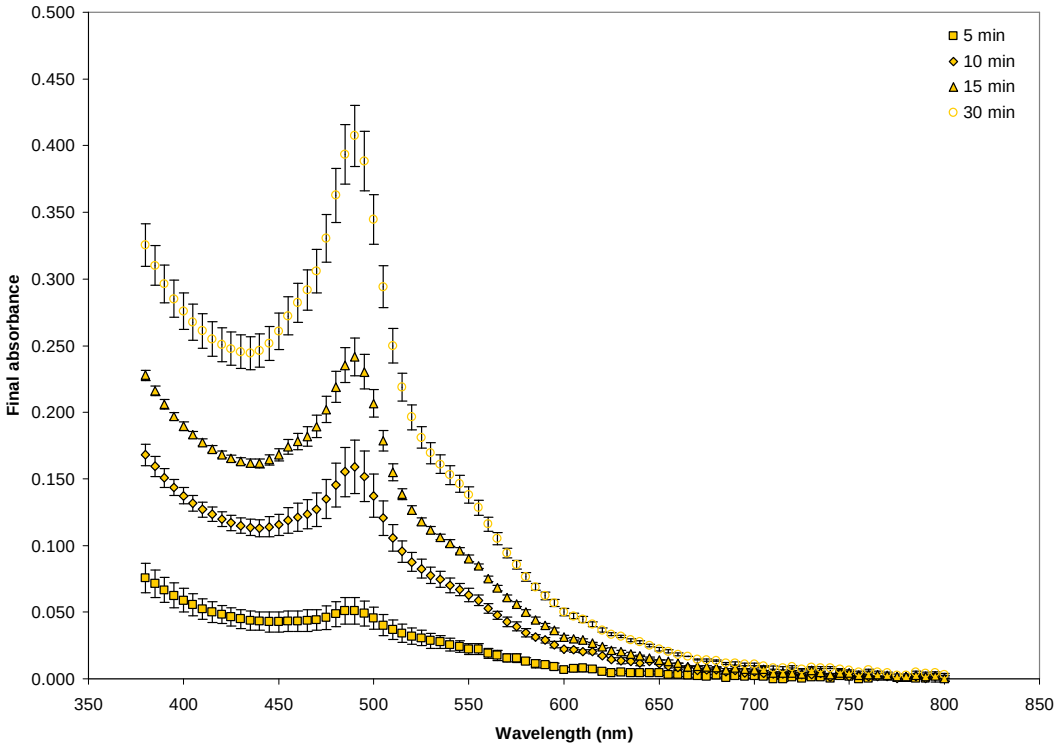


Figure 3.4: Absorbance spectrum of product formed from resorcinol at pH 6, using laccase 51003.

It can be hypothesized that the enzymatically formed products could be of anthocyanine, polymethine, or carotenoid structure. Guaiacol could also form a tetramer, which is known to have a maximum absorbance at 470 nm (16).

3.1.2 Characterization

3.1.2.1 Ultraviolet – Visible Spectroscopy

Substrate affinity of substituted phenols for laccase was monitored by following the changes in maximum absorbance over time (Figure 3.5). A graph demonstrating the increase in product is presented in Figure 3.5 for guaiacol. If the maximum absorbance ($AU_{\lambda max}$) is graphically correlated with the reaction time, the slope of the resulting

straight line can be used to estimate the colorant formation rate in $AU_{\lambda max}$/min. Rates were not reported as change in concentration per minute.

Although the formed colorants were cleaned of salts and enzyme, it is likely they still consist of a mixture of components; thus, a standard curve could not be obtained, and therefore, rates of product formation were reported as $AU_{\lambda max}$/min. Nonetheless, it is possible to relate absorbance to concentration, since absorbance is directly proportional to it, according to Beer – Lambert law ($A=\varepsilon bc$; where A equals absorbance, b the path length, c the concentration, and ε molar absorbtivity). Hence, an increase in maximum absorbance corresponds to an increased amount of formed product.

Enzymatic reactions were initially performed with both types of laccases (51002 at pH 5.0 and 51003 at pH 6.0) and results for colorant production with either enzyme are listed in Table 3.1. Overall, it was found that both enzymes worked equally well, and that the differences in product formation rate or properties were minor. Thus, for most subsequent experiments laccase 51002 was arbitrarily chosen as the catalyst.

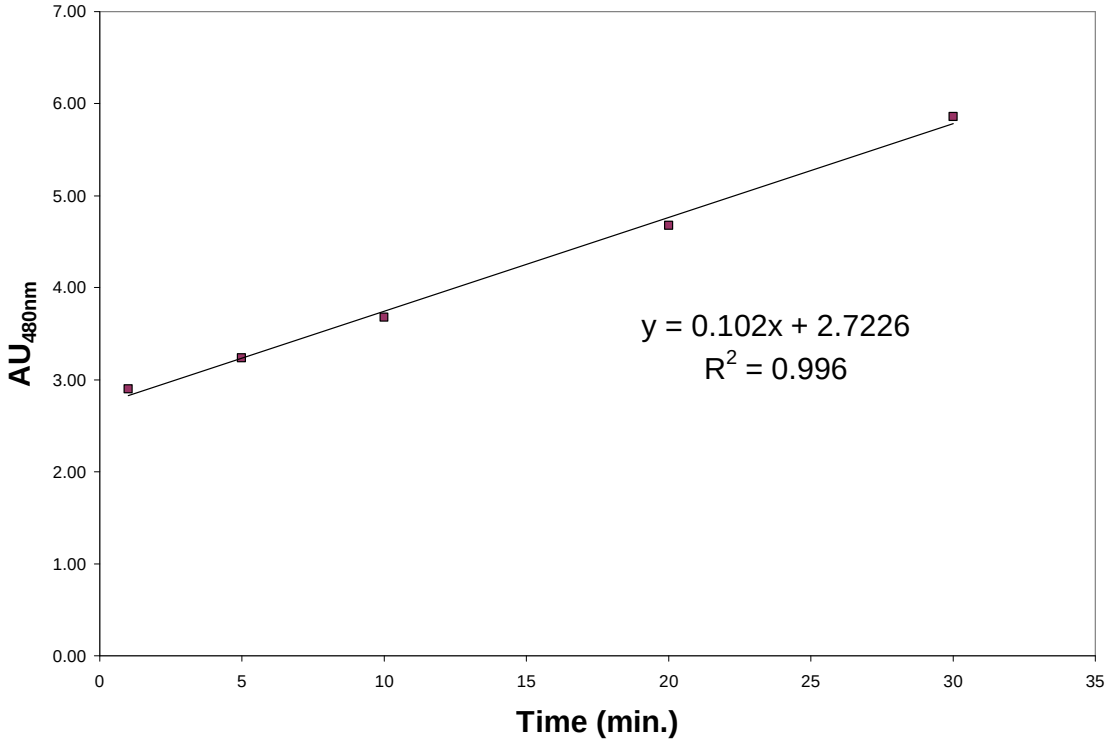


Figure 3.5: Increase in guaiacol product quantity monitored at maximum absorbance with laccase 51003.

It was found that both laccase 51002 and 51003 formed products at a faster rate for guaiacol with AU_{480}/min rates of 0.065 and 0.102 (Table 3.1). It seems that, as more substituents are added onto the benzene ring, the amount of product decreases.

Table 3.1: Rate of colorant formation for substituted phenols by measurement of increase in maximum absorbance per minute with two types of laccases.

Phenol	Laccase 51002		Laccase 51003	
	$AU_{\lambda max}$/min	R^2	$AU_{\lambda max}$/min	R^2
Guaiacol	0.0654	0.9956	0.102	0.996
Resorcinol	0.0217	0.9998	0.0138	0.975
Vanillic acid	0.0139	0.9526	0.0085	0.9883
Vanillyl alcohol	0.0105	0.9775	0.0077	0.9956
Isovanillyl alcohol	0.004	0.988	0.0009	0.971

Guaiacol and resorcinol clearly formed the highest amount of product in the shortest time. Therefore compounds obtained from these phenols were studied more in depth than from the other phenols.

3.1.2.2 Differential Scanning Calorimetry

In a solid, molecules are held closely together by strong intermolecular attractive forces. As temperature is raised, disorder within the structure increases; vibrations within the structure gradually rise until they overcome the attractive forces holding the solid together. The solid shows melting at a specific temperature, referred to as the T_m.

In order for the T_m of a compound to increase, the contribution of intermolecular forces and/or a restriction of vibrational, rotational, or transational motions of the overall structure must occur. Therefore, it could be possible, that an increase in T_m of a product could indicate the formation of a higher molecular weight structure, a more crystalline structure, or a combination of both.

With the help of the DSC, measurements of temperature related changes in physical state, such as melting (T_m) or freezing point, as well as non-thermodynamically defined transitions, such as glass transition, can be determined by rising the temperature of a sample and a standard at the same heating profile. The difference in energy needed by the sample for these transitions will be reflected in peaks in the DSC diagram while the standard maintains a straight increase in temperature. If by modification a compound increased in molecular stiffness, crystallinity or molecular weight, sample transitions will appear at higher temperatures as compared to a control.

DSC measurements were performed for guaiacol, resorcinol and their enzymatically formed products. The results are presented in Figures 3.6 and 3.7.

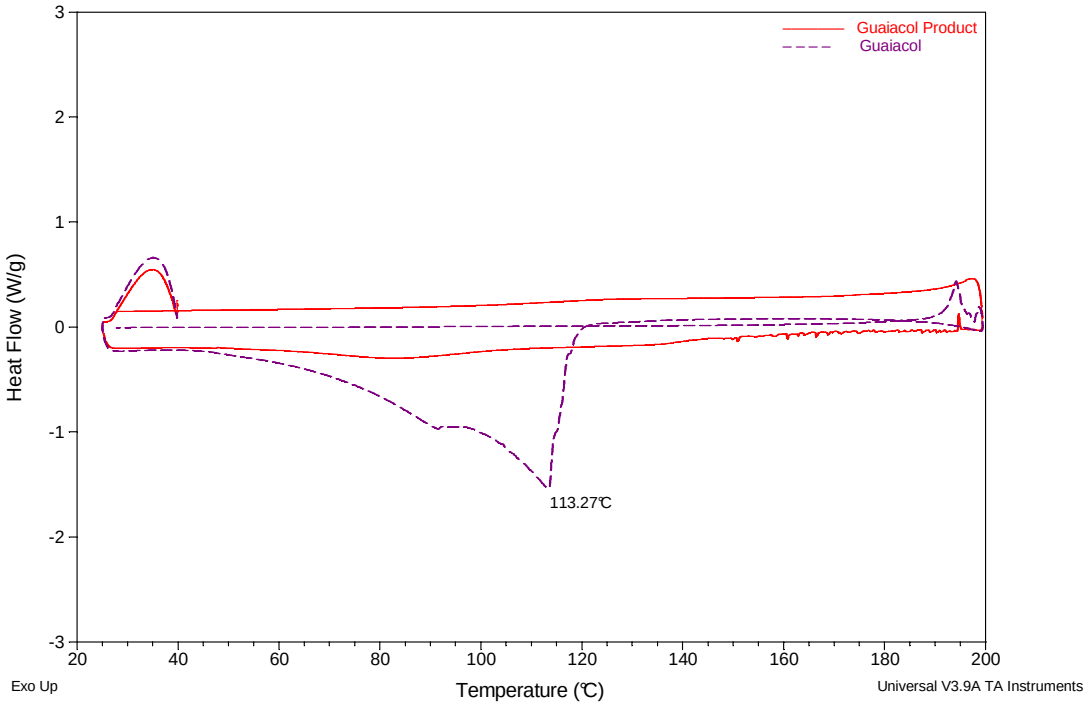


Figure 3.6: DSC of guaiacol and guaiacol colorant formed by laccase.

