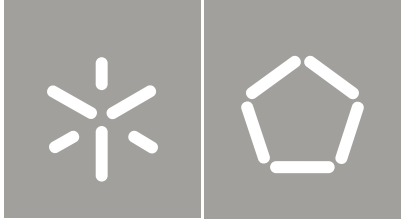
Universidade do Minho
Escola de Engenharia

Andrea Zille

Laccase Reactions for Textile Applications

Setembro de 2005



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Escola de Engenharia

Andrea Zille

Laccase Reactions for Textile Applications

Tese de Doutoramento em Engenharia Têxtil,
Área de Conhecimento de Química Têxtil

Trabalho efectuado sob a orientação de
Professor Doutor Artur Cavaco-Paulo

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DECLARAÇÃO

Nome: ANDREA ZILLE
Endereço Electrónico: azille@det.uminho.pt **Telefone:** 253 510 100

N.° do Bilhete de Identidade: AG9317764 (Itália)

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Abstract

Laccase reactions for textile applications

The release of azo dyes into the environment is deleterious, not only because of their color, but also because they are not easily degraded by aerobic bacteria and under action of anaerobic reductive bacteria they can form toxic or mutagenic compounds. As common physical or chemical methods for dye removal are expensive and sometimes generate secondary pollution, the biodegradation by the use of a *Trametes villosa* laccase appears to be an attractive alternative.

A rapid and cheap method for predicting the potential of enzymatic dye biodegradation in the effluents was studied. It was demonstrated that the redox potential of the azo dyes is a reliable preliminary tool to predict the decolorization capacity of oxidative and reductive biocatalysts. A linear relationship was found during the initial period of decolorization with laccase and a laccase/mediator system between the percentage decolorization of each dye and the respective anodic peak potential. The less positive the anodic peak of the dye, the more easily is decolorized oxidatevely with laccase.

Since several limitations prevent the use of free enzymes in bioremediation, an immobilized laccase was used to decolorize a reactive Black 5 industrial dyeing effluent. Surprisingly the immobilized enzyme showed lower stability than the free form in dyeing effluents (194 h free and 79 h immobilized). The stability of the enzyme depended on the dyeing liquor composition and the chemical structure of the dye. In the decolorization experiments with immobilized laccase, two phenomena were observed: decolorization due to adsorption on the support (79%) and dye degradation due to the enzyme action (4%). Adsorption appears to be the most important factor in decolorization. However, both immobilized and free laccase showed a good decolorization degree and re-dyeing in the enzymatically recycled effluent provided consistency of the color with both bright and dark dyes.

For a better understanding of the dye degradation mechanisms, laccase was used for phenolic and non-phenolic azo dye degradation and the reaction products that accumulated after 72 hours of incubation were analyzed.

Chemical pathway for azo dye degradation was proposed. LC-MS analysis shows the formation of phenolic compounds that can recombine with undegraded products, as well as a large amount of polymerized products that retain the azo group integrity. Reactions of amino-phenols were also investigated by ^{13}C-NMR and LC-MS analysis and the polymerization character of laccase was shown. These polymerized products provide unacceptable color levels in effluents limiting the application of laccases as bioremediation agents.

The direct laccase decolorization of effluent in free and immobilized form and the coupling/polymerization laccase reactions in the azo reductase pretreated effluent are compared on the basis of the kinetic parameters using a HBT/laccase system. The addition of 1-hydroxybenzotriazole (HBT) as mediator considerably improves the catalytic efficiencies in all systems. Laccase was coupled with an azo reductase that can cleave a wider range of azo dyes into corresponding amines. It can be concluded that the laccase-mediated coupling/polymerization of the aromatic amine with catechol is a promising alternative method in dye removal.

The ability of laccases to polymerize was also used to generate color "*in situ*" as effluent reutilization technique and as alternative dyeing process. Wool dyeing was performed in a dye bath prepared with a dye precursor (2,5-diaminobenzenesulfonic acid), dye modifiers (catechol and resorcinol) and laccase, without any dyeing auxiliaries at mild temperature and pH. Darker coloration of the samples could be obtained by increasing the reaction time and minimizing the enzyme and modifiers loading. This makes laccase dyeing an economically attractive alternative to the conventional dyeing process. Resorcinol should be used in low concentration to attain deep-shade dyeing. Microscopic observation of the cross-section of the enzymatically dyed wool showed penetration of the colorant into the mass of the fibers.

The research presented in this thesis shows the limitation of the direct azo dye enzymatic degradation in both free and immobilized forms and makes clear that the catalyzed-laccase polymerization reactions can be studied as a promising methodology in textile wastewater treatment and recycling.

Resumo

Reacções com lacase para aplicações têxteis

A libertação dos corantes azo no meio ambiente é prejudicial, não somente por causa da sua cor, mas também porque estes não são facilmente degradados pelas bactérias aeróbias e sob a acção das bactérias anaeróbias redutivas podem dar origem a compostos mutagénicos. Devido ao facto dos tradicionais métodos físico-químicos para a eliminação dos corantes serem caros e às vezes causarem problemas de polução secundaria, a biodegradação com uma lacase de *Trametes villosa* parece ser uma alternativa atractiva. Foi desenvolvido um método rápido e económico para prever o potencial dos corantes azo para serem biodegradados. Demonstrou-se que o potencial redox dos corantes azo é uma ferramenta útil para prever a capacidade de descoloração na biocatálise oxidativa e redutiva. Encontrou-se uma relação linear entre a percentagem de descoloração inicial de cada corante e o respectivo potencial do pico anódico quer com lacase quer com um sistema lacase/mediador. Quanto menor é o potencial do pico anódico do corante, mais facilmente o mesmo é oxidado pela lacase.

Devido às limitações do uso de enzimas livres na biodegradação, uma lacase imobilizada foi utilizada para descolorar um efluente têxtil. A estabilidade da enzima depende da composição do efluente e da estrutura química do corante. Contráriamente ao esperado a enzima imobilizada mostrou no efluente uma estabilidade mais baixa do que a enzima livre (194 h livre e 79 h imobilizada). Nas experiências de descoloração com lacase imobilizada, observaram-se dois fenómenos: a descoloração do corante devida à absorção no suporte, que revelou ser o factor mais importante para a descoloração (79%), e a descoloração devida à acção da enzima (4%). Tanto a enzima imobilizada como a livre mostraram um aceitável grau de descoloração e o tingimento com o efluente reciclado teve uma boa consistência da cor com corantes claros e escuros.

Para uma melhor compreensão dos mecanismos da degradação dos corantes, a lacase foi usada para degradar corantes azo fenólicos e não-fenólicos e os produtos da degradação que se acumularam após 72 horas de incubação foram também analisados. Os mecanismos químicos da

degradação foram assim propostos. A análise por LC-MS mostrou a formação de compostos fenólicos que se podem recombinar-se com os produtos não-degradados, assim como uma grande quantidade de produtos polimerizados que retêm a integridade do grupo azo. A capacidade da lacase em catalizar a polimerização dos compostos amino-fenólicos foi também demonstrada através das análises com ^{13}C-NMR e LC-MS. Estes produtos polimerizados criam níveis inaceitáveis de cor nos efluentes limitando assim a aplicação da lacase para o tratamento deste tipo de efluente.

A descoloração dos efluentes com lacase livre e imobilizada e as reacções de polimerização da lacase nos efluentes pré-tratados com azoreductase foram comparados num sistema de HBT/lacase com base nos parâmetros cinéticos. A adição de 1-hydroxybenzotriazole (HBT) como mediador melhora consideravelmente a eficácia catalítica em todos os sistemas. A lacase foi associada a uma azoreductase capaz de converter uma extensa gama de corantes azo nas correspondentes aminas. Pode concluir-se que a polimerização das aminas aromáticas com catechol catalizada pela lacase e HBT é um método alternativo promissor para a remoção dos corantes nos efluentes.

A capacidade da lacase de catalizar a polimerização foi também usada como um processo alternativo de tingimento "in situ". O tingimento da lã com lacase foi executado sem auxiliares e a temperatura e pH moderados, utilizando ácido 2,5-diaminobenzenosulfónico, catechol e resorcinol. Aumentando o tempo de reacção e diminuindo as doses da enzima e dos químicos obtiveram-se colorações mais escuras nas amostras. O tingimento com lacase mostrou ser uma alternativa economicamente atractiva em comparação a os processos convencionais de tingimento. Demonstrou-se que para obter colorações escuras o resorcinol deve ser usado em baixas concentrações. A observação microscópica da secção transversal da lã tingida com lacase demonstrou a penetração do corante nas fibras.

A pesquisa apresentada nesta tese demonstrou as limitações da degradação enzimática directa dos corantes azo na forma livre e imobilizada e torna claro que as reacções de polimerização catalizadas pela lacase podem ser estudadas como uma metodologia promissora no tratamento e na reciclagem das águas residuais da indústria têxtil.

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"Progress is impossible without change, and those who cannot change their minds cannot change anything."

George Bernard Shaw

1

General Introduction

1. General Introduction

The pollution problems due to textile industry effluents have increased in the last years. The dyeing processes have in general a low yield, and the percentage of the lost dye in the effluents can reach up to 50% (Pierce 1994, Pearce *et al.* 2003). From the available bibliography it can be estimated that approximately 75% of the dyes, discharged by Western European textile-processing industries, belong to the classes of the reactive (~36%), acid (~25%) and direct dyes (~15%) (Øllgaard *et al.* 1999). In these classes, the azo dyes (aromatic moieties linked together by azo (-N=N-) chromophores) are the most important chemical class of synthetic dyes and pigments, representing between 60 to 80 % of the organic dyes referenced in the Color Index (Vandervivere 1998). This was the reason of the use of azo dyes in this work.

The textile effluents, usually highly colored, when discharged in open waters present an obvious aesthetic problem. Moreover, the dyes without an appropriate treatment can persist in the environment for extensive periods of time and are deleterious not only for the photosynthetic processes of the aquatic plants but also for all the living organisms since the degradation of these can lead to carcinogenic substances (Hao *et al.* 2000, Pinheiro *et al.* 2004). The European community has not been indifferent to this problem and in September 2003 the European directive 2002/61/EC came into force. This directive forbids the use of some products, derivatives of a restricted number of azo dyes. However, these restriction measures are not enough to solve the problems due to the huge amount of dyes discharged in the environment every year.

In the last years, new processes for the degradation of dyes and reutilization of wastewater have been developed. Due to the high amounts of water used by the textile industries (~100 liters for 1 kg of cotton in a continuous process), the control of water waste with recycling technologies is an important factor for limiting the amount of effluent and the costs of production (Diaper *et al.* 1996). Among them, systems based in biological processes allow degradation and

mineralization with low environmental impact and without the use of potentially toxic chemical substances, in mild pH and temperature conditions (Robinson *et al.* 2001b).

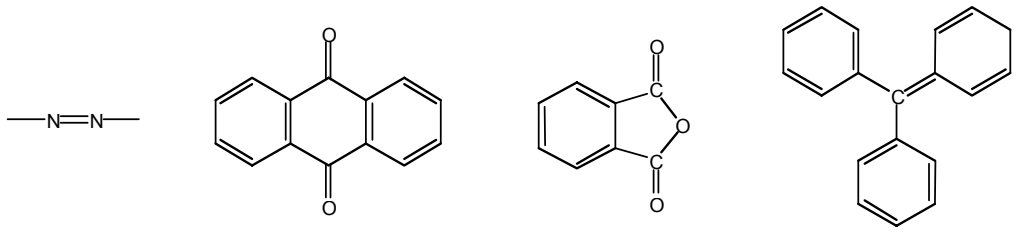
Many studies on the biological degradation of dyes are focused on the identification and characterization of the enzymes that can degrade them (Abadulla *et al.* 2000, Nyanhongo *et al.* 2002, Blümel and Stolz 2003). One of these enzymes, the *Trametes villosa* laccase, was chosen for studying in detail the processes of oxidative degradation of azo dyes, without forgetting the effluent recycling processes. Laccase is an oxidoreductive ligninolytic enzyme used in various biotechnological and environmental applications (Section 1.6). In the last years its capacity to degrade synthetic dyes has been extensively studied (Mayer and Staples 2002). In comparison to other oxidoreductases, as for example the peroxidades that need H_2O_2 in its catalytic process, laccase only uses oxygen for the oxidation of its reduced state (Spadaro and Renganathan 1994). Laccase also degrades azo dyes without the direct breaking of the azo bond, through a non-specific free radical mechanism that prevents the formation of aromatic amines as degradation products (Chivukula and Renganathan 1995). Beside the application, this work also reveals the limitations of the laccase dye degradation mechanism and the potential of the laccase polymerization reactions as a promising effluent treatment method. The laccase-catalyzed polymerization reactions can be applied not only to enhance pollutant precipitation from the effluents but also for generating color "in situ", allowing useful effluent reutilizations. Laccase's versatility in the degradation and polymerization reactions, and its ecological and economic advantages in comparison with other enzymes, have been the reasons for its choice for this work.

1.1. Dye history

The use of natural dyes for painting and dyeing has been known since ancient times. The recent discovery in the Chauvet-pont-d'arc caves in France of 30000-year-old Paleolithic rock paintings provide the ancientest testimony of the millenary use of inorganic pigments such as hematite, manganese oxide, soot and ochre (Chippindale 1998). Organic natural colorants have also a long history, especially as textile dyes. Most dyeing techniques in use until the XIXth century were established by the ancient Egyptians, who developed methods using plant extracts, sometimes in association with a mordant (Carr 1995). Also other civilizations developed dyeing methods using not only plants, as the Indigo from Dyer's woad (*Tinctoria isatis*) or the red alizarin from Madder (*Rubia tinctorum*), but also from insects (Persian scarlet), mollusks (Tyrian purple), fungi and lichens. Due to the fact that these plants and materials were usually native from the regions where they were used in the dyeing processes the diffusion of these methods has not been possible for a long time (Carr 1995). Until the XVIth century the dyeing processes were well kept secret, but with the growth of commercial trips and the expansion of the knowledge they have had a rapid increment and diffusion. In 1548, Giovanventura Rossetti published the "Plichto dei tintori" in which not only described some dyeing and active constituent extraction methods but also chemical preparations such as hydrochloric acid (Welham 2000). In 1671, Colbert in France established the first regulations for the control of the dyeing quality. In 1737 Dufay de Cisternay published the first truly scientific account about systematic fastness testing and quality classification in dyeing processes based on physical chemical ideas (Welham 2000). In 1856 the young English chemist W.H. Perkin, in the attempt to synthesize quinine, discovered and patented a substance with excellent dyeing properties that later would come to be known as aniline purple. In the following years other dyes have been developed, but it was only in 1865, with the Kekule's discover of the molecular structure of the benzene, that the research followed a less empirical and more systematic approach. In the beginning of the XXth century the synthetic dyes had almost completely supplanted the natural dyes (Welham 2000).

1.2. Dye classification

All molecules absorb electromagnetic radiation, but differ in the specific wavelengths absorbed. Some molecules have the ability to absorb light in the visible spectrum (400-800 nm) and, as a result, they are themselves colored. The dyes are molecules with delocalized electron systems with conjugated double bonds that contain two groups: the chromophore and the auxochrome. The chromophore is a group of atoms, which controls the color of the dye, and it is usually an electron-withdrawing group. The most important chromophores are -C=C-, -C=N-, -C=O, -N=N-, $-NO_2$ and -NO groups. The auxochrome is an electron-donating substituent that can intensify the color of the chromophore by altering the overall energy of the electron system and provides solubility and adherence of the dye to the fiber. The most important auxochromes are $-NH_2$, $-NR_2$, -NHR, -COOH, $-SO_3H$, -OH and $-OCH_3$ groups (Rocha Gomes 2001). Based on the chemical structure or chromophore, 20-30 different dye groups can be identified. Azo (monoazo, disazo, triazo, polyazo), anthraquinone, phthalocyanine and triarylmethane dyes are quantitatively the most important chromophores (Figure 1.1).



Azo Anthraquinone Phthalocyanine Triarylmethane

Figure 1.1 - The most important chromophores.

Most of the commercial dyes are classified in terms of color, structure or method of application in the Color Index (C.I.), which is edited every three months since 1924 by the "Society of Dyers and Colourists" and the "American Association of Textile Chemists and Colorists". The last edition of the Color Index lists about 13000 different dyes. Each dye is assigned to a

C.I. generic name determined by its application and color. The 15 Color Index different application classes are listed in Table 1.1.

Table 1.1 - Color Index application classes (Christie 2001)

Application Class	Characteristics
Acid dyes	Highly water-soluble due to the presence of sulphonic acid groups. Form ionic interactions between the protonated functionalities of the fibers ($-NH_3^+$) and the negative charge of the dyes. Also Van-der-Waals, dipolar and hydrogen bonds are formed. The most common structures are azo, anthraquinone and triarylmethane.
Reactive dyes	Form covalent bonds with -OH, -NH or -SH groups in cotton, wool, silk and nylon. The problem of colored effluents associated to the use of these dyes is due to the hydrolysis of the reactive groups that occurs during the dyeing process. The most common structures are azo, metal complex azo, anthraquinone and phthalocyanine.
Direct dyes	Their flat shape and length enables them to bind along-side cellulose fibers and maximize the Van-der-Waals, dipole and hydrogen bonds. Only 30% of the 1600 structures are still in production due to their lack of fastness during washing. The most common structures are almost always sulphonated azo dyes.
Basic dyes	Basic dyes work very well on acrylics due to the strong ionic interaction between dye functional groups such as $-NR_3^+$ or $=NR_2^+$ and the negative charges in the copolymer. The most common structures are azo, diarylmethane, triarylmethane and anthraquinone.
Mordant dyes	Mordants are usually metal salts such as sodium or potassium dichromate. They act as “fixing agent” to improve the color fastness. They are used with wool, leather, silk and modified cellulose fibers. The most common structures are azo, oxazine or triarylmethane.
Disperse dyes	Non-ionic structure, with polar functionality like $-NO_2$ and –CN that improve water solubility, Van-der-Waals forces, dipole forces and the color. They are usually used with polyester. The most common structures are azo, nitro, anthraquinones or metal complex azo.

Table 1.1 – Continue

Application Class	Characteristics
Pigment dyes	These insoluble, non-ionic compounds or salts, representing 25% of all commercial dye names, retain their crystalline or particulate structure throughout their application. The most common structures are azo or metal complex phthalocyanines.
Vat dyes	Vat dyes are insoluble in water, but may become solubilized by alkali reduction (sodium dithionite in the presence of sodium hydroxide). The produced *leuco* form is absorbed by the cellulose (Van-der-Waals forces) and can be oxidized back, usually with hydrogen peroxide, to its insoluble form. The most common structures are anthraquinones or indigoids.
Ingrain dyes	The term ingrain is applicable to all dyes formed *in situ*, in or on the substrate by the development, or coupling, of one or more intermediate compounds and a diazotized aromatic amine. In the Color Index the sub-section designated Ingrain is limited to tetra-azaporphin derivatives or precursors.
Sulphur dyes	Sulphur dyes are complex polymeric aromatics with heterocyclic S-containing rings representing about 15% of the global dye production. Dyeing with sulphur dyes (mainly on cellulose fibers) involves reduction and oxidation processes, comparable to vat dyeing.
Solvent dyes	Non-ionic dyes that are used for dyeing substrates in which they can dissolve as plastics, varnish, ink and waxes. They are not often used for textile processing. The most common structures are diazo compounds that undergo some molecular rearrangement, triarylmethane, anthraquinone and phthalocyanine.
Other dye classes	Food dyes are not used as textile dyes. Natural dyes use in textile-processing operations is very limited. Fluorescent brighteners mask the yellowish tint of natural fibers by absorbing ultraviolet light and weakly emitting blue light. Not listed in a separate class in the Color Index, many metal complex dyes can be found (generally chromium, copper, cobalt or nickel). The metal complex dyes are generally azo compounds.

1.3. Azo dyes

Azo dyes are the largest group of synthetic dyes and pigments with industrial application due to their relatively simple synthesis and almost unlimited number and types of substituents (McCurdy 1991). The worldwide production of these organic dyes is currently estimated at 450000 tons/year, with almost 50000 tons/year lost in effluent during application and manufacture (Lewis 1999). Azo dyes contain at least one N=N double bond and many different structures are possible. Monoazo dyes have only one N=N double bond, while diazo, triazo and polyazo dyes contain two, three or more N=N double bonds, respectively. The azo groups are generally connected to benzene and naphthalene rings, but can also be attached to aromatic heterocyclic or enolizable aliphatic groups (Zollinger 2003). The general structure of the azo dye molecule can be seen in Figure 1.2.

Figure 1.2 - General structure of azo dyes (where R can be an aryl, heteroaryl or - CH = C(OH) - alkyl derivative).

These lateral groups are necessary for obtaining colors with different shades and intensities. Azo colorants range in shade from greenish yellow to orange, red, violet and brown. The colors depend largely on the chemical structure, whereas different shades rather depend on physical properties. However, the important disadvantage, limiting their commercial application, is that most of them are red and none are green (Øllgaard *et al.* 1999). Synthesis of most azo dyes involves diazotization of a primary aromatic amine to give a diazonium salt. The diazonium compound is then coupled with one or more nucleophiles. Amino- and hydroxyl- groups are commonly used coupling components. The coupling reaction is generally in *para* position in respect to the amino- or hydroxyl- groups (Zollinger 2003). The general scheme of azo dye synthesis is shown in figure 1.3.

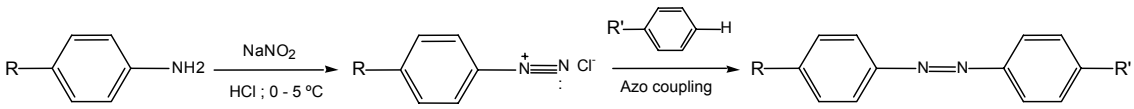


Figure 1.3 - Azo dye synthesis.

The azo linkage is considered the most labile portion of an azo dye. The linkage easily undergoes enzymatic breakdown, but thermal or photochemical breakdown may also take place. Degradation of azo dyes can be obtained by reduction or by oxidation. The reduction releases the colorless component-amines (Figure 1.4).

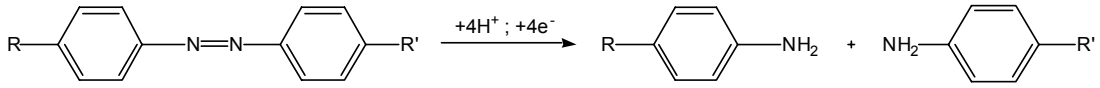


Figure 1.4 - Azo dye reduction.

A large variety of azo dyes can be reduced by many different bacteria, which suggest the non-specific nature of this reaction. The potentiality to reduce the azo dyes can therefore be considered an universal property of the anaerobic bacteria. An accepted distinction of the different reduction mechanisms of the azo dyes can be made among direct enzymatic reduction, indirect enzymatic reduction (needing mediators) and chemical reduction (Figure 1.5) (Van der Zee 2002).

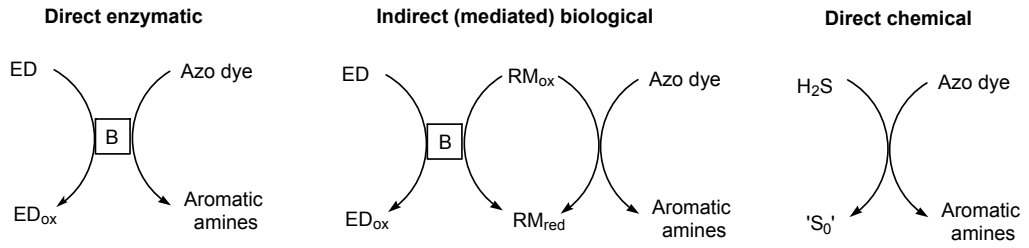


Figure 1.5 - Schematic representation of the different mechanisms of the azo dye reduction (ED = electron donor; B = bacteria (enzyme system); RM = redox mediator) (adapted from Van der Zee 2002).

The general oxidative mechanism is more difficult to establish due to the high reactivity of the free radicals normally involved in the degradation process. The chemical oxidation of an azo dye (Orange I) by chlorine in acidic media is represented in Figure 1.6 (Oakes and Gratton 1998). A similar pathway was observed in enzymatic oxidation (Chivukula and Renganathan 1995). The electron-withdrawal character of azo-groups generates electron deficiency. Thus it makes the compounds less susceptible to oxidative catabolism, and as a consequence many of these chemicals tend to persist under aerobic environmental conditions (Knackmuss 1996).

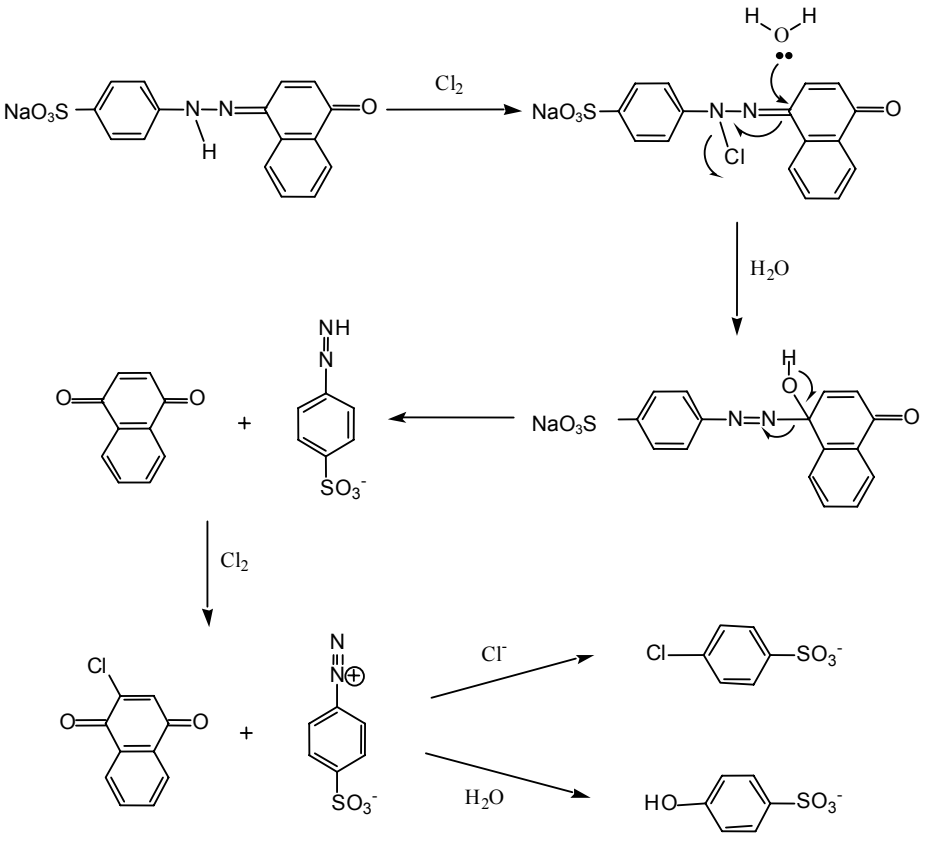


Figure 1.6 - Oxidation of azo dye "Orange I" with chlorine in acidic media (reproduced from Oakes and Gratton 1998).

