BIOMIMICKING OF ENZYMES FOR TEXTILE PROCESSING

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BIOMIMICKING OF ENZYMES FOR TEXTILE PROCESSING

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BIOMIMICKING OF ENZYMES FOR TEXTILE PROCESSING

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Xuehong Ren, son of Anbang Ren and Xinglan Wu, was born April 15, 1971 in Shaanxi Province, People's Republic of China. He entered Suzhou Institute of Silk Textile Technology in 1989, and graduated with Bachelor of Engineering (Dyeing & Finishing Engineering) in July 1993. He worked as teaching assistant and later lecturer at Jiangnan College from 1993 to 1999. After six years work he attended the graduate program of College of Material Engineering at Soochow University, and got his Master degree in Textile Chemistry in July 2002. He entered the PhD program at the Department of Polymer and Fiber Engineering, Auburn University in August 2002. He married Yalan Wu, daughter of Linxing Wu and Shuizheng Hua, in 1997. He is the father of two sons, Steven K. Ren and Jon Ren.

DISSERTATION ABSTRACT

BIOMIMICKING OF ENZYMES FOR TEXTILE PROCESSING

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Enzymes are very large protein compounds. A small portion of their structure constitutes their highly specific active site which is fundamentally responsible for the catalytic capability of the enzymes. To mimic the active site of different oxidoreductases the components of the active site of were studied regarding their functionality when isolated without the complete protein structure. Oxidoreductases investigated include glucose oxidases, peroxidases, and laccase. Glucose oxidases belong to oxidoreductase enzymes and are capable of generating hydrogen peroxide for bleaching of cellulosic textile materials and pulp fibers. Their active site contains flavin adenine dinucleotide (FAD) as cofactor. Compounds similar to the active site were used to mimic the reactions

of the intact enzyme. The mimics' dosage, pH value and the source of oxygen play a role for the reaction to occur as well as the exposure to light. The influence of amino acids that are in direct contact with the cofactor of the intact enzyme on the biomimetic reactions was also explored. The mimics of glucose oxidase applied for bleaching cotton fabric could achieve whiteness levels of about 70% compared to whiteness levels reached with glucose oxidase or commercial H_2O_2.

Lignin and manganese peroxidase and laccase are enzymes that are not capable of generating peroxide, however have proved to play important roles in the degradation of ligninic compounds in pulp. The application of these oxidoreductases to unbleached linen was investigated regarding their bleaching effectiveness. Glucose oxidase can also be used for bleaching of linen. Laccase was found effective for delignification of linen fibers to increase the whiteness of linen fabric. The combination of laccase and glucose oxidase for bleaching of linen fabric showed higher effectiveness regarding whiteness than either one of the enzymes applied alone. The whiteness increase of treated fabric might be related to the decrease of lignin content of enzyme-treated linen.

The surface properties of scoured unbleached linen fibers and enzymatically treated linen fibers were investigated by inverse gas chromatography (IGC), and the dispersive component free energy γ_S^d as well as surface acidity constant (K_a) and basicity constant (K_b) were determined. The decrease of both K_a and K_b of enzymatically treated linen can be explained by the change of surface chemical groups of the linen fibers. For the linen fabric high in lignin weight the K_a and K_b values of enzyme-treated fabric are inconclusive and not directly related to the whiteness and lignin content of the fabric.

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CHAPTER 1. INTRODUCTION

Introduction

Enzymes are protein catalysts produced by living cells that catalyze specific chemical or biochemical reactions. The use of enzymes in textile processes has gained increased interest due to the advantages of enzymes being non-toxic, biodegradable, and environmentally friendly. Enzymes can be safely used in textile wet processing like desizing, scouring, bleaching, dyeing, and finishing, while traditional chemicals can cause many problems including pollution of effluents when disposed into the environment. Advances in enzyme technology use in the textile industry have made it possible to explore the potential of single enzymes or enzyme mixtures for specific applications. Hydrolases (e.g., amylases, cellulases, pectinases, proteases) and oxidoreductases have been employed for fabric preparation and finishing.

Enzymes are large high-molecular weight protein structures with highly specific active sites within the molecule that perform the catalytic reaction. Replacing enzymes with simpler compounds that mimic the behavior of these biocatalysts could significantly increase the reaction rate, facilitate the enzymatic process and decrease costs. The key point in this project is whether enzymes could be replaced with simpler compounds that mimic the behavior of these biocatalysts. This work focuses on reactions of oxidoreductases in connection with oxidative bleaching of cellulosic materials. Enzymes investigated in this research include glucose oxidase (GO), laccase, and peroxidases. The

approach offers the opportunity to study the enzyme reaction in depth and to gain insight into unsolved questions regarding the mode of action of these interesting compounds as well as opens doors to new future directions.

Bleaching is one of the preparatory processing steps routinely performed for cotton and other cellulosic fabrics with the purpose of removing natural pigments and other noncellulosic impurities. Currently, hydrogen peroxide, applied at high pH and under high temperature, is the most common bleaching agent. However correct control is essential. Alternatively, the enzyme glucose oxidase has been used to produce hydrogen peroxide for bleaching of cotton fabrics. Ligninolytic enzymes like lignin peroxidase, manganese peroxidase, and laccase have been shown to play a role in lignin degradation in pulp bleaching and decolorization of textile dyehouse effluent.

Glucose oxidases belong to oxidoreductase enzymes and are capable of generating hydrogen peroxide for bleaching of cellulosic textile materials and pulp fibers. Their active site contains flavin adenine dinucleotide (FAD) as cofactor. Compounds similar to the active site were used to mimic the reactions of the intact enzyme.

Peroxidases like lignin peroxidase and manganese peroxidase are another type of oxidoreductases. They have ferriprotoporphyrin IX as their prosthetic group. Simple chemical compounds similar to their prosthetic group were used to mimic the catalytic behavior of these enzymes.

Objective

The overall goal of this work was to explore the potential of oxidoreductases for textile applications, their possible mimics that might simplify enzymatic processes. Specific objectives included:

- To develop enzymatic systems using glucose oxidase for bleaching of scoured unbleached 100% cotton fabric (control)
- To explore enzyme combinations of glucose oxidase and peroxidases and laccase for bleaching of scoured unbleached 100% cotton fabric
- To find possible mimics for glucose oxidase to simulate the function of the active site of glucose oxidase and mimic the behavior of this catalyst without the bulk of the proteins
- To explore enzyme combinations of glucose oxidase and peroxidases and laccase for bleaching of scoured unbleached 100% linen fabric with high and low lignin content
- To find possible mimics for peroxidases to simulate the catalytic reaction of peroxidases without the bulk of the proteins
- To evaluate the effect of these enzymes on fiber surface properties

Chapter arrangement

This dissertation is divided into five chapters. Chapter 1 introduces the topic and objective. In Chapter 2 reviews the background of the bleaching process of cellulosic fibers, enzymes used in this process, and mimics of these enzymes. Chapter 3 covers the materials and experimental section including chemicals used, enzymes, mimics, and testing methods. Chapter 4 is devoted to discussion of results. Chapter 5 presents the summary of this dissertation.

CHAPTER 2. LITERATURE REVIEW

2.1 Conventional chemical bleaching processes for cotton and linen fabrics

Cotton comes from seed hairs and is a very important textile fiber. As a natural fiber, cotton contains different noncellulosic impurities such as pectin, waxes, sugars, proteins, organic acids, ashes, and coloring matter. Flax fibers are obtained from the plant stem. Flax fibers processed and made into textile structures are called linen. Flax fibers also contain a variety of different impurities such as hemicelluloses, pectin, lignin, waxes, water solubles, and coloring matter.

Cotton and linen fabrics require pretreatments before they can be dyed and finished. The purpose of fabric preparation is to remove most or all of the contaminants and impurities from fabrics and thus produce clean, white, smooth, and evenly absorbent fabric ready for later wet processes like dyeing and finishing. Preparation processes include desizing, scouring, and bleaching.

Impurities in cotton and flax fibers may absorb light which might cause the fibers to have a yellowish or dull appearance. Thus after the scouring processing, cotton and linen must be bleached to remove the coloring matter and destroy other impurities by the use of oxidizing agents and to obtain a desirable uniform white fabric surface. The bleaching process must be carefully controlled so that the damage to the fabric is minimized and coloring matter is completely removed. There are three major chemical

bleaching agents used in the textile industry: sodium hypochlorite, sodium chlorite, and hydrogen peroxide. Hydrogen peroxide is the chemical most commonly used because the decomposition products of hydrogen peroxide bleaching process are environmentally-friendly compared to the other two chemical bleaching agents. The best bleaching whiteness can be obtained at pH 10.5-11. The temperature and time varies with the process and types of equipment being used in peroxide bleaching. Stabilizers such as sodium silicate are usually added to the bleaching bath to control the decomposition of peroxide and to achieve optimum bleaching results.

The chemical structures of natural pigments in cotton and flax fibers are still unknown. Color in organic substances results from the presence of mobile electrons within the colorant's chromophoric system of conjugated double bonds. The conjugated double bonds might be broken and some of the double bonds are saturated during bleaching which limits the delocalization of π electrons. Thus, the resulting break in the chromophoric system of the colorants produces colorless products. The mechanism of hydrogen bleaching is complicated and still not fully understood. Both free radical and ionic mechanisms have been proposed to explain hydrogen peroxide bleaching of fibers. The ionic mechanism suggests that the active species in hydrogen bleaching is the perhydroxyl ion. Hydrogen peroxide is a weak acid and ionizes in water according to Figure 2.1. Higher alkalinity of the bleaching bath increases the bleaching rate since a higher concentration of perhydroxyl ions is formed with higher alkalinity.

Figure 2.1 Ionization of hydrogen peroxide in water

The free radical mechanism proposes that the conjugated double bonds are attacked by free radicals produced by reaction of hydrogen peroxide with a donor substance. The mechanism of decolorization or bleaching process is shown in Figure 2.2.

The electron donor may be a perhydroxyl ion or metal ion. The reaction mechanism producing radicals is shown in Figure 2.3.

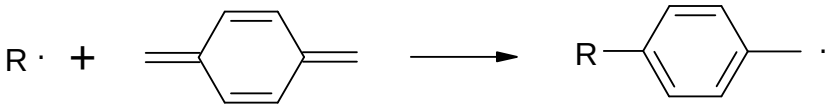


Figure 2.2 Mechanism of bleaching process

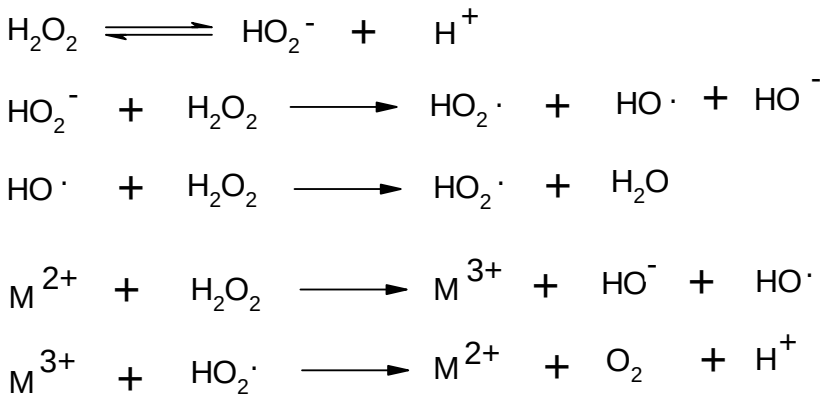


Figure 2.3 Reaction mechanism to produce radicals (M = metal) [1]

2.2 Enzymes used for bleaching

2.2.1 Introduction to Enzymes

Enzymes are high-molecular weight proteins that consist of intertwined chains of amino acids. Enzymes act as catalysts for chemical or biological reactions. Compared with common chemical catalysts, enzymes are more efficient and increase the reaction

rate by 10^7 – 10^{13}. Compared to general chemical catalysts, enzymes have the added advantage to make a reaction occur under mild conditions such as fairly low temperature, normal pressure, and in neutral aqueous solution. They also have the advantage of being non-toxic, bio-degradable, and environmentally-friendly. Enzymes are highly substrate-specific. They react with their substrates at a region within the protein molecule which is called active site. The active site of the enzyme must have the necessary structure characteristics to recognize the right substrate and the proper chemical environment to make the reaction happen.

According to the Enzyme Commission (EC) all enzymes are grouped into six classes on the basis of the types of reaction they catalyze. They are categorized into oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases.

The enzymes most commonly involved in textile applications are hydrolases and oxidoreductases. They can be safely used in the textile industry in fabric preparation and finishing processes. Table 2.1 shows the applications of hydrolases and oxidoreductases. Oxidoreductases are very important enzymes in bleaching wood pulp and nature cellulosic fibers.

Table 2.1 Textile applications of hydrolases and oxidoreductases

Enzyme	Substrate	Application
Hydrolases		
Amylase	Starch	Removal of starch-based size
Cellulase	Cellulose	Bio-polishing, bio-finishing
Pectinase	Pectin	Scouring
Proteases	Protein	Silk degumming, wool bio-antifelting
Catalase	Peroxides	Peroxide decomposition
Lipases	Fats	Hydrolysis PET
Oxidoreductases		
Glucose oxidase	Pigments	Bleaching cotton
Laccase	Coloring matter	Bleaching wood pulp and fibers with lignin, discoloration of effluent, bleaching indigo dyed denim
Peroxidases	Coloring matter	Bleaching of wood pulp
Azo reductase	Coloring matter	Discoloration of azo dyes
Peroxidase ostreatus	Coloring matter	Discoloration of basic dye

2.2.2 Oxidoreductases

Oxidoreductases are a class of enzymes that oxidize or reduce a substrate by transfer of hydrogen(s) and/or electron(s). Many of these enzymes are commonly known as oxidases, reductases, peroxidases, hydrogenases, oxygenases, or dehydrogenases.

Important oxidoreductases addressed in this study include glucose oxidase, laccase, and peroxidases. The reactions involved in oxidoreductases are shown in Figure 2.4.

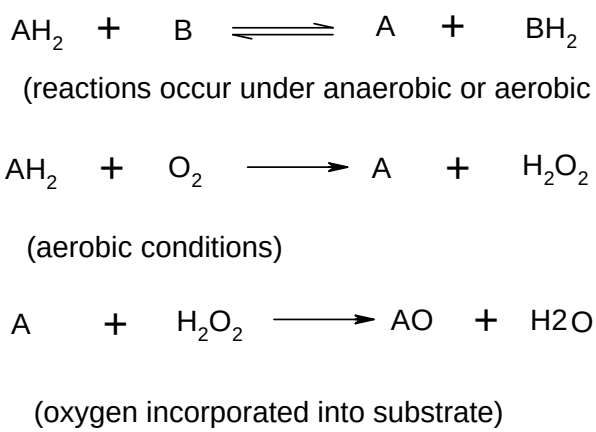


Figure 2.4 Catalytic reactions of oxidoreductases and their substrate

2.2.2.1 Glucose oxidase

Glucose oxidase (β-D-glucose: oxygen-1-oxidoreductase, EC1.1.3.4) (GO) belongs to the group of oxidoreductases with flavin prosthetic groups. The enzyme is found in certain fungi, such as Aspergillus oryzae, Aspergillus niger, Penicillium amagasakiense, and others. Common to all glucose oxidases is their fairly high molecular weight of 150 to 190 kDa and their high specificity for β-D-glucose. Each subunit of the enzyme contains one mole of flavin adenine dinucleotide (FAD) (Figure 2.5). There are two distinct domains in each GO monomer: one very tightly binds the FAD moiety but not covalently, and another binds the β-D-glucose substrate. The enzyme is highly specific for β-D-glucose, and can generate hydrogen peroxide in the presence of oxygen in aqueous solution by using β-D-glucose as substrate [2,3,4,5] (Figure 2.6). β-D-glucose is oxidized to gluconic acid with the transfer of two protons and two electrons from the

substrate to the flavin moiety. The produced hydrogen peroxide can then act as bleaching agent for decomposition of any yellowing compounds in cotton or other cellulosics. Glucose oxidase is an alternative to hydrogen peroxide bleaching being non-toxic, biodegradable, and eco-friendly [2, 4].

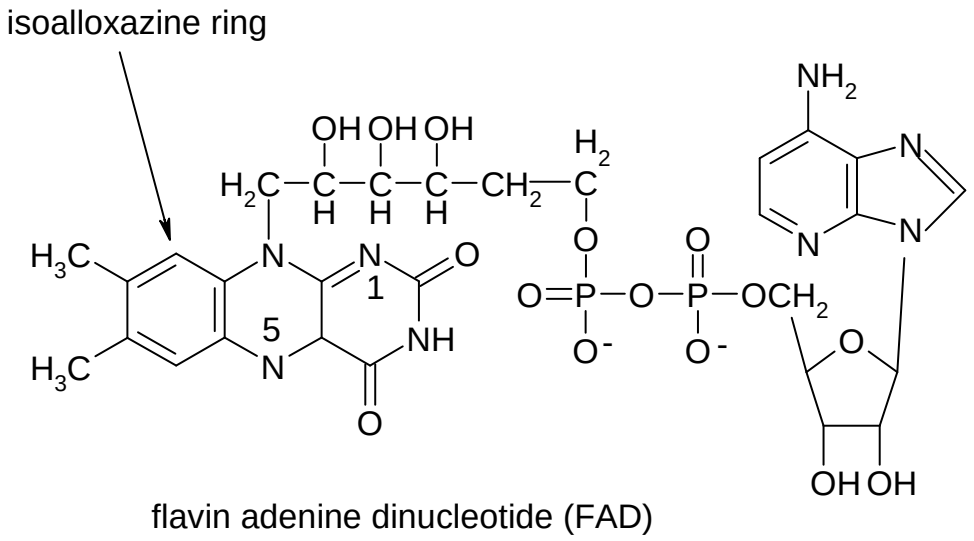


Figure 2.5 Chemical structure of the active site of glucose oxidase, FAD

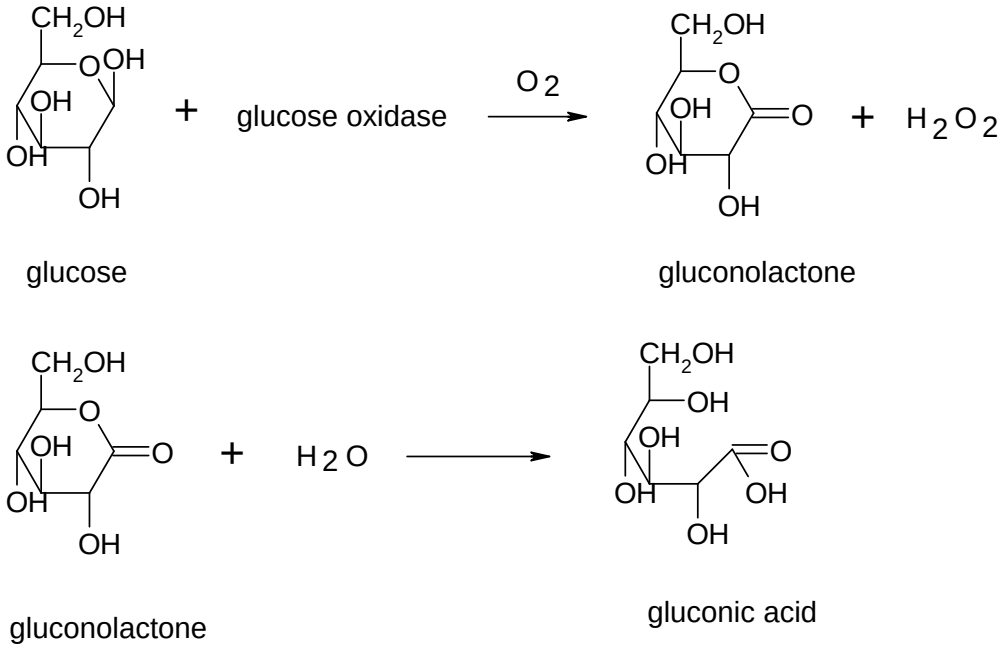


Figure 2.6 Reaction between glucose oxidase and glucose

Glucose oxidase is a very large complex protein molecule with a highly specific active site that catalyzes selected redox reactions. The active site, consisting of the prosthetic group FAD and amino acid residues adjacent to FAD, determines the specificity of the glucose oxidase in redox reactions. Flavins can undergo either two sequential one-electron transfers, or a simultaneous two-electron transfer through the semiquinone state (Figure 2.7). The reaction of GO with glucose can be seen as a reductive half-reaction (hydride transfer from C-H of glucose to FAD) and an oxidative half-reaction in which the reduced FAD ($FADH^-$) is oxidized by O_2 generating H_2O_2. The apoprotein of glucose oxidase most likely plays an important role in the catalytic reaction due to the pH dependency of the reduction potentials [6]. Of the amino acid residues, histidine seems to be most influential on the catalytic reaction of the reduced $FADH^-$ and oxygen [7, 8].

Meyer [9] reported that the protonated His 516 and His 559 under acidic conditions increases the rate of the reaction between GO and glucose. It has been stated that His 516 is largely responsible for catalyzing the oxidation of $FADH^-$ by dioxygen [8, 10].

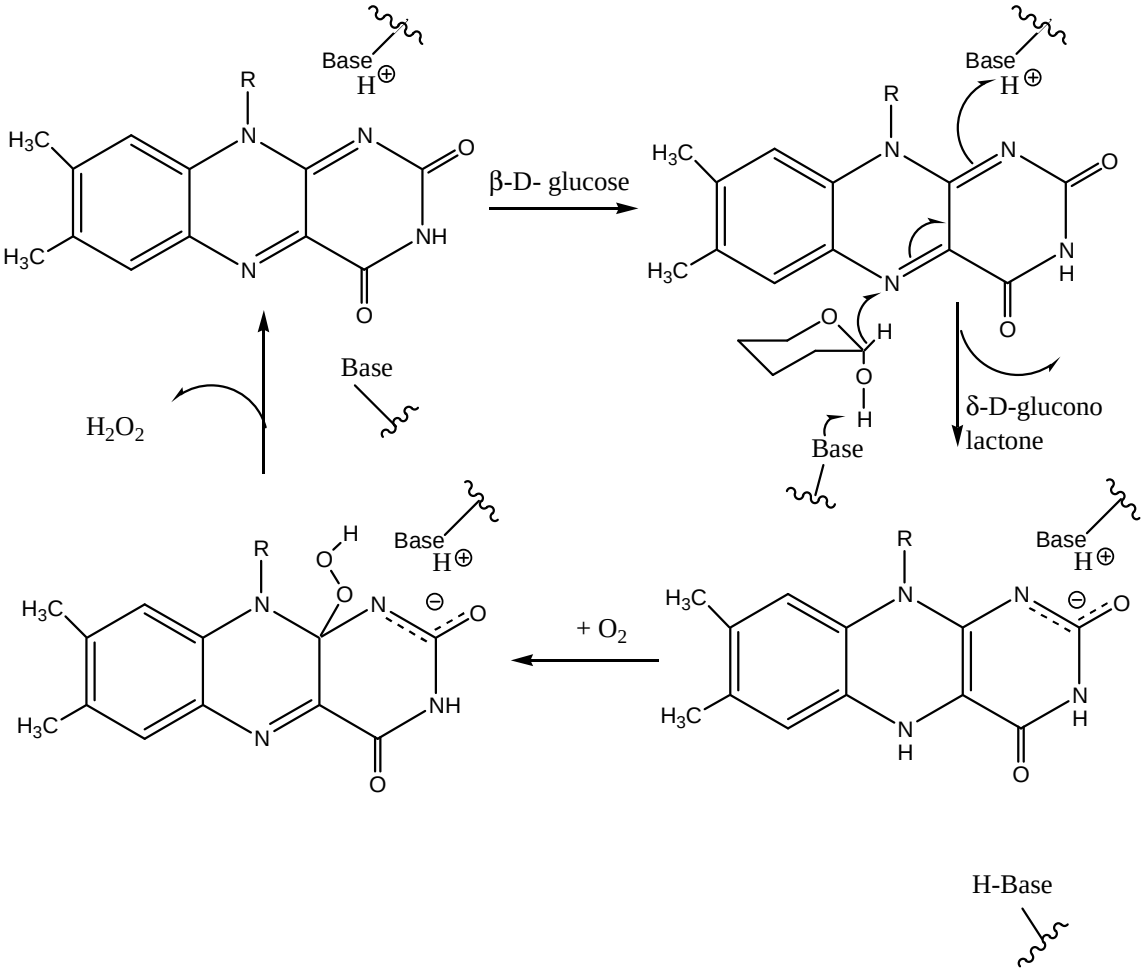


Figure 2.7 Reaction mechanism of flavins by two sequential one-electron transfers [7]

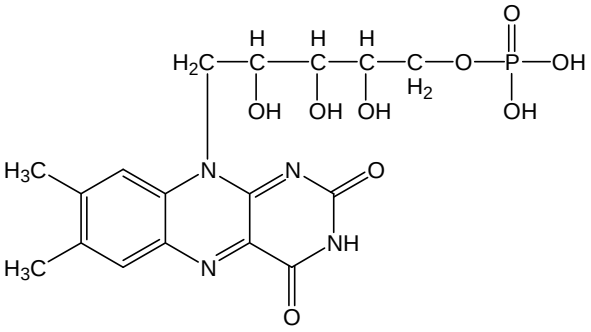
2.2.2.2 Mimics of glucose oxidase

It is well-known that vitamin B_2 (riboflavin) plays an important role regarding light sensitivity in biological systems [11]. Further research showed that hydrogen peroxide could be produced with flavin compounds and, to a lesser extend, methylene blue in the presence of visible light [12]. Catalase was used to demonstrate that hydrogen peroxide clearly was one product of the system. In earlier studies [13, 14] it was found that riboflavin, flavin mononucleotide, or lumiflavin could act as photosensitizer, but the

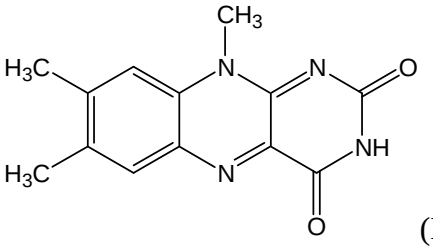
addition of compounds such as thiourea, EDTA, or semicarbazide were needed to increase the effeciency of reducing the oxidized isoalloxazine ring. It was assumed that the flavin semiquinone formed in the process transferred the acquired electron to molecular oxygen and to the superoxide anion, to produce hydrogen peroxide.