The T_m of guaiacol was observed to be 113.2 ºC, which agrees with the literature value. As Figure 3.6 indicates, guaiacol product did not exhibit a T_m between 20 - 270ºC; this could be due to an increase in the melting point temperature above the measured temperature range or a decomposition of the compound at elevated temperatures. A higher T_m would indicate an increase in molecular weight. However, from a preliminary experiment of simply heating the sample above 400ºC, some color loss was observed. When both profiles are compared, the enzymatically synthesized product of guaiacol seemed to be somewhat more thermally stable.

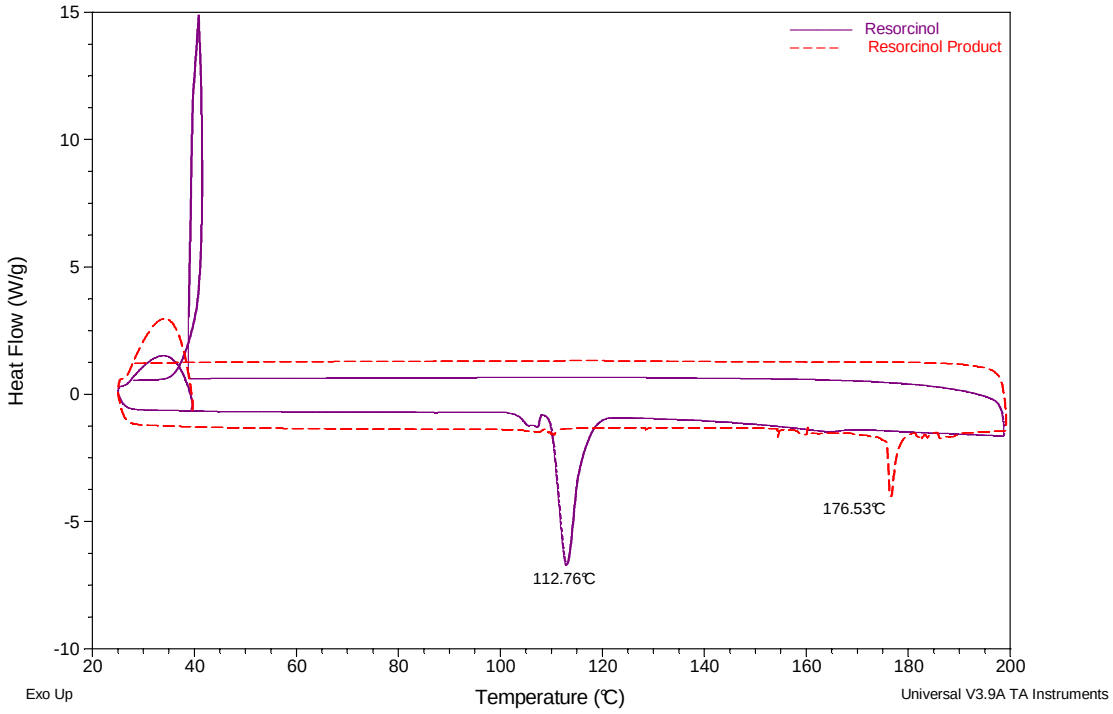


Figure 3.7: DSC of resorcinol and resorcinol colorant formed by laccase.

The T_m of resorcinol was measured to be 112.8ºC, also in agreement with the literature. The colored product obtained from laccase synthesis using resorcinol, showed a T_m value of 176.3ºC, indicating an increase in molecular weight.

3.1.2.3 Infrared Spectroscopy

To evaluate the structural properties of substrate and product, FT-IR spectra were taken of resorcinol, resorcinol product, guaiacol and guaiacol product, presented in Figures 3.8 and 3.9.

In the upper spectrum in Figure 3.8 (resorcinol), the strong peak around 3175 cm^{-1} belongs to the O–H stretching vibrations of resorcinol. O–H in and out of plane bending vibrations can be seen by absorption peaks at 1375 cm^{-1} and 679 cm^{-1}, respectively. The absorption peaks between 1487 cm^{-1} and 1606 cm^{-1} can be attributed to aromatic C = C stretching vibrations, and four bands found between 739 cm^{-1} and 900 cm^{-1} are due to arene C–H bending vibrations. The C – O stretching vibrations of resorcinol can be found at 960 cm^{-1} and 1144 cm^{-1}(10, 18, 19, & 20). In the lower spectrum in Figure 3.8, which corresponds to the resorcinol product, the broadening of the absorption peak at 3207 cm^{-1} could be assigned to polymeric O–H stretching frequencies. Broadening of absorption bands was observed between 400 cm^{-1} to 1600 cm^{-1}.

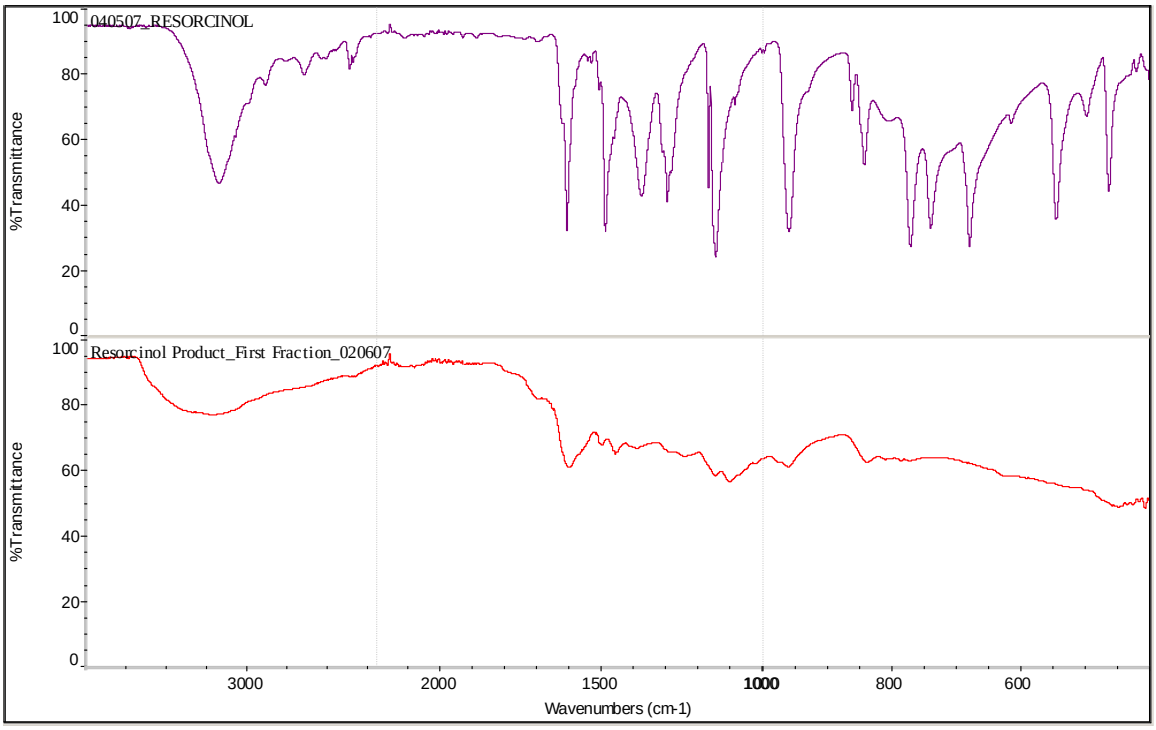


Figure 3.8: FT-IR spectra of resorcinol (upper) and resorcinol product (lower).

In Figure 3.9 (upper spectrum), the strong peak around 3506 cm^{-1} belongs to the O–H stretching vibrations of guaiacol. The two bands around 2949 cm^{-1} and 2841 cm^{-1} are due to $–CH_3$ and aromatic $–OCH_3$ stretching vibrations, respectively. O–H in-plane bending vibrations can be seen by the absorption peak at 1360 cm^{-1}. The absorption peaks at 1498 cm^{-1} and 1596 cm^{-1} can be attributed to aromatic C = C stretching vibrations. Bands found between 739 cm^{-1} and 916 cm^{-1} could be due to arene C–H bending vibrations. The C–O stretching vibrations of guaiacol can be found between 1038 cm^{-1} and 1171 cm^{-1}. Peaks at 1256 cm^{-1}, 1205 cm^{-1}, and 1020 cm^{-1} can be attributed to aromatic and vinyl ethers (18, 19, & 20). In the bottom spectrum of Figure 3.9, which corresponds to guaiacol product, the broadening of the absorption peak at 3418 cm^{-1} could arise from polymeric O – H stretching frequencies. The loss of the prominent band

at 739 cm^{-1} could be the effect of increased rigidity within the structure. If a network were to form, aromatic C–H would find it more difficult to bend.

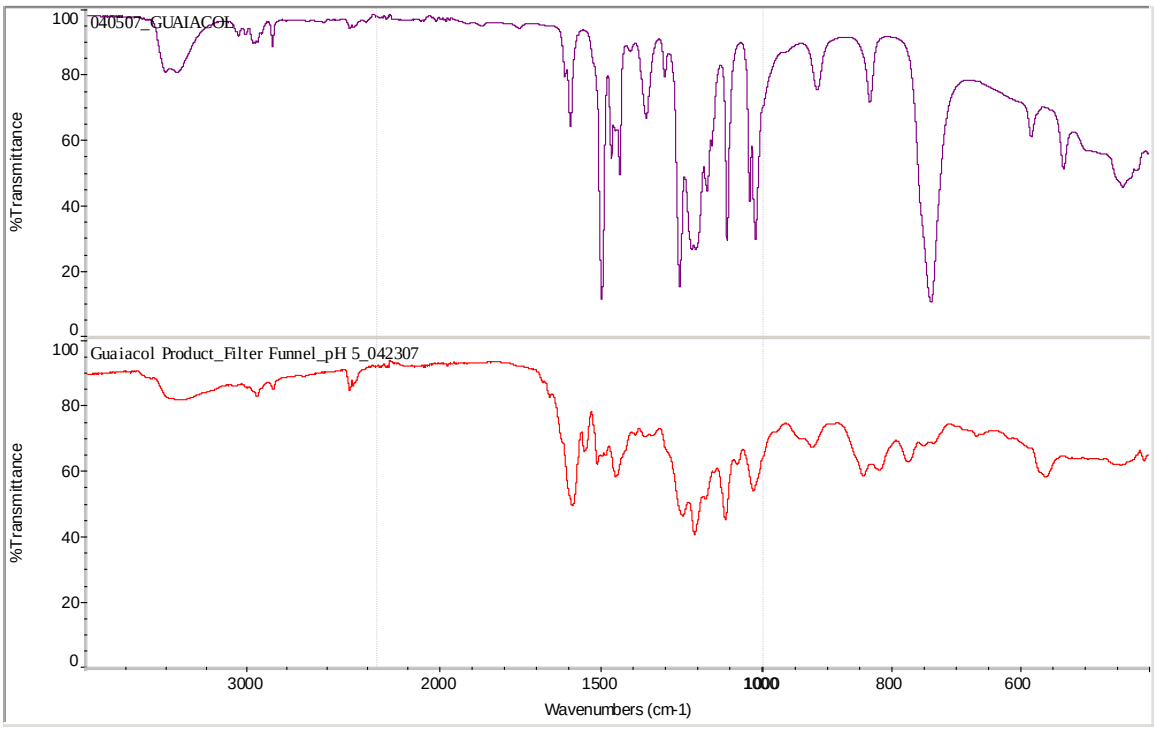


Figure 3.9: FT-IR spectra of guaiacol (upper) and guaiacol product (lower).

An overall broadening of absorption bands is observed between 400 cm^{-1} to 1600 cm^{-1}, as well as broadening of the phenolic O–H stretch, for resorcinol and guaiacol products; this weakening of intensity could originate from higher symmetry within the structure and intermolecular hydrogen bonding, respectively. It is possible that the overlapping of band frequencies is a result of an increase in the number of neighboring atoms, due to the formation of either an oligomeric or polymeric structure. This could affect the vibrational modes of near-by groups; resulting in a broadening of absorption intensities.

