Universidade de Aveiro Departamento de Química
Ano 2014

Andreia de Freitas Silva

Aplicação de novas oxidorredutases no branqueamento de pasta kraft

Application of new oxidoreductases in bleaching of kraft pulp

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Química, realizada sob a orientação científica do Professor Doutor Dmitry Victorovitch Evtuguin, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro e do Doutor Pedro Emanuel Garcia Loureiro, investigador no Departamento de Produtos Florestais da Novozymes A/S, Dinamarca.

Dedico este trabalho aos meus pais e ao meu irmão.

o júri

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Palavras-chave *Eucalyptus*, pasta kraft, branqueamento, ECF, TCF, sequências, lacase, mediador, peroxidase, brancura ISO, propriedades da pasta

Resumo

O branqueamento da pasta de papel é um dos processos mais importantes e dispendiosos da indústria da pasta e papel. Ao longo dos anos esta tecnologia atravessou várias alterações no sentido de reduzir os riscos ecológicos e os custos de produção.

O objetivo deste trabalho é efetuar um estudo sobre a aplicação de uma nova oxidorredutase, produzida pela Novozymes A/S, em branqueamento ECF e TCF usando pasta kraft de *Eucalyptus* pré-branqueada com oxigénio e, posteriormente comparar o desempenho desta enzima com o de outras oxidorredutases já estudadas ou comercializadas pela empresa.

A oxidorredutase usada neste estudo foi primeiramente a lacase NS-51002 (e as suas variantes), cujo meio de cultura foi purificado. O sistema lacase-mediador e as condições de incubação foram otimizados. De seguida, o desempenho da lacase NS-51002 no branqueamento foi comparado com o de outras enzimas, tais como a lacase Novozym 51003 e as peroxidases NS-51004 e NS-51113. A peroxidase NS-51113 demonstrou ser a melhor enzima, assim sendo também foi otimizada em termos de condições de aplicação. Para além dos estágios enzimáticos, também foi otimizado a carga de químicos do estágio de extração alcalina. Seguidamente, a lacase NS-51002 e a peroxidase NS-51113 peroxidase foram implementadas em várias sequências de branqueamento ECF e TCF. De modo a estudar o efeito das enzimas e químicos no branqueamento, foram determinadas algumas propriedades em diferentes partes da sequência, tais como: brancura ISO; número kappa; teor de ácidos hexenuronicos; viscosidade; pH, teor residual de peróxido de hidrogénio e CQO nos filtrados da pasta.

Usando a sequência DE_pDP (90,5%) como referência, foi possível concluir que a implementação de um estágio enzimático com xilanase no início da sequência, i.e. XDE_pDP (91,1%), aumentou a brancura da pasta. Além disso, algumas sequências que usam a peroxidase NS-51113 como estágio L provaram ser bastante eficazes sob o ponto de vista do branqueamento, permitindo alcançar uma brancura ISO de 90±0,5, em particular a sequência XLE_pDP (89,5%).

Keywords

Eucalyptus, kraft pulp, bleaching, ECF, TCF, sequences, laccase, mediator, peroxidase, ISO brightness, pulp properties

Abstract

Pulp bleaching is one of the most important and expensive processes in the pulp and paper industry. Along the years this technology has gone through several changes in order to reduce the ecological risks and the production costs.

The aim of this work is to study the application of a new oxidoreductase, produced by Novozymes A/S, in ECF and TCF bleaching of oxygen pre-bleached *Eucalyptus* kraft pulp and then to compare the bleaching performance of this enzyme with other oxidoreductases already studied or commercialized by the company.

The oxidoreductase used in this study was primarily the NS-51002 laccase (and its variants) of which culture broths were purified. The laccase-mediator system and the incubation conditions were optimized. Then the NS-51002 laccase bleaching performance was compared with other enzymes like Novozym 51003 laccase and NS-51004 and NS-51113 peroxidases. The best enzyme proved to be the NS-51113 peroxidase, which was also optimized in terms of application conditions. Besides the enzymatic stages, the dosage of chemical used in the alkaline extraction stage was also optimized. Afterwards, the NS-51002 laccase and the NS-51113 peroxidase were implemented in several ECF and TCF bleaching sequences. To study the effect of the enzymes and chemicals on bleaching a number of properties were measured in different parts of the sequence, such as: ISO brightness; kappa number; hexenuronic acid content; viscosity; pH, residual hydrogen peroxide and COD in the pulp filtrates.

Using the sequence DE_pDP (90,5%) as reference, it was concluded that the implementation of an enzymatic stage with xylanase in the beginning of the sequence, i.e. XDE_pDP (91,1%), enhanced the pulp brightness. Furthermore, some sequences using the NS-51113 peroxidase as the L stage proved to be quite effective from the bleaching point of view, allowing to reach 90±0,5 of ISO brightness, in particular the sequence XLE_pDP (89,5%).

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Nomenclature

List of Abbreviations

A – Hot acid stage

AS – Acetosyringone

BOD – Biochemical oxygen demand

BR – Britton-Robinson

C – Chlorine stage

CED – Cupriethylenediamine

COD – Chemical oxygen demand

D – Chlorine dioxide

DTT – Dithiothreitol

E – Alkaline extraction stage

ECF – Elemental chlorine free

E_o – Alkaline extraction stage reinforced with oxygen

E_p – Alkaline extraction stage reinforced with hydrogen peroxide

EP – Enzyme protein

EPR – Electron paramagnetic resonance

ES – Enzyme solution

G – Guaiacyl unit

H – p-Hydroxyphenyl unit or hypochlorite stage

HexA – Hexenuronic acid

HIC – Hydrophobic interaction chromatography

HOBT – 1-Hydroxybenzotriazole

IEC – Ion exchange chromatography

L – Laccase or peroxidase stage

LACU – Laccase activity unit

L_c – Laccase or peroxidase control stage

LCC – Lignin-carbohydrate complex

LMS – Laccase mediator system

M – Transition metals

MeS – Methylsyringate

MES – 2-(N-morpholino)ethanesulfonic acid

MTP – Microtiter plate

NHA – N-hydroxyacetanilide

od – Oven-dried

odp – Oven-dried pulp

P –Hydrogen peroxide stage

PCR – Polymerase chain reaction

PEG – Polyethylene glycol

PHL – Pre-hydrolysis liquor

PPT – Phenothiazine-10-propionic acid

S –Syringyl unit

SA – Syringaldehyde

SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoreses

TCF – Total chlorine free

TEMPO – (2,2,6,6-tetramethylpiperidin-1-yl)oxy

Tris – Trisaminometano

UF – Ultra-filtration

VA – Violuric acid

X – Xylanase stage

List of symbols

Symbol	Meaning	Unit
A	Absorbance	
c	Viscometer constant	
C	Concentration	g/L or kg/t_{odp} or $mmol/kg_{odp}$
CF	Correction factor	
d	Density of the pulp solution	g/cm^3
DM	Dry matter	%
DF	Dilution factor	
KN	Kappa number	
KF	Kappa factor	
l	Cuvette pathway length	cm
m	Mass of wet pulp	g
M_w	Molecular weight	Da
n	Number of moles	mol
t	Average efflux time	s
v	Viscosity	cP
V	Total volume	mL
V_{Lac}	Laccase volume	mL
x	Blank volume	mL
Y	Kappa number guess	
w	Weight of oven dried pulp	g
ε	Molar absorption coefficient	$M^{-1}cm^{-1}$

Introduction

The paper is an ancient object that was invented in China in the II century. Before the invention of paper, the primitive people used parchment, palm leaves and papyrus instead. From these times, the history of paper evolved exponentially, but the main goal of the paper was and always will be the same: transmit a message.

In Europe, the paper manufacturing started in the Iberian Peninsula in the X century by using a water-powered mill. The printing revolution in the XV century and the development of Fourdrinier machine in the early XIX century boosted the modern methods of papermaking. The evolution continued since then regarding to the raw materials, techniques and uses of paper. Nowadays pulp and paper production is a complex and multifaceted process of great dimension composed by several steps and techniques, starting by wood handling, pulping, bleaching and paper production.

In order to understand the behaviour of wood during the production steps mentioned above, it is fundamental to have knowledge about its structural and chemical features. Therefore, the early part of this work will focus in these aspects. Thereafter, it will be made a brief approach regarding the kraft pulping process, seeing that the pulp utilized in this study was produced using this pulping process.

The kraft pulping represents more than 90% of the worldwide pulp production. It was conceived in 1879 in Germany and it was first implemented in a Swedish mill in 1890.[1]

The pulp bleaching process is also very important and will be the main topic of this work. Bleaching of pulp had suffered several changes along the years, mainly related to the process, the equipment and especially related to the chemicals utilized. These changes were caused by economical and legislative factors, and especially by constant pressure coming from the global environmental protection organizations that were concerned about emissions of dangerous and toxic effluents from the mills.

Chlorine usage in pulp bleaching appeared in the early 1900s and was one of the first and efficient bleaching agents used in the industry. However, it is harmful to the environment due to emissions of chlorinated organic compounds in the water effluents. Therefore, chlorine was substituted by chlorine dioxide in the middle of the XX century, which originated the Elemental Chlorine Free (ECF) bleaching.

Later on appeared Totally Chlorine Free (TCF) bleaching, which would solve the problem of chlorinated organic compounds in the effluents through substitution of chlorine dioxide by e.g. oxygen, ozone and hydrogen peroxide. Although TCF bleaching is beneficial for the environment,

it is not so efficient in terms of bleaching, yield and pulp quality. Actually, TCF bleaching only represented 25% of the European market in 1999 and it is now considered as a niche market.

It is therefore still a challenge to efficiently remove lignin and chromophores from the unbleached pulp without affecting polysaccharides. In this context, the use of a delignification system based on lignolytical enzymes would be of high interest to this industry. These enzymes already proved to be efficient in delignification and brightening when used with an appropriate mediator and with process-optimization.

Novozymes A/S is a biotech company focused on the production and commercialization of enzymes being the world leader in bio-innovation. Therefore, the R&D part of the organization is very important to deliver everyday products to the market with enhanced sustainability and performance allowing e.g. energy cost savings and decrease of raw material costs. The selection of an enzyme comprises the exploration of nature by the collection of fungi and bacteria, selection of the strains, enzyme modifications by genetic engineering, enzyme production and application performance in different areas (e.g. detergents, forest products, food, etc.).

The present work attempts to contribute for the development of enzymatic pulp bleaching by optimizing a new experimental oxidoreductase produced by Novozymes A/S, namely a laccase. This enzyme will be compared with other oxidoreductases (experimental and commercial), that already have been tested for bleaching purposes. Finally, the best enzymes and optimized conditions will be introduced in several ECF and TCF bleaching sequences with different pre-bleaching approaches including hot acid stages (A) and xylanase stages (X).

The conclusions from this work will be based on the application performance of the several enzyme candidates and sequences tested in terms of pulp and filtrate analysis, such as ISO brightness, viscosity, kappa number, HexA content, filtrate COD, pH and chemicals consumption. These analyses will be performed in different points of the sequences, which enable a more wide-ranging study of the enzymes and synergies with other chemicals employed along the sequences including savings and substitution of bleaching chemicals, such as chlorine dioxide.

Chapter 1: Literature review

1.1. Wood Structure and Chemistry

The wood is a complex element that can be divided into three main types: softwood, hardwood and grasses. Macroscopically the wood is composed by the bark, the cambium, the xylem, the pith and the rays, which have functions of mechanical support and nutrient storage and transport.

In a microscopic level, the wooden tissues consist of several types of cells that differ in shape and size, which can be divided into prosenchyma (thin and longer cells, narrower towards the end, e.g. tracheids and libriformes) and parenchyma (short cells, rectangular or round).

In a chemical level, the wood is an organic material composed by carbon (49%), oxygen (44%), hydrogen (6%), nitrogen (<1%) and inorganic elements (<<1%).[2] All these elements combined together originate different polymers that comprise the cell wall of wood such as cellulose, hemicelluloses and lignin. Low molecular-weight substances like extractives and ash can also be found in the cell wall. The proportions of these components differ according to wood type and species.

The cellulose is the main component of wood and consists of a long and strictly linear homopolymer of several β-D-glucopyranose units linked by β(1→4) glycosidic bonds. The general formula of cellulose is $(C_6H_{10}O_5)_n$, where *n* represents the polymerization degree.[3]

The hydroxyl groups are free to form hydrogen bonds with oxygen atoms. Intramolecular bonds are responsible for the chain stiffness and strength, whereas intermolecular bonds are responsible for the supramolecular structure of cellulose.[2] The cellulose chains assemble into microfibrils with high tensile strength, which in turn assembles into fibrils and these into the cellulosic fibers, originating a complex structure.[4]

The hemicelluloses are polysaccharides of non-cellulosic origin, consisting of several sugar moieties, such as D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, glucuronic acid and L-rhamnose; acetyl groups are also a part of this polymer. Hemicelluloses are mostly branched and have a molecular weight lower than cellulose.[5] They can be classified into pentosanes and hexosanes: xylans and arabinans belong to the first group while mannans, glucans and galactans belong to the second. All of these compounds can be found either in softwood and hardwood, but there are major differences related to their structure and amounts.[2,4]

The lignin is a complex aromatic polymer specialized in mechanical strength and in keeping the fibers linked to each other. Lignin has an amorphous and highly branched structure; it

is predominantly hydrophobic and has low reactivity being largely insoluble in regular solvents. Therefore, the wood delignification process requires extreme operating conditions.[6]

The precursor units of lignin are three hydroxycinnamyl alcohols or monolignols: p-coumaryl, coniferyl and sinapyl alcohol, which are synthesized via the phenylpropanoid pathway and differ only in the number of methoxy groups attached to the aromatic ring. These monolignols undergo radical coupling reactions originating three lignin units respectively: p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S). The reactivity increases and selectivity decreases from unit H to unit S.[6]

These units are covalently linked to one another in a complex and random way resulting in a large variety of lignin molecules and for this reason lignin is the most heterogeneous wood component. The predominant bonds in lignin are: ether bonds (β-O-4 and α-O-4), which are easier to cleave; β-5, 4-O-5 and β-1 bonds; and β-β and 5-5 or C-C bonds, which are the hardest to cleave.[7]

1.2. Process of pulp production

The knowledge of wood structure and chemistry is very important to understand the transformation process of wood into paper. This process occurs in pulp mills through a series of stages and techniques, as shown in Figure 1. This section will focus on wood handling in general and kraft pulping. The bleaching step will be discussed in 1.3.

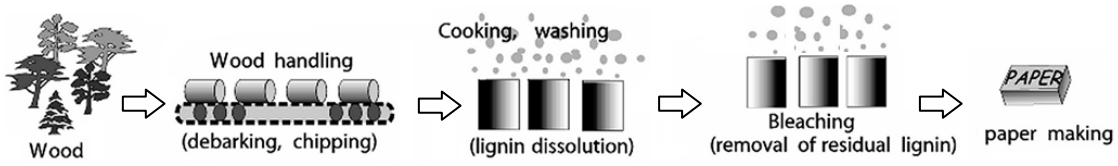


Figure 1 – Main steps of the process of chemical pulp production (Adapted from [8]).

The raw material, in the form of logs is received in the wood yard. Before pulping, the bark is removed from the logs in a process called debarking. Otherwise, the bark presence will promote a low yield, equipment damage, high content of extractives, among others.[9] The peeled logs need to be cut into small and uniform pieces called wood chips, in order to reduce fiber damage, while allowing good penetration of chemicals and heat into the wood during the cooking process.[10] However, not all of the produced chips have the required size and shape; hence the chips have to be screened before pulping. Oversized chips are rejects and are re-chipped and sent to the screener, while the fines and dust are used as fuel in the bark boiler.[9]

Then the chips are ready for cooking or pulping, which is the process that allows the release of the fibers from the wood matrix, through either mechanical or chemical treatments, or even a combination of both. Therefore, the resulting set of fibers (pulp) will have different properties that will be used according to distinct applications.[11]

Mechanical pulping requires a lot of electrical power, but provides high process yield since practically the whole wood material is used. On the other hand, in chemical pulping only half of the wood is transformed into pulp and the other half is dissolved but without demanding of external energy. Moreover, chemical pulp fibers are more flexible than mechanical, which offers better strength properties to the pulp.[11]

On a global scale, chemical pulping is the predominant type of pulp production and this process occurs in big pressurized vessels called digesters, that are designed to operate continuously or in batch mode.[12] The chemical pulping process can be divided as well into different pulping methods, which have different characteristics and operating conditions, such as kraft, soda, acid sulphite and bisulphite.[13]

In this work it was used chemical pulp cooked by the kraft process. In this process, the active cooking species are hydroxide and hydrogen sulphide anions; the hydrogen sulphide is the main delignifying agent while the hydroxide accelerates delignification and keeps the lignin fragments in solution. These species are present in the so-called white liquor, composed by an aqueous solution of sodium hydroxide and sodium sulphide.[1]

The kraft process includes a recovery cycle, whose functions are: reduce the chemical demand, minimize the environmental impact of waste material, and generate steam and power.[14] Besides this advantages, the kraft cooking can handle almost all wood species, being tolerant to bark and producing high strength pulp. However, kraft pulps are more difficult to bleach when compared to sulphite pulps. The kraft process have low yields and the sulphur in its reduced form provides air emissions with a pungent smell.[12]

1.3. Bleaching Process

1.3.1. General

Bleaching is one of the most important and expensive processes in the pulp and paper industry, especially for the production of printing and writing and tissue grades. Bleaching increases pulp brightness to a desired level and reduces the propensity for lignin-derived [1]colour reversion, as it removes residual lignin of the unbleached pulp, which is highly coloured, especially

[1] Colour reversion is the pulp yellowing caused by exposures to air, light, heat, fungi and certain metallic ions, due to modifications of the residual lignin and chromophores formation.[17]

kraft-lignin. In fact, after the kraft cooking the pulp still contains residual lignin (3-6% in softwoods and 1,5-4% in hardwoods), and also contains other coloured impurities that need to be removed, such as extractives and dirt.[15,16]

There are two ways to achieve an acceptable level of brightness: by removing the major part of residual lignin or by reducing light-absorbing groups, as known as chromophores, while preserving the lignin. The type of bleaching depends on the intended brightness level for a given application. Nevertheless, pulp bleaching requires a multistage process since lignin is a complex molecule (heterogeneous nature) possessing different degrees of reactivity with the typical bleaching chemicals. The bleaching process alternates between delignification stages and alkaline extraction stages where the pH normally shifts from acidic to alkaline.[17]

The multistage bleaching process reduces the amount of chemicals required while providing a good synergy to the process, since each stage uses specific conditions and chemicals with distinct characteristics, reactivity and functions. The early part of the sequence uses chemicals to remove the major part of the residual lignin in pulp (delignification-stages). In the later stages chemicals are used to eliminate the residual lignin and other chromophores in order to attain a high brightness level (bleaching-stages). Usually, there is inter-stage washing to remove water-soluble products resultant from bleaching reactions, which in turn improves the efficiency of the subsequent bleaching stages.[15,16]

The cost of chemicals and of waste treatment, the chemical selectivity with regard to the preservation of pulp and paper properties are very important aspects to consider when establishing the proper bleaching sequence. The bleaching chemicals are intended to be more specific to lignin removal than to carbohydrate degradation compared with the cooking chemicals. In spite of that, some bleached pulp properties are affected by the bleaching process: the fiber flexibility and strength can be increased by the removal of lignin but the fiber swelling degree and the bonding ability of the fiber surface can be decreased by a too extensive removal of hemicelluloses. If bleaching conditions are too severe, fiber damage and cellulose depolymerisation are likely harmed, which will affect pulp strength in different ways.[16]

To evaluate the progress along the bleaching sequence and the effect of bleaching chemicals on pulp, it is important to measure and analyze several pulp quality parameters: brightness, viscosity and kappa number are some of the most common parameters.

Pulp brightness is defined as the reflectance of visible blue light from a pad of pulp sheets, using a defined spectral band of light having an effective wavelength of 457 nm. The most common method used to measure brightness is the ISO standard method.[15]

Pulp viscosity is a measure of the average degree of cellulose polymerization and indicates the extension of pulp degradation during pulping and bleaching. It is determined in a viscometer

after dissolving the pulp in a cupriethylenediamine (CED) solution. Higher viscosity means higher average degree of cellulose polymerization.[17]

The kappa number (KN) is usually considered a measure of the lignin content in pulp and is used to monitorize the extent of delignification. It corresponds to the volume of a 0,1 N potassium permanganate ($KMnO_4$) solution consumed per gram of oven-dried pulp (odp) in specific standardized conditions.[17] However, it is not only lignin that consumes $KMnO_4$, but also hexenuronic acids (HexA), and so the total kappa number can be described by equation (1). It is therefore important to measure the HexA content in parallel to have a more accurate estimate of lignin content in pulp. The HexA are generated during alkaline pulping from 4-*O*-methylglucuronic acid side-groups in the xylan backbone by methanol elimination.

$$KN_{total} = KN_{lignin} + 0{,}086\ C_{HexA} \quad \textbf{(1)}$$

1.3.2. Bleaching equipment

In a pulp mill the generic bleaching equipment per stage consists of a feed pump, mixer, ascendant or descendent reaction vessel depending on the consistency, blow tank and post-stage washing.[15]

The pulp occurs in a two-phase system together with liquor. The proportion between the two phases is characterized by the consistency (mass fraction of odp based on the total amount of pulp and liquor). Depending on the consistency level, the type of equipment has to be adjusted: low consistency (<3-4%), medium consistency (6-14%) and high consistency (30-40%) feed pumps and mixers are available to mix the pulp with the chemicals and to load them into the reaction vessel. Today, the industries operate mostly at medium consistency, where the pulp slurry presents non-Newtonian flow behaviour. It is in the reaction vessel that the bleaching reactions occur and when there is gas present after the reactions, it can be separated from the pulp suspension in the blow tank. At the end, the pulp is normally pumped to the washing equipment before going to the next bleaching stage.[15]

1.3.3. Bleaching chemicals

Table 1 provides an overview of the different bleaching chemicals and stages.

Table 1 – Overall characteristics of the main bleaching chemicals and stages.[16]

Stage	Chemical	Medium	Function	Advantages	Disadvantages
C	Cl_2	Acidic	Oxidation	Effective and economical	Can cause loss of pulp strength, chlorinated effluents
H	$Ca(OCl)_2$ or NaOCl	Alkaline	Oxidation, brightening and solubilisation	Easy to make and use	Expensive, can cause loss of pulp strength
D	ClO_2	Acidic	Oxidation, brightening and solubilisation	High brightness without pulp degradation	Expensive, must be made on-site
O	O_2	Alkaline	Oxidation and solubilisation	Low chemical cost	Expensive equipment, can cause loss of pulp strength
P	H_2O_2	Alkaline	Oxidation and brightening	Easy to use, high yield	Expensive, poor particle bleaching
E	NaOH	Alkaline	Solubilisation and hydrolysis	Effective and economical	Darkens pulp
Z	O_3	Acid	Oxidation, brightening and solubilisation	Effective, chloride-free effluent	Expensive, poor particle bleaching

As a result of the concern regarding the formation of chlorinated organic compounds during chlorine bleaching, the conventional bleaching concepts (e.g. CEDED and CEHEH) were rapidly replaced by the ECF bleaching (e.g. DEDED, ODED and DEDP), which involves replacement of all molecular chlorine forms by chlorine dioxide. Then, the TCF bleaching appeared with absolutely no chlorine usage (e.g. OZEP and OQPZP sequences), which solve the problem of chlorinated organic compounds in the mills effluents. [15,16]

Currently, ECF bleaching is the dominant bleaching technology, corresponding to about 90% of all bleached wood pulp. TCF bleaching has become notable in central European sulphite mills, but corresponds only to 5% of the worldwide bleached wood pulp produced.[18]

The main bleaching chemicals can be divided into three categories as referred by Machenal and Muguet, based on their reactivity towards residual lignin structures (Table 2). An efficient bleaching sequence should contain at least one chemical from each category.

Table 2 – Oxidative bleaching chemicals categories referred by Machenal and Muguet.[15]

Bleaching chemicals	Category 1	Category 2	Category 3
Chlorine-containing	Cl_2	ClO_2	NaOCl
Chlorine-free	O_3	O_2	H_2O_2
Type of reaction	Electrophilic	Electrophilic	Nucleophilic
Medium	Acidic	Acidic/ alkaline	Alkaline
Reaction sites in Lignin	Olefinic and aromatic	Free phenolic groups and double bounds	Carbonyl groups, conj. double bonds
Reaction sites in Carbohydrates	HexA	HexA (only in ClO_2 stage)	

Nucleophilic agents decolorize the pulp but are less efficient regarding to delignification as compared to electrophilic agents.

Most bleaching reactions of chemical pulps are equivalent to oxidation reactions. Oxidants accept electrons from the substrate and are thereby reduced. With the concept of oxidation equivalent (OXE), the oxidation capacity of any bleaching chemical can be expressed. An OXE is equal to 1 mol of electrons being transferred during oxidative bleaching.[15]

1.3.4. Oxygen (O) stage

The oxygen stage was developed in the late 1960s, and is usually a pre-bleaching and delignification stage applied after cooking. It has the advantages of reducing the kappa number without expensive bleaching chemicals, while providing higher yields compared with pulping and the possibility of effluent recycling, thereby reducing COD, BOD, toxic compounds and effluent colour. Therefore, the O stage is environmentally beneficial.[15,19]

For TCF bleaching, oxygen is essential to obtain a major decrease in lignin while for ECF bleaching is essential to reduce the chemical consumption and to control the pollution.[19]

Bleaching with oxygen is carried out at 100ºC with a pH around 10-11. This process is usually conducted under pressure and the delignification is normally in the range of 19 to 55% for hardwoods.[15] Further delignification would cause excessive cellulose degradation, contributing to pulp viscosity and strength losses. To overcome this drawback, magnesium salts are added to the O stage which helps to preserve strength properties.[20]

1.3.5. Chlorine dioxide (D) stage

Chlorine dioxide is a very strong oxidant that reacts with lignin without significantly degrading the cellulose or hemicelluloses, thus preserving pulp strength while providing high and

stable brightness. It is an efficient chemical either for pulp delignification or brightening, by eliminating residual lignin and chromophores and it can also decolorize dirt specks (high level of cleaning).[21]

Chlorine dioxide is more environmentally acceptable than molecular chlorine, since reduces the quantity of chlorinated organic compounds produced during bleaching. Chlorine dioxide has more oxidizing power on a molar basis than chlorine (2,63 times on a pound per pound basis). Chlorine dioxide accepts five electrons, whereas chlorine only accepts two during oxidation reactions, according to equations (2) and (3) respectively.[21]

$$ClO_2 + 2\,H_2O + 5\,e^- \rightarrow Cl^- + 4\,OH^- \quad \textbf{(2)}$$

$$Cl_2 + 2\,e^- \rightarrow 2\,Cl^- \quad \textbf{(3)}$$

At room temperature, chlorine dioxide is an unstable gas and must be manufactured at the place where it is used (on-site production). The typical operation conditions for the D stage are present in Table 3.