The mechanism of the light-sensitized reaction has been explained by Hellis et al. [15] and by Fontes et al. [14] by a one-electron transfer. The flavin semiquinone form produced might move the acquired electron to molecular oxygen and to the superoxide anion, to produce hydrogen peroxide. The photoreduced dihydroflavin (H at N1 and N10 position) can either be oxidized by the presence of oxygen directly or indirectly via a radical route [16]. A high pH value and the disproportionation of superoxide to hydrogen peroxide might favor the reaction via superoxide. The reaction of dioxygen with the anionic flavin radical should be faster than with semiquinone in neutral radical form [17].

Replacing enzymes with simpler compounds that mimic the behavior of these biocatalysts might help to better understand the mechanism of the enzymatic process. In this paper the active site of glucose oxidase was mimicked by using flavin mononuleotide (FMN), riboflavin, and lumiflavin (Figure 2.8). Semicarbazide, originally used as electron donor, was replaced by amino acids that are either adjacent to FAD in the original enzyme or that could assist the reaction of glucose oxidase and glucose. The effect of a light source during the mimicking reaction was taken into account.



(A)



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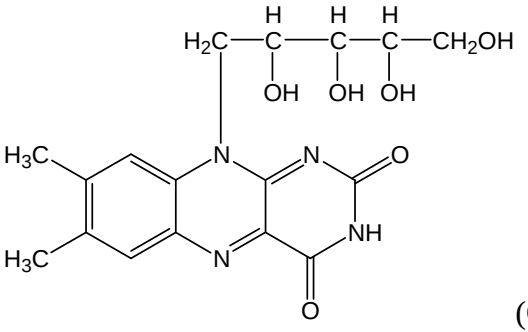

(C)

Figure 2.8 Structure of flavin mononucleotide (A), lumiflavin (B), and riboflavin (C).

2.2.2.3 Laccase

Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) is a blue multi-copper containing oxidase and found in fungi growing on fruit, vegetables or trees, lignolytic white-rot fungus, and bacteria. The four copper atoms act as cofactor of laccase and are classified as one Type I, one Type II and an antiferromagnetically-coupled Type III pair

of Cu^{2+} [18]. Laccase is an important oxidant for aromatic rings with electron withdrawing groups. The enzyme reacts with many substrates under H-atom abstraction (i.e. $1H^+ + 1e^-$) forming reactive radicals which may undergo further enzymatic oxidation resulting in quinine-like structures or non-enzymatic reactions leading to the often observed polymerized products.

In laccase-catalyzed reactions one-electron substrate oxidations are coupled with one four-electron reduction of oxygen to water, and electrons from the oxidation steps have to be stored in order to reduce molecular oxygen. The reduction of molecular oxygen to two molecules of water with exchange of four electrons can be written as shown below (Figure 2.9) [18]:

Figure 2.9 Reduction of oxygen to water by laccase

Laccase can oxidize not only phenolic compounds such as lignin-related compounds, coniferyl alcohol, vanillic acid, and p-cresol, but also ascorbic acid, and p-phenylenediamine [19]. Laccase oxidizes benzenediol into benzoquinone in the presence of oxygen (Figure 2.10). Laccase is one of the most important enzymes in lignin degradation. Removing lignin is an important way to improve the properties of pulps and the brightness of the paper.

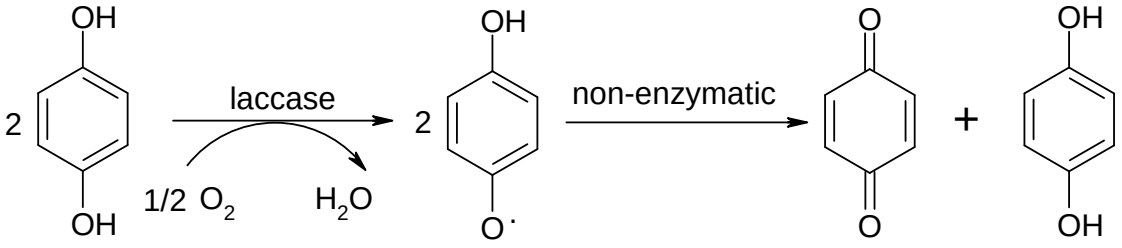

Figure 2.10 Oxidization of benzenediol to benzoquinone by laccase

In some cases enzymatic systems need mediators (substrate of laccase) to make reactions more effectively. The mediators of laccase include 2, 2′ -azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotrialzole (HBT), and syringaldazine. The reason to introduce low molecular weight redox mediators is to produce stable radicals, which make laccase more specific for different textile or pulp and paper applications. Biobleaching of pine kraft pulp with dioxygen in the laccase-mediator system is an example for a potential environmentally benign bleaching process for the degradation of residual lignin [20]. The laccase-mediator system allows the development of totally chlorine-free bleaching sequences for pulp from kraft and flax non-wood fibers [21, 22]. These mediators enhance the capabilities of laccase for oxidation of non-phenolic lignin [23]. Laccase has been used for pulping and kraft and flax pulp bleaching. High-quality paper pulps can be achieved by using a laccase-mediator system [24]. The enzymatic removal of lignin results in high final brightness values.

A widely accepted mechanism for mediated laccase oxidation and depolymerization of lignin is presented in Figure 2.11 [19]. According to this mechanism, laccase oxidizes the mediator via the four-electron reduction of oxygen to water. The oxidized mediator, being able to diffuse through the fiber wall, oxidizes lignin and depolymerizes it.

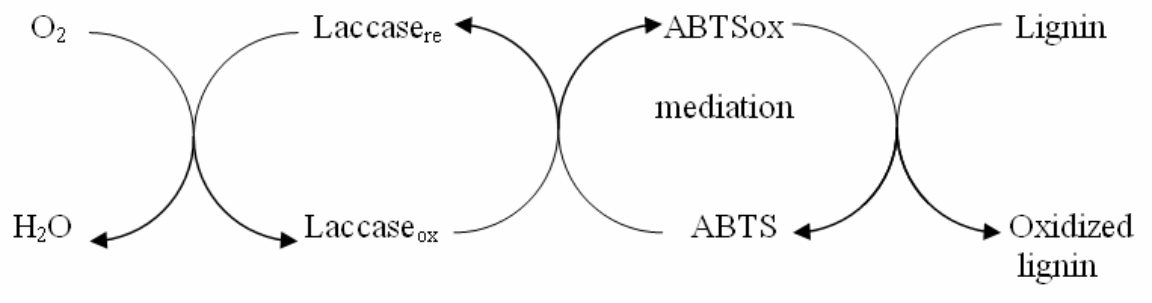

Figure 2.11 ABTS-mediated oxidation of lignin catalyzed by laccase

In addition to delignification, laccases have also found applications in textile processes. Lignin probably plays an important role in all reaction mechanisms involving laccase. Since cotton has basically no lignin, treatment of unbleached cotton with laccase alone could not yield satisfactory bleaching results. Laccase scouring of flax fibers as an alternative to chemical scouring has the advantage of being a mild reaction and obtaining better quality yarn [25]. Bleached-out looking jeans were created by the application of laccase together with a suitable mediator. The indigo chromophore was transferred into isatin and backstaining was avoided in the process [26]. The above process worked well for indigo dyed jeans while laccase was found to be ineffective for bleaching of undyed cotton. Laccase pre-treatment of scoured cotton fabric before the traditional hydrogen peroxide bleaching was found to improve the whiteness of fabric [27]. A reasonable explanation might be that laccase transformed the cotton coloring matter into different colored substrates which are easier to remove during peroxide bleaching.

Besides lignin degradation, laccase can promote polymerizations as well. It was found that laccase preferentially polymerizes lignin-related substrates resulting in the formation of lignin-analogue polymers [28]. Poly(phenylene oxide) was synthesized by laccase-catalyzed oxidative polymerization of syringic acid in an aqueous organic solvent (Figure 2.12) [29,30]. Laccase produced from *Trametes versicolor* was utilized to synthesize poly(pyrogallol) in aqueous solution [31]. Laccases have also been shown to catalyze polymerization of monomers such as phenols and their derivatives into polymers [32-37]

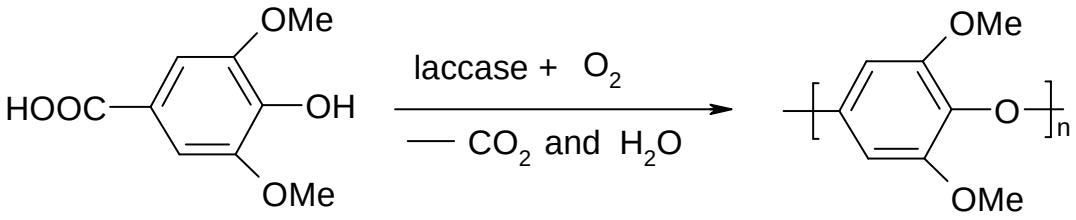

Figure 2.12 Laccase-catalyzed oxidative polymerization of syringic acid

Oxidative polymerization of catechol catalyzed by laccase enzyme from *Trametes versicolor* in aqueous solution containing acetone was reported [38, 39]. The proposed chemical structure of the resulting poly(catechol) is shown in Figure 2.13. α-naphthol was polymerized in the presence of laccase from *Trametes versicolor* [40]. Laccase utilizes molecular oxygen to oxidize α-naphthol. This oxidation produces α-naphthol radicals which react with other radicals to form poly-naphthol. Laccase-catalyzed synthesis of poly(catechin) without using hydrogen peroxide in aqueous organic solvents was used for development of polymeric antioxidants to amplify the beneficial physiological properties of flavonoids [41].

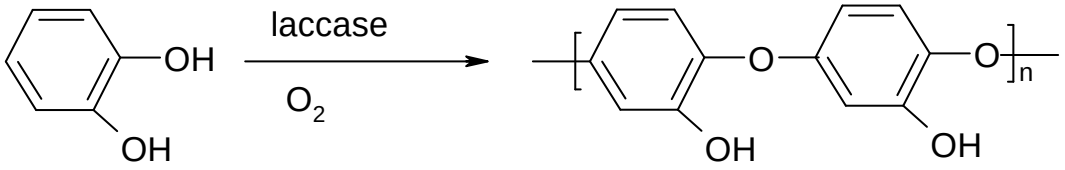

Figure 2.13 Oxidative polymerization of catechol catalyzed by laccase

Laccase from *Coriolus hirsutus* was used in the synthesis of water-soluble conducting polyaniline in the presence of molecular dioxygen [42]. The laccase-catalyzed polymerization of aniline was performed in the presence of sulfonated polystyrene (SPS)

as a template. The advantages of laccase over horseradish peroxidase in the synthesis of conducting polyaniline were its high activity and stability under acidic conditions.

There are reports on polymerization of vinyl monomers using laccase as catalyst. For example, the polymerization of acrylamide and methyl methacrylate was catalyzed by laccase and the polymer efficiently produced with high molecular weight [43, 44].

2.2.2.4 Mimics of laccase

Laccase belongs to the copper-containing enzymes. Copper (II) complexes can mimic laccase. Polyoxometalate (POM) has been reported to be promising for bleaching of kraft pulps. It requires oxygen as oxidant. The reaction system is reminiscent of the biomimetic system of laccase [45]. It is not easy to mimic laccase because of the difficulty in finding proper copper complexes.

2.2.2.5 Peroxidases

Peroxidases are a group of enzymes that catalyze oxidation-reduction reactions. Peoxidases including horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, HRP, EC 1.11.1.7), lignin peroxidase (LiP, EC.1.11.1.14), and manganese peroxidases (MnP, EC.1.11.1.13) are widely found in higher plants, e.g. in horseradish, tobacco, potato, turnip, and in microorganisms. The use of these peroxidases requires the presence of hydrogen peroxide. On the other hand, the problem is that excess hydrogen peroxide inhibits the enzyme activities. Theoretically, hydrogen peroxide can be supplied by glucose oxidase and glucose at an appropriate rate to match its consumption rate which alleviates the inhibition of these enzymes.

Horseradish peroxidase

HRP is a hydrogen peroxide oxidoreductase and belongs to a group of hemoproteins having ferriprotoporphyrin IX as prosthetic group (Figure 2.14). This enzyme has oxygenase activity activating dioxygen for incorporation into the substrate and peroxidase activity using peroxide for oxidation of the substrate. Ferriprotoporphyrin IX is made up of four nitrogens of the pyrrole ring coordinated to the ferric iron. The fifth coordination is located on the proximal side of the heme and occupied by an imidazole side chain of His170.

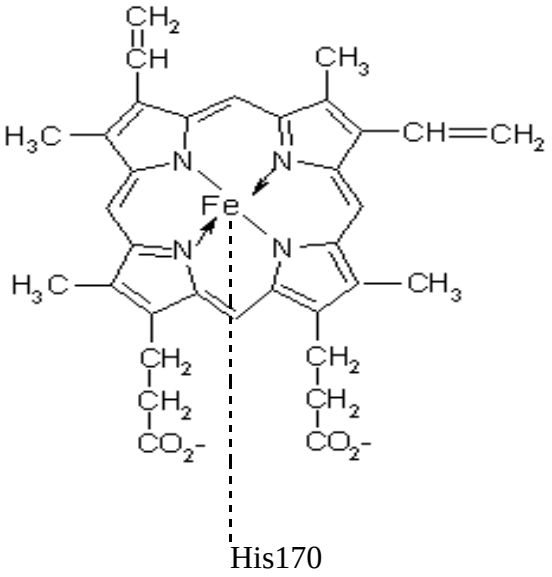

Figure 2.14 Structure of ferriprotoporphyrin IX, prosthetic group of peroxidases

The reaction of HRP involves two steps: (1) the formation of compound I intermediate by a two-electron oxidation with the hydrogen peroxide cleaved at the O-O bond; (2) the formation of HRP via compound II intermediate by a two-electron reduction of compound I (substrate is electron donor). There are several possible

mechanisms for the peroxidase reaction according to enzyme-substrate complexes. The following mechanism is universally accepted [46]:

$$HPR + H_2O_2 \longrightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + AH_2 \longrightarrow \text{Compound II} + \cdot AH$$

$$\text{Compound II} + AH_2 \longrightarrow HRP + \cdot AH + H_2O$$

The sum of the above three reactions is:

$$H_2O_2 + AH_2 = 2\,H_2O + 2\cdot AH$$

Lignin peroxidase

Lignin peroxidases have been identified as hemoprotein produced by many wood degrading fungi as a family of isoenzymes. These hemoproteins utilize hydrogen peroxide and organic peroxides to oxidize a variety of substrates. Lignin peroxidase can catalyze oxidations of both phenolic and nonphenolic aromatic compounds. The obtained aryl cationic radicals from oxidation of these substrates can lead to the following reactions: demethoxylation; C_α-C_β cleavage of lignin model compounds; benzylic alcohol oxidation; hydroxylation of aromatic rings and side chains. Lignin peroxidase has been reported to degrade lignin in wheat straw [47]. Veratryl alcohol (VA) has been found to be used as substrate or possible mediator in catalysis reactions (Figure 2.15) [48, 49]. Lignin peroxidase uses VA radical mediator to attack lignin at a distance rather than to attack the insoluble lignin substrate directly.

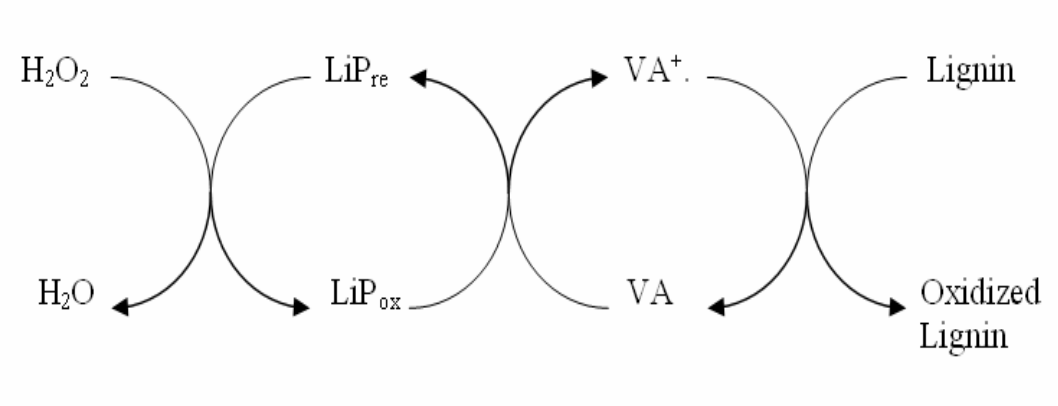

Figure 2.15 Proposed mechanism for the oxidation of lignin veratryl alcohol cation radical as a redox mediator [50]

Manganese peroxidase

Manganese peroxidase also belongs to hemoproteins. Manganese peroxidases are also called Mn-dependant peroxidases and used in the oxidative degradation of lignin. Manganese acts as a redox mediator like VA. Manganese peroxidase can catalyze the oxidation of Mn^{2+} in the presence of hydrogen peroxide to produce Mn^{3+} (stabilized by organic acids such as oxalate, malonate chelating the oxidized manganese ions) as a direct oxidant. Chelated Mn^{3+} is then reduced back to Mn^{2+} while at the same time phenolic rings in lignin are oxidized to phenoxy radicals, resulting in the decomposition of these structures (Figure 2.16). The formation of phenoxy radicals by MnP activity may subsequently lead to the cleavage of some bonds between aromatic rings and C_α carbon atoms. MnP systems appear to be more important than LiP for practical application. However, chelated Mn^{3+} is not strong enough to oxidize recalcitrant non-phenolic units of lignin.

Manganese peroxidases have been applied to bleach kraft and wood pulps [51-53]. MnP is a H_2O_2 dependent enzyme. H_2O_2 was added to the treatment solutions and Mn^{2+} was added as the redox mediator. Bermek [54] suggested that unsaturated fatty acids such as linoleic and linolenic acid significantly enhance the bleaching effects of MnP.

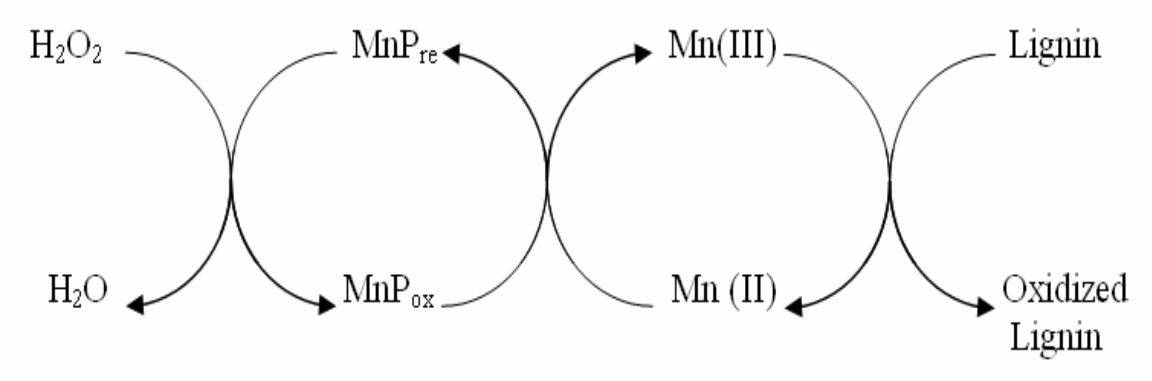

Figure 2.16 Proposed mechanism for the oxidation of lignin catalyzed by MnP in the presence of H_2O_2 and Mn^{2+} [55]

2.2.2.6 Mimics of peroxidases

Natural and synthetic iron-porphyrins can mimic LiP and MnP enzymes, and simple metalloporphyrins such as protoporphyrin iron chloride and meso-tetraphenylporphyrin iron chloride can mimic the function of lignin peroxidase in degrading lignin. Kraft pulps were treated with a variety of natural and synthetic porphyrins in the presence of peroxide [56]. The pulps treated with porphyrins were lignin free and the cellulose content in the pulps decreased somewhat. The problems of metallporphyrins as biomimetic catalysts are stability, redox potential and catalytic efficiency. The stability of the metalporphyrins was related to many factors, including type of solvent, type of the oxidant, and the presence or absence of substrate [57-61].

Metallophthalocyanines can replace metalporphyrins as lignin peroxidase model, but the problem of metallophthalocyanines is their insolublility. Another problem of metallophthalocyanine is that they are degraded rapidly under oxidative conditions. For metallophthalocyanines with Fe(III) or Mn(III) stability is also dependent on the pH of the solution as well as the type of oxidant used. Water-soluble synthetic metallporphyrins have been used for lignin degradation [62, 63]. Fe(III) and Mn(III) phthalocyanines could mimic the function of lignin peroxidase in the oxidation of veratryl alcohol and β-1 and β-O-4 lignin model compounds.

A MnP mimetic system containing Mn(II) and peracetic acid degraded LiP-resistent β-O-4 model substrates and bleached kraft pulps. Other models are H_2O_2 in combination with binuclear Mn(III/IV) complex or systems containing Mn(II), oxalate and DMSO [45]. Manganese complexes have been used as catalysts in bleaching textiles in the presence of hydrogen peroxide [64]. These catalysts assisted bleaching processes for the removal of stains from clothes by hydrogen peroxide at low temperatures.

Peroxidases are more complex in their mode of action than glucose oxidase and may contain one or more active sites with metallized porphyrin as their prosthetic group. The above potential biomimetic compounds including hemin and metallo phthalocyanines are colored compounds in most cases. This fact adds to the complexity of the mimetic system and creates a problem when used in bleaching of cotton and linen.