1.4. Ecotoxicity of azo dyes

Due to the fact that the dyes are synthesized to be chemically and photolytically stable, they are highly visible (some can be detected in concentration < 1 mg/l) and persistent in natural environments (Nigam *et al.* 2000, Rieger *et al.* 2002). Consequently, the release of potentially hazardous dyes in the environment can be an ecotoxic risk and can affect man through the food chain (Van der Zee 2002).

The acute toxicity of azo dyes is rather low. Algae growth and fish mortality are not affected by dye concentrations below 1 mg/l. The most toxic dyes for algae and fishes are basic and acid dyes. In the mammal tests only a few azo dyes showed LD_{50} values below 250 mg/kg body weight, whereas a majority showed LD_{50} values between 250 and 2000 mg/kg body weight (Van der Zee 2002). Sulphonation of azo dyes appears to decrease toxicity by enhancing urinary excretion of the dye and its metabolites (Brown and DeVito 1993).

Sensitization to azo dyes has been seen in textile industry since 1930, when 20% of the workers dyeing cotton with red azoic dyes, developed occupational eczema (Giusti *et al.* 2004). The majority of sensitizing dyes, present in clothes, practically all belong to the group of disperse dyes. The explanation is probably that the attachment of molecules from disperse dyes is weak, as they are more easily available for skin contact (Seidenari *et al.* 2002).

The azo dyes propensity to bioaccumulate has been extensively investigated in fish. The uptake rates are influenced by the partition coefficient (Erickson and McKim 1990). However other factors may be important for the uptake as diffusion resistance, molecular size, respiratory volume and gill perfusion (Niimi *et al.* 1989). The elimination rates for hydrophobic chemicals are low. For hydrophobic chemicals it has often been shown that uptake and clearance between fish and water is a first-order exchange process (Van Hoogen and Opperhuizen 1988). Water-soluble dyes like acid, reactive and basic dyes generally are not bioaccumulated. Also for the poor soluble disperse dyes the bioaccumulation values are much lower than expected (Van der Zee 2002). It is concluded that the ionic dyes do not have, in general, a significant bioaccumulation potential, but, at least some acid dyes, may bioaccumulate.

Non-ionic dyes and pigments, on the other hand, have a high bioaccumulation potential (Anliker *et al.* 1988).

In general, the correlation between the results of mutagenicity tests and carcinogenicity seen in animal experiments of azo dyes is poor. The lack of correlation is probably due to the rather complex metabolic pathways, which azo dyes undergo in mammalian organisms (Brown and DeVito 1993). The majority of azo dyes, if highly purified are not mutagenic. However, many of the commercial available azo dyes may, due to impurities, show metabolic activation and mutagenic activity in vitro (Arcos and Argus 1994). For increasing the solubility of the dyes used in the textile industry, they generally contain one or more sulphonated groups. Due to this fact, sulphonic containing dyes generally have a low genotoxic potential (Jung *et al.* 1992). The labile azo linkage may easily undergo enzymatic breakdown in mammalian organisms, including man. After cleavage of the azo-linkage, the component aromatic amines are absorbed in the intestine and excreted in the urine (Brown and DeVito 1993). Many studies have been conducted showing the toxic potential of aromatic amines from azo dyes (Weisburger 2002, Pinheiro *et al.* 2004). The aromatic amine toxicity and carcinogenicity depends on the three-dimensional structure of the molecule and on the location of the amino groups. Moreover the nature and the position of other substitutents can increase (nitro, methyl or methoxy) or lower (carboxyl or sulphonate) the toxicity (Chung and Cerniglia 1992).

1.5. Dye removal techniques

The majority of physical, chemical and biological color removal techniques work either by concentrating the color into sludge, solid supports, or by the complete destruction of the dye molecule. It is expected that decoloration systems involving destruction technologies will prevail, as the transfer of pollution from one part of the environment to another is prevented (Vandevivere *et al.* 1998, Hao *et al.* 2000, Robinson *et al.* 2001a). Currently, the major methods of textile wastewater treatment involve physical and/or chemical processes as membrane filtration, coagulation/flocculation, precipitation, flotation, adsorption, ion exchange, ion pair extraction, ultrasonic mineralization, electrolysis, chemical reduction and advanced chemical oxidation (Gogate and Pandit 2004a). The advanced oxidation processes include chlorination, bleaching, ozonation, Fenton oxidation, photocatalytic oxidation and wet-air oxidation (Slokar and Le Marshal 1998, Robinson *et al.* 2001a, Pizzolato *et al.* 2002, Alaton and Ferry 2003, Kusvuran *et al.* 2004, Gogate and Pandit 2004b). Such methods, that use compounds with an oxidation potential (E_0) higher than that of oxygen (1.23 V) as hydrogen peroxide (E_0 = 1.78 V), ozone (E_0 = 2.07 V) and the hydroxyl radical (E_0 = 2.28 V), are often very costly and accumulation of concentrated sludge creates a disposal problem (Robinson *et al.* 2001a). There is also the possibility that a secondary pollution problem will arise due to excessive chemical use. Biological and/or mixed treatment systems that can effectively remove dyes from large volumes of wastewater at a low cost are a preferable alternative (Robinson *et al.* 2001a). Biological techniques include biosorption and biodegradation in aerobic, anaerobic, anoxic or combined anaerobic/aerobic treatment processes with bacteria, fungi, plants, yeasts, algae and enzymes (Heinfling *et al.* 1998, Rafiie and Coleman 1999, Semple *et al.* 1999, Nyanhongo *et al.* 2002, Ramalho *et al.* 2002, Mohan *et al.* 2002, Pearce *et al.* 2003, Blümel and Stolz 2003, Ramalho *et al.* 2004, Forgacs *et al.* 2004, Acuner and Dilek 2004, Aubert and Schwitzguebel 2004, Mbuligwe 2005, Christian *et al.* 2005, Mohan *et al.* 2005, Shrivastava *et al.* 2005). Textile dye effluents are complex, containing a wide variety of dyes, natural impurities extracted from the fibers and other products such as dispersants, levelling agents, acids, alkalis, salts and sometimes heavy metals (Laing 1991). In general, the effluent is highly

colored with high biological oxygen demand (BOD) and chemical oxygen demand (COD), has a high conductivity and is alkaline in nature.
For this reason, several factors determine the technical and economic feasibility of each single dye removal technique as dye type, wastewater composition, dose and costs of required chemicals, operation costs (energy and material), environmental fate and handling costs of generated waste products. Usually, the use of one individual process may not be sufficient to obtain complete decolorization because each technique has its limitations. Dye removal strategies consist therefore mostly of a combination of different techniques (Van der Zee 2002). In the following chapters an overview of the most important techniques is presented.

1.5.1. Physical methods

Membrane filtrations. Nanofiltration and reverse osmosis can be applied as main or post treatment processes for separation, purification and reuse of salts and larger molecules including dyes from dyebath effluents and bulk textile-processing wastewaters (Crossley 1995, Van't Hul 1997, Sójka-Ledakowicz *et al.* 1998, Koyuncu et al 2004, Kim *et al.* 2005). In reverse osmosis the effluent is forced under moderate pressure across a semipermeable membrane to form a purified permeate and a concentrate. The process can remove up to approximately 98% of the impurities in the water with a relative molecular mass higher then 100 (Southern 1995). In nanofiltration the membrane effectively acts as a molecular filter retaining material with a relative molecular mass greater than about 200 (Southern 1995). In spite of its degree of efficiency, reverse osmosis and nanofiltration present some disadvantages. The membranes have to be cleaned on a regular basis and may be attacked by the dye materials or other constituents of the effluent changing their surface characteristics. Moreover, these techniques have high capital and relatively high running costs (Cooper 1993, Vandevivere *et al.* 1998, Hao *et al.* 2000). Filtration with bigger pores (ultrafiltration and microfiltration) is generally not suitable as the membrane pore size is too large to prevent dye molecules from passing through but it can be successful as pre-treatment (Rozzi *et al.* 1999, Marcucci *et al.* 2001, Koyuncu 2003, Ciardelli *et al.* 2003).

Coagulation and flocculation. The inorganic coagulants - lime, aluminum, magnesium and iron salts – have been used for coagulation in the treatment of textile-processing wastewater to partly remove total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD) and color over many years. (Sarasa *et al.* 1998, Semerjian and Ayoub 2003, Allegre *et al.* 2004, Peres *et al.* 2004, Aguilar *et al.* 2005, Golob *et al.* 2005). The principle of the process is the addition of a coagulant followed by a generally rapid association between the coagulant and the pollutants. The thus formed coagulates or flocks subsequently precipitated are then removed by either flotation, settling, filtration or other physical technique to generate a sludge that is normally further treated to reduce its water content and toxicity (Aguilar *et al.* 2002, Semerjian and Ayoub 2003, Papiç *et al.* 2004, Golob and Ojstrsek 2005, Mishra and Bajpai 2005). Organic anionic, cationic or non-ionic coagulant polymers have been developed in the last years for color removal treatments and in general they offer advantages over inorganics: lower sludge production, lower toxicity and improved color removal ability (Al-Mutairi *et al.* 2004, Zouboulis *et al.* 2004).

Sorption. The use of any adsorbent, whether ion-exchanger, activated carbon or high-surface-area inorganic material, for removing species from a liquid stream depends on the equilibrium between the adsorbed and the free species. Dye effluents are multicomponent mixtures with different absorption degrees and concentrations. In same cases weaker bounds are formed with the adsorbent and some material can be released back into the stream (Southern 1995). The range of adsorbents described in the literature for this application covers the range of activated carbons, high-surface-area inorganic materials, synthetic ion-exchange resins and cellulose-based adsorbents such as chitin (poly-N-acetylglucosamine), synthetic cellulose and other fiber-based bioadsorbents. Standard ion exchange systems have not been widely used for treatment of dye effluents due to the high cost of organic solvents to regenerate the ion-exchanger, and due to the extremely large inorganic load of the effluent (Southern 1995, Slokar and Le Marechal 1998, Robinson *et al.* 2001a). Activated carbon is reasonably effective at removing many different dyes from aqueous streams (Slokar and Le Marechal 1998, Robinson *et al.* 2001a). However, the effective cost

of the high-temperature regeneration process, including the replacement cost and the waste sludge yield, makes their regeneration unattractive to the small companies (Pereira *et al.* 2003, Faria *et al.* 2004, Forgacs et. 2004, Golob and Ojstrsek 2005). Therefore, various low-cost adsorbents have been investigated as an alternative to activated carbon. The use of inorganic adsorbents, such as high-surface-area silica, cinder ash and clays, has been tried for a range of dyes. Their effectiveness depends on the types of dye in the effluent stream or, more particularly, on the relative charge on the dye molecule. Bioadsorbents are cheap naturally biodegradable occurring polymers (or their synthetic derivatives) that have a high dye-binding capacity and can act as ion-exchangers. A wide range of biomaterials can be used as bioadsorbent: corn, wheat, rice husks, wood chips, sawdust, bark, bagasse pith, cotton waste, cellulose, bacterial biomass, fungal biomass, yeast biomass, etc. (Fu and Viraraghavan 2001, Fu and Viraraghavan 2002, Dönmez 2002, Robinson *et al.* 2002, Woolard *et al.* 2002, Waranusantigul *et al.* 2003, Shawabkeh and Tutunji 2003, Guo *et al.* 2003, Aksu and Dönmez 2003, Ho and McKay 2003, Sun and Yang 2003, Walker *et al.* 2003, Malik 2003, Malik 2004, Garg *et al.* 2004, Wibulswas 2004, Wu *et al.* 2004, Gong *et al.* 2005, Delval *et al.* 2005, Janos *et al.* 2005, Gupta *et al.* 2005, Alkan *et al.* 2005, Aksu and Dönmez 2005, Özacar and Engil 2005). Recently published work refers to the use of purified chitin or chitosan due to their extremely high acid and reactive dye adsorption and binding capacity (Jocic *et al.* 2004, Cestari *et al.* 2005).

1.5.2. Chemical methods

Electrolysis. The electrochemical technique is very efficient to remove the color from a wide variety of dyes and pigments. Biological oxygen demand (BOD) and chemical oxygen demand (COD) reduction and coagulation of the total suspended solids present in the wastewater are also obtained (Vlyssides *et al.* 2000, Gürses *et al.* 2002, Daneshvar *et al.* 2004, Bayramoglu *et al.* 2004, Cerón-Rivera *et al.* 2004, Fernandes *et al.* 2004, Shen *et al.* 2005, Alinsafi *et al.* 2005, Carneiro *et al.* 2005). The process very simply is based on applying an electric current through to the wastewater by using sacrificial iron electrodes to produce ferrous hydroxide in solution. These sacrificial iron electrodes generate Fe(II)-ions and -OH. The $Fe(OH)_2$ is formed and soluble and insoluble acid dyes are removed from the effluent. Moreover Fe(II) can reduce azo dyes to arylamines. Water can also be

oxidized resulting in the formation of O_2 and O_3. The efficiency of the electrochemical system in pollutant removal can often reach 90%. However, the process is expensive due to large energy requirements, limited lifetime of the electrodes and uncontrolled radical reactions (Hao *et al.* 2000, Van der Zee 2002, Cerón-Rivera *et al.* 2004).

Ozone. Ozone is a very powerful and rapid oxidizing agent that can react with most species containing multiple bonds (such as C=C, C=N, N=N, etc.) and with simple oxidizable ions such as S^{2-}, to form oxyanions such as SO_3^{2-} and SO_4^{2-} (Gogate and Pandit 2004a). Ozone rapidly decolorizes water-soluble dyes but with non-soluble dyes (vat dyes and disperse dyes) react much slower. Furthermore, textile-processing wastewater usually contains other refractory constituents that will react with ozone, thereby increasing its demand (Özbelge *et al.* 2003, Pera-Titus *et al.* 2004, Muthukumar *et al.* 2005). Decomposition of ozone requires high pH values (pH >10). In alkaline solutions ozone reacts almost indiscriminately with all compounds present in the reacting medium (Aplin and Wait 2000, Chu e Ma Chi 2000) converting organic compounds into smaller and biodegradable molecules (Peralta-Zamora *et al.* 1999a). Consequently, after ozone treatment seems logical the use of biological methods for reaching a complete mineralization (Krull *et al.* 1998, Krull and Hempel 2001). A major limitation of the ozonation process is the relatively high cost of ozone generation process coupled with its very short half-life (Gogate and Pandit 2004a).

Fenton reagents. The oxidation system based on the Fenton's reagent (hydrogen peroxide in the presence of a ferrous salt) has been used for the treatment of both organic and inorganic substances. The process is based on the formation of reactive oxidizing species, able to efficiently degrade the pollutants of the wastewater stream but the nature of these species is still under discussion (Walling 1998, MacFaul *et al.* 1998, Moura *et al.* 2005, Wang *et al.* 2005). The main reactive radical species involve the presence of hydroxyl radicals whereas higher oxidized iron species may be formed (Aplin e Wait 2000, Kang *et al.* 2002b, Hsueh *et al.* 2005). It is accepted that both hydroxyl as well as ferryl complexes coexist in Fenton's mechanism and depending on the operating conditions (substrate nature, metal–peroxide ratio, scavengers addition etc.),

one of them will predominate (Bossmann *et al.* 1998). The oxidation system can be effectively used for the destruction of non-biodegradable toxic waste effluents and render them more suitable for a secondary biological treatment (Bigda 1996, Chen and Pignatello 1997, Nesheiwat and Swanson 2000). Fenton oxidation process can decolorize a wide range of dyes and in comparison to ozonation, the process is relatively cheap and results generally in a larger COD reduction (Fernandes *et al.* 1999, Ince and Tezcanli 1999, Park *et al.* 1999). Fenton oxidation is limited to the fact the textile process wastewaters usually have high pH, while the Fenton process requires low pH. At higher pH, large volumes of waste sludge are generated by the precipitation of ferric iron salts and the process loses its effectiveness (Van der Zee 2002).

Photocatalytic methods. The photocatalytic or photochemical degradation processes are gaining importance in the area of wastewater treatment, since these processes result in complete mineralization with operation at mild conditions of temperature and pressure. The photo-activated chemical reactions are characterized by a free radical mechanism initiated by the interaction of photons of a proper energy level with the molecules of chemical species present in the solution, with or without the presence of the catalyst (Gogate and Pandit 2004a). The radicals can be easily produced using UV radiation. UV light has been tested in combination with H_2O_2, TiO_2, Fenton reagents, O_3 and other solid catalysts such as for the decolorization of dye solutions (Hao *et al.* 2000, Gogate and Pandit 2004b). While the UV/H_2O_2 process appeared too slow, costly and little effective for potential full-scale application, the combination UV/TiO_2 seems more promising. With UV/TiO_2 treatment, a wide range of dyes can be oxidized and generally not only decolorized but also highly mineralized (Gonçalves *et al.* 1999, Peralta-Zamora *et al.* 1999a, Gomes de Moraes *et al.* 2000, Bauer *et al.* 2001, Konstantinou and Albanis 2004, Forgacs *et al.* 2004, Hasnat *et al.* 2005, Toor *et al.* 2006). Because UV penetration in dye solutions is limited due to the highly colored nature of the effluents, the best use of UV technology is a post-treatment after ozonation (Vandervivere 1998).

1.5.3. Biological methods

Bacterial. The ability of bacteria to metabolize azo dyes has been investigated by a number or research groups (Cao *et al.* 1993, McMullan *et al.* 2001, Claus *et al.* 2002, Bhaskar *et al.* 2003). Under aerobic conditions azo dyes are not readily metabolized, although the ability of bacteria with specialized reducing enzymes to aerobically degrade certain azo dyes was reported (Stolz 2001). In contrast, under anaerobic conditions many bacteria reduce azo dyes by the activity of unspecific, soluble, cytoplasmatic reductase, known as azo reductases. The anaerobic reduction degrades the azo dyes that are converted into aromatic amines (Blümel *et al.* 2002), which may be toxic, mutagenic, and possibly carcinogenic to mammalians (Pinhero *et al.* 2004). Therefore, to achieve complete degradation of azo dyes, another stage that involves aerobic biodegradation of the produced aromatic amines is necessary (Haug *et al.* 1991, Seshadri *et al.* 1994, O'Neill *et al.* 2000, Kalyuzhnyi and Sklyar 2000, Lourenço *et al.* 2001, Shaw *et al.* 2002, Isik and Sponza 2003, Isik and Sponza 2004, Libra *et al.* 2004, Supaka *et al.* 2004, Sponza and Isik 2005). Bacterial biodegradation of non-azo dyes has only recently been studied. It has been observed that several bacteria can degrade anthraquinone dyes (Seignez et. 1996, Walker and Weatherley 2000, Fontenot *et al.* 2001). Aerobic decolorization of triphenylmethane dyes has also been demonstrated (Azmi *et al.* 1998, Sarnaik and Kanekar 1999, Sani and Banerjee 1999). In phtalocyanine dyes, reversible reduction and decolorization under anaerobic conditions have been observed (Beydilli *et al.* 2000, Van der Zee 2002).

Fungal. The most widely researched fungi in regard to dye degradation are the ligninolytic fungi. White-rot fungi in particular produced enzymes as lignin peroxidase, manganese peroxidase and laccase that degrade many aromatic compounds due to their non-specific activity (Stolz 2001, Robinson *et al.* 2001b, Hatakka 2001, McMullan *et al.* 2001, Hofrichter 2002, Wesenberg *et al.* 2003, Forgacs *et al.* 2004, Ehlers and Rose 2005, Srebotnik and Boisson 2005, Harazono and Nakamura 2005, Pazarlioglu *et al.* 2005b, Toh *et al.* 2005). Large literature exists regarding the potential of these fungi to oxidize phenolic, non-phenolic, soluble and non-soluble dyes (Field *et al.* 1993, Pasti-Grigsby *et al.* 1992, Chao and Lee 1994, Bumpus 1995, Conneely *et al.* 1999, Kapdan *et al.* 2000, Borchert and Libra 2001, Heinfling-Weidtmann *et al.* 2001, Tekere *et al.* 2001, Kapdan and Kargi 2002, Martins *et al.* 2002b, Libra *et al.* 2003). In particular laccase from *Pleurotus ostreatus,*

Schizophyllum commune, Sclerotium rolfsii and *Neurospora crassa*, seemed to increase up to 25% the degree of decolorization of individual commercial triarylmethane, anthraquinonic, and indigoid textile dyes using enzyme preparations (Abadulla *et al.* 2000). On the contrary, manganese peroxidase was reported as the main enzyme involved in dye decolorization by *Phanerochaete chrysosporium* (Chagas and Durrant 2001) and lignin peroxidase for *Bjerkandera adusta* (Robinson *et al.*, 2001b). Some non-white-rot fungi that can successfully decolorize dyes have also been reported (Kim *et al.* 1995, Kim and Shoda 1999, Cha *et al.* 2001, Abd El-Rahim *et al.* 2003, Ambrósio and Campos-Takaki 2004, Tetsch *et al.* 2005). Fungal degradation of aromatic structures is a secondary metabolic event that starts when nutrients (C, N and S) become limiting (Kirk and Farrel 1987). The influence of the substitution pattern on the dye mineralization rates and between dye structure and fungal dye biodegradability is a matter of controversy (Fu and Viraraghavan 2001). However, these difficulties are even greater if one considers that complex mixed effluents are extremely variable in composition even from the same factory, as is often the case of the textile industry. Other important factors for cultivation of white-rot fungi and expression of ligninolytic activity are the availability of enzyme cofactors and the pH of the environment (Swamy and Ramsay 1999). Although stable operation of continuous fungal bioreactors for the treatment of synthetic dye solutions has been achieved, application of white-rot fungi for the removal of dyes from textile wastewater faces many problems as the nature of synthetic dyes, the control of the produced biomass and the great treating volumes (Palma *et al.* 1999, Nigam *et al.* 2000, Zhang e Yu 2000, Robinson *et al.* 2001b, Mielgo *et al.* 2001, Stolz 2001, Van der Zee 2002).

1.6. Laccases

Enzymes exhibit a number of features that make their use advantageous, as compared to conventional chemical or microbial catalysts such, as the high level of catalytic efficiency, the high degree of specificity and the absence of side-reactions. In addition, enzymes are biodegradable, easily removed from contaminated streams, easily standardized in commercial preparations and generally operate at mild conditions of temperature, pressure, and pH. These characteristics provide substantial process energy savings and reduced manufacturing costs. However, the unstable nature of enzymes, when removed from their natural environment, and the high cost of enzyme isolation and purification still discourages their extensive use, especially in areas which currently have an established alternative procedure. In spite of these disadvantages, the research on enzyme applications is in steady development and the technological problems are constantly being overcome (Chaplin and Bucke 1990). In contrast to the generally high specificity of enzymes, laccases are rather unspecific. Laccase was first discovered by Yoshida in plants (Yoshida 1883). He observed that the latex of the Chinese or Japanese lacquer trees (*Rhus sp.*) was rapidly hardened in the presence of air. The enzyme was named laccase about 10 years later after its isolation and purification (Bertrand 1894). Laccases have been examined since the mid seventies and results are reviewed extensively (Mayer and Staples 2002, Claus 2004).

Laccases (EC 1.10.3.2) are multi–copper oxidases, which catalyze one electron oxidation of a wide range of inorganic and organic substances, coupled with one four-electron reduction of oxygen to water (Xu 1996). Laccases not only catalyze the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols, *ortho*- and *para*-diphenols, but also can oxidize other substrates such as aromatic amines, syringaldazine, and non-phenolic compounds, to form free radicals (Bourbonnais *et al.* 1997, Li *et al.* 1999, Robles *et al.* 2000). After long reaction times there can be coupling reactions between the reaction products and even polymerization. It is known that laccases can catalyze the polymerization of various phenols and halogen, alkyl- and alkoxy-substituted anilines (Hoff *et al.* 1985, Kobayashi *et al.* 2001, Kobayashi

and Higashimura 2003). In soils, natural and xenobiotic phenolics or aromatic amines can thus be bound to the organic humic matrix. In the case of substituted compounds, the reaction can be accompanied by partial demethylation and dehalogenation (Durán and Esposito 2000). In higher plants, the cross-linking of phenolic precursors by laccases is one part in the lignification process. Only recently has positively been demonstrated that plant laccases are able to polymerize monolignols within the plant cell wall matrix, in the complete absence of peroxidase (Sterjiades *et al.* 1992, Liu *et al.*1994, Richardson *et al.* 2000). These studies show that laccases are involved only in the early stages of lignification, while peroxidases are involved later. However, a definitive conclusion on the role of laccase in the lignification process remains an unsolved matter (Bao *et al.* 1993, Wallace and Fry 1999, Boudet 2000). Among other roles, laccase can protect the fungal pathogen from the toxic phytoalexins and tannins in the host environment (Pezet *et al.* 1992, Johansson *et al.* 1999, Brasier and Kirk 2001, Pipe *et al.* 2000). Some fungal secreted laccase act as a detoxifying enzyme to protect the fungus from toxic metabolites (Schouten *et al.* 2002, Gil-ad *et al.* 2000, Gil-ad *et al.* 2001, VanEtten *et al.*, 2001, Schoonbeek *et al.* 2001), and to reduce lignification activities by the host (Bar-Nun Tal *et al.* 1988, VanEtten *et al.* 1994). In insects, the laccase-catalyzed oxidative coupling of catechols with proteins may be involved in cuticle sclerotization (Kramer *et al.* 2001). Laccases are also involved in the degradation of complex natural polymers, such as lignin or humic acids (Claus and Filip 1998). The reactive radicals generated lead to the cleavage of covalent bonds and to the release of monomers. Because of steric hindrance, the enzyme might not come directly into contact with the polymers. However, small organic compounds or metals (mediators) can also be oxidized and activated by laccases and degrade the substrate (Claus *et al.* 2002). Laccases in both free and immobilized form, as well as in organic solvents, have found various biotechnological and environmental applications, such as analytical tools-biosensors for phenols, development of oxygen cathodes in biofuel cells, textile dye degradation, organic synthesis, immunoassays labeling and delignification, demethylation, and thereby bleaching of craft pulp (Bourbonnais and Paice 1992, Bourbonnais *et al.* 1995, Ghindilis *et al.* 1995, Xu 1996, Gardiol *et al.* 1996, Bourbonnais *et al.* 1997, Call and Mucke 1997, Schneider and Pedersen 1998, Schneider and Pedersen 1998, Li *et al.* 1999, Hublik and Schinner 2000, Lante *et al.* 2000, Durán and Esposito 2000,

Kuznetsov *et al.* 2001, Barton *et al.* 2001, Mayer and Staples 2002, Haghighi *et al.* 2003, Karamyshev *et al.* 2003, Wesenberg *et al.* 2003, Martins *et al.* 2003, Blanquez *et al.* 2004, Maximo and Costa-Ferreira 2004, Novotny *et al.* 2004, Camarero *et al.* 2004, Ciecholewski *et al.* 2005). Laccase in nature can be found in eukaryotes, as fungi, plants and insects (Mayer and Staples 2002). However in the last years there is an increasing evidence for the existence in prokaryotes of proteins with typical features of the multi-copper oxidase enzyme family (Alexandre and Zhulin 2000, Claus 2003). Corresponding genes have been found in gram-negative and gram-positive bacteria, including species living in extreme habitats (Freeman *et al.* 1993, Givaudan *et al.* 1993, Claus and Filip 1997, Sanchez-Amat and Solano 1997, Diamantidis *et al.* 2000, Sanchez-Amat *et al.* 2001, Endo *et al.* 2002, Suzuki *et al.* 2003). Very recently a laccase-like enzyme activity was found in thermostable spores of different Bacillus strains (Hullo *et al.* 2001, Martins *et al.* 2002a, Hirose *et al.* 2003).