Table 3 – Typical operating conditions for the D stage at medium consistency (9-13%).[20]

Stage	Temperature (ºC)	pH	Time (min)	Dosage (kg/t_{odp})
D_0	40-70	2-3	30-60	$KF \times KN$
D_1	55-75	3-4	60-360	5-15
D_2	60-85	3,5-4,5	60-360	2-8

The temperature and pH are the main control variables. During the reaction occurs a rapid decrease in the pH due to the organic and hydrochloric acids formation. The optimal end pH for the D stage is in the range of 3,5 to 4 in order to obtain maximum brightness. If the D stage is very acidic, it may occur cellulose hydrolysis and the viscosity will decrease. On the other hand at a pH greater than 5, chlorite is stable in pulp suspensions and therefore the bleaching does not occur. In addition, long retention times are desirable to achieve maximum brightness.[21]

Chlorine dioxide is converted to chlorite (ClO_2^-) and chloride (Cl^-) ions by a number of reducing agents, such as hydrogen peroxide and sulphurous acid. Thus, the bleaching efficiency is rather low due to formation of these ions. Moreover, chlorate (ClO_3^-) has been shown to be toxic for algae, thus many studies focus on minimizing the formation of chlorate and chlorite.[15]

Chlorite does not react with lignin, contributing in this way for a large loss of oxidation potential. However, this loss can be recovered by the regeneration of chlorine dioxide through chlorite oxidation. Chlorite oxidizes via a dichlorodioxide intermediate, which can undergo a

number of possible reactions: if the media contain an excess of hypochlorous acid, it favours the chlorate formation according to equation (4); on the other hand, a chlorite excess favours the generation of chlorine dioxide according to equation (5). In contrast to chlorite, the oxidation potential of chlorate cannot be reactivated by adjusting the reaction conditions.[15]

$$ClO_2^- + HClO + H_2O \longrightarrow ClO_3^- + H^+ + Cl^- + H_2O \quad \textbf{(4)}$$

$$2\,ClO_2^- + HClO \longrightarrow 2\,ClO_2 + Cl^- + OH^- \quad \textbf{(5)}$$

The chlorine dioxide efficiency also decreases with pH, especially at pH below 3,4 since chlorite concentration is low and so the chlorate production dominates.[15]

1.3.6. Hot Acid (A) stage

In order to remove HexA and save chlorine dioxide, hot acid stage is implemented in some mills after oxygen delignification. However, only half of the HexA can be removed without severe losses of viscosity and yield, which does not justify the investment in additional equipment. Therefore, the A stage is usually combined with a D stage without washing in between, resulting in a A/D or D/A stage.[15]

For the treatment of hardwood pulp, the A stage is performed at 90-95ºC, with a pH below 3,5 during 2 h. These conditions are propitious to rapid hydrolysis of HexA, which occurs also in the presence of electrophilic bleaching agents, such as in chlorine dioxide and ozone stages. Besides the HexA content, the A stage reduces also the amount of transition metals in pulp; therefore, the A stage is also beneficial in TCF bleaching.[15]

The reduction of HexA has the additional advantages of increasing the brightness stability and preventing the brightness reversion. The disadvantages of this stage are related to an increased steam demand, viscosity loss if the conditions are not well controlled, and the more difficult handling of the acidic filtrates.[15]

1.3.7. Alkaline extraction stage reinforced with hydrogen peroxide (E_p)

The aim of the alkaline extraction (E) stage is to solubilise and extract degraded lignin compounds with sodium hydroxide, which would otherwise increase the chemical usage in subsequent bleaching stages.[17]

Applying a high amount of sodium hydroxide has a limited effect on lignin removal. Instead, the E stage is often reinforced with oxygen and/or hydrogen peroxide (E_o, E_p or E_{op}), as these chemicals provide delignification and brightening effects during the extraction while reducing the demand of sodium hydroxide. Other advantages of adding hydrogen peroxide is the

significant decrease in the effluent colour and the ability to bleach shives. In this case, stabilizers like magnesium sulphate should be added to prevent the peroxide decomposition.[15]

The reinforced extraction occur typically at 60-85°C, during 1-2 h, the initial pH is around 11 and the final pH around 10. The hydrogen peroxide dosage in the first E_p stage is ca. 0,2–0,7% and in the second E_p stage is ca. 0,1-0,2% on odp basis.[22]

1.3.8. Hydrogen peroxide (P) stage

Hydrogen peroxide is a good brightening agent but it is not so good for pulp delignification, so it is usually applied in the end of bleaching sequences in order to increase brightness to a maximum level and improve brightness stability. The residual hydrogen peroxide is very low, so its effect on effluents and environment is minimal. Mills also use peroxide to reduce the chlorine dioxide applied in the final D stage.[23]

This bleaching agent reacts under alkaline conditions with hydroxide ion to yield the perhydroxyl ion (HOO^-), according to equation (6). The perhydroxyl ion, a nucleophile intermediate, is responsible for the oxidation of chromophores in lignin through the cleavage of side chains, but it cannot attack the electron-rich aromatic rings of the residual lignin, and so the delignification effect is limited. The perhydroxyl ion concentration increases with pH, however the optimal pH of the P stage is around 10,5-11,5, because very high pH will also induce peroxide decomposition (equation (7)).[20]

$$H_2O_2 + OH^- \rightarrow HOO^- + H_2O \quad \textbf{(6)}$$

$$HOO^- + H_2O_2 \rightarrow H_2O + O_2 + OH^- \quad \textbf{(7)}$$

Hydrogen peroxide has a single bond between oxygen atoms, so it is easily decomposed into water and oxygen, equation (8), especially when the conditions are too severe and in the presence of transition metals, acting as catalysts.[20]

$$H_2O_2 \xrightarrow{Fe^{3+},Mn^{2+},Cu^{2+}} H_2O + \frac{1}{2}O_2 \quad \textbf{(8)}$$

Hydrogen peroxide can also be decomposed into reactive intermediates, through single electron transfer reactions catalyzed by transition metals (M), equations (9) to (12).[15]

$$H_2O_2 + M^+ \rightarrow OH^- + HO^\bullet + M^{2+} \quad \textbf{(9)}$$

$$H_2O_2 + HO^\bullet \rightarrow HOO^\bullet + H_2O \quad \textbf{(10)}$$

$$M^+ + HOO^\bullet \rightarrow O_2 + H^+ + M \quad \textbf{(11)}$$

$$H_2O + HOO^{\bullet} \rightarrow OO^{-\bullet} + H_3O^{+} \quad \textbf{(12)}$$

The hydroxyl radical ($HO^{\bullet}$), the hydroperoxy radical ($HOO^{\bullet}$) and the superoxide anion radical ($OO^{-\bullet}$) are important intermediates since they cause side reactions in the bleaching, with delignification being a positive and cellulose depolymerisation being a negative effect. In general, radicals produce more negative than positive effects, so the amount of metals in the bleaching process should be controlled. In order to control the metal content, it is often applied a chelating or acid stage before the P stage. On the other hand, ions like magnesium, calcium and silicates may be added into the P stage, because they complex transition metal ions through oxy or hydroxy bridges and decrease their catalytic effect, resulting in a stabilizing and buffering effect on hydrogen peroxide.[15,23]

Temperature and pH also influence the decomposition and performance of hydrogen peroxide. At temperatures over 95ºC (non-pressurized) the pulp reactions with hydrogen peroxide will not have a market bleaching effect and it will occur thermal decomposition of hydrogen peroxide, consequently the pulp quality will decrease. The typical temperature range is between 70-90ºC and the normal residence time is between 1,5-3 h.[15]

To avoid high alkalinity and to have best stabilizer performance the chemicals should be added in the following order: magnesium sulphate, sodium hydroxide and at the end hydrogen peroxide.[24]

1.3.9. Bleaching with enzymes

Enzymes are complex macromolecules of protein origin with a specific three-dimensional structure. They are produced inside living cells and can act as a biological catalyst in intracellular or extracellular level, under controlled conditions of the media (temperature, pH, time, etc.).

A catalyst is an element/substance responsible for reducing the activation energy of a reaction in order to increase the reaction rate, without participating neither as a product nor reactant. As a result, reactions reach quickly their equilibrium state and the products are formed faster, which can mean significant energy savings in a given process.

Enzymes are organized in different classes and subclasses according e.g. to the catalyzed reactions. For pulp bleaching applications the enzymes of interest are lignin degrading enzymes (also called ligninolytic enzymes) and hemicellulases, like xylanases and mannanases. All of these enzymes have been extensively investigated.[25] Europe and North America already incorporate some of these enzymes, specifically xylanases, in bleaching processes since they constitute environmentally friendly alternatives to the use of chlorinated chemicals.[26]

Ligninolytic enzymes catalyze oxidation or reduction reactions, hence categorized as oxidoreductases. Within this class the most studied are laccases and peroxidases. Both of these enzymes are large molecules and cannot penetrate the cell wall, thus the enzymatic action occurs only at the surface. In order to overcome this limitation, laccases or peroxidases are often combined with appropriate low molecular weight compounds called mediators, which can penetrate into the pores of lignified cell walls after being activated by the enzyme. In this way, the enzymatic action is extended inside the cell wall.[27]

Unlike peroxidases, laccases are unable to oxidize non-phenolic lignin units (>1,5 V) due to its low oxidation potential (0,5 to 0,8 V). In the case of laccases, the use of specific mediators with higher oxidation potential also extends the oxidation reaction to non-phenolic lignin units, which constitutes over 80% of the lignin.[28]

In the peroxidase/laccase mediator system (LMS), the mediator is oxidized by the enzyme and then is able to diffuse into the cell wall, therefore oxidizing phenolic and non-phenolic lignin units, according to Figure 2.[27,28] The oxidation can follow an electron transfer (ET), a radical hydrogen transfer (HAT) or an ionic mechanism, depending on the mediator.[29] Oxygen is the electron acceptor for laccases and hydrogen peroxide for peroxidases.

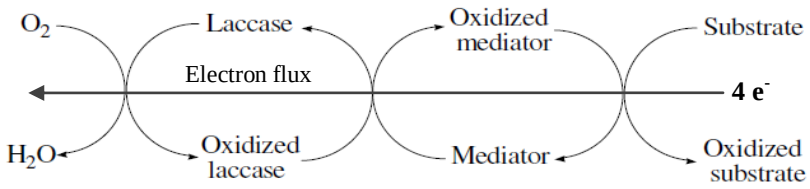


Figure 2 – Schematic representation of the LMS (Adapted from [27]).

The activity of the LMS towards lignin depends essentially on the enzyme and the mediator oxidation potential and also on the stability and reactivity of the mediating radical, resultant from the oxidation by the laccase or the peroxidase. For example, some mediating radicals degrade rapidly and sometimes can attack the enzyme and decrease its activity.[27,30]

In summary, oxidoreductases have shown to be favourable enzymes for industrial use in wood applications, such as wood fiber modification and bleaching, but it requires the presence of an ideal oxidation mediator.

1.3.9.1. Laccases

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) were discovered in 1883, and are a group of multi-copper oxidases mainly produced by white-rot fungi, specially *Basidiomycetes* such as *Trametes versicolor*.[31,32] It can also be found in bacteria like *Bacillus*

sphaericus, in high plants and insects.[33] Fungal laccases are extracellular and monomeric glycoproteins, typically with a molecular weight between 60 and 80 kDa and an isoelectric point around 4,0.[27,34]

Laccases act on aromatic substrates, particularly phenols and anilines, by catalyzing the oxidation of their phenolic hydroxyl or aromatic amino groups to phenoxy radicals and amino radicals, respectively, using oxygen as electron acceptor. Meanwhile, occurs the reduction of oxygen to water, according to equation (13).[35,36] The formed radicals can undergo numerous reactions, such as ring cleavage of aromatics, cross-linking of monomers and degradation of polymers.[37]

$$4\,PhOH + O_2 \xrightarrow{Laccase} 4\,PhO^{\bullet} + 2H_2O \qquad \textbf{(13)}$$

The active site of laccases comprises four copper atoms in three distinct groups. These atoms are all in the 2+ oxidation state but they differ in their electron paramagnetic resonance (EPR) signals. Type 1 copper (T1 Cu) exists in its oxidized form and confers the blue colour (at 600 nm) characteristic of laccases and is EPR detectible; type 2 copper (T2 Cu) is colourless and is EPR detectible; type 3 copper (T3 Cu) exhibits a weak absorption at 600 nm and has no EPR signal.[38,39] T2 Cu is strategically positioned close to T3 Cu in order to form a trinuclear cluster that is involved in the electron transfer catalytic mechanism, which occurs in three steps, according to Figure 3.[37]

1 – The substrate is oxidized to a radical in T1 Cu, since this copper has a high oxidation potential (+0,79 V).

2 – The electron is transferred from T1 Cu to the trinuclear cluster, formed by T2 Cu and T3 Cu.

3 – In the trinuclear cluster occurs the reduction of oxygen to water.

In this way, a one-electron substrate oxidation is coupled to a four-electron reduction of oxygen.

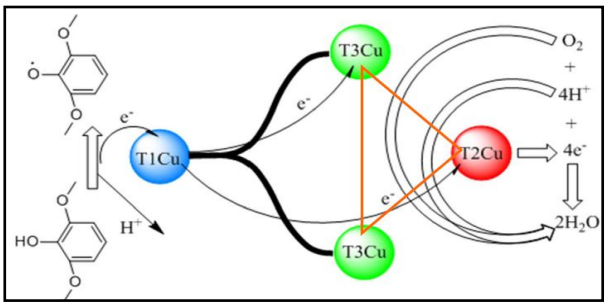


Figure 3 – Schematic representation of the electron transfer catalytic mechanism of laccases.[40]

The application of the LMS in bleaching can occur at 40-65ºC, pH 4-7, consistency 1-20%, pressure 1-14 bar, during 1-4 h and the consumption of mediator is typically around 40-50%, so it can be regenerated *in-situ* and recycled. Such an enzymatic treatment can reduce 5,6-64% of kappa number depending on the pulp type and on the enzyme and mediator dosages.[25]

The synthetic/artificial laccase mediators most studied are phenoxazines, phenothiazines and N-hydroxy compounds, such as N-hydroxybenzotriazole (HOBT), violuric acid (VA), (2,2,6,6-tetramethylpiperidin-1-yl)oxy (TEMPO), and N-hydroxyacetanilide (NHA).[41] In spite of its proven efficiency, the high cost and toxicity of these mediators have hindered so far their industrial implementation.[42,43] Hence the interest for natural mediators has increased, some examples are p-coumaric acid and syringyl-type compounds like syringaldehyde (SA), acetosyringone (AS), methyl syringate (MeS), etc.[28] These phenolic compounds are obtained from spent chemical pulping liquors or plant materials, which can be easily extracted at relatively low cost.[44] In fact, some of these compounds are capable of mediating the oxidation of non-phenolic compounds with similar or improved performances compared with the synthetic mediators.[45]

Table 4 – Main advantages and disadvantages of LMS.[46]

Advantages	Disadvantages
- Requires milder conditions; - Easy to manipulate comparing to other enzymes; - Great lignin removal without damaging cellulose; - Savings of energy and chemicals.	- Low enzyme availability in an industrial level; - Enzyme stability; - Cost of mediators; - Toxicity of synthetic mediators.

To solve the problem of LMS commercialization for bleaching applications, it is important to overcome the disadvantages present in Table 4, by discovering new laccases with higher oxidation potential or finding an effective, cheaper and environmental safe laccase mediators.[47]

1.3.9.2. Peroxidases

There are a vast number of peroxidases, most are glycoproteins containing N-linked oligosaccharide chains and protoporphyrin IX (heme) as prosthetic group. The peroxidases can be divided into three classes depending on its origin: class I consist of intracellular peroxidases, class II of secretory fungal peroxidises and class III of secretory plant peroxidises. [48] Class II peroxidases have the designation EC 1.11.1.-. The most common in the literature are manganese peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.1.14) and versatile peroxidises (EC 1.11.1.16). The molecular weight of these enzymes is between 35-100 kDa.[49]

In this work the focus will be on the heme peroxidase class II and class III. Heme peroxidases are heme-containing enzymes that use hydrogen peroxide as electron acceptor to catalyze oxidation reactions, using iron ions in the heme group, in which two electrons are derived from substrate molecules to reduce the enzyme, followed by releasing of two water molecules.[50]

The catalytic cycle is based on three consecutive and distinct redox steps, involving two high-valent intermediates (compound I and compound II). Initially, a molecule of hydrogen peroxide binds to the active site and undergoes a two-electron reduction to water and the formation of compound I through oxidation of the enzyme. The compound I has an oxyferryl centre and an organic cation radical located either on the heme or on a protein residue. Then, the cation radical undergoes a one-electron reduction, oxidising one substrate molecule to give a substrate radical and forming compound II. Finally, compound II is reduced by a second substrate molecule to the resting ferric state.[49]

The main limitation of all heme-containing peroxidases is their low operational stability, mostly due to their rapid deactivation by hydrogen peroxide.[27]

1.3.9.3. Xylanases

Biobleaching is not restricted to the use of oxidative enzymes. The application of xylanases in enzymatic bleaching was first reported in 1986 to reduce substantially the chlorine usage. In ECF and TCF bleaching xylanases are used to improve the final brightness of pulp or to reduce the demand of bleaching chemicals. In fact, for expensive chlorine-free alternatives, xylanase pre-treatment may allow them to become cost effective. Another advantage of this enzyme is the ease of application.[25]

This enzyme can be obtained from fungal and bacterial sources. The strains used for commercial production include for example *Trichoderma reesei*, *Thermomyces lanuginosus* and *Aureobasidium pullulans*.[25]

Xylanases hydrolyse xylans, in particular reprecipitated xylan from the alkaline cooking and also as part of LCCs, thereby improving pulp bleachability. In other words, lignin becomes susceptible to removal by the subsequent stages since xylans are linked to lignin and constitutes a barrier to chemicals accessibility to lignin. For that reason xylanases are in fact a bleaching-aiding enzyme rather than delignification agents.[51]

Xylanases can be divided into: endo-xylanases (EC 3.2.1.8), that attacks the main chain (xylan backbone); β-xylosidases (EC 3.2.1.37), which hydrolyze xylo-oligosaccharides to xylose; 1,2-α-D-glucuronidases (EC 3.2.1.139) and 1,3-α-L-arabinosidases (EC 3.2.1.55), that remove the side chains; and esterases (EC 3.1.1.72), which hydrolyze the ester linkages between xylan and the acetic or phenolic acids. [52]

Chapter 2: Experimental procedures

The Forest Products Research & Development department at Novozymes A/S received eleven unpurified NS-51002 laccase variants, including the wild type. The variants had modifications in their amino acid sequence in one Protein Chemistry department. The performance of these variants will be studied in forest products applications and one of them is on bleaching which will be the focus of the present work.

This chapter is reserved for all the experiments conducted with this and with other enzymes, since the enzyme purification and optimization work to the application on bleaching. It also reveals the techniques for the characterization of the pulp and filtrates from the bleaching stages.

2.1. Purification of the NS-51002 laccase variants

The NS-51002 laccase variants were purified through a series of techniques represented in Figure 33 from Attachment A, in order to isolate the laccase from the culture broth, obtained from another department.

The first step consisted of an ultra-centrifugation of each laccase variant (100 mL) at 4000 rpm during 20 min; then the supernatants were collected in 50 mL tubes, this way the heavy impurities were removed. Each supernatant from samples 2 to 9 was submitted to an ultra-filtration (UF) in the BVP-Z machine, through a permeable membrane in order to remove iron and other small components. After the sample run out, the tubes were washed twice with 100 mL of MilliQ-water and once with 150 mL of 25 mM Tris buffer pH 7. The conductivity of the resulting filtrate was measured; its value need to be below 5 mS/cm otherwise the ultra-filtration shall be repeated. Then, an activity test was made in the filtrates in order to detect laccase losses. The test consists of adding in a well of the MTP 100 μL of BR buffer pH 5,0, 20 μL of syringaldazine and 10 μL of filtrate. The colourless mixture turned to violet, so it was lost some laccase to the filtrate. In this case the ultra-filtration was not an efficient technique.

Thus, a different procedure was implemented on samples 1, 10 and 11, which consists of adding 1,2 M of ammonium sulphate to the samples and stir. Then the mixture was submitted to a sterilized filtration, in order to remove iron and small proteins without losses of laccase. Hereafter, a hydrophobic interaction chromatography (HIC) was performed in a Toyopearl –phenyl 650 M column. The column was equilibrated with 50 mM Tris buffer pH 7,5 plus 1,2 M ammonium sulphate (buffer A), with a flow rate of 10 mL/min. Then the sample was injected with a flow rate of 10 mL/min. After the sample run out the column was washed with buffer A (approximately two times more of the sample volume). The last part of the HIC is based on an elution step with 50 mM

Tris buffer pH 7,5 (buffer B), it was applied a step gradient (100% B during 0 min) with a flow rate of 10 mL/min. During this part the liquid leaving the column was fractionally collected in test tubes.

Then, all the samples, either processed by ultra-filtration or sterilized filtration and HIC, were submitted to ion exchange chromatography (IEC) that separates proteins based on their charge to the column. The procedure was the same as the HIC, the only differences were the column, which was a Q-sepharose HP column (20 cm^2), and the buffers: buffer A was 25 mM Tris pH 7 and buffer B was 25 mM Tris pH plus 0,5 M NaCl. In the elution step it was applied a ramp gradient (100% B during 30 min).

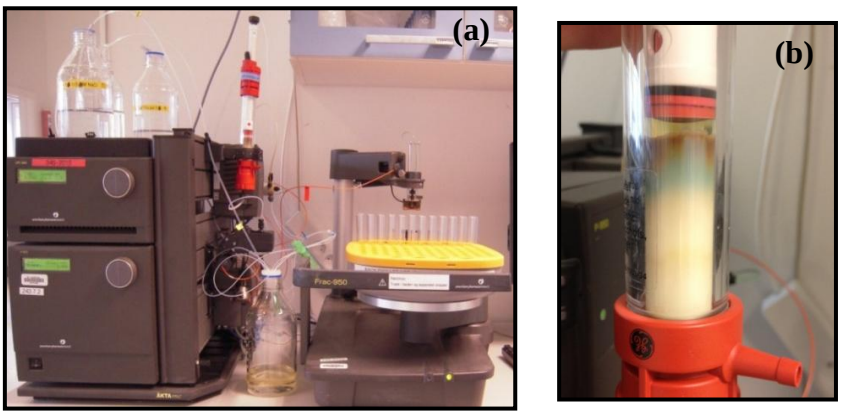


Figure 4- Setup of the IEC and HIC (a) and the Q-sepharose HP column (b) in the middle of the purification process.

The fractions collected in the IEC were diluted 100 times in order to do a kinetic analysis, based on the absorbance measurement at 550 nm for 5 min on the Microplate Reader. In each well of the MTP were added 100 µL of BR buffer pH 5,0, 75 µL of MilliQ-water, 15 µL of syringaldazine and 10 µL of the diluted sample. The fractions with high slopes values (absorbance as function of time) were consider very active, so they were transferred to centrifugal filter tubes and the other fractions were thrown away. Then, those tubes were submitted to centrifugation for 12 min at 4000 rpm and the solution with the concentrated enzyme, correspondent to the non-filtrate content, was saved in small containers and numbered from 1 to 11.

2.2. SDS-PAGE

An SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis was made on the purified and crude laccase variants, with the purpose of evaluating the efficacy of the purification process.

The procedure consisted of adding in the PCR plate 24 µL of sample, crude or purified (the purified samples were previously diluted to a concentration of 1 g/L) and 6 µL of sample buffer (constituted by 250 mM Tris-HCl pH 6,8; 500 mM DTT; 10% SDS; 0,1% bromophenol blue and 10% glycerol). The PCR plate was stirred and placed it in the PCR boiling machine at 99°C for 5 min. Then the samples were ready to be transferred to the SDS gel, which was Criterion^{tm} XT Precast Gel 10% Bis-Tris.

First, the pre-casted gel was removed from the box (the box was saved for later use) and washed with MilliQ-water. Afterwards, the comb was removed, the gel was placed into the chamber and the slots were carefully washed with 400 mL of running buffer (constituted by 1X of XT- MES buffer 20X). With a pipette 5 µL of SDS marker (Kaleidoscope: 260 – 150 – 100 – 75 – 50 – 37 – 25 – 20 – 15 – 10 kDa) was set on the selected slots and 18 µL of the samples was set on the other free slots. The gel run at 200 V and 400 mÅ for 30 min, after that time the gel was removed from the chamber and washed three times with MilliQ-water in the saved box. Then, the gel was stained for 1 hour with Imperial Protein stain and de-stained in MilliQ-water over night, hereafter the gel was ready for scanning.

2.3. Absorbance of the NS-51002 laccase variants.

The absorbance of the crude and purified variants was measured in the nanodrop machine, across a wavelength range of 200 to 800 nm and using a pathway of 0,1 and 1 mm. A drop (2 µL) of enzyme was placed in a specific spot where the light passes through. The nanodrop was cleaned with MiliQ-water between each measurement.

2.4. Pre-screening of the NS-51002 laccase variants with dye

The screening was carried out in a MTP, indigo carmine was the dye used and the mediators were MeS, VA, PPT, HOBT and TEMPO.

The procedure consists of adding into the MTP 20 µL of enzyme solution, previously diluted to a concentration of 1,5 µg/mL; 100 µL of 100 mM BR buffer pH 3 to 7 and 0,20 µL of 10 mM mediator solution. The reaction is started by adding 100 µL of 0,43 mM indigo carmine solution with a multi-pipette. The plate was then quickly transferred to the MTP reader; the absorbance was measured at 610 nm for 5 min, with premixing of the plate every 5 sec. After the absorbance measurement the MTP were scanned from time to time.

2.5. Screening with oxygen pre-bleached *Eucalyptus* kraft pulp – 1,5% consistency

2.5.1. Preparing the sample

It was first necessary to determine the DM of the oxygen pre-bleached *Eucalyptus* kraft pulp, using the HG53 Halogen Moisture Analyser from Mettler Toledo, with a drying temperature range from 50 to 200°C. Then 60 mg odp was weighted into test tubes, small magnets (4,5 mm x 15 mm) and 2 mL of 0,1 M acetate buffer pH 5,0 were added. After 30 min (to ensure enough time for the buffer to fully penetrate into the pulp) a certain amount of water was added. The amount of water was previously calculated in order to attain the right final concentration for all components and the right consistency.

2.5.2. Laccase incubation

The tubes were placed into the heat block with stirring at 50°C and the right amount of enzyme was added followed by the mediator. The tubes were stirred in the vortex mixer between each addition. At the end, all the tubes had 4 mL of liquid volume, which correspond to 1,5% consistency. The oxygen tubes were then placed into the mixture, with glass lids on. When the oxygen valve was open the clock started counting until 1 hour. After that time the oxygen valve was closed and the lids were removed.

(Note 1: The control assay was performed in the same way and with the same conditions as the laccase incubation but without any laccase or mediator; instead it was used water.)

(Note 2: The laccase incubation was done in duplicate, i.e. there were two tubes for the same assay with the same characteristics.)

Figure 5 – Setup of the screening assay with 1,5% pulp consistency.

2.5.3. Washing

Afterwards, the tubes were immediately centrifuged at 4000 rpm during 5 min in the Eppendorf Centrifuge 5810 and the supernatants were collected to measure the pH. The pulp was washed with 6 mL of MilliQ-water. The centrifugation and washing steps were repeated twice in order to ensure removal of the enzyme and the mediator.

2.5.4. Alkaline Extraction stage reinforced with hydrogen peroxide

After the laccase treatment and washing of the pulp, the E_p stage was performed with 0,5% of $MgSO_4$, 1,0% of NaOH and 0,8% of H_2O_2 on odp basis in each tube, which corresponds to 0,056

g/L of $MgSO_4$, 1,1g/L of NaOH and 0,89 g/L of H_2O_2. After adding the chemicals, the tubes were stirred and closed with glass lids in order to be placed in the water bath at 85°C during 1,5 h. After that time, the tubes were centrifuged and the supernatants were collected to measure the pH.

2.5.5. Making handsheets for measurement of reflectance

The next step consisted of forming small pulp handsheets (20 mm of diameter). For that purpose, the pulp was washed with 8 mL of MilliQ-water and filtrated under vacuum, with a polyester fabric between the top of a plastic bottle and the lid. The sheets were placed on a metallic plate and pressed twice with 2 dry blotters on the bottom of the plate and other two on the top of the handsheets. The first press was during 5,5 min and the second during 3,3 min, with a pressure around 300-400 kPa. Then the metallic plate with pulp handsheets was placed in a dark place to dry overnight. In the next day the reflectance of the pulp sheets was measured at 460 nm using a MacBeth ColourEye spectrophotometer.