2.3 Surface characterization

Untreated and enzyme-treated linen fabrics contain different functional groups on accessible surfaces due to modified lignin content and therefore differ in surface

characteristics. The acid-base properties of fiber surfaces play an important role in all wet processing steps due to surface Lewis acid-base interactions between solids (fiber) and any type of encountered liquids. Inverse gas chromatography (IGC) is a tool to investigate surface properties, especially the electron acceptor (acidity) and donor (basicity) capabilities. Since oxidoreductases do not affect cellulose, the study of surface properties may lead to a better understanding of the enzymatic treatment of linen fibers. Thus, in this study linen fabric was enzymatically bleached with oxidoreductases and the resulting surface characteristics evaluated with IGC.

2.3.1 Surface characterization by inverse gas chromatography

Gas chromatography (GC) is one of the most important and widely used analytical separation methods in modern chemistry. It provides rapid and very high resolution separations of volatile compounds [65]. A typical GC consists of a mobile phase, a stationary phase in column, an injection port, and a detector.

Inverse gas chromatography (IGC) is an extension of conventional GC. For IGC small amounts of volatile probe molecules are injected into columns carrying the unknown solid polymeric stationary phase. This stationary phase is characterized by the known properties of probe molecules as they pass through the column by means of an inert gas.

IGC method has received much attention over recent years since its invention in 1967 [66] and subsequent theory and methodology development in 1976 [67]. IGC data can be collected rather rapidly and conveniently over extended temperature ranges and a variety of probes can be used in the mobile phase to characterize the stationary phase.

Besides its earlier application for determining glass transition temperatures, IGC is now mostly used to study surface energy properties and acid-base interaction in material.

The surface properties obtained from IGC data are the dispersive component of the surface free energy, the enthalpy and entropy, and the surface electron acceptor (acidity) and donor (basicity) constants (K_a and K_b, respectively). The dispersive component of surface free energy of fibers calculated from the IGC is difficult to measure by other methods such as dynamic contact angle [68, 69]. Materials such as polymers, textile and industrial fibers, wood and pulp fibers and composites have been characterized by IGC. The surface characteristics of fibers usually play an important role in their applications. For composites the chemical nature of the fiber surface determines the degree of adhesion of the reinforcing fiber to the matrix. Cellulosic materials are the common cost-cutting fillers of composites. IGC has been used successfully for studying the surface of cellulosic materials by several research groups [70, 71, 72, 73].

CHAPTER 3. EXPERIMENTAL

3.1 Materials

100% scoured unbleached cotton fabric was obtained from Testfabrics, Inc., New Jersey. The fabric weight per unit area was 109.2g/m^2.

For reason of comparison two types of linen fabric were used for this research. A light-weight 100% scoured unbleached linen fabric with low lignin content 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. A coarse heavy-weight 100% linen fabric with high lignin content was bought from Inotex Company, Czech Republic. The fabric weight per unit area was 510.06g/m^2.

Glucose oxidase (202 U/mg), lumiflavin, riboflavin, flavin mononucleotide (FMN), semicarbazide, L-histidine, L-lysine, L-asparagine, and L-arginine were purchased from Sigma-Aldrich Chemicals. Sodium acetate, sodium hydroxide, glucose, and hydrogen peroxide were purchased from Fisher Chemicals (analytical grade).

Laccase (250 units/g), MnP (20 units/g), LiP (12.5 units/g) were purchased from Tienzyme Inc., Salt Lake City, UT.

The analytical grade probes used for IGC were purchased from Fisher. They were used as received without further purification. The apolar probes are n-pentane, n-hexane,

n-heptane, n-octane, and n-nonane. The polar probes were acetone, chloroform, and tetrahydrofuran (THF).

3.2 Glucose oxidase bleaching of cotton fabric

A series of samples (control samples) was prepared by using the intact enzyme. The conditions for producing hydrogen peroxide by glucose oxidase (GO) were set to 35^{o}C and pH 5.1 (0.05 M aqueous sodium acetate buffer). Glucose dosage was 10 g/L and reaction time was set to 2 h. The concentration of glucose oxidase was varied. The bleaching process included two steps. First, hydrogen peroxide was produced by glucose oxidase at optimum conditions for the enzyme. Second, the pH was adjusted to 7 or 10.5, and bleaching performed at 90-95 ^{o}C for 2 h with the hydrogen peroxide generated by the enzyme. Unbleached desized 100% cotton fabric samples (1.5g) were treated with bleaching solutions. The samples were neutralized with dilute acetic acid, washed in water, air-dried.

3.3 Mimics of glucose oxidase for bleaching of cotton fabric

Aqueous solutions (60 mL) containing 10 mL 4.0 x 10^{-4}M mimicking compounds (lumiflavin, riboflavin or FMN) were prepared. As electron donors either semicarbazide (SC, 15 mM) or one or more of the amino acids L-histidine (His), L-lysine (Lys), L-asparagine (Asp), and L-arginine (Arg) were added (concentration varied, see text). For experiments with amino acid(s) the treatment time was 8 h and pH 5.1 (adjusted by mixing of a solution consisting of 0.20 M boric acid and 0.05 M citric acid solution with a solution of 0.10 M tertiary sodium phosphate). The glucose dosage was set to 10 g/L.

Oxygen was supplied in gaseous form and bubbled through the solutions simultaneously ensuring adequate mixing. The aqueous solutions were irradiated with a 75 W white light.

For experiments involving semicarbazide the solutions were adjusted to pH 12.3 or 13.3 with sodium hydroxide and irradiated with a 60 W halogen light or 75W white light for various lengths of time. The concentration of riboflavin and semicarbazide were varied. Oxygen was supplied in the same way as mentioned above. The concentration of hydrogen peroxide in the bleach bath was determined by an iodometric method [74]. For the actual bleaching step, the pH was adjusted to 7 or 10.5, and the reaction performed at 90-95^{o}C for 2 h with the hydrogen peroxide generated by the mimics. After bleaching, the samples were thoroughly washed in water and air-dried. Their color coordinates were compared with those of the control samples.

3.4 Laccase and glucose oxidase bleaching of cotton fabric

Cotton fabrics were pretreated with laccase (from Sigma) with or without syringadazine followed by the bleaching process with hydrogen peroxide produced by glucose oxidase. The pretreatment conditions were pH 6.0 (0.05M sodium acetate buffer) and 30 - 35^{o}C for 2h. The concentration of laccase was 0.1 g/L. The treated fabrics were thoroughly washed with boiling distilled water for 10 min to deactivate the enzymes. The conditions of hydrogen peroxide producing process with glucose oxidase were pH 5.1 (0.05M aqueous sodium acetate buffer) and 35^{o}C for 2h. The concentrations of glucose and glucose oxidase were 10 g/L and 10 U/mL, respectively. The bleaching was

performed at 90-95ºC for 2 h with the amount of hydrogen peroxide generated in the above step.

3.5 Laccase and glucose oxidase bleaching of linen fabric

The fabrics were pretreated with laccase followed by the bleaching process with glucose oxidase. The pretreatment conditions were pH 6.0 (0.05 M sodium acetate buffer) and 50^{o}C for 24 h. The concentration of laccase was varied. The treated fabrics were thoroughly washed with boiling distilled water for 10 min to deactivate the enzymes. The bleaching process included two steps. First, hydrogen peroxide was produced by glucose oxidase at the enzyme optimum conditions (35ºC at pH 5.1 (0.05M aqueous sodium acetate buffer) for 2 h). The concentration of glucose oxidase was 20 U/mL. Second, the pH was adjusted to 10.5, and bleaching performed at 90-95ºC for 2 h with the amount of hydrogen peroxide generated in the first step.

For reasons of comparison, a set of linen samples was bleached with commercial hydrogen peroxide. Triplicate sets of scoured unbleached 100% linen fabric samples (1.5g) were treated in the bleaching solutions. The samples were washed in water and air-dried. The bleaching bath consisted of 8% owf (on weight of fabric) 35% commercial hydrogen peroxide, 3% owf sodium silicate, and 1% owf sodium hydroxide. The liquor ratio was 50:1 and bleaching was carried out at boiling temperatures for 1 h.

3.6 Testing methods

3.6.1 Determination of hydrogen peroxide concentration produced by glucose oxidase

The concentration of hydrogen peroxide produced by glucose oxidase was determined by AATCC Test Method 102: Determination of Hydrogen Peroxide by Potassium Permanganate Titration. The volume of the aqueous hydrogen peroxide specimen was 10.0 ± 0.1 mL. 20 ± 0.1 mL distilled water and 20± 0.1 mL of 20% sulfuric acid was added to the flask while gently stirring. The above solution was titrated with 0.588 N standardized potassium permanganate ($KMnO_4$) to faint pink color lasting 30 s. The concentration of hydrogen peroxide was calculated by the following equation:

Concentration of H_2O_2 (mg/L) = V_t x 100

where V_t is the volume of titrant in mL.

3.6.2 Determination of the activity of commercial hydrogen peroxide

In industry, commercial hydrogen peroxide in aqueous solution is used to bleach cotton fabric. In order to compare the activity of commercial hydrogen peroxide and hydrogen peroxide produced from GO, the amount of active peroxide was determined by the above method at several different concentrations of commercial hydrogen peroxide ranging from 0.5 g/L to 2.0 g/L were titrated by 0.582 N $KMnO_4$. The active peroxide corresponding to each concentration as titrated by 0.582 N $KMnO_4$ is shown in Figure 3.1. A linear line was obtained following the equation: y = 708.52 x

where y is active hydrogen peroxide in mg/L, x is concentration of commercial hydrogen peroxide (g/L).

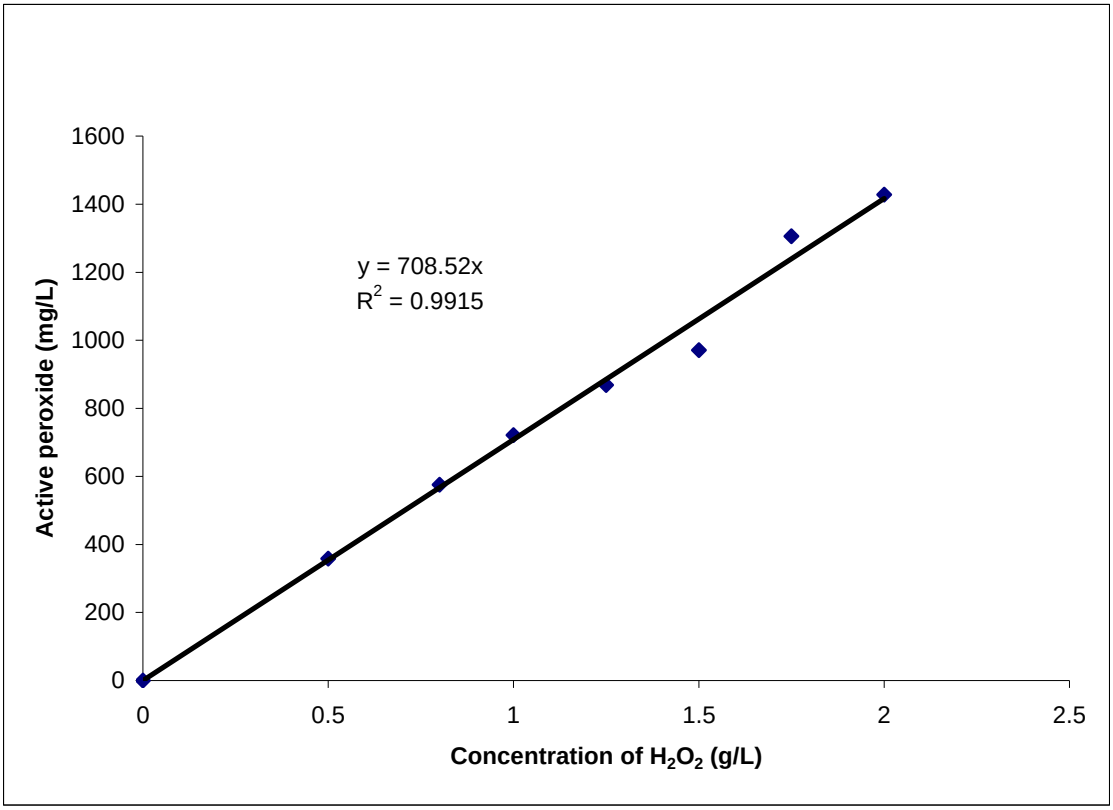

Figure 3.1 Relationship between commercial H_2O_2 and active peroxide (titration with 0.582N $KMnO_4$)

3.6.3 Measurement of hydrogen peroxide concentration produced by mimics of glucose oxidase

The concentration of hydrogen peroxide produced by the mimics was determined by an iodometric method. 10 mL aqueous hydrogen peroxide solution was added to 1 g of potassium iodiode in 100 mL sulphuric acid (1:20). The mixture was allowed to stand for 15 min and the liberated iodine was titrated with standard 0.1 N-sodium thiosulphate until the iodine has been nearly discharged. The concentration of hydrogen peroxide was calculated by the following equation:

Concentration of H_2O_2 (mg/L) = V_t x 68

where V_t is the volume of titrant in mL.

3.6.4 Whiteness

The level of whiteness of control and treated samples were measured with a colorspectrophotometer (CS-5 Chroma Sensor, Datacolor International) using DL* to represent the difference in whiteness of the treated fabric compared to the control. Several measurements at different spots of the fabric were taken and the results averaged.

3.6.5 Weight per unit area of fabric

Weight per unit area of fabric was measured following ASTM D-3776: Standard Test Method for Mass Per Unit Area (Weight) of Fabric.

3.6.6 Lignin content

The lignin content of the untreated and treated linen samples was determined by TAPPI Method #222. The fibers were ground in a Wiley mill with a 40 mesh screen. Sulfuric acid was used for carbohydrate hydrolysis. 40 mL 72% sulfuric acid was added to the oven-dried samples (2 ± 0.1g) in 100 mL beakers with gradual stirring. The samples were macerated with a glass rod while keeping the beaker temperature at 2 ± 1^{o}C. After dispersion of the sample, the beaker was kept in a bath at 20 ± 1^{o}C for 2 h while being stirred frequently to ensure complete solution. The material in the beaker was transferred to a flask containing 300 to 400 mL water. Additional water was used to rinse the beaker, dilute the flask content to 3% sulfuric acid and adjust to a total volume of 1540 mL. The solution was boiled for 4 h and the insoluble lignin allowed to settle

overnight. The supernatant solution was siphoned off through a glass filter without stirring up the precipitate. The lignin was transferred quantitatively to the filter using hot water and washed with hot water to remove residual acid. The glass filter with lignin was dried in an oven at 105 ± 3^{o}C to constant weight, cooled in a desiccator and weighed. The lignin content was calculated as weight of lignin per oven-dried sample weight as follows:

Lignin, % = W x 100/W_0

Where W = weight of lignin, g; and W_0 = oven-dry weight of samples, g.

3.6.7 Inverse gas chromatography

A Hewlett Packard 5700A series gas chromatograph equipped with flame ionization detection (FID) was used. The columns were cut from stainless steel of ¼-inch (o.d.) to a length of 0.5 m. The materials used for packing the IGC columns in this study were linen fibers from the above fabrics ground in a Wiley mill to mesh size 40. 1.5 g linen fibers were packed into the column evenly with the help of a vibrator. As carrier gas helium was used at a flow rate of 15 mL/min, measured by a soap bubble flow meter at room temperature. The injector temperature was set at 150^{o}C and the FID detector was heated to 200^{o}C. The oven temperature ranged from 40^{o}C to 70^{o}C and increased by 10^{o}C increments. Methane was used as a non-interacting reference probe to measure the dead volume of the column. The probes were injected manually by using 1.0 µl SGE microvolume syringes, All measurements were repeated several times with minute quantities of probe vapor (0.1 µL) for each injection to show for the elution peaks to be reproducible. The retention times of each probe and of methane were obtained from their respective peak maxima and averaged for further calculations.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Glucose oxidase and its mimics for bleaching of cotton fabric

4.1.1 Glucose oxidase bleaching of cotton fabric

For commercial hydrogen peroxide bleaching of cotton fabric the amount of hydrogen peroxide added to the bleaching bath is related to the impurities of the fabric and the whiteness level requirement. The pH value of the bleach bath generally ranges from 10 to 11 and the temperature from 90 to 100^{o}C. These conditions are fairly harsh and fiber damage could occur, especially in the presence of metal ions introduced by rusty equipment.

For glucose oxidase bleaching, in order to achieve the desired whiteness, sufficient hydrogen peroxide must be produced by GO and glucose under aerobic conditions. The amount of glucose and GO dosage plays an important role in producing hydrogen peroxide. In previous work glucose dosage was determined to be sufficient at 10 g/L [2]. Figure 4.1 shows the relationship between GO dosage and hydrogen peroxide concentration with or without fabric in the treatment bath. Within increasing GO dosage from 2 to 10 U/mL higher concentrations of peroxide could be achieved. Above 10 U/mL GO the rate of peroxide production decreased as previous research had demonstrated [2]. It is interesting that the presence of fabric during this step played a considerable role in

producing peroxide. Without the fabric the amount of peroxide produced was between 800 and 950 mg/L, while with fabric 1120-1260 mg/L peroxide was generated.

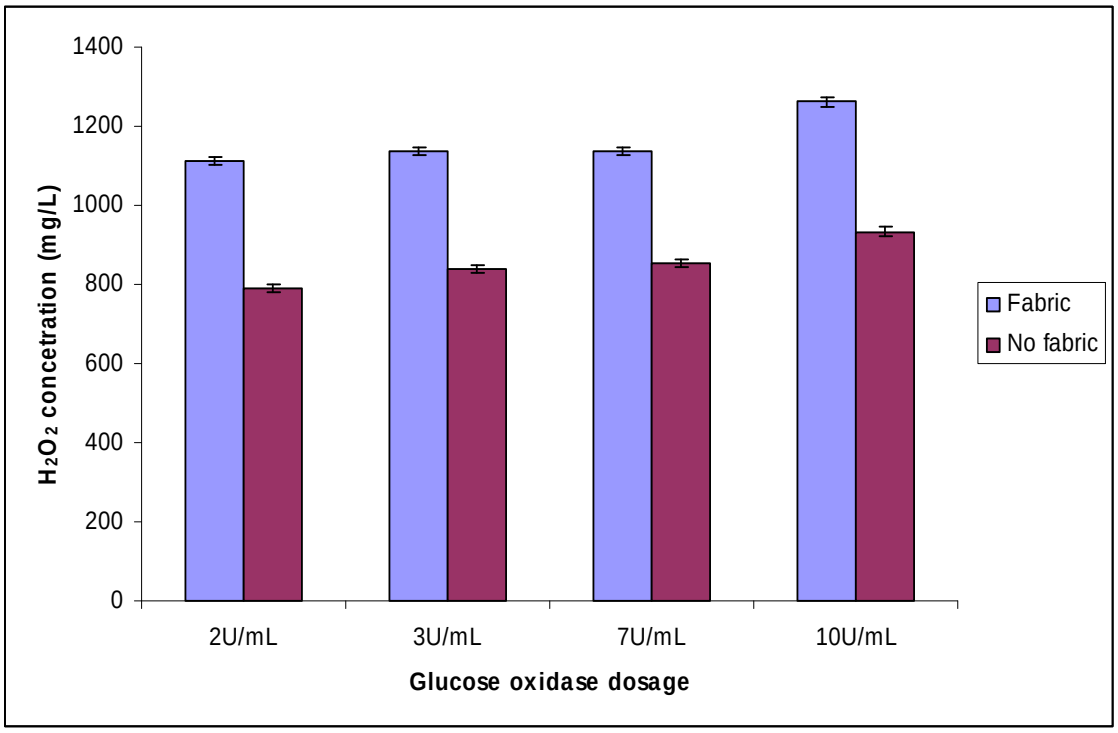

Figure 4.1 Enzymatic production of hydrogen peroxide with and without fabric by glucose oxidase on dosage

The pH of the treatment bath is generally crucial for all enzymes to perform at their optimum. In the case of the interaction of GO with glucose slightly acidic conditions (pH 5.1) are optimal. Hydrogen peroxide for bleaching, on the other hand, requires for the pH to be adjusted to 7.0 [2] or 10.5 (industrial conditions) to be effective. In Figure 4.2 the relationship between whiteness of enzymatically bleached fabric, GO dosage, and pH setting is shown. The fabric whiteness was higher at increased GO concentration due to more available hydrogen peroxide produced at higher GO dosage. The bleaching effect of the bath at 10.5 was enhanced compared to pH 7 as could be expected.

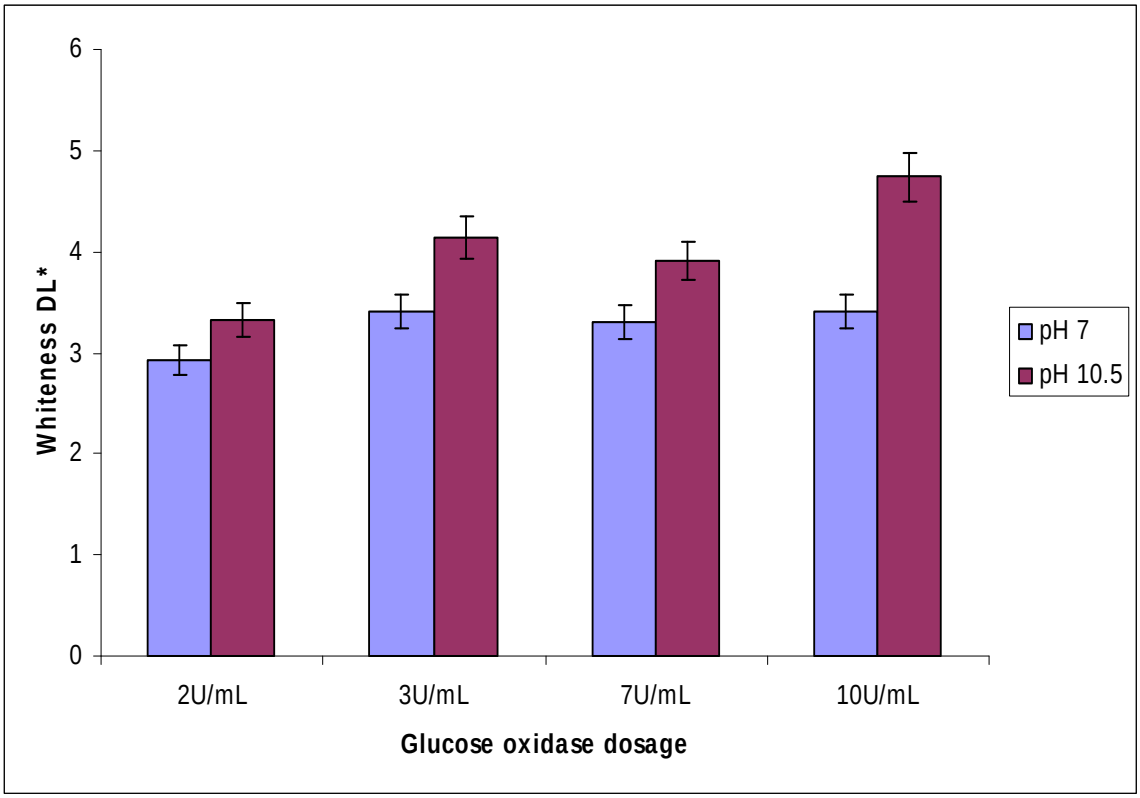

Figure 4.2 Whiteness increase of cotton fabric compared to unbleached control when bleached with GO at various levels and two pH values (7, 10.5). The fabric was added during the previous bleaching step.

The amount of active peroxide was determined with the method described above. It was found that more active hydrogen peroxide was generated by enzymatic means than was contained in commercial hydrogen peroxide. Thus, 1000 mg/L hydrogen peroxide produced by GO equal 1410 mg/L commercial hydrogen peroxide necessary to generate the same amount of active peroxide.

4.1.2 Peroxidases and laccase in GO bleaching cotton fabric

Manganese peroxidase (MnP) belong to lignin-degrading enzymes and showed some applications for pulp bleaching. In this study, MnP has been added to promote the GO bleaching effect for cotton. Various treatment methods were explored. The results of the different procedures are summarized in Figure 4.3.

