BIOVALORIZATION OF TECHNICAL LIGNINS FOR ADDED-VALUE PRODUCTS AND APPLICATIONS

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ABSTRACT: Lignin is an abundant non-toxic amorphous natural polymer. Nowadays it is available a great variety and large amounts of technical lignins as by-products from the pulp and paper industries. Some successful biotechnological applications of enzymatically modified lignins are described in the literature, namely for the production of lignin based copolymers, binders for wood composites, chelating agents, compositions for treating porous materials, coatings, paintings and others. From a new species from *Bjerkandera genus* which exhibits high decolourisation activity on Poly R-478 and Remazol Brilliant Blue R (RBBR) dyes, was isolated, purified and identified the main enzyme responsible for Remazol Brilliant Blue R dye decolourisation. Such an enzyme is able to oxidise manganese, as well as VA and DMP in manganese-independent reactions; the enzyme substrate range for oxidation of several phenolic and non-phenolic aromatic compounds was determined. This enzyme was tested for transformation of a lignin fraction obtained from straw pulping. Characterisation by gel filtration chromatography of the evolution of the molecular mass distribution of the lignin fragments generated by said enzyme indicated that this enzyme is able to interact directly with lignin in the absence of other mediators.
Keywords: bio-materials, enzymes, lignin

1 INTRODUCTION

Prevention, reduction and elimination, as far as possible, of environmental contamination stands out as a major issue on the agenda for the 21st century. In addition, it is steadily increasing the consumers' demand for products complying with consumers' health, safety and environmental requirements. As a result, it is occurring worldwide intensive R&D aiming cleaner industrial practices, more eco-friendly products, a higher industrial usage of renewable raw materials and, finally, to improve the technologies for by-products up-grading, contributing for wastes minimisation.

Technical lignins are by-products from the pulp and paper industry, as well as from other biomass-based industries. They possess highly reactive locations that can be surprisingly modified through a selection of chemical, physical and/or enzymatic reactions, which gives them a great potential for their exploitation as industrial raw materials.

A particularly promising solution for up-grading technical lignins is through Biotechnology, and extensive research efforts have been dedicated to produce added-value products presenting higher quality and biodegradability.

This paper aims to provide a general picture of the variety of the new products that were already developed through enzyme-based technologies and using technical lignins as raw materials. In addition, lignin modification by a novel ligninolytic enzyme (a versatile peroxidase) isolated from a new *Bjerkandera (Bjerkandera paranensis)* is here described. Results show the high potential of this new enzyme for industrial applications, in order to develop new and environmental friendly products.

2 TECHNICAL LIGNINS

The cheapest and most abundant source of technical lignins are the materials derived from leftovers in pulp and paper processes. Their macromolecular structure present a high heterogeneity, which is caused by variations in lignin composition, size, cross-linking and functional groups due to differences in raw material, pulping and isolation conditions [1]. For example, the material derived from the sulphite and the kraft processes [respectively, lignosulphonates (LS) and kraft lignin] present sulphur-containing groups, such as thiols and sulphuric acids.

But a large variety of other industrial lignin types are available nowadays. This is mainly due to the implementation of new pulping sequences, to the use of other non-woody pulp raw materials, and also to the recent technologies that use lignocellulosics for other proposals (e.g. ethanol production by steam-explosion).

There is still a very small market for the large quantities of technical lignins produced (only about 2% of the lignins available from the pulp and paper industry are commercialised), being the rest of it burned to generate energy and to recover chemicals. The existing markets are either for very low value products (LS mainly in dispersing and binding applications) or limited to very narrow market segments (high quality dispersants from chemically modified kraft lignin) [2]. Thus, there is lack of a wider range of value-added applications, which is mainly caused by the low-purity standards, heterogeneity, smell and colour problems of the existing commercial lignins [1, 2]. But, the great efforts that are actually being done to develop new techniques for lignin modification and to increase the sensitiveness of the analytical methods used to follow-up those modifications, will soon give rise to technically more useful materials and will certainly contribute to turnover this situation.

3 LIGNIN MODIFICATION BY ENZYMES

Enzymes bring undoubting advantages to industrial processes, justifying the intensive research that has been done in this field for the last three decades.

For those technical processes requiring modifications on the lignin structure, research has focused on the oxidative enzymes produced by ligninolytic fungi.

In Nature, the initial steps of lignin biodegradation consist in introducing new functional groups into its macromolecular structure by oxidative enzymes, rendering lignin more susceptible towards its subsequent degradation by other enzymes. There are a wide variety of fungi and bacteria that can degrade lignin through a battery of different enzymes, but the so-called white-rot fungi (WRF) are the only known organisms that can completely break down the lignin molecule to CO_2 and H_2O (see Table I). They owe their name to the specific bleaching process that occurs during the fungal degradation of wood [3].

Table I: Lignin-degrading organisms [3]

Organism	Subdivision	Lignin degradation	Genera (e.g.)
WRF[1