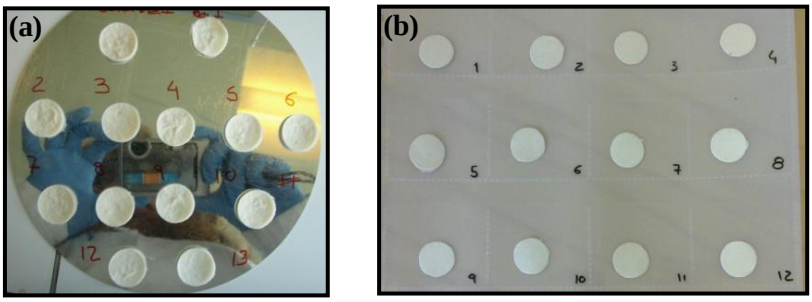


Figure 6 – Small pulp handsheets before (a) and after (b) pressing.

2.6. Screening with oxygen pre-bleached *Eucalyptus* kraft pulp – 10% consistency

2.6.1. Preparing the sample

The DM of the pulp was determined in advance and 5 g odp were weighted into small stomacher bags. Then 50 mM of acetate or phosphate buffer was added, depending on the pH and on the enzyme applied. The bags were manually thoroughly mixed and pre-heated in the water bath for 30 min at 50°C.

2.6.2. NS-51002 laccase incubation

After preparing the sample, MilliQ-water previously heated at 50°C, laccase and mediator were added in this order to the pulp. In between each addition, the bags were again thoroughly mixed, sealed and placed in a "paint shaker" for 2-3 min, thereby providing a better mixing of the pulp suspension. Then the pulp was transferred into small beakers, where 4 bar of oxygen was added through a valve existing on the beakers which were then placed in the water bath for 3 h at

50°C. After the incubation the pressure of the beakers was released by pressing the end point of the valve.

(Note 3: The control assay was performed in the same way and with the same conditions as the laccase incubation but without enzyme and mediator; instead it was used water. It was not added oxygen neither.)

2.6.3. NS-51004 and NS-51113 peroxidase incubation

After preparing the sample, MilliQ-water previously heated at 50 °C, peroxidase, mediator and a small dosage of hydrogen peroxide were added in this order. In between each addition, the bags were again thoroughly mixed, sealed and placed it in a "paint shaker" for 2-3 min, thereby providing a better mixing of the pulp suspension. Then the bags were placed in the water bath for 3 h at 50°C. From time to time (around 30 min), the bags were refilled with small dosages of hydrogen peroxide and placed in the paint shaker again and then in the water bath.

(Note 4: The control assay was performed in the same way and with the same conditions as the peroxidase incubation but without enzyme, mediator and hydrogen peroxide; instead it was used water.)

2.6.4. Washing

After the incubation, the pulp was drained in a magnet-funnel, with a binding clamp polyester fabric in between, and washed three times with approximately 300 mL of de-ionized water (the first 300 mL were used to wash the beaker or the bag).

2.6.5. Alkaline Extraction stage reinforce with hydrogen peroxide

The pulp was manually disintegrated into small pieces and weighted. Then, MilliQ-water, previous heated at 85°C, was added until the total weight was 45,5 g. Hereafter, the bags were carefully pressed by hand for the chemical addition into the top liquid phase then thoroughly mixed between each addition: first 2,5 mL of 1,0 g/L of $MgSO_4$ stock solution, then 1 mL of 50 g/L of NaOH and 1 mL of 40 g/L of H_2O_2 solution; in order to attain 0,5% of $MgSO_4$, 1,0% of NaOH and 0,8% of H_2O_2 on odp basis in each bag. The final consistency was 10%.

The bags were manually thoroughly mixed between each chemical/enzyme addition, sealed and placed in a paint shaker for 2 min. Then, they were placed in the water bath for 90 min at 85°C.

After this reaction time, it was carried out the washing step and the filtrates were collected in test tubes to measure the pH and then were frozen for further analyses.

Finally, the pulp was disintegrated by hand into small pieces and saved in the fridge in labelled plastic bags.

2.6.6. Making handsheets for measurement of ISO brightness

The pulp handsheets were made based on the ISO standard 3688:1999 ("Pulps — Preparation of laboratory sheets for the measurement of diffuse blue reflectance factor (ISO brightness)").

The DM of each treated pulp was first determined and 4 g odp was weighted, in order to produce two sheets with 200 g/m^2 of basis weight. The pulp, along with 800 mL of de-ionized water, was disintegrated in the disintegrator with 10 000 rotations and then 200 mL of de-ionized water was used to wash the stirrer and the disintegrator. The pH of the suspension was adjust to a value between 4,0 and 5,5 with solutions of 0,1 M of NaOH and 0,05 M of H_2SO_4. Then, the suspension was well mixed and divided into two equal portions.

The next step consists of a vacuum filtration of the suspension in a büchner funnel with around 150 mm of diameter and a thick filter paper. The sheet was removed from the funnel and placed in a blotter paper.

Then the handsheets prepared were pressed during 80 sec. with a pressure around 300 kPa in the sheet press with the following sequence: metallic plate, 2 blotter paper, blotter paper with the sheet covered by the filter paper, 2 blotter paper, metallic plate and so on. Hereafter, the sequence was disassembled and the filter paper was detached and placed on top of the sheet in order to protect it from dust. The sheets were placed on the metallic plate with metal rings and dried on the climate room, with controlled temperature and humidity, during at least 3-4 h until a moisture of 5-15% was reached. The sheets were pressed again for 50 sec, with a pressure between 300-500 kPa and dried for 1 h in the climate room (the total drying time shall not exceed 24 h). Then the ISO brightness was measured using Technidyne Colour Touch PC spectrophotometer.

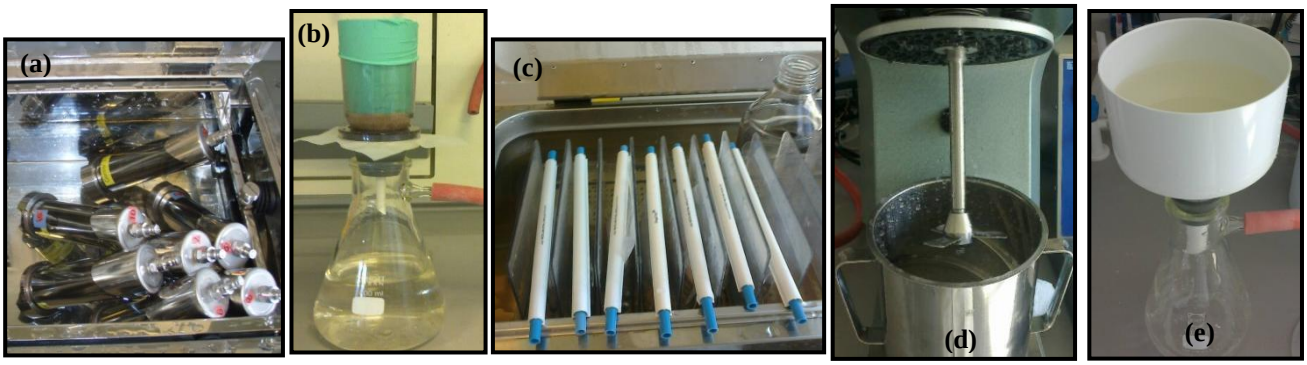


Figure 7 – Screening assay at 10% pulp consistency: laccase stage (a), washing (b), alkaline extraction stage with H_2O_2 (c), disintegration (d) and filtration (e) of pulp in order to make handsheets.

2.7. Determination of the NS-51002 laccase activity

The laccase activity test was performed in a quartz cuvette where the chemicals and enzyme were added in the following order:

- 1000 µL of 24,7 mM MES buffer pH 5,5 preheated at 30°C;
- 25 µL of diluted enzyme sample (the diluent solution was a mixture of 25,0 g of PEG 6000, 5,0 g of Triton X-100 in 500 mL of MilliQ-water);
- 75 µL of 0,28 mM syringaldazine substrate solution (syringaldazine was dissolved in a mixture of 96% ethanol and water in a proportion of 1:2).

The reaction started by adding the syringaldazine to the cuvette. Then, the cuvette was briefly mixed and immediately placed into the spectrophotometer. The absorbance was measured in the mode "kinetics" at 530 nm for up to 120 seconds and the A_{530}/min was determined by the software as the slope of A_{530} versus time from 50 to 110 seconds, multiplied by 60 seconds. The operable range for this method is between 0,100 and 0,400 $A_{530\ nm}$/min.

A unit of laccase activity (LACU) represents the amount of laccase that can degrade 1 µmol of substrate per minute and can be calculated by equation (14).

$$LACU/mL = \frac{A_{530}/min \times V \times DF}{\varepsilon \times V_{Lac}} \quad \textbf{(14)}$$

Where DF is the dilution factor, ε is the product molar absorption coefficient, V_{Lac} is the laccase sample volume added to the cuvette and V is the total volume.

2.8. Bleaching stages (10% consistency)

Nine ECF and eight TCF bleaching sequences were tested using combinations of several stages, either enzymatic or chemical, of which experimental procedures are described next. The bleaching sequences performed were designated by the letters of the stages as follows: DE_pDP, LE_pLP, LAE_pLP, LAE_pDP, ALE_pLP, ALE_pDP, ADE_pDP, XLE_pLP, XLE_pDP and XDE_pDP. For all of the sequences pulp handsheets were produced after each stage according to 2.6.6.

2.8.1. Enzymatic stage with laccase or peroxidase (L)

The procedure for enzymatic stages with NS-51002 laccase no. 10 and NS-51113 peroxidase is the same as described in 2.6. The only difference was the quantity of pulp used, 30 g and 20 g, and so it was used 600 mL beakers (laccase) or big stomacher bags (peroxidase). The

enzyme dosage used was 0,0005 kg ES/t_{odp} and 0,0007 kg ES/t_{odp} for the NS-51002 laccase no.10 and NS-51113 peroxidase, respectively.

2.8.2. Xylanase (X) stage

Phosphate buffer (50 mM; pH 8,0) was added to 30 g odp in big stomacher bags, which remained 30 min in the water bath at 75ºC. After that time, they were removed and were added 0,00043 kg ES/t_{odp} of NS-51121 xylanase, which is an endo-xylanase. Then, the bags were sealed, placed in the paint shaker for 2-3 min and were replaced in the water bath for 2h.

2.8.3. Hot acid (A) stage

It was weighted 30 g odp into big stomacher bags, MilliQ-water at 95ºC was added until the right consistency and some drops of 2 M H_2SO_4 solution in order to achieve a pH of 3,5. The bags were manually mixed and the pH was checked several times. Then the bags were sealed and put in the water bath at 95ºC for 2 h.

2.8.4. Chlorine dioxide (D) stage

Some drops of 2 M H_2SO_4 solution were added to the MilliQ-water to achieve a solution with a pH around 2,0 and the solution was added to the pulp in a big stomacher bag at room temperature. The pulp was then manually thoroughly mixed and the pH was checked several times and adjusted to a value of 2,5 with NaOH or H_2SO_4 solutions. Afterwards, the bags were sealed while a small cut was made in the bag's corner in order to add the chlorine dioxide solution at ca. 5ºC. After adding ClO_2 the bag was sealed, manually thoroughly mixed and put in the water bath for 1,5 h.

For the first D stage of the sequences the temperature was 70ºC and for the second D stage of the sequences it was 85ºC. The ClO_2 dosages for the first D stage was estimated according to equation (15) and they were around 0,4-0,8% on odp basis, which corresponds to 0,5-0,9 g ClO_2/L and to 4,4-8,0 kg ClO_2/t_{odp}. The ClO_2 dosage for the second D stage was 0,08% on odp basis, which corresponds to 0,09 g ClO_2/L and to 0,89 kg ClO_2/t_{odp}. This dosage was estimated based on the ISO brightness of the incoming pulp from the previous stage.

$$ClO_2(\%\ on\ odp\ basis) = \frac{ClO_2\ in\ active\ Cl_2\ (\%\ on\ odp\ basis)}{2{,}63} = \frac{KF \times K}{2{,}63} \qquad \textbf{(15)}$$

Where KF is the kappa factor and K is the kappa number of the incoming pulp.

2.8.5. Alkaline extraction stage reinforced with hydrogen peroxide (E_p)

It was added MilliQ-water to the pulp at 85ºC (previously calculated) and then the chemicals in the following order: 0,05% of $MgSO_4$, 0,8% of NaOH and 0,8% of H_2O_2 on odp basis, which corresponds to 0,056 g/L of $MgSO_4$ and 0,89 g/L of NaOH and H_2O_2. The bags were manually thoroughly mixed between each addition. At the end the bags were sealed, mixed in the paint shaker for 2 min and placed in the water bath at 85ºC for 1,5 h.

2.8.6. Hydrogen peroxide (P) stage

It was added MilliQ-water to the pulp at 70ºC previously calculated and then the chemicals in the following order: 0,05% of $MgSO_4$, 0,7% of NaOH and 1,5% of H_2O_2 on odp basis. The bags were manually thoroughly mixed between each addition. At the end they were sealed, mixed in the paint shaker for 2 min and placed in the water bath at 70ºC for 2h. For the P stage of the sequences DE_pDP, ADE_pDP and XDE_pDP the dosages used were 0,05% of $MgSO_4$, 0,6% of NaOH and 0,5% of H_2O_2 on odp basis. The dosage of chemicals was estimated based on the ISO brightness of the incoming pulp from the previous stage.

2.8.7. Washing

When big amounts of pulp were used the washing step was made in a multi-filtration apparatus consisting of several filtration funnels under the vacuum produced by a pump coupled to this unit. The pulp was washed three times with 1 L of de-ionized water. At the end, the pulp was kept in labelled plastic bags at ca. 5ºC in the fridge.

2.9. Methods for pulp characterization

2.9.1. ISO Brightness

The ISO Brightness of the sheets made in 2.6.6. and 2.8. was determined following the ISO standard 2470:2009 ("Paper, board and pulps — Measurement of diffuse blue reflectance factor - Part 1: Indoor daylight conditions (ISO brightness)").

The measurements were performed with the Technidyne Colour Touch spectrophotometer using the program "Brightness L*a*b Whiteness AVG". The procedure consisted of loading a pad of sheets into the machine, by gently pulling down the arm of the equipment and then releasing it to press the sample. There were made four readings on different points of the filter side of each pulp handsheet produced.

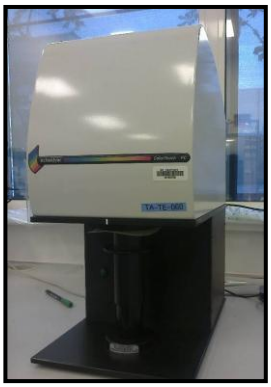


Figure 8 – Technidyne Colour Touch spectrophotometer for ISO brightness measurement.

2.9.2. Pulp viscosity

The pulp viscosity was determined according to TAPPI T 230 om-04 standard ("Viscosity of pulp (capillary viscometer method)"), but instead of using 0,25 g odp was used 0,15 g odp. The DM of the samples was determined in advance, then 0,15 g odp was weighted into 50 ml plastic flasks and it was added some glass beads with 6 mm diameter together with a few copper pieces, a magnet and 25,00 mL of MilliQ-water. Then, one flask at a time was placed in a magnetic stirrer for 20 min. Hereafter, 25,00 mL of CED solution was added, the air bubbles inside the flask were removed by capping it with a special lid and the flask was mixed in rotary mixer for 25 min. After that time, the flask was placed into the water bath at 25°C for 1 min.

In order to measure the efflux time, the viscometer was first cleaned with de-ionized water and with sample solution. Then, the sample solution was pumped under suction into the capillary viscometer, by immersing the bottom point of the viscometer into the solution and applying suction with a pipette bulb in the top end. The efflux time is the time that the sample takes to flow from the first upper mark in the viscometer until the second mark (1 mL) and it was measured with a stopwatch. The waste was discarded in a proper container.

To calculate the viscosity it was used equation (16).

$$v = c.t.d \qquad \textbf{(16)}$$

Where v is the viscosity of CED at 25ºC (cP); c is the viscometer constant found through calibration, which was 0,0572; t is the average efflux time (s) and d is the density of the pulp solution (g/cm^3).

2.9.3. Kappa number

2.9.3.1. Sample preparation

The pulp was disintegrated by hand into small pieces and the DM was determined. To calculate how much sample is needed to weigh it was used equations (17), (18) and (19), after making an educated guess of the expected kappa number.

$$\text{Kappa} > 10 \longrightarrow m = \frac{50 \times 25 \text{ ml } KMnO_4}{Y \times DM} \quad \textbf{(17)}$$

$$\text{Kappa} < 10 \longrightarrow m = \frac{50 \times 12{,}5 \text{ ml } KMnO_4}{Y \times DM} \quad \textbf{(18)}$$

$$\text{Kappa} < 5 \longrightarrow m = \frac{50 \times 6{,}25 \text{ ml } KMnO_4}{Y \times DM} \quad \textbf{(19)}$$

Where m represents the amount of wet pulp to weight and Y is the guess of the kappa number. It was used an analytical balance to weigh out the amount of pulp determined into the 250 mL cups while taking note of the exact values.

2.9.3.2. Preparation of Metter Toledo 58 titration system

It was necessary to check the volume of all reagents and to put an empty cup and a cup filled with de-ionized water in the indicated right positions.

First, it was made a flush and then 2 blanks according to the kappa number (<5, <10 or >10) with the software "LabX titration". The blank volumes used should be in the following intervals in order to continue and run the samples.

$$\text{Kappa} > 10 \longrightarrow 24{,}4\ mL < x < 24{,}9\ mL$$
$$\text{Kappa} < 10 \longrightarrow 12{,}3\ mL < x < 12{,}6\ mL$$
$$\text{Kappa} < 5 \longrightarrow 6{,}1\ mL < x < 6{,}3\ mL$$

2.9.3.3. Running samples

The cups with the weighted samples were placed in the Metter Toledo 58 titration machine in the clockwise direction. The appropriate method in the "LabX titration" software was chosen, for example "Kappa <5 TAPPI", and the pulp identification, DM and the exact weighed pulp was introduced before the samples were run.

When the measurement finished, the "alfa value" was cheeked and had to be between 30 and 70%, otherwise a new kappa guess had to be made.

Figure 9 – Metter Toledo 58 titration system for kappa number measurement.

2.9.4. HexA content

The HexA content determination was based on the HUT method reported by Vuorinen et al.[53], which consists of an UV photometric measurement based on acid hydrolysis of the HexA from pulp.

The DM of the pulp was first measured, 2 g odp was weighted into 200 mL vessels using an analytical balance and the value was noted. Then 150 mL of 0,01 M formate buffer pH 3,5 was added, the vessels were sealed and placed in the Labomat (Figure 10). The hydrolysis reaction occurred for 60 min at 110ºC and 5 rpm (30 sec left and 30 sec right). After that time, the hot vessels were removed from the Labomat and placed in a bucket with ice for cooling. Once cooled, the vessels were open, the pulp slurry was well mixed with a spoon and 7 mL of pulp slurry were withdrawn from the vessel to be filtered using a 10 mL lur-loc syringe coupled to a 0,45 µm filter. All the filtrate/hydrolysate was collected in test tubes and further diluted ten times with MilliQ-water, for absorbance measurement at 245 and 480 nm. The blank was 0,01 M formate buffer also diluted ten times.

Figure 10 – Labomat setup.

In order to calculate the HexA content it was used equation (20).

$$HexA\ (mol/kg\ odp) = \frac{n_{HexA}}{w} = \frac{A.V}{\varepsilon.l.w} = \frac{(A_{245} - A_{480})\ (150 + CF).DF}{\varepsilon.l.w} \quad \textbf{(20)}$$

Where n_{HexA} is the number of HexA moles (mol), w is the weight of od sample (kg), A is the absorbance at 245 nm (2-furoic acid) with background correction at 480 nm, V is the volume of

liquid, CF is the correction factor accounting for the amount of water in the pulp (mL), DF is the dilution factor, l is the cell pathway (cm) and ε is the molar absorption coefficient of 2-furoic acid at 245nm with respect to HexA in hexenuronoxylo-oligosacharides ($mM^{-1}cm^{-1}$).

2.10. Methods for filtrate characterization

2.10.1. COD determination

The filtrates were thawed and diluted five times with MilliQ-water. The COD determination was performed in specific test cells, with H_2SO_4 and $K_2Cr_2O_7$, for COD measurement (COD Cell Test from Merck). First, the cells were swirled to suspend the bottom sediment. Then 3 mL of the diluted sample was added into the cell (the cell quickly became hot). The reaction cell was tightly closed, mixed vigorously in the vortex mixer and placed into the thermo reactor at 148ºC for 2 h. After that time, the cells were removed and placed into a test tube rack to cool down. After cooling 10 min, the cells were swirled and placed again in the tube rack for complete cooling to room temperature. Then, the cells were cleaned with a dry cloth before being positioned into the cell compartment of the photometer NOVA 60 (Figure 11 (a)) while aligning the cell mark with the one on the photometer. The COD have to be measured within 60 min after the reaction finished and the value should be in a range between 25 and 1500 mg/L for the test kit used.

2.10.2. Hydrogen peroxide consumption

To evaluate the total consumption of hydrogen peroxide in the alkaline extraction stage and in the peroxidase stage, it was roughly measured its residual value in the filtrates collected from these stages with peroxide strips from Quantofix (Figure 11 (b)).

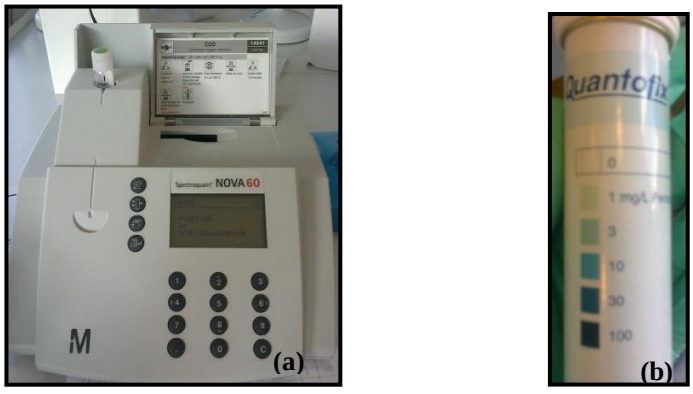


Figure 11 – Photometer NOVA 60 (a) for COD measurement and Quantofix strips (b) for residual hydrogen peroxide measurement.

Chapter 3: Results and discussion

In this chapter, it is presented the main results of this work, followed by a brief analysis and discussion spanning from the enzyme purification until the enzymatic optimization and application experiments in pulp bleaching.

3.1. Laccase variants purification

The fungal laccases undergo a series of processes before becoming a commercial product that could be applied in the pulp and paper industry or even in other industries like detergents, textile or food industry.

The first step consists of collecting the fungi and e.g. soil from the nature, analyzed and identified the strains, and then the enzymes have to be isolated from the media and cultivated/fermented. Before application, the fermented media is typically purified, which allows the separation of the laccase from other enzymes.

Sometimes the amino acids sequence of the enzyme is modified by genetic engineering in order to improve some features like enzyme-mediator/substrate affinity and enzyme stability in terms of pH and temperature conditions, thereby creating different variants of the same enzyme.

The NS-51002 laccase variants under study had already been isolated from the fungi and cultivated, thus consisting of a culture of several proteins that have to be separated. There are many methods for enzyme purification that are based on:

- Enzyme molecular weight via size exclusion chromatography or SDS-PAGE;
- Enzyme polarity/hydrophobicity via high performance liquid chromatography or reversed-phase chromatography;
- Enzyme isoelectric point via ion exchange chromatography.

In this case, the purification was based on enzyme isoelectric point and enzyme hydrophobicity. The methods used are illustrated in Figure 33 (Attachment A).

After purification, an SDS-PAGE was made in order to evaluate the efficiency of this technique. The SDS-PAGE allows the separation of proteins according to molecular weight across a polyacrylamide gel; in this way it is possible to conclude if the content of the enzyme solution is well purified. The scan of the gel is present in Figure 12.

According to Figure 12 it is possible to verify that the purification was efficient, since the bands from the purified samples are located almost exclusively in the same area, which is around 55 kDa, and in fact it is the molecular mass of the NS-51002 laccase in study (ca. 53 kDa). Laccase variant no. 11 is the only exception since there are bands correspondent to other molecular weights,

but are not that accentuated. In general, it is possible to conclude that the purification processes used (UF followed by IEC or sterilized filtration followed by HIC and dialysis) were both efficient in terms of purification performance. However, in terms of laccase losses, the second process was more efficient than the first one.

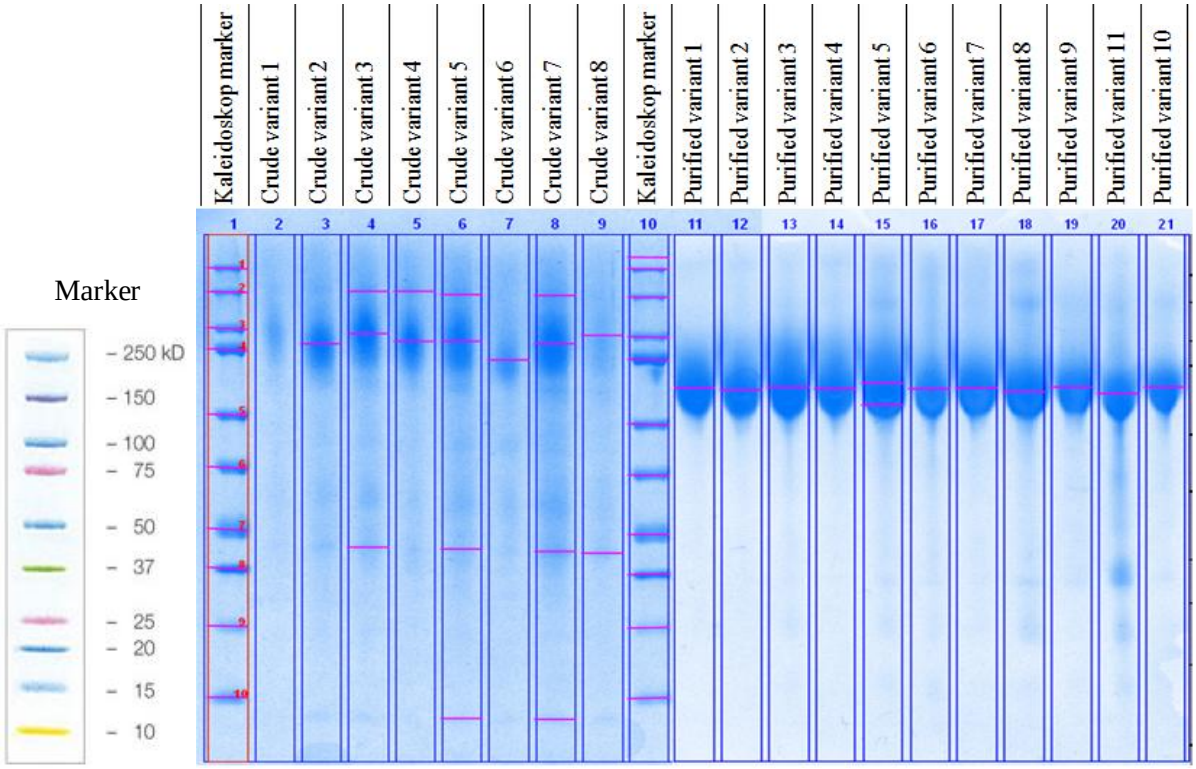


Figure 12 - SDS gel of the laccase variants, using kaleidoscope as a marker.