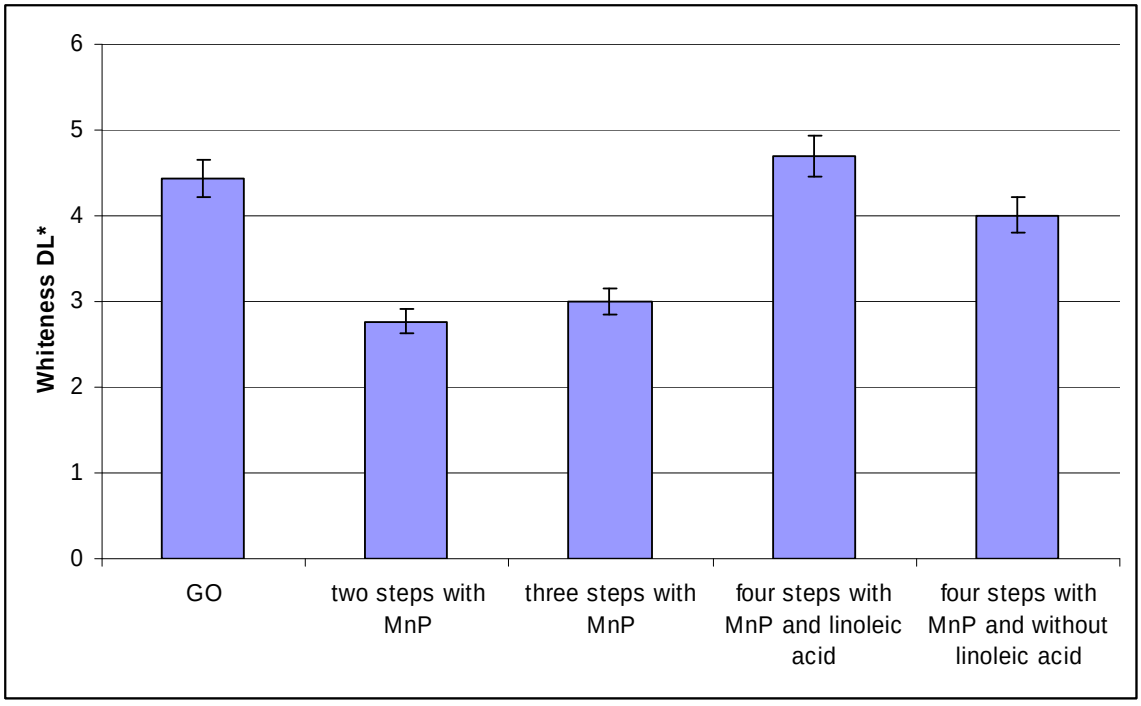

Figure 4.3 Comparison of whiteness of fabric bleached with MnP and GO (see text for explanation)

As the easiest approach, a two-step process was developed in which cotton fabric was initially treated with GO as described before (GO dosage 10 U/mL). Subsequently, MnP (700 ppm), $MnSO_4$ (0.5 mM), and linoleic acid as a mediator (1 μL/mL) were added to the solution. The result was insufficient (DL* = 2.77) compared to the control (DL* = 4.44). It could be speculated that the high bleaching temperature and pH were not suitable

for MnP which performs best at temperatures around 25-30^{o}C and pH 4.5. Furthermore, the addition of the metal ion Mn^{2+} could destabilize the peroxide and decompose it too fast to lose its activity.

Based on this result, another processing step was added (third column in Figure 4.4). After hydrogen peroxide production by GO, the fabric is immersed in a solution containing 700 ppm MnP and 0.05 mM Mn^{2+} at pH 4.5 and treated for 24 h at 30^{o}C. Then, the fabric was bleached at alkaline condition and high temperature, simultaneously deactivating the enzyme. Fabric whiteness slightly improved (DL* = 3.00), however not satisfactorily. The existence of Mn^{2+} may still account for the loss of peroxide activity.

Consequently, two pretreatment steps were developed (fourth column in Figure 4.4). The cotton fabric was immersed in pH 4.5 (0.05 mM malonate) solutions containing MnP (700 ppm), $MnSO_4$ (0.5mM), GO (1.0 U/ml), glucose (20 mM) with/without linoleic acid (1 μL/mL). The fabric was treated for 24 h at 30°C. The treated fabric was then transferred to a solution containing 1.2 % o.w.f EDTA and treated at pH 5 and 50°C for 30 min. The purpose of EDTA was to overcome the potential negative impact of Mn^{2+} by chelation. The third and four steps in this process were the same as in the original GO/MnP bleaching process. The whiteness increased to 4.70 and 4.01 with and without linoleic acid, respectively. MnP may oxidize the carbon-carbon double bond (C=C) in linoleic acid producing a peroxyl radical. The produced radical might act as oxidant for on non-phenolic compounds in lignin [75].

Horseradish peroxidase (HRP) uses hydrogen peroxide for oxidation of aromatic compounds. Theoretically, HRP can act as hydrogen peroxide activator. The application of HRP in GO bleaching was explored by using different combination and treatment

conditions. The process developed for HRP and GO bleaching resembled that of GO/MnP bleaching with the only difference being that HRP was added during the first stage since this stage was performed under mild reaction condition. Figure 4.4 shows the effect of HRP on the whiteness of fabric. The addition of HRP in the first stage influenced the whiteness of fabric to a certain extent.

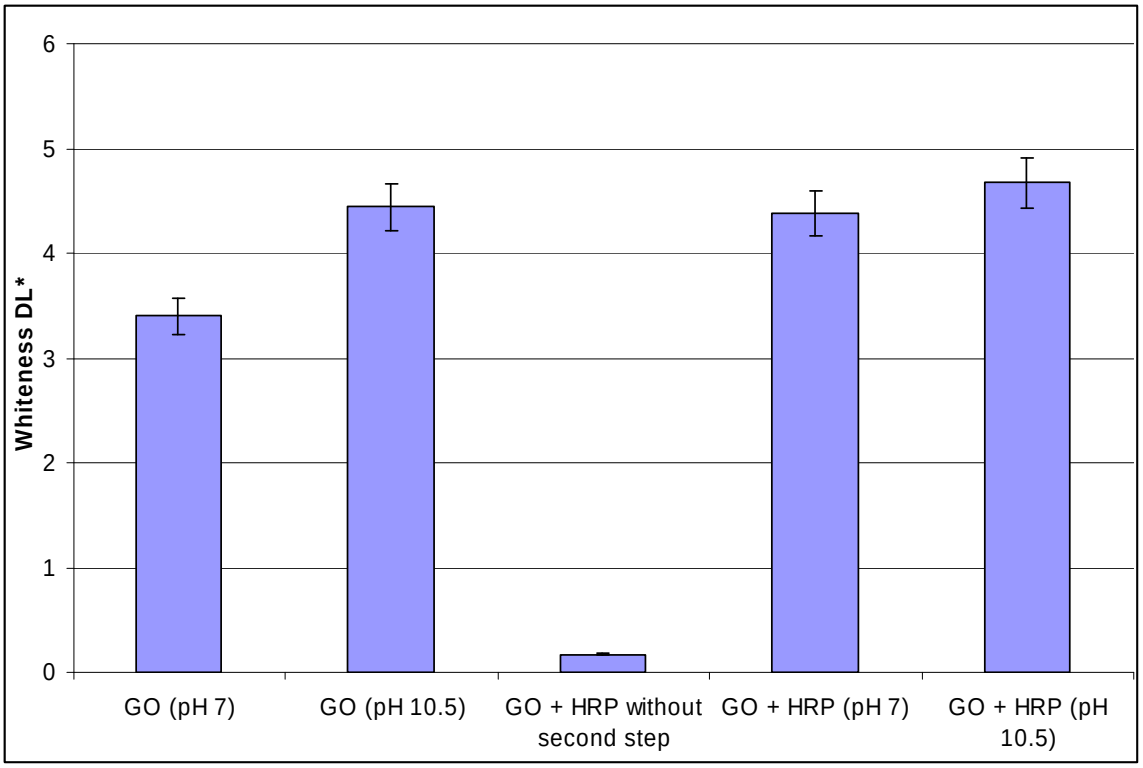

Figure 4.4 Comparison of whiteness of fabric bleached with HRP under different methods and pH (see text)

Using HPR/GO at pH 10.5 and 7, the whiteness increases were 4.67 and 4.38, respectively (columns 4 and 5 in Figure 4.4). Compared with the whiteness of fabric bleached by GO only at these pH values (4.44, 3.40) a slightly higher whiteness increase was observed at pH 10.5 while at pH 7 the increase is more pronounced with HRP in the

system. If the process was conducted without increasing the pH and/or temperature, the addition of HRP did not improve the whiteness (DL* = 0.17; third column in Figure 4.4).

Subsequently, combinations of GO and laccase were explored for bleaching cotton fabric. The influence of laccase, mediator, and oxygen on the whiteness of GO bleaching was studied. Table 4.1 shows some of the results.

Table 4.1 Whiteness increase DL* of fabric bleached with Laccase and GO (syringaldazine as mediator)

Enzyme treatment; with/without mediator		Whiteness DL*
GO		4.44 ± 0.22
Laccase, then GO	Syringaldazine	3.89 ± 0.19
	No mediator	4.38 ± 0.22
Laccase with GO	Syringaldazine	3.72 ± 0.19
	No mediator	3.90 ± 0.20
Laccase + O_2, then GO	Syringaldazine	2.70 ± 0.14
	No mediator	4.70 ± 0.24
Laccase with Cellulase, then GO		4.55 ± 0.23

Laccase treatment alone did not improve fabric whiteness although it is possible that the structure of aromatic noncellulosic compounds in cotton changed somewhat. With the combination of GO and laccase the results could be improved. The fabric was first pretreated in a pH 6.0 buffer solution containing laccase (0.1 g/L) for 2 h at 35^{o}C,

followed by the GO bleaching procedure described before. The pretreatment did not yield a major improvement in whiteness (DL* = 4.38). Laccases are known to use molecular oxygen as co-substrates. Thus, oxygen was supplied in gaseous form and bubbled through the pretreatment solution. With oxygen the laccase pretreatment lead to an increase in fabric whiteness (DL* = 4.70). Redox mediators were suggested by other research groups to improve the efficiency of laccases for they can enlarge their redox potential by generating stable radicals. Syringaldazine (0.5 M) was used in this study. However, from the results shown in Table 4.1 it can be seen that the whiteness of fabric actually decreased after laccase-mediator system was introduced even with oxygen in the system. Thus, the mediator concept was not applicable for this process. The possible reason might be the yellow color of syringaldazine which adds a yellowish tint to the fabric.

4.2 Mimics of glucose oxidase for bleaching of cotton fabric

FAD, the active site of GO, and amino acid residues adjacent to FAD, catalyze transformation of glucose into gluconic acid, in the process of which hydrogen peroxide is generated. The structures of FMN, riboflavin, and lumiflavin are similar to FAD. Thus, these compounds were selected to mimic the behavior of FAD in reaction. Based on work of De la Rosa et al. [13, 14] on light-sensitized hydrogen peroxide production by flavin compounds, as electron-donor, semicarbazide was initially selected for these experiments as it had proved to be most efficient. Additionally, a light source with defined energy output was installed. It had also been established [13] that the photochemical formation of hydrogen peroxide is greatly influenced by the pH value.

In this work, the concentration of flavins and the amount of semicarbazide were varied. Higher pH values favored the rate of hydrogen peroxide production. Oxygen was supplied in gaseous form and bubbled through the solutions. The relationship between the riboflavin concentration, and semicarbazide and hydrogen peroxide concentration is shown in Figures 4.5 and 4.6, respectively. The more riboflavin was used for the reaction, the higher concentration of hydrogen peroxide could be produced. The semicabazide dosage also played a role in the production of hydrogen peroxide. The flavin produced hydrogen peroxide increased with the increase of semicarbazide dosage and reached maximum at 0.1 g.

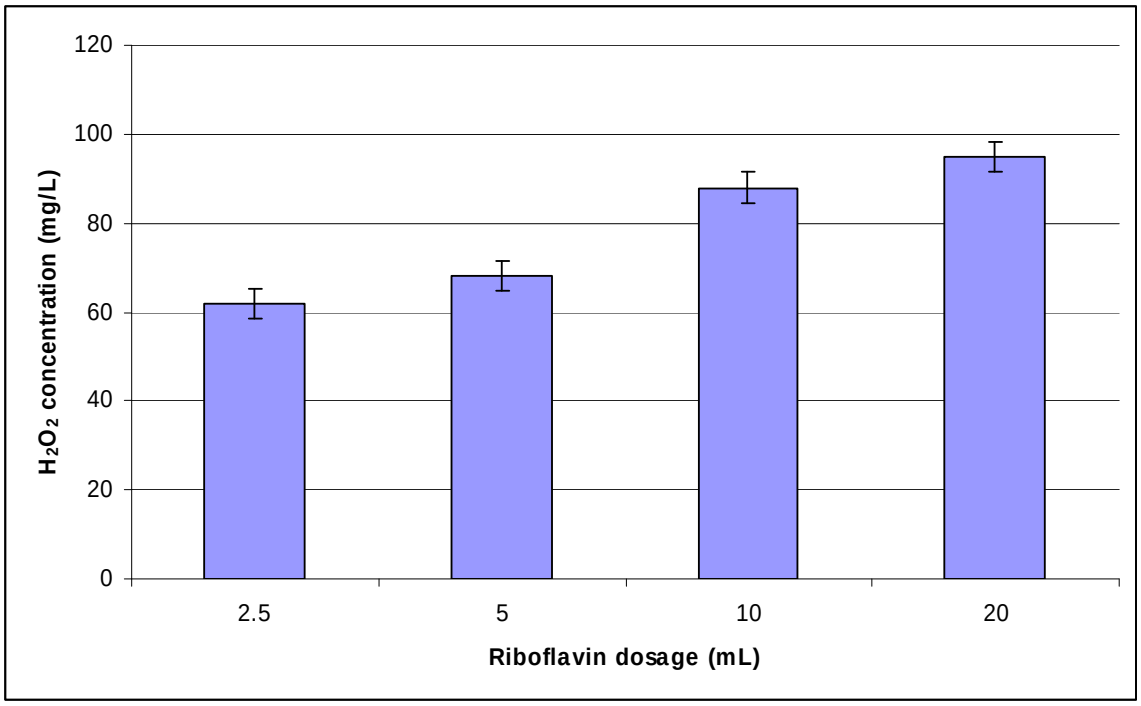

Figure 4.5 Effect of riboflavin concentration on hydrogen peroxide concentration (0.1 g semicarbazide; pH 12.3; 75 W light lamp; riboflavin concentration 4.0 x 10^{-4} M; 8 h)

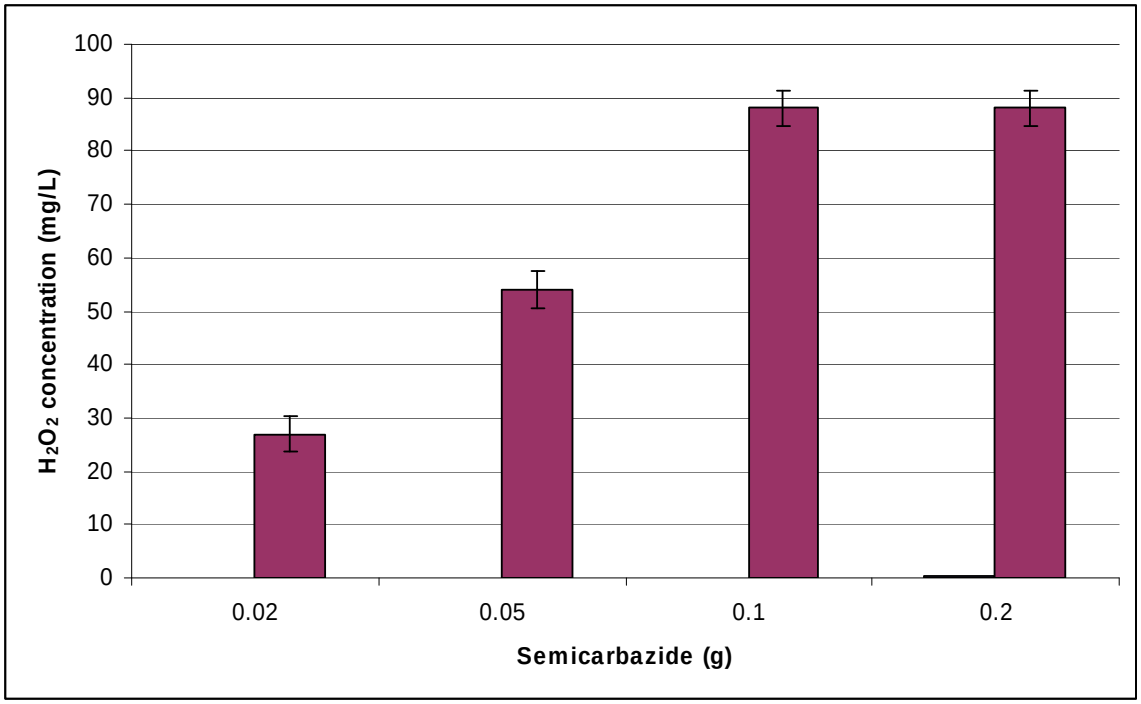

Figure 4.6 Effect of semicarbazide dosage on hydrogen peroxide concentration (10 ml 4.0 x 10^{-4} M riboflavin; pH 12.3; 75 W light lamp; 8 h)

The photochemical formation of hydrogen peroxide is also greatly influenced by the pH value. Higher pH value greatly enhanced the rate of hydrogen peroxide production as mentioned before. In Figure 4.7 the amount of photochemically produced hydrogen peroxide by riboflavin is presented in relation to the irradiation time at pH 12.3 and 13.3 (white lamp 75 W). At pH 12.3 the concentration of hydrogen peroxide increased during the first four hours of the reaction, then leveled off. Hydrogen peroxide production at pH 13.3 was almost three times higher than at pH 12.3 and kept increasing with reaction time.

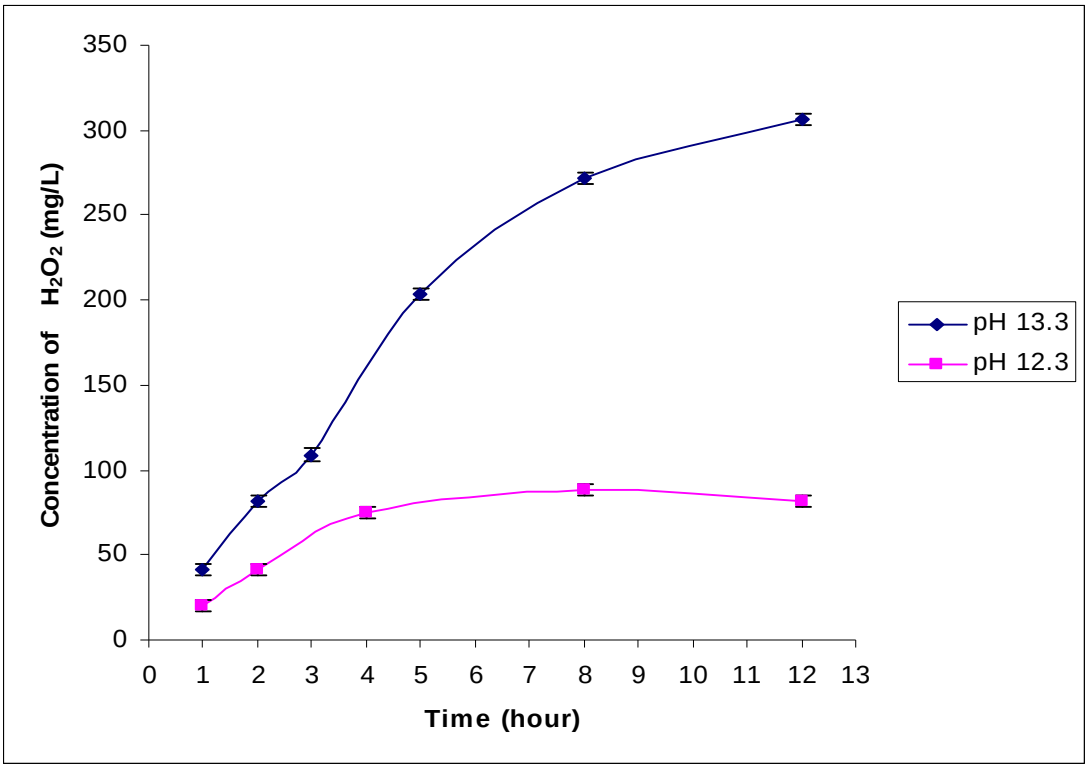

Figure 4.7 Production of hydrogen peroxide by riboflavin with the extension of time at pH 12.3 and 13.3 (10 ml 4.0 x 10^{-4} M riboflavin; 0.1 g semicarbazide; 75 W light lamp)

The mechanism of light-sensitized biomimicking might account for the reason that high pH values benefit the hydrogen peroxide production. A one-electron transfer reaction has been widely accepted for the mode of reactivity of excited flavins with the electron donors [76]. The flavin semiquinone form produced may transfer the acquired electron to molecular oxygen and to the superoxide anion, to produce hydrogen peroxide. The photoreduced dihydroflavin (H at N1 and N10 position) can either be oxidized by the presence of oxygen directly:

$$\text{Flavin}H_2 + O_2 \longrightarrow \text{Flavin} + H_2O_2$$

or go through a radical route indirectly:

$$\text{Flavin}H_2 + O_2 \longrightarrow \text{Flavin}H^{\cdot} + O_2^{-\cdot} + H^+$$

$$\text{Flavin}H_2 + O_2^{-\cdot} + H^+ \longrightarrow \text{Flavin}H^{\cdot} + H_2O_2$$

$$\text{Flavin}H^{\cdot} + O_2 \longrightarrow \text{Flavin} + O_2^{-\cdot} + H^+$$

$$2O_2^{-\cdot} + H^+ \longrightarrow O_2 + H_2O_2$$

The high pH value and the disproportionation of superoxide to hydrogen peroxide might favor the reaction via superoxide. The reaction of oxygen with the anionic flavin radical should be faster than with semiquinone in neutral radical form [17].

Since the pH value producing most hydrogen peroxide by the mimics was 13.3 under the applied conditions and the proper pH for hydrogen peroxide bleaching is 10-11, it was important to adjust the pH. The photochemically produced hydrogen peroxide was thus used to bleach cotton fabric after lowering the pH. In Figure 4.8 results for fabric whiteness are presented obtained with riboflavin at pH 10.5 and 7. At pH 7 a steady increase of fabric whiteness with time was observed, while at pH 10.5 whiteness reached a maximum at 8 h, corresponding to the amount of available hydrogen peroxide. However, even with such extended treatment times, only approximately 70% of the whiteness levels of the enzymatically (GO) treated control could be achieved.

The efficiency of the photochemical system might be related to the type of mimicking compound as well as the light source with its specific energy output (Figures 4.9 and 4.10). Compared to riboflavin and lumiflavin, FMN together with the 60 W halogen light source was more effective regarding whitening the cotton fabric, even with the concentrations of hydrogen peroxide being slightly lower, and lumiflavin performed best with the 75 W white light. Overall, the results of both light sources were similar.