1.7. Molecular and active site properties of laccase

The laccase molecule, as an active holoenzyme form, is a dimeric or tetradimeric glycoprotein, usually containing four copper atoms per monomer, bound to three redox sites (T_1, T_2 and T_3 Cu pair). The molecular mass of the monomer ranges from about 50 to 100 kDa. An important feature is a covalently linked carbohydrate moiety (10–45%), which may contribute to the high stability of the enzyme (Durán *et al.* 2002).

The four Cu atoms differ from each other in their characteristic electronic paramagnetic resonance (EPR) signals. For the catalytic activity a minimum of four copper atoms per active protein unit is needed. One belongs to the paramagnetic "blue" T_1 copper site that has a strong electronic absorbance at 610 nm. Another belongs to the T_2 paramagnetic 'non-blue' copper site. The other two belong to the diamagnetic spin-coupled copper-copper pair type 3 site that has a weak UV absorbance at 330 nm. The T_2 and T_3 copper atoms form a trinuclear cluster site, which is responsible for oxygen binding and its reduction to water. T_2 copper is coordinated by two histidines and T_3 copper pair by six histidines. The strong anti-ferromagnetic coupling between the two T_3 copper atoms is maintained by a hydroxyl bridge (Claus 2004). The function of the T_1 site in this type of enzyme involves electron abstraction from reducing substrates (electron donors) with a subsequent electron transfer to the T_2/T_3 copper cluster (Figure 1.7).

The redox potential of the T_1 site has been determined for many laccases using different mediators and varies from 430 mV for the laccase from *Rhus vernicifera* tree up to 780 mV for fungal laccase from *Polyporus versicolor* (Reinhammar and Vanngard 1971, Reinhammar 1972, Xu *et al.* 1996, Xu *et al.* 1999, Schneider *et al.* 1999, Xu *et al.* 2000, Koroleva *et al.* 2001, Klonowska *et al.* 2002). It was previously found that the catalytic efficiency (k_{cat}/K_m) of laccases for some reducing substrates depended linearly on the redox potential of the T_1 copper, in the sense that the higher the potential of the T_1 site the higher the catalytic efficiency (Xu *et al.* 1996, Xu *et al.* 2000). That is why laccases with a high redox potential of the T_1 site are of special interest in biotechnology, e.g., for efficient bleaching and bioremediation processes (Reinhammar and Vanngard 1971, Reinhammar 1972).

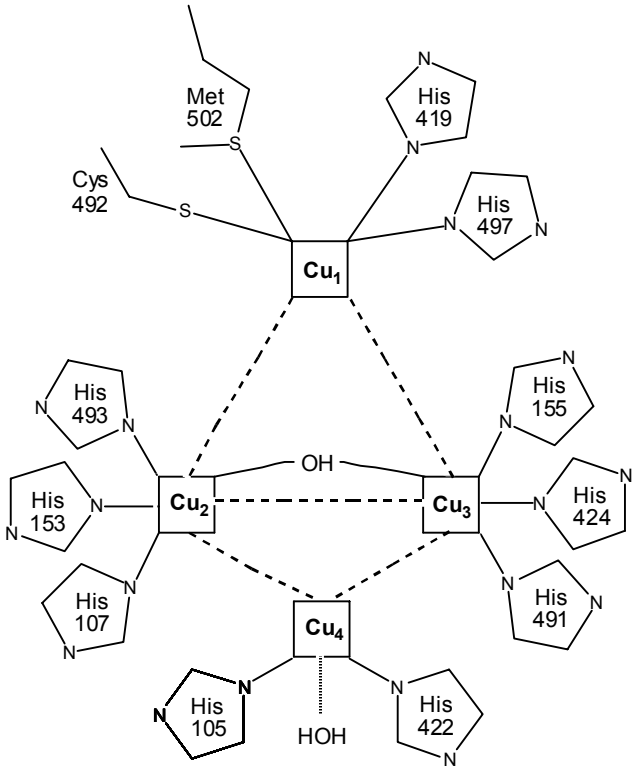


Figure 1.7 - Copper centers of the laccase (adapted from Claus 2004).

Kinetic data of laccases from different sources were reported (Yaropolov *et al.*1994). K_m values are similar for the co-substrate oxygen (about 10^{-5} M), but V_{max} varies with the source of laccase (50–300 M/s). The turnover is heterogeneous over a broad range depending on the source of enzyme and substrate/type of reaction. The kinetic constants differ in their dependence on pH. K_m is pH-independent for both substrate and co-substrate, while the catalytic constant is pH-dependent. Independently on the source, laccase can be very strongly inhibited by many anions, which are able to interact with the copper sites like azide, cyanide, thiocyanide and fluoride. Complexing agents removing copper from the active site exert a reversible activity inhibition. Activities of current interest include screening of laccase sources, studying new laccases (Shin and Kim 1998, Koroljova-Skorobogatko *et al.* 1998, Shin and Lee 2000, Smirnov *et al.* 2001, Kumar *et al.* 2003, Xu *et al.* 2000, Koroleva *et al.* 2001, Klonowska *et al.* 2002, Martins *et al.* 2002a, Palmer et al, 2003), investigating the structure of the enzyme (Antorini *et al.* 2001, Hakulinen *et al.* 2002, Piontek *et al.* 2002, Enguita *et al.* 2003), elucidating

the mechanism of the internal electron transfer as well as the mechanism of oxygen reduction to water (Lee *et al.* 2002, Palmer *et al.* 2002), investigating the electrochemical properties of laccases (Johnson *et al.* 2003, Christenson *et al.* 2004) among others. In Table 1.2 some important properties of laccase, in general and from *Trametes* in detail, are summarized.

Table 1.2 - Some properties of laccases in general and from *Trametes* laccase (adapted from Call and Mücke 1997)

Property	Range of laccases	*Trametes* laccase
pH-Optimum	3.0–7.5	3.6 – 5.3
Temperature-Optimum (°C)	40–80	60
Molecular mass (kDa)	60–390	60–65
Copper content (atoms per molecule)	2-16	4
Redox potential (mV)	180–800 (T_1 in different proteins, not only laccase)	T_1 (pH 5.5) 785 T_3 (pH 5.5) 782
Number of isoenzymes	Up to 5	Two or three chromatographic forms; different genes
Isoelectric points	2.6–7.6	3.1, 3.3, 4.6–6.8
Inhibitors	CN-; N3 -; F -; other halides and anions; pH (formation of Cu (II) OH- complex), Dithioethylcarbamic acid, thioglycolic acid, phenylthiourea EDTA, coniferyl alcohol	
Reactions catalyzed	Demethylation, demethoxylation decarboxylation, formation of phenoxy radicals, Cα-Cβ cleavage, alkylaryl cleavage, Cα-oxidation (in β-1-lignin model substrates)	

1.8. Catalytic mechanism of Laccase

The mechanism of electron transfer and the mechanism of dioxygen reduction to water are not fully understood for laccase. However, a number of mechanistic schemes have been proposed (Figure 1.8), which are consistent with the kinetic and structural data currently available (Shleev *et al.* 2005). In the catalytic cycle of laccase, the substrate reduces the T_1 site, which in turn transfers the electron to the trinuclear cluster. Two possible mechanisms for the reduction of the trinuclear cluster are possible. The T_1 and T_2 sites together reduce the T_3 pair (A in Figure 1.8) or each copper in the trinuclear cluster is sequentially reduced by electron transfer from T_1 site (B in Figure 1.8), in which case the T_3 no longer acts as a two-electron acceptor. Slow decay of the "native intermediate" leads to the resting fully oxidized form. In this form, the T_1 site can still be reduced by substrate, but electron transfer to the trinuclear site is too slow to be catalytically relevant (Solomon *et al.* 1996). The structural model of bridging between the T_2 and T_3 has provided insight into the catalytic reduction of oxygen to water (Cole *et al.* 1990, Sundaran *et al.* 1997, Palmer *et al.* 1999). It has been elucidated that the T_2 copper is required for the reduction of oxygen since bridging to this center is involved in the stabilization of the peroxide intermediate (Cole *et al.* 1990). Reduction of oxygen by laccase appears to occur in two $2e^-$ steps. In this T_2/ T_3 bridging mode for the first $2e^-$ reduced, the peroxide-level intermediate would facilitate the second $2e^-$ reduction (from the T_2 and T_1 centers) in which the peroxide is directly coordinated to reduce T_2 copper, and the reduced T_1 is coupled to the T_3 by the covalent Cys–His linkages (Clark and Solomon 1992). This demonstrates that the T_2/ T_3 trinuclear Cu site represents the active site for binding and multi-electron reduction of dioxygen. T_1 Cu is clearly not necessary for reactivity with dioxygen, and in its absence, an intermediate is formed which shares some properties with the oxygen intermediate in native laccase.

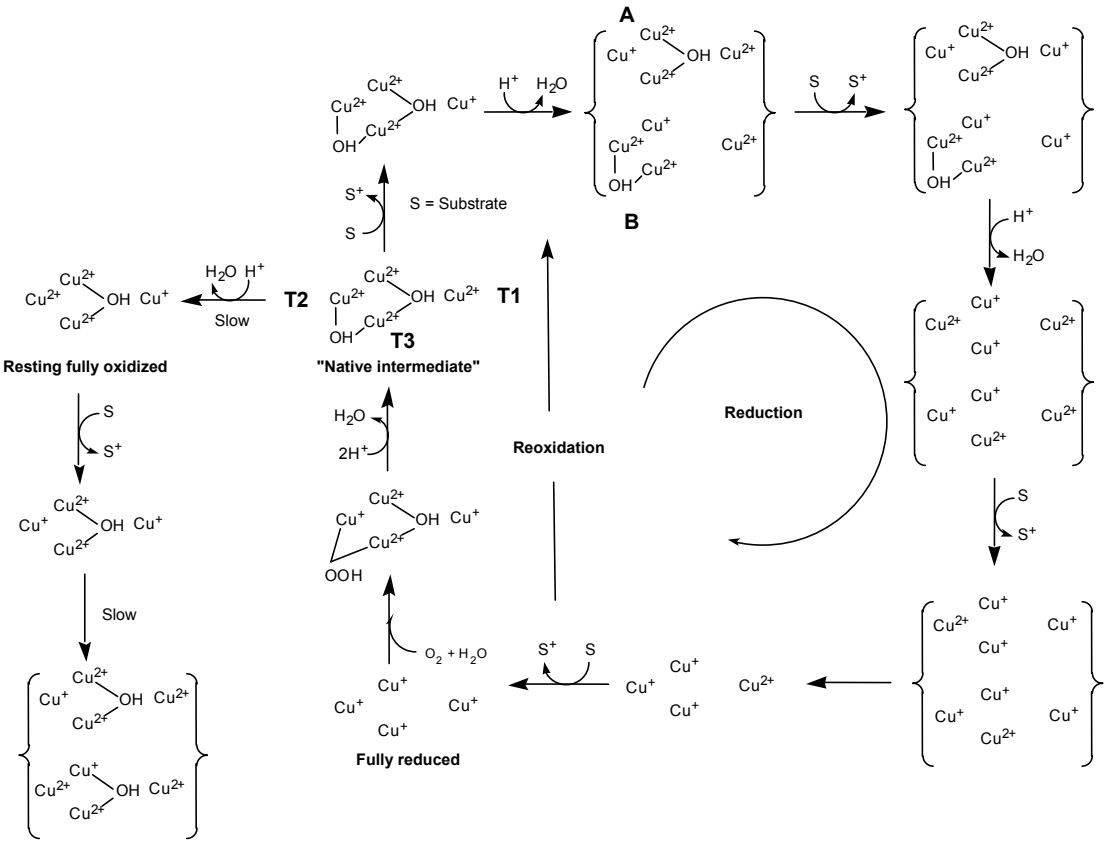


Figure 1.8 - Proposed catalytic cycle of laccase showing the mechanism for reduction and oxidation of the copper sites (adapted from Shleev *et al.* 2005).

1.9. Laccase mediators

Due to the random polymer nature of lignin and to the laccase lower redox potential, with respect to other ligninolytic enzymes, laccase can oxidize only phenolic fragments of lignin (Kersten *et al.* 1990, Evans and Hedger 2001). Small natural low-molecular weight compounds with high redox potential (>900 mV) called mediators may be used to oxidize the non-phenolic residues from the oxygen delignification (Eggert *et al.* 1996). In the last years the discovery of new and efficient synthetic mediators extended the laccase catalysis towards xenobiotic substrates (Bourbonnais and Paice 1990, Hammel and Moen 1991, Bourbonnais and Paice 1992, Hammel 1996, Eggert *et al.* 1996, Bourbonnais *et al.* 1997, Kuhad *et al.* 1997, Crestini and Agryropoulos 1998, Van Aken and Agathos 2001, Van Aken and Agathos 2002, Camarero *et al.* 2005). A mediator is a small molecule that acts as a sort of 'electron shuttle': once it is oxidized by the enzyme generating a strongly oxidizing intermediate, the co-mediator (Med_{ox}), it diffuses away from the enzymatic pocket and in turn oxidizes any substrate that, due to its size, could not directly enter the enzymatic pocket (Figure 1.9) (Banci *et al.* 1999).

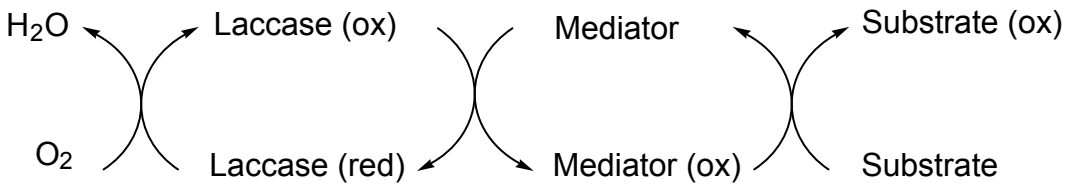


Figure 1.9 - Catalytic cycle of a laccase-mediator oxidation system (reproduced from Banci *et al.* 1999).

Alternatively, the oxidized mediator could rely on an oxidation mechanism not available to the enzyme, thereby extending the range of substrates accessible to it (Hildén *et al.* 2000). It is therefore of primary importance to understand the nature of the reaction mechanism operating in the oxidation of a substrate by the Med_{ox} species derived from the corresponding mediator investigated. In the laccase-dependent oxidation of non-phenolic substrates, previous evidence suggests an electron-transfer (ET) mechanism with mediator ABTS,

towards substrates having a low oxidation potential. Alternatively, a radical hydrogen atom transfer (HAT) route may operate with N-OH-type mediators, if weak C-H bonds are present in the substrate (Cantarella *et al.* 2003).

Over 100 possible mediator compounds have been described but the most commonly used are the azine 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and the triazole 1-hydroxybenzotriazole (HBT) (Figure 1.10) (Bourbonnais *et al.* 1995, Bourbonnais *et al.* 1997, Camarero *et al.* 2005). Various laccases readily oxidize ABTS, by free radicals, to the cation radical $ABTS^{+\cdot}$ and the concentration of the intensely colored, green-blue cation radical can be correlated to the enzyme activity. It is well known that cation radicals represent an intermediate oxidation step in the redox cycle of azines and, upon extended oxidation and abstraction of the second electron, the corresponding dications can be obtained. The redox potentials of $ABTS^{+\cdot}$ and $ABTS^{2+}$ were estimated as 0.680 V and 1.09 V respectively (Scott *et al.* 1993).

1-Hydroxybenzotriazole (HBT) belongs to the *N*-heterocyclics coumpounds bearing *N*–OH–groups mediators (Call 1994). Consuming oxygen HBT is converted by the enzyme into the active intermediate, which is oxidized to a reactive radical ($R–NO^{\cdot}$) (Bourbonnais *et al.* 1997).

Mediated laccase catalysis has been used in a wide range of applications, such as pulp delignification (Bourbonnais and Paice 1996, Call and Mücke 1997, Crestini and Argyropoulos 1998, Sealey and Ragauskas 1998, Li *et al.* 1999), textile dye bleaching (Schneider and Pedersen 1995, Wesenberg *et al.* 2003, Camarero *et al.* 2005), polycyclic aromatic hydrocarbon degradation (Johannes *et al.* 1996, Majcherczyk *et al.* 1998), pesticide or insecticide degradation (Amitai *et al.* 1998, Kang *et al.* 2002a), and organic synthesis (Fritz-Langhals and Kunath 1998, Potthast *et al.* 1996). In paper and pulp industry, novel enzymatic bleaching technologies are attracting increasing attention because of concerns regarding the environmental impact of the chlorine-based oxidants currently being used in delignification or bleaching (Paice *et al.* 1989, Fujita *et al.* 1991, Bourbonnais and Paice 1996, Call and Mücke 1997, Balakshin *et al.* 2001, Camararero *et al.* 2004, Sigoillot *et al.* 2005).

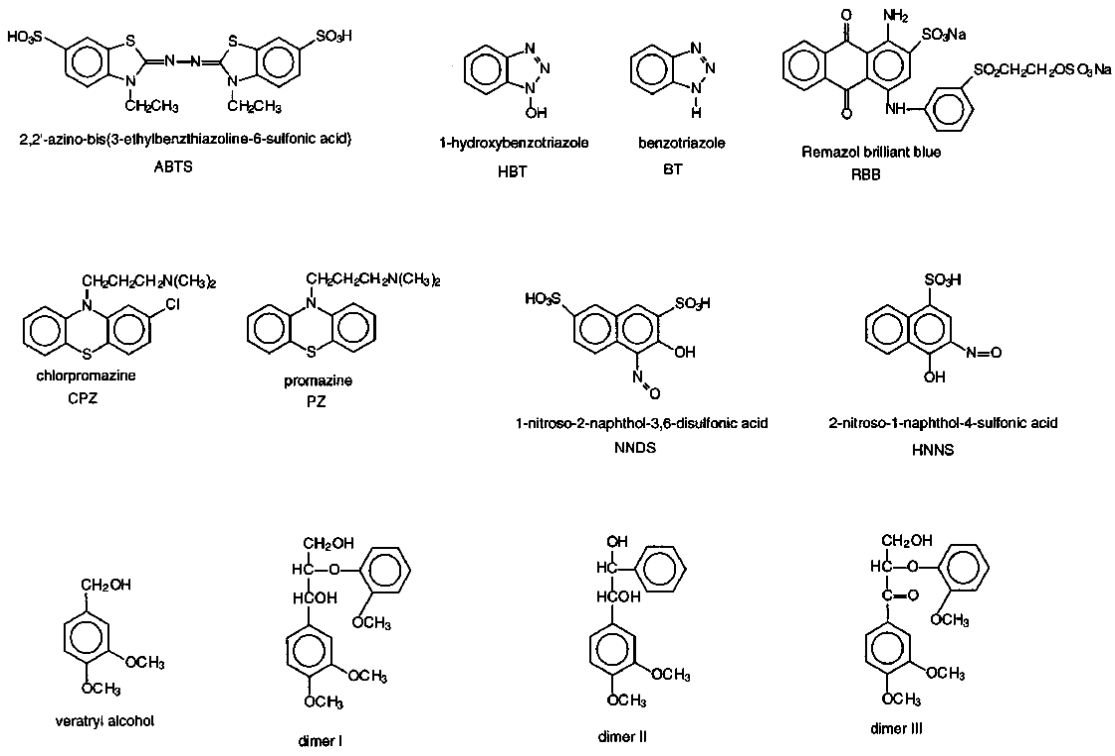


Figure 1.10 – Some natural and synthetic mediators (reproduced from Bourbonnais *et al.* 1997).

1.10. Laccase immobilization

Enzymes exhibit a number of features that make their use advantageous as compared to conventional chemical catalysts. However, a number of practical problems exist that reduce their operational lifetime, such as their high cost of isolation and purification, their non-reusability, the instability of their structures and their sensitivity to process conditions. Many of these undesirable limitations may be overcome by the use of immobilized enzymes (Taylor 1991). Immobilization is achieved by fixing enzymes to or within solid supports, as a result of which heterogeneous immobilized enzyme systems are obtained. By mimicking the natural mode of occurrence in living cells, where enzymes for the most cases are attached to cellular membranes, the systems stabilize the structure of enzymes, hence their activities. In the immobilized form enzymes are more robust and more resistant to environmental changes allowing easy recovery and multiple reuse (Krajewska 2004). Compared with the free enzyme, the immobilized enzyme has usually its activity lowered and the Michaelis constant increased (Durán *et al.* 2002). These alterations result from structural changes introduced to the enzyme by the applied immobilization procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution. Enzymes may be immobilized by a variety of methods (Table 1.3) mainly based on chemical and/or physical mechanisms. Since the methods for the immobilization procedures greatly influence the properties of the resulting biocatalyst, immobilization strategy determines the process specifications for the catalyst (Hartmeier 1988).

Laccase immobilization was extensively studied with a wide range of different methods and substrates (Durán *et al.* 2002, Haghighi *et al.* 2003, Tarasevich *et al.* 2003, Krajewska 2004, Moeder *et al.* 2004, Dodor *et al.* 2004, Quan and Shin 2004, Kandelbauer *et al.* 2004, Kiiskinen *et al.* 2004, Ehlers and Rose 2005, Pazarlioglu *et al.* 2005b, Delanoy *et al.* 2005, Mazmanci and Ünyayar 2005). The adsorption of chromophoric-oxidized products on the surface of the immobilization support often leads to enzyme inactivation phenomena (Peralta-Zamora *et al.* 1999b, Cordi *et al.* 2000a, Cordi *et al.* 2000b, D'Annibale *et al.* 1999, D'Annibale *et al.* 2000).

Table 1.3 - Principal immobilization methods for enzymes (adapted from Scouten *et al.* 1995)

Method	Advantages	Disadvantages
Adsorption on insoluble matrices (e.g. by van der Waals forces, ionic binding or hydrophobic forces)	Simple, mild conditions, less disruptive to enzyme protein	Enzyme linkages are highly dependent on pH, solvent and temperature
Entrapment in a gel (eventually behind a semipermeable membrane)	Universal for any enzyme, mild procedure	Large diffusional barriers, loss of enzyme activity by leakage, possible denaturation of the enzyme molecules as a result of free radicals
Crosslinking by a multifunctional reagent (such as glutaraldehyde his-isocyanate derivatives or bis-diazobenzidine)	Simple procedure, strong chemical binding of the biomolecules; widely used in stabilizing physically adsorbed enzymes or proteins that are covalently bound onto a support	Difficult to control the reaction, requires a large amount of enzyme, the protein layer has a gelatinous nature (lack of rigidity), relatively low enzyme activity
Covalent bonding onto a membrane, insoluble supports	Stable enzyme-support complex, leakage of the biomolecule is very unlikely, ideal for mass production and commercialization	Difficult and time-consuming: possibility of activity losses due to the reaction involving groups essential for the biological activity (can be minimized by immobilization in the presence of the substrate or inhibitor of the enzyme)

The formation of insoluble laccase reaction products, due to non-enzymatic reactions is another important technical limitation. A prolonged and repeated use of immobilized laccase results in the accumulation of a precipitate on the outlet filter of the reactor (fouling), leading to significant reductions in the flow rates. A recent and particularly promising approach is to combine the use of immobilized laccase with cationic polymers, such as chitin and chitosan cross-linked with epichlorohydrin, which are able to promote the coagulation of oxidized reaction products (Wada *et al.* 1995, Krajewska 2004). Several *Trametes* laccase as well as several supports and immobilization methods are summarized in table 1.4.