3.2. Absorbance and concentration of the laccase variants

For a further evaluation of the purification efficiency, it was made a comparative study of absorbance between crude and purified NS-51002 laccase variants, for a pathway of 0,1 mm and 1,0 mm and for wavelengths of 260 nm (referent to DNA), 280 nm (representative of proteins) and 610 nm (referent to the blue colour of copper atoms present in laccases).

According to the spectra for crude and purified variants present in Figure 34 (Attachment B), it was possible to verify that the pathway 0,1 and 1,0 mm did not show much sensitivity for high and low wavelengths, respectively, since the signals were not detected or were unreadable. Thus, the absorbance values for 260 nm and 280 nm were taken from the pathway 0,1 mm and the values for 610 nm from the pathway 1,0 mm. In Figure 13, it is presented the absorbance ratio between wavelengths of 280 nm and 260 nm for all variants. This absorbance ratio is an indicator of how well purified are the enzymes: if its value is close to 2,0, the enzymes are well purified.

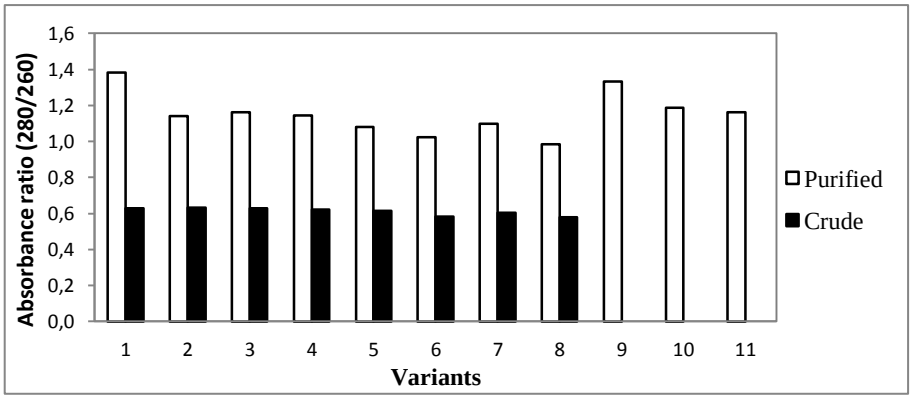


Figure 13- Absorbance ratio between wavelengths of 280 nm and 260 nm for all variants (crude variants no. 9, no. 10 and no. 11 were not available).

In general, it is possible to conclude that the purification was good, especially for variants no. 1 and no. 9. Comparing with the crude absorbance ratio, the purified values are almost the double, so a great improvement was reached in the purification process.

By knowing the absorbance of the purified variants in these wavelengths, it was possible to determine their concentration using the Beer-Lambert law, equation (21).

$$A.M_w = C.l.\varepsilon \quad \textbf{(21)}$$

Where A represents the absorbance, M_w the molecular weight of the laccase (53313 Da), C the concentration (g/L), l the cuvette pathway length (0,1 cm) and ε the molar absorption coefficient (4460 $M^{-1}cm^{-1}$)[54].

The concentrations were determined for 610 nm. These concentration values are important to prepare the necessary stock solutions for enzymatic bleaching. Moreover, the concentration ratio between 610 nm and 280 nm (Table 5) is useful to conclude once again about the purification effectiveness.

Table 5 - Concentration ratio between 610 nm and 280 nm of the purified laccase variants.

Laccase variant	1	2	3	4	5	6	7	8	9	10	11
C_{610}/ C_{280}	0,503	0,386	0,315	0,346	0,240	0,222	0,262	0,203	0,444	0,341	0,291

3.3. Pre-screening of the laccase variants with dye

The pre-screening assay with dye is a very useful and fast method for assessment of laccase activity and it is suited for multi-parameter screening. This method is based on the kinetic

analysis of decolourization/oxidation of a dye in the presence of molecular oxygen, catalyzed by the LMS. In this case the dye used was indigo carmine, which is easily oxidized by laccases.

The aim of this particular pre-screening was to select the most actives NS-51002 laccase variants, as well as the best mediators and pHs for each variant. Therefore, it was tested 5 different pHs from 3 to 7 and 5 different mediators, which are present in Figure 35 (Attachment C).

The best pH for each LMS is compiled in Table 6. Based on this table, it was built the graph of Figure 14 with the different combinations for each variant. The scans of the MTP used in the screening are present in Figure 36 (Attachment D).

Table 6 - Best pH for each laccase-mediator system.

Best combinations of mediator-pH	Variants	1	2	3	4	5	6	7	8	9	10	11
	Mediator	Best pH										
Combination A	MeS	3	5	5	3	4	5	5	4	5	5	5
Combination B	VA	4	3	3	5	5	5	5	5	5	3	5
Combination C	PPT	5	5	4	5	4	5	5	4	5	5	5
Combination D	HOBT	3	5	5	5	3	3	6	3	5	3	5
Combination E	TEMPO	3	5	5	5	5	5	4	4	4	3	4

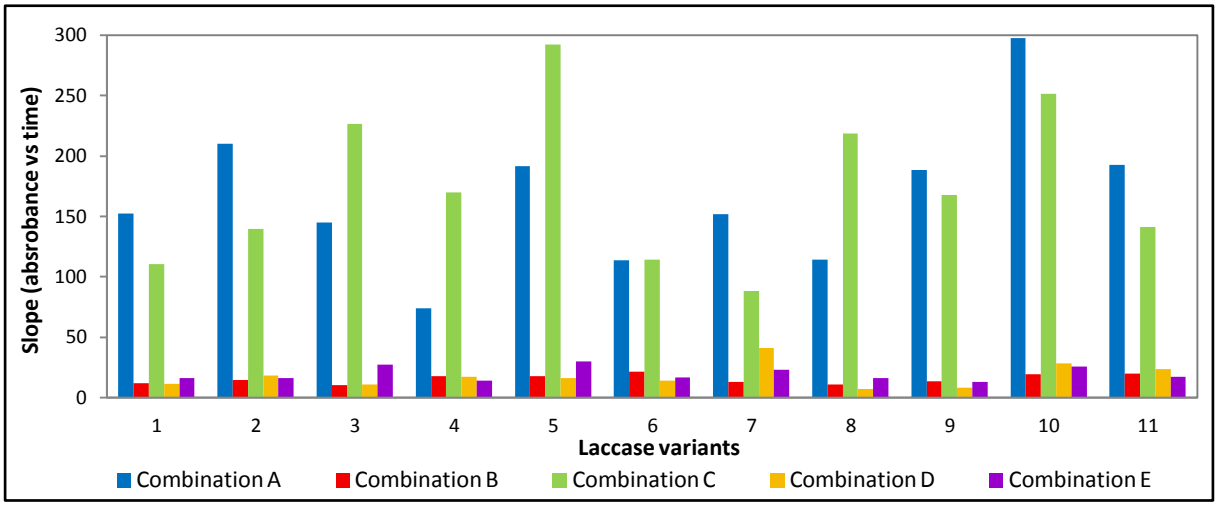


Figure 14 - Best combinations for each NS-51002 laccase variant (the analysis of the graph is supported by Table 6).

Through the analysis of Figure 14 along with Table 6 it is possible to conclude that NS-51002 laccase variants no. 5 and no. 10 are the most actives with indigo carmine. For variant no. 10 the best combination is MeS and pH 5,0 and for variant no. 5 is PPT and pH 4,0. The wild type is no. 8 and is also very active, especially for the combination PPT and pH 4,0.

The pre-screening assay is indeed a very easy and useful method that gives an idea of the most active enzyme variants and the best conditions. However, the conclusions of this study cannot

be directly transferred to their action on the pulp, since indigo carmine is a very different model substrate compared to the complexity of lignin and of pulp components in general. Therefore, the next step was to test the variants in pulp bleaching. Nevertheless, from this first screening assay it was possible to select variants no. 5 and no. 10 as the most promising to be subsequently analysed on pulp together with the wild type (no. 8). This first selection is important because it is not feasible to test all the variants with a high throughput at the scale used in the pulp bleaching assays.

3.4. Laccase activity

The activity of Novozym 51003 laccase and of some NS-51002 laccase variants was determined using the LACU method, since these enzymes will be used in further assays. The LACU method is based on the analysis of syringaldazine oxidation in the presence of a laccase. Syringaldazine is a colourless compound and oxidizes to tetramethoxy-azo bismethylene quinon, which has violet colour (530 nm). Accordingly, the reaction was followed by change in absorbency at 530 nm and it is represented in Figure 15.

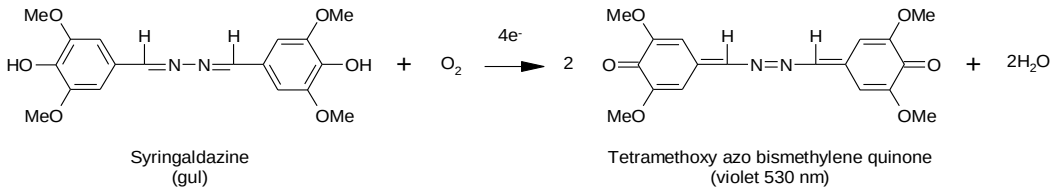


Figure 15 – Oxidation of syringaldazine by molecular oxygen, catalyzed by laccases.

In this work, it is important to know the activity of the enzymes that will be studied in order to compare their performance bearing in mind the resulting specific activity. However, this does not avoid the evaluation on the final substrate, i.e. pulp. The activity results are present in Table 7.

Table 7- Laccase activity determined by LACU method.

Laccase	NS-51002 variants				Novozyme 51003
	No. 5	No. 6	No. 8	No. 10	
Activity (U/g EP)	0,088	0,209	0,221	0,129	0,071

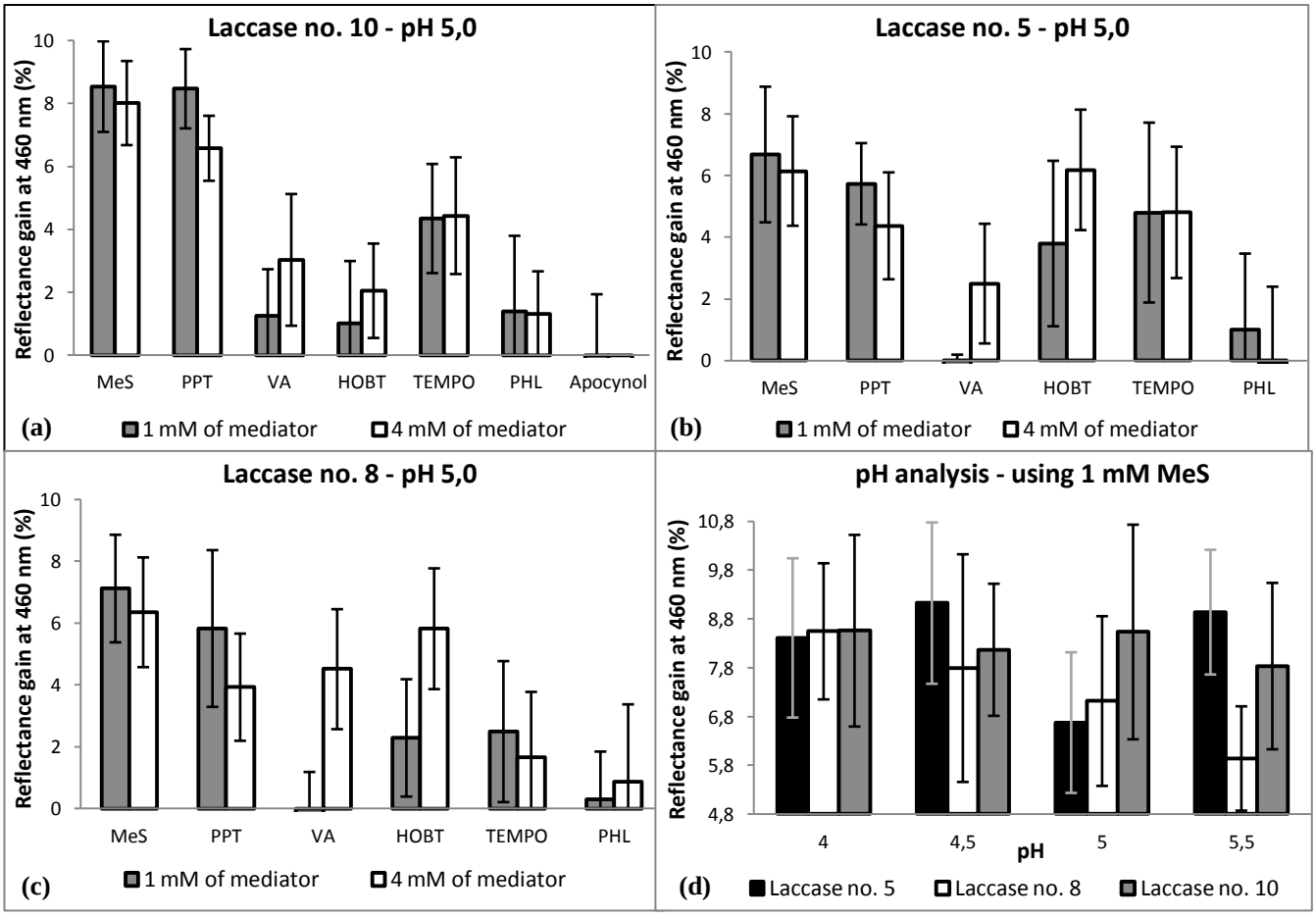
The results from Table 7 show that the NS-51002 laccase no. 6 and no. 8 are the most actives and that the less active is Novozyme 51003, which is a commercial laccase.

3.5. Screening with oxygen pre-bleached *Eucalyptus* kraft pulp – 1,5% consistency

The screening with pulp at 1,5% consistency is based on a bleaching sequence with two stages: an enzymatic stage with laccase, and an alkaline extraction stage reinforced with hydrogen peroxide, both performed in test tubes. In order to evaluate the enzymatic bleaching performance it was measured the pulp reflectance at 460 nm at the end of the LE_p sequence, in the MacBeth ColourEye spectrophotometer. The raw oxygen pre-bleached *Eucalyptus* kraft pulp had a reflectance at 460 nm of 46%.

This type of small-scale screening assay is a fast method of optimizing enzymatic systems since it enables to test different characteristics extensively, such as concentrations, mediators, pH and temperature; without requiring significant amounts of pulp or chemicals. In this screening it was studied three NS-51002 laccases: variants no. 5 and no. 10 and the wild type no. 8 (wild type I), and several mediators that are represented in Figure 35 (Attachment C).

The most important screening results are compiled in Figure 16 in the form of reflectance gain to allow a relative comparison among the samples, since all trials were not made at the same time. The gain in reflectance is the difference between the reflectance of the sample treated with LMS and the reflectance of the control sample, which was not treated with LMS.



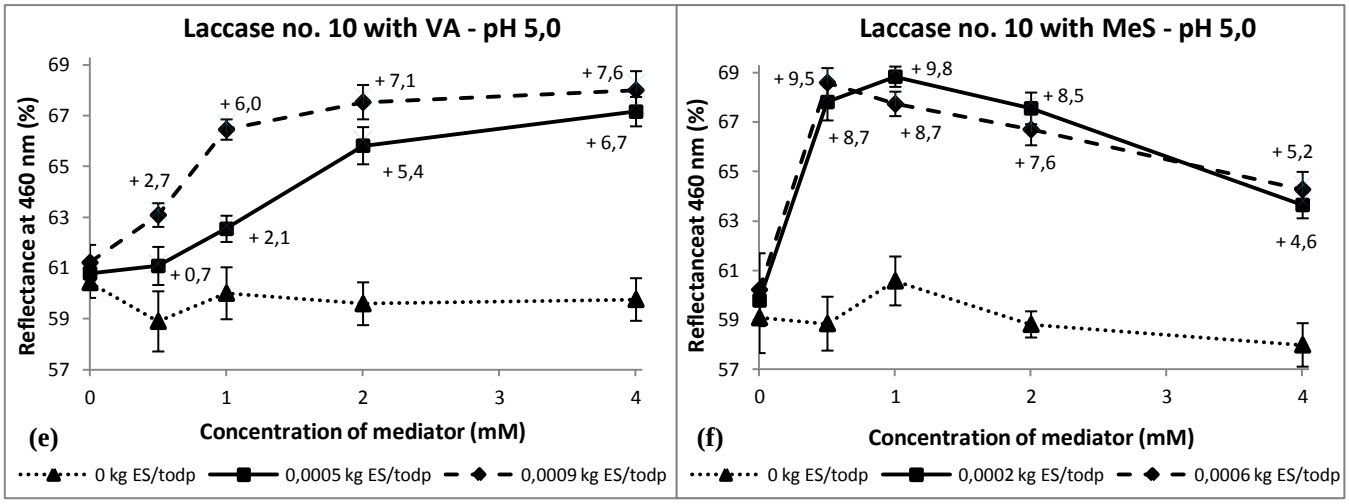


Figure 16 – Reflectance or reflectance gain at 460 nm after LEp bleaching: mediator screening for NS-51002 laccases no. 10 (a), no. 5 (b) and no. 8 (c); pH screening (d); screening of enzyme and mediator concentrations using VA (e) and MeS (f) for laccase no. 10 (the reflectance gain values are presented next to the dots).

In the screenings assays from Figure 16 (a), (b), (c) and (d) it was used the same enzyme protein concentration (EP). In turn, in the Figure 16 (e) and (f) it was used different concentrations, which are expressed in terms of dosage of enzyme solution (ES) per ton of odp.

The mediators that were used can be grouped based on their structure and oxidation mechanism towards non-phenolic lignin units.[43,55] This classification may help understand the differences and similarities in the results from the mediator screening, presented in Figure 16.

- N-hydroxy mediators, like VA (0,92V) and HOBT (1,08V) undergo a radical hydrogen transfer mechanism and are known for low stability and rapid degradation.
- Aminoxyl radicals mediators like TEMPO (0,73V) oxidizes via an ionic mechanism.
- Natural mediators (phenols derivatives), such as MeS and apocynol undergo an electron transfer mechanism, where the oxidation is mostly dependent on a negatively charged residue (e.g. aspartate) close to the substrate binding site of the fungal enzyme.[55]
- Phenothiazine derivatives, like PPT also undergoes an electron transfer mechanism.

From figures 16 (a), (b), (c) and (d), it is possible to observe significant error bars, not only due to the fact that the procedure comprises several steps but also due to the small diameter of the handsheets produced in this assay. These conditions do not allow a good repeatability as seen with the duplicates. However, it is possible to analyse tendencies between the results regarding the LMS performance on pulp bleaching.

For the NS-51002 laccases no. 5, no. 8 and no. 10 the best mediators were MeS and PPT, followed by TEMPO, HOBT and VA. MeS, PHL and apocynol can be considered natural

mediators; but only MeS result as an efficient laccase mediator. This mediator also proved to be a very efficient mediator in previous laccase studies for oxidation of veratryl alcohol[45] and non-phenolic lignin units[55].

In general, the application of LMS with MeS, PPT and TEMPO as mediators show that it is preferable a small dosage of mediator. In fact, for higher mediator concentrations, the mediating radicals formed could react with the enzyme, inactivating it.

On the other hand, the application of LMS with VA and HOBT as mediators indicates that it is preferable to increase the mediator dosage. In fact, N-OH compounds usually require high amounts to enhance the catalytic effect. Due to their instability, the mediating radical formed may undergo further reactions other than oxidizing the lignin units, and so it is often needed an excess of mediator.[56]

Despite its good performance, PPT is a dangerous and very expensive chemical so its application is not preferable. Actually, this mediator is more common in detergents applications than in pulp bleaching. In turn, MeS is more affordable and VA is the one who has a great interest due to its cost.

It was studied the concentration effect of these two mediators, jointly with the enzyme concentration effect. The results are presented in figures 16 (e) and (f). In the case of the NS-51002 laccase no. 10 with MeS, it is possible to observe that a small concentration of mediator is better and that the increase in the enzyme concentration does not have much influence in the results. In contrast, in the case of the NS-51002 laccase no. 10 with VA, a high concentration of this mediator is advantageous as well as a high concentration of enzyme, but only to a certain limit. Also from figures 16 (e) and (f), it is possible to visualize the laccase effect without mediator (0 mM). In this case the reflectance gain is almost none, which indicates that the mediator is essential for a good bleaching performance.

According to Figure 16 (d) a pH range of 4,0 to 5,5 is acceptable for these enzymes, but there is a slightly preference for pH 4,5 for laccase no. 5; pH 4,0 for laccase no. 8 and pH 5,0 for laccase no. 10, which is in concordance with the pre-screening results from Table 6. Moreover, the Figure 16 (d) suggests that the laccase no. 10 is slightly better compared with the studied variants.

3.6. Screening with oxygen pre-bleached *Eucalyptus* kraft pulp – 10% consistency

Since the results obtained from the screening at 1,5% consistency were not totally reliable because of the error associated to the procedure, it was then studied some parameters at bigger scale, namely 10% consistency. This type of assay has the disadvantages of requiring more pulp and chemicals and being more laborious, but in turn it allows producing larger handsheets, which

benefits the precision of the results. Moreover, the consistency used is within the typical range at industrial scale, medium consistency. Hence, the screening at 10% consistency was used to optimize not only the enzymatic stage but also the following E_p stage.

In these experiments, the enzymatic bleaching performance in terms of brightness measurements was done in the Technidyne Colour Touch PC spectrophotometer, at the end of the LE_p sequence. It was used two oxygen pre-bleached *Eucalyptus* kraft pulps coming from distinct mills, that will be identified in this work as "pulp 1" and "pulp 2" which are characterized by a brightness of 50,6% and 53,4%, respectively.

3.6.1. Optimization of the enzymatic stage

It was first analyzed the NS-51002 laccase no. 10 with Mes and the laccase no. 6 with VA, since it was the most promising enzyme towards VA, according to the screening with dye presented in Figure 14. These enzymes were compared always with the NS-51002 wild type I. It was also introduced in this study the NS-51002 wild type II, which comes from a different batch. It was later study the commercial laccase, Novozym 51003, with MeS. The results for the individual enzymes are present in Figure 37 (Attachment E), while the best results for each enzyme are compiled in Figure 17.

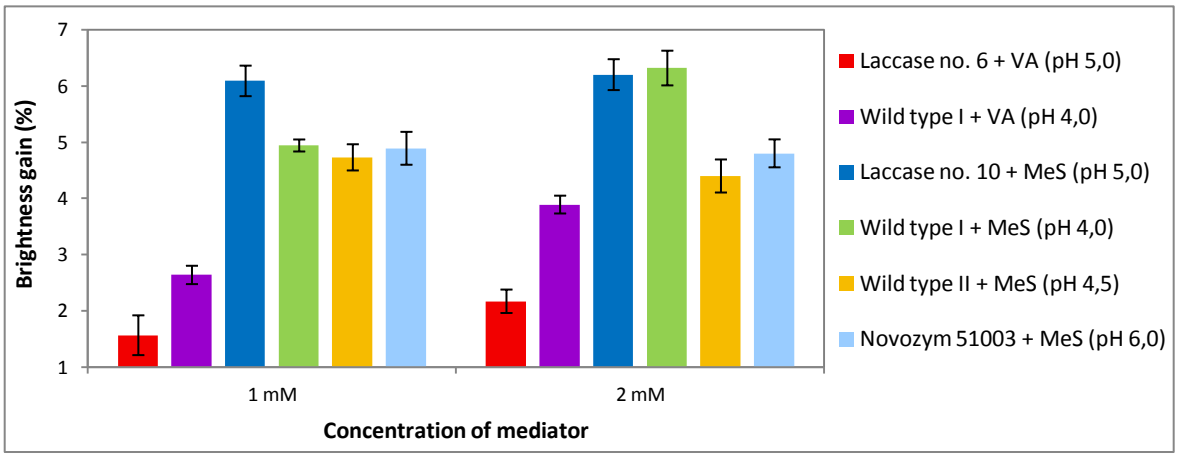


Figure 17 – Brightness gain results after LEp bleaching for the enzyme screening with the same EP concentration and with two different mediator concentrations at 50ºC, during 3h and using pulp 1.

According to Figure 17, the best enzymatic system is the NS-51002 laccase no. 10 with MeS and is better than the Novozym 51003 with MeS. For these systems an increase in the mediator concentration does not add benefits, as previous noted in 3.5.

Although VA is a mediator with particular interest, the performance of the NS-51002 laccase no. 6 or the wild type I with this mediator was not satisfactory, since the results from Figure 17 show only a small brightness gain when compared with systems that uses MeS as mediator,

which was also previous noted in 3.5. Therefore, the VA was not used in further assays with the NS-51002 laccase.

Afterwards, it was studied the enzyme concentration effect of the two best enzymes: NS-51002 laccase no. 10 and the wild type I. The results are presented in Figure 18.

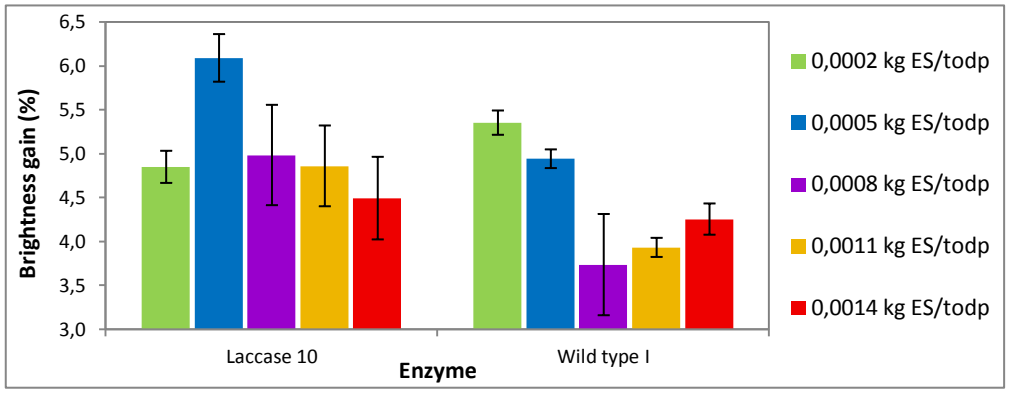


Figure 18 – Brightness gain results after LEp bleaching for the NS-51002 laccase no. 10 and the wild type I concentration screening with 1 mM of MeS, at 50ºC, during 3h and using pulp 1.

(Note 5: The columns of the same colour correspond to the same EP concentration. The concentrations presented in the Figure 18 are based on the ES of the laccase no. 10. The ES concentrations are the triple for the wild type I.)

The Figure 18 shows that an increase of the enzyme concentration does not bring improvements to the LMS, for 1 mM of MeS. Once again the NS-51002 laccase no. 10 is better than the wild type I and it is preferable a concentration of 0,0005 kg ES/t_{odp}.

It was studied as well the incubation time effect for the NS-51002 laccase no. 10 and the results are presented in Figure 19, which shows an increase in brightness along with an increase in the incubation time.

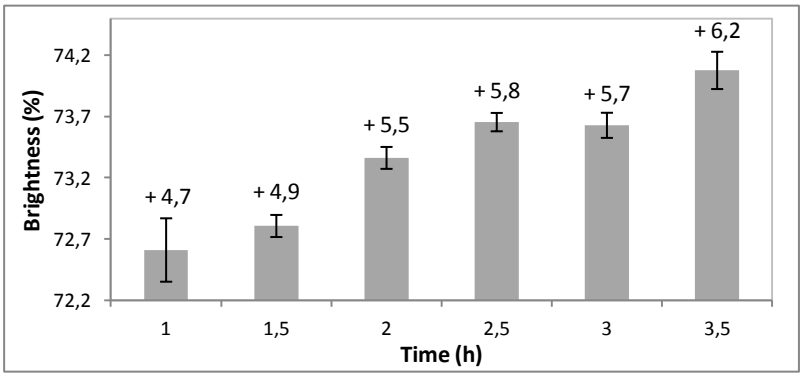


Figure 19 – Brightness results after LEp bleaching for the incubation time screening using 0,0003 kg ES/t_{odp} of NS-51002 laccase no. 10 with 1 mM of MeS, at 50°C and using pulp 1 (the brightness gain is represented on top of the bars).