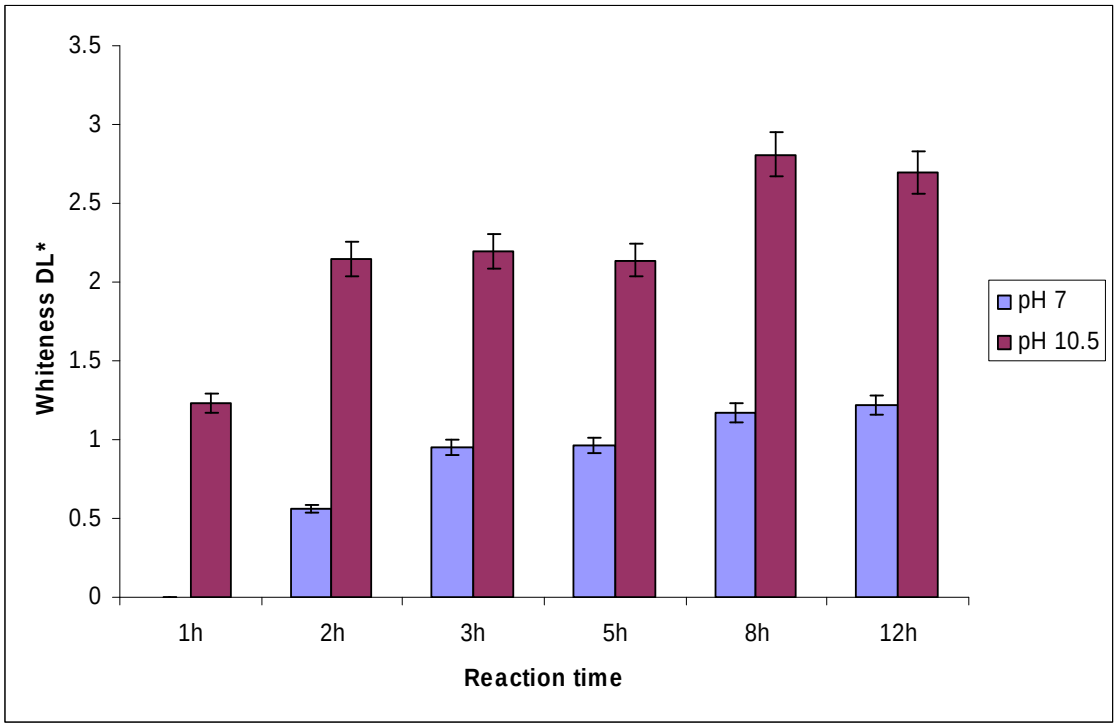

Figure 4.8 Whiteness increase of cotton fabric treated with riboflavin as a function of treatment time and pH compared to the scoured control (10 ml 4.0 x 10^{-4} M riboflavin; 0.1 g semicarbazide; pH 13.3; 75 W light lamp); bleaching of cotton fabric was performed at 90-95^{o}C for 2 h at pH 7 or 10.5

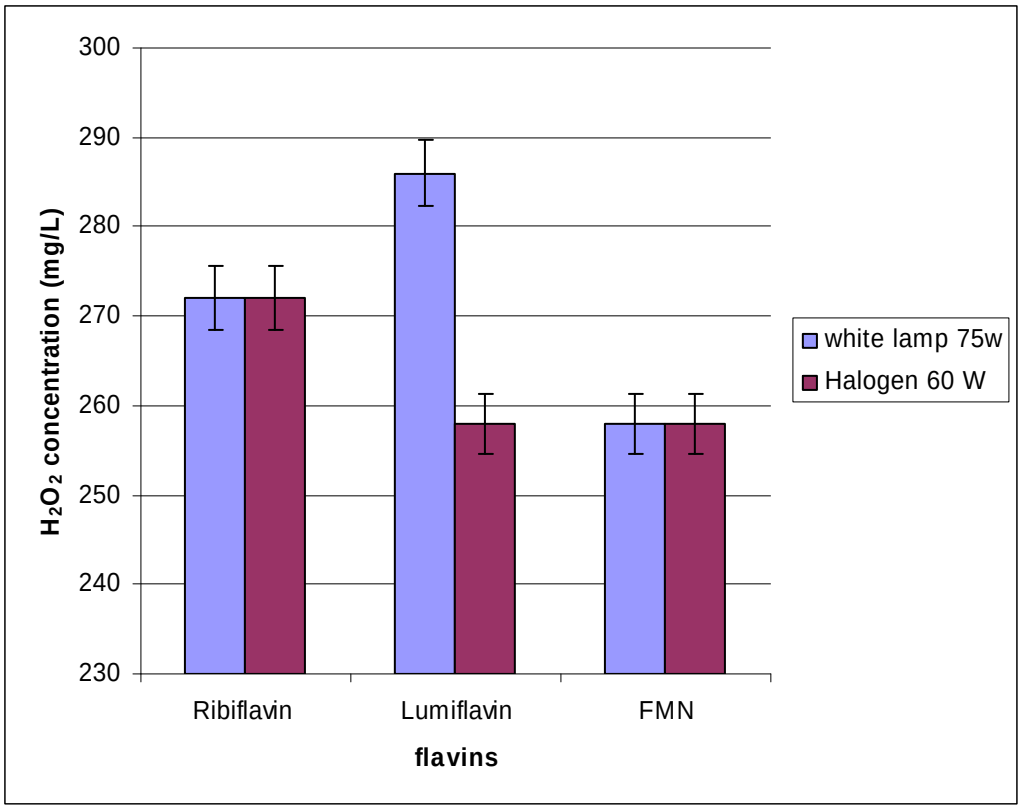

Figure 4.9 Effect of different flavins and light sources on H_2O_2 concentration (10 ml 4.0 x 10^{-4} M riboflavin; 0.1 g semicarbazide; pH 13.3; 75 W light lamp; 8 h)

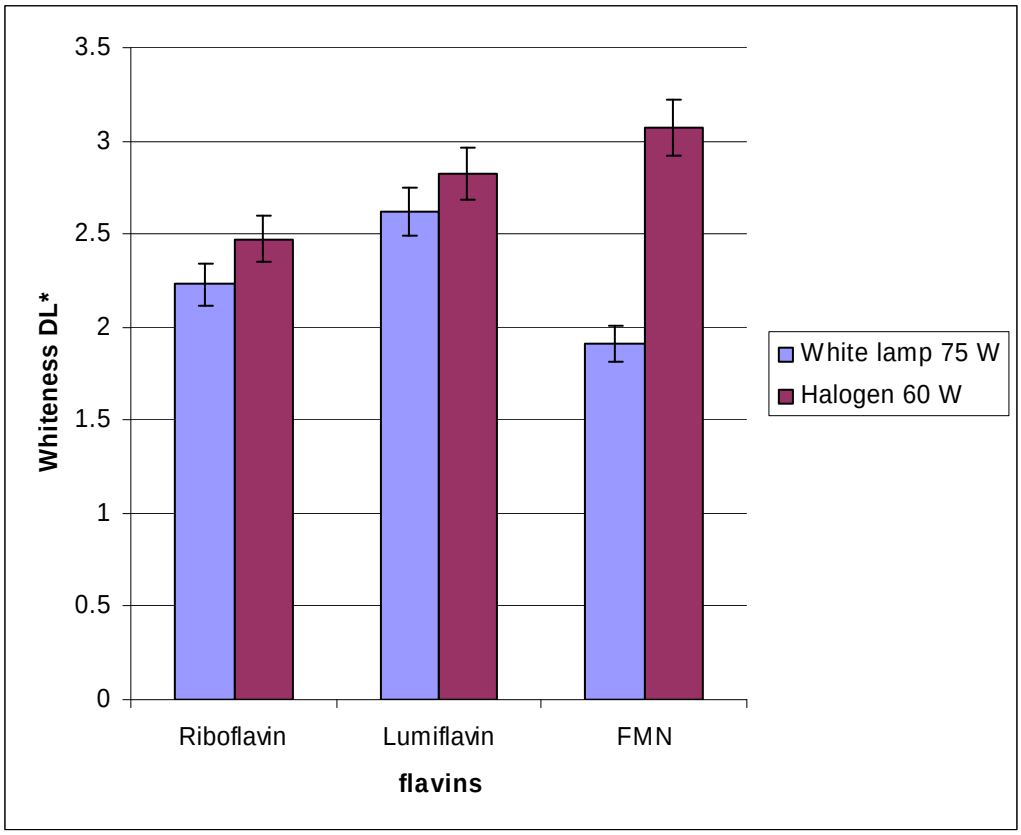

Figure 4.10 Whitening effect of different flavins and lights using semicarbazide. Bleaching was performed at 90-95^{o}C for 2 h at pH 10.5

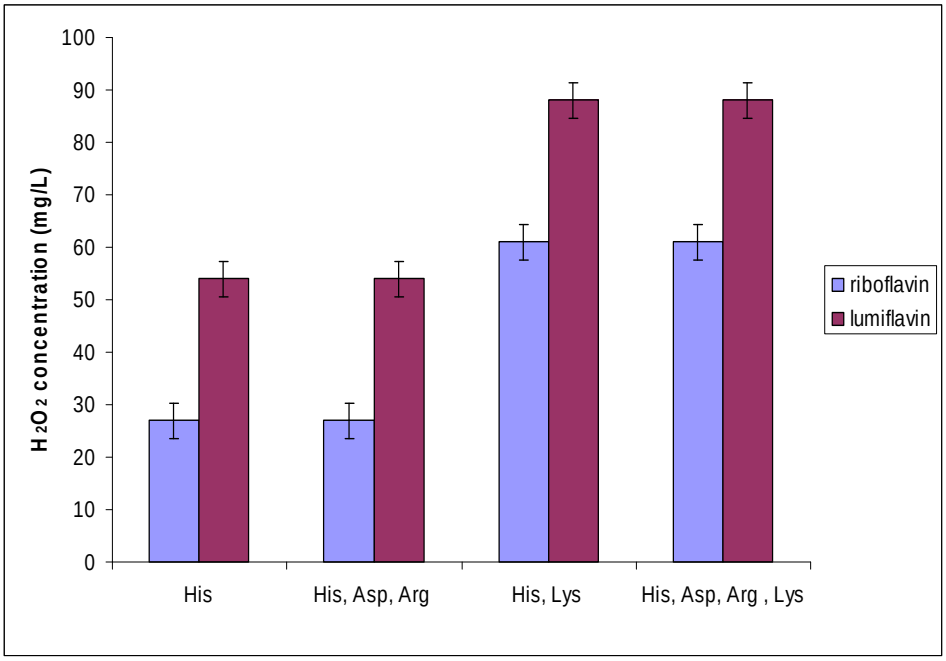

Figure 4.11 Production of hydrogen peroxide by riboflavin and lumiflavin with various combinations of His, Lys, Asp, and Arg in equal molar ratio. The amount of each amino acids used in experiment is 3.2×10^{-4} mole (10 ml 4.0×10^{-4} M lumiflavin; pH 5.1; 75 W light lamp; 8 h)

Amino acid residues in direct neighborhood to FAD are believed to play a vital role in the reaction as electron donors. For this research, histidine (His) and lysine (Lys) were used to mimic the environment of FAD and to support the electron transfer. In addition, the effect of aspartic acid (ASP) and arginine (Arg) was studied. The pH was originally varied in the range of 4 to 10, including pH 5.1 which is the optimum pH for the intact GO used in the control experiments. Higher or lower pH values resulted in lower performance of the mimics; thus, only results at pH 5.1 are reported here. The energy output of the light source was also varied, but only results obtained with a 75 W white light for irradiation are presented here since the difference between light sources was insignificant.

In Figure 4.11 the effect of peroxide concentration and different combinations of these amino acids and mimics on hydrogen peroxide production is presented. Equal molar ratios of each amino acid in combination were used. The results indicate that His and Lys were more effective than His alone or the combination of His, Asp, and Arg in producing peroxide.

The addition of Arg and Asp to His and Lys did not significantly improve peroxide production. Regarding the mimics, lumiflavin seemed to be more effective than riboflavin. In Figure 4.12 the relationship of peroxide concentration and the different flavin mimics under otherwise same conditions are compared.

Since the combination of His and Lys seemed to work best to mimic the environment of FAD and His 516 and His 559 are in direct vicinity of FAD of the intact GO [8], different molar ratios of His and Lys were studied further.

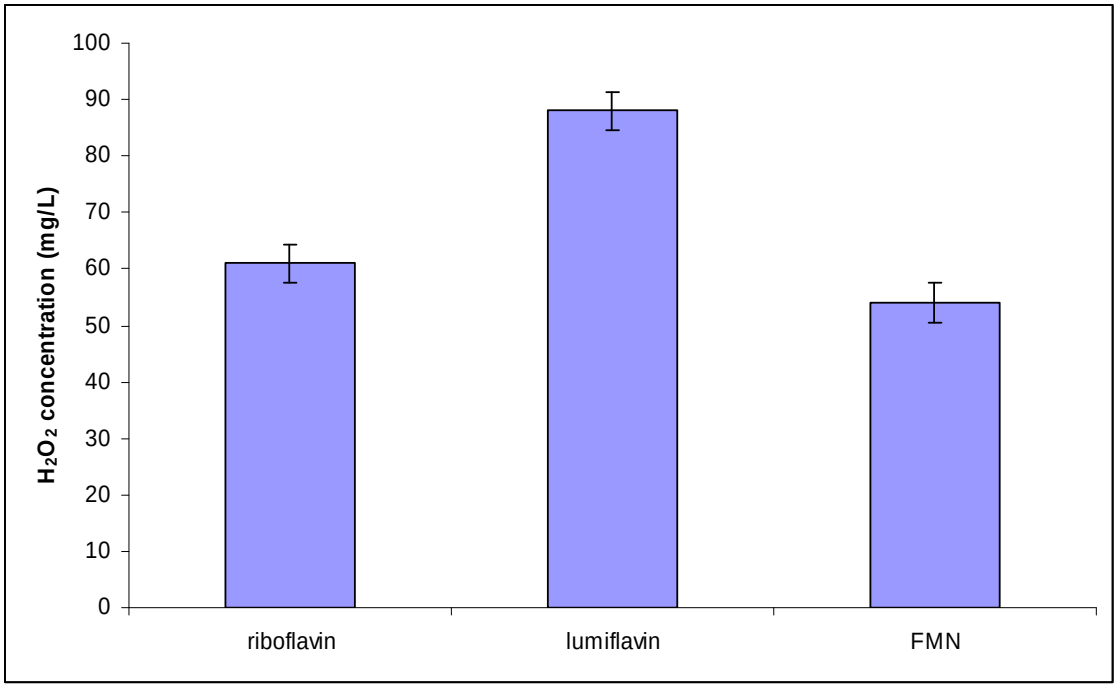

Figure 4.12 Production of hydrogen peroxide by lumiflavin, riboflavin, and FMN with mole ratio of His and Lys of 1:1(10 ml 4.0 x 10^{-4} M flavins; pH 5.1; 75 W light lamp; 8 h)

Table 4.2 Production of hydrogen peroxide by lumiflavin with varied dosage of His and Lys (mole ratio 2:1)

His (mole)	Lys (mole)	H_2O_2 (mg/L)
2 x 10^{-4}	1 x 10^{-4}	40 ± 3.4
5 x 10^{-4}	2.5 x 10^{-4}	102 ± 3.4
6.4 x 10^{-4}	3.2 x 10^{-4}	107 ± 1.7
9 x 10^{-4}	4.5 x 10^{-4}	122 ± 1.7
1.8 x 10^{-3}	9 x 10^{-4}	150 ± 3.4

In Table 4.2 the amounts of hydrogen peroxide are listed that were generated in presence of lumiflavin with increasing dosage of His and Lys at a 2:1 mole ratio. Hydrogen peroxide concentration produced by this mimic system increased with the increase of amino acid dosage.

Even though hydrogen peroxide could be produced by the flavin mimics/amino acid system, the relative amount was lower than the intact enzyme could generate (see Figure 4.1). It was assumed that the electron donor capacity of the free amino acids under the chosen experimental conditions was not quite sufficient. In this aspect, semicarbazide was more effective. The hydrogen peroxide production however occurred at more benign conditions (pH 5.1) with amino acids while semicarbazide only functioned well at highly alkaline pH values.

The intact glucose oxidase enzyme in oxidized form shows an absorbance maximum at 280 nm and two additional maxima at 377 nm and 455 nm which originate from FAD. Basicity at the active site in the intact enzyme, necessary for the interaction with the substrate, is most likely provided by histidine side groups. Exposure to light causes the FAD mimics to become more reactive, which is expressed in a shift of the absorption maximum to shorter wavelengths and lower intensity. The absorption spectra of riboflavin, lumiflavin, and flavin mononuleotide are shown in Figures 4.13, 4.14 and 4.15, respectively. Riboflavin, for example, exhibits two maxima (376 and 454 nm) which are almost at the same wavelength as those of FAD. The peaks shift to 358 and 449 nm upon light exposure. For lumiflavin and flavin mononuleotide the wavelengths of the absorbance maxima decreased upon exposure time. The possible reason of additional maxima might be found in the reduction of the flavins by irradiation.

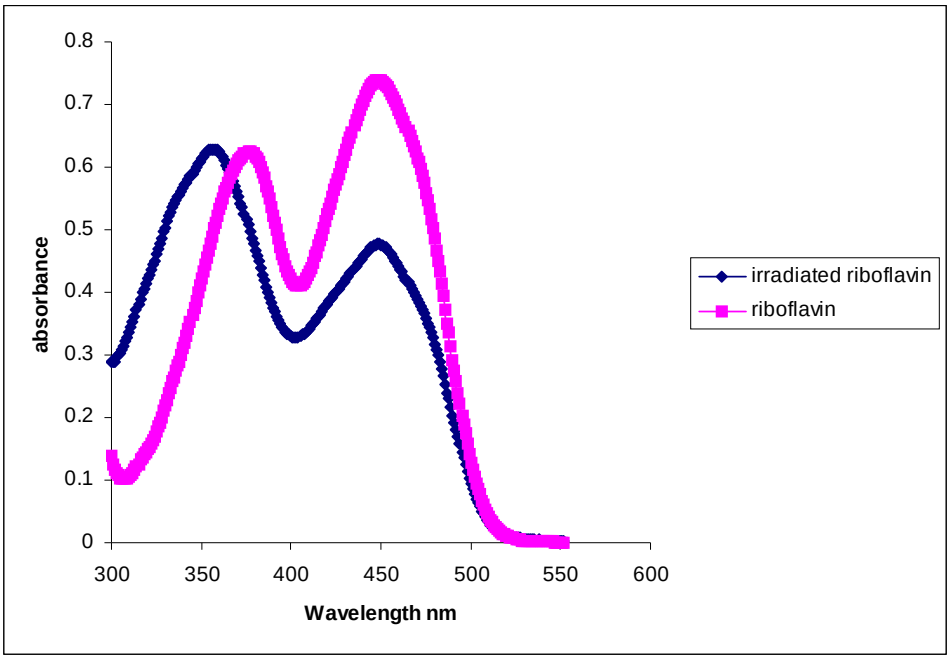

Figure 4.13 Absorption spectra of RF before and after irradiation

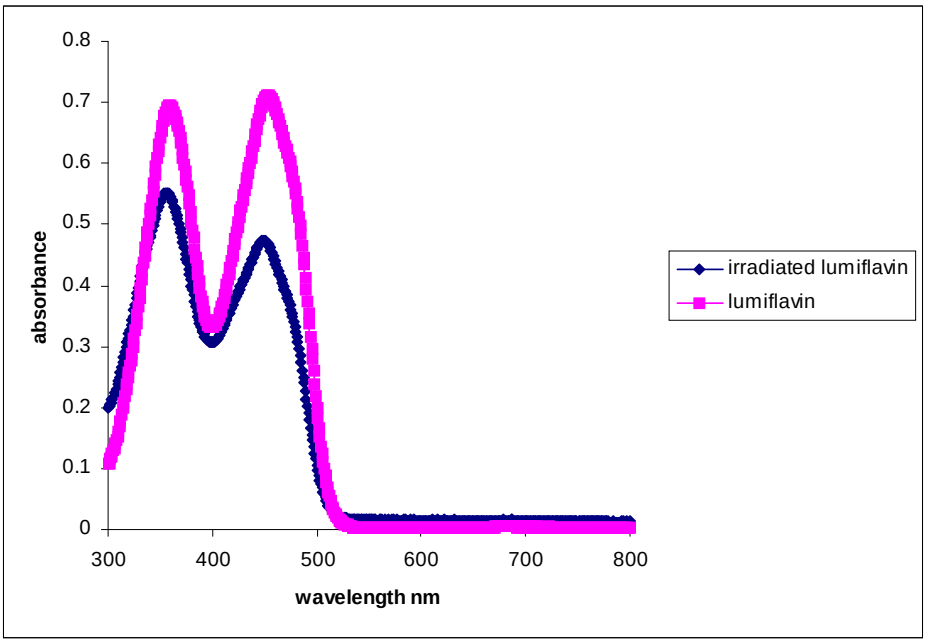

Figure 4.14 Absorption spectra of LF before and after irradiation

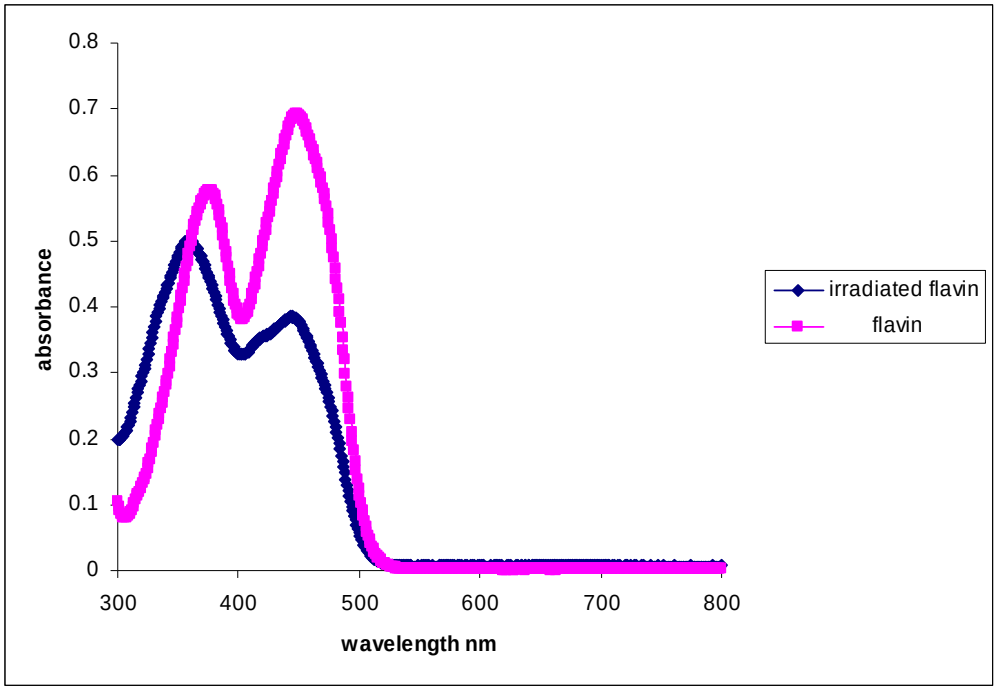

Figure 4.15 Absorption spectra of FMN before and after irradiation

4.3 Mimics for peroxidases

Some peroxidases contain ferriprotoporphrin IX as their prosthetic group. An example is horseradish peroxidase. Its prosthetic group consist of hemin, ferriprotoporphyrin IX (iron III). There are a large variety of compounds based on metalloporphyrins available with different types of metal ions, some of which are found in natural dyes, chlorophyll, blood, etc. However, only few of these compounds are suitable as mimics of peroxidases due to their low solubility or their inherent color. Possible mimics include porphyrin iron, hemin, and substituted phthalocyanines.

Hemin was selected as potential mimic for the catalytic behavior of peroxidases in this research. The conditions of producing hydrogen peroxide were same as used for the

bleaching process for cotton fabric with the glucose oxidase concentration at 10 U/mL at high temperature and pH 10.5. Three different process variations were investigated: hemin was added to the GO-containing solution; linen fabric was pretreated with hemin before high temperature bleaching and after GO production of hydrogen peroxide; hemin was added during the high temperature bleaching process. The concentration of hemin in all process variations was 0.001 g/ml. The results of these approaches are listed in Table 4.4. Compared with the whiteness of cotton fabric (DL* = 4.44) bleached with hydrogen peroxide produced by GO the addition of hemin decreased the whiteness of the fabric, especially when hemin was added to the GO solution (DL* = -0.33). It is possible that the yellowish color of hemin interfered with fabric whiteness as well as reduced the amount of available bleaching agent.