3.1.2.4 Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy experiments were performed on purified resorcinol and guaiacol products. Peaks were observed between chemical shifts of 3.4 – 3.9 ppm, 6.5 – 7.5 ppm, and 8.4 – 9.4 ppm in the proton NMR spectrum of guaiacol colorant (Figure 3.10) which had not appeared in the NMR spectrum of the starting compounds. Peaks between 3.4 – 3.9 ppm indicate the presence of either an alcohol or ether proton (21). The peaks occurred as a set of multiple signals with varying intensity and broad overlapping (signals were not separate and well defined). This broadening could be due to different types of alcohols or ether protons producing signals around the same frequency. Peaks between 6.4 – 9.4 ppm indicate the presence of aryl and phenolic protons. The more downfield signals could also be the result of aldehyde or carboxylic protons. Each cluster of peaks denotes that different types of hydrogen are present; thus, it can be speculated that the structure of the formed compound is fairly complex.

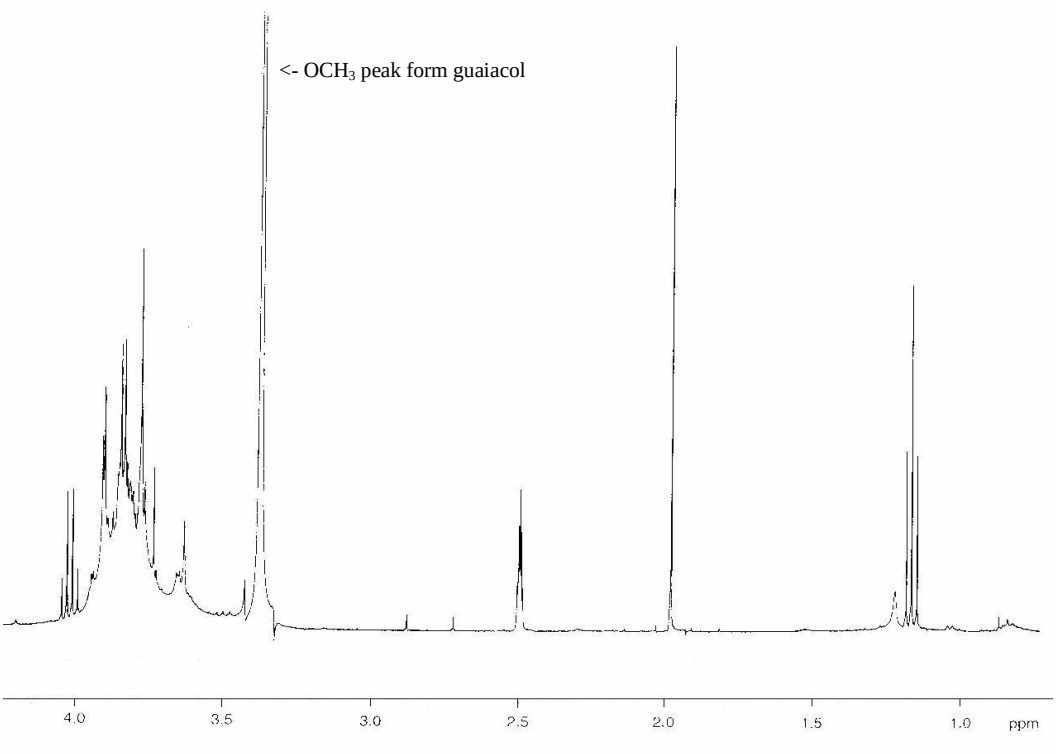


Figure 3.10: Proton NMR of guaiacol product, chemical shift between 1.0 – 4.2 ppm.

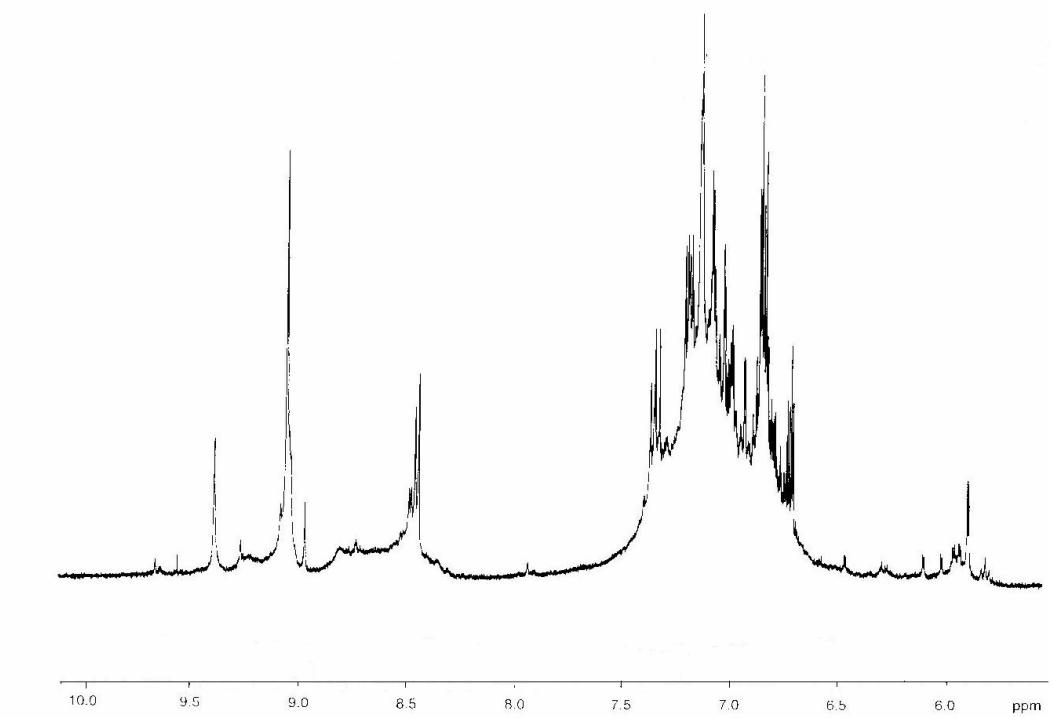


Figure 3.11: Proton NMR of guaiacol product, chemical shift between 5.5 – 10 ppm.

It was difficult to interpret the proton NMR signals and clearly assign them to a specific product as they were rather weak in comparison to the reference peaks. It seemed that a relatively small amount within the sample was actually responsible for the color. A carbon spectrum of guaiacol colorant was also obtained; other than signals from guaiacol carbons, only a few new peaks could be seen very close to background noise. Carbon NMR spectra did therefore not appear to be valuable enough to further pursue.

Both proton and carbon NMR of resorcinol product were taken; comparable results were obtained for the resorcinol colorant as discussed for the guaiacol colorant (multiple peaks with overlap).

3.1.2.5 Gas Chromatography/Mass Spectroscopy

Samples of guaiacol product, resorcinol product, and standards were run on the GC/MS. Using the internal software, the area under the peaks was calculated and a plot generated for the standard curve of concentration versus area; a standard curve for guaiacol is shown in Figure 3.12. Regression analysis indicated that less than 0.5 mg/mL of substrate remained for both guaiacol and resorcinol. Initial reactions were set up with 2.0mg/mL of substrate. Considering that some of the phenol could be lost during the purification process, one can approximate that between 50 - 75% of the substrate has been used by laccase in the synthesis of the colored compounds. This yield could possibly be increased by the addition of more laccase over the course of the reaction.

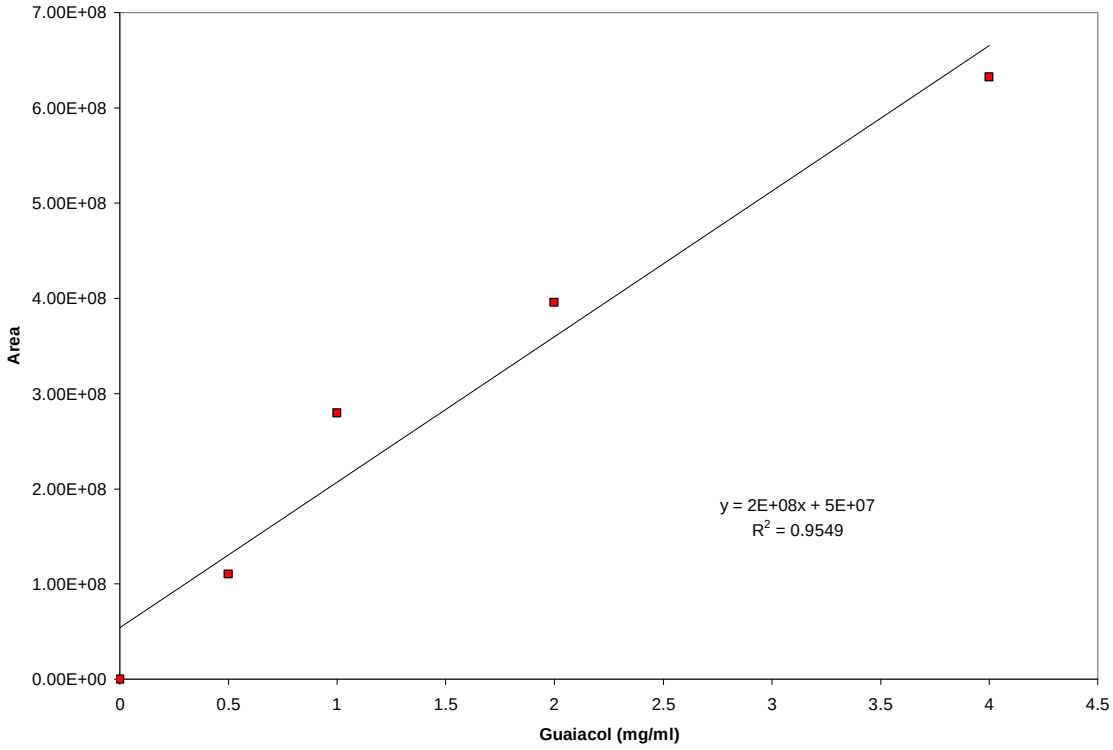


Figure 3.12: Guaiacol Standard Curve on GC-MS, concentration versus area of peak.

3.2 Application of Colorant

Although it is significant that colorants can be synthesized via green processes, it is both necessary and important that applications for these colorants can be found. Hence, coloration experiments were conducted on linen fabric, as well as for films from poly(vinyl alcohol) PVA and carboxymethyl cellulose (CMC). For this purpose colorant was incorporated into polymer solutions before film forming.

Guaiacol, resorcinol, and vanillic acid were used most extensively for the coloration experiments. Based on initial tests, they proved to be the most effective phenols in dyeing of linen fabric. The colored fabrics showed sufficient contrast in shade between various treatments and the control, so that the results could be clearly demonstrated and compared between samples.

3.2.1 Coloration of Linen

Linen fabric was incubated in a buffered media with laccase and a suitable substrate. Substrate concentrations were either 20% or 80% based on fabric for 1, 5, and 24 h incubation periods.

Figure 3.13 shows the effects of enzymatic coloration of linen with guaiacol and laccase. Color absorption onto linen fabric increased over time when laccase was provided 20% (owf) of substrate. This trend was not observed when laccase was given 80% (owf) of substrate; the maximum absorption occurred after 5 h of incubation, and the CIEL*a*b* values were similar to those of incubation with 20% (owf) of substrate for 5 h. This could be due to either the lack of oxygen within the container relative to the substrate concentration, or substrate inhibition effects. It was observed that the best

absorption of colorant occurred by providing laccase at 20% (owf) of substrate with an incubation of 24 h.