Besides the laccases, peroxidases were also a part of this study in terms of pulp bleaching application. Therefore, it was additionally studied two peroxidases: NS-51004 and NS-51113, which belong to the class II and class III peroxidases, respectively. The results are present in Figure 20. These peroxidases are not unknown, as previous studies had been already done with pulp in the Forest Products department which have pointed to a good performance in bleaching when using VA as mediator.

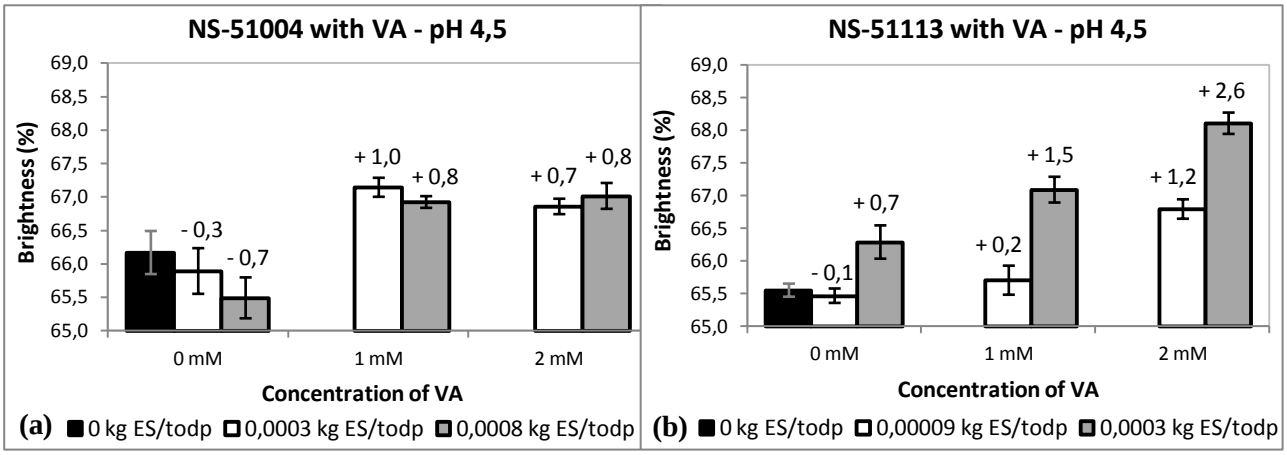


Figure 20 – Brightness and brightness gain results (on top of the bars) after LEp bleaching for the screening of enzyme and mediator concentrations, using NS-51004 (a) and NS-51113 (b) peroxidases, performed at 50ºC for 3h and using pulp 1 (one-time addition of hydrogen peroxide).

The assays presented in Figure 20 were performed with three different EP concentrations. Although the ES concentration is different for the two enzymes, it corresponds to the same EP concentration.

In the beginning of these bleaching assays it was added 2 mM and 4 mM of hydrogen peroxide, respectively for the NS-51004 and for the NS-51113 peroxidases, since the NS-51004 peroxidase had shown in previous studies to be more sensitive to this chemical than NS-51113 peroxidase. For both these assays was not refilled hydrogen peroxide over time.

According to figures 20 (a) and (b) it is possible to conclude that the best peroxidase is the NS-51113 and that a high concentration of enzyme and mediator seem to be advantageous. It was therefore studied the enzymatic effect for higher concentrations of enzyme (Figure 21) and mediator (Figure 22) in order to determine if the NS-51002 laccase no. 10.

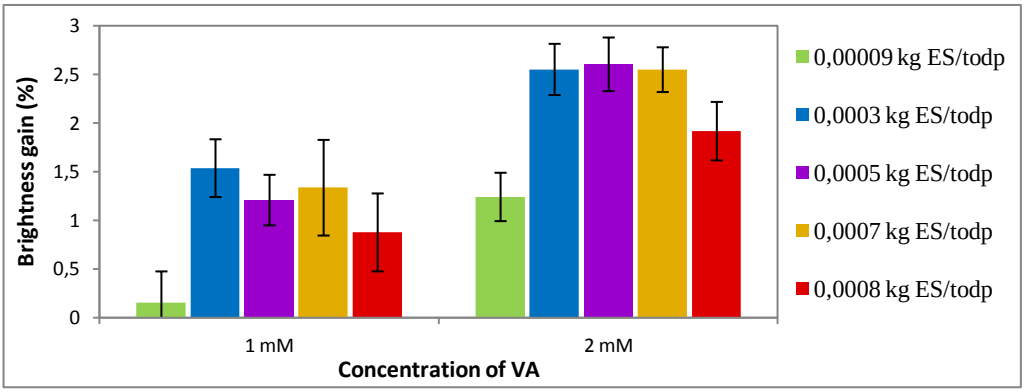


Figure 21 – Brightness gain results after LEp bleaching for the NS-51113 peroxidase concentration screening, using VA as mediator, performed at 50ºC, pH 4,5, for 3h and using pulp 1.

For the bleaching assays presented in Figure 21, it was initially added 3 mM of hydrogen peroxide and after 1,5 h of incubation it was added more 2 mM. In general, the consumption of hydrogen peroxide was around 53% of the total added.

The mediator concentration screening, presented in Figure 22, was performed for two incubation temperatures, that are normally used and for which the enzyme proved to be very active in previous studies performed in the Forest Products department. For these bleaching assays it was made a more detailed study about the consumption of hydrogen peroxide. The temperature profile for the NS-51113 peroxidase is represented in Figure 38 (Attachment F).

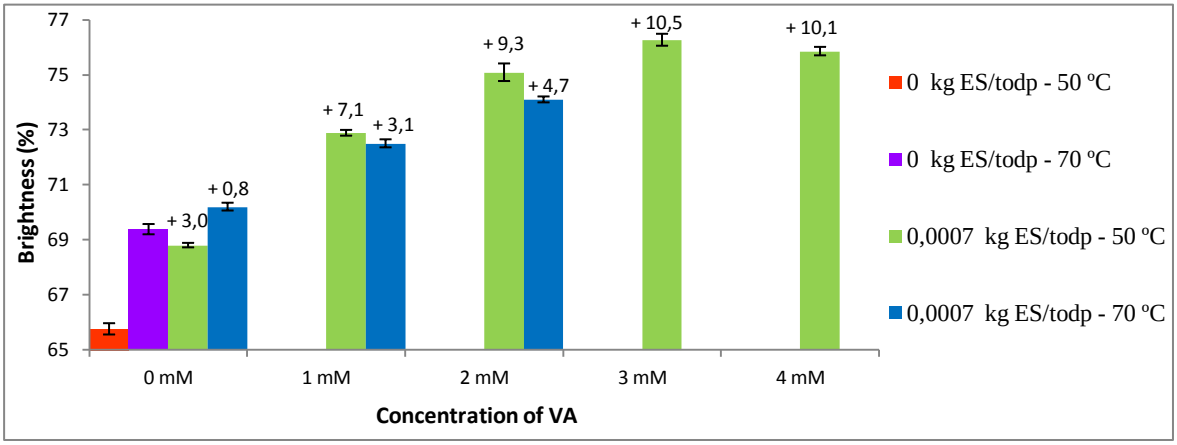


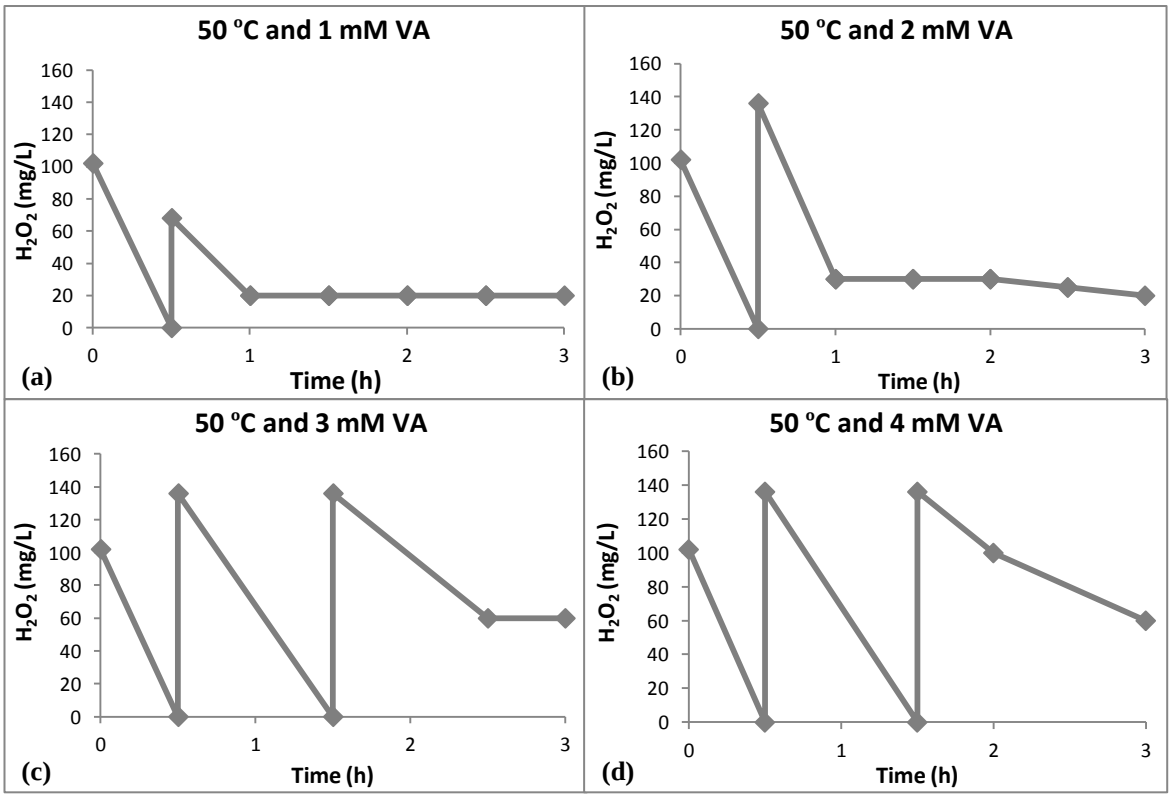
Figure 22 – Brightness and brightness gain results (on top of the bars) after LEp bleaching for temperature and mediator concentration screening, for the NS-51113 peroxidase with VA, pH 4,5, for 3h and using pulp 2 (The assays for 70 ºC using 3 and 4 mM of VA were not performed).

From figures 21 and 22 it is possible to conclude that high concentrations of enzyme and mediator are preferable but only to a certain limit, which in this case is not more than 0,0007 kg ES/t_{odp} and 3 mM of mediator.

In Figure 22 it was also represented the brightness gain (based on the control assay without enzyme and mediator) and it is possible to observe that the values are very different when comparing the assays for 50ºC and 70ºC, which is expected since the control brightness for the two temperatures differ in 3% units. This big difference in the controls may result from the temperature effect on the transition metals present in the pulp. Temperatures high as 70ºC in the enzymatic treatment can decrease the metal content and improve this way the bleaching performance of the subsequent E_p stage, resulting in an increase of brightness as shown in Figure 22. However, even disregarding the brightness gain values, the absolute brightness suggests that it is preferable to perform the peroxidase incubation at 50ºC in comparison with 70ºC.

The peroxidases have a particularity of requiring a constant hydrogen peroxide refill over time, since they are not very tolerable to high initial concentrations. Therefore, in intervals of 30 min, it was measured the residual hydrogen peroxide of the sample and added small quantities of hydrogen peroxide, if the residual value was too low. This refill was in very small volumes, in order to not change the pulp consistency.

The evolution of hydrogen peroxide concentration with time is shown in Figure 23 and the total consumption of hydrogen peroxide in the several assays is shown in Figure 24.



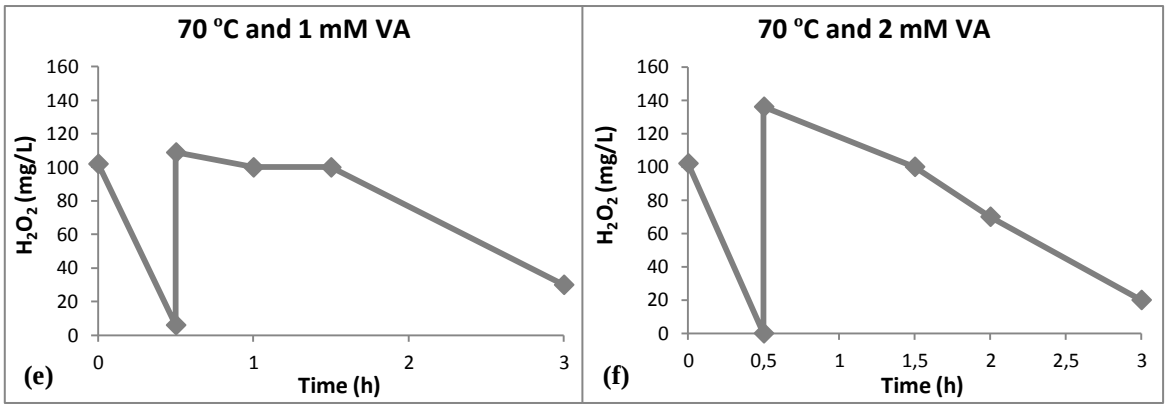


Figure 23 – Hydrogen peroxide concentration over time, for the assays performed with the NS-51113 peroxidase.

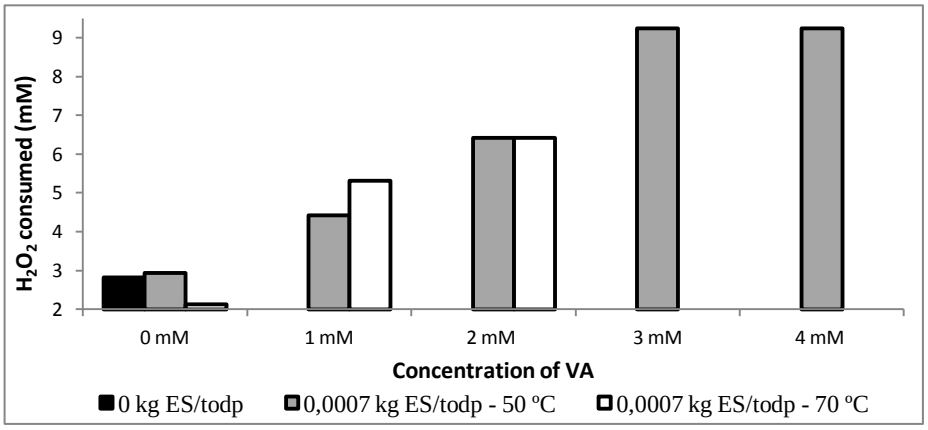


Figure 24 – Overall consumption of hydrogen peroxide for the assays performed with NS-51113 peroxidase.

According to Figure 24, the assays with higher mediator concentration require more hydrogen peroxide. Through Figure 23, it is possible to visualize that, regardless the temperature and the mediator dosage, for all the assays the hydrogen peroxide is depleted within the first 30 minutes of reaction.

For lower mediator concentrations, figures 23 (a), (b), (e) and (f), it is only necessary to refill once with hydrogen peroxide. For this situation it was added 2-4 mM, correspondent to 68-136 mg/L. On the other hand, for higher mediator concentration, figures 23 (c) and (d), the pulp bags were refilled twice. For this case, it was added 4 mM (or 136 mg/L) in each refilling and it is noted that the residual hydrogen peroxide (H_2O_2 content for 3h) increased, which indicates that the concentration of the second refilling could be lower, resulting in savings of hydrogen peroxide.

From the results of the consumption of this chemical, it can also be noted a temperature effect on the enzymatic system. Figure 23 (a) and (b) show that after 1 h of reaction at 50ºC the peroxide content almost does not change, which means that the enzymatic reaction occurred only in the first hour. In contrast, in figures 23 (e) and (f), for the reaction at 70ºC, the amount of peroxide is decreasing continuously, so the enzymatic reaction is still occurring over the 3 hours. In conclusion, for the same enzyme and mediator concentrations and for the same consumption of peroxide, the peroxidase requires a longer incubation time at 70ºC than at 50ºC in order to have a similar performance, which means that the reaction rate is lower for 70ºC.

In short, the best enzymes were the NS-51002 laccase no. 10 and the NS-51113 peroxidase. The overall optimized conditions performed in this work for the application of these enzymes are present in Table 8, which were selected to be applied further in the bleaching sequences.

Table 8 – Optimized conditions for the NS-51002 laccase no. 10 and the NS-51113 peroxidase, for oxygen pre-bleached *Eucalyptus* kraft pulp bleaching.

Enzyme	NS-51113 peroxidase	NS-51002 laccase no. 10
Enzyme concentration (kg ES/t_{odp})	*0,0007	0,0005
Mediator	VA	MeS
Mediator concentration (mM)	3	1
Total H_2O_2 concentration (mM)	9-10	n.a.
Oxygen pressure (bar)	n.a.	4
pH	4,5	5,0
Temperature (ºC)	50	50
Time (h)	3	3,5
Brightness gain after LEp (% units)	10,5	6,1

* The concentration of the NS-51113 peroxidase corresponds, in terms of EP concentration, to more than the double when comparing to the EP concentration of the NS-51002 laccase no.10.

3.6.2. Optimization of the E_p stage

After optimizing the enzymatic stage, it was also optimized the E_p stage in terms of chemicals dosage. The applied dosages were chosen according to a preliminary test with pulp at 1,5% consistency, presented in Figure 39 (Attachment G). The optimization results are present in figures 25 and 26.

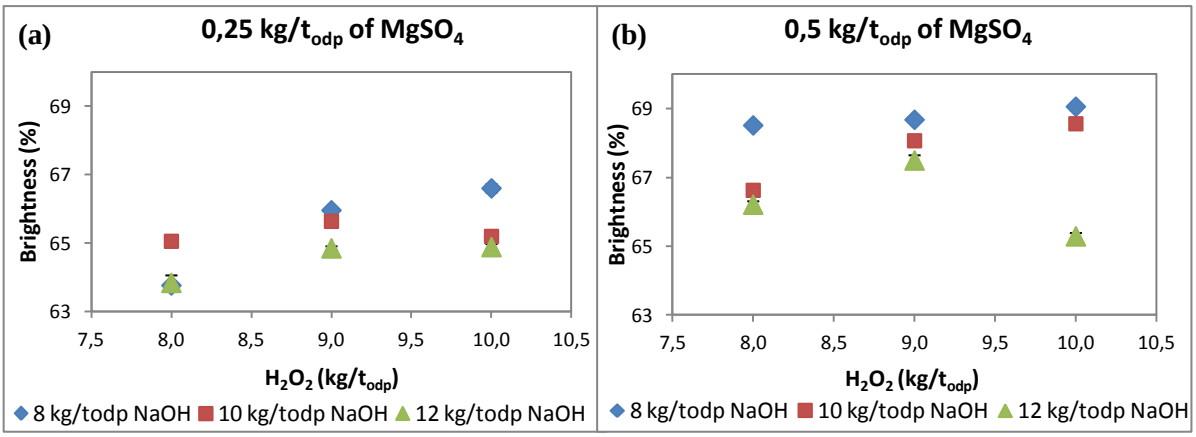


Figure 25 – Brightness results for E_p stage optimization, using 0,25 kg/t_{odp} (a) and 0,5 kg/t_{odp} (b) of $MgSO_4$, at 85°C, during 1,5 h and using pulp 2.

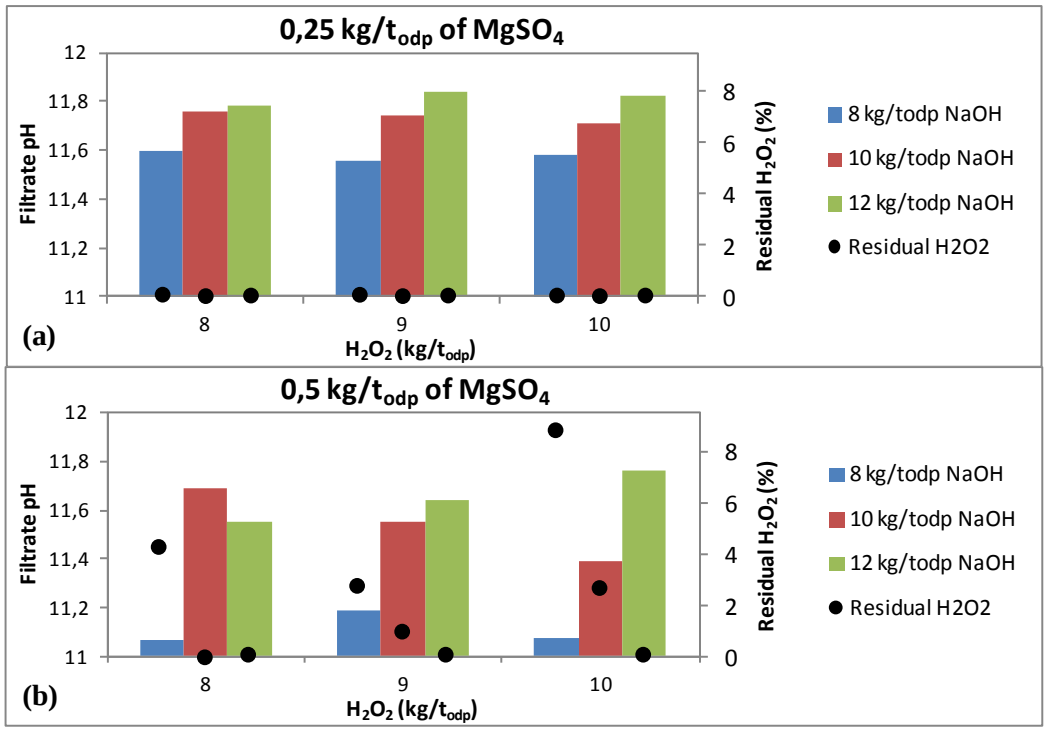


Figure 26 – Filtrate pH and residual H_2O_2 results for E_p stage optimization, using 0,25 kg/t_{odp} (a) and 0,5 kg/t_{odp} (b) of $MgSO_4$, at 85°C, during 1,5 h and using pulp 2.

The results from Figure 25 suggest that an increase of the dosage of $MgSO_4$ and a decrease of the dosage of NaOH enhance the pulp brightness. In the E_p stage the chemicals react with each other according to equations (22) and (23).

$$H_2O_2 + HO^- \rightleftharpoons HOO^- + H_2O \quad \textbf{(22)}$$

$$Mg^{2+} + OH^- \rightleftharpoons Mg(OH)_2 \quad \textbf{(23)}$$

It is expected that for a small magnesium sulphate dosage, the reaction (23) is not so extensive (forward direction) and then less consumption of sodium hydroxide will be obtained. For a high dosage of magnesium sulphate, Figure 26 (b), lower pHs are obtained and a better peroxide stabilizing effect is achieved as seen in the lower consumption and higher brightness obtained. Too much soda is not favourable and can be explained by alkali-induced decomposition of peroxide.

Regarding to the hydrogen peroxide dosage, the analysis of the brightness alone, in Figure 25, is inconclusive since an increase of the dosage is beneficial, but not significantly. Through Figure 26 (b), it is possible to note that an increase in the dosage of the hydrogen peroxide leads to a high residual value at the end, which indicates that an excess of this chemical did not react. Moreover, an increase of the peroxide dosage is not just another expense but could also contribute for loss of pulp viscosity. Summarizing, the best dosages for the E_p stage, that will be used in the bleaching sequences, are: 0,5 kg $MgSO_4/t_{odp}$, 8 kg $NaOH/t_{odp}$ and 8 kg H_2O_2/t_{odp}.

3.7. Bleaching sequences (ECF and TCF)

As mentioned before, the pulp bleaching process evolved quite a lot over the years. However, in the XXI century most mills still choose ECF sequences like DEDD or DEDED as bleaching technologies. Nevertheless, the replacement of the D stages for P stages has been studied and implemented for instance in final bleaching as it comprises an enhancement in pulp brightness, lower brightness reversion, savings in chlorine dioxide, elimination of chlorinated organic compounds in the effluents and enhance in pulp strength properties. However, this stage requires a control of the transition metals present on the pulp in order to be efficient.[57]

Today the implementation of the hot acid stage (A) in the beginning of sequences like $A/DE_{op}DP$ is a reality that allows reducing the demand of chlorine dioxide due to a decrease of the HexA content after the A stage. The target for the future is to reduce even more the usage of chlorine dioxide in bleaching with the use of enzymatic stages in the bleaching sequences.

In this work, the performed bleaching sequences consisted of 17 sequences, 8 TCF and 9 ECF, all of them with a final P stage, using oxygen pre-bleached *Eucalyptus* kraft pulp with a ISO brightness of 53,4% (pulp 2). It was also implemented enzymatic stages with the enzymes optimized in 3.6.1 and xylanases. All bleaching stages were performed at 10% pulp consistency (medium consistency). Despite of simulating well the actual bleaching conditions, medium consistency assays executed in the laboratory might not provide a very homogeneous mixing and might cause mass transfer problems that can affect the bleaching quality.

During the bleaching it was sampled pulp and filtrate in different points of the sequences for the analysis of ISO brightness, viscosity, kappa number and HexA content in pulps and the

analysis of COD, pH and residual H_2O_2 in the filtrates. These analysis were useful to evaluate the overall efficiency of the bleaching sequences and it also had other goals, such as comparing the performance of the two enzymes in a full sequence; evaluate the substitution, partial or total, of D stages (ECF) for enzymatic stages (TCF); compare the effect of the A and the X stages in the early part of the sequence, and evaluate the effect of individual stages during the sequence.

The main results corresponding to the full sequences are presented in figures 27, 28 and 29 while in Table 9 it is shown some data characterizing the pulp that was used in these experiments.

Table 9 – Untreated and control pulp ISO brightness, kappa number and HexA content.

Pulp	ISO Brightness (%)	Kappa number	HexA (mmol/kg_{odp})	Viscosity (cP)
Laccase control pulp 2	53,7	8,9	49,8	---
Peroxidase control pulp 2	54,1	9,1	49,6	---
Raw oxygen pre-bleached pulp 2	53,5	12,5	62,9	9,9

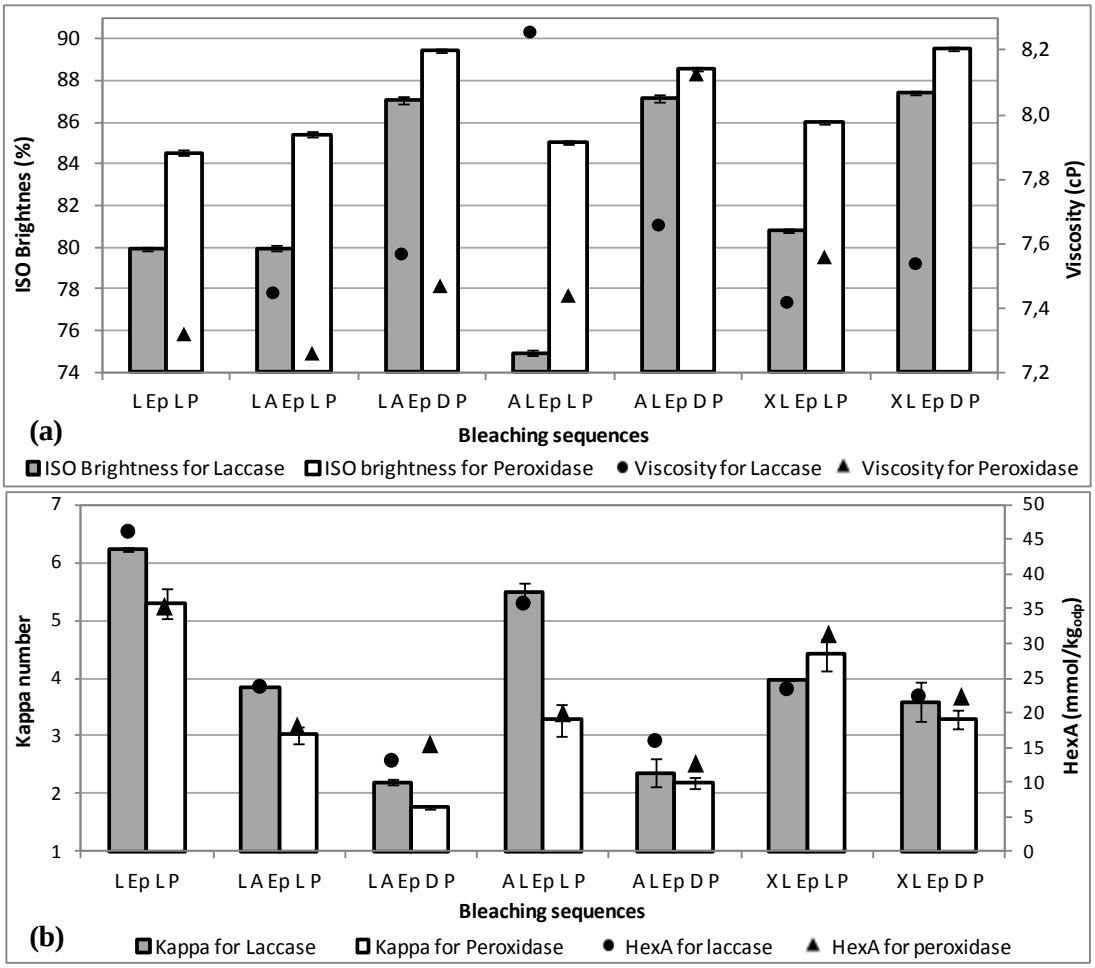


Figure 27 – ISO brightness (a), viscosity (a), kappa number (b) and HexA content (b) at the end of the bleaching sequences with oxidoreductases, L means stages with NS-51002 laccase no.10 or NS-51113 peroxidase, using pulp 2.