Table 1.4 - *Trametes* laccases immobilized on different supports (adapted from Durán *et al.* 2002)

Origin	Substrate	Support	Immobilization
Trametes hirsuta	Textile dyes, effluent	Alumina	Adsorption
Trametes sp.	Phenols, catechins	Porous glass beads	Covalent-aminopropyltriethoxysilane (APTES)-glutaraldehyde (GLUTAL)
Trametes versicolor	Syringaldazine, ferulic acid, sinapic acid, phenols, catechols, 2,6-dimethyl phenol, 2,4-dichlorophenol, amines, azide, 4-Methyl-3-hydroxy anthranilic acid, Phenylurea pesticide	Porous glass, kaolinite, carbon fibers, gels, montmorillonite, sepharose, osmium, resin, vitroceramics, redox hidrogel, polyacrilamide, polyvinylidenefluoride	Covalent-APTES-GLUTAL, adsorption, entrapped, covalent hydroxysuccinimide, ester derivatives cross-linking agarose gel, adsorption-polyethyleneimine/GLUTAL, reverse micelles
Trametes villosa	Textile dyes, effluent	Alumina	Covalent-APTES-GLUTAL

1.11. Laccase applications

1.11.1. Dye degradation

Real textile effluents are extremely variable in composition since they contain not only dyes but also salts, sometimes at very high ionic strength and extreme pH values, chelating agents, precursors, by-products and surfactants that can inhibit enzyme activity and thereof decolorization (Abadulla *et al.* 2000). Therefore, decolorization of textile effluents requires an appropriate choice of the type of enzyme as well as of reactor environment (Wesenberg *et al.* 2003). The capability of laccases to act on chromophore compounds such as azo, triarylmethane, anthraquinonic and indigoid dyes leads to the suggestion that they can be applied in industrial decolorization processes (Damsus *et al.* 1991, Pedersen and Schmidt 1992, Pedersen and Kierulff 1996, Morita *et al.* 1996, Abadulla *et al.* 2000, Kirby *et al.* 2000, Chagas and Durrant 2001, Robinson *et al.* 2001b, Jarosz-Wilkolazka *et al.* 2002, Peralta-Zamora *et al.* 2003, Wesenberg *et al.* 2003, Martins *et al.* 2003, Blanquez *et al.* 2004, Maximo and Costa-Ferreira 2004, Novotny *et al.* 2004). Recent studies propose several degradation mechanisms for phenolic and non-phenolic azo dyes (Chivukula and Renganathan 1995, Soares *et al.* 2002). In the proposed model azo dyes are degraded without direct cleavage of the azo bond through a highly non-specific free radical mechanism forming phenolic type compounds, thereby avoiding the formation of toxic aromatic amines, which might be useful to control environmental pollution (Wong and Yu 1999, Gianfreda *et al.* 1999). However, some substrate specificity can be found in laccase reactions, which limits the number of azo dyes that can be degraded. To solve this problem laccase/mediator systems are normally used to broaden the range of azo dyes and to increase the decolorization rates (Bourbonnais *et al.* 1997, Srebotnik and Hammel 2000, Fabbrini *et al.* 2002, Rodríguez Couto *et al.* 2005, Camarero *et al.* 2005). However, the capacity to evaluate the laccase degradation potentials remains incomplete since there is not a complete knowledge on dye decolorization pathways, dye mineralization mechanisms and formation of potentially toxic accumulating intermediates. Small differences in dye electron distribution, charge density and steric factors can affect enzymatic decolorization (Wesenberg *et al.* 2003).

1.11.2. Bioremediation

In addition to the previously discussed dye degradation, laccases have also shown to be useful for the removal of toxic compounds through oxidative enzymatic coupling of the contaminants, leading to insoluble complex structures (Dawel *et al.* 1997, Wang *et al.* 2002). The degradation of a variety of persistent environmental pollutants, in particular phenols, was also observed. Phenolic compounds are present in wastes from several industrial processes, as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others (Aggelis *et al.* 2003). Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants (Hublik and Schinner 2000, Ehlers and Rose 2005). Laccase was found to be responsible for the transformation of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone (Leontievsky *et al.* 2000). Laccases from white-rot fungi have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene in the presence of HBT and ABTS as mediators (Niku and Viikari 2000, Bressler *et al.* 2000). Isoxaflutole is an herbicide activated in soils and plants to its diketonitrile derivative, the active form of the herbicide. Laccases are able to convert the diketonitrile into the acid (Mougin *et al.* 2000).

1.11.3. Delignification and pulp bleaching

In the industrial preparation of paper the separation and degradation of lignin in wood pulp are conventionally obtained using ClO_2 and O_3. Oxygen delignification process has been industrially introduced in the last years to replace conventional and polluting chlorine-based methods. In spite of this new method, the pre-treatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose (Barreca *et al.* 2003, Sigoillot *et al.* 2005, Gamelas *et al.* 2005). Lignocellulose is a common substrate for laccase and the laccase ability to break down non-phenolic ligno-cellulose is provided by certain phenolic compounds acting as mediators (Bourbonnais *et al.* 1997). More recently, the potential of this enzyme for cross-linking and functionalizing lignaceous compounds was discovered.

Laccases can be used for binding fiber-, particle- and paper-boards (Gübitz and Cavaco-Paulo 2003). However, different wood-decaying basidiomycetes have shown a highly variable pattern of laccase formation, and this subject requires more detailed experiments (Mayer and Staples 2002).

1.11.4. Organic synthesis

Recently, increasing interest has been focused on the application of laccase as a new biocatalyst in organic synthesis (Milstein *et al.* 1989, Mayer and Staples 2002). Laccase provided an environmentally benign process of polymer production in air without the use of hydrogen peroxide (Kobayashi and Higashimura 2003, Mita *et al.* 2003). Laccase-catalyzed cross-linking reaction of new urushiol analogues for the preparation of "artificial urushi" polymeric films (Japanese traditional coating) was demonstrated (Ikeda *et al.* 2001). More recently, the potential of this enzyme for crosslinking and functionalizing lignaceous compounds was discovered (Grönqvist *et al.* 2003). It is also mentioned that laccase induced radical polymerization of acrylamide with or without mediator (Ikeda *et al.* 1998). It has also been used for the chemo-enzymatic synthesis of lignin graft-copolymers (Gübitz and Cavaco-Paulo 2003). Laccases are also known to polymerize various amino and phenolic compounds (Ikeda *et al.* 1996, Aktas *et al.* 2000, Aktas and Tanyolaç 2003, Karamyshev *et al.* 2003, Güreir *et al.* 2005). The ability of laccases to generate color "in situ" from originally non-colored low-molecular substances makes their use an alternative to the conventional dyeing processes (Barfoed *et al.* 2001, Pilz *et al.* 2003). These abilities of laccase for the synthesis of new compounds can be also used for surface modifications of the fabrics. The enzymatic modification and dyeing processes can be applied in several natural substrates like cotton, sisal, wool, flax and wood (Tzanov *et al.* 2003a). Recently, to improve the production of fuel ethanol from renewable raw materials, laccase was expressed in *Saccharomyces cerevisiae* to increase its resistance to phenolic inhibitors in lignocellulose hydrolyzates (Larsson *et al.*, 2001).

1.11.5. Wine and beer stabilization

Wine stabilization is one of the main applications of laccase in the food industry as alternative to physical-chemical adsorbents (Minussi *et al.* 2002). Musts and wines are complex mixtures of different chemical compounds, such as ethanol, organic acids (aroma), salts and phenolic compounds (color and taste). Polyphenol removal must be selective to avoid an undesirable alteration in the wine's organoleptic characteristics. Laccase presents some important requirements when used for the treatment of polyphenol elimination in wines, such as stability in acid medium and reversible inhibition with sulphite (Plank and Zent 1993, Servili *et al.* 2000, Tanrıöven and Eksi 2005). Laccases are also used to improve storage life of beer. Haze formation in beers is a persistent problem in the brewing industry. Nucleophilic substitution of phenolic rings by protein sulphydryl groups may lead to a permanent haze that does not re-dissolve when warmed. As an alternative to the traditional treatment to remove the excess of polyphenols, laccase could be added to the wort. (Mathiasen 1995, Minussi *et al.* 2002)

1.11.6. Food improvement

The flavor quality of vegetable oils can be improved with laccase by eliminating dissolved oxygen (Petersen and Mathiasen 1996). Laccase can also deoxygenate food items derived partly or entirely from extracts of plant materials. Cacao was soaked in solutions containing laccase, dried and roasted in order to improve the flavor and taste of cacao and its products (Takemori *et al.* 1992). The reduction of odors with laccase is documented in the literature (Tsuchiya *et al.* 2000). Treatment with a fungal laccase can also be performed to enhance the color of a tea-based product (Bouwens *et al.* 1999). It is also used to perform the cross-link of ferulic acid and sugar beet pectin through oxidative coupling to form gels for food ingredients (Micard and Thibault 1999). Various enzymatic treatments have been proposed for fruit juice stabilization, among which the use of laccase (Piacquadio *et al.* 1998, Minussi *et al.* 2002, Alper and Acar 2004). Laccase is added to the dough used for producing baked products, to exert an oxidizing effect on the dough constituents and to

improve the strength of gluten structures in dough and/or baked products (Minussi *et al.* 2002, Figueroa-Espinoza *et al.* 1999, Labat *et al.* 2001)

1.11.7. Textile finishing

Laccase is used in commercial textile applications to improve the whiteness in conventional bleaching of cotton and recently biostoning (Tzanov *et al.* 2003a). Cellulases were used to partially replace the load of pumice stones and laccases could bleach indigo dyed denim fabrics to lighter shades (Campos *et al.* 2001, Pazarlioglu *et al.* 2005a).

1.11.8. Biosensors

A biosensor is an integrated biological-component probe with an electronic transducer, thereby converting a biochemical signal into a quantifiable electrical response that detects, transmits and records information regarding a physiological or biochemical change (D'Souza 2001). A number of biosensors containing laccase have been developed for immunoassays, glucose determination, aromatic amines and phenolic compound determinations (Simkus *et al.* 1996, Bauer *et al.* 1999, Huang *et al.* 1999, Ghindilis 2000, Freire *et al.* 2002, Gomes *et al.* 2004).

1.11.9. Medical applications

Laccase can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, sedatives, etc. (Nicotra *et al.* 2004). Recently a new enzymatic method based on laccase has been developed to distinguish morphine from codeine simultaneously in drug samples injected into a flow detection system (Bauer *et al.* 1999).

1.12. Research objectives and thesis outline

The aim of this thesis is not only to study the dye degradation mechanism by laccase, kinetic properties, and operative azo dye degradation conditions but also all the aspects involved in a potential industrial application of laccase in order to reduce the waste of enzyme, and encourage its extensive use. Other important objectives of this thesis are to show the limitation of the direct laccase-catalyzed azo dye degradation and to highlight the laccase-catalyzed polymerization reactions as an alternative methodology for effluent bioremediation.

The first part of the thesis (Chapter 1) presents the state of the art on textile dyes, particularly azo dyes, and on laccase enzymes, covering the aspects of wastewater ecotoxicological concerns, dye removal techniques and degradation mechanisms.

Chapter 2 is focused on characterization of laccase through activity assays and voltammetric techniques. The best work conditions for laccase catalysis are established and the voltammetric measurements of the dyes are used to predict the azo dye decolorization ability of *Trametes villosa* laccase.

In chapter 3 stability and dye degradation ability of free and immobilized laccase are investigated. The results suggest that the immobilization technique is important for the control of the catalysis and the economy of the process. However, it is not always beneficial for the stability and the performances of the enzyme.

Chapters 4 and 5 investigate the mechanistic and kinetic features of azo dye degradation. Chapter 4 describes LC/MS analysis on the characterization of degradation products and its role in defining the enzymatic degradation mechanism of phenolic and non-phenolic azo dyes. Chapter 5 investigates the kinetics of the degradation and polymerization reactions, and the role of redox mediators in the enzymatic catalysis. The kinetic parameters, obtained from amperometric methodologies, are expressed through the Michaelis-Menten equation. This part of the thesis proposes an alternative dye removal methodology, based on the laccase property to catalyze polymerization of some compounds. It is suggested the possibility of removing the degradation

products by filtration of the precipitate, optimizing and amplifying the laccase catalyzed polymerization conditions.

Chapter 6 proposes an alternative laccase application for the recycling of dyeing effluents. The laccase's properties to catalyze polymerization and coupling reactions with phenolic compounds were used in the effluents to synthesize new dyes. The resulting dyes were used to dye wool.

Finally, in chapter 7, the findings of the previous chapters are organized in the general conclusions and suggestions for future work are given.

"An expert is a person who has made all the mistakes that can be made in a very narrow field."

Niels Bohr

2

Use of redox potential in predicting azo dye biodegradation with a *Trametes villosa* laccase

2. Use of redox potential in predicting azo dye biodegradation with a *Trametes villosa* laccase

2.1. Introduction

The improvement of rapid and cheap methods for predicting the potential of enzymatic dye biodegradation in effluents is important to promote enzyme industrial applications and to reduce enzyme waste. The question targeted in this chapter is whether the redox potential of azo dyes is a preliminary tool to predict the decolorization capacity of oxidative and reductive biocatalysts. The ability of the bio-agents to degrade azo dyes depends on the structural characteristics of the dye, temperature and pH of treatment, presence of intermediates, and difference between the redox potentials of the biocatalyst and the dye (Xu 1996, Goyal *et al.* 1998, Xu *et al.* 2001,). Two biological approaches for biodegradation under aerobic conditions of azo sulfonated dyes are performed: the *ascomycete* yeast *Issatchenkia occidentalis* with reducing activity and an oxidative *Trametes villosa* laccase enzyme with or without 1-hydroxybenzotriazole (HBT) as mediator. These two processes have been compared on the basis of the electrochemical properties of dyes and bio-agents.

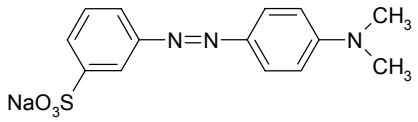
2.2. Materials and methods

2.2.1. Enzyme characterization

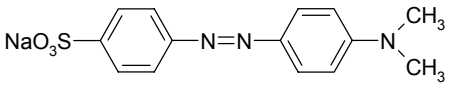
Trametes villosa laccase (EC 1.10.3.2) (5.3 mg protein/ml, 600 U/ml, supplied by Novo Nordisk, Denmark) activity was determined using ABTS [2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] as substrate (Leonowicz *et al.* 1988). The amount of protein was determined using the Bradford method (Bradford 1976). The reaction mixture contained 0.5 mmol ABTS and 1 ml of sample, diluted in 0.1 M sodium acetate (pH 5), in a total volume of 2 ml. Oxidation of ABTS was followed spectrophotometrically at 420 nm. The enzyme activity was calculated using the molecular extinction coefficient of $3.6*10^4$ 1/(mM*cm) and expressed in µmol/min. The temperature profile was calculated in 0.1 M Na-acetate buffer at pH 5 in the temperature range 30-70 ºC. The pH profile was studied with a Britton-Robinson universal buffer (constant ionic strength type) in the range of pH 2-9 and in these experiments the temperature was set at 45 ºC. Due to the inhibitory effect of the universal buffer on the laccase activity, another experiment with different buffers was performed. The 0.1 M buffer systems used in these experiments were tartaric acid-NaOH (pH 2-3.5), acetic acid-NaOH (pH 3.5-5.5), phosphoric acid-NaOH (pH 5.5-7.5), tris-HCl (pH 7.5-9).

2.2.2. Dyes and reagents

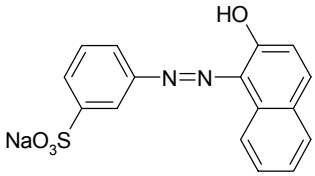
The structure of dyes and mediator tested in the present work are depicted in Figure 2.1. Dyes I and III (minimum 90% dye content) were synthesized by the conventional method of coupling the diazonium salt of methanilic acid with either N,N-dimethyl-p-phenylenediamine or 1-amino-2-naphtol (Furniss *et al.* 1989). The structures of the isolated dyes, as sodium salts, were confirmed by ^{1}H NMR spectroscopy in dimethylsulfoxide (DMSO). All other reagents and dyes were purchased from Sigma-Aldrich and used without further purification.



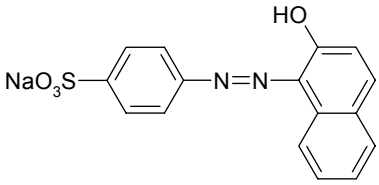
I) 3-(4-dimethylamino-phenylazo)-benzene sulfonic acid sodium salt



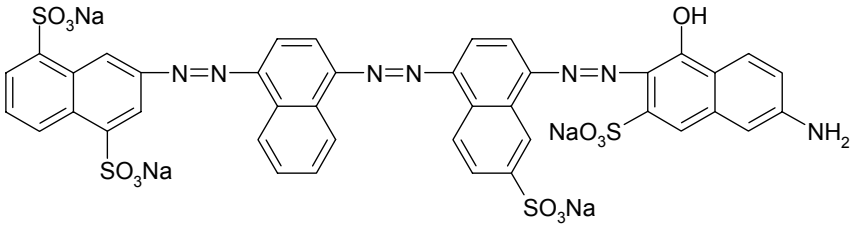
II) Acid Orange 52



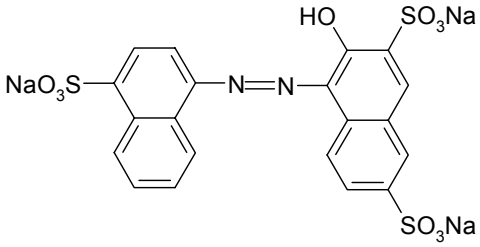
III) 3-(2-hydroxy-naphthalen-1-phenylazo)-benzene sulfonic acid sodium salt



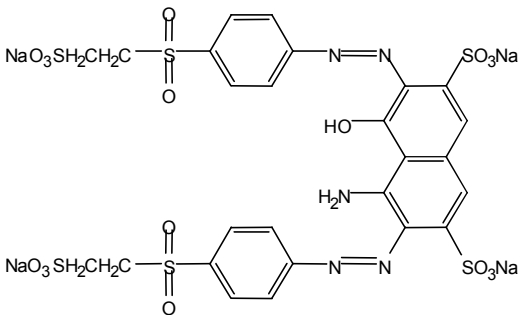
IV) Acid Orange 7



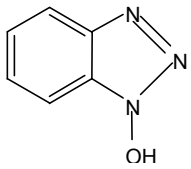
VI) Direct blue 71



V) Acid red 27



VII) Reactive Black 5



HBT – 1-Hydroxybenzotriazole

Figure 2.1 - Dye and mediator structures.

2.2.3. Microorganism

The *ascomycete* yeast *Issatchenkia occidentalis* (Portuguese Yeast Culture Collection 5770), was isolated on the basis of its capacity to decolorize agar plates containing Yeast Extract/Peptone/Glucose 0.5:1:2 (%w/v) and the azo dye Acid orange 7 (dye IV), as described in a previous publication (Martins *et al.* 1999).

2.2.4. Decolorization with laccase and laccase/mediator system

Dye solutions (0.1 mM; 2.5 ml) buffered with 0.1 M Na-acetate buffer, pH 5, were incubated with 20 µl of laccase (5.3 mg protein/ml, 600 U/ml) and 0.5 ml distilled water in a standard stirred cuvette at 45ºC. Dye absorbance was measured at different times during the experiment and the percentage of effluent decolorization was calculated thereof. In the case of experiments with mediator the water volume (0.5 ml) was replaced by 0.1 mM aqueous solution of 1-hydroxybenzotriazole (HBT).

2.2.5. Dye decolorization with microorganism

Decolorization experiments by growing cultures of *I. occidentalis* were typically performed in 250 ml cotton-plugged Erlenmeyer flasks with 100 ml of sterile medium (normal decolorization medium, NDM) containing 2% of glucose, as carbon and energy source, and 0.2 mM of the tested dye, in a mineral salts base, as previously described (Ramalho *et al.* 2002). Dissolved oxygen was measured as oxygen partial pressure using a Clark-type polarographic electrode, with an ATI RUSSEL model RL 400, according to the manufacturer instructions (detection level 0.1 mg/l). The flasks were incubated under orbital shaking (120 rpm) at 26 ºC. Dye concentration was monitored by absorbance readings of centrifuged medium aliquots at the dye λ_{max}. The assay cuvette contained 0.3 ml of 1 M acetate buffer (pH 4.0), sample and

water to 3.0 ml; the blank was prepared with the same dilution of buffer in distilled water.

2.2.6. Electrochemical measurements

Cyclic voltammetry of the azo dyes was performed using a Voltalab 30 Potentiostat (Radiometer Analytical, France), controlled by the Voltamaster 4 electrochemical software, at 100 mV/s scan rate. The working, counter and reference electrodes were respectively: glassy carbon electrode (0.07 cm^2), coiled platinum wire (23 cm) and an Ag|AgCl electrode filled with 3M NaCl, all purchased from BAS, USA. The glassy carbon electrode was successively polished with 5, 1, 0.3 and 0.05 µm alumina polish (Buehler Ltd, USA) and then rinsed with 8 M nitric acid and distilled water before use. The experiments were performed in 0.1 M acetate buffer pH 5 at dye concentration of 0.1% w/v. Prior to analysis all solutions were purged with nitrogen for 15 min. The redox potentials recorded *vs.* Ag|AgCl reference electrode were corrected by 0.206 V to the Normal Hydrogen Electrode (NHE). Redox potentials of *Trametes villosa* laccase*,* 1-hydroxybenzotriazole and nicotinamid adenine dinucleotide phosphate (NADH) were provided from the literature and are as follows: laccase +780 mV, HBT +1.084 mV and NADH -320 mV vs. NHE (Clark 1960, Xu 1997, Bourbonnais *et al.* 1998).

2.3. Results and discussion

2.3.1. Temperature and pH activity profiles

The optimal temperature treatment for laccase at 1 h of incubation is 45 ºC. The optimal temperature was investigated through assays performed in the range of 30 ºC to 70 ºC (Figure 2.2). The optimal pH for laccase is pH 5, but a good activity (90%) is retained in the pH range of 4 to 6. The experiments were performed whit a different type buffer for pH 2 to pH 9. The use of Britton-Robinson buffer with constant ionic strength (μ=0.3 M) induces a severe reduction of the laccase activity but enhances the determination of the optimum pH point (Figure 2.3).

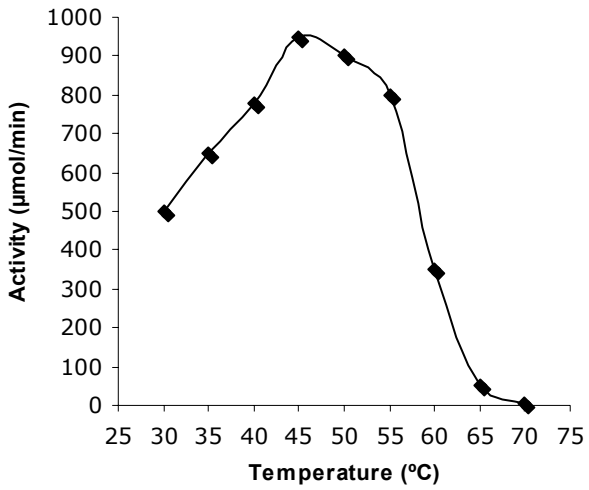


Figure 2.2 – Temperature profile in 0.1 M Na-acetate buffer at pH 5 in the temperature range of 30-70 ºC.

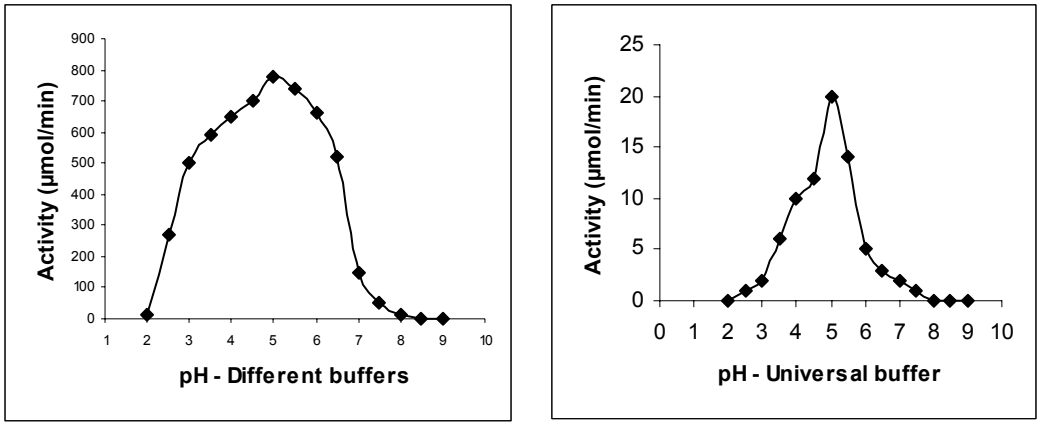


Figure 2.3 - pH profile at 45 ºC in the pH range of 2 - 9 with different buffers and constant ionic strength universal buffer.

2.3.2. Cyclic voltammetry of azo dyes

The azo dyes tested in this study presented similar cyclic voltammograms illustrated by the voltammogram of dye I (Figure 2.4), in both positive and negative scans. In the first positive scan of dye I an irreversible anodic peak (II_a) in the potential range of +0.9 to +1.3 V vs. NHE was observed. All dyes displayed an irreversible reduction peak in the range of -0.13 to -0.48 V vs. NHE (II_r). In the following scans an apparently semi-reversible redox couple (I_a, I_r) was detected. The reductive wave I_r of the semi-reversible redox couple did not appear in the first negative scan. These redox couple peaks appear to be associated with the formation of unstable amine products, which were oxidized in the range of +0.15 to +0.58 V vs. NHE and reduced in the potential range of -0.1 to +0.3 V vs. NHE. The redox peaks II_a and II_r can be associated with irreversible redox reactions leading to cleavage of the azo bonds. In the voltammograms of dyes VI (tri-azo) and VII (bi-azo) the number of oxidation peaks was higher than that observed for monoazo dyes. These peaks resulted from the oxidation of the amine products generated during the disruption of more than one azo bond in these dye molecules. To confirm this theory the cyclic voltagrams of the pure amine product solutions were performed separately. The results peaks could be overlaid respectively to the peaks I and II in the azo dye voltammograms (data not shown).

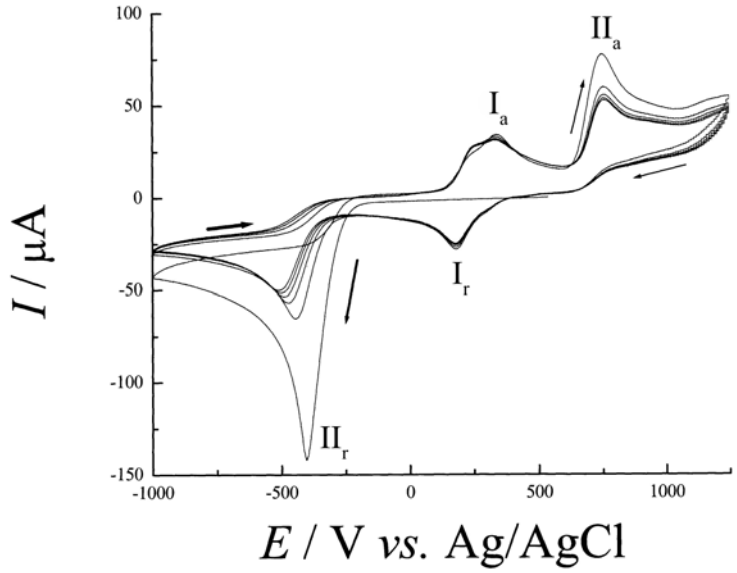


Figure 2.4 - Cyclic voltammogram of dye I: (thin line) positive to negative, (thick line) negative to positive; 6 cycles at 100 mV/s scan rate.