Table 4.3 Whitening effect of hemin and Mn phthalocyanime

Mimics	Whiteness DL*
Hemin	
GO + hemin, bleaching	-0.33
GO, hemin, bleaching	0.31
GO, bleaching (hemin)	1.42
Mn phthalocyanine	
Phthalocyanine, GO, bleaching	-2.19

As another potential mimic, phthalocyanine was selected for bleaching of cotton fabric. Fabric was pretreated with 0.01 g/ml Mn phthalocyanime at pH 4.5, 25^{o}C for 24 h.

However, the experiment failed (DL* = -2.19). Mn phthalocyanine is blue and has some affinity for fibers, thus functioned as a dye rather than as a bleaching agent. Other phthalocyanine based compounds showed similar problems in addition to solubility issues.

4.4 Use of laccase, MnP, LiP and GO for bleaching of linen fabric

4.4.1 Laccase and GO combinations for bleaching linen fabric with low lignin content

As mentioned before, excellent results for bleaching could be achieved with GO. The whiteness of the cotton fabric was comparable to that achieved with conventional chemical agents [2, 75]. The combination of GO and peroxidases was explored to further improve the whiteness level. On cotton peroxidases without GO, however, performed poorly, and the combination of GO and peroxidases did not yield the desired enhancement.

Peroxidases have successfully been applied in delignification processes for wood pulp. The presence of lignin appears to have a great influence on the whitening process. Thus, scoured unbleached linen fabric containing fair amounts of lignin was used as the substrate for experiments with peroxidases to replace cotton. Laccase was then studied in more detail due to availability and overall performance. Two types of linen fabric were used for these experiments, one of which was a light-weight plain-weave fabric with low lignin content obtained from China, and the other a heavy-weight coarse fabric with high amounts of lignin from the Czech Republic. The following section will focus on linen containing a small amount of lignin.

The experimental conditions for the treatment of fabric with laccase were based on the influence of pH and temperature on laccase activity. The optimum temperature for the treatment was 50^{o}C and the pH 6.0 as suggested by the manufacturer. The concentration of laccase ranged from 1 to 20 U/g fabric. Two kinds of enzymatic processes were applied in order to study the influence of laccase on the whiteness of fabric: in one process only laccase in different concentrations was used to treat the fabric. The second process consisted of three steps. First, fabric was treated with different concentration of laccase. Second, hydrogen peroxide was produced by reaction of GO (10 U/mL) and glucose. Third, hydrogen peroxide generated in the previous step was used to bleach the fabric.

The relationship between laccase concentration and whiteness of fabric is shown in Figure 4.16. The treatment of fabric with laccase alone could slightly improve the whiteness of fabric but not sufficiently, and the concentration of laccase did not have much influence on fabric whiteness. For the fabric treated with the combination of laccase and GO, however, the whiteness of fabric increased with the increase of laccase concentration and reached maximum at 10 U/g. Overall, the treatment of fabric with laccase might remove some of the lignin present which in turn improves the whiteness of GO bleached fabric.

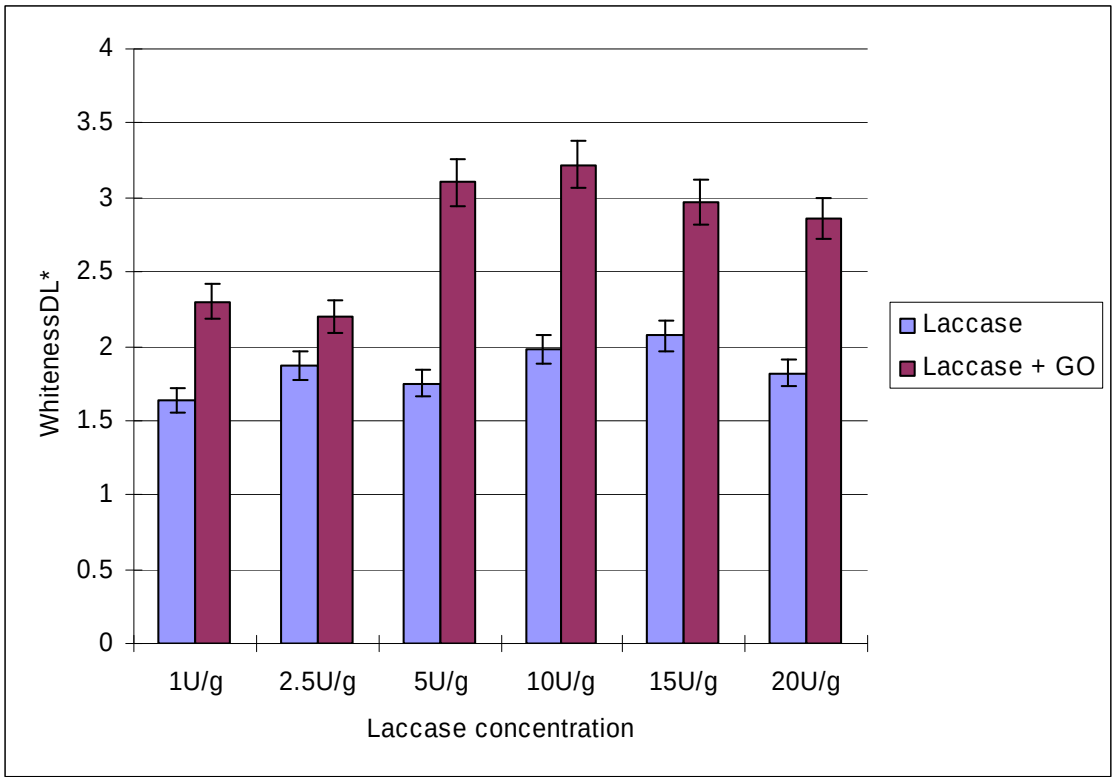

Figure 4.16 Relationship between laccase concentration and whiteness increase DL*

The optimum conditions for producing hydrogen peroxide by glucose oxidase were 35ºC and pH 5.1 with concentrations of GO between 2 and 20 U/mL. The influence of glucose oxidase concentration on the whiteness of fabric is shown in Figure 4.17. The whiteness of fabric increased with increase in GO dosage for both fabrics treated with GO and fabric pre-treated with laccase. At a GO concentration of 20 U/mL the whiteness of fabric was very close to that of chemically bleached fabric. Higher concentrations of GO produced higher concentrations of hydrogen peroxide which resulted in a higher whiteness of the fabric.

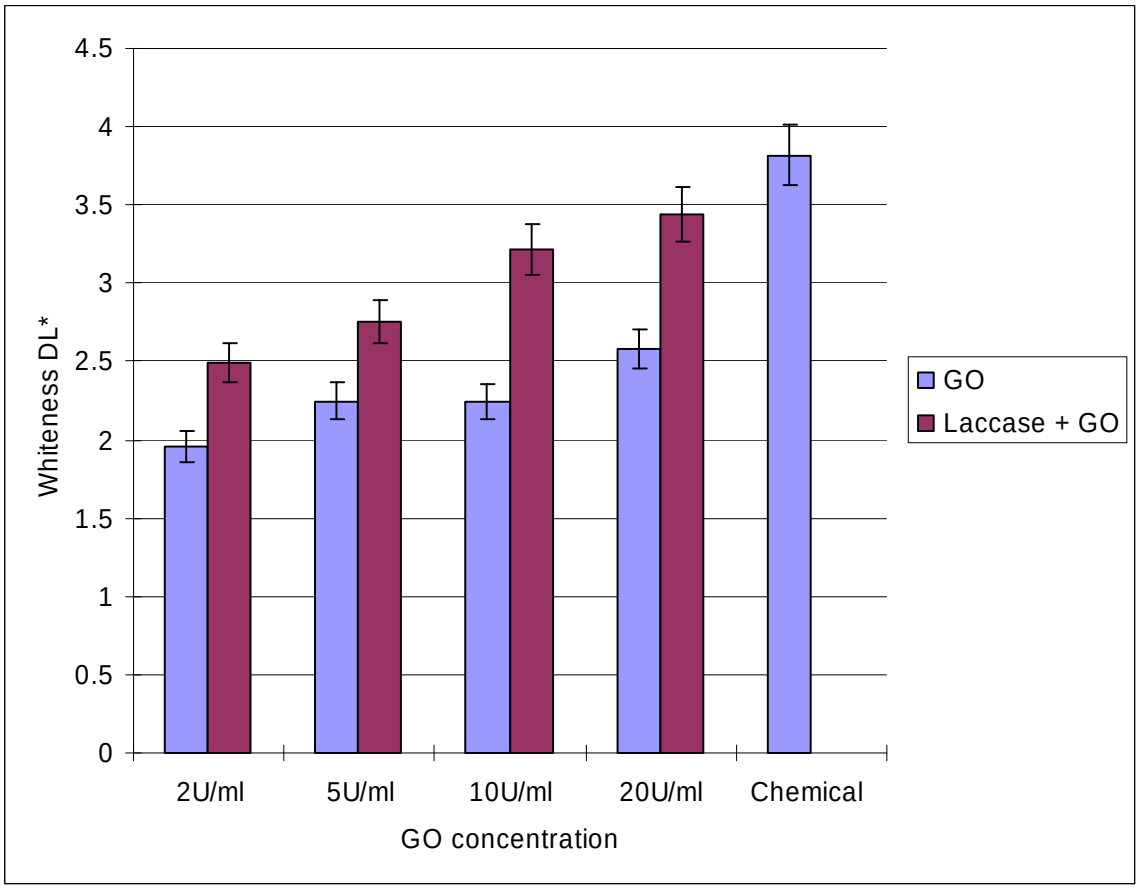

Figure 4.17 Relationship between GO concentration and whiteness increase DL*

Lignin in linen fabric might play an important role in enzymatic whitening processes. Lignin is the most complex natural polymer. The exact structure of lignin is still unknown. Model structures have been elucidated by the degradation products from lignin. As an example, Figure 4.18 shows a structure model for softwood lignin [77] which is thought to be closely related to the structure of lignin in the flax fiber. The chemical structure of lignin may affect the pulping and bleaching process of flax fibers.

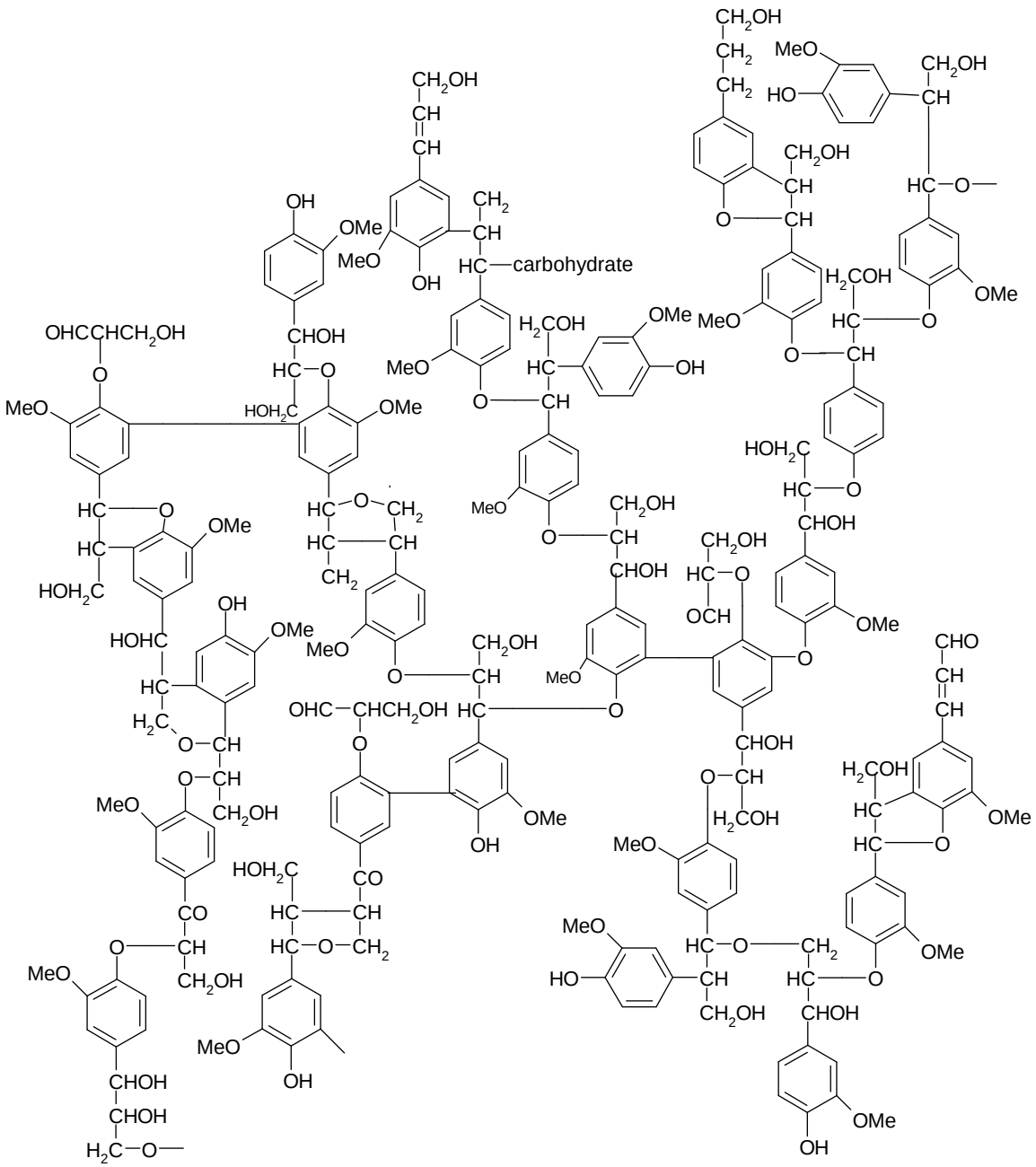

Figure 4.18 Chemical structure model for lignin (softwood) [77]

The whiteness increase (DL*) of the light-weight linen fabric was only 2.24 (see Table 4.3) after bleaching with hydrogen peroxide produced *in-situ* with GO, thus much lower than after chemical bleaching (3.82). Fabric containing a more substantial amount of lignin emphasized these results even more. An increase in whiteness of only 7.23 (GO process) as opposed to 19.18 for chemical bleaching was achieved with the coarse linen fabric. It is possible that the dense structure of this fabric obstructed or slowed the effective access of enzymatically produced hydrogen peroxide to incorporated natural pigments, and thus in conjunction with the higher lignin content, did not allow for better results.

Table 4.4 Increase in whiteness of enzyme-treated linen fabric compared to the scoured control

Samples	Whiteness increase DL*	
	Linen fabric (light-weight)	Linen fabric (heavy-weight)
Laccase	1.9 ± 0.1	-0.95 ± 0.05
GO	2.24 ± 0.11	7.23 ± 0.36
Laccase and GO	3.44 ± 0.17	11.37 ± 0.57
Chemical	3.82 ± 0.19	19.18 ± 0.96

Lignin, present in linen fabric, is a potential substrate for peroxidases, such as laccase. Thus, the fabrics were treated with laccase under optimum conditions for the enzyme (pH, temperature, etc.). The light-weight linen fabric, containing a small amount of lignin (4.4%, see Figure 4.19), showed an increase in fabric whiteness DL* of 1.9. The

coarser fabric from the Czech Republic could not be bleached by means of a laccase treatment alone. With lignin (7.8%; Figure 4.20) embedded in the walls of the ultimate cells of the fibers and between fiber bundles the coarse structure of the linen fabric might not have been open enough for the reaction with laccase. Common mediator systems for laccase [75] did not markedly influence the outcome of the laccase reaction.

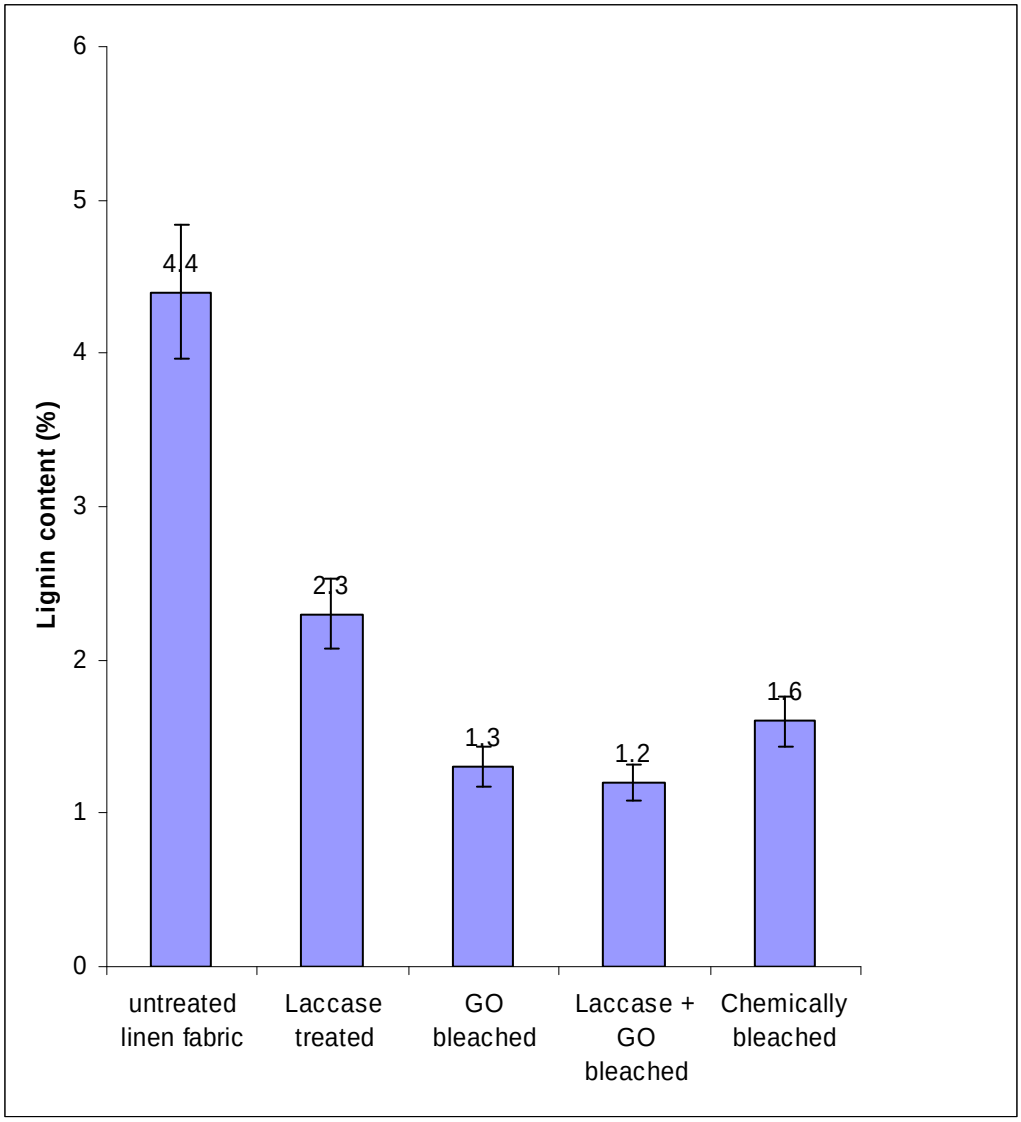

Figure 4.19 Lignin content (%) of enzyme-treated light-weight linen

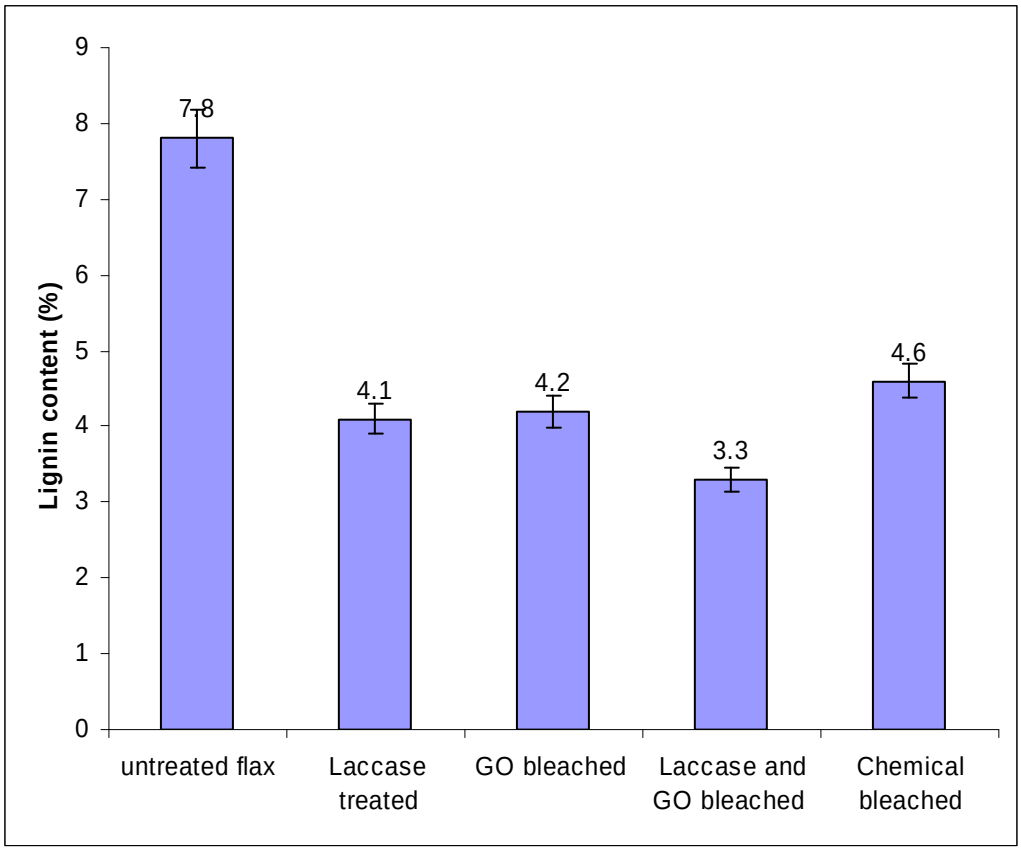

Figure 4.20 Lignin content (%) of enzyme-treated heavy-weight linen

A combined treatment using both enzymes showed much better results. Laccase and the flavoprotein GO seemed to work in a synergistic manner. The combination of a laccase pretreatment followed by peroxide production *in-situ* by GO could significantly improve the whiteness of the coarse fabric (DL* = 11.37), which equals approximately 60% of the whiteness achieved by a chemical bleach. It was thus much higher than the whiteness increase (7.23) gained by GO alone. For the light-weight fabric a whiteness increase of 90% was observed compared to the chemically bleached fabric (whiteness considered as 100%).

From the above presented analysis it appears that the whitening process is influenced by the presence of lignin, however fabric whiteness and lignin removal could not be directly correlated. In Figure 4.20 the lignin analysis of the light-weight linen fabric treated with different enzymes is presented. After treatment with laccase the lignin content dropped by 48% and with GO by 70%. Chemical bleaching caused a decrease of about 64% and combinations of different enzymes with GO treatments yielded a 73% reduction in lignin under the most favorable conditions. The whiteness index of the treated linen samples improved accordingly, though without direct correlation. It again became clear from these results that best conditions could be obtained by the combination of oxidoreductases.

In the case of the coarse linen fabric, after laccase treatment the lignin content decreased by 56% and the GO treatment led to a 55% decrease (Figure 4.21). Chemical bleaching caused a decrease of about 51% and combinations of laccase with glucose oxidase treatments yielded a 65% reduction in lignin under the optimum conditions.

Laccase is an important enzyme in lignin degradation processes and potential reactions with lignin functional groups are numerous due to lignin's complex structure [78-81]. For example, phenolic subunits in lignin could react with laccase via a one-electron oxidation, followed by further enzymatic or non-enzymatic reactions of the formed radicals (Figure 4.21).