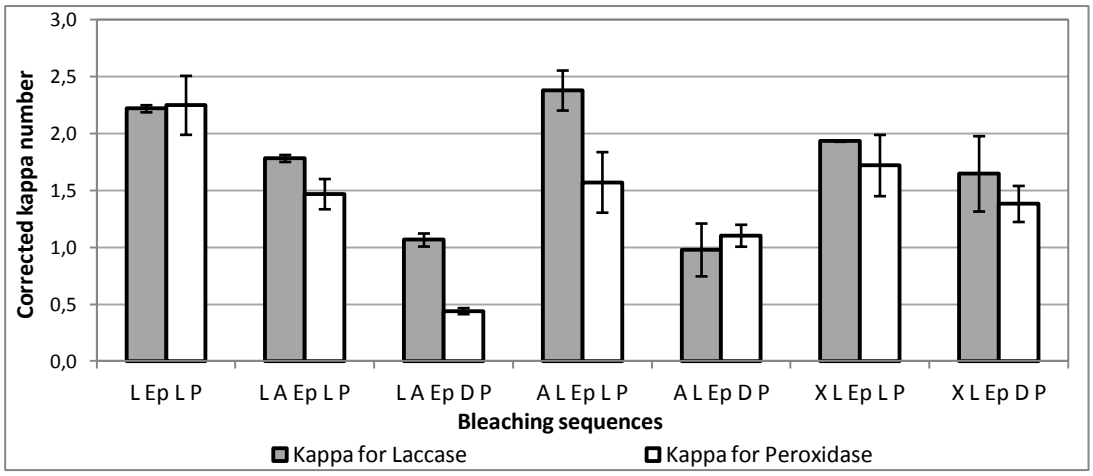


Figure 28 –Corrected kappa number correspondent only to the lignin content, at the end of the bleaching sequences with oxidoreductases, L means stages with NS-51002 laccase no.10 or NS-51113 peroxidase, using pulp 2.

3.7.1. Enzyme comparison

The Figure 27 corroborates the results obtained in 3.6.1. in the way that the NS-51113 peroxidase performance is better than the NS-51002 laccase no. 10, but now this conclusion is ensured for a full bleaching sequence. Regardless of the bleaching sequence, Figure 27 (a) and (b) shows that the use of the NS-51113 peroxidase provides the highest final brightness and lowest kappa number. Both the ISO brightness and the kappa number difference between the peroxidase and the laccase is less accentuated in sequences with D stage in the 4th stage (e.g. ALE$_p$DP and XLE$_p$DP) than in sequences with two enzymatic stages (e.g. ALE$_p$LP and XLE$_p$LP).

The final viscosity and the HexA content are similar for laccase and peroxidase. In general, the viscosity is slightly lower for peroxidase as well as the HexA content.

As previously described in 1.4.1, kappa number is a function of the lignin and the HexA contents, thus to conclude about the chemicals/enzymes effect on delignification is preferable to analyse the corrected kappa number correspondent only to the lignin content, calculated by equation (1), which is presented in Figure 28. According to this figure, in general, the NS-51113 peroxidase improves more the delignification than NS-51002 laccase no. 10.

3.7.2. Total and partial substitution of D stage for enzymatic stage

To gain a better understanding on the effect that the substitution of D stages for enzymatic stages has on the pulp properties, it was compiled in Figure 29 the results for sequences with NS-51113 peroxidase, which proved to be the best enzyme, and sequences with chlorine dioxide.

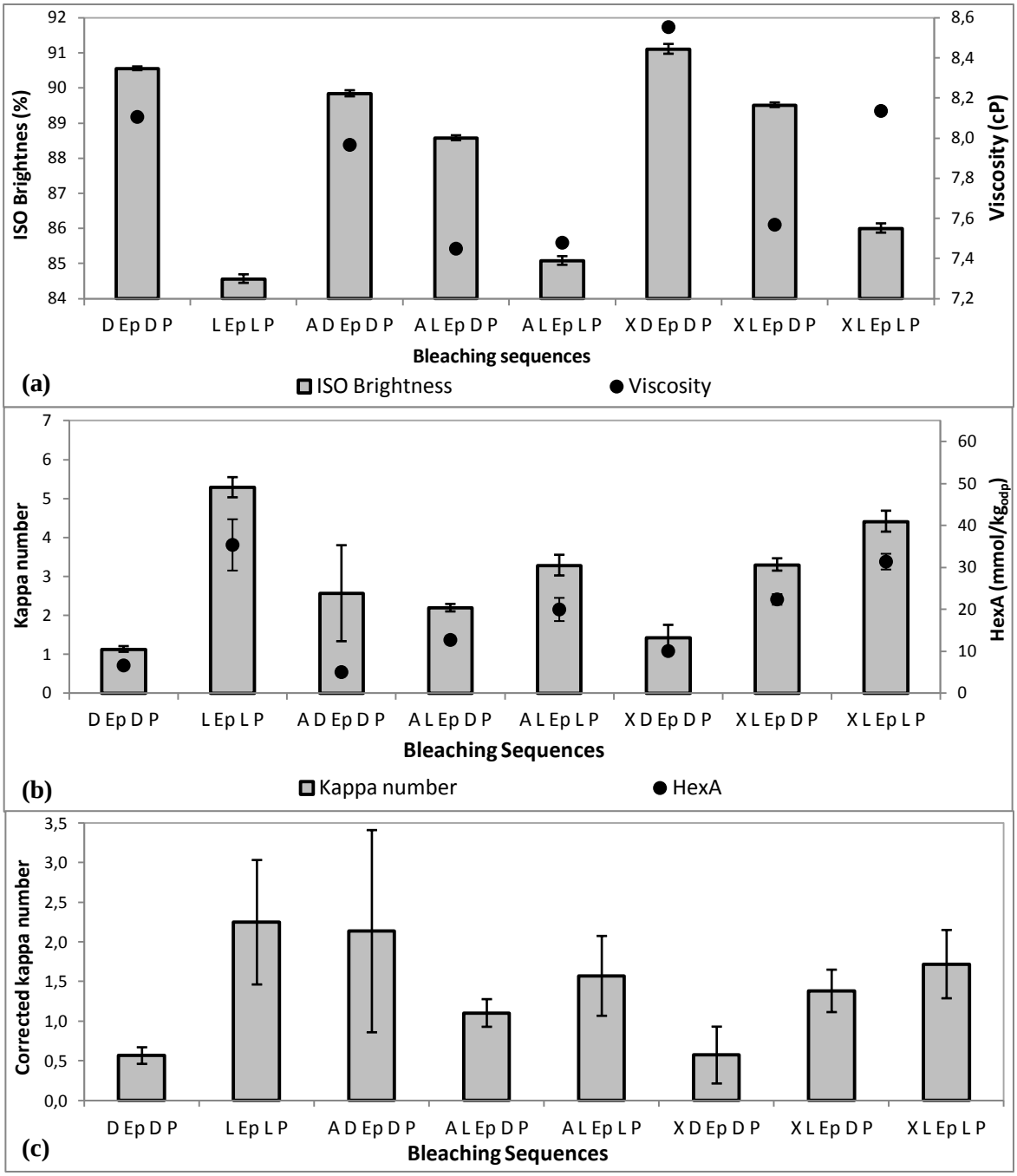


Figure 29 - ISO brightness (a), viscosity (a), kappa number (b), HexA content (b) and corrected kappa number correspondent only to the lignin content (c), for ECF bleaching sequences, using pulp 2.

From Figure 29 (a) it is possible to note that the partial and the total substitution of D stage for peroxidase stage decreases the final brightness and the viscosity, independently if the sequence starts with A stage, X stage or none of these.

The final kappa number, shown in Figure 29 (b), is lower for sequences with two D stages, like DE_pDP, ADE_pDP and XDE_pDP. However, the kappa number measurements in highly bleached pulps are not accurate. Although the kappa results were represented with the respective error bars, the most correct is to consider a kappa number below 1 for these cases.

The HexA content is also represented in Figure 29 (b), which shows lower HexA for sequences with two D stages, followed by sequences with one D stage in the 4^{th} stage (e.g. ALE_pDP and XLE_pDP), which is expected since the HexA structures are extensively degraded when exposed to chlorine dioxide.[58] The sequences in which D stages are substituted by peroxidase stages (e.g. ALE_pLP and XLE_pLP) exhibit the highest HexA content. However, these sequences still can reach more than 50% reduction in the HexA content.

The corrected kappa number corresponding only to the lignin content is presented in Figure 26 (c). The results suggest that for ECF sequences the delignification was higher than for TCF sequences and these parameter was slightly higher for sequences that start with the X stage, like XDE_pDP and XLE_pDP rather than for sequences that start with the A stage, like ADE_pDP and ALE_pDP.

The overall data corroborates the conclusion that the chlorine dioxide is difficult to be totally replaced by the NS-51113 peroxidase with the same performance for similar amount of stages. However, the replacement of the first D stage for the peroxidase stage in the sequence XLE_pDP allowed reaching an ISO brightness of 89±0,5%, a kappa number of 4±0,5 and a chlorine dioxide saving of 8,0 kg ClO_2/t_{odp}.

3.7.3. A stage and X stage effect in the beginning of a bleaching sequence

It has been reported that hot acid and xylanase stages in the beginning of a bleaching sequence could result in major improvements for the bleaching. Accordingly, it was analysed the effect of these stages, combined with both ECF and TCF sequences.

As for the A stage, when comparing the TCF sequences (LE_pLP and ALE_pLP where L represents the NS-51113 peroxidase) from figures 27, 28 and 29, it is observable that the A stage favoured the kappa number reduction, but the brightness only increased a little. For these sequences, the viscosity was similar. Comparing now ECF sequences (DE_pDP and ADE_pDP) from Figure 29 (a), it shows that brightness and viscosity is slightly lower in the sequence with the A stage.

Moreover, it was studied the placement of the A stage in the early part of the sequence (1st or 2nd stages) and the results suggest that it does not affect much the pulp properties, especially for sequences with the NS-51113 peroxidase.

Nevertheless, in both TCF and EFC sequences, the implementation of the A stage in the beginning had significant impact on decreasing the HexA content which is, in fact, the main advantage of this stage. So, the kappa number drop in this stage is caused essentially by HexA removal and not so much by the delignification itself.

A reduction in the HexA content is also very important to prevent brightness reversion besides chlorine dioxide savings.

According to figures 27, 28 and 29, the introduction of the X Stage in the beginning of both TCF (LE_pLP and XLE_pLP) and ECF sequences (DE_pDP and XDE_pDP) increased the brightness and the viscosity. In the case of TCF sequences the kappa number decreased and the HexA content decreased only slightly. In contrast, for ECF sequences the kappa number was similar.

According to papers that studied the ECF bleaching performance of similar sequences using laccases from the *Trametes* specie as the L stage[59], the implementation of a xylanase stage before the laccase stage also enhance the pulp brightness and reduces the chlorine dioxide demand.

Comparing directly the A and the X stages it is possible to conclude that sequences with the A stage promote more the HexA and kappa number reduction than the X stage. When evaluating the corrected kappa number the values are similar for both cases. However, the pulp brightness and viscosity are slightly higher for sequences with the X stage.

The COD analysis performed at the end of the bleaching sequences also allowed comparing the sequences with A and X stages. The COD is an indirect measure of the amount of organic compounds in a liquid sample, in this case the pulp bleaching filtrates. It is based on the fact that nearly all organic compounds can be fully oxidized to carbon dioxide with a strong oxidizing agent, under acidic conditions. High COD values imply that more organic compounds were removed from pulp. These components may be products resultants from the lignin solubilisation, but it can also be products related to cellulose and hemicellulose degradation.

The COD of the full sequence was determined and the results for the sequences without oxidoreductases are represented in Table 10. The sequences with oxidoreductases were not represented since the acetate buffer used in the enzymatic stages would contribute for a high COD, which hinders an accurate analysis.

Table 10 – COD, ISO brightness, KN and viscosity results for bleaching sequences without oxidoreductases.

Bleaching sequence	DE_pDP	ADE_pDP	XDE_pDP
COD (mg/kg_{odp})	11250	9855	12240
ISO brightness (%)	90,5	89,8	91,1
KN	1,1	2,6	1,4
Viscosity (cP)	8,1	8,0	8,6

According to Table 10 and having the sequence DE_pDP as reference, it is possible to conclude that the implementation of the X stage in the beginning of the sequence enhance the COD. On the contrary, the implementation of the A stage decreased this property.

In the case of the sequence with the X stage, the enzymatic hydrolysis of xylan and the release of lignin-xylan complexes would contribute to increase the COD. In the case of the sequence with the A stage, as has a lower COD, it could not reach the same brightness level and kappa number of the sequence with the X stage and also had a lower viscosity.

3.7.4. Effect of each individual stage in the full bleaching sequence

In order to evaluate the individual effect of each stage, it was measured some properties in different points of the sequences: brightness was measured in every stage; kappa number was measured at the beginning, middle and end of the sequences; COD was measured along the sequence being the filtrates of each stage mixed together to give a sequence filtrate. The results are present in figures 30, 32 and 33, respectively.

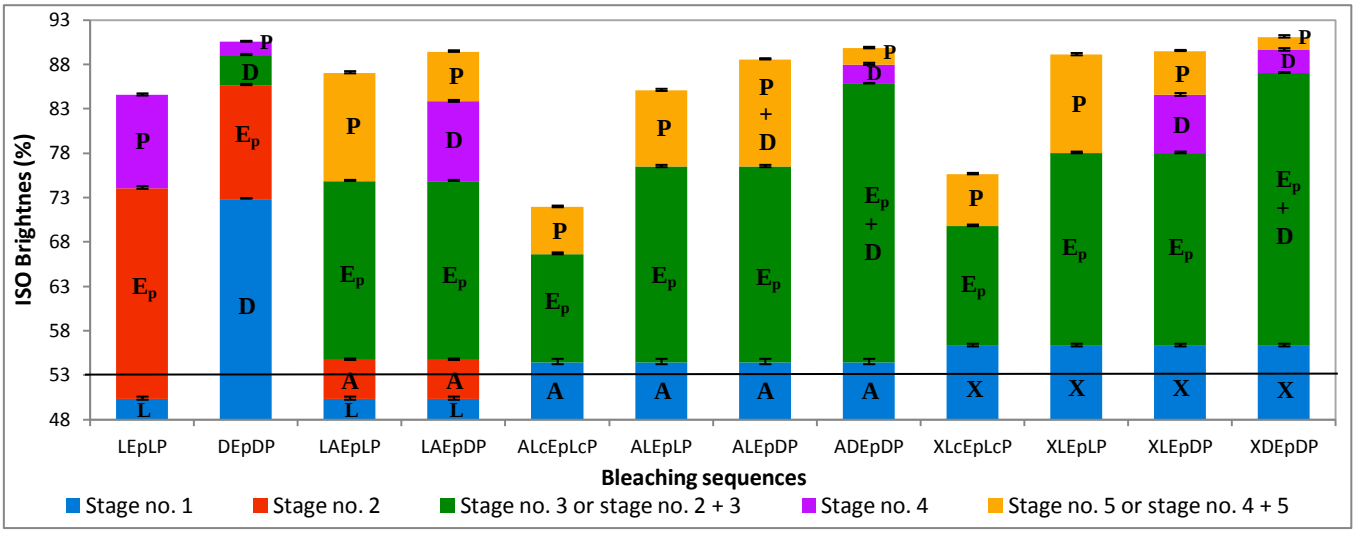


Figure 30 – ISO brightness in every stage of the sequences; here L means the stages with the NS-51113 peroxidase and Lc means the peroxidase control. The black line on 53% marks the raw pulp ISO brightness.

In Figure 30 it is possible to observe the brightness evolution along the different ECF and TCF sequences, using NS-51113 peroxidase as the enzymatic stage (L stage). During the experimental procedure it was visible that both the laccase and peroxidase stages gave a darker colour to the pulp, as seen in Figure 31 (a). In fact, the oxidation of phenolics generates phenoxy radicals which consequently form quinones (Figure 31 (b)) by molecular rearrangements. These conjugated cyclic structures exhibit absorption in the visible region of the spectrum, being responsible for the dark colour in pulp.[35,60]

Figure 31 –Pulp before (bag no. 4) and after (bag no. 5) the enzymatic stage with peroxidase (a) and *para*-quinone structure (b).

In fact, the results from the sequences LE_pLP, LAE_pLP and LAE_pDP present in Figure 30 showed that the brightness of the first L stage was lower than the raw pulp brightness. This is also the reason why the L stage brightness present in the 4th stage does not appear, since the ISO brightness gain is negative. Thus, it is possible to conclude that the enzymatic stage alone do not have an improving effect on the pulp brightness.

It was not possible to measure the brightness in all stages, so some stages like no. 3 and no. 5 have incorporated the brightness gain from the previous stage, respectively no. 2 and no. 4.

Regarding the effect of the individual stages on the brightness, it is observed that the highest brightness gain for the first stage occurs for the D stage. However, in general, the highest increase in brightness occurs after the third stage, which is the combined effect of peroxidase and alkaline extraction stage (LEp) or of chlorine dioxide and alkaline extraction stage (DE_p), depending on the sequence. As expected, the highest increase occurs for sequences with DE_p stages incorporated. This also happens in the last stage (P stage), but in this case the brightness gain is not so high. With this study it is confirmed that the enzymatic effect on brightening can only be validated after the E_p or the P stage.

The sequence that provided the highest brightness was XDE$_p$DP (91,1%), followed by DE$_p$DP (90,5%) and ADE$_p$DP (89,8%). Sequences with NS-51peroxidase that demonstrated to be quite good were XLE$_p$DP (89,5%) and LAE$_p$DP (89,4%), providing high brightness and savings of chlorine dioxide.

The kappa number and HexA content correspondent to different parts of the sequences are represented in Figure 32. However, for the sequences LE$_p$LP, ALcE$_p$LcP, ADE$_p$DP and XLcE$_p$LcP it was not measured these pulp properties after the E$_p$ stage and for the sequence DE$_p$DP it was not measured the HexA content after stage no. 1.

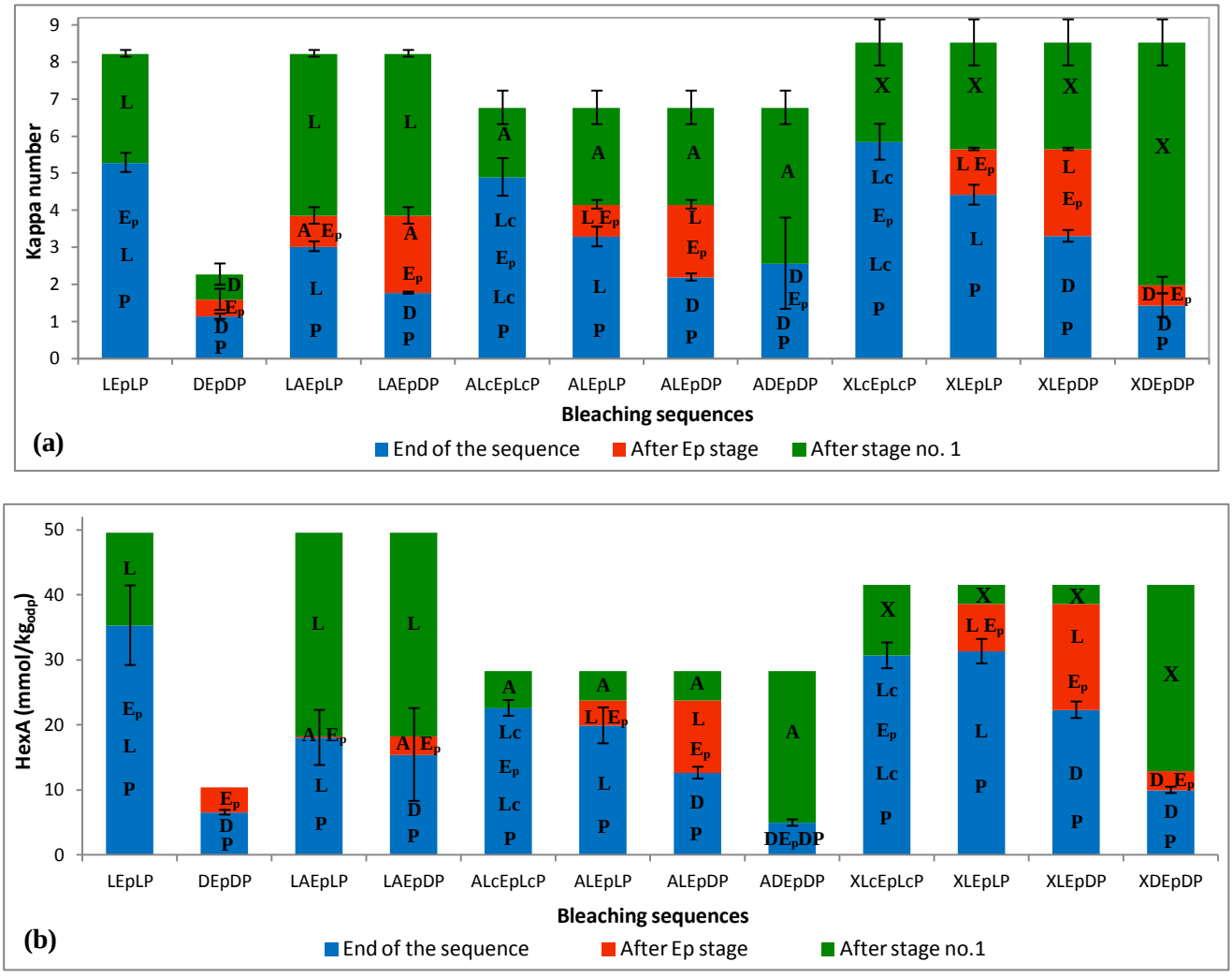


Figure 32 – Kappa number (a) and HexA content (b) results for different parts of the sequences, here L means the stages with peroxidase and Lc means the peroxidase control.

Having in mind that the kappa number of the raw pulp was 12 and the HexA content was 62,9 mmol/kg$_{odp}$, from Figure 32 (a) it is possible to verify that the first bleaching stage allowed a

significant reducing in kappa number: approximately 3,5 units when using the X stage, 4 units for the peroxidase stage and 5 units for the A stage. Figure 32 (b) suggest that the large kappa reduction resultant from the A stage is associated to the reduction of the HexA content by approximately half also in the first stage. The peroxidase and the X stages in the first position also reduces the HexA but in small quantities.

Once again it is confirmed through Figure 32 that the A stage position, in stages no. 1 or no. 2, does not affect the pulp parameters, since after the E_p stage the kappa number and the HexA values are the same for sequences like LAE_pLP and ALE_pPLP.

Sequences with two D stages decrease quite a lot the HexA content (reduction of 84-92% in relation to the raw pulp HexA content) and the decrease is highest in the early part of the sequence, namely after the E_p stage (reduction of 80-84%), rather than in the final part (reduction of 23-37%). In turn, sequences with D stage in the 4^{th} stage, like ALE_pDP, LAE_pDP and XLE_pDP, promote the kappa number and the HexA reduction in end of the sequence, more than stages with peroxidase, like ALE_pLP, LAE_pLP and XLE_pLP.

In Figure 32 it is also represented the control sequences (ALcEpLcP and XLcEpLcP). In the enzymatic control stages the pulp was treated under the same conditions as the peroxidase stage but without enzyme, mediator and hydrogen peroxide. These sequences are useful to understand the effect of peroxidases alone in the sequences ALE_pLP and XLE_pLP.

When comparing the peroxidase sequences with the control sequences it is possible to verify that the peroxidases alone helped to reduce the kappa number in 1,5 units and the HexA in 1-2,5 $mmol/kg_{odp}$. However, as shown in Table 9, the peroxidase control stage (mild acidic stage) demonstrated to have also a positive impact on these properties. This aspect cannot be disregarded, and therefore the whole effect of the first peroxidase stage, for the sequence LAE_pLP, or the effect of the first peroxidase stage combined with the E_p stage, for the sequences ALE_pLP and XLE_pLP, was estimated considering the results in Figure 32, that are also present in Table 13 (Attachment H), in relation to the raw pulp properties. The same study was made for the sequences with the NS-51002 laccase no. 10 stages in order to have a quantitative estimate of the differences between the enzymes even though it is already known that peroxidase is the most efficient. These results are present in Table 11.

Table 11 – Kappa number and HexA content reduction after (in absolute units and percentage) for the selected sequences incorporating peroxidase and laccase stages, in relation to the raw pulp.

	Kappa number		HexA (mmol/kg$_{odp}$)	
Sequence	**Peroxidase**	**Laccase**	**Peroxidase**	**Laccase**
LAE$_p$LP	5,0 (54%)	4,7 (55%)	13,5 (30%)	10,6 (27%)
ALE$_p$LP	3,5 (38%)	1,3 (18%)	8,5 (20%)	8,4 (19%)
XLE$_p$LP	4,1 (51%)	4,5 (53%)	10 (32%)	17,8 (45%)

According to Table 11, stages with NS-51113 peroxidase in the sequences LAE$_p$LA and ALE$_p$LP contributed for a higher reduction in the kappa number and HexA content, when compared to the stages with NS-51002 laccase no.10. On the other hand, in the sequence XLE$_p$LP the reduction of these properties was slightly higher for the NS-51002 laccase no.10.

Overall, the sequences with lower kappa number are ECF sequences: DE$_p$DP, XDE$_p$DP and ADE$_p$DP, followed by LAE$_p$DP and ALE$_p$DP.

During the sequential bleaching process, the filtrates were collected from several stages, for pH and residual H_2O_2 measurement. The results for all stages are present in Table 13 (Attachment H) and the range of results is present in Table 12.

Table 12 – Residual hydrogen peroxide and final pH range for the collected filtrates.