2.3.3. Decolorization with laccase

It has been reported that the chemical structures of dyes largely influence their decolorization rates with laccase and that its decolorization efficiency was limited to several azo dye structures (Chivukula and Renganathan 1995, Pasti-Grigsby *et al.* 1992). A correlation between the enzyme redox potential and its activity towards the substrates has also been described (Xu *et al.* 1996, Call and Mucke 1997). The driving force for a redox reaction is expected to be proportional to the difference between the redox potentials of oxidant and reductant. For laccase–mediated oxidations, an increase in the substrate redox potential should therefore decrease the efficiency of the reaction. This hypothesis was tested by measuring the percentage of decolorization of each dye in the presence of laccase alone or laccase+HBT after 1h incubation. The observed results are summarized in Table 2.1, together with the respective anodic peak potential. The potential of the anodic peak gives the “degradation potential” in an irreversible redox reaction. As seen in Figure 2.5, a remarkably good linear correlation was found, in both systems, between the percentage of decolorization of each dye and the respective anodic peak

potential. The linear relationship was preserved for up to 2 h, during the initial period of decolorization. When the maximum of decolorization was reached the linearity disappeared. An important observation is that the anodic peak potentials of all the dyes were higher than the reported redox potential for *Trametes villosa* laccase (+0.780 V vs. NHE) and, even so, most of them were extensively decolorized by laccase. The exceptions were dyes V and VII, for which high oxidation potentials were found (Table 2.1). Concerning the positive effect of HBT on the decolorization degree, this can be rationalized considering that the laccase/HBT system, which is also effective through the formation of a free radical, is a stronger oxidant than laccase itself (+1.084 V vs. NHE) (Johannes and Majcherczyk 2000). Thus in the oxidative dye decolorization approach using laccase or laccase/mediator, the redox potential difference between the biocatalyst and the dye is, as expected, a relevant indicator of the ability of the enzyme to decolorize the dye.

Table 2.1 - Decolorization percentages with laccase or laccase+HBT and oxidation peak potentials (vs. NHE) of the tested azo dyes

Dye	% Decolorization (± S.D.)		Oxidation peak (V)[1]
	Laccase	Laccase+HBT	
I	71 ± 3	95 ± 6	+ 0.961
II	76 ± 6	93 ± 5	+ 0.965
III	90 ± 5	89 ± 7	+ 0.952
IV	91 ± 5	94 ± 7	+ 0.996
V	15 ± 3	66 ± 4	+ 1.260
VI	50 ± 4	87 ± 5	+ 1.091
VII	0,6 ± 0.2	65 ± 4	+ 1.305

[1] Potentials (vs. Ag/AgCl (3M NaCl) and corrected to NHE) were recorded in both laccase and laccase/mediator system without significative change in potential

S.D. – Standard deviation

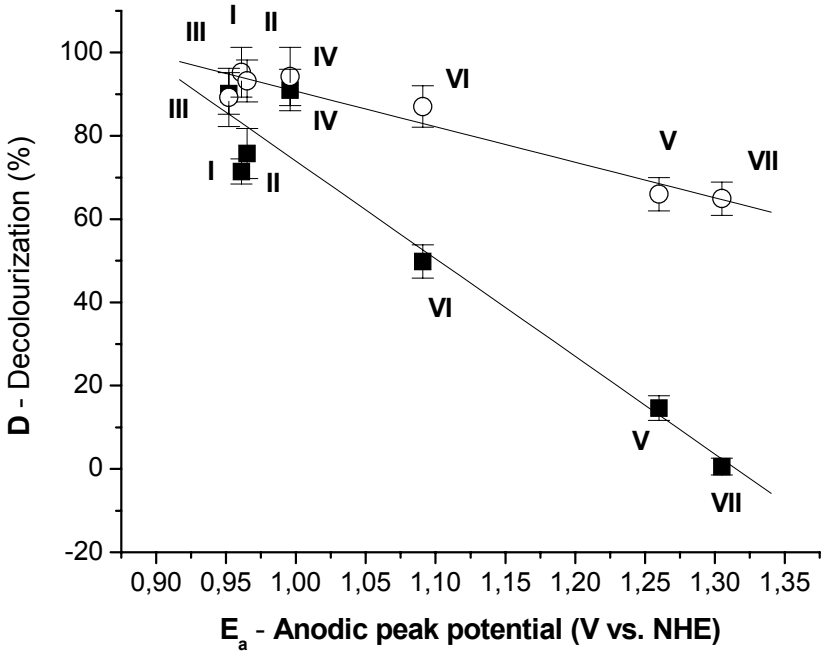


Figure 2.5 - Correlation between anodic peak potential (E_a) and % of decolorization of azo dyes after 1 h with (■) laccase and (○) laccase/HBT mediator system. Correlation: D (■) = (308.6 ± 28.9) – (234.6 ± 26.6) E_a, r^2 = 0.97, S.D. = ±9.7; D (○) = (176.1 ± 10.8) – (85.4 ± 9.9) E_a, r^2 = 0.97, S.D. = ±3.6.

2.3.4. Decolorization by *I.occidentalis*

Ionisable azo dyes are impermeant to cell membranes and their transformation by living microbial cells must thus occur in the extracellular medium (Pearce *et al.* 2003). Azo dyes can be reduced by two or four electrons to produce usually colorless hydrazo compounds or amines, respectively (Hu 1994). In the case of bisazo dyes the reduction of the azo bonds occurs consecutively (Goyal and Minocha 1985). The substituents next to the azo bond affect the rate of azo dyes reduction (Suzuki *et al.* 2001). The process is also facilitated by redox mediators (Keck *et al.* 1997). Previous work with yeasts has shown that azo dyes are reduced to amines (Ramalho *et al.* 2002). In this work we investigated the possibility of using data obtained by cyclic voltammetry to predict relative decolorization rates of azo dyes by *I. occidentalis*. Our

approach was to measure the times required for ≥98% decolorization of the dyes (Table 2.2). As it can be seen in Figure 2.6, an approximately linear correlation was observed between the decolorization times and the cathodic peak potentials of the tested dyes. Concerning cell mediated reductions, NAD(P)H is generally assumed to be the primary electron donor. The driving force for the reduction reactions promoted by NAD(P)H will therefore be proportional to the difference between the reduction potentials of the donor and acceptor species: the less negative the redox potential of the azo dye, the more favorable (and faster) will be its reduction (Bragger *et al.* 1997, Semde *et al.* 1998). We confirmed these principles in our observations.

Table 2.2 - Times for maximum decolorization (≥ 98%) by the yeast strain *I.occidentalis* and reduction peak potentials (vs. NHE) of the tested azo dyes

Dye	Time for max decolorization (≥98%) (h ± S.D.)	Reduction peak (V)[1]
I	8 ± 1	- 0.191
II	8 ± 1	- 0.131
III	24 ± 3	- 0.315
IV	30 ± 4	- 0.354
V	15 ± 2	- 0.270
VI	38[2] ± 5	- 0.408
VII	45[3] ± 5	- 0.478

[1] Potentials were recorded vs. Ag/AgCl (3M NaCl) and corrected to NHE

[2] Conc. 0.97 mM

[3] Conc. 1.01 mM

S.D. – Standard deviation

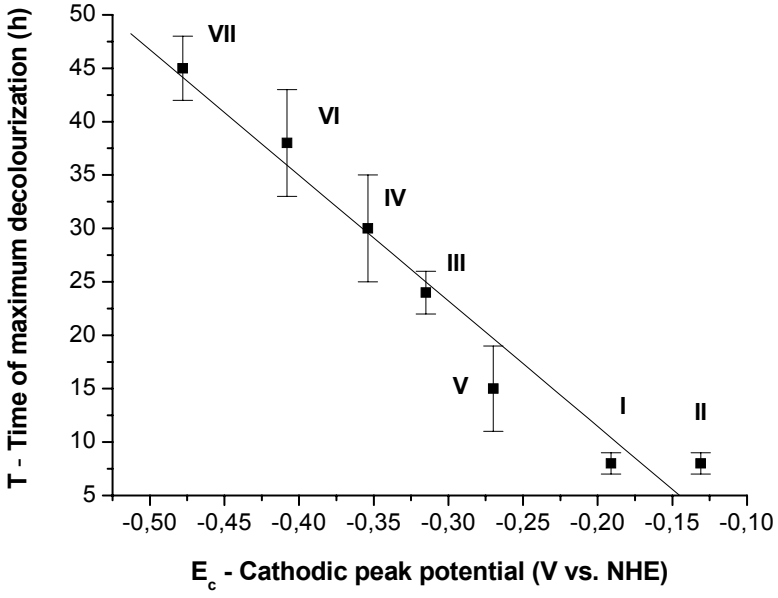


Figure 2.6 - Correlation between cathodic peak potential (E_c) and time of maximum decolorization of dyes (≥ 98%). Correlation: T (■) = (12.1 ± 3.7) + (-117.6 ± 11.3) E_c, r^2 = 0.97, S.D. = ±3.

2.4. Conclusion

A linear relationship was found during the initial period of decolorization with laccase and a laccase/mediator system between the percentage of decolorization of each dye and the respective anodic peak potential. The less positive the anodic peak of the dye is, the more easily is oxidatively degraded with laccase. Contrary to the laccase system, *I. occidentalis* decolorizes azo dyes through a reductive mechanism, but also in this system a linear relationship between the cathodic peak potentials and the time of maximum decolorization of the azo compounds was observed. The more positive the cathodic peak of the dye is, the more rapidly the dye molecule is reduced with yeast. The redox potential differences between the biocatalysts and the dyes are a relevant indicator whether the enzyme is able to decolorize the dye.

"It is a good morning exercise for a research scientist to discard a pet hypothesis every day before breakfast. It keeps him young".

Konrad Lorenz

3

Immobilized and free *Trametes villosa* laccase for decolorization of azo dye effluents

3. Immobilized and free *Trametes villosa* laccase for decolorization of azo dye effluents

3.1. Introduction

The stability and the catalytic ability of free enzymes are dramatically decreased by highly polluted wastewaters due to the instability of their structures and their sensitivity to the process, apart from being non-reusable (Taylor 1991). Therefore, additional measures to increase enzyme operational lifetime and reduce enzyme waste are required. The use of immobilized enzymes can overcome some of these limitations and provide stable catalysts with longer life times (Krajewska 2004). In particular, immobilization of laccases by covalent coupling usually provide enzymes with high stability and is proved to be effective in removing phenolic compounds and color over wide ranges of pH and temperature (Davis and Burns 1992, Rogalski *et al.* 1995). Valuable information can be obtained about the performance of enzymes in industrial applications determining the enzyme half-life time under the process conditions. The objective of this work is to investigate the stability and decolorization efficiency of free and immobilized laccase in a Reactive Black 5 industrial effluent and respective pure dye solution. The decolorization of the effluent would enable its reuse in dyeing processes providing water and energy savings in textile wet processing.

3.2. Materials and methods

3.2.1. Enzyme, dye and effluent

Trametes villosa laccase (EC 1.10.3.2) was used for dye decolorization as previously described (Chapter 2.2.1). Reactive Black 5 (RB5 - 0.04 g/l in 0.1 M acetate buffer, pH 5) dye from Sigma (Dye VII in Figure 2.1) and the respective dyeing effluent (wavelength of maximum dye adsorption in both dyeing effluent and pure dye solution was 595 nm) were substrates for enzymatic decolorization. The composition of the RB5 dye-bath, from which the corresponding effluent was discharged, was 1 g RB5/l and 30 g NaCl/l.

3.2.2. Laccase immobilization

Alumina (Al_2O_3) spherical pellets (3 mm diameter) from Sigma were silanized with 2.5% (v/v) α-aminopropyltriethoxy silane (Sigma) in acetone at 45 ºC for 24 h (Cho and Bailey 1979). The silanized carriers were washed with distilled water and treated with 2% (v/v) aqueous glutaraldehyde (Aldrich) for 2 h at room temperature, washed again and dried at 60 ºC for 1 h. Modified support (10 g) was immersed in 50 ml laccase preparation (0.8 g protein/l) in 0.1 M acetate buffer (pH 5), for 5 h at room temperature (Leonowicz *et al.* 1988, Costa *et al.* 2002). The amount of protein in the supernatant solution after immobilization was determined using the Bradford method (Bradford 1976). Bound protein was determined as a difference between initial and residual protein concentrations (immobilization yield ~ 50%, 0.02 g of protein on the support).

3.2.3. Immobilized laccase stability in dyeing effluent

RB5 effluent sample and respective pure dye solution (100 ml) were adjusted to pH 5 and incubated with 10 g of alumina support with immobilized enzyme at 45 ºC in a shaker bath (90 rpm). The support was previously saturated in a concentrated solution of RB5 (1 g/l in 0.1 M acetate buffer pH 5, for 1 h) in

order to minimize the decolorization due to dye adsorption on the support. The immobilized enzyme was removed at different times (1, 24, 48 and 72 h) and used to decolorize another solution of Reactive Blue 19 (RB19 from Sigma, 50 ml, 0.1 g/l in 0.1 M acetate buffer pH 5, 45 ºC; wavelength of maximum dye adsorption is 595 nm) for 40 min. The percentage of RB19 decolorization as a function of the time was used to define the relative enzyme activity. The relative enzyme activity was plotted vs. time and from the derived exponential equation ($Y = A_i * \exp(-k*X)$; where Y = relative activity; X = time; A_i = initial activity; k = rate constant) the rate constant was obtained. The half-life was calculated as ln2/k.

3.2.4. Free enzyme stability in dyeing effluent

RB5 dye solution and the effluent solution (100 ml) were adjusted to pH 5 and individually incubated with enzyme (0.2 g protein/l) in a shaker bath, at 45 ºC. Sample (1 ml) were removed from the reaction mixture at 1, 24, 48 and 72 h, and used to decolorize RB19. The relative enzyme activity and the half-life were calculated as previously described.

3.2.5. Decolorization experiments

RB5 dye solution and effluent (100 ml, pH 5) were individually incubated with free enzyme (0.2 g protein/l) in a shaker bath (45 ºC, for 24 h). The above experiment was repeated using 10 g alumina with immobilized enzyme (2 mg protein/g support). Samples of the reaction mixture were collected at different times to measure the dye absorbance, and the percentage of effluent decolorization was calculated. In the case of free enzyme, samples were collected at 1, 2, 3, 4, 5, 24, 48, 72 and 140 h. Measurements of the decolorization with the immobilized enzyme were performed at 1, 2, 3, 4, 5 and 24 h, during 4 cycles.

3.2.6. Dye/protein/support interaction

Bovine serum albumine (BSA) was immobilized on alumina support (~2mg protein/g support) saturated with RB5 (1 g/l for 1 h) to evaluate the effect of the support and protein adsorption in the decolorization process. Alumina support with and without immobilized BSA was incubated with RB5 pure dye solution and effluent (100 ml, pH 5, 45 ºC) for 4 cycles of 24 h. Decolorization was measured at 1, 2, 3, 4, 5 and 24 h.

3.2.7. Re-dyeing experiments

Re-dyeing experiments using the enzymatically decolorized RB5 effluent were carried out in bright and dark colors, respectively – Reactive Orange 70 and Reactive Blue 214. The dyes were applied on bleached cotton fabrics in two concentrations 0.25g/l and 1.5 g/l, in the presence of 20 g Na_2CO_3/l and 60 g Na_2SO_4/l. The dyeing was performed in an Ahiba Spectradye dyeing apparatus (Datacolor) at 80 ºC, for 1 h. Dyed fabrics were thoroughly washed afterwards by boiling to remove any unfixed dye. The color differences (E^*) on the fabrics dyed using enzymatically recycled effluent and fresh water were determined using a reflectance-measuring apparatus Spectraflash 600 (Datacolor), according to the CIELab color difference concept at standard illuminant D65 (LAV/Spec. Excl., d/8, D65/10º). We assumed a color difference tolerance interval of one CIELab unit as acceptable.

3.3. Results and discussion

3.3.1. Laccase stability

The immobilized laccase had a higher stability than the free laccase in buffer and salt solutions (Table 3.1). Comparatively, the stability of the free laccase in the effluent, containing both dye and salt, increased. The industrial dyeing effluent contained not only unfixed and hydrolyzed dyestuff (initially 1 g RB5/l) but also NaCl (0.5 M). The ionic strength of the enzymatic solutions is one of the most important factors affecting the biocatalyst performance. The relatively high amounts of salt in the dyeing effluent enhance the electrostatic coupling of the anionic dyes and the positively charged proteins, thereby forming stable dye/enzyme aggregates. Various authors have reported enzyme stabilization above 0.5 M $(NH_4)_2SO_4$ and NaCl (Göller and Galinski 1999, Dötsch *et al.* 1995, Carpenter and Crowe 1988). Such stabilization occurred with both free and immobilized enzyme in the RB5 effluent compared to the RB5 pure solution and in the salt solution compared to the buffer (see Table 3.1). In the presence of dye the stability of the immobilized enzyme unexpectedly decreased. The RB5 is a di-azo sulphonic dye (see Figure 2.1 in Chapter 2) that binds to enzyme molecules forming ion pairs between negatively charged sulphonic groups and positively charged protein groups. Anionic sulphonic dyes are known to protect the enzymes from inactivation (Matulis *et al.* 1999). Sulphonic dye stabilization was effective only on free laccase. The sulphonate dye and the salt present in RB5 dyeing effluents probably exert a synergistic stabilization effect on free laccase. Surprisingly, the immobilized enzyme showed lower stability than the free form in dyeing effluents. Normally the enzyme immobilization is expected to provide stabilization effect restricting the protein unfolding process as a result of the introduction of random intra- and intermolecular crosslinks (Rogalski *et al.* 1995). The immobilization procedure has a variety of effects on protein conformation as well as on the state of ionization and dissociation of the enzyme and its environment (Emine and Leman 1995). The laccase structure became possibly less available after the immobilization for interaction with anionic dyes. The immobilization process,

depending on the environment, might have a stabilization/destabilization effect on the enzyme.

Table 3.1 - Half-life (h) of free (0.2 g protein/l) and immobilized laccase (10 g support, 0.002 g protein/g support) in 100 ml Reactive Black 5 pure solution (0.04 g/l) and respective dyeing effluent in 0.1 M acetate buffer pH 5, 45 ºC, with shaking at 90 rpm

	Half-life (h) ± S.D.	
	Immobilized laccase	**Free laccase**
Pure dye RB5 solution (0.04 g/l)	57 ± 8	105 ±16
Effluent RB5 solution (~0.04 g/l)	79 ± 3	194 ± 38
Acetate buffer (0.1 M, pH5)	110 ± 26	85 ± 9
NaCl solution (30 g/l)	148 ± 34	122 ±19

S.D. – Standard deviation

3.3.2. Decolorization of pure dyes and colored effluents with free and immobilized laccase

The enzymatic decolorization of RB5 (~90%) took 24 h. This relatively slow decolorization can be explained by the hydrophilic nature of the RB5, which favors the equilibrium distribution towards the aqueous phase (Churchley *et al.* 2000). The decolorization was in all cases higher for the pure dye solution than for the effluent, with both free and immobilized laccase. The immobilized laccase, even after the 4th cycle of reuse, showed greater decolorization efficiency than the free enzyme (Figure 3.1). The higher decolorization performance of the immobilized enzyme in comparison to the free enzyme could be explained by a high dye adsorption on the alumina support. The difference in the decolorization capacity of immobilized laccase in pure and effluent solutions, from the first to the last cycle of utilization might be attributed to the presence of unfixed or hydrolyzed dyestuff and salt in the dyeing effluent.

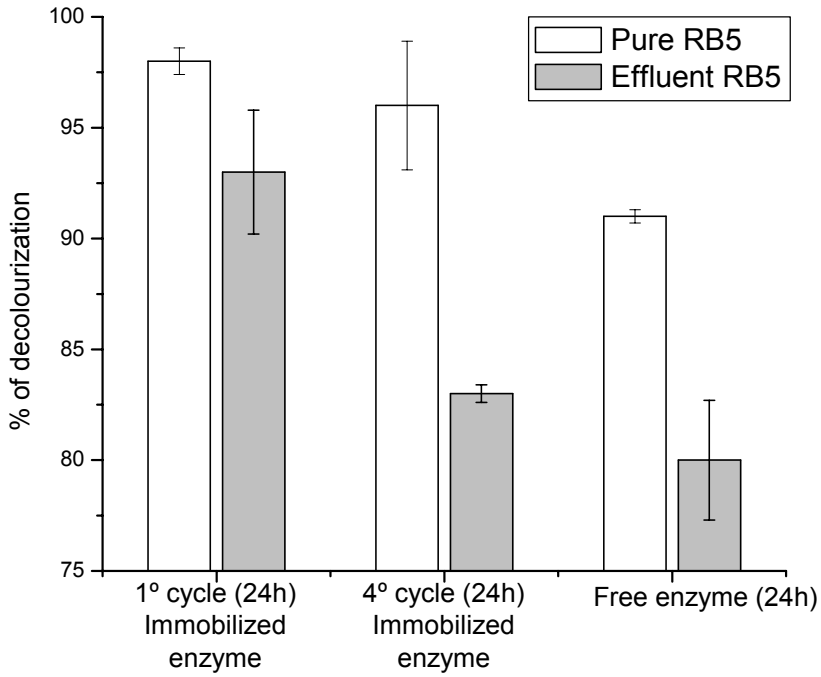


Figure 3.1 - Decolorization (%) of 100 ml Reactive Black 5 pure dye (0.04 g/l) and respective dyeing effluent with immobilized (10 g support, 0.002 g protein/g support) and free laccase (0.2 g protein/l) in 0.1 M acetate buffer pH 5, 45 ºC, shaker bath (90 rpm), 4 decolorization cycles of 24 h each. Decolorization was followed spectrophotometrically at 595 nm.

3.3.3. Dye/protein/support interactions in decolorization

A series of experiments were carried out to evaluate the effect of the support/protein/dye interactions in the decolorization process. Alumina support, immobilized BSA and immobilized laccase were used for decolorization experiments in RB5 solutions. This would allow the effects of support adsorption, dye/protein interaction and enzymatic degradation of the dye to be quantified. In the first cycle of decolorization with immobilized laccase, the color removal was mostly due to adsorption on the support and on the protein (Figure 3.2). In the next cycles, partial saturation of the support occurred; the extra dye adsorption, due to the BSA protein decreased and the contribution of laccase increased. Even though the support was saturated with dye, further adsorption occured and appeared to be an important factor for

decolorization. After 24 h it was still difficult to distinguish the laccase decolorization from the alumina adsorption of RB5. Decolorization due to adsorption on the support continued even after loss of the enzymatic activity. The decolorization with immobilized laccase proved to be a complex process, consisting of concomitant dye-support adsorption, dye-protein adsorption and enzymatic dye degradation.

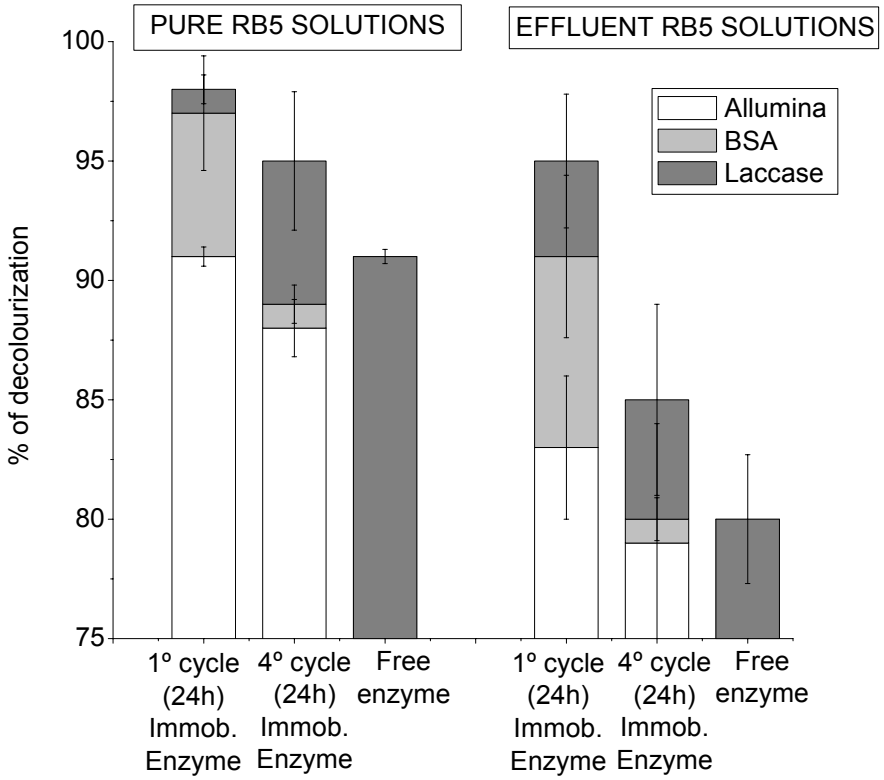


Figure 3.2 - Alumina (10 g), BSA (0.002 g protein/g support) and laccase (0.002 g protein/g support) contribution to the decolorization of 100 ml Reactive Black 5 pure solution (0.04 g/l) and dyeing effluent in 0.1 M acetate buffer pH 5, 45 ºC, shaker bath (90 rpm), 4 decolorization cycles of 24 h each. Decolorization was followed spectrophotometrically at 595 nm.

3.3.4. Dyeing using enzymatically recycled dyeing effluents

The dyeing in dye-baths prepared with laccase decolorized RB5 effluent, showed E^* values for both dyes and dye concentrations, within the acceptable

range of one unit. As might be expected dyeing in dark color with decolorized dyeing liquor yielded slightly better results than dyeing in bright color (Table 3.2).