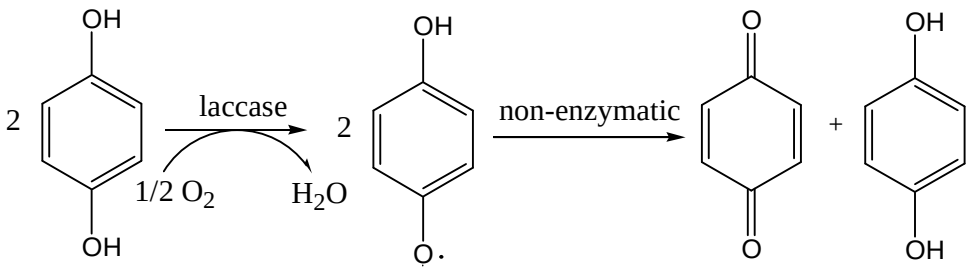

Figure 4.21 One-electron oxidation mechanism performed by laccase and subsequent non-enzymatic reactions

Both phenolic and non-phenolic lignin groups can be subject to degradation by peroxide oxidation [82]. The hydroxyl groups in phenols and/or in α - carbons are oxidized to the corresponding aldehyde or ketone functional groups (Figure 4.22). Applied to the linen fabrics used in this research, some oxidized lignin residues might have become water-soluble, leading to the observed decrease in lignin content, and allowing for access to embedded natural pigments by glucose oxidase produced hydrogen peroxide. As a consequence the fabric whiteness is increased.

Alkaline hydrogen peroxide can also result in oxidation of lignin components. Lignin is degraded by nucleophilic attack of hydroxyl radicals generated from hydrogen peroxide on the side-chain of the aromatic ring followed by ring-opening reactions. Low molecular weight products [82] possibly obtained from the oxidation of lignin by hydrogen peroxide at high temperature are shown in Figure 4.23. Most of them are aromatic and aliphatic carboxylic acid compounds. If many carboxylic acid groups are obtained, the hydrophilicity of lignin will be enhanced which facilitates the dissolution of lignin. The removal of oxidized and water-soluble residues of lignin leads to the decrease of lignin content in linen fibers and thus concomitantly to a whiteness increase.

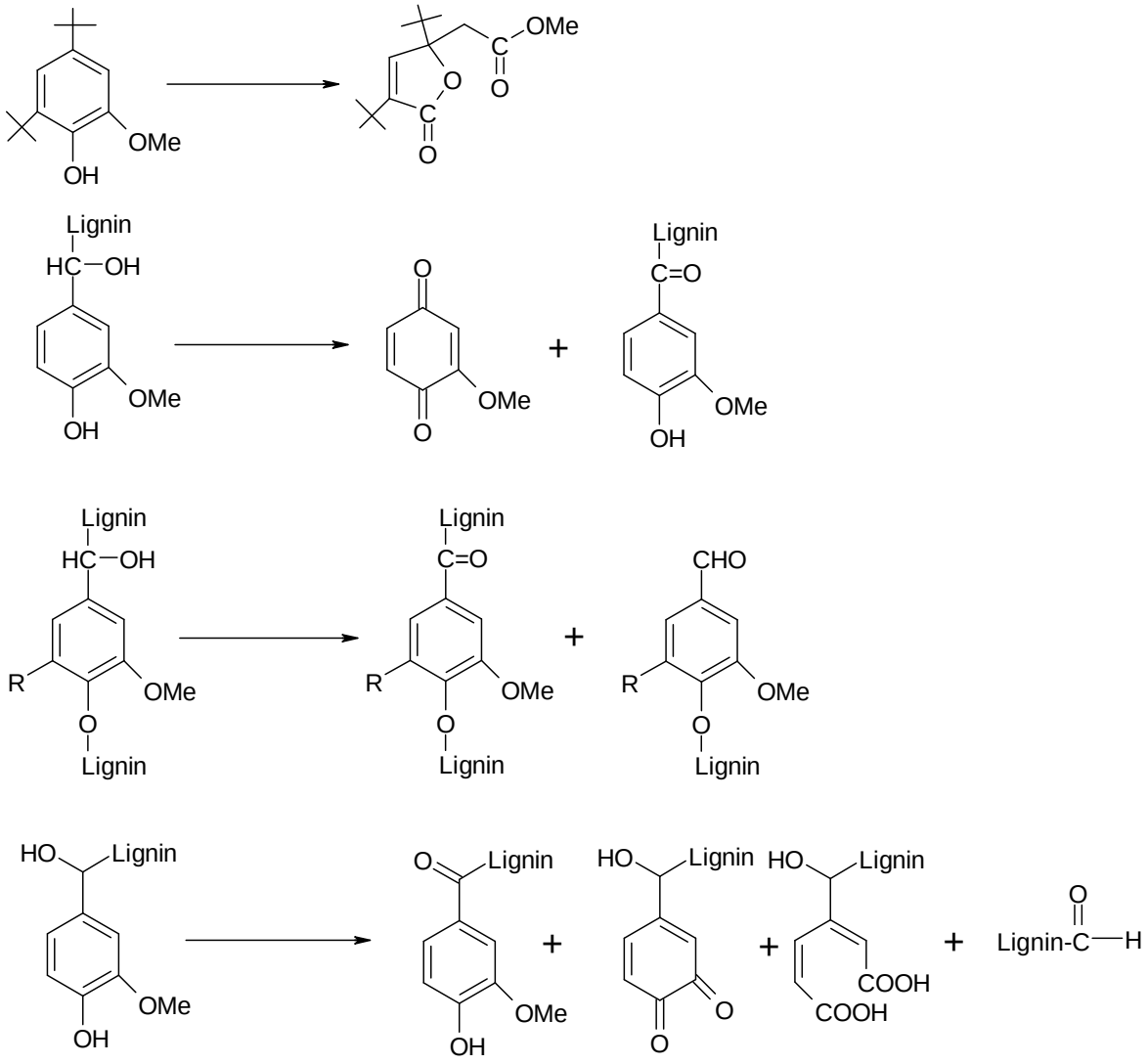

Figure 4.22 Examples of possible reactions of lignin with laccase [78-81]

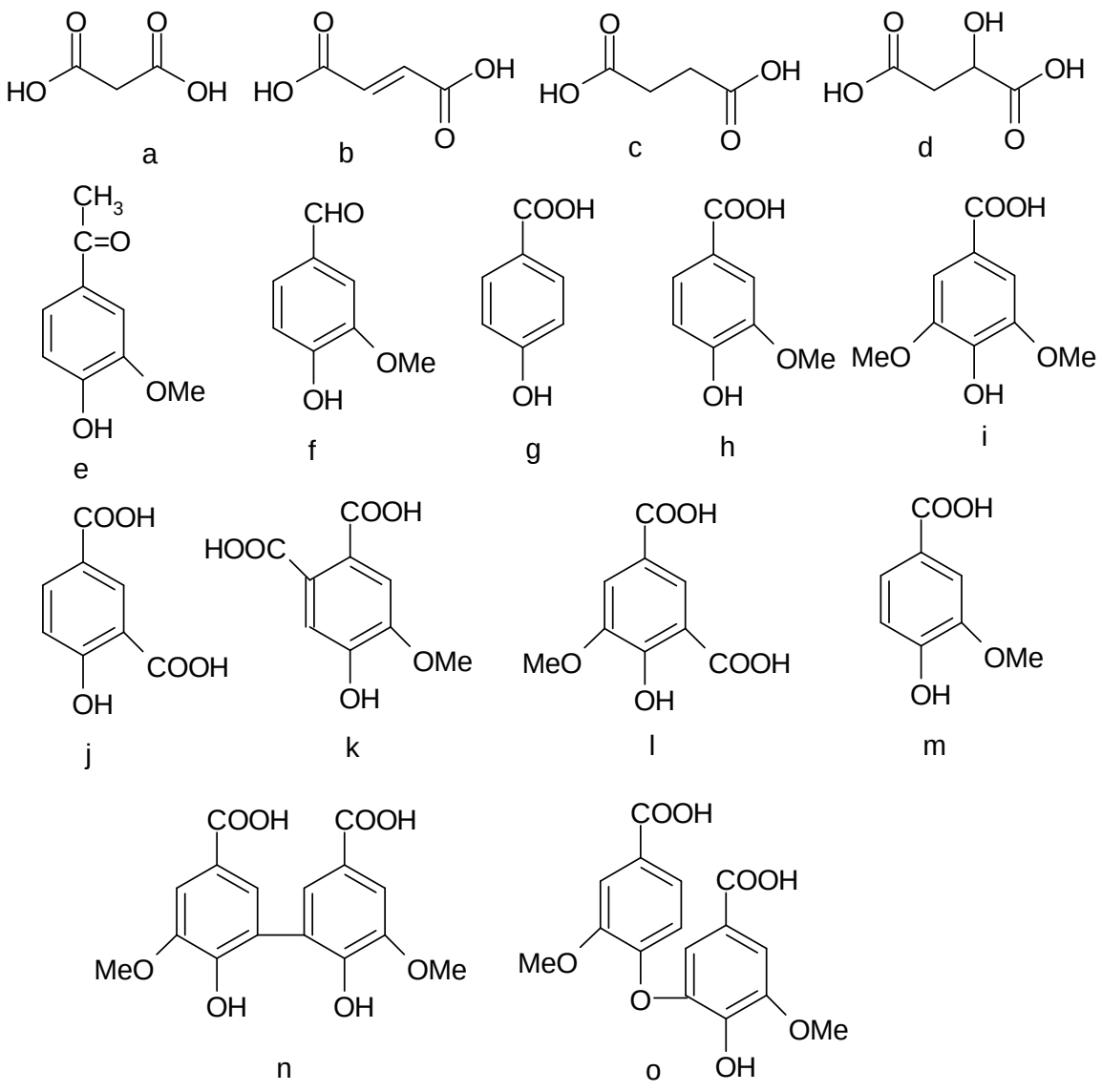

Figure 4.23 Potential low molecular weight products of lignin obtained by oxidation with hydrogen peroxide [73]

4.4.2 Application of MnP, LiP and GO for bleaching linen with high lignin content

GO has shown to perform synergistic reactions with peroxidases, especially with laccase, when used on linen (see previous sections), while peroxidases alone did not have a sufficiently pronounced effect. Peroxidases such as LiP and MnP are lignin degrading enzymes. While MnP is able to oxidize phenolic lignin moiety but not nonphenolic lignin units, LnP is capable of oxidizing of nonphenolic aromatic compounds.

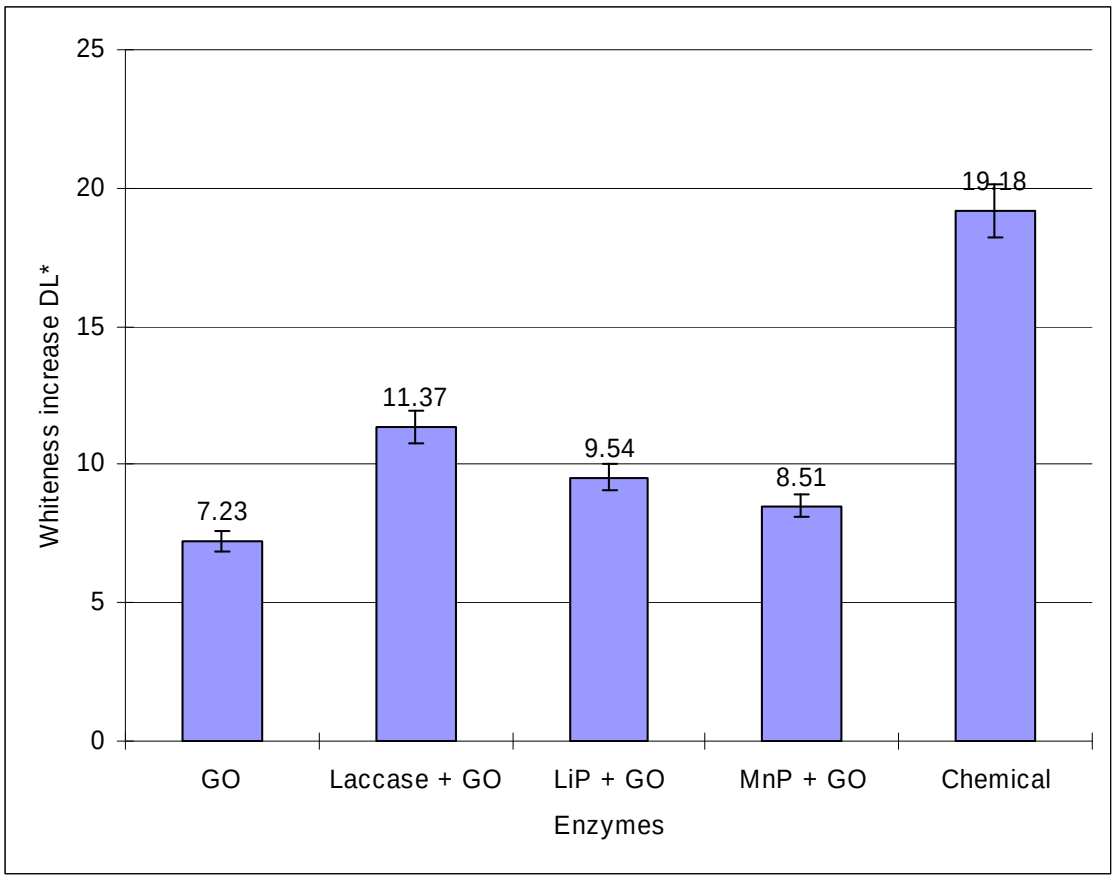

Figure 4.24 Whiteness increase of coarse linen fabric treated with peroxidases and GO.

In a set of experiments it was investigated whether fabric with higher lignin content could be bleached with LiP or MnP alone to sufficient whiteness or whether a

combinational treatment might also be necessary. The results are shown in Figure 4.24. Compared to GO bleached fabric, laccase was more effective in regard to whitening the fabric than LiP or MnP. Whiteness increased by 57% (GO sample used as control), followed by whiteness levels observed after treatment with combinations of LiP and GO (32%), and MnP and GO (18%). Again, laccase seemed to be the most effective peroxidase in this aspect. However overall, levels of whiteness obtained by chemical means could not be achieved by any of the enzymatic methods.

4.5 Surface characterization of enzyme-treated linen fabric by IGC

4.5.1 Dispersive component of the surface free energy (linen with low lignin content)

An attempt was made to characterize the modified surface of the fabrics after the oxidoreductase treatment, using IGC. Since cellulose is not a substrate for oxidoreductases all detected surface changes should monitor changes in accessible lignin. Experiments were performed for the light-weight linen samples only.

IGC data analysis involves measuring the retention times of the probes injected into the columns packed with ground linen fibers. The value of the net retention volume, V_n, was calculated by the following equation [83, 84, 85]:

$$V_n = JF(t_r - t_o) \qquad (1)$$

Where F is the carrier gas flow rate, J is a correction factor for any pressure drop across the column, t_r and t_o are the retention times of the probe and non-interacting probe such as methane determined by peak maximum for a symmetrical peak. V_n is defined as the amount of carrier gas required to elute the injected volume of probe molecules from the column and is a measure of the interaction between probe and materials in the column.

The net retention volume is related to the thermodynamic functions through equation (2):

$$\Delta G_a = -RT \ln V_n + C \qquad (2)$$

where ΔG_a is the free energy of adsorption, R is the ideal gas constant, T is the column temperature, and C is a constant. Probes used for these experiments and their properties are listed in Table 4.4.

Table 4.5 Characteristics of IGC probes used [85, 87]

Probe molecule	a $(Å)^2$	γ_L^d (mJ/ m^2)	AN	DN (kcal/mol)	Specific characteristic
C_5H_{12}	45.55	16.1	0	0	neutral
C_6H_{14}	51.5	18.4	0	0	neutral
C_7H_{16}	57.0	20.3	0	0	neutral
C_8H_{18}	62.8	21.3	0	0	neutral
C_9H_{20}	68.9	22.7	0	0	neutral
acetone	42.5	16.5	17	12.5	amphoteric
$CHCl_3$	45.0	25.9	23.1	0	acid
THF	45	19.2	8	20	base

The surface energy is the sum of the energies arising from many different sources including dispersive component from van der Waals and London forces, and acid/base component from Lewis acid/base interactions and hydrogen bonding. When neutral probes such as n-alkanes were used, the acid-base interaction between the probes and the fibers can be neglected. The free energy of adsorption can be written through the Fowkes'

approach [86]:

$$RT\ln(V_n) = 2N \cdot a \cdot \sqrt{\gamma_L^d \cdot \gamma_S^d} + C \qquad (3)$$

where N is Avogadro's number, a is the surface area of the adsorbed probe molecules, γ_L^d and γ_S^d are the dispersive components of the surface free energy of the probe and the solid, respectively, and C is a constant.

Plotting $RT\ln(V_n)$ against $a(\gamma_L^d)^{0.5}$ for the adsorption of n-alkanes yielded a straight line, the value of γ_S^d can be obtained from the slope of the straight line (see Figure 4.25).

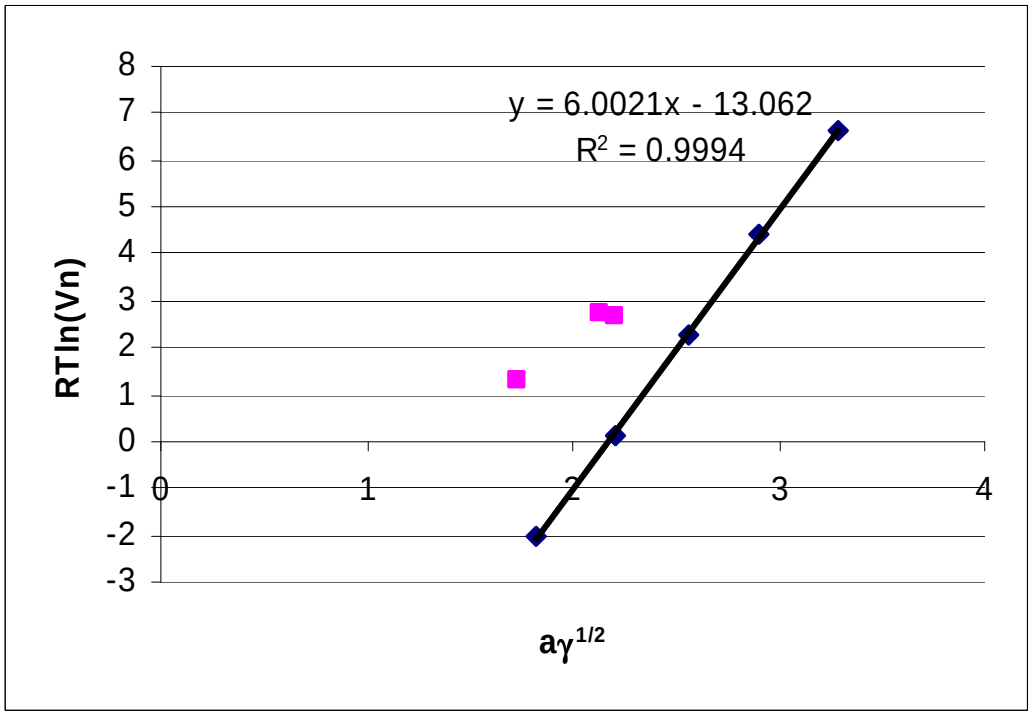

Figure 4.25 Example of IGC data used for the determination of γ_S^d and ΔG_a for linen fibers treated with laccase and GO at 70°C

The values of γ_S^d at four different temperatures are presented in Table 4.5. Plots of γ_S^d as a function of temperature for the substrates are shown in Figure 4.26. The

dispersive component of the surface free energy of all substrates decreased with increase of temperature from 40 to 70°C. This means all the substrates showed a negative temperature coefficient in this temperature range and the dispersive component of the energy of adsorption was exothermic. At lower temperatures (40°C and 50°C), the γ_S^d values of untreated linen fiber were a little bit higher than those of enzyme and chemically treated linen. There was no big difference among γ_S^d values of unbleached fiber and enzyme-treated fibers. The greatest difference was only 3.27 mJ/m^2. The average values of γ_S^d for all substrates were very close except for the industrially bleached linen sample with a slightly lower value. This indicates that the interactions (London forces) between substrates and nonpolar probes are almost same. The values of γ_S^d agree with data found in literature. The enzyme treatment might only touch the small molecular components and lignin on the surface of substrates while the major cellulose was untouched.

Table 4.6 Disperse components of surface free energies γ_S^d (mJ/ m^2) of linen fibers at different temperatures (R^2 =1)

T (°C)	Unbleached control	Lac.	GO	Lac. + GO	Chemical	Industry
40	47.41	44.84	45.22	46.43	44.39	41.77
50	43.75	40.48	42.37	43.35	40.52	40.07
60	38.04	36.66	40.10	43.11	38.22	34.79
70	35.20	36.03	36.77	36.02	35.45	31.94
Ave.	41.1	39.50	41.12	42.23	39.65	37.14

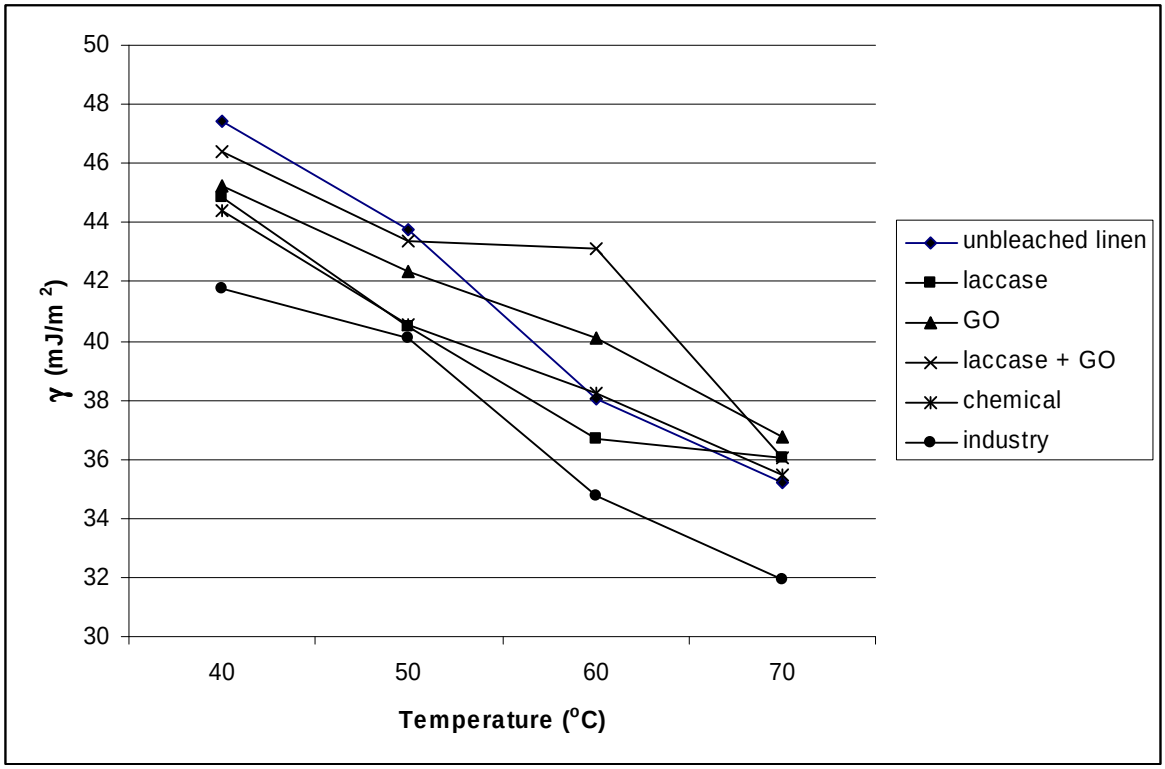

Figure 4.26 Values of surface free energies γ_S^d at different temperatures

4.5.2 Free energy of adsorption, free enthalpy and entropy of adsorption (linen with low lignin content)

If polar probes are used in IGC experiments, the interaction of polar probes with the substrate includes both dispersive interactions and acid-base interactions. In the plot of $RT\ln(V_n)$ versus $a(\gamma_L^d)^{0.5}$, the acidic, amphoteric, and basic probes will deviate from the straight line. Polar probes are above the line formed by the n-alkane probes. The contribution of the acid-base interaction to the free energy of adsorption can be determined by the difference between the value of $RT\ln(V_n)$ for a polar probe and the value of $RT\ln(V_n)$ on the reference line for non-polar probes with the same $a(\gamma_L^d)^{0.5}$

value. The free energy of adsorption, ΔG_a^{AB} corresponding to the acid/base interactions, is given by the equation [88]:

$$\Delta G_a^{AB} = -RT \ln(\frac{V_n}{V_n^{ref}}) \qquad (4)$$

where V_n is the retention volume of the polar probe and V_n^{ref} of a corresponding non-polar reference probe [89]. Values of the free energy of adsorption corresponding to surface acid-base interactions, ΔG_a^{AB}, are summarized in Table 4.6.