Stage	Laccase	Peroxidase	A	X	D	Ep	P
pH	4,9-5,3	4,5-4,8	3,2-3,4	7,7	2,2-2,9	10,4-11,2	10,4-11,1
Residual H_2O_2 (mg/L)	n.a.	1st stage: 3-25 4th stage: 30-150	n.a.	n.a.	n.a.	10-300	300-800

The pH values were in the normal range. The residual H_2O_2 was high for the latter part of the sequence, like the P stage or peroxidase in the 4th stage. In the case of the E$_p$ stage, the highest values of residual H_2O_2 are correspondent to sequences where the previous stage is D stage, which was expected since chlorine dioxide is a very efficient bleaching agent, thus the dosage of chemicals in the following E$_p$ stage could be lower in order to have savings in chemicals.

Chapter 4: Conclusions

This work had the purpose of studying new oxidoreductases, namely NS-51002 laccase variants, produced in Novozymes A/S, with regard to the assessment of their ECF and TCF bleaching of oxygen pre-bleached *Eucalyptus* kraft pulp and by comparing with other benchmark enzymes already tested in pulp bleaching applications.

Firstly, the NS-51002 laccase variants were purified using different procedures: UF followed by IEC or sterilized filtration followed by HIC and dialysis. The SDS-PAGE showed that both procedures were efficient towards purification, but the UF followed by IEC was not so efficient in terms of laccase losses.

In addition, the laccase variants were tested in indigo carmine with different mediators and pHs in order to evaluate the enzymes performance in decolourization. The most active enzymes were selected to be tested in pulp in screening assays at 1,5% consistency. This assay could not give an accurate output regarding the best mediators, pH and enzyme and mediator concentrations since the screening demonstrated high error bars associated to the experimental procedure. Therefore, it was performed a selected number of screening assays at 10% consistency which revealed small error and pointed that the NS-51002 laccase no. 10 had the best performance in bleaching, being even better than the benchmark laccase, Novozym 51003.

Then, it was optimized the incubation conditions for NS-51002 laccase no.10. The best conditions were: 0,0005 kg ES/t_{odp}, 1 mM of MeS, 4 bar of molecular oxygen, at pH 5,0 and 50 C during 3,5 h.

Peroxidases have also great potential for bleaching applications. Thus it was study two peroxidases, NS-51004 and NS-51113, previously analysed by the Forest Products department at Novozymes A/S, which demonstrated good affinity with VA, the cheapest mediator. In this study NS-51113 peroxidase proved to have a better performance in bleaching and the enzymatic system was also optimized. The best conditions were: 0,0007 kg ES/t_{odp}, 3 mM of VA, 9-10 mM (refilled 2-3 times over time), at pH 4,5 and 50ºC during 3,0 h.

It is noteworthy that the NS-51113 peroxidase provide a higher brightness gain (+10,5%) than NS-51002 laccase no. 10 (+6,1%).

It was also optimized the chemical dosages of the E_p stage, through a study of brightness, filtrate pH and residual H_2O_2. The best dosages were: 0,5 kg $MgSO_4/t_{odp}$, 8 kg NaOH /t_{odp} and 8 kg H_2O_2/t_{odp}.

NS-51113 peroxide and NS-51002 laccase no. 10 were then applied in several TCF and ECF bleaching sequences, in order to evaluate its effect in a full sequence and to compare its bleaching performance with chlorine dioxide (D stage), which is the most efficient bleaching agent,

though toxic and dangerous for the environment. It was also introduced a xylanase (X) and a hot acid (A) stage in the beginning of the sequences.

To evaluate the bleaching efficiency, pulp properties were determined, such as ISO brightness, kappa number, viscosity, HexA content as well as filtrate properties like pH, residual H_2O_2 and COD. Some of these properties were measured not only in the end but also in different points of the sequence, in order to have a broader picture of the pulp properties evolution along the sequences. From this study it was possible to achieve the following conclusions:

- The application of the NS-51113 peroxidase is better than the NS-51002 laccase no. 10 for the full sequence, not only related to brightening but also to delignification and HexA reduction. In terms of viscosity, the values are slightly lower for the peroxidase.
- The effect of the enzymes, laccase or peroxidase, in brightness is only shown after an alkaline stage with hydrogen peroxide (E_p or P stage). The use of enzyme alone does not improve the brightness; on the contrary, it gives a darker colour to the pulp. However, the use of enzyme alone has significant impact in the kappa number and HexA content reduction.
- The ECF sequences have a higher positive impact on brightness, viscosity, kappa number and HexA content, than the TCF sequences.
- In TCF sequences, like LE_pLP and ALE_pLP, the introduction of the A stage in the first stage demonstrate a reduction in HexA and kappa number, but in terms of bleaching the improvements were small.
- In ECF sequences, like DE_pDP and ADE_pDP, the introduction of A stage in the first stage demonstrates a slightly decrease in brightness and viscosity.
- The positioning of the A stage in the first or second stage does not influenced much the studied pulp parameters.
- The application of the X stage in the first stage improved the brightness and viscosity but the influence on kappa number and HexA was minimal.
- The COD was slightly higher for sequences that begin with the X stage instead of the A stage.
- The A stage promotes more the HexA reduction than the X stage. In contrast, the X stage promotes more the kappa number reduction and the enhancement of the pulp brightness and viscosity.
- The highest increase in brightness occurs after the third stage, which is the combined effect of enzymatic treatment with the alkaline extraction with hydrogen peroxide or, in some

sequences, the effect of chlorine dioxide with the alkaline extraction with hydrogen peroxide.

In conclusion, the sequences that provide the highest ISO brightness were: XDE_pDP (91,1%), DE_pDP (90,5%), ADE_pDP (89,8%), XLE_pDP (89,5%) and LAE_pDP (89,4%). The sequences with lower kappa number were DE_pDP, XDE_pDP, ADE_pDP (around 1), followed by LAE_pDP (1,8) and ALE_pDP (2,2). Here the L represents the NS-51113 peroxidase.

Although ISO brightness is one of the best parameters to evaluate the bleaching performance it is also important other properties such as the colour reversion of the bleached pulps as well as the papermaking properties (physical, structural and optical), which were not performed in this work.

References

(1) Sixta, H.; Potthast, A.; Krotscheck, A. W. Chemical pulping processes. In *Handbook of pulp*; Sixta, H., Ed.; Walter de Gruyter GmbH & Co: Weinheim, 2006; pp. 109–510.

(2) Koch, G. Raw Material for Pulp. In *Handbook of pulp*; Sixta, H., Ed.; Wiley-VCH: Weinheim, 2006; pp. 21–68.

(3) Sjöström, E. Wood polysaccharides. In *Wood Chemistry: Fundamentals and Applications*; Sjöström, E., Ed.; Academic Press, 1981; pp. 49–67.

(4) Biermann, C. J. Wood and fiber fundamentals. In *Handbook of Pulping and Papermaking*; Biermann, C. J., Ed.; Academic Press, 1996; pp. 13–54.

(5) Teleman, A. Hemicelluloses and pectins. In *Wood Chemistry and Wood Biotechnology (Pulp and Paper Chemistry and Technology) V1*; Ek, M.; Gellerstedt, G.; Henriksson, G., Eds.; Walter de Gruyter GmbH & Co: Berlin, 2009; pp. 101–120.

(6) Henriksson, G. Lignin. In *Wood Chemistry and Wood Biotechnology (Pulp and Paper Chemistry and Technology) V1*; Ek, M.; Gellerstedt, G.; Henriksson, G., Eds.; Walter de Gruyter GmbH & Co: Berlin, 2009; pp. 121–146.

(7) Santos, R. B.; Hart, P.; Jameel, H.; Chang, H. Wood Based Lignin Reactions Important to the Biorefinery and Pulp and Paper Industries. *BioResources* **2013**, *8*, 1456–1477.

(8) Downloadable graphs to Chapter 13. http://www.balticuniv.uu.se/environmentalscience/ch13/chapter13_g.htm (accessed Sep 24, 2014).

(9) Brännvall, E. Wood handling. In *Wood Chemistry and Wood Biotechnology (Pulp and Paper Chemistry and Technology) V2*; Ek, M.; Gellerstedt, G.; Henriksson, G., Eds.; Walter de Gruyter GmbH & Co: Berlin, 2009; pp. 13–34.

(10) Ressel, J. B. Wood yard operations. In *Handbook of pulp*; Sixta, H., Ed.; Wiley-VCH: Weinheim, 2006; pp. 69–108.

(11) Brännvall, E. Overview of pulp and paper processes. In *Wood Chemistry and Wood Biotechnology (Pulp and Paper Chemistry and Technology) V2*; Ek, M.; Gellerstedt, G.; Henriksson, G., Eds.; Walter de Gruyter GmbH & Co: Berlin, 2009; pp. 1–12.

(12) Biermann, C. J. Pulping fundamentals. In *Handbook of Pulping and Papermaking*; Biermann, C. J., Ed.; Academic Press, 1996; pp. 55–100.

(13) Sjöström, E. Pulping chemistry. In *Wood Chemistry: Fundamentals and Applications*; Sjöström, E., Ed.; Academic Press, 1981; pp. 104–145.

(14) Tran, H.; Vakkilainnen, E. K. The kraft chemichal recovery process. http://www.tappi.org/content/events/08kros/manuscripts/1-1.pdf (accessed Sep 24, 2014).

(15) Sixta, H.; Süss, H.-U.; Potthast, A.; Schwanninger, M.; Krotscheck, A. W. Pulp bleaching. In *Handbook of pulp*; Sixta, H., Ed.; Walter de Gruyter GmbH & Co: Weinheim, 2006; pp. 609–932.

(16) Bajpai, P. General Background. In *Environmentally Benign Approaches for Pulp Bleaching*; Bajpai, P., Ed.; Elsevier, 2012; pp. 5–18.

(17) Biermann, C. J. Pulp bleaching. In *Handbook of Pulping and Papermaking*; Biermann, C. J., Ed.; Academic Press, 1996; pp. 123–136.

(18) Bajpai, P. ECF and TCF bleaching. In *Environmentally Benign Approaches for Pulp Bleaching*; Bajpai, P., Ed.; Elsevier, 2012; pp. 263–286.

(19) Bajpai, P. Oxygen delignification. In *Environmentally Benign Approaches for Pulp Bleaching*; Bajpai, P., Ed.; Elsevier, 2012; pp. 19–58.

(20) Teeri, T.; Henriksson, G. Bleaching of pulp. In *Wood Chemistry and Wood Biotechnology (Pulp and Paper Chemistry and Technology) V2*; Ek, M.; Gellerstedt, G.; Henriksson, G., Eds.; Walter de Gruyter GmbH & Co: Berlin, 2009; pp. 240–276.

(21) Bajpai, P. Chlorine Dioxide Bleaching. In *Environmentally Benign Approaches for Pulp Bleaching*; Bajpai, P., Ed.; Elsevier, 2012; pp. 135–166.

(22) Hart, P. W.; Rudie, A. W. Extraction and Oxidative Extraction. In *The bleaching of pulp*; Hart, P. W.; Rudie, A. W., Eds.; TAPPI Press, 2012.

(23) Bajpai, P. Hydrogen peroxide bleaching. In *Environmentally Benign Approaches for Pulp Bleaching*; Bajpai, P., Ed.; Elsevier, 2012; pp. 97–134.

(24) Lapierre, L.; Berry, R.; Bouchard, J. The Effects of the Order of Chemical Addition on the Peroxide Bleaching of an Oxygen-Delignified Softwood Kraft Pulp. *Holzforschung* **2000**, *54*.

(25) Bajpai, P. Enzymatic Pre-bleaching. In *Environmentally Benign Approaches for Pulp Bleaching*; Bajpai, P., Ed.; Elsevier, 2012; pp. 189–223.

(26) Viikari, L.; Kantelinen, A.; Sundquist, J.; Linko, M. Xylanases in bleaching: From an idea to the industry. *FEMS Microbiol. Rev.* **1994**, *13*, 335–350.

(27) Kunamneni, A.; Plou, F. J.; Ballesteros, A.; Alcalde, M. Laccases and their applications: a patent review. *Recent Pat. Biotechnol.* **2008**, *2*, 10–24.

(28) Widsten, P.; Kandelbauer, A. Laccase applications in the forest products industry: A review. *Enzyme Microb. Technol.* **2008**, *42*, 293–307.

(29) Barreca, A. M.; Sjögren, B.; Fabbrini, M.; Galli, C.; Gentili, P. Catalytic Efficiency of some Mediators in Laccase-Catalyzed Alcohol Oxidation. *Biocatal. Biotransformation* **2004**, *22*, 105–112.

(30) Rochefort, D.; Leech, D.; Bourbonnais, R. Electron transfer mediator systems for bleaching of paper pulp. *Green Chem.* **2004**, *6*, 14.

(31) Couto, S. R.; Toca-Herrera, J. L. Laccase production at reactor scale by filamentous fungi. *Biotechnol. Adv.* **2007**, *25*, 558–569.

(32) Bourbonnais, R., M.G. Paice, I. D. Reid, P. L. and M. Y. Lignin oxidation by laccase isozymes from T.versicolor and role of the mediator ABTS in kraft lignin depolymerization. *Appl. Environ. Microbiol* **1995**, *61*, 1876–1880.

(33) Claus, H.; Filip, Z. The evidence of a laccase-like enzyme activity in a Bacillus sphaericus strain. *Microbiol. Res.* **1997**, *152*, 209–216.

(34) Thurston, C. . The structure and function of fungal laccases. *Microbiology* **1994**, *140*, 19–26.

(35) Leonowicz A, Cho NS, Luterek J, Wilkolazka A, Wojtas-Wasilewska M, Matuszewska A, et al. Fungal laccase: properties and activity on lignin. *J Basic Microbiol* **2001**, *41*, 185–227.

(36) Bourbonnais, R.; Paice, M. G. Oxidation of non-phenolic substrates. *FEBS Lett.* **1990**, *267*, 99–102.

(37) Claus, H. Laccases: structure, reactions, distribution. *Micron* **2004**, *35*, 93–96.

(38) Bertrand, T., C. Jolivalt, P. Briozzo, J. N. Caminade, C. M. and C. M. Crystal structure of four-copper laccase complexed with an arylamide: insights into substrate recognition and correlation with kinetics. *Biochem.* **2002**, *41*, 7325–7333.

(39) Piontek, K., Antorini, M. and Choinowski, T. Crystal structure of a laccase from the fungus T.versicolor at 190 A Resolution containing a full complement of coppers. *J. Biol.Chem.* **2002**, *277*, 633–669.

(40) Kosa, M. Laccase Biobleaching Review. http://www.ipst.gatech.edu/faculty/ragauskas_art/technical_reviews/Laccasea.pdf (accessed Sep 27, 2014).

(41) Galli, C.; Gentili, P. Chemical messengers: mediated oxidations with the enzyme laccase. *J. Phys. Org. Chem.* **2004**, *17*, 973–977.

(42) Xu, F.; Kulys, J. J.; Duke, K.; Li, K.; Krikstopaitis, K.; Deussen, H. J.; Abbate, E.; Galinyte, V.; Schneider, P. Redox chemistry in laccase-catalyzed oxidation of N-hydroxy compounds. *Appl. Environ. Microbiol.* **2000**, *66*, 2052–2056.

(43) Astolfi, P.; Brandi, P.; Galli, C.; Gentili, P.; Gerini, M. F.; Greci, L.; Lanzalunga, O. New mediators for the enzyme laccase: mechanistic features and selectivity in the oxidation of non-phenolic substrates. *New J. Chem.* **2005**, *29*, 1308.

(44) Gutiérrez, A.; Rencoret, J.; Ibarra, D.; Molina, S.; Camarero, S.; Romero, J.; Del Río, J. C.; Martínez, A. T. Removal of lipophilic extractives from paper pulp by laccase and lignin-derived phenols as natural mediators. *Environ. Sci. Technol.* **2007**, *41*, 4124–4129.

(45) Nousiainen, P.; Maijala, P.; Hatakka, A.; Martínez, A. T.; Sipilä, J. Syringyl-type simple plant phenolics as mediating oxidants in laccase catalyzed degradation of lignocellulosic

materials: Model compound studies 10th EWLP, Stockholm, Sweden, August 25–28, 2008. *Holzforschung* **2009**, *63*.

(46) Bajpai, P. Enzymatic Pre-bleaching. In *Environmentally Benign Approaches for Pulp Bleaching*; Bajpai, P., Ed.; Elsevier, 2012; pp. 189–223.

(47) Ibarra, D.; Romero, J.; Martínez, M. J.; Martínez, A. T.; Camarero, S. Exploring the enzymatic parameters for optimal delignification of eucalypt pulp by laccase-mediator. *Enzyme Microb. Technol.* **2006**, *39*, 1319–1327.

(48) Hiraga, S. A Large Family of Class III Plant Peroxidases. *Plant Cell Physiol.* **2001**, *42*, 462–468.

(49) Fu, J.; Nyanhongo, G. S.; Gübitz, G. M.; Cavaco-Paulo, A.; Kim, S. Enzymatic colouration with laccase and peroxidases: Recent progress. *Biocatal. Biotransformation* **2012**, *30*, 125–140.

(50) D'Aix-Marseille, U. Cazy - Carbohydrate Active Enzymes Database. http://www.cazy.org/AA2.html (accessed Sep 24, 2014).

(51) Bajpai, P.; Bhardwaj, N. K.; Bajpai, P. K.; Jauhari, M. B. The impact of xylanases on bleaching of eucalyptus kraft pulp. *J. Biotechnol.* **1994**, *38*, 1–6.

(52) Teeri, T.; Henriksson, G. Enzymes degrading wood components. In *Wood Chemistry and Wood Biotechnology (Pulp and Paper Chemistry and Technology) V1*; Ek, M.; Gellerstedt, G.; Henriksson, G., Eds.; Walter de Gruyter GmbH & Co: Berlin, 2009; pp. 245–272.

(53) Chai, X.-S.; Junyong, Z.; Li, J. A simple and rapid method to determine hexeneuronic acid groups in chemical pulps. *J. Pulp Pap. Sci.* **2001**, *27*, 165–170.

(54) Jaouani, A.; Guillén, F.; Penninckx, M. J.; Martínez, A. T.; Martínez, M. J. Role of Pycnoporus coccineus laccase in the degradation of aromatic compounds in olive oil mill wastewater. *Enzyme Microb. Technol.* **2005**, *36*, 478–486.

(55) Rosado, T.; Bernardo, P.; Koci, K.; Coelho, A. V; Robalo, M. P.; Martins, L. O. Methyl syringate: an efficient phenolic mediator for bacterial and fungal laccases. *Bioresour. Technol.* **2012**, *124*, 371–378.

(56) Bourbonnais, R.; Leech, D.; Paice, M. G. Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochim. Biophys. Acta - Gen. Subj.* **1998**, *1379*, 381–390.

(57) Loureiro, P. E.; Santos, A.; Evtuguin, D. V; Carvalho, M. The Influence of ECF Bleaching History on the Performance of a Final Hydrogen Peroxide Stage. **2011**, *64*, 83.

(58) Brogdon, B. N. A Fundamental Reveiw and Critical Analysis of Hexenuronic Acids and Their Impact in Elemental Chlorine-Free Bleaching. In *TAPPI Engineering, Pulping, Environmental Conference*; 2009.

(59) Bajpai, P.; Anand, A.; Sharma, N.; Mishra, S. P.; Bajpai, P. K.; Lachenal, D. Enzymes improve ECF bleaching of pulp. *BioResources* **2007**, *1*, 34–44.

(60) Lundquist, K.; Kristersson, P. Exhaustive laccase-catalysed oxidation of a lignin model compound (vanillyl glycol) produces methanol and polymeric quinoid products. *Biochem. J.* **1985**, *229*, 277–279.

Attachment A:

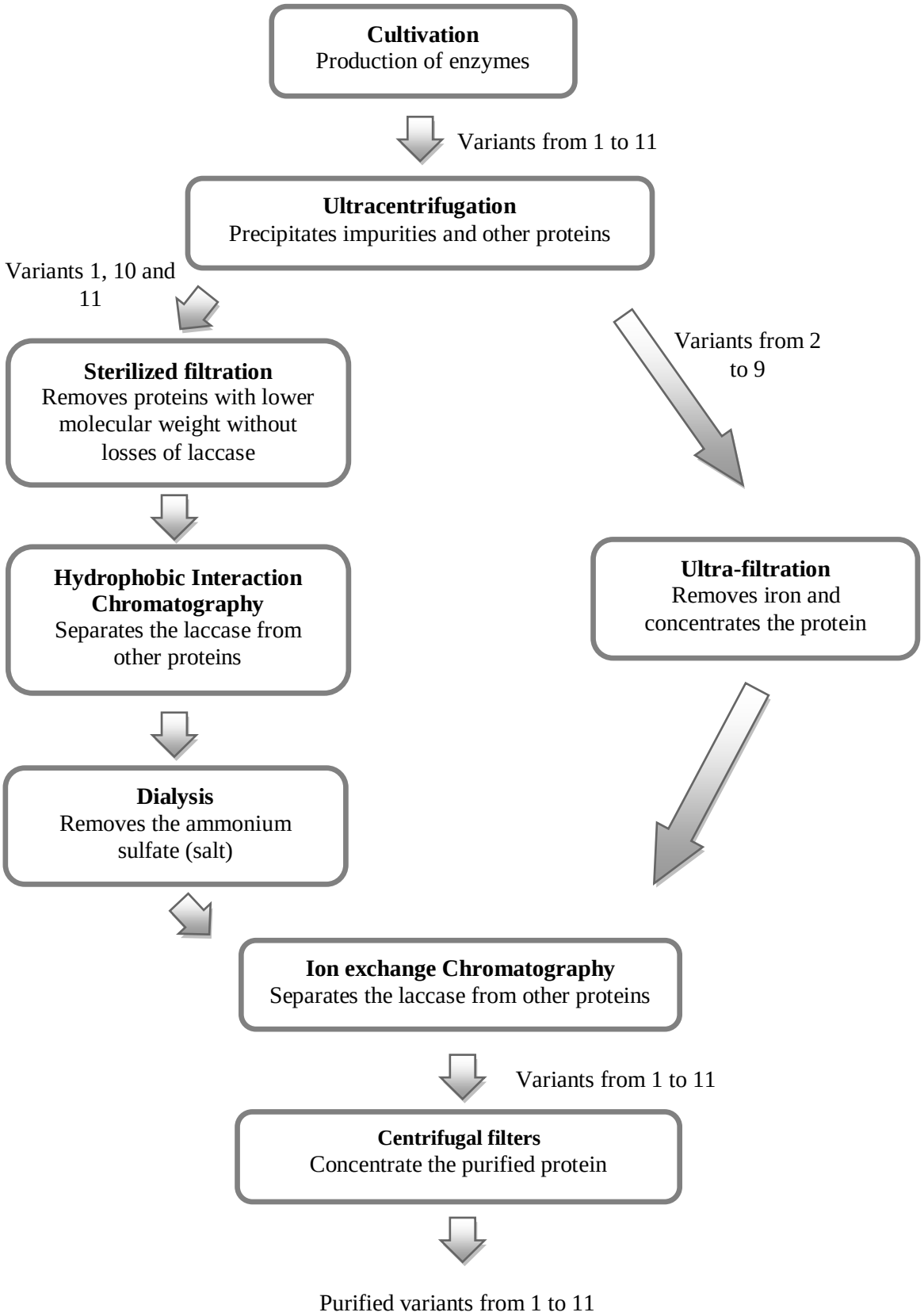


Figure 33 - Scheme of the laccase variants purification.

Attachment B:

The spectrum for crude and purified variants are present in Figure 34, for pathways of 0,1 and 1,0 mm, which can help the analysis of the results from 3.2.

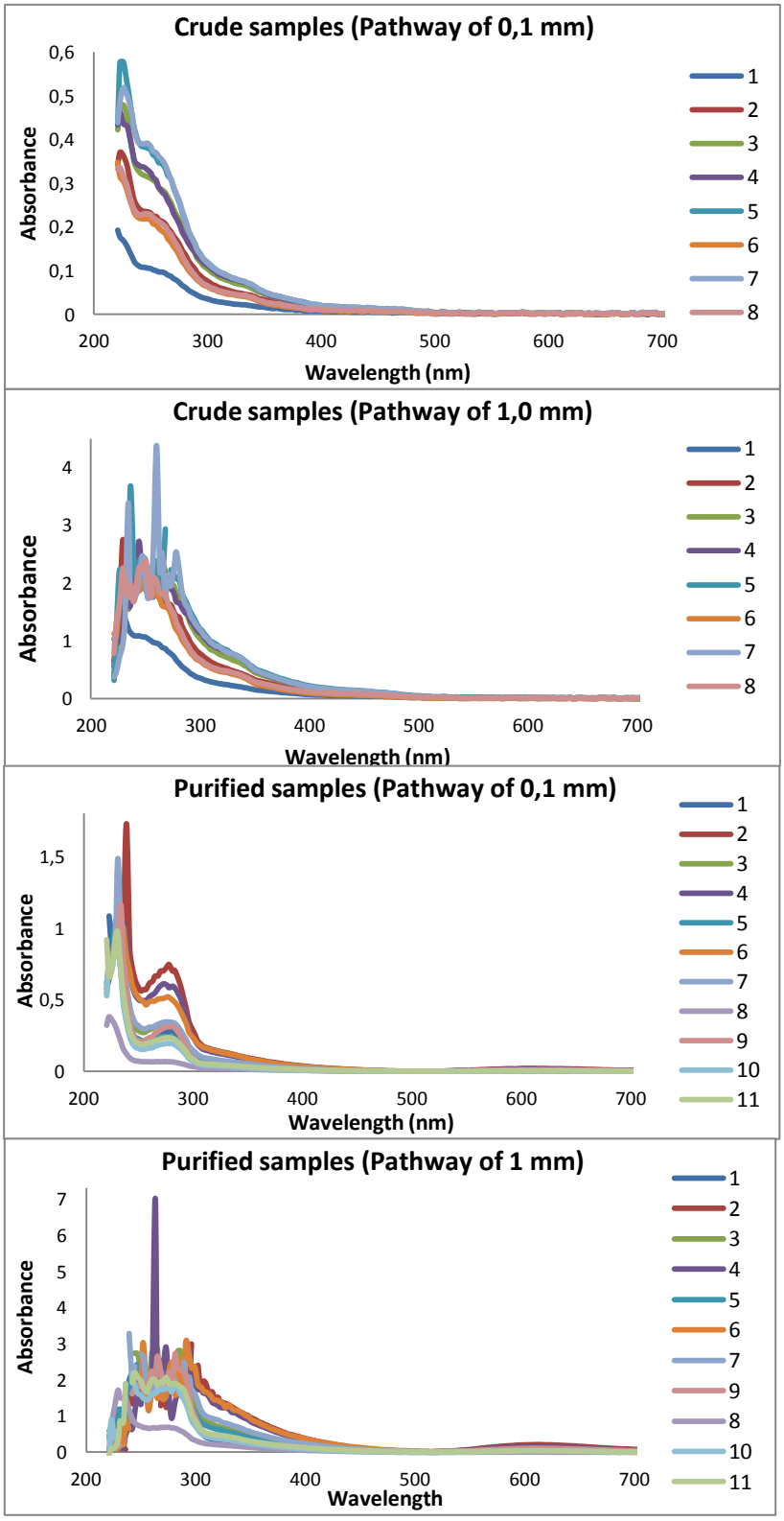


Figure 34 –Spectrum for crude and purified laccase variants, for a pathway of 0,1 and 1,0 mm.

Attachment C:

Some information about the mediators used in the screening with dye (3.3) and with pulp (3,5 and 3,6) are represented in Figure 35.