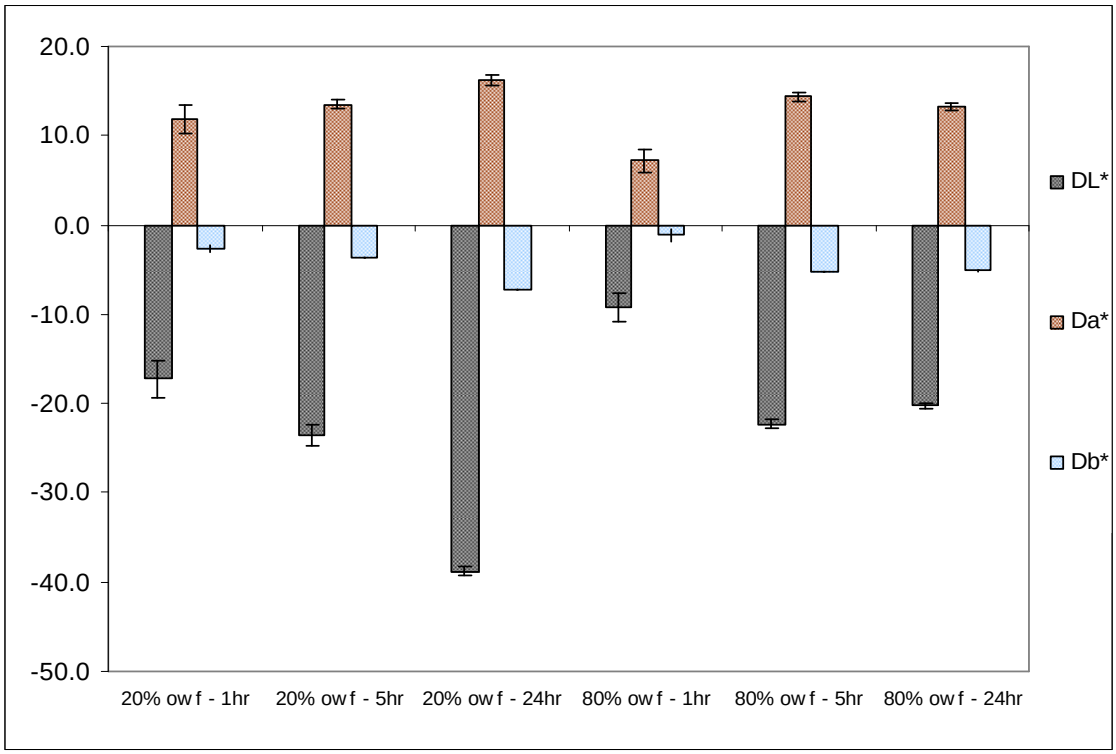


Figure 3.13: Change in CIEL*a*b* values of linen fabric at various incubation times with 20 % or 80% guaiacol substrate in the presence of laccase.

Figure 3.14 shows the effects of coloration of linen with resorcinol and laccase. Color absorption onto linen fabric increased over time when laccase was provided either 20% or 80% owf of substrate, with the maximum absorption occurring after 24 hours of incubation. There seems to be a slight benefit in coloration of linen with resorcinol at 20% owf of substrate rather than 80% at all incubation times. This could be due to substrate inhibition occurring at higher concentrations, which results in slowing reaction rates; hence, less colorant produced given equal amounts of reaction times.

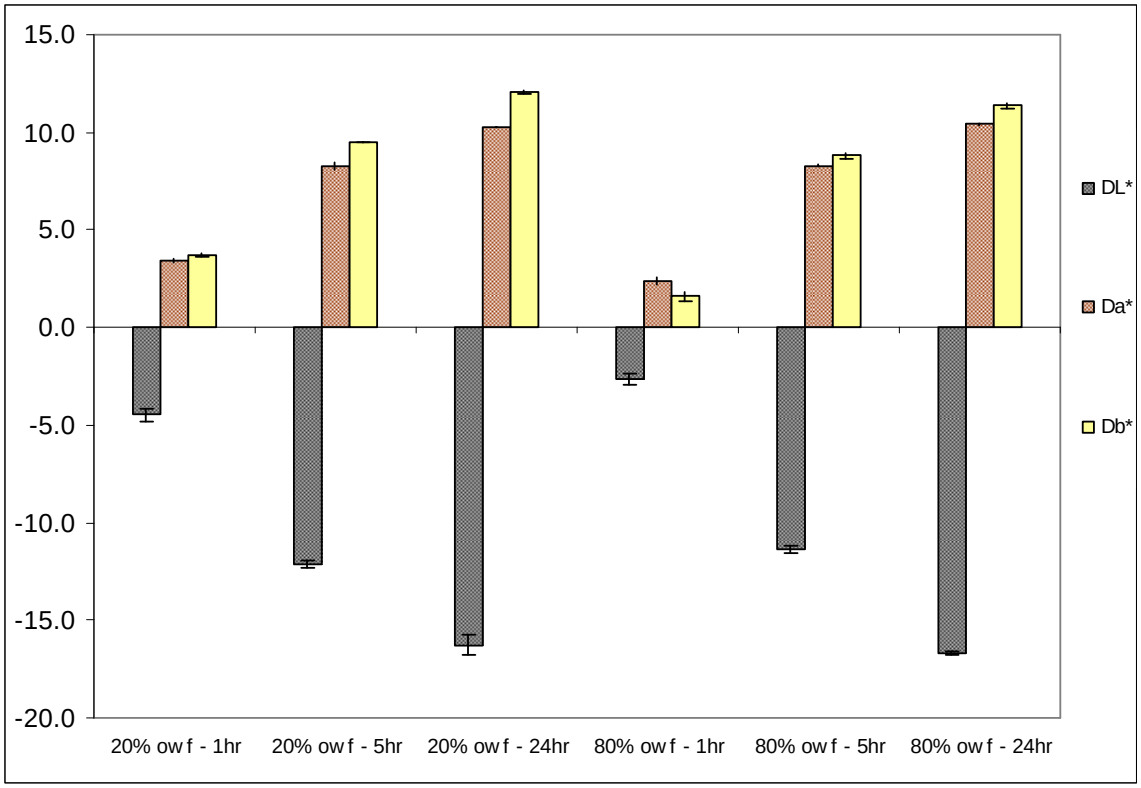


Figure 3.14: Change in CIEL*a*b* values of linen fabric at various incubation times with 20 % or 80% resorcinol substrate in the presence of laccase.

Since it is known that lignin plays an important role in the enzymatic synthesis with laccase, the coloration experiments were repeated with the addition of free lignin and sulfonated lignin present in the batch. This was to observe whether the additional unbound lignin could enhance in the process or whether only lignin enclosed in the linen fibers was effective.

It was noted that, in the presence of free lignin (Figures 3.15 and 3.16), coloration of linen occurred with either guaiacol or resorcinol; however, the depth of shade was reduced. This could be due to free lignin adsorbing at the surface of the fabric without actually reacting with guaiacol or resorcinol under the influence of laccase. Thus the

surface would be partially blocked and the formation of colorant hindered to a certain extent.

In the presence of free sulfonated lignin (Figures 3.15 and 3.16), the shade of the fabric changed to a light brownish-grey; this was observed for both guaiacol and resorcinol coloration processes. This indicates that sulfonated lignin either formed a competitive inhibitor in the reaction of both guaiacol and resorcinol, or that interactions occurred between the phenols and free sulfonated lignin, which could reduce its substrate affinity for laccase. Control experiments of free lignin and sulfonated lignin with laccase in absence of phenols were also performed under the same conditions. Color barely developed on the linen fabric samples, even less with the sulfonated form of lignin. These experiments made clear that additional lignin did not enhance the coloration process, at least not in the form used in this research.

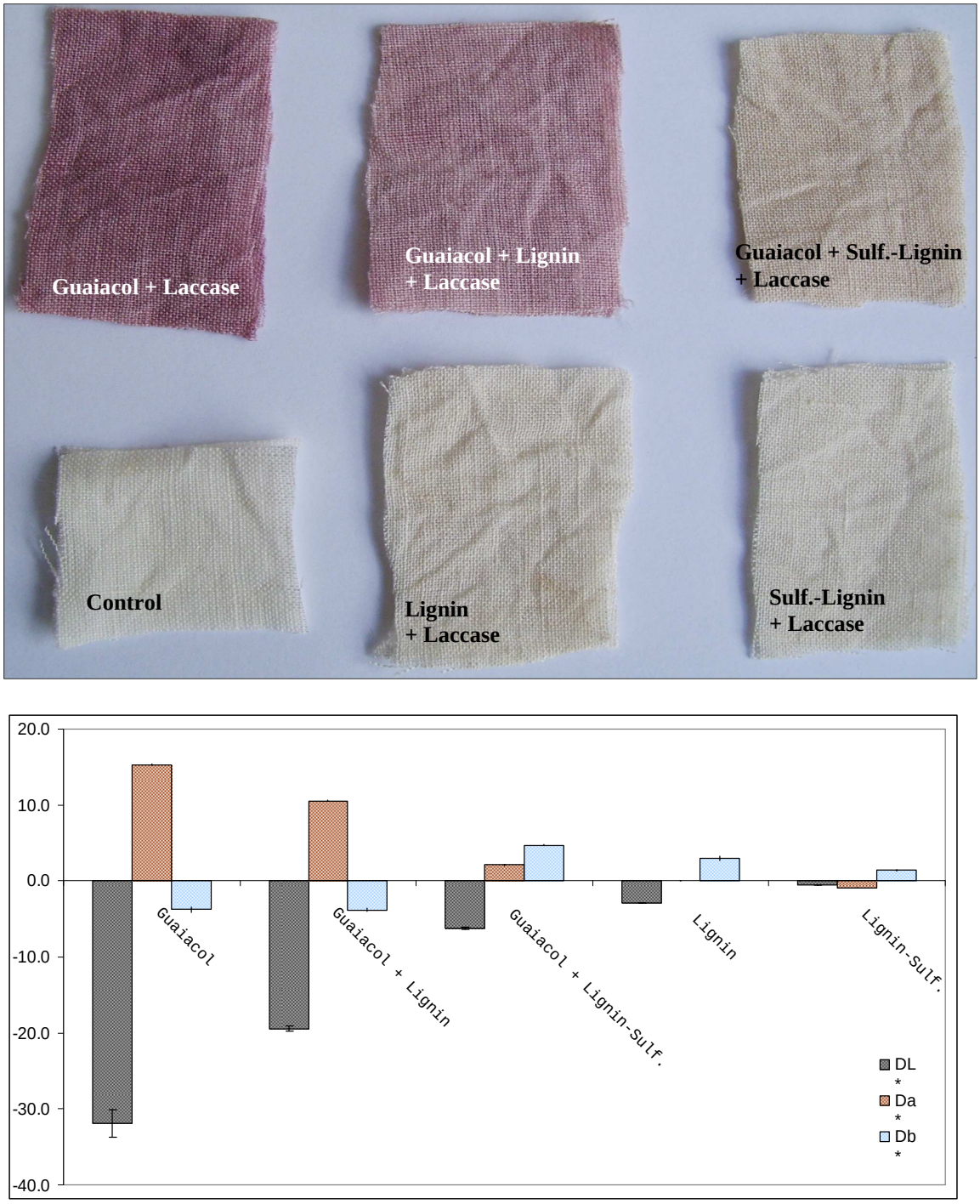
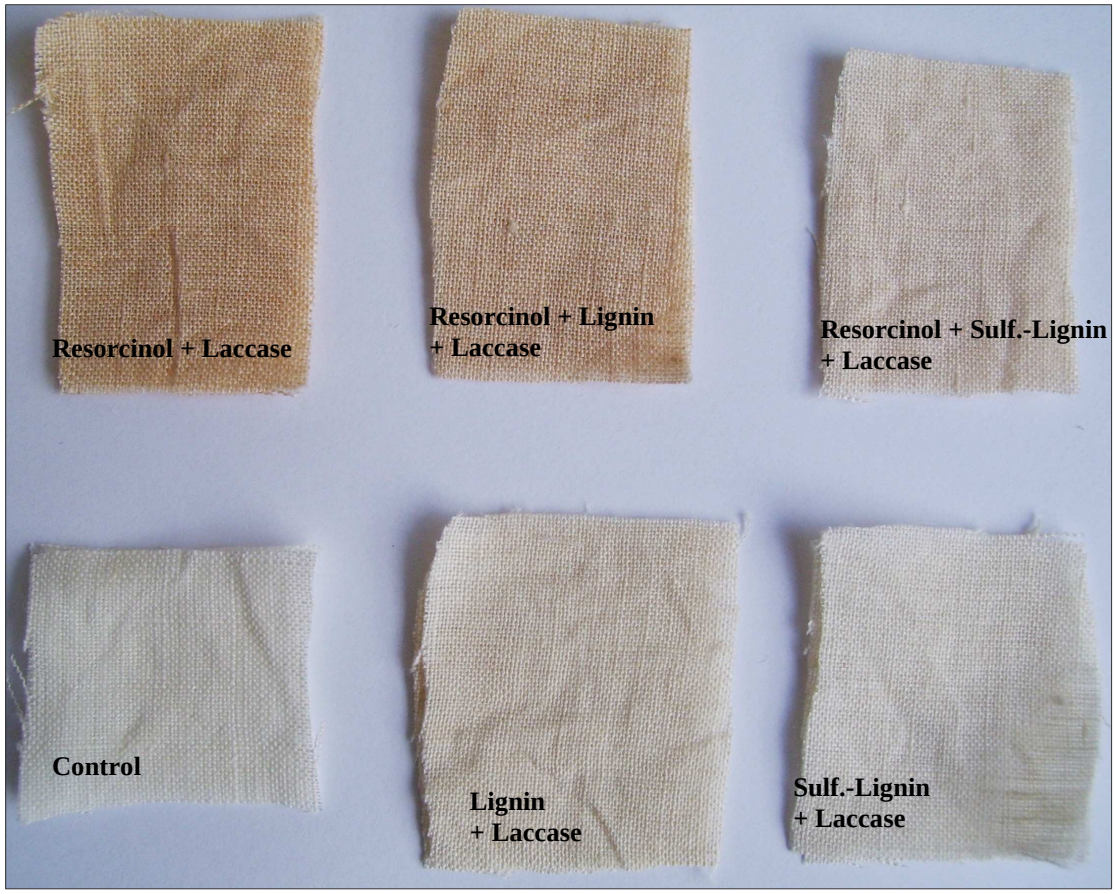