Table 3.2 - Color differences (E^*) on fabrics dyed (1 h, at 80 ºC) in dye-baths (20 g Na_2CO_3/l, 60 g Na_2SO_4/l and 0.25 ÷ 1.5 g/l Reactive Orange 70 or Reactive Blue 214), prepared with laccase-recycled Reactive Black 5 dyeing effluent

	Reactive Orange 70		**Reactive Blue 214**	
Dye concentration (g/l)	0.25	1.5	0.25	1.5
E^* ± S.D.	0.91 ± 0.02	0.34 ± 0.01	0.17 ± 0.03	0.13 ± 0.02

S.D. – Standard deviation

3.4. Conclusion

The immobilization procedure has a variety of effects on protein structural conformation as well as on the state of ionization and dissociation of the enzyme and its environment. Thus, the immobilized enzyme has generally its activity lowered and its stability increased. However, in the present work the immobilized enzyme showed both lower stability and decolorization ability than the free form in dyeing effluents. The lower stability of the immobilized laccase in dyeing liquors may be due to the enzyme's structure being less accessible for interaction with salts and anionic dyes. Among the solutions tested with the immobilized enzyme, highest stability was attained for the control salt solution. This fact suggests that laccase's structure became less available after the immobilization for interaction with anionic dyes. Reactive Black 5 dye exerts a destabilizing effect on the alumina-laccase complex that makes its use less attractive for industrial applications. The low stability in dye liquor is not the single limitation in the alumina immobilization methodology. Due to the alumina high porosity the adsorption of the dye appears to be the most important factor for decolorization. Even if the support is previously saturated with dye, the adsorption continues even after loss of enzymatic activity. The dye adsorbed on alumina is not degraded and an additional step for the treatment of the exhausted alumina is required. Although these limitations, both immobilized and free laccase showed a good decolorization degree and the re-dyeing experiments using the enzymatically decolorized RB5 effluents are comparable with conventional dyeing processes.

"All truths are easy to understand once they are discovered; the point is to discover them."

Galileo Galilei

4

Degradation of azo dyes by *Trametes villosa* laccase under long time oxidative conditions

4. Degradation of azo dyes by *Trametes villosa* laccase under long time oxidative conditions

4.1. Introduction

The main drawback of azo dyes is that they are not easily degraded by aerobic bacteria and under action of anaerobic or microaerobic reductive bacteria they can form toxic and/or mutagenic compounds (Chung and Cernigla 1992). Laccases can decolorize azo dyes without the formation of toxic aromatic amines. However most of the bioremediation systems in textile mills are applied in dilution pools where effluents stay for several days before being discharged. In these conditions degradation products will recombine yielding darker products. In the literature, there is a large number of papers reporting decolorization of azo dyes. However, the fate of the products of azo dye laccase reactions and their possible polymerization is ignored (Chagas and Durrant 2001, Jarosz-Wilkolazka *et al.* 2002, Robinson *et al.* 2001b, Tauber *et al.* 2005). Therefore, in this work, the laccase-catalyzed azo dye degradation and aminophenols polymerization were performed for several days in order to study the azo dye degradation and the coupling/polymerization reactions. Catechol, a diphenolic compound, was also added to the system to enhance the degree of polymerization and to simulate the reaction between the degradation products and the substances naturally present in the environment, since catechol is already existent in the humic substances of the soil. The formed soluble products were studied by LC-MS and the polymerized insoluble products were studied by ^{13}C -NMR. As a result, the mechanistic chemical model of the azo dye degradation reactions for phenolic and non-phenolic azo dyes was proposed. The studies of these types of reactions that can occur during and after dye degradation are important, since the resulting polymers can limit the laccase batch application as bioremediation agent in textile effluents.

4.2. Materials and methods

4.2.1. Dyes, reagents and enzymes

The investigated dyes 3-(4-dimethylamino-1-phenylazo) benzene sulfonic acid sodium salt and 3-(2-hydroxy-naphthalen-1-phenylazo)-benzene sulfonic acid sodium salt (Dye I and III in Figure 2.1) were synthesized as described in Chapter 2.2.2. All other reagents and dyes were purchased from Sigma-Aldrich, St. Louis, MO, USA and used without further purification. Laccase (EC 1.10.3.2) from *Trametes villosa* (5.3 mg protein/ml, 600 U/ml) was kindly provided by Novo Nordisk, Denmark.

4.2.2. Dye decolorization with laccase

Stirred azo dye solutions (10 mM; 50 ml) buffered with 0.1 M Na-acetate buffer, pH 5, were incubated with 20 µl of *Trametes villosa* laccase (5.3 mg protein/ml, 600 U/ml) at room temperature. The dye decolorization was measured in a UV-visible spectrophotometer (Unicam, Cambridge, England) at different times in the course of the experiment and the percentage of effluent decolorization was calculated thereof.

4.2.3. Polymerization reactions with laccase

Stirred equimolar solutions of 2,5-diamino benzene sulfonic acid (DBSA) and catechol (10 mM; total volume 50 ml) buffered with 0.1 M Na-acetate buffer, pH 5, were incubated with 20 µl of laccase (5.3 mg protein/ml, 600 U/ml) at 25 ºC. The same experiments were performed with catechol and 2,5-diamino benzene sulfonic acid separately.

4.2.4. LC-MS and ^{13}C NMR analyses

LC-MS analyses were performed in negative ion mode on an Agilent 1100 HPLC system (degasser, binary pump, column compartment, DAD) coupled

with a Q-trap LC-MS from Applied Biosystems, Canada. The flow coming from the HPLC was split 1:28 and introduced to the ESI (electrospray ionization) turbo spray, which was operated at 350 ºC. A scan rate of 4.000 amu/s was performed under negative ionization in the enhanced scan mode. All gases consisted of nitrogen produced from gas generator from PEAK Science, Scotland. Further MS-settings were: Ion spray voltage: -4.500 V, declustering potential: -50 V, entrance potential: -10 V, collision energy: -90÷-10 V, curtain gas: 40 psi, nebulizer gas: 45 psi, turbo gas: 80 psi. The chromatographic separation was performed by following chromatographic columns: Synergi Hydro, 150 x 4.6 mm, 4 μm (Phenomenex, USA), ProntoSIL AQ, 60 x 4 mm, 3 μm (Bischoff, Deutschland), Nucleosil HD, 70 x 4 mm, 3 μm (Macherey-Nagel, USA). The DAD performed a scan range of 200-800 nm with a sampling rate of 1.25 Hz at a slit width of 4 and a step width of 2 nm. A 753-suppressor module from Methrom, Switzerland, was used for cation suppression. The ^{13}C CP/MAS NMR spectra were recorded as described before (Del Arco *et al.* 2004).

4.3. Results and discussion

4.3.1. Spectrophotometric analysis

The degradation of azo dyes was followed by UV-Vis analysis. The UV-Vis spectrum showed dramatic changes in the enzymaticaly treated solutions. It was observed that this reaction was a multi-step process with a decrease of absorbance of the visible peak in the first stages of treatment, and a general increase of absorbance in all UV-Vis spectra due to darkening of enzymatic treated solutions after longer treatment times, i.e., 72 hours.

The decrease in the intensity of the visible peak (465 nm) of dye I is indicating that the degradation of the molecule was not complete and some undegraded dye was still present in the solution after 24 h of treatment with laccase. In the UV spectra two new peaks emerged at 250 and 320 nm (Figure 4.1). The spectrophotometric analysis of the dye I, in the UV region, showed peaks that could be attributed to the conversion of the degraded dye into unknown reaction products. Furthermore the increased absorption in the visible spectra could be good evidence that polymerization reaction might have occurred. The breaking down of the dye into smaller fragments, including the breakage of the azo bond can lead to a decrease in the absorbance of the visible spectra and to a colorless solution. However, according to the literature, from this reaction a product could result that is simply losing the color due to a shift of the UV spectrum, rather than a direct degradation of the molecule into smaller fragments (Novotny *et al.* 2004). This slow degradation model based on a demethylation processes could explain the initial loss of color as a simple shift in the UV spectrum, and the persistence of the yellowish color in the solution, which remained even in the presence of very low concentrations of Dye I (Novotny *et al.* 2004). Bianco Prevot *et al.* suggested that after the demethylation reaction a non-enzymatic oxidation disrupts the azo bond (Bianco Prevot *et al.* 2004). Based on this model (Bianco Prevot) and on the results we obtained by LC-MS, the absence of the N,N-dimethylaniline and 4-hydroxy-N,N-dimethylaniline from the reaction products can be explained.

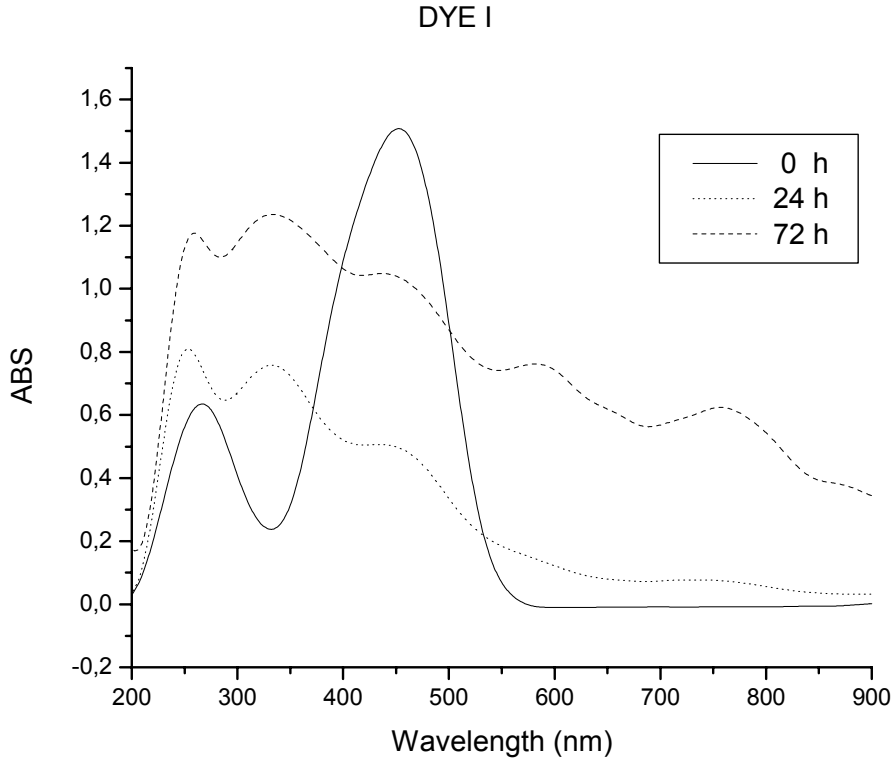


Figure 4.1 - UV-Vis spectra of dye I (10 mM; 50 ml in 0.1 M Na-acetate buffer, pH 5) before and after laccase (20 µl; 5.3 mg protein/ml, 600 U/ml) decolorization at room temperature.

In the case of the dye III we observed a very rapid decrease in intensity of the peak in the visible absorption spectrum (465 nm) indicating an almost complete decolorization with the disruption of the chromophoric group (azo bond disruption). The decrease of the absorbance of the two peaks in the UV region (242, 307 nm) and the formation of a new peak at 250 nm suggests changes in the aromatic group (Figure 4.2). The dye III peak near 250 nm is normally associated with the presence of phenolic and naphthoquinone groups (Mielgo *et al.* 2001). These findings support, the model oxidation pathway, by laccase, of the hydroxy-naphthylazo dyes, where the laccase action allowed the direct and rapid dye degradation (Chivukula and Renganathan 1995).

Furthermore, the two dyes showed after 48 hours a general increase in the absorption bands in the visible spectra indicating the formation of coupling products, which retain the azo group integrity in their molecules. Both dyes retained some color, especially dye I that appeared darker than before after decolorization. The products of the laccase degradation were participating in the coupling reaction with the unreacted and reacted dye. Thus the formation of polymerized products stopped the degradation processes under laccase action, leading to an incomplete decolorization of the dye solutions.

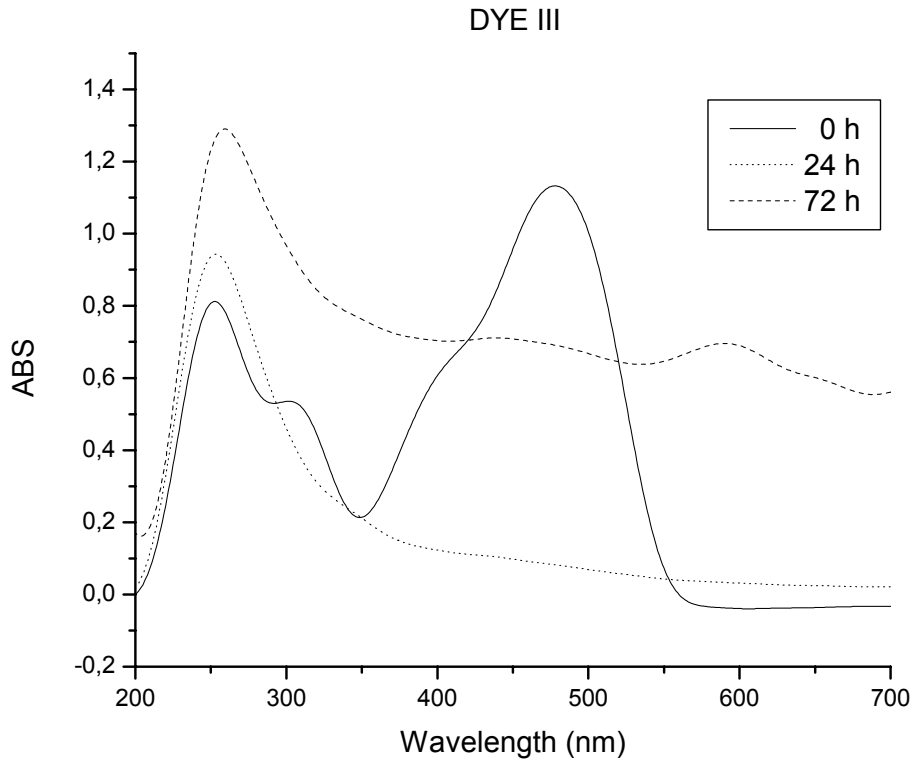


Figure 4.2 - UV-Vis spectra of dye III (10 mM; 50 ml in 0.1 M Na-acetate buffer, pH 5) before and after laccase (20 µl; 5.3 mg protein/ml, 600 U/ml) decolorization at room temperature.

4.3.2. LC-MS/MS analysis of the degradation products of dye I

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a versatile system which combines both selectivity and sensitivity, and it is generally considered as the most reliable technique to quantify chemical compounds in complex matrices (De Hoffman *et al.* 2001).

During the LC-MS/MS-analysis of dye I, 10 compounds have been detected and 7 of them have been identified. The mass spectra of the detected compounds were performed using the Enhanced Ion Scan and Enhanced Product Ion Scan by various level of collision energies (Table 4.1).

The identified compounds are products of the cleavage of N-C-bond in the dye molecule as well as polymeric products of coupling of these products with undegradated dye molecules. Compound I has been identified as benzenesulfonic acid and compound II as hydroxy-benzenesulfonic acid. Compound III was tentatively identified as 3-diazenyl-benzenesulfonic acid but this cannot be stated unambiguously. Compounds IV and V are dimeric coupling products of dye I and benzenesulfonic acid. Compound IV is a dimeric coupling product of dye I and one molecule of benzenesulfonic acid, while compound V is a dimeric product of coupling two molecules benzenesulfonic acid and dye I (Figure 4.3). Compound VII has been identified as the product of the coupling reaction of two 3-(4-methylamino-phenylazo)-benzenesulfonic molecules with one nitrogen molecule. In the sample a coupling product between a contaminant of the sample with benzenesulfonic acid (compound VI) was identified.

The other three products that appear at m/z 327, 366 and 480, respectively, could not be identified. It should be noted that these products were not stable and disappeared when samples were frozen.

Table 4.1 - Mass spectra of dye I degradation products

Compound Number	Enhanced mass spectrum	Enhanced product ion mass spectrum of quasi molecular ion	
	m/z (relative intensity, %)	Collision energy (V)	m/z (relative intensity, %)
I	157 (100); 93 (5)	-50	157 (8,1); 139 (9,5); 93 (27,5); 80 (100); 65 (11)
II	173 (100); 172 (56); 171 (1,5); 109 (2); 80 (1)	-25	173 (100); 155 (5); 109 (15); 93 (5); 80 (22)
III	185 (?); 93 (?)**a**	-30	185 (100); 121 (99); 93 (6); 80 (31)
IV	1382 (<0,5); 922 (0,6) 921 (2); 462 (9); 461(16); 460 (68); 327 (14); 328 (20); 327 (100);	-35	921 (76); 786 (13); 761 (5); 592 (3); 474 (5); 446 (18); 431 (5); 415 (3); 380 (12); 340 (14); 327 (100)
V	1233 (<0,5); 618 (17); 617 (26); 616 (100); 329 (16); 328 (15); 327 (53);	-35	1233 (18); 904 (11); 616 (100); 602 (18); 573 (12); 536 (33); 482 (26); 327 (63); 246 (25); 166 (30)
VI	549 (6); 365 (3,5); 364 (16); 274 (78); 263 (3,5); 262 (13); 261 (27); 232 (3,5); 231 (8,5); 172 (7,5); 171 (19); 158 (2,5); 157 (3,5); 156 (100)	-35	549 (100); 521 (1,5); 364 (23); 336 (1,5); 260 (18); 232 (2,5); 171 (8,5); 156 (28,5)
VII	448 (4); 447 (9,5); 446 (73); 261 (16); 260 (5); 172 (4); 171 (1,5); 157 (3); 156 (100)	-35	446 (13); 260 (11); 171 (29); 156 (100); 80 (10)

a not stated because of weak chromatographic separation

The literature on chemical oxidation of azo dyes and the products found during the LC-MS/MS analysis could be helpful to understand the degradation pathways of the dye I after enzymatic treatment for a long period of time (Chivukula and Renganathan 1995, Galindo *et al.* 2000). Even though dye I is not a phenol azo dye that would be a typical substrate for laccase, extensive degradation was observed. Earlier reports on the chemical oxidation of methyl azo dyes

suggest that one-electron extraction from the amino substituent occurs in the initial step of the dye degradation. The resulting radical cation undergoes an oxidation and leads to the formation of an iminium ion, and then the secondary amine can be formed through solvolysis processes. Loss of both N-methyl groups has also been reported (Darwent and Lepre 1986). In this case the mechanism could follow a similar pathway, performing one–electron oxidation of the tertiary amine and subtraction of hydrogen radical, with a subsequent demethylation and followed by the oxidation of the secondary amine by laccase. Nucleophilic attack by water on the phenolic ring carbon bearing the azo linkage causes N-C-bond cleavage and produces 3-diazenyl-benzenesulfonic acid and 4-methylimino-cyclohexa-2,5-dienone. The 3-diazenyl-benezenesulfonic acid loses a nitrogen molecule to produce benzenesulfonic acid radical, which could further undergo hydrogen radical addition or polymerization with dye I molecule. However, this mechanism is not fully elucidated.

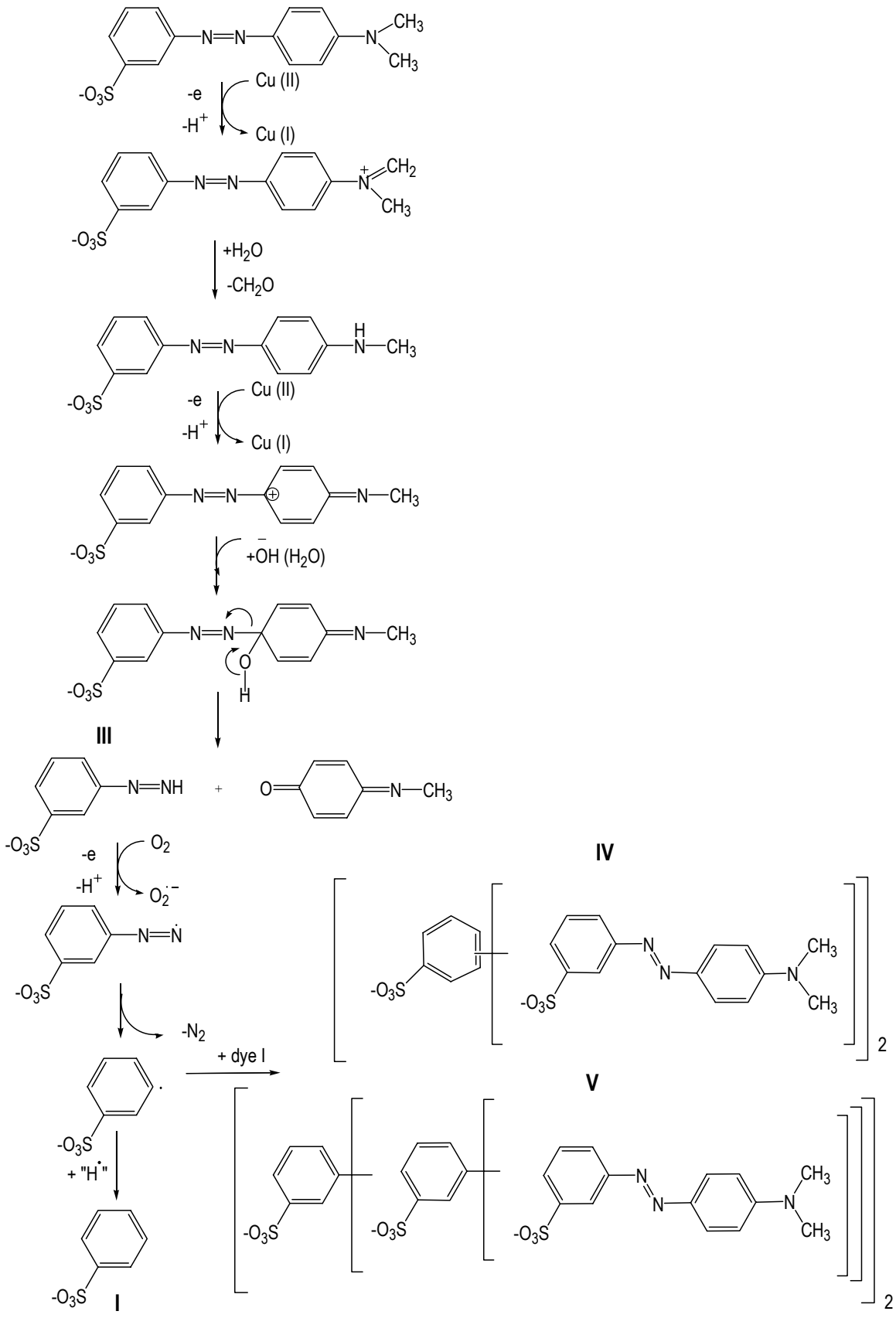


Figure 4.3 - Proposed mechanism of degradation of dye I by laccase.

4.3.3. LC-MS analysis of the degradation products of dye III

During the analysis of dye III 7 compounds were identified. The mass spectra of identified compounds were recorded in the same way as for dye I using the Enhanced Ion Scan and Enhanced Product Ion Scan by various values of collision energies. The mass spectra of compounds I, II and III are listed in Table 4.1, and the mass spectra of the compounds VIII, IX, X, XI are listed in Table 4.2.

The products of the degradation of dye III, namely hydroxy-benzene sulfonic acid (II) and benzene sulfonic acid (I), have been found. Unfortunately, the identification of 3-diazenyl-benzenesulfonic acid (III), could not be stated unambiguously.

The products obtained from the coupling processes of dye III with products obtained from azo dye oxidation by laccase were identified. Compound VIII is a coupling product of dye III with one 1,2-naphthoquinone molecule while compound IX is obtained from coupling of dye III with 2 molecules of 1,2-naphtoquinone. In addition, another two compounds were identified, denominated as compound X and compound XI, which are the reaction products of coupling the dye with 1,2-naphthodiol, and of the hydroxy-benzenesulfonic acid with 1,2-napthoquinone, respectively.

Table 4.2 - Mass spectra of dye III degradation products

Compound Number	Enhanced mass spectrum	Enhanced product ion mass spectrum of quasi molecular ion	
	m/z (relative intensity, %)	Collision energy (V)	m/z (relative intensity, %)
VIII	484 (26); 483 (100); 457 (4,5); 456 (24); 455 (80); 429 (<1); 428 (2,5); 427 (7); 158 (7); 157 (8); 156 (73)	-30	483 (26); 455 (28); 427 (7); 347 (0,5); 312 (0,5); 172 (6,5); 156 (100); 80 (11,5)
IX	641 (1); 640 (3); 639 (12,5); 612 (3); 611 (5,5); 456 (7); 455 (21), 454 (100); 399 (1); 398 (5); 397 (19); 362 (3); 361 (8); 158 (6); 157 (6,5); 156 (84); 145 (22)	-50	639 (2); 611 (38); 583 (7,5); 531 (12); 503 (11,5); 454 (12,5); 441 (7); 425 (5); 397 (4); 326 (14,5); 282 (7,5); 172 (4); 156 (100); 80 (9,5)
X	487 (9); 486 (26,5); 485 (100); 458 (0,5); 457 (8)	-50	485 (72); 457 (70); 441 (6); 405 (13); 385 (7); 373 (70); 361 (14); 349 (100); 301 (9); 273 (7); 245 (6); 156 (16); 80 (6)
XI	331 (1,5); 330 (18,5); 329 (100); 302 (4); 301 (27)	-35	329 (100); 301 (51,5); 285 (11); 273 (68,5); 249 (64,5); 221 (70)

In the case of dye III, the presence of the coupling products of 1,2-naphthoquinone confirms the most accepted model of azo dyes degradation by laccase (Chivukula and Renganathan 1995, Soares *et al.* 2002).

According to this model, laccase oxidizes the phenolic group of the azo dye with the participation of one electron generating a phenoxy radical which is sequentially followed by the oxidation to a carbonium ion. The nucleophilic attack by water on the phenolic ring carbon bearing the azo linkage to produces 3-diazenyl-benzenesulfonic acid (III) and 1,2-naphthoquinone than takes place. Quinones can form stable structures by addition reactions to the radicals in reaction environment (Thurston 1994, Ulbricht 1992). The reaction

pathways earlier published by Chivukula and co-workers allows us to explain the formation of the coupling products of 1,2-naphthoquinone and its derivative, 1,2-naphthodiol obtained in the laccase degradation of dye III (Chivukula and Renganathan 1995). In the present case, it is possible that the radicals, which have been formed in the one-electron oxidation of dye III by laccase, would react with 1,2-naphthoquinone, rather than be oxidized. The formed radicals can undergo oxidation to yield compound VIII, reduction to yield compound X or further polymerization and again oxidation to form compound IX. Compound XII can be produced in the process of one electron oxidation of hydroxy-benzenesulfonic acid, which can undergo coupling reaction with 1,2-naphthoquinone and followed then by further oxidation. The proposed reaction pathway of dye III degradation by laccases is shown in Figure 4.4. The position of 1,2-naphthoquinone and 1,2-naphthodiol in the identified oligomeric molecules cannot be stated at present. Bollag *et al.* observed dimerization and polymerization of phenoxy radicals during *Rhizoctonia practicola* laccase treatment of organic compounds (Bollag 1992). According to their investigation, the cross-coupling between the reactive species results in the formation of C-C and C-O bonds, between phenolic molecules and C-N and N-N between aromatic amines. By phenolic cross-coupling an electron is removed from the hydroxyl group, generating an alkoxy radical. The alkoxy free radical forms dimer in the *ortho*- and *para*-position to the hydroxyl groups. Phenolic radicals can be further oxidized to yield oligomeric products. Under certain conditions the C-C formed dimers can take part in coupling reactions to form extended quinines.