The free enthalpy of adsorption ΔH_a^{AB} corresponding to the specific acid/base interactions can be determined by studying the temperature dependence of ΔG_a^{AB} according to the following equation:

$$\Delta G_a^{AB} = \Delta H_a^{AB} - T\Delta S_a^{AB} \qquad (5)$$

where ΔS_a^{AB} is the free entropy of adsorption corresponding to specific acid-base interactions. The ΔH_a^{AB} value is determined as the slope of the plot of $\Delta G_a^{AB}/T$ versus $1/T$ (see Figure 4.27). The corresponding values of enthalpy of adsorption, ΔH_a^{AB} and entropy of adsorption, ΔS_a^{AB} are presented in Tables 4.7 and 4.8, respectively.

By contrast, the values of ΔH_a^{AB} for enzyme and chemical bleached linen fibers were lower than those for corresponding unbleached control. Similar results were observed with values for ΔS_a^{AB}. It could be interpreted as the treated linen fibers having weaker acid and basic properties than the unbleached fiber. This can be confirmed by comparing of K_a and K_b. The values of ΔH_a^{AB} for acetone, THF, and chloroform are very close. Thus, the fiber samples used in this study were amphoteric.

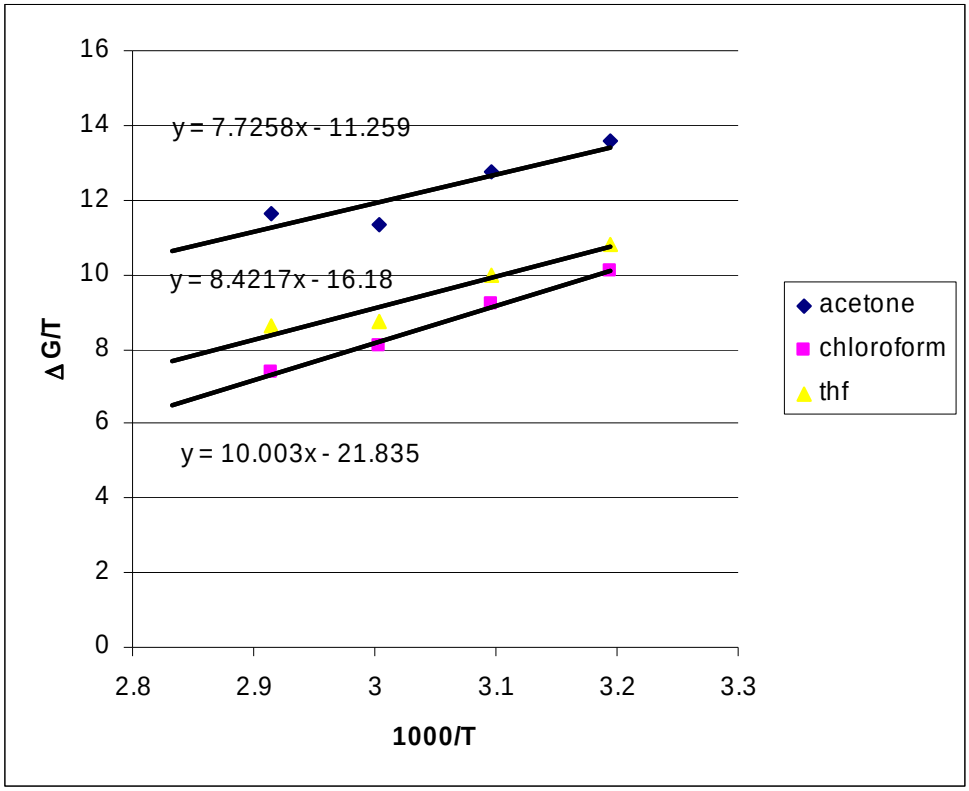

Figure 4.27 Example of IGC data interpretation for the determination of ΔH_a^{AB} for linen fibers treated with laccase and GO

Table 4.7 Free energies of adsorption ΔG^{AB} (KJ/mol)

Substrate/probe	- ΔG^{AB} (KJ/mol)			
	40°C	50°C	60°C	70°C
Scoured unbleached				
Acetone	5.70	5.88	5.53	5.23
Chloroform	3.14	2.71	2.71	2.43
THF	3.65	3.61	3.35	3.06
Laccase treated				
Acetone	4.24	4.12	3.78	4.00
Chloroform	3.16	2.98	2.70	2.53
THF	3.37	3.23	2.91	2.95
GO bleached				
Acetone	4.40	4.54	4.64	4.01
Chloroform	3.05	2.96	2.84	2.44
THF	3.28	3.23	3.29	2.93
Laccase + GO bleached				
Acetone	4.10	4.30	4.40	3.67
Chloroform	2.88	2.80	2.79	2.24
THF	3.15	3.15	3.22	2.73
Chemically bleached				
Acetone	5.35	5.48	5.39	5.20
Chloroform	2.73	2.55	2.50	2.27
THF	3.46	3.36	3.27	3.14

Industrially bleached				
Acetone	3.38	3.41	3.18	2.99
Chloroform	1.19	1.22	0.95	0.77
THF	2.28	2.29	2.09	1.95

Table 4.8 Free enthalpies of adsorption of linen fibers ΔH^{AB} (KJ/mol)

Probe	- ΔH^{AB} (KJ/mol)					
	unbleached	Laccase	GO	Lac + GO	Chemically bleached	Industrially bleached
Acetone	11.17	7.73	7.61	7.64	7.04	7.74
Chloroform	10.82	8.42	6.39	6.80	6.75	6.08
THF	9.92	10.00	9.11	8.82	7.17	5.98

Table 4.9 Free entropies of adsorption of linen fibers ΔS^{AB} (KJ/mol)

Probe	- ΔS^{AB} (J/mol)					
	unbleached	Laccase	GO	Lac. + GO	Chemically bleached	Industrially bleached
Acetone	17.03	11.26	9.80	10.75	5.15	13.70
Chloroform	19.82	16.18	9.75	11.38	10.50	11.97
THF	24.36	21.84	19.16	18.71	14.21	15.08

4.5.3 Acid-base characteristics of surfaces of linen fibers with low lignin content

The free enthalpy of adsorption ΔH_a^{AB} is related to the electron acceptor (acidity) and donor (basicity) constants of the solid materials, K_a and K_b. The values of K_a and K_b can be obtained by the following expression [86, 90]:

$$\Delta H_a^{AB} = K_a \cdot DN + K_b \cdot AN \qquad (6)$$

where DN (kcal/mol) and AN (kcal/mol) are the donor and acceptor numbers of the acid and base probes as defined by Gutmann [87].

Gutmann developed a new approach to the acid-base concept. He studied acid-base self interactions which lead to the characterization of the strength of both acid and base functionalities. According to Gutmann, a Lewis acid is considered as an electron acceptor and a Lewis base as an electron donor. Antimony pentachloride ($SbCl_5$), a strong Lewis acid was used as a reference acceptor. The basic donor strength was determined by calorimetric heats of acid-base interactions of basic compounds with $SbCl_5$. A neutral solvent 1, 2-dichloroethane was used in the measurement. The donor number (DN) was defined as the negative of the enthalpy of reaction between the basic compound investigated and the reference acid $SbCl_5$.

$$DN_{base} = -\Delta H_{SbCl_5 - base}$$

The acceptor number (AN) was defined by the magnitude of induced chemical shift of ^{31}P NMR spectra of the base triethylphosphine oxide (Et_3PO) while dissolved in the acid under investigation. A scale was provided by assigning zero to the shift in n-hexane and 100 to the shift generated by Et_3PO in the solution of 1, 2-dichloroethane with the reference acid. The acceptor number is determined according to

$$AN_{base} = 100(\delta_{corr})\delta_{corr}(SbCl_5 - Et_3PO)$$

where δ_{corr} is the ^{31}P NMR chemical shift of Et_3PO at infinite dilution in solvent S.

Plotting of $\Delta H_a^{AB} / AN$ versus DN/AN, a straight line can be obtained with slope K_a and intercept K_b (Figure 4.28). Table 4.9 lists the K_a and K_b values for the linen samples. After the enzyme treatment as well as after the chemical bleaching process K_a and K_b values decreased. This is consistent with the overall decrease of lignin content. However, it was surprising to find that the glucose oxidase treatment by itself yielded a larger drop in K_a and K_b than the laccase or the combined enzyme treatment, especially regarding surface acidity, while lignin content was lowest and whiteness highest for the combined treatment.

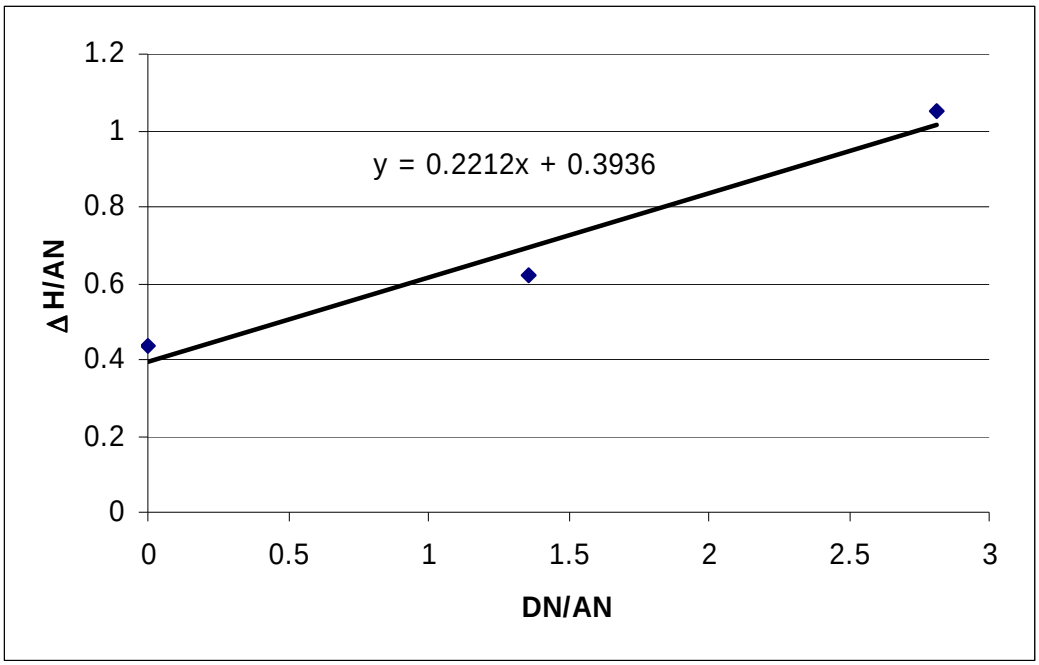

Figure 4.28 Plot of $\Delta H_a^{AB} / AN$ against *DN/AN* for determination of electron acceptor constant K_a and electron donor constant K_b of laccase and GO treated linen fibers

Table 4.10 Acid-base constants, K_a and K_b of linen fibers

Substrate	Lignin content (%)	K_a	K_b	R^2
Scoured control	4.4	0.2739	0.4863	0.9939
Laccase treated	2.3	0.2212	0.3936	0.9567
GO bleached	1.3	0.1435	0.4009	0.997
Laccase + GO bleached	1.2	0.1663	0.3828	0.9999
Chemically bleached	1.6	0.1896	0.3088	0.9999
Industrially bleached	--	0.1772	0.2994	0.9306

Glucose oxidase-produced hydrogen peroxide seemed to more effectively decompose hydroxyl-group containing compounds, lowering the overall acidity of the surface. Some of these compounds might have been components without color and different from pigments or lignin.

Laccase and alkaline hydrogen peroxide treatments, on the other hand, can lead to the oxidization of lignin components into compounds with carbonyl groups instead of hydroxyl groups, thus reducing surface basicity in a more pronounced manner. As mentioned above, the structure of lignin is very complex and contains phenolic and aliphatic hydroxyl groups as well as ether-oxygen groups which also add to surface basicity. In any case compounds that are more easily dissolved in water have been formed as the decreased lignin content after all treatments attests for.

Chemical bleaching using alkaline conditions and commercial hydrogen peroxide considerably lowered the surface basicity of the samples. No major difference was found

between samples bleached in the industry and samples bleached in the laboratory under industrial conditions. Although whiter than all enzymatically treated samples, the lignin content left in the fabric was slightly higher after the chemical bleach.

4.5.4 Surface Characterization of enzyme-treated coarse linen fabric with high lignin content

4.5.4.1 Dispersive component of the surface free energy

The values of γ_S^d at four different temperatures are listed in Table 4.10. Plots of γ_S^d as a function of temperature for the substrates are shown in Figure 4.29. The dispersive component of the surface free energy of all substrates decreased slightly with increase in the temperature except industrially treated fabric at 70°C. The decrease of γ_S^d is due to the entropic contribution to the surface free energy. There was no big difference among γ_S^d values of untreated and enzyme-treated fibers. The greatest margin was 1.75 mJ/ m^2.

Table 4.11 Disperse components of surface free energies γ_S^D (mJ/ m^2) of linen fibers at different temperatures (R^2 =1)

T (°C)	untreated	Laccase	Lac. + GO	Chemical
40	48.12	49.23	47.48	48.01
50	44.16	45.37	45.09	44.42
60	41.76	42.16	42.19	42.21
70	39.62	40.48	40.19	44.44
Ave.	43.42	44.31	43.74	44.77

The average value of γ_S^d (43.42 mJ/ m^2) for untreated fibers was very close to that of enzymatically treated and chemically bleached linen. The values of γ_S^d of the enzyme treated coarse linen fabric were higher that those of the unbleached control.

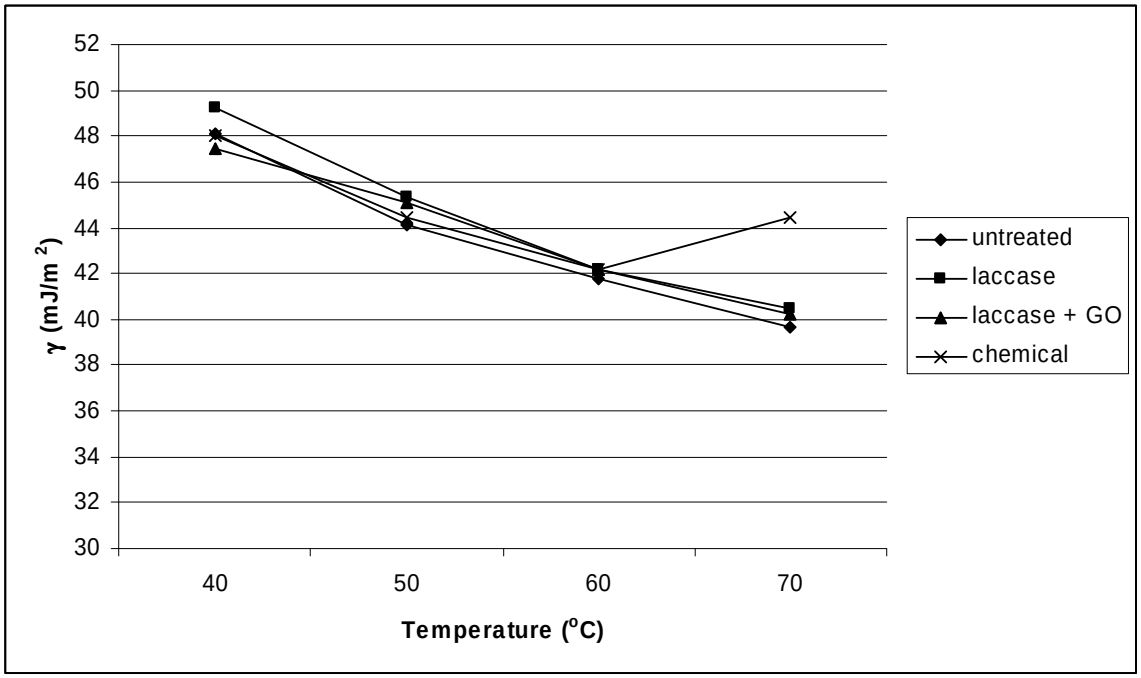

Figure 4.29 Surface free energies γ_S^d of treated linen samples at different temperatures

4.5.4.2 Free energy of adsorption, free enthalpy and entropy of adsorption

Values of the free energy of adsorption corresponding to surface acid-base interactions, ΔG_a^{AB}, are summarized in Table 4.11. Compared with the untreated control the ΔG_a^{AB} values of laccase treated fibers for acetone, chloroform, and THF were higher. For the fiber treated with laccase and GO the ΔG_a^{AB} values for chloroform and THF increased while ΔG_a^{AB} for acetone decrease. Chemical bleach caused a decrease in ΔG_a^{AB} for all three probes.

The ΔH_a^{AB} value is determined as the slope of the plot of $\Delta G_a^{AB}/T$ versus $1/T$. The corresponding values of the enthalpy of adsorption, ΔH_a^{AB}, and the entropy of adsorption ΔS_a^{AB}, are presented in Tables 4.12 and 4.13, respectively.

The values of ΔH_a^{AB} for laccase and chemically bleached linen fibers were lower than those for untreated control for acid, base, and amphoteric probes. The values of ΔS_a^{AB} showed a similar trend. These results indicate that laccase and chemical treated fibers had weaker acid and basic properties than the untreated control. The values of ΔH_a^{AB} and ΔS_a^{AB} of laccase and GO bleached fibers depicted a different trend. For acetone and chloroform both ΔH_a^{AB} and ΔS_a^{AB} values increased but for THF the two values decreased. Thus all linen fibers investigated were amphoteric.

Table 4.12 Free energies of adsorption ΔG^{AB} (KJ/mol)

Substrate/probe	ΔG^{AB} (KJ/mol)			
	40°C	50°C	60°C	70°C
Untreated control				
Acetone	7.81	7.75	7.98	7.71
Chloroform	3.33	3.19	3.10	2.80
THF	4.10	4.01	4.01	3.76
Laccase				
Acetone	7.93	8.11	8.22	8.32
Chloroform	3.57	3.47	3.35	3.24
THF	4.49	4.43	4.30	4.29
Lac. + GO bleached				
Acetone	7.22	7.26	7.07	6.67
Chloroform	3.60	3.52	3.48	3.21
THF	4.43	4.34	4.32	4.06
Chemically bleached				
Acetone	6.61	6.60	6.68	6.82
Chloroform	3.05	2.95	2.84	2.66
THF	3.75	3.67	3.67	3.52

Table 4.13 Free enthalpies of adsorption ΔH^{AB} of linen fibers

Probe	ΔH (KJ/mol)			
	untreated	Laccase	Lac. + GO	Chemical
Acetone	8.02	3.96	12.88	4.43
Chloroform	7.17	6.77	8.00	5.93
THF	8.55	7.05	7.33	7.00

Table 4.14 Free enthalpies of adsorption ΔS^{AB} of linen fibers

Probe	-ΔS (KJ/mol)			
	Control	Laccase	Lac. + GO	Chemical
Acetone	0.62	-12.75	17.77	-6.85
Chloroform	9.76	7.29	11.3	6.93
THF	16.62	11.1	11.84	12.57

4.5.4.3 Acid-base characteristics of the linen surfaces

K_a and K_b values for samples are presented in Table 4.14. The K_a value of the laccase treated fibers was very close to that of the untreated fiber, while the K_b value decreased. The decrease in aliphatic hydroxyl groups and ethers might have led to the lower surface basicity. In the case of the chemically bleached fiber both K_a and K_b decreased. The oxidation of linen fibers by hydrogen peroxide in alkaline condition resulted in a decrease in lignin content which could have caused a lower amount of surface phenolic and aliphatic hydroxyl groups. K_a and K_b values of laccase and GO bleached linen fibers both increased after treatment which is difficult to interpret. In this

case the data strongly fluctuated (see R^2 value). It is possible that the surface groups showed some instability under the applied test conditions. The correct coefficient (R^2) for acidity and basicity is also not very good.

Table 4.15 Acid-base constants, K_a and K_b of coarse fibers

Substrate	Lignin content (%)	K_a	K_b	R^2
Control	7.8	0.1869	0.376	0.9987
Laccase	4.1	0.1943	0.2192	0.7826
Laccase + GO bleached	3.3	0.2398	0.4493	0.6994
Chemically bleached	4.6	0.1569	0.2478	0.8508

CHAPTER 5. CONCLUSIONS

Hydrogen peroxide produced by the reaction of glucose oxidase and glucose was successfully used for bleaching of scoured unbleached cotton fabric to get the desired whiteness. The active site of glucose oxidases contains FAD as cofactor which plays a key role in catalyzing the reactions. Compounds such as flavin mononucleotide (FMN), riboflavin, and lumiflavin having structures similar to FAD were used to mimic the active site of glucose oxidase. The amino acids adjacent to FAD were found being important in producing hydrogen peroxide in the biomimicking system. The combination of His and Lys was very effective to mimic the environment of FAD especially when the mole ratio of His and Lys was 2:1, the reason being that this ratio of His and Lys mirrors the vicinity of FAD in the intact glucose oxidase. Hydrogen peroxide concentration produced by this mimic system increased with the increase of amino acids dosage. The more effective electron donor semicarbazide was used in photochemical biomimicking process to produce hydrogen peroxide. The influence of pH and light sources with different energy output was explored. The higher pH value favored hydrogen peroxide production. FMN was the most effective mimic in whitening cotton fabric in combination with a halogen lamp as light source. The whiteness achieved with the mimics was about 60-70% of which could be reached with glucose oxidase under comparable conditions.

The combination of laccase, peroxidases and glucose oxidase for bleaching of cotton fabric was not very effective regarding fabric whiteness. However, the combination of laccase and glucose oxidase for bleaching of linen fabric with low lignin content was significantly more effective than either one of the enzymes applied alone. The whiteness increase of linen correlated to some degree with the decrease in lignin content. With the help of IGC it could be shown that there was no big difference among the dispersive component of the surface free energy γ_S^d of all substrates as a result of the enzyme treatments. This indicates that the enzymatic treatment only touch the small molecular components and lignin on the surface of linen while the major cellulose substrate was unaffected. The enzymatic treatment also reduced the acidity and basicity (K_a and K_b, respectively) of the fiber surfaces indicating a change in the amount and possibly type of lignin functional groups. The decrease of lignin content and oxidation of phenolic and aliphatic hydroxyl groups into carbonyl groups resulted in the decrease of K_a and K_b. Since enzymes do not affect cellulose the study of K_a and K_b may lead to a better understanding of the enzymatic treatment of linen fibers. The acid-base properties of fiber surfaces also play an important role in all wet processing steps due to surface Lewis acid-base interactions between solids (fiber) and any type of encountered liquids. However, a direct correlation between whiteness, lignin content and surface energies was difficult to establish.

When the same conditions were applied to bleach the coarse linen fabric with high lignin content the combination of laccase and glucose oxidase was also significantly more effective than either one of the enzymes applied alone. Laccase, manganese and lignin peroxidases alone did not improve the whiteness of the coarse linen fabric. However, if

used together with glucose oxidase the whiteness of linen fabric improved considerably. Laccase showed a more pronounced whitening effect than other peroxidases. IGC was also used to analyze the surface properties of these laccase and glucose oxidase treated linen samples. The values of γ_S^d of enzyme treated coarse linen fabric were higher that those of the unbleached linen fabric. The K_a value of laccase treated fiber was very close to that of control. However the K_b value decreased. The K_a and K_b values of laccase and GO bleached fiber increased after the treatment.

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