Figure 3.15: Image and CIEL*a*b* values of colored linen fabric in the presence of guaiacol and laccase with and without the 20% (owf) of free lignin and sulfonated lignin.



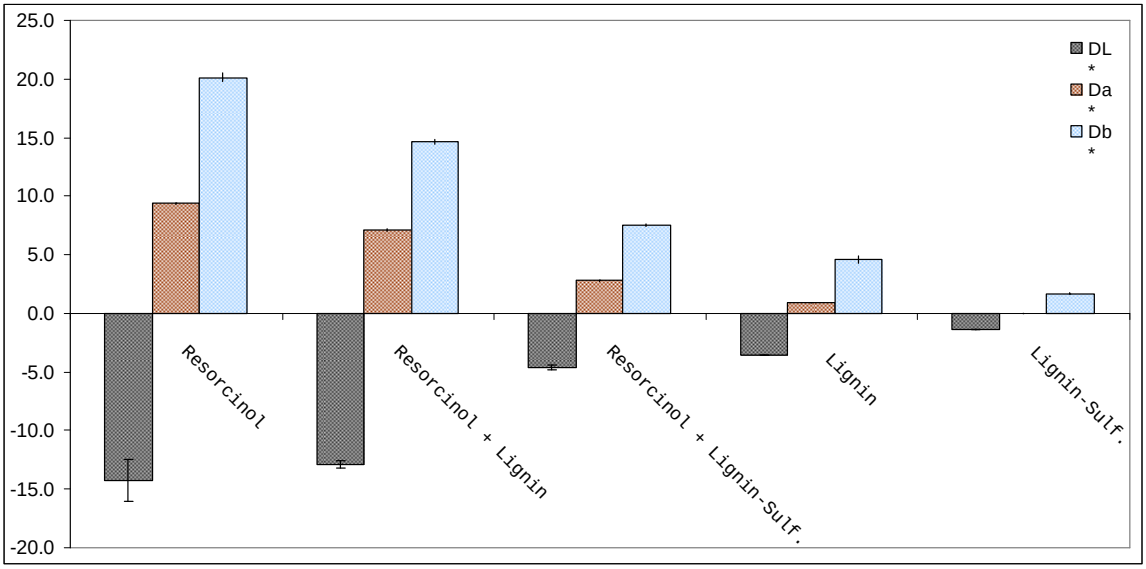


Figure 3.16: Image and CIEL*a*b* values of colored linen fabric in the presence of resorcinol and laccase with and without the free lignin.

3.2.1.1 Streaming Potential Measurements

The surface properties of both treated and untreated linen fabrics were evaluated using electrokinetic analysis (EKA). Zeta potential values were monitored from a pH of 3.0 to approximately pH of 9.0.

When two surfaces are in contact with one another (such as a solid and liquid) a surface charge develops which depends on the pH value of the liquid and the functional groups of the solid. Directly at the interface, a rigid electronic double layer forms, of immobile counterions adsorbed at the surface functional groups, which is referred to as the Stern layer. With increasing distance from the Stern layer, the influence on ionic movement in the liquid is lessened (diffuse layer) until finally ions move freely in the bulk of the liquid. The shear plane between diffuse layer and free flow in the bulk is not defined thermodynamically, but can be determined by streaming potential measurements (EKA). The resulting electrokinetic or zeta potential is an indication of the charge and thus the surface functionality of the solid.

The linen control sample showed the expected profile with varied pH values (Figure 3.17, 21). With exposure to laccase, the negative surface potential was significantly reduced (BE in Figure 3.17). Since laccase has no substantivity for cellulose, only groups of the incorporated lignin could have reacted with it, and all effects observed can only be assigned to functional groups of lignin. It is interesting, although fairly small, that it made a difference whether guaiacol or resorcinol was used to produce colorants in regard to the surface acidity or basicity of the linen fabric. The enzymatic reaction in presence of resorcinol resulted in a surface more similar to that of the

untreated linen, while the surface after reaction with guaiacol resembled that of the laccase sample (RE and GE in Figure 3.17, respectively). In both cases, the surface was less negative than that of the untreated control.

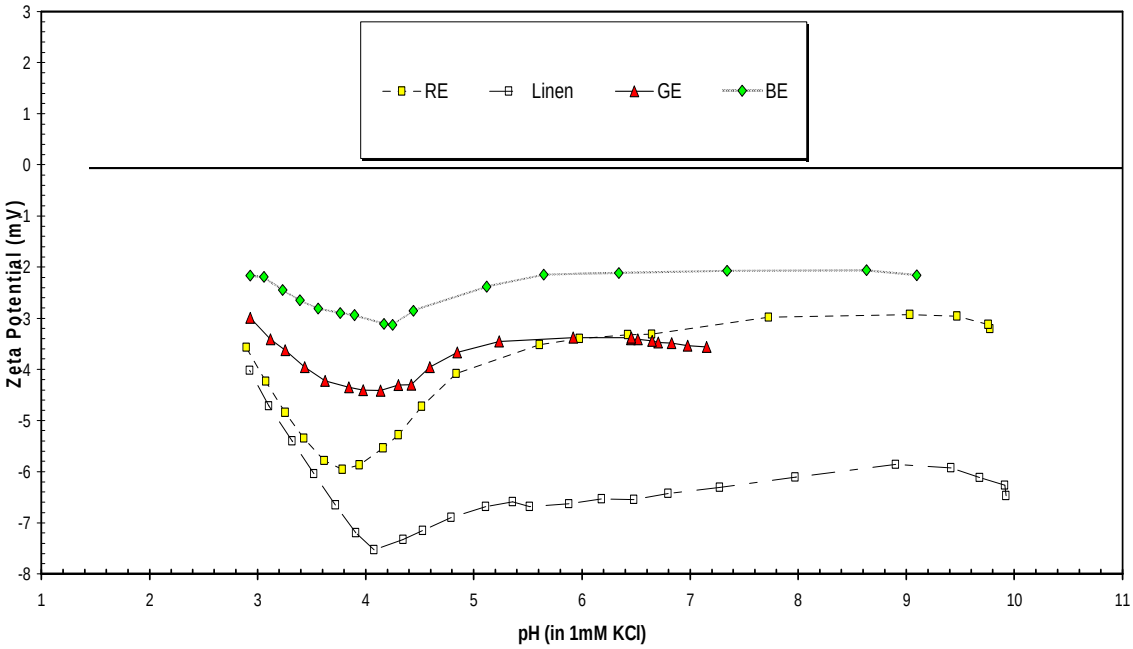


Figure 3.17: Zeta potentials of enzyme treated linen fabric corresponding to the pH value. RE = resorcinol + laccase + linen (colored square), GE = guaiacol + laccase + linen (triangle), BE = laccase + linen (diamond), and Linen (square).

When free lignin was added into the process, zeta potentials fell between those of the guaiacol reaction alone (GE) and the untreated linen samples (Figure 3.18). If amount of added free lignin was increased, the negative zeta potential was slightly lower (G20LE versus G10LE). As mentioned above, free lignin did not specifically increase the formation of colorant on the linen fabric. It was speculated that a weak interaction between free lignin and surface groups on the linen fabric occurred, which hindered the process more than it assisted (by blocking available interaction sites for the colorant). The initial interaction between free lignin and surface groups was weak and unstable;

hence, the final rinsing step in the dyeing process could disrupt this interaction, releasing the free lignin and exposing the negatively charged groups.

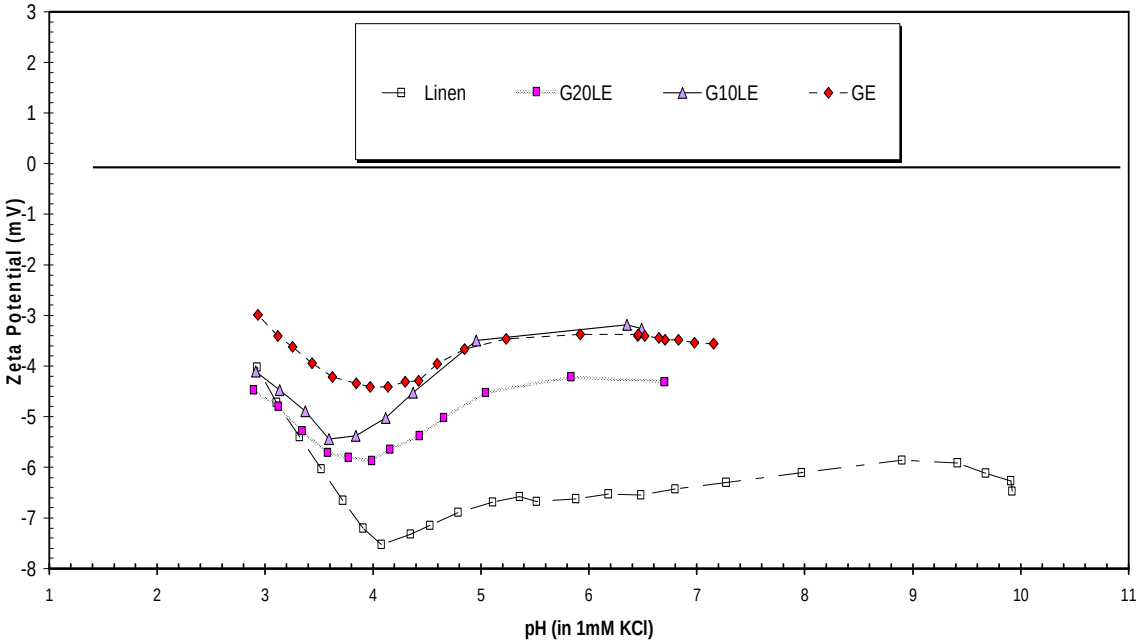


Figure 3.18: Impact of the addition and concentration of free lignin on the enzymatic color formation with guaiacol. GE = guaiacol + laccase + linen (diamond), G10LE = guaiacol + laccase + linen + 10% free lignin (owf) (triangle), G20LE = guaiacol + laccase + linen + 20% free lignin (owf) (colored square), and Linen (square).