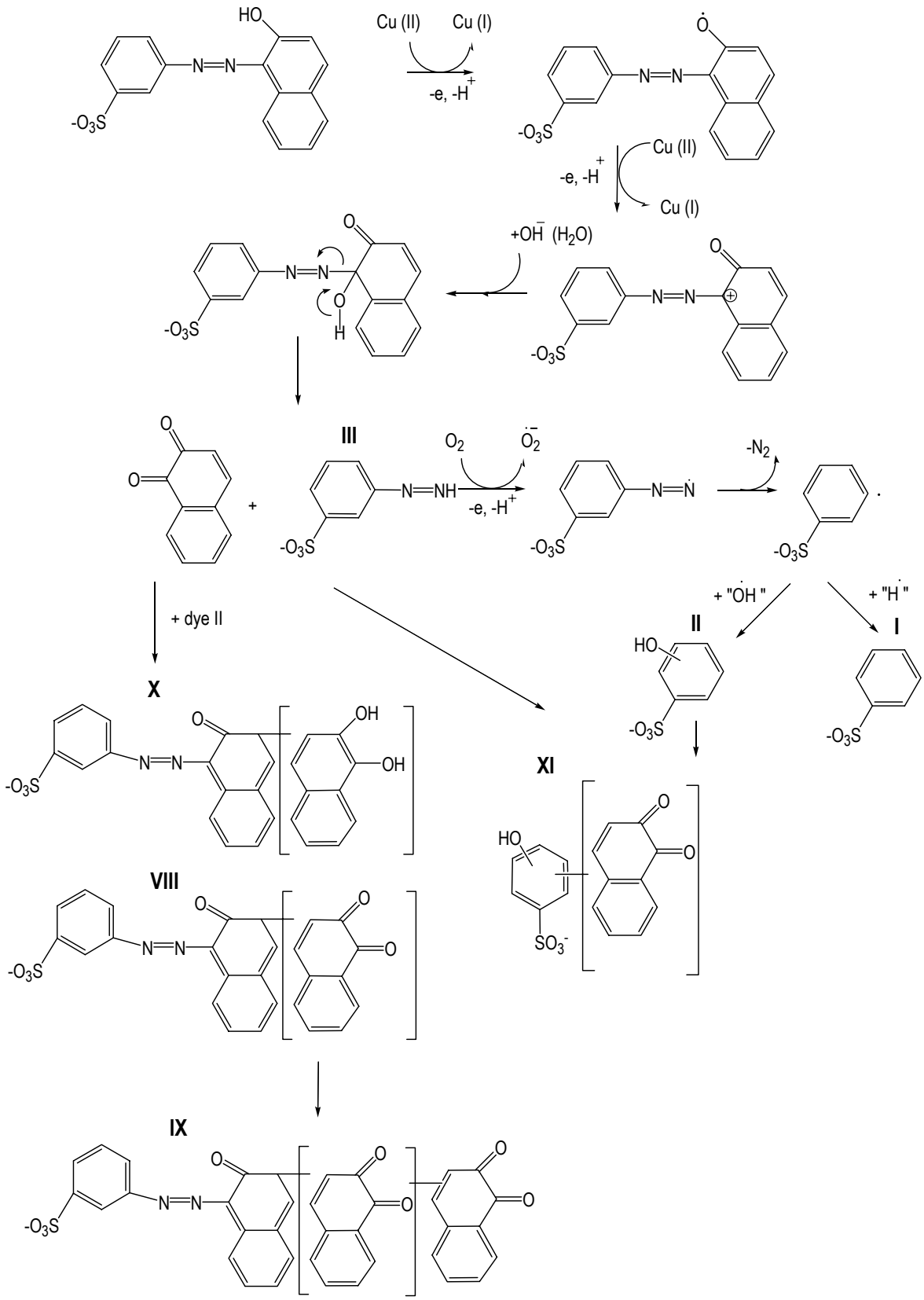


Figure 4.4 - Proposed mechanism of degradation of dye III by laccase.

4.3.4. Polymerization experiments

In order to confirm the efficiency of the laccase polymerization, a preliminary study of the ability of *Trametes villosa* laccase to catalyze polymerization and coupling reactions was performed.

The reaction between a phenol (catechol) and an aromatic amine (2,5-diamino benzene sulfonic acid - DBSA) was performed in the presence of *Trametes villosa* laccase. The insoluble polymer and the effluent were investigated by ^{13}C-NMR and LC-MS analysis.

The LC-MS analysis of the products showed the presence in the solution of coupling (m/z 293) and oligopolymeric products (m/z 325, 369, 672, 525, 408, 259, 391, 647). The structure of the oligopolymer was not yet fully elucidated and will be subject of future studies. The coupling product found in small amounts, was identified as 2-amino-5-(3-hydroxy-4-oxo-cyclohexa-2,5-dienylideneamino)-benzenesulfonic acid (m/z 293) and the oligopolymeric product, was identified as poly(catechol) (m/z 325) (Figure 4.5).

The ^{13}C-NMR spectra, of the precipitated catechol polymer, showed in the aromatic range two large peaks at 144.7 and 122.5 ppm (Table 4.3). These peaks correspond to the carbons linked with the OH groups and to the carbon in position *meta*- and *para*-, respectively, confirming a structure that is related to the catechol polymer structure (Aktas and Tanyolaç 2003). The DBSA polymer spectrum showed a series of peaks in the aromatic range, different from the noticeable unreacted structure of the diamine, that confirm the oligomerization of the DBSA under laccase action (111.7, 113.5, 123.5, 125.8, 128, 129, 131.8, 134.6 ppm). In the spectra of the polymer obtained in the reaction between DBSA and catechol in presence of the laccase intense peaks were observed at 144 and 116.2 ppm.

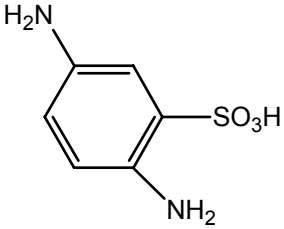
Usually laccase is able to catalyze polymerization reactions of various substituted anilines, performing an oxidative oligomerization established by a non-enzymatic coupling reaction between substituted anilines (Hoff *et al.* 1985). The catechol is a known substrate for laccase that polymerizes forming a poorly soluble product (Aktas and Tanyolaç 2003). In the present study the presence of a phenolic compound in the system, in this case catechol, offers the

advantage of enhancing the degree of polymerization. It was earlier suggested that the presence of catechol in the reaction media disfavored the aromatic amine self-coupling and enhanced the coupling between catechol and the amines (Anderson 2000, Thorn *et al.* 1996). We assumed that the major part of the coupling product was included in the identified polymeric matrix of catechol and precipitates from the solution as a copolymer. In the present studies, it was observed that the amounts of polymers obtained in the laccase catalyzed processes of DBSA or catechol alone in solution or of DBSA and catechol both present in the reaction media were significantly different, confirming the earlier published work (Thiele *et al.* 2002).

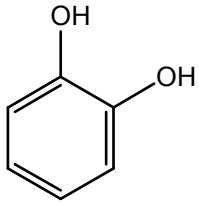
The ^{13}C-NMR analysis confirmed the difference in the structure of the polymers obtained when both reactants are in solution, and of the polymers obtained when catechol and DBSA were reacted separately. The differences in the peaks positions between catechol, DBSA and DBSA/catechol polymers allowed the interpretation of the inhibition effect of the catechol on the aromatic amine self-coupling with the formation of a copolymer between the oxidized anilines and catechol, from the simultaneous non-enzymatic coupling and enzymatic polymerization reactions (Klibanov *et al.* 1983, Simmons *et al.* 1989).

Table 4.3 - Chemical shifts in the CP/MAS ^{13}C NMR spectra of the samples treated with laccase

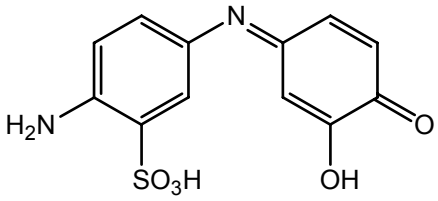
Description	Catechol	DBSA	Catechol+DBSA
C or CH ring	144.0	111.7	144.7
	122.5	113.5	116.2
		123.5	
		125.8	
		128	
		129	
		131.8	
		134.6	



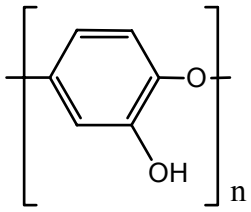
2,5 diaminobenzene sulfonic acid (DBSA)
(m/z 187)



Catechol
(m/z 109)



Identified couple product :
2-Amino-5-(3-hydroxy-4-oxo-cyclohexa
-2,5-dienylideneamino)-benzenesulfonic acid
(m/z 293)



Chemical structure of
laccase-catalyzed
poly(catechol)
(m/z 325; n=3)

Figure 4.5 - Identified catechol polymer and couple product between DBSA and catechol.

4.4. Conclusion

Phenolic azo dyes are easily degraded by laccase. However, due to the non-specific laccase reactions, several non-phenolic substrates are degraded by laccase even without mediators. In the present chapter a mechanism of laccase dye degradation is proposed for both phenolic and non-phenolic azo dyes. The dyes were, in the first hours of the enzymatic treatment, rapidly decolorized and it was observed a decrease of the absorbance, especially in the peak of the maximum wavelength. However, after 24 hours, an increase in the absorbance in all the range of the visible spectrum was observed, due to polymerization reactions, leading to the darkening of the solution. Under longer times of oxidation, the products obtained during the degradation, can undergo further reactions so that they can react between themselves or with the unreacted dye, producing a large amount of coupled and polymeric products. The laccase-catalyzed reaction of the phenolic and aminophenolic compounds is a coupling/polymerization reaction, which occurs in the same manner as described in this chapter. The presence of laccase in solution leads to the oxidation of all the compounds in the system, due to the fact that this enzyme has a high oxidative potential. In the batch decolorization processes of the azo dyes, in the presence of laccase, the polymerization reactions must be considered, since that in several cases acceptable dye degradation cannot be attained limiting factor the application of laccases as bioremediation agents.

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed."

Albert Einstein

5

Kinetics of dye degradation and coupling/polymerization reactions mediated by *Trametes villosa* laccase

5. Kinetics of dye degradation and coupling/polymerization reactions mediated by *Trametes villosa* laccase

5.1. Introduction

In this chapter, the "traditional" direct decolorization of effluents using a free and immobilized form of laccase and the coupling/polymerization reactions in an azo reductase pretreated effluent are compared, on the basis of the kinetic parameters using a HBT/laccase system.

The conclusions of the previous chapters are that the immobilized laccase is not stable in dyeing liquors and that the laccase-catalyzed polymerization reactions can seriously interfere in batch dye bioremediation. Therefore, an alternative method that takes advantage of the laccase characteristic to polymerize phenol and amine compounds was developed. Laccase was associated with an azo reductase that under microaerophilic conditions can cleave a wider range of azo dyes into corresponding amines (Ramalho *et al.* 2002, Hoff *et al.* 1985). The *Trametes villosa* laccases is able to polymerize various substituted anilines through an oxidative oligomerization established by a non-enzymatic coupling reaction (Karamyshev *et al.* 2003). However, in order to enhance the degree of polymerization, catechol, a diphenolic compound, was added to the effluent (Aktas and Tanyolac 2003). The presence of catechol disfavors the aromatic amine self-coupling and enhances the coupling between catechol and the amines (Anderson 2000, Thiele *et al.* 2002, Pilz *et al.* 2003). The formation of the insoluble products brings the advantage that they can be removed from effluents in the form of a precipitate by further treatment processes.

5.2. Materials and methods

5.2.1. Chemicals

Methyl orange (3-(4-dimethylamino-1-phenylazo) benzene sulfonic acid sodium salt) (Dye I in Figure 2.1, Chapter 2.2.2) was synthesized as described in chapter 2.2.2. 1-Hydroxybenzotriazole (Figure 2.1 in Chapter 2.2.2), 2,5-diaminobenzene sulfonic acid and catechol (Figure 4.5 in Chapter 4.3.4) were purchased from Sigma, St. Louis, MO. All chemicals were of high purity and used as received. Laccase (EC 1.10.3.2) from *Trametes villosa* (5.3 mg protein/ml, 600 U/ml) was kindly provided by Novo Nordisk, Denmark.

5.2.2. Electrode preparation

For the experiments with laccase in solution a glassy carbon electrode was used as working electrode. Prior to the experiments the surface of the glassy carbon electrode was successively polished with 5, 1, 0.3 and 0.05 μm alumina polish (Buehler Ltd, USA) and then rinsed with 8 M nitric acid and distilled water before use. The laccase-modified electrodes were prepared using rods of solid spectroscopic graphite (SGL Carbon, Werke Ringsdorff, Bonn, Germany, type RW001, 3.05 mm diameter). The graphite rods were first polished on wet fine-structured emery paper (grit size: P1200) and then additionally polished on paper to obtain a mirror-like surface. The electrode rods were carefully rinsed with deionized water and allowed to dry at room temperature. A 5 μl aliquot of the enzyme solution was added to each of the polished ends of the graphite rods and the electrodes were then placed at 4 °C for 1 h in a glass beaker covered with sealing film, to allow the enzyme to adsorb slowly and to prevent rapid evaporation of the droplet of enzyme solution. The enzyme electrodes were then thoroughly rinsed with 0.1 M sodium citrate buffer, pH 5.0, and if not immediately used, they were stored in the same buffer at 4 °C. Weakly adsorbed laccase was desorbed before measurements, by rotating the electrode in buffer for at least 30 min.

5.2.3. Electrochemical experiments

All the electrochemical experiments were performed using a Voltalab 30 Potentiostat (Radiometer Analytical, France), controlled by the Voltamaster 4 (version 5.6) electrochemical software. The working, counter and reference electrodes were respectively: glassy carbon electrode or the modified graphite electrode (0.07 cm^2), coiled platinum wire (23 cm) and an Ag|AgCl electrode filled with 3M NaCl (BAS, Bioanalytical Systems, West Lafayette, IN, USA). The supporting electrolyte used in the electrochemical cell was a solution of 0.1 M sodium-citrate buffer pH 5.0. All solutions were deoxygenated through bubbling nitrogen for 20 min before measurements. All experiments were performed in bulk using amperometric detection (each experiment was repeated 5 times). The applied potential was -50 mV *vs.* Ag|AgCl. The experiments were performed using a glassy carbon (laccase in solution) or a graphite electrode (laccase adsorbed onto the electrode surface).
The ability of the laccase to decolorize the azo dye was investigated through addition of a freshly prepared dye solution to the electrolyte solution.

5.2.4. Dissolved oxygen consumption rate

Experiments were carried out in a Pyrex flask with a net volume of 250 cm^3. A galvanic oxygen sensor (WTW-InoLab Oxi level 2, Weilheim, Germany, precision of 0.01 mg/l) was used to measure the dissolved oxygen concentration in the reaction medium. To assure a constant temperature, the reactor was immersed in a thermostated water bath operating at 20 ºC with a precision of ±0.1 ºC. The measurements (duplicates) were done under stirring, using a magnetic stirrer at 250 rpm. The monitoring of the degradation started after addition of 20 μl laccase, and the concentration of the dissolved oxygen was monitored continuously for 15 min. The registered response was corrected towards the response obtained for the blank samples (with buffer only).

5.2.5. Decolorization of the azo dye using laccase in the presence and in the absence of a mediator

Azo dye solution (10 mM; 2.5 ml) in 0.1 M sodium-citrate buffer pH 5.0 was incubated with 20 µl of laccase and 0.5 ml of 0.1 M sodium-citrate buffer pH 5.0 in a standard cuvette at 25ºC. The absorbance was measured at different incubation times during the experiment and the percentage of effluent decolorization was calculated thereof. In the case when the mediated degradation of the dye was investigated, then the buffer volume (0.5 ml) was replaced with 10 mM buffered solution of 1-hydroxybenzotriazole (HBT).

5.2.6. Coupling experiments

Equimolar solutions of 2,5-diamino benzene sulfonic acid (DBSA) and catechol (10 mM; total volume 2.5 ml) buffered with 0.1 M sodium-citrate buffer pH 5.0, were incubated with 20 µl of laccase and 0.5 ml of buffer in a standard stirred cuvette at 25ºC. In the case of experiments with mediator the buffer volume (0.5 ml) was replaced by 10 mM buffered solution of HBT. Another experiment was performed with a DBSA and laccase premixed solution and the catechol was added successively. The same experiments were performed with catechol and DBSA separately.

5.3. Results and discussion

5.3.1. Methyl orange degradation

Laccase catalyzes the oxidation of organic substrates such as phenolic compounds by molecular oxygen in homogeneous solutions (Leonowicz *et al.* 2001, Shin 2004, Rodriguez Couto *et al.* 2004, Baldrian 2004, Cameselle *et al.* 2003). When laccase is adsorbed on graphite, bioelectrocatalytic reduction of oxygen occurs and is observed as a reduction current caused by direct (mediatorless) electron transfer (DET) from the electrode to the immobilized laccase and then further to molecular oxygen in solution. In the presence of soluble electron donors, laccase can be reduced in a mediated electron transfer (MET) mechanism (see Figure 1.9 in Chapter 1.9). In this mechanism the electron donor (substrate) penetrates the active site of the enzyme where it is oxidized in a single electron oxidation step often producing an electrochemically active compound (possibly a radical) that in turn can be re-reduced at the electrode surface in a mediated electron transfer (MET) step.

The responses are dependent on the concentration of the azo dye in the solution of interest. At higher azo dye concentrations the current-concentration dependence gradually reached saturation (Figure 5.1).

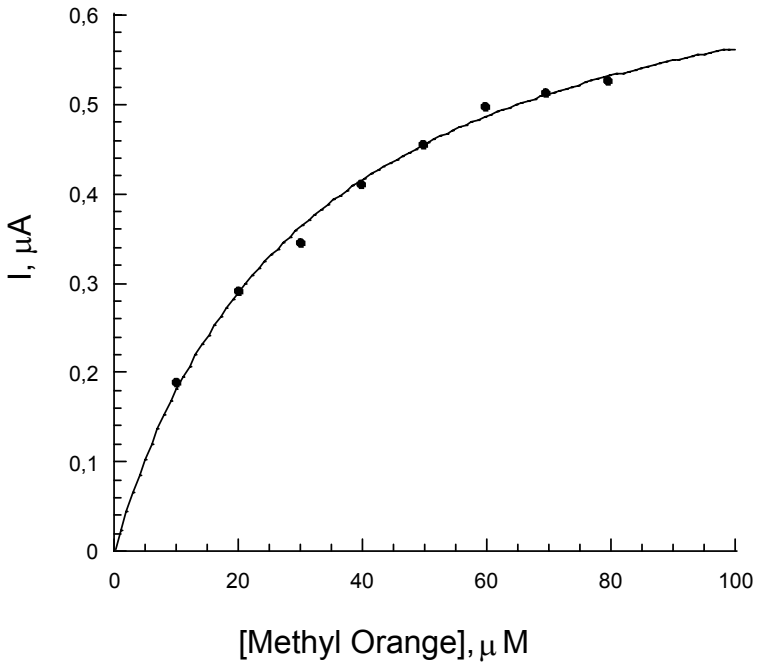


Figure 5.1 - Calibration graph for methyl orange obtained with a laccase modified graphite electrode in 0,1 M citrate buffer pH 5.0, at -50 mV vs. Ag|AgCl electrode filled with 3 M NaCl.

The apparent Michaelis–Menten constants (K_m^{app}) and maximal currents (I_{max}) have been calculated by fitting the variation of current–concentration dependencies of the analyzed compounds to the electrochemical Michaelis–Menten equation (Shu and Wilson 1976):

$$I = \frac{I_{max}[S]}{[S] + K_m^{app}} \quad (1)$$

In this equation S is the substrate concentration, I_{max} the maximum current and K_m^{app} the apparent Michaelis–Menten constant. K_m^{app} is an indicator of the affinity that an enzyme has for a given substrate, and hence the stability of the enzyme-substrate complex. The calculated values of K_m^{app} (calculated

from Hanes–Woolf linearization of the equation (1)) and the catalytic efficiencies are presented in Table 5.1.

Table 5.1 - Results obtained for the oxidation of the methyl orange by laccase (average of 5 indipendent experiments)

	I_{max} (μA) ± S. D.	K_m^{app} (μM) ± S. D.	I_{max}/K_m^{app}(μA/μM) ± relative S.D.
Azo dye and immobilized laccase	0.793±0.002	31.497±0.075	0.025±0.003
Azo dye and immobilized laccase + HBT	1.510±0.009	0.699±0.004	2.160±0.008
Azo dye and laccase in solution	29.442±0.187	8.206*±0.052	3.588±0.009
Azo dye and laccase in solution + HBT	1.781±0.012	0.377±0.003	4.724±0.009

* value comparable with the K_m^{app} (7,40 μM) obtained in the oxygen consumption experiments.

S.D. – Standard deviation

The experiments with immobilized and free laccase suggest that the immobilized laccase is less accessible than the free enzyme for interaction with the dye (Emine and Leman 1995). This fact is also confirmed by comparing the catalytic efficiencies of the oxidation reactions, values that for the adsorbed laccase were found to be about three hundred times lower than for the system with laccase in solution.

The presence of HBT in the system led to a lower K_m^{app} (between 20 and 50 times lower) than in the mediatorless system. The kinetics of mediated laccase catalyzed reactions is firstly affected by the affinity between enzyme and the mediator. An estimation of this influence can be done by amperometric measurements of the I_{max}/K_m^{app} ratio. Lower K_m^{app} values at similar catalytic currents involve higher effectiveness of the enzyme at lower mediator concentrations.

From the results obtained with free laccase in solution and with laccase adsorbed onto the graphite electrodes it can be concluded that the best

system is the one with laccase in solution because it shows a higher catalytic efficiency and a more narrow dynamic range as a consequence of a higher I_{max} and a lower K_m^{app} value.

It is interesting to note that the presence of the HBT in this system led to a 15 times lower I_{max} value than the one obtained for the mediatorless system. This result might be explained considering that an electrode fouling might occur due to the initial step that is the oxidation of HBT to $HBT^{\bullet+}$ by laccase, followed by the deprotonation of $HBT^{\bullet\cdot+}$ with formation of a nitroxyl radical. The latter eventually abstracts the benzylic hydrogen from the substrate, thereby giving rise to the aldehyde and producing HBT back (Cantarella *et al.* 2003).

5.3.2. Coupling experiments

The feasibility of oxidative coupling between xenobiotics in the presence of oxidoreductive enzymes for the remediation of environmental pollution has been described by various researchers (Bollag and Myers 1992, Klibanov *et al.* 1983, Simmons *et al.* 1989).

In these studies catechol was used as coupler to enhance the possibility of removal of the aromatic amines formed during the azo dye degradation. At the same time DBSA was chosen since it is one of the most studied precursors of the coupling reactions (Anderson 2000).

It was observed that in presence of DBSA the addition of catechol or of laccase to the system gave no change in the current, even if HBT (as mediator) was added to the solution. The absence of a measurable signal at the used concentration of DBSA permitted us to run further experiments in order to study the unmediated and mediated coupling of the catechol with DBSA in presence of laccase.

Firstly the response of the catechol oxidation in presence of laccase was monitored in the absence and in the presence of 100 µM HBT. Since the response of the sensor is proportional to the concentration of the catechol in solution, then if the catechol is consumed in the coupling reaction with DBSA this will be observed as decay in the current.

When the coupling reaction was studied in the absence of HBT the current measured was due to the oxidation of catechol by laccase (data not shown). In the coupling reactions it could be observed that the signal measured with the addition of catechol was lower if DBSA was present in the electrolyte solution. The same low response was observed if catechol was added after previous mixing with DBSA (equimolar ratio). As can be seen from Table 5.2, for this case the values of I_{max} are decreased in both cases when DBSA is present in the electrolyte solution, and, moreover an increase in the values of K_m^{app} is observed leading us to the conclusion that a competitive reaction (coupling of the catechol with DBSA) might take place.

Table 5.2 - Results obtained for the coupling reaction of the DBSA with catechol (average of 5 independent experiments)

	I_{max} (μA) ± S. D.	**K_m^{app} (μM) ± S. D.**	**I_{max}/K_m^{app}(μA/μM) ± relative S. D.**
Catechol and laccase	2.399±0.014	146.970±0.884	0.0163±0.008
DBSA and laccase	n.d.*	n.d.*	n.d.*
DBSA and catechol	n.d.*	n.d.*	n.d.*
DBSA/laccase premixed and catechol	1.741±0.011	174.750±1.091	0.0099±0.002
DBSA/catechol premixed and laccase	1.935±0.017	226.110±1.988	0.0086±0.001
Catechol and laccase + **HBT**	1.535±0.011	260.700±1.841	0.0059±0.007
DBSA and laccase + **HBT**	n.d.*	n.d.*	n.d.*
DBSA and catechol+ **HBT**	n.d.*	n.d.*	n.d.*
DBSA/laccase premixed and catechol + **HBT**	3.128±0.006	133.470±0.274	0.0234±0.002
DBSA/catechol premixed and laccase + **HBT**	1.113±0.004	117.630±0.498	0.0094±0.005

* n.d. - Not detectable. DBSA in presence of catechol or in the presence of laccase gave no change in the current even in the presence of HBT.