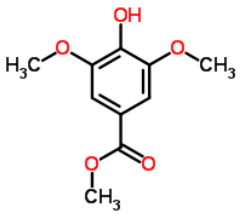
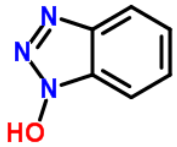
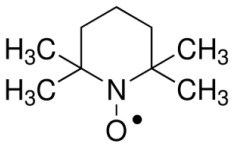
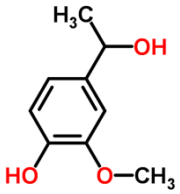
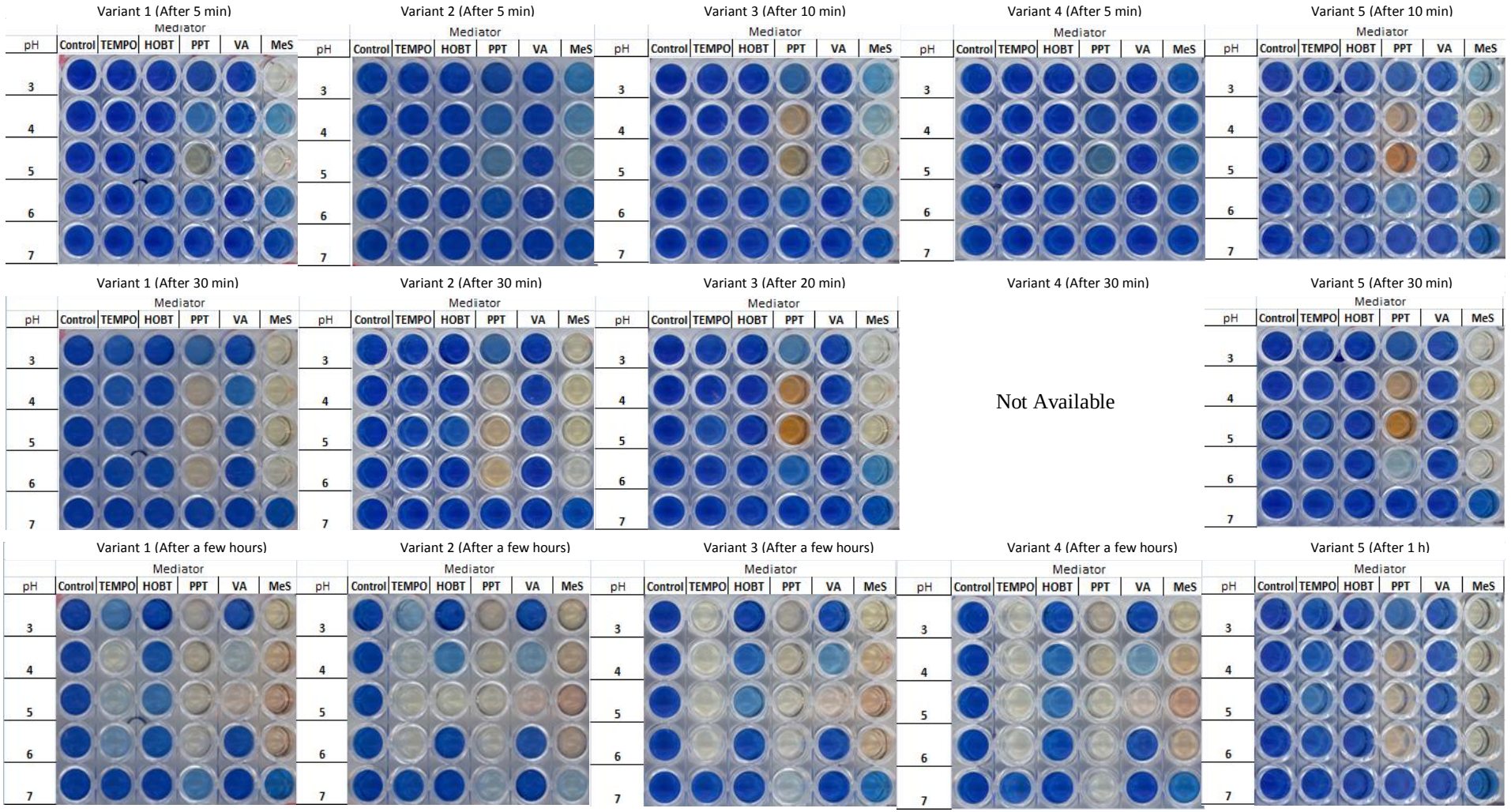
Mediators			
MeS Methyl syringate $C_{10}H_{12}O_5$ M_w = 212,204		**HOBT** 1-hydroxybenzotriazole $C_6H_5N_3O$ M_w = 135,12	
VA Violuric acid $C_4H_3N_3O_4$ M_w = 175,1		**TEMPO** (2,2,6,6-tetramethylpiperidin-1-yl)oxy $C_9H_{18}NO$ M_w = 156,25	
PPT Phenothiazine-10-propionic acid $C_{15}H_{13}NO_2S$ M_w = 271,3		**Apocynol** $C_9H_{12}O_3$ M_w = 168,19	

Figure 35 - Mediators used in the screening assays along with some info.

Attachment D:

The scans of the MTP from the pre-screening study of the NS-51002 laccase variants with indigo carmine are present in Figure 36, for different times after the kinetic analysis in the spectrophotometer.



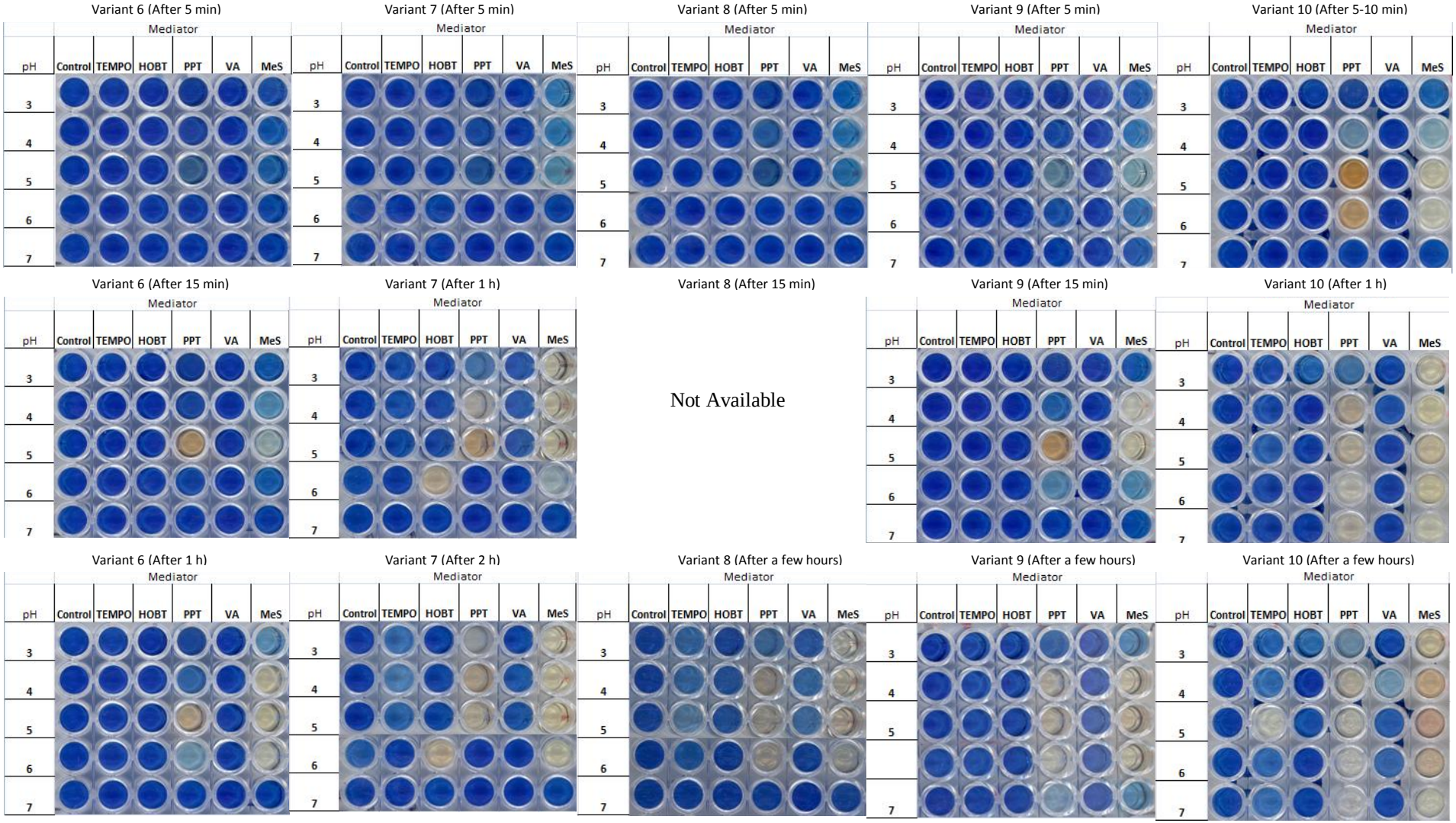


Figure 36 – Scans of the MTP, for each NS-51002 laccase variant, a few minutes and a few hours after the kinetic analysis in the spectrophotometer.

Attachment E:

In Figure 37 is represented the brightness results for each LMS study at 10% consistency. The best results for each LMS were used to build the Figure 17.

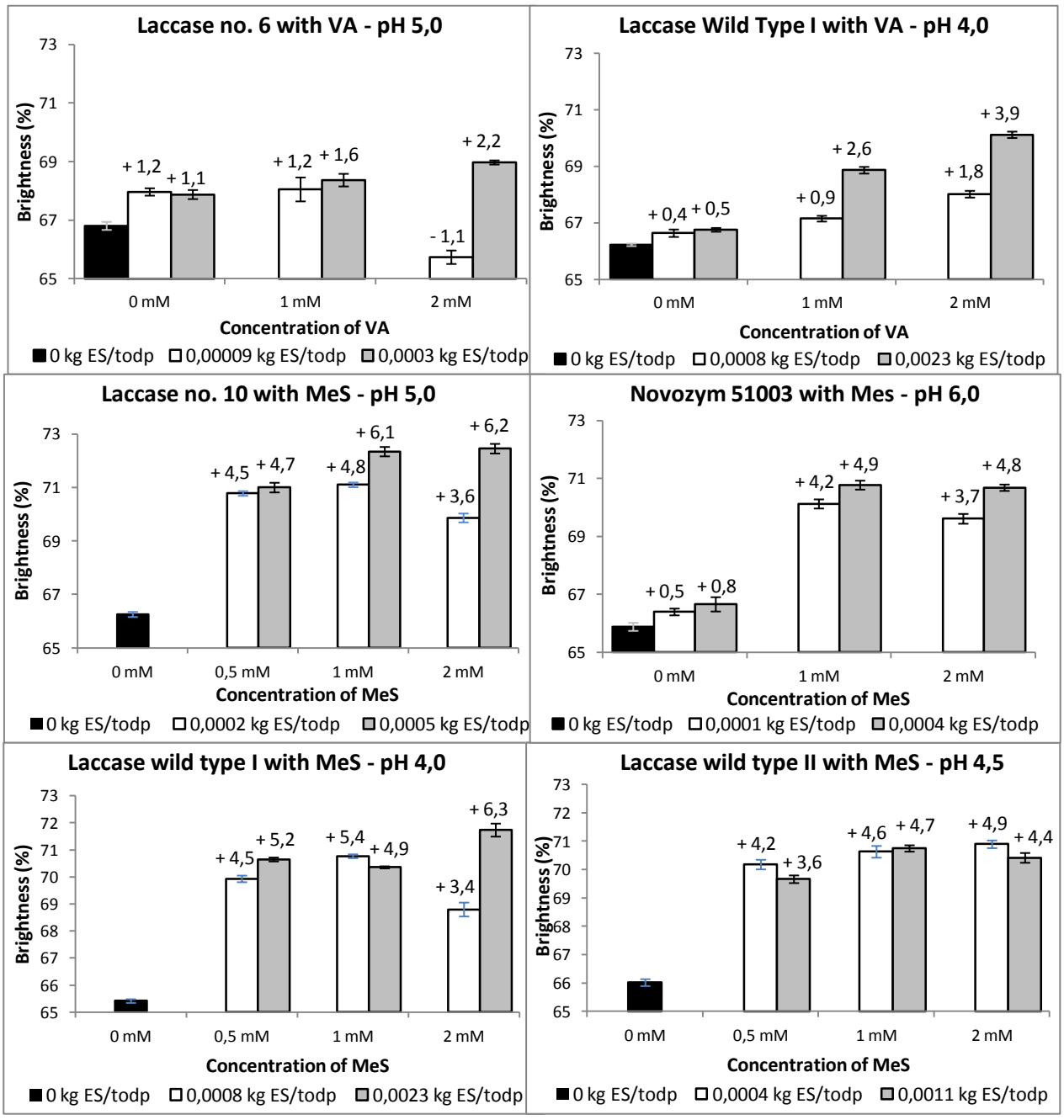


Figure 37 – Enzyme and mediator concentration screening for different LMS, performed at 50ºC, during 3h and using pulp 1.

Attachment F:

In Figure 38 is represented the temperature profile for both peroxidases studied in this work. This data were collected by scientist from the Forest Products Department of Novozymes.

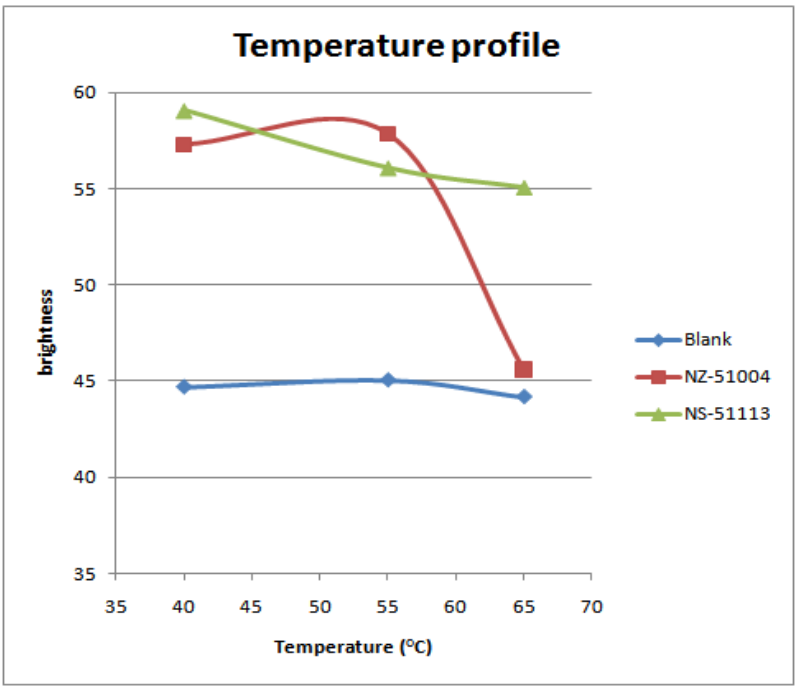


Figure 38 – Temperature profile for the NS-51113 and the NS-51004 peroxidases, performed at 1,5% consistency by other scientists in the Forest Products Department.

Attachment G:

The Figure 39 shows the absorbance results for the E_p stage optimization performed at 1,5% consistency. These results provided the basis for the E_p stage optimization performed at 10% consistency present in 3.6.2.

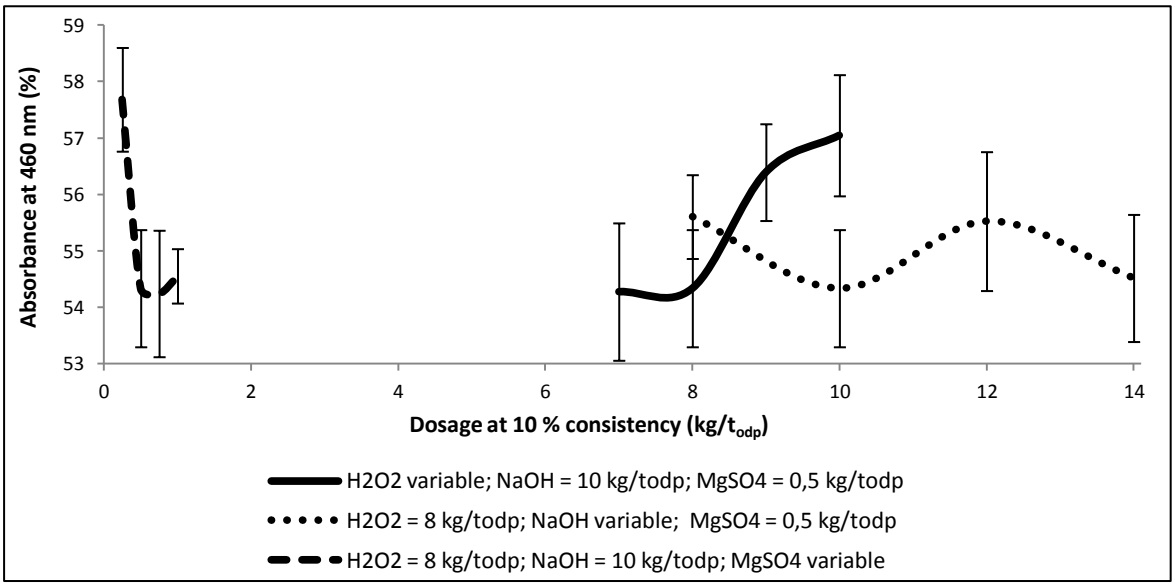


Figure 39 –E_p stage optimization absorbance results, using different dosages of chemicals, performed at 1,5% consistency, 85ºC, for 1,5 h and using pulp 1.

In Figure 39 there is a tendency that shows that high dosages of H_2O_2 and small dosages of $MgSO_4$ improve the brightening. In turn, the line correspondent to the dosage of NaOH is inconclusive due to the errors of the low scale procedure.

Attachment H:

Table 13 – Pulp and filtrate properties measured after each stage of the different sequences.

Stage	pH	H_2O_2 residual (mg/L)	COD (mg/L)	ISO Brightness (%)	St. dev. (%)	Kappa	Kappa error	HexA (mmol/kg odp)	St. dev. (mmol/kg odp)	Viscosity (cP)
Lac. control	--	--	--	53,68	0,22	8,91	0,13	49,81	0	--
Per- control	--	--	--	54,12	0,07	9,05	0,09	49,57	0	--
Raw pulp	--	--	--	53,45	0,20	12,45	0,30	62,94	0	9,92

Stage	pH	H_2O_2 residual (mg/L)	COD (mg/L)	ISO Brightness (%)	St. dev. (%)	Kappa	Kappa error	HexA (mmol/kg odp)	St. dev. (mmol/kg odp)	Viscosity (cP)
L - Lac.	5,3	--	--	51,31	0,15	8,91	0,02	49,49	0,00	--
E_p	11,1	60	--	70,30	0,38	--	--	--	--	--
L - Lac.	5,2	--	--	--	--	--	--	--	--	--
P	11,1	500	--	79,92	0,06	6,23	0,03	46,67	0,00	--

L - Lac.	5,3	--	--	51,31	0,15	8,91	0,02	49,49	0,00	--
A	3,4	--	--	52,56	0,31	--	--	--	--	--
E_p	10,4	0,5	--	70,02	0,19	5,04	0,02	21,24	0,00	--
L - Lac.	5,2	--	--	--	--	--	--	--	--	--
P	10,9	500	--	79,95	0,11	3,85	0,03	24,05	0,55	7,45

L - Lac.	5,3	--	--	51,31	0,15	8,91	0,02	49,49	0,00	--
A	3,2	--	--	52,56	0,31	--	--	--	--	--
E_p	10,8	10	--	70,02	0,19	5,04	0,02	21,24	0,00	--
D	2,5	--	--	--	--	--	--	--	--	--
P	10,7	800	--	87,10	0,18	2,21	0,06	13,26	3,77	7,57

Stage	pH	H_2O_2 residual (mg/L)	COD (mg/L)	ISO Brightness (%)	St. dev. (%)	Kappa	Kappa error	HexA (mmol/kg odp)	St. dev. (mmol/kg odp)	Viscosity (cP)
L - Per.	4,6	3	--	50,34	0,18	8,23	0,09	49,57	0,00	--
E_p	10,7	1	--	74,07	0,15	--	--	--	--	--
L - Per.	4,8	0	--	--	--	--	--	--	--	--
P	10,9	500	435	84,56	0,12	5,28	0,26	35,28	6,12	--

L - Per.	4,6	3	--	50,34	0,18	8,23	0,09	49,57	0,00	--
A	3,3	--	--	54,73	0,09	--	--	--	--	--
E_p	10,7	5	--	74,88	0,06	3,85	0,22	18,25	0,00	--
L - Per.	4,8	100-150	--	73,24	0,15	--	--	--	--	--
P	11,1	500	467	85,42	0,13	3,02	0,13	18,02	4,23	7,33

L - Per.	4,6	3	--	50,34	0,18	8,23	0,09	49,57	0,00	--
A	3,3	--	--	54,73	0,09	--	--	--	--	--
E_p	10,8	20	--	74,88	0,06	3,85	0,22	18,25	0,00	--
D	2,6	--	--	83,83	0,11	--	--	--	--	--
P	10,8	800	--	89,44	0,09	1,77	0,03	15,40	7,13	7,27

D	2,2	--	--	72,86	0,02	2,27	0,29	--	--	--
E_p	10,8	300	--	85,68	0,07	1,59	0,11	10,38	0,00	--
D	2,8	--	--	89,06	0,07	--	--	--	--	--
P	11,0	300	250	90,55	0,05	1,13	0,07	6,52	0,37	8,10

Stage	pH	H_2O_2 residual (mg/L)	COD (mg/L)	ISO Brightness (%)	St. dev. (%)	Kappa	Kappa error	HexA (mmol/kg odp)	St. dev. (mmol/kg odp)	Viscosity (cP)
A	3,3	--	--	54,47	0,30	6,77	0,45	28,28	0,00	--
L - Lac.	4,9	--	--	--	--	--	--	--	--	--
E_p	11,0	50	--	72,03	0,11	4,82	0,58	22,30	0,00	--
D	2,5	--	--	--	--	--	--	--	--	--
P	10,4	500	981	87,16	0,09	2,37	0,23	16,14	2,19	7,66

A	3,3	--	--	54,47	0,30	6,77	0,45	28,28	0,00	--
L - Lac.	4,9	--	--	--	--	--	--	--	--	--
E_p	11,0	50	--	72,03	0,11	4,82	0,58	22,30	0,00	--
L - Lac.	5,3	--	--	--	--	--	--	--	--	--
P	11,7	0	--	74,94	0,14	5,49	0,18	36,19	5,48	8,25

A	3,3	--	--	54,47	0,30	6,77	0,45	28,28	0,00	--
Control	4,9	--	--	--	--	--	--	--	--	--
E_p	10,2	50	--	67,61	0,19	--	--	--	--	--
Control	5,2	--	--	--	--	--	--	--	--	--
P	10,9	650	--	72,95	0,15	5,07	0,16	20,26	6,11	8,43

A	3,3	--	--	54,47	0,30	6,77	0,45	28,28	0,00	--
L - Per.	4,5	15	--	--	--	--	--	--	--	--
E_p	10,9	20	--	76,49	0,13	4,15	0,12	23,72	0,00	--
D	2,5	--	--	--	--	--	--	--	--	--
P	10,7	800	299	88,57	0,07	2,19	0,10	12,61	0,91	7,45

Stage	pH	H_2O_2 residual (mg/L)	COD (mg/L)	ISO Brightness (%)	St. dev. (%)	Kappa	Kappa error	HexA (mmol/kg odp)	St. dev. (mmol/kg odp)	Viscosity (cP)
A	3,3	--	--	54,47	0,30	6,77	0,45	28,28	0,00	--
L - Per.	4,5	25	--	--	--	--	--	--	--	--
E_p	10,9	20	--	76,49	0,13	4,15	0,12	22,80	0,00	--
L - Per.	4,8	40	--	--	--	--	--	--	--	--
P	10,9	800	--	85,07	0,12	3,28	0,27	19,88	2,77	7,48

A	3,3	--	--	54,47	0,30	6,77	0,45	28,28	0,00	--
Control	4,5	--	--	--	--	--	--	--	--	--
E_p	11,2	150	--	66,66	0,11	--	--	--	--	--
Control	5,0	--	--	--	--	--	--	--	--	--
P	11,0	800	313	71,95	0,09	4,89	0,51	22,56	1,21	7,39

A	3,3	--	--	54,47	0,30	6,77	0,45	28,28	0,00	--
D	2,5	--	--	--	--	--	--	--	--	--
E_p	10,8	300	--	85,82	0,03	--	--	--	--	--
D	2,4	--	--	88,01	0,12	--	--	--	--	--
P	11,0	500	219	89,84	0,09	2,56	1,23	47,64	4,64	7,96

X	7,7	--	--	56,32	0,15	8,52	0,62	41,55	0,00	--
L - Lac.	5,2	--	--	--	--	--	--	--	--	--
E_p	11,0	50	--	74,20	0,11	5,94	0,70	39,51	0,00	--
D	2,5	--	--	80,03	0,35	--	--	--	--	--
P	10,4	400	1045	87,45	0,07	3,59	0,33	22,66	3,40	7,54

Stage	pH	H_2O_2 residual (mg/L)	COD (mg/L)	ISO Brightness (%)	St. dev. (%)	Kappa	Kappa error	HexA (mmol/kg odp)	St. dev. (mmol/kg odp)	Viscosity (cP)
X	7,7	--	--	56,32	0,15	8,52	0,62	41,55	0,00	--
L - Lac.	5,2	--	--	--	--	--	--	--	--	--
E_p	11,0	50	--	74,20	0,11	5,94	0,70	39,51	0,00	--
L-PpL	5,3	--	--	68,46	0,13	--	--	--	--	--
P	10,7	800	1510	80,84	0,04	3,97	0,00	23,69	1,87	7,42

X	7,7	--	--	56,32	0,15	8,52	0,62	41,55	0,00	--
Control	5,2	--	--	--	--	--	--	--	--	--
E_p	11,4	150	--	68,69	0,08	--	--	--	--	--
Control	--	--	--	--	--	--	--	--	--	--
P	11,1	500	--	75,28	0,07	5,74	0,40	25,03	3,59	--

X	7,7	--	--	56,32	0,15	8,52	0,62	41,55	0,00	--
L - Per.	4,7	5	--	--	--	--	--	--	--	--
E_p	10,5	0,5	--	78,03	0,10	5,64	0,03	38,61	0,00	--
D	2,9	--	--	84,56	0,16	--	--	--	--	--
P	10,1	800	360	89,51	0,07	3,30	0,16	22,28	1,27	7,57

X	7,7	--	--	56,32	0,15	8,52	0,62	41,55	0,00	--
L - Per.	4,7	5	--	--	--	--	--	--	--	--
E_p	10,5	0,5	--	78,03	0,10	5,64	0,03	38,61	0,00	--
L - Per.	4,7	30	--	74,93	0,09	--	--	--	--	--
P	10,7	220	--	86,00	0,13	4,41	0,27	31,28	1,88	8,13

Stage	pH	H_2O_2 residual (mg/L)	COD (mg/L)	ISO Brightness (%)	St. dev. (%)	Kappa	Kappa error	HexA (mmol/kg odp)	St. dev. (mmol/kg odp)	Viscosity (cP)
X	7,7	--	--	56,32	0,15	8,52	0,62	41,55	0,00	--
Control	--	--	--	--	--	--	--	--	--	--
E_p	11,2	90	--	69,81	0,10	--	--	--	--	--
Control	--	--	--	--	--	--	--	--	--	--
P	10,8	300	--	75,64	0,08	5,84	0,48	30,64	1,98	--

X	7,7	--	--	56,32	0,15	8,52	0,62	41,55	0,00	--
D	2,9	--	--	--	--	--	--	--	--	--
E_p	10,7	300	--	87,03	0,05	1,97	0,22	12,85	0,00	--
D	2,8	--	--	89,63	0,14	--	--	--	--	--
P	11,0	300	272	91,10	0,14	1,43	0,32	9,96	0,48	8,55