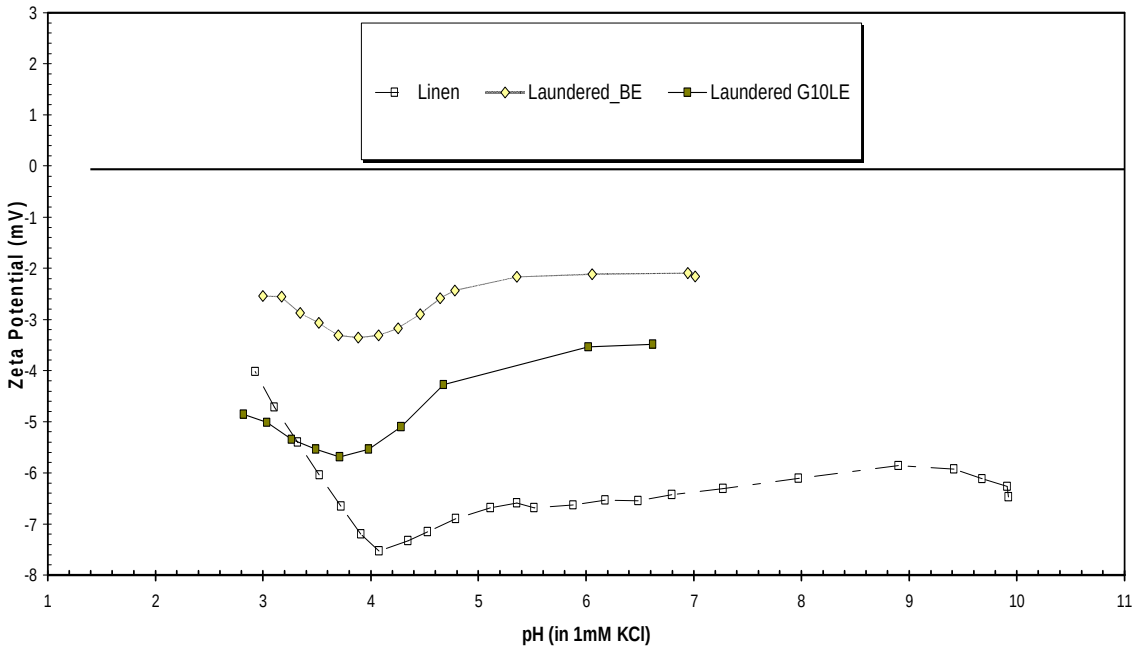


Figure 3.19: Change in zeta potentials upon laundering. Laundered G10LE = guaiacol + laccase + linen + 10% free lignin (owf) (colored square), Laundered BE = laccase + linen (diamond), and Linen (square).

Washfastness of colorants is an important aspect. Therefore laundering was performed on the linen samples and the zeta potentials were determined (Figure 3.19). Laundering of the treated fabrics did only slightly reduce the negative surface potential compared to the non-laundered counterparts, which could be interpreted as verification of the stability and washfastness of the produced colorants.

3.2.2 Coloration of Film

The enzymatically produced colorants were successfully incorporated into PVA and CMC films; complete dissolution occurred and an even blend was obtained, as can be seen in Figures 3.20 and 3.21. The darkest hue was obtained with guaiacol for both PVA and CMC films, as expressed in an increased L* value of the samples. Overall the films

were very thin and hence, the coloration fairly faint. These experiments however showed that it was feasible to integrate the enzymatically produced colorants uniformly in coatings and films. This observation could be very important for future applications in the food packaging industry.

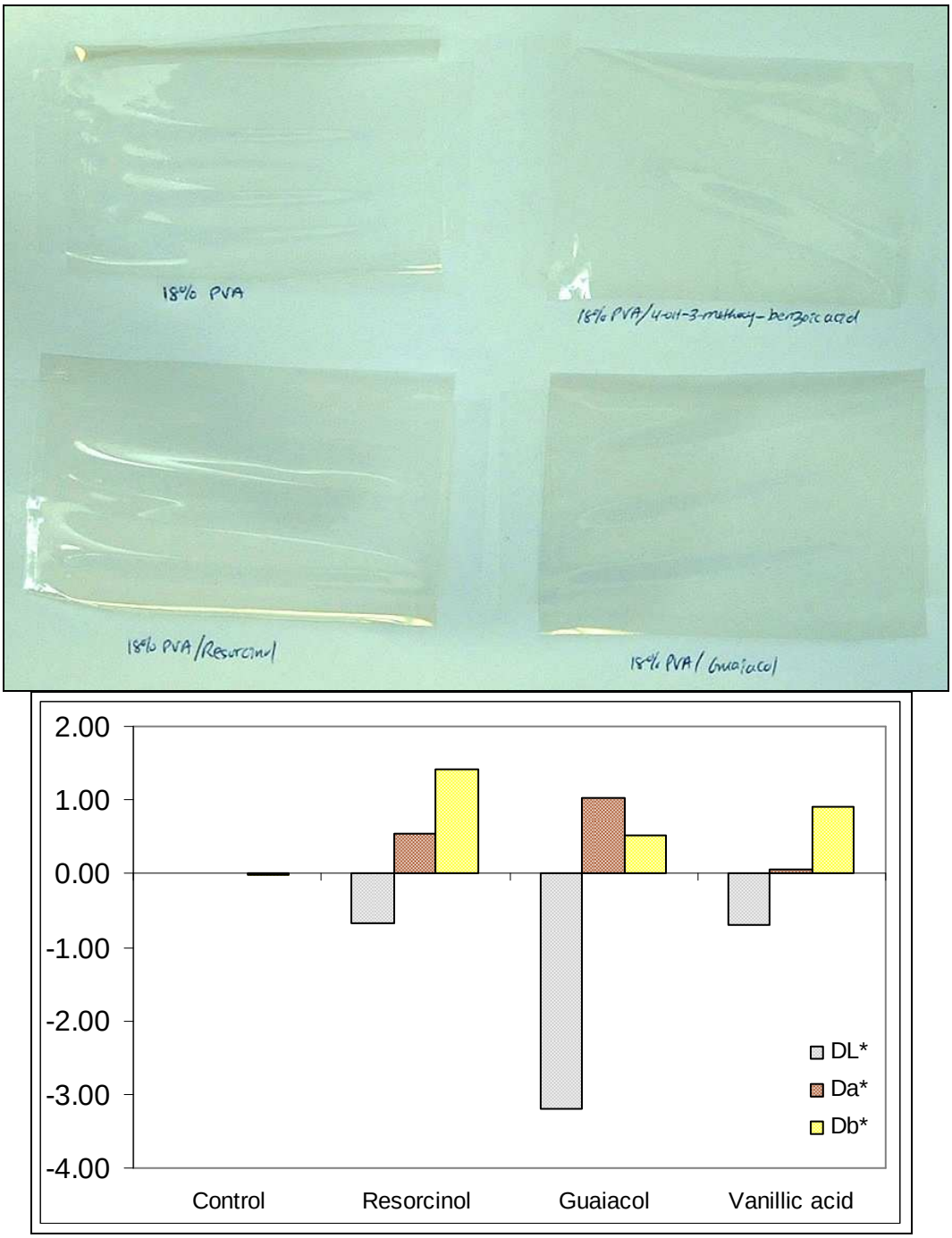


Figure 3.20: Image of PVA colored films (top), CIEL*a*b* values of 18% PVA films incorporated with colorant (bottom).

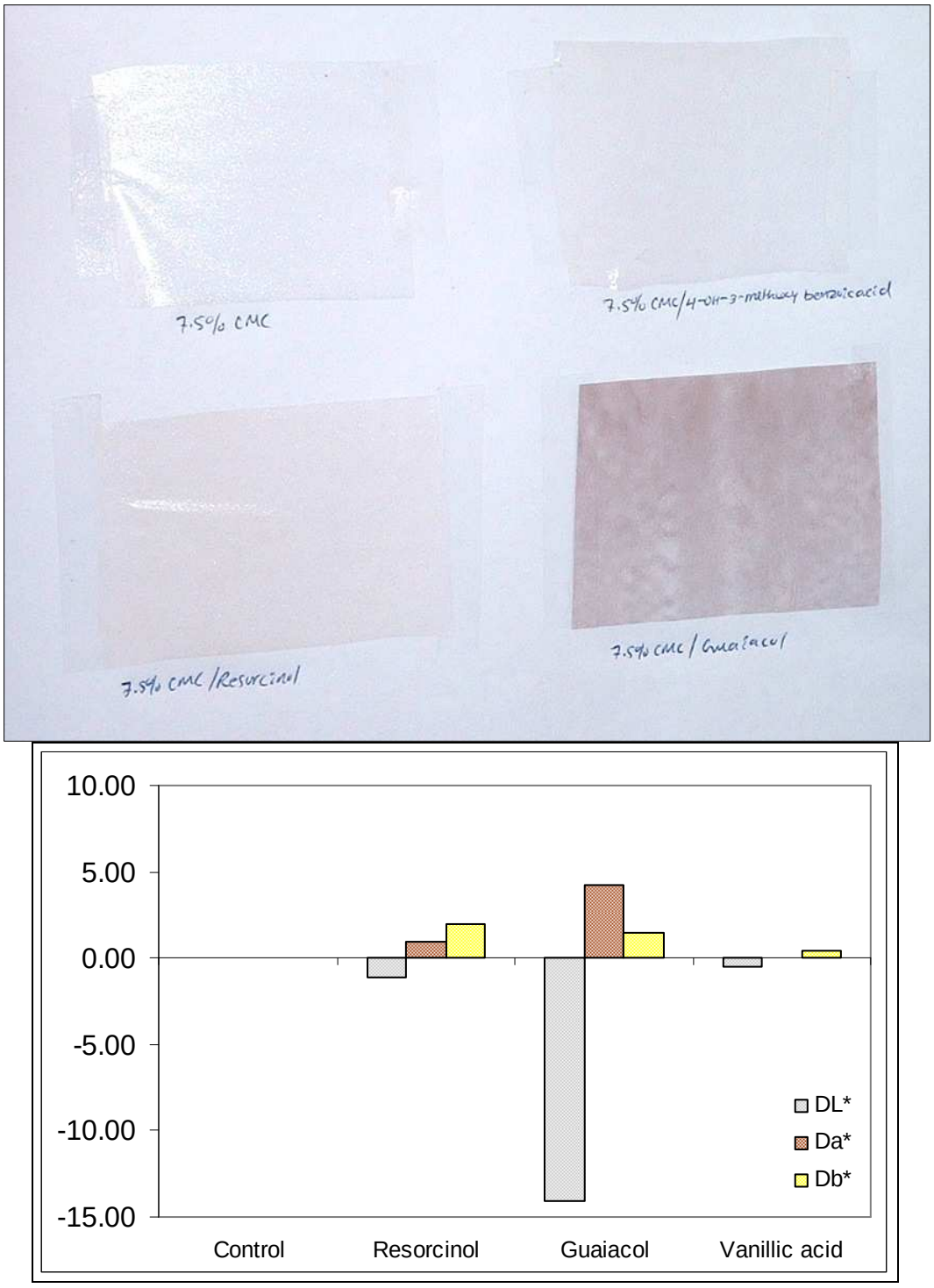


Figure 3.21: Image of CMC colored films (top), CIEL*a*b* values of 7.5%% CMC films incorporated with colorant (bottom).

CHAPTER FOUR

CONCLUSION

Synthesis of colorants and the dyeing of textile fibers and other materials are processes where large amounts of waste are generated. Issues with waste products, toxicity of materials, safe working environment, and pollution control are all concerns for industries dealing with coloration; to resolve these issues, there is a pursuit for novel green methods. A variety of ways can be used to approach this matter; however, the best approach would be to make the overall process more energy efficient with a minimum amount of waste generation. The use of enzymes as biocatalysts is a novel solution to this matter; their reactions occur under mild conditions and are highly regio-, stereo-, and enantiospecific, making the process more efficient and less wasteful.