S.D. – Standard deviation

In the presence of HBT as mediator it was also observed a reduction in the current registered for the case when the catechol and DBSA were mixed (equimolecular ratio) prior to the addition to the electrolyte solution (• in Figure 5.2) in respect to the response obtained when the catechol addition was made just in the presence of laccase and of the HBT (■ in Figure 5.2). Surprisingly when the addition of catechol to the system was made after addition of the HBT and DBSA it was observed that the registered currents for catechol (▲in Figure 5.2) were higher than in the absence of DBSA (amplification factor of 2).

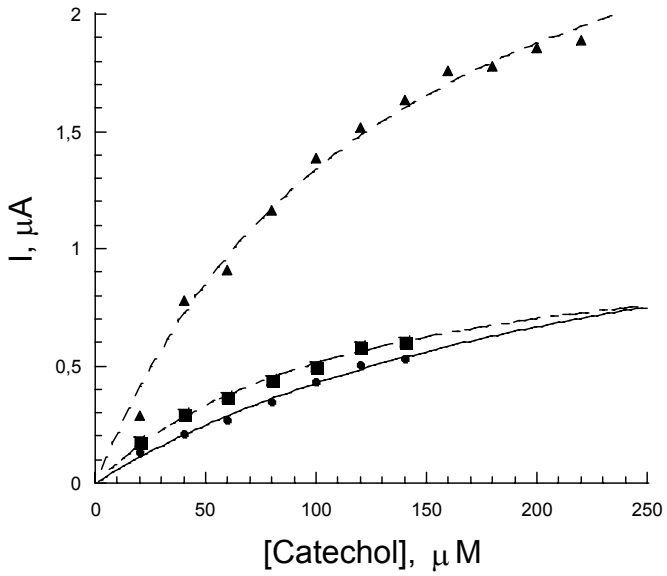


Figure 5.2 - Results for the oxidation of catechol by laccase in presence of HBT. • - catechol premixed with DBSA, ■ - catechol alone, ▲ - catechol added after previous addition of DBSA to the system, in 0,1 M citrate buffer pH 5.0, at -50 mV vs. Ag|AgCl electrode filled with 3 M NaCl.

In the premixed solution of DBSA and catechol a coupling product might be formed before the addition of laccase to the bulk solution and the presence of HBT favored the copolymerization reaction (Anderson 2000, Thorn *et al.* 1996). From Table 5.2 it can be seen that the values obtained for K_m^{app} for the premixed solutions of catechol and DBSA, are decreasing when the reaction occurs in presence of HBT. At the same time when catechol was added after addition of

laccase to the solution of DBSA, the values for K_m^{app} showed the same tendency to decrease in presence of HBT. However, the best coupling system seems to be the premixed solution of DBSA and catechol in the presence of HBT. A full understanding of the interaction between catechol and DBSA especially in the presence of HBT and its implications on the Michaelis-Menten kinetics still needs to be elucidated.

5.4. Conclusion

The addition of 1-hydroxybenzotriazole (HBT) as a mediator improved the degradation of methyl orange using laccase. Indeed the results obtained with free laccase in solution seemed to be better than with the immobilized form, but the differences are not so significant. Besides, the good results obtained when laccase was adsorbed onto the electrode surface provide excellent promises for using of these systems on online monitoring of the enzyme activity. The most important feature revealed in this chapter is the possibility of removal of aromatic amines obtained in the reductive degradation of the azo dyes. This removal was processed by the coupling/polymerization reactions of laccase with catechol, also enhanced by the presence of HBT in the system. The copolymerization between the oxidized anilines and catechol in the effluent, performed by simultaneously non-enzymatic coupling and enzymatic polymerization, yielded products with low solubility. These reaction products were not observed when the anilines and catechol were reacted separately in the presence of laccase in the same conditions. In conclusion the main advantages of these reactions are in the fact that the method, if used for removing of aromatic amines from polluted waters or soils, does not require any further addition of catechol to the system, since it already exists in the humic substances of the soil.

"Change everything so that everything can remain the same"

Giuseppe Tomasi di Lampedusa

6

An alternative application of laccase-catalyzed coupling and polymerization reactions: Enzymatic dyeing of wool

6. An alternative application of laccase-catalyzed coupling and polymerization reactions: Enzymatic dyeing of wool

6.1. Introduction

The ability of laccases to polymerize phenolic and aminic compounds is used to generate color "*in situ*" from originally non-colored, low-molecular substances. This method can be applied for effluent reutilization and as an alternative to the conventional dyeing processes. In the last few years, various patents reported on coloration achieved with laccase (Aaslyng *et al.* 1997, Aaslyng *et al.* 1999, Sørensen 1999, Rørbæk *et al.* 1998, Rørbæk *et al.* 1997, Martin *et al.* 1994, Barfoed *et al.* 2001, Shin *et al.* 2001). Small colorless aromatic compounds such as diamines, aminophenols, aminonaphtols, and phenols, described as dye precursors, are oxidized by laccase to aryloxyradicals. The formed free (cation) radical, may undergo further nonenzymatic reactions resulting in colored dimeric, oligomeric, and polymeric molecules. Dye precursors can be used alone or in combination with a suitable modifier (coupler), in order to enlarge the color palette achieved in the enzymatic dyeing. Knowledge about the process parameters for the enzyme application is, however, quite limited. Implementation of biotechnology in the textile industry aims at replacing traditional chemicals, energy and water high-consuming operations, with appropriate enzymatic processes at milder conditions. The objective of the present study was to investigate the dyeing ability of laccases as an alternative to conventional acid dyes for wool, and to define the optimal experimental conditions to perform the enzymatic dyeing process. Process parameters such as modifier, laccase concentration, and time of dyeing, would influence simultaneously the dyeing result (Tzanov *et al.* 2003b).

6.2. Materials and methods

6.2.1. Enzymatic Dyeing

The textile material used in the experiments was scoured and washed 100 % wool fabric. The dyeing was carried out in 0.1 M acetate buffer pH 5, at 50°C with 2.5 – 10 ml/l *Trametes villosa* laccase from Novo Nordisk (6.8 g protein/l), 0.1 M dye precursor (2,5-diaminobenzene sulfonic acid), 5 – 50 mM dye modifiers (cathehol or resolcinol), for 1 - 9 hours. All reagents were from analytical grade, provided by Sigma. After dyeing the fabrics were thoroughly washed by boiling in non-ionic detergent Lutensol ON 30 (BASF) until no more dye was released in the washing bath. Transmission optic microscope (Olympus BH2) with magnification 40 X was used to observe the dye distribution across the fibers.

6.2.2. Measurement of color differences

A series of experiment were carried out to evaluate the effect on the color of the fabrics of the modifier concentration (mM), laccase amount (ml/l) and dyeing time (h). The responses analyzed were the color characteristics: K/S, L*, a*, b*. K/S is the Kubelka-Munk relationship, in which K is an adsorption coefficient and S is a scattering coefficient. This relationship is applied to textiles under the assumption that light scattering is due to the fibers, while adsorption of light is due to the colorant. L*, a*, and b* are the coordinates of the color in the cylindrical color space, based on the theory that color is perceived by black-white (L* = lightness), red-green (a*), and yellow-blue (b*) sensations. The color of the dyed fabrics was evaluated using a reflectance measuring Datacolor apparatus at standard illuminant D_{65} (LAV/Spec. Incl., d/8, D_{65}/10°). Five areas on each sample were measured in various positions, and the results represent average values with up to 1% variation.

6.3. Results and discussion

Screening experiments were conducted to identify which process parameters influence the color (in terms of K/S, L*, a*, and b*) of the enzymatically dyed fabrics. The experimental matrix and the results are presented in Table 6.1. The first step in the process of seeking optimal conditions for the enzymatic dyeing is to identify the input variables with greatest influence on the responses. K/S and lightness values of the dyed fabrics at the dye maximum absorption wavelenght varied considerably (Table 6.1).

Table 6.1 - Dyeing results with modifiers cathehol and resorcinol (A= modifier concentration (mM), B=laccase amount (ml/l) and C=dyeing time (h))

	Variable			Catechol response				Resorcinol response			
Run	A (mM)	B (ml/l)	C (h)	K/S	L*	a*	b*	K/S	L*	a*	b*
1	5	2.5	1	2.61	48.89	6.58	7.17	2.17	52.57	8.90	8.22
2	50	2.5	1	5.14	39.04	5.91	7.46	2.40	52.64	8.40	11.91
3	5	10	1	3.43	45.01	6.54	7.41	2.49	51.52	8.84	10.26
4	50	10	1	3.34	45.14	6.66	7.13	2.21	53.76	8.24	11.81
5	5	2.5	9	14.16	26.42	6.45	7.85	15.22	23.99	11.29	3.16
6	50	2.5	9	14.94	25.72	5.72	7.93	9.04	33.55	10.76	10.18
7	5	10	9	19.49	22.47	6.11	7.30	18.35	22.78	11.17	5.50
8	50	10	9	23.91	18.30	10.00	3.01	13.97	27.05	10.65	8.05
9	30	7.5	4.5	11.50	28.72	6.31	7.56	7.95	33.75	10.04	7.25
10	30	7.5	4.5	12.86	27.24	6.17	7.42	8.89	32.47	10.29	7.49
11	30	7.5	4.5	14.36	25.77	6.10	7.16	9.60	31.11	10.01	6.80

The function K/S is directly proportional to the concentration of the colorant on the substrate and indicates dye adsorption and fixation. In these dyeing experiments, K/S reflected the amount of fixed dye, since all the unfixed dyestuff was presumably washed off. For the modifier catechol, K/S varied from 2.61 to 23.91 and L* from 48.89 to 18.30. For the other modifier, K/S values ranged from 2.17 to 18.35 and L* from 53.76 to 22.78. The highest K/S value for catechol was achieved at the uppermost levels of the three variables. Interestingly, for resorcinol, the highest K/S value was attained when the modifier was applied at the lowest concentration, while the amount of enzyme and the time of treatment were at their highest levels. Catechol and resorcinol are, respectively, *ortho*- and *meta*-substituted diphenols. Considering runs 7 and 8, the fabrics dyed with catechol appeared redder and bluer with the increase in modifier concentration, while the samples dyed with resorcinol became yellower and greener. Obviously the position of the second OH group in the molecule of the modifier was responsible for the different coloration behavior and the change in hue. This could be explained by the different pathway of the enzymatically-catalyzed reaction between the dye precursor and modifier. Laccase oxidizes the phenolic compounds, converting them to reactive quinone species, which subsequently react nonenzymatically with amines forming 1,4-Michael-type adducts (Figure 6.1).

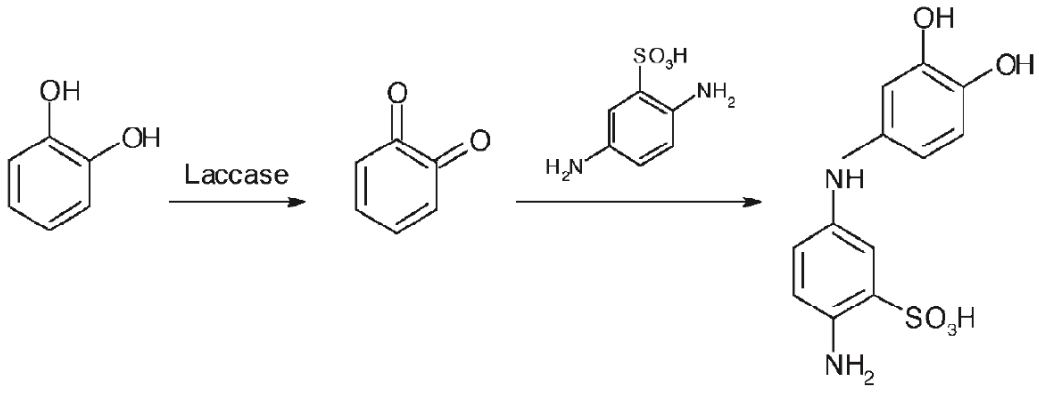


Figure 6.1 - Expected mechanism of reaction between dye precursor and modifier (adapted from Anderson 2000).

The *ortho*-substituted diphenol-catechol, can further undergo another Michael's addition of amine, developing deeper color than that of the resorcinol (Table 6.1). The *ortho*-diphenols were reported as better substrates for laccase than the *meta*-substitutes (Thurston 1994). Apart from reacting with the dye precursor, the phenol modifiers could undergo an oxidatively induced polymerization. Thus, the laccase-mediated oxidation of the dye precursor and modifiers results in highly reactive radicals, which can undergo either self- or cross-propagation with the respective monomers in a way very complex for characterization. Independently of the other variables, increasing the dyeing time from 1 to 9 h (dyeing temperature of 50°C, pH 5.0) drastically increased K/S and decreased L* values. By comparison, the duration of the conventional chemical dyeing process is normally 3 to 4 h, at boiling temperature in highly acidic medium. Problems have been experienced in all attempts to introduce low-temperature methods for wool dyeing, necessitating application of various auxiliaries or solvents to facilitate diffusion of the dye into the fibers, and even then the temperatures were in the range of 60–80 °C. This high time-dependent increase in K/S suggested that a deeper color could be achieved simply by prolonging the contact time between the textile material, enzyme, dye precursor, and modifier, in contrast to the conventional dyeing of wool, in which the depth of the color is proportional to the amount of dye. It is not clear, however, whether the dye was formed in the solution and then was adsorbed on the textile material, or whether it was formed directly on the fabric. Both possibilities exist, since the reactive colored compounds adsorbed on the fabric could continue to interact non-enzymatically. The presence of sulpho-groups in the molecule of the dye precursor provides both solubility of the dye and substantivity toward the wool material. The increase in K/S also indicated a higher dye presence on the fabric. The cross-section image of the enzymatically dyed fibers in Figure 6.2 shows penetration of the dye into the interior of the keratin fiber.

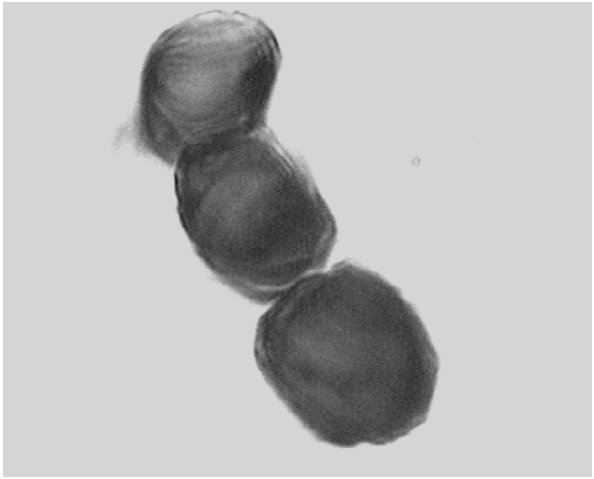


Figure 6.2 - Microscopic photograph of cross-section of wool fibers (original magnification: x40) dyed according to trial 8 from the adopted full factorial design (with catechol).

This image suggests that the small molecules of the dye precursor and modifiers could penetrate beyond the wool cuticles and some portion of the color was formed in the fiber itself. The small size of the dyeing molecules provides levelness of the dyeing.

6.4. Conclusions

Wool dyeing was performed in a dye bath prepared with a dye precursor (2,5-diaminobenzenesulfonic acid), dye modifiers (catechol and resorcinol) and laccase, without any dyeing auxiliaries. By increasing the reaction time and minimizing the enzyme and modifiers loading it is possible to obtain darker coloration of the samples. This fact renders laccase dyeing an economically attractive alternative to the conventional use of high water, dyes, auxiliaries, and energy consuming acid in wool dyeing. Additionally, the enzymatic reaction was carried out at pH and temperature values safe to the textile material. The dyeing experiments with two modifiers having the same molecular weight but with different position of the substituents revealed the potential of the enzymatic approach for achieving a large diversity of colors and hues on the fabrics, varying the starting compounds. Comparison of the two modifiers showed that the concentration was not significant for the color depth in the case of catechol, but very significant in the case of resorcinol. Resorcinol should be used in low concentration to attain deep-shade dyeing. Microscopic observation of the cross-section of the enzymatically dyed wool demonstrated penetration of the colorant into the mass of the fibers.

"The reward for work well done is the opportunity to do more”.

Jonas Salk

7

General discussion and future perspectives

7. General discussion and future perspectives

7.1. General discussion

The aim of this thesis was the study of the dye degradation mechanism by laccase, the enzyme kinetic properties, and operative conditions for azo dye degradation. The limitations of direct laccase-catalyzed azo dye degradation were shown and the laccase-catalyzed polymerization reactions as an alternative effluent bioremediation methodology were highlighted.

The best working conditions for the catalysis by laccase were established with activity measurements and voltammetric techniques. The optimal temperature for 1 h treatment with laccase is 45 ºC and the optimal pH 5. The voltammetric measurements showed a remarkably good linear correlation between the percentage decolorization of each dye and the respective anodic peak potential. It was demonstrated that the redox potential differences between the biocatalysts and the dyes are a relevant preliminary tool to predict the decolorization capacity of oxidative and reductive biocatalysts.

Although redox potentials can help to predict dye biodegradation, in bioremediation with free enzymes, there are also other parameters that can limit their use. Therefore, the stability and the dye degradation ability of free and immobilized laccase were investigated. Relatively high amounts of salt in the dyeing effluent enhance the electrostatic coupling of anionic dyes with positively charged proteins, thereby forming stable dye/enzyme aggregates (Göller and Galinski 1999, Dötsch *et al.* 1995, Carpenter and Crowe 1988). Such stabilization occurred with both free and immobilized enzyme in Reactive Black 5 (RB5) effluent (comparatively to the RB5 pure solution) and in the salt solution (comparatively to the buffer). The sulphonate dye and the salt present in RB5 dyeing effluents probably exert a synergistic stabilization effect on free laccase (Matulis *et al.* 1999). Surprisingly, the immobilized laccase showed lower stability and lower decolorization than the free form in dyeing effluents. The immobilization procedure has a variety of effects on protein conformation as well as on the state of ionization and dissociation of the enzyme and its environment (Rogalski *et al.* 1995, Emine and Leman 1995). RB5 dye exerts a

destabilizing effect on the alumina-laccase complex. Moreover, due to the alumina high porosity the adsorption of the dye appears to be the most important factor in decolorization. The immobilized laccase, even after the 4th cycle of reuse, showed greater decolorization efficiency than the free enzyme due to the high dye adsorption on the alumina support. In spite of these limitations both immobilized and free laccase showed an acceptable decolorization degree and the re-dyeing experiments using decolorized RB5 effluents are comparable with conventional dyeing processes.

The mechanism of laccase dye degradation is proposed for both phenolic and non-phenolic azo dyes. Even though dye I is not a phenol azo dye that would be a typical substrate for laccase, extensive degradation was observed. Earlier reports on the chemical oxidation of methyl azo dyes suggest that one-electron extraction from the amino substituent occurs in the initial step of dye degradation (Bianco Prevot *et al.* 2004). The resulting radical cation undergoes an oxidation and leads to the formation of an iminium ion, and then the secondary amine can be formed through solvolysis processes (Galindo *et al.* 2000, Darwent and Lepre 1986). In this case the mechanism could follow a similar pathway, performing one–electron oxidation of the tertiary amine and subtraction of hydrogen radical, with a subsequent demethylation and followed by the oxidation of the secondary amine by laccase. Nucleophilic attack by water on the phenolic ring carbon bearing the azo linkage causes N-C-bond cleavage. In the case of dye III, the presence of the coupling products of 1,2-naphthoquinone confirms the most accepted model of azo dye degradation by laccase (Chivukula and Renganathan 1995, Soares *et al.* 2002). According to this model, laccase oxidizes the phenolic group of the dye with the participation of one electron, generating a phenoxy radical, which is sequentially followed by the oxidation to a carbonium ion. The nucleophilic attack by water on the phenolic ring carbon bearing the azo linkage causes N-C-bond cleavage. The LC-MS and ^{13}C –NMR data showed that, under long times of oxidation, the products obtained during the azo dye degradation reactions, can undergo further reactions. These products can be polymerized or coupled among themselves or with the unreacted dye producing a large amount of coupled and polymeric products leading to a darkening of the solution. In order to confirm the efficiency of the laccase polymerization, a

preliminary study of the ability of *Trametes villosa* laccase to catalyze polymerization and coupling reactions between amines and phenols was performed. Usually the laccase is able to catalyze the polymerization reaction of various substituted anilines, performing an oxidative oligomerization established by a non-enzymatic coupling reaction between substituted anilines (Hoff *et al.* 1985). Catechol is a known substrate for laccase that polymerizes forming a poorly soluble product (Aktas and Tanyolaç 2003). It was earlier suggested that the presence of catechol in the reaction media disfavors the aromatic amine self-coupling and enhances the coupling between catechol and the amines (Anderson 2000, Thorn *et al.* 1996). We assumed that the major fraction of the coupling product was included in the identified polymeric matrix of catechol and precipitates from the solution as a copolymer (Klibanov *et al.* 1983, Simmons *et al.* 1989). In the laccase catalyzed azo dye decolorization, the polymerization reactions must be considered, since in several cases acceptable dye degradation could not be attained, limiting its application as a bioremediation agent.

The “traditional” direct laccase decolorization of effluent in free and immobilized forms and the coupling/polymerization laccase reactions in the azo reductase pretreated effluent were compared on the basis of the kinetic parameters using an HBT/laccase system. The kinetic parameters showed that with the addition of the mediator it was possible to improve dye degradation and polymerization using laccase. Indeed the kinetic results obtained with the laccase in solution seem to be better than the ones with immobilized laccase, confirming previous observations (Emine and Leman 1995). The catalytic efficiencies values for the adsorbed laccase were found to be about three hundred times lower than for the system with free laccase. The presence of HBT in the system led to lower K_m^{app} (between 20 and 50 times lower) than in the mediatorless system. The most important feature disclosed in this work is the possibility of physical removal of the aromatic amines eventually obtained in the reductive degradation of the azo dyes processes by coupling/polymerization reactions, also enhanced by the presence of catechol and HBT in the system. In the premixed solution of DBSA and catechol a coupling product might be formed before the addition of laccase to the bulk solution and the presence of HBT favored the copolymerization reaction

(Anderson 2000, Thorn *et al.* 1996). The values obtained for the K_m^{app} for the premixed solutions of catechol and DBSA, are lower when the reaction occurs in presence of HBT. At the same time when the catechol was added after addition of laccase to the solution of DBSA, the values for K_m^{app} show the same tendency to decrease in presence of HBT. This method may be extended to the removal of aromatic amines from polluted soil, without any further addition of catechol to the system, since it already exists in the humic substances of the soil. However, a better understanding of the laccase/polymerization and laccase/mediator mechanisms is required.

It was proved that the ability of laccases to polymerize phenolic and aminic compounds can be successfully applied as an economically attractive alternative to conventional water, dyes, auxiliaries, and energy high-consuming wool dyeing processes. Screening experiments were conducted to identify which process parameters influence the color of the enzymatically dyed fabrics. The highest coloration value for catechol was achieved at the highest levels of the three variables (modifier concentration, laccase amount and dyeing time). Interestingly, for resorcinol the highest coloration value was attained when the modifier was applied at the lowest concentration, while the amount of enzyme and the time of treatment were at their highest levels. Independently of the other variables, increasing the dyeing time from 1 to 9 h drastically increased coloration values. Analysis showed that by increasing the reaction time and minimizing the enzyme and modifiers loading darker coloration of the samples could be obtained.

In summary, it is concluded that compared to the common and expensive physical or chemical ways for dye effluent remediation, the biodegradation by the use of a immobilized *Trametes villosa* laccase appears to be an attractive alternative even if it is quite unstable in dyeing liquors. It is also clear that the laccase-catalyzed polymerization reactions can seriously interfere in batch dye bioremediation. Therefore, color removal or color application alternative methods that take advantage on laccase's proprieties to polymerize phenol and amine compounds, also in presence of a mediator, seem to be very attractive technologies.

7.2. Future perspectives

The work described in the present thesis has allowed important information about the use of laccase in textile applications. Significant steps forward are possible and efforts are already being made in that direction.

The promising voltammetric tool should be extended to further studies with other classes of dyes and with different oxidoreductase enzymes. Preliminary experiments with different dye classes show that basic dyes also display a linear correlation between anodic peak potential and dye decolorization.

The limitations of the alumina as an efficient immobilization support for laccase in dye decolorization were demonstrated. Therefore, different and more efficient immobilization supports and techniques should be investigated in future to improve immobilized-laccase stability in dyeing liquors. The adsorption of oxidized products on the surface of the alumina support shows that enzyme inactivation occured. This effect, when reversible, can be partially overcome by using washing procedures with high ionic strength buffered solutions or using low-adsorbing supports such as vitroceramic materials. However the most promising approach is to combine laccase with cationic polymers, such as chitosan, which are able to promote the coagulation of oxidized reaction products. In the present work the free laccase shows better activity and kinetic performances in comparing with the immobilized form. Therefore, a soluble/insoluble support like Eudragit™ that is reversibly soluble depending on the pH of the medium, can be used for improved enzyme stability, particularly at high temperatures, without major loss of specific activity. This approach presents all the advantages of the enzymes in solution and, additionally, the biocatalyst can be recovered and reused.

It is clear that further studies should be performed in order to better understand the laccase mediated degradation mechanism of non-phenolic substrates. *In vivo* experiments will be necessary to understand the complex pathways of dye degradation products in the natural environmental. LC-MS and ^{13}C–NMR data showed the formation of products that can be polymerized or coupled among themselves or with the unreacted dye, producing a large amount of coupled and polymeric product. The LC/MS technique, in particular,

revealed to be a powerful tool to understand the complexity of the free radical reactions catalyzed by laccase. Therefore, the study of laccase degradation mechanisms should also be extended to anthraquinone, indigoid and triarylmethane dyes that can be oxidized through laccase-mediated reactions.

The kinetic parameters showed that with the addition of a mediator it is possible to improve dye degradation and polymerization using laccase. Further study should consider different and less polluting mediators such as the natural mediators produced by laccase in natural environment during lignin degradation. However, the most important feature disclosed in this work is the possibility of physical removal of aromatic amines by coupling/polymerization reactions with phenols. Preliminary experiments were performed in an attempt to associate the polymerization proprieties of laccase with an azoreductase. The reductive enzymatic degradation of the azo dyes produces amines that can be subsequently coupled or polymerized with laccase and phenols. This method may be also extended in the future for the removal of aromatic amines from the polluted soil.

It was proved that the ability of laccases to polymerize phenolic and aminic compounds could be successfully applied as an alternative dyeing process. Further enzymatic polymerization applications may be performed not only for dyeing but also for surface modification and functionalization. These enzymatic applications are a promising technology especially for the coating of natural and synthetic materials.

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