By using laccase, colorants were synthesized in an aqueous medium by the coupling of various lignin model compounds which, at the same time, can be part of a colorant's chromophore. It was found that the shade of the product depended upon the initial substrate chosen. Colors ranged in hue from an orange-yellow, to tan, to burgundy. The major peak in the absorbance spectrum of the colorants did not shift significantly with the duration of the enzymatic reaction, which means that the main chromophore contributing to the shade was not altered, but the amount of colorant produced increased as the reaction progressed

As a consequence of the developed color shade the original phenols most likely formed oligomeric or polymeric products with laccase catalysis. However, it was also found that the products did not consist of one single compound, but rather of several in more or less small amounts which were difficult to separate. A number of methods were employed to isolate these products into distinct components.

To characterize the colorants a combination of spectroscopic techniques was employed. DSC analysis of resorcinol and guaiacol products indicated either an increase in melting point temperature of the product (for the former) or higher thermal stability (for the latter) when compared to the starting phenolic compound. This denotes an increase in either molecular weight, crystallinity, or both. NMR results were not conclusive; however, the spectra suggested that different kinds of aryl hydrogen peaks are present. It can be speculated, that aromatic groups are a major part of the overall structure, a fact that would also justify the observed color shade of the products. From the IR spectra of the products, broadening of peaks was observed; this can result from molecules being in close proximity to one another, which could be a result of an oligomeric or polymeric structure.

Linen fabric was dyed using the enzymatically produced colorants. Maximum absorption of both guaiacol and resorcinol colorants occurred with 20% (owf) substrate, at 5 h incubation for the former and 24 h incubation for the latter. In both cases, increasing substrate concentration or addition of free lignin into the system, did not aid in the coloration processes. Surface analysis of the various treated fabrics indicated that surface properties changed after the coloration process and that the colored fabrics proved to be washfast.

In addition, enzymatically synthetized colorants were successfully incorporated into PVA and CMC solutions and films formed. Though thin, the films showed uniform colorant distribution. Under the given experimental conditions the highest amount of colorant enclosed was the enzymatic product from guaiacol, which, not surprisingly, resulted in the darkest shade.

In conclusion, this work showed that laccase is capable of catalyzing reactions with simple phenols to give colored solutions. Colorants could be isolated and added to polymeric solutions for biodegradable films. Enzymatic dyeing of cellulosic materials, such as linen fabrics, was also feasible. Enzymatic oligo- or polymerizations therefore have a great potential to lead to a “green”, more sustainable chemistry in the future.

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APPENDIX

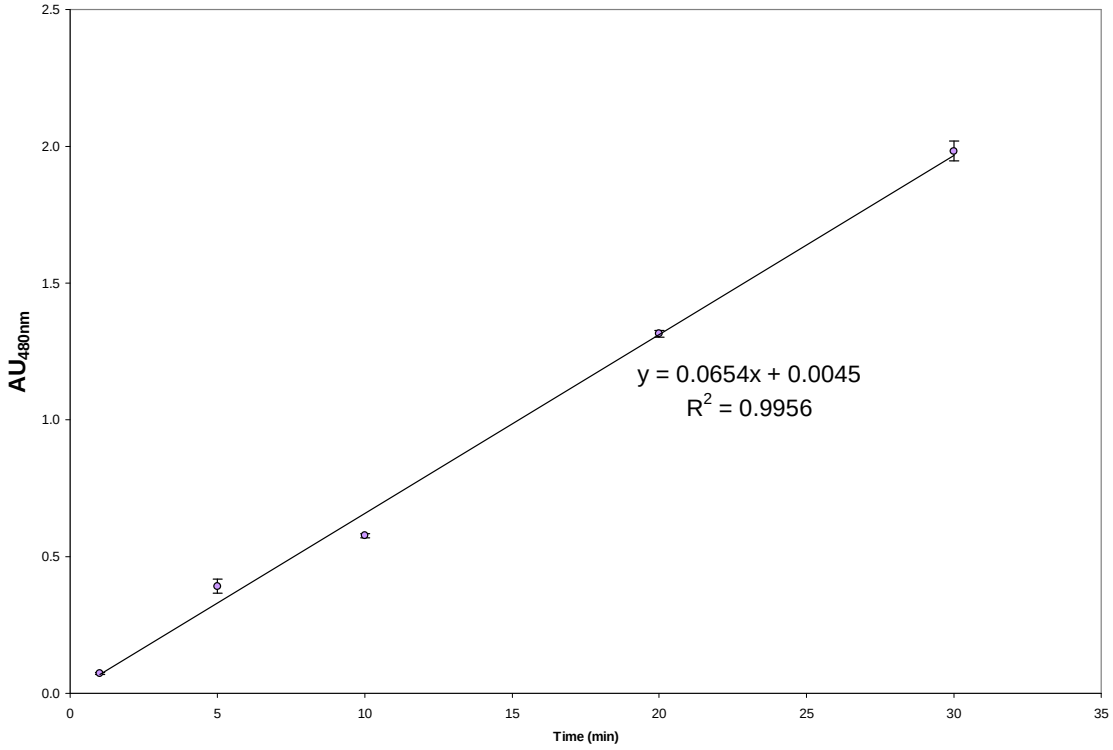

Figure A.1: Change in $AU_{\lambda max}$ over time of guaiacol with laccase 51002.

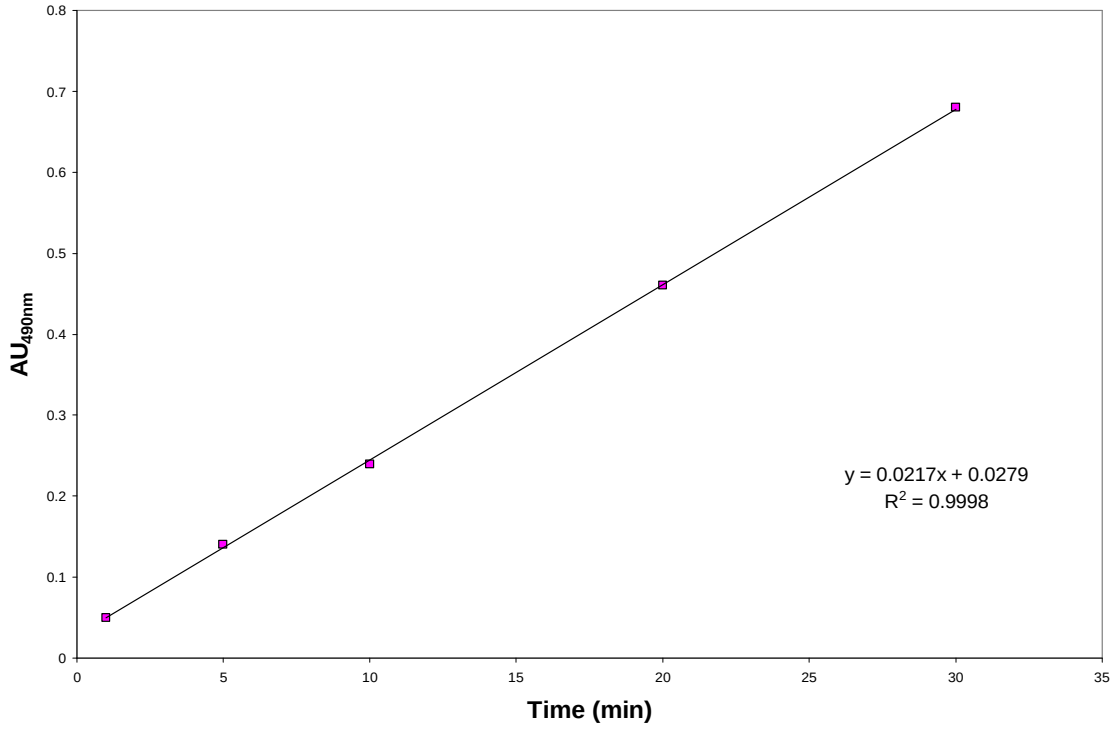

Figure A.2: Change in AUλmax over time of resorcinol with laccase 51002.

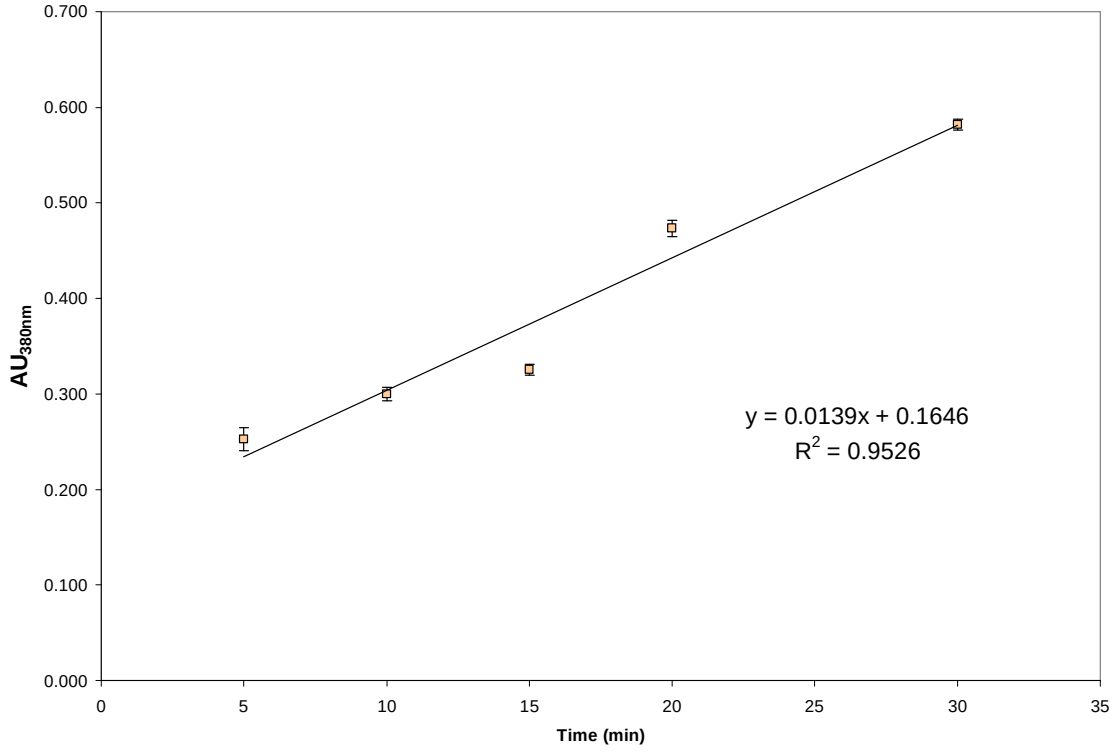

Figure A.3: Change in $AU_{\lambda max}$ over time of vanillic acid with laccase 51002.

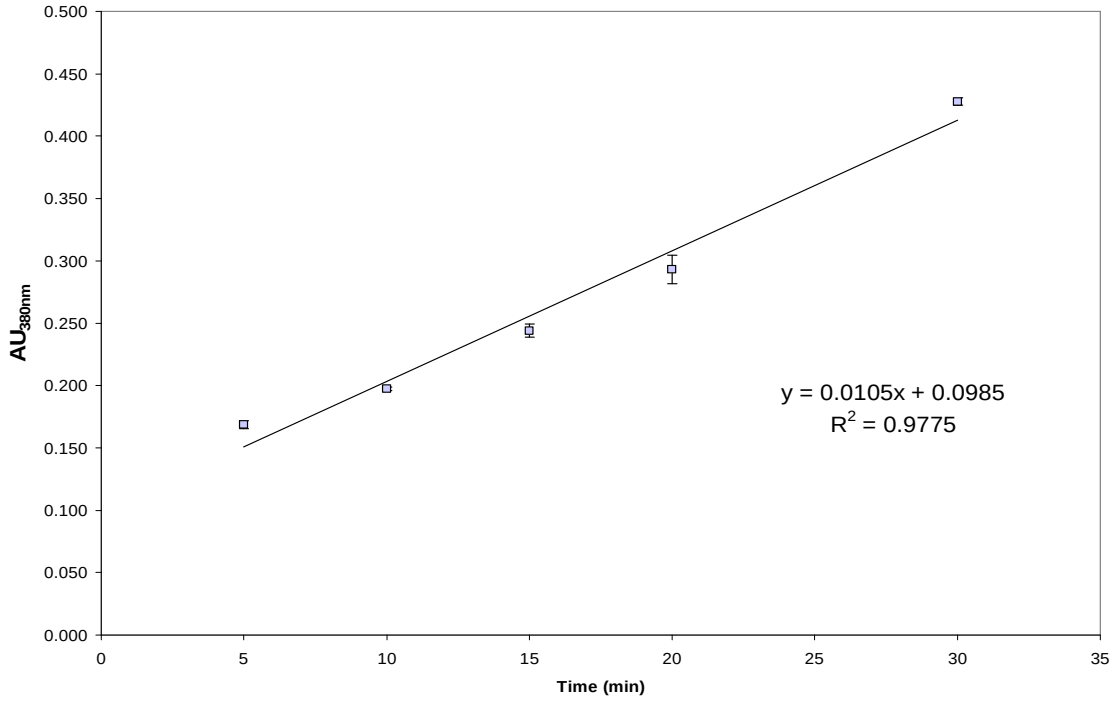

Figure A.4: Change in $AU_{\lambda max}$ over time of vanillyl alcohol with laccase 51002.

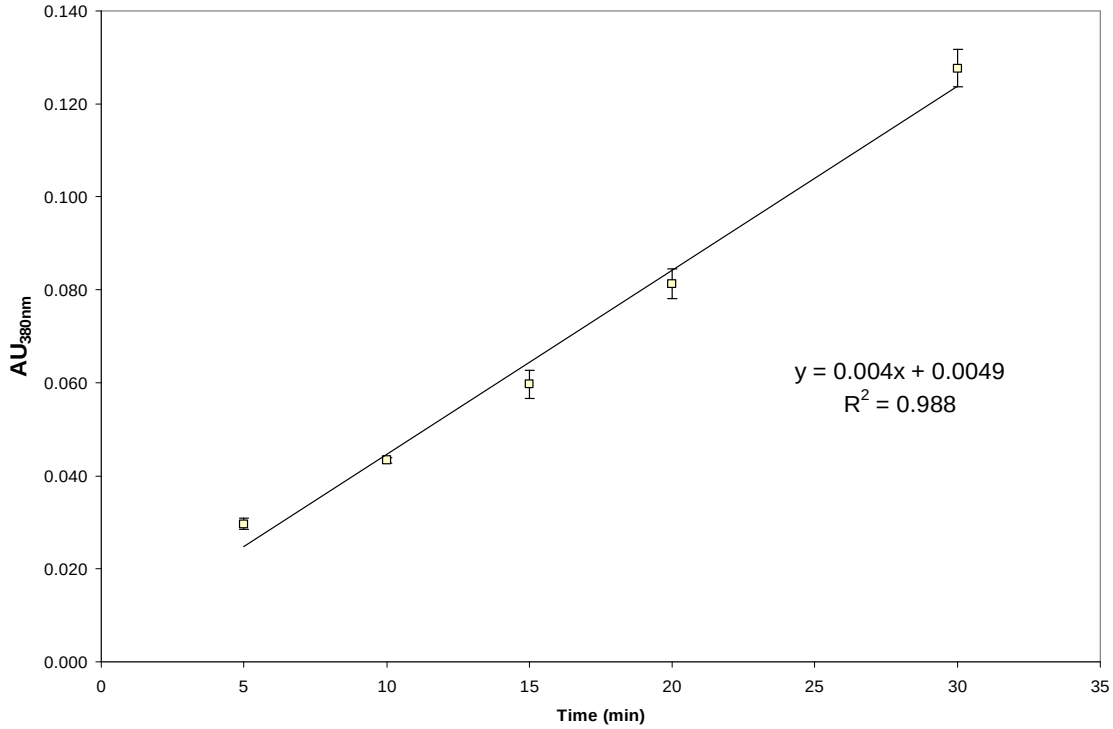

Figure A.5: Change in $AU_{\lambda max}$ over time of isovanillyl alcohol with laccase 51002.

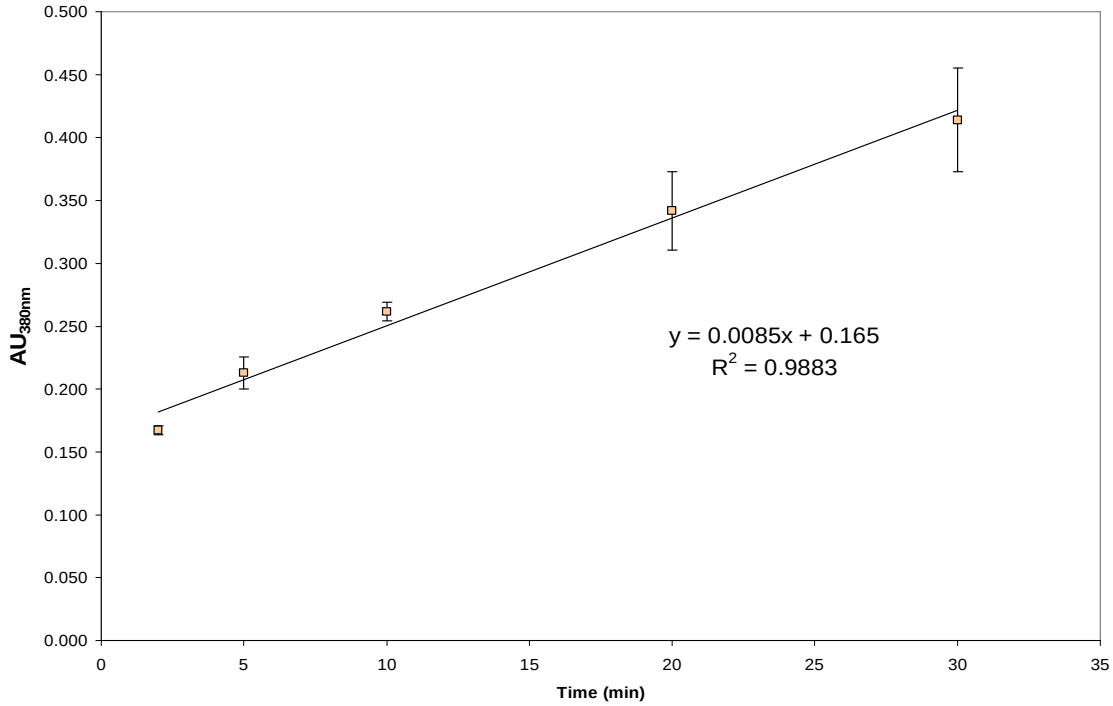

Figure A.6: Change in $AU_{\lambda max}$ over time of vanillic acid with laccase 51003.

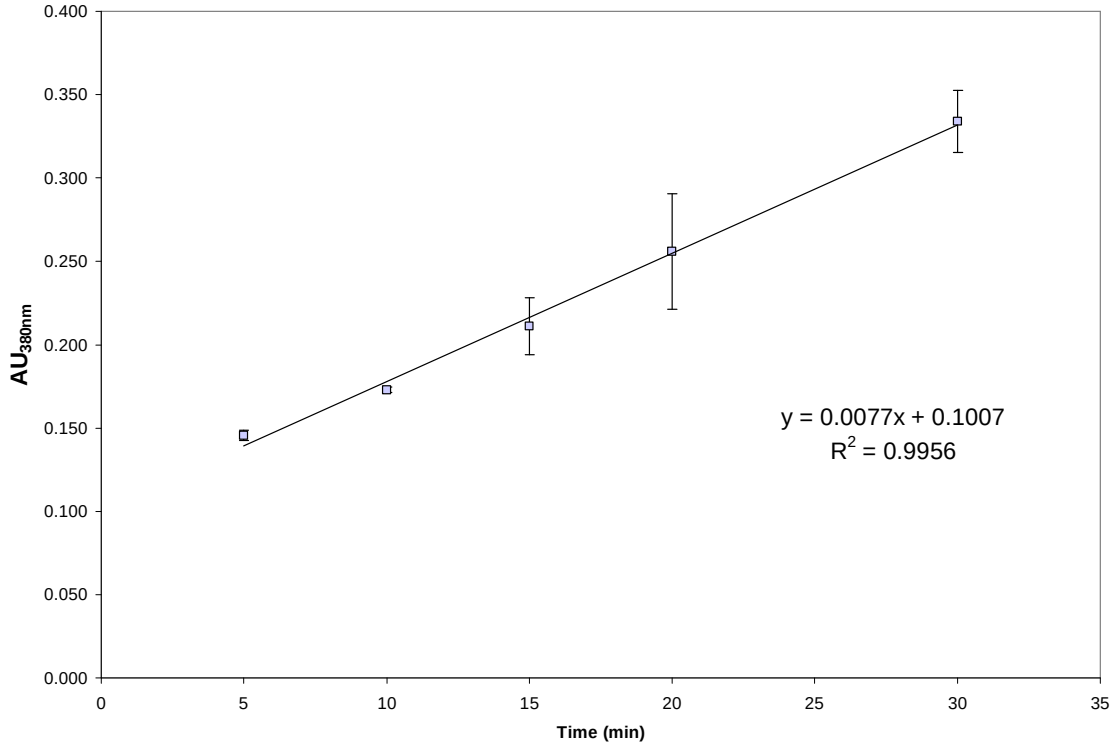

Figure A.7: Change in $AU_{\lambda max}$ over time of vanillyl alcohol with laccase 51003.

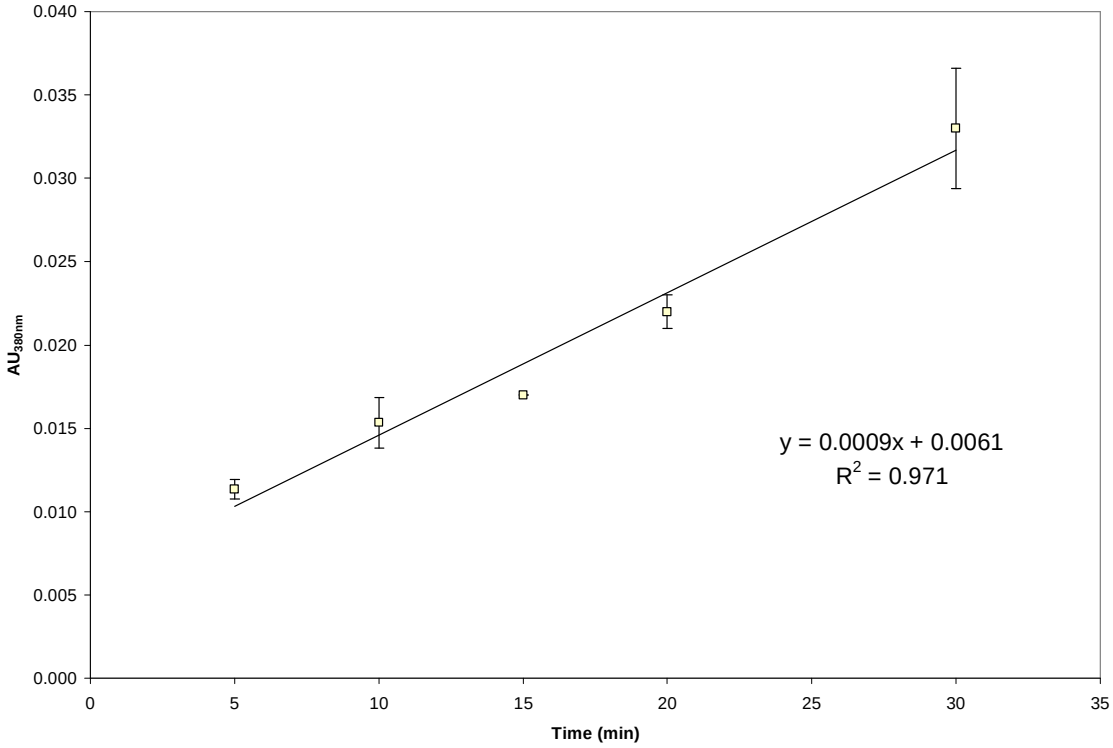

Figure A.8: Change in $AU_{\lambda max}$ over time of isovanillyl alcohol with laccase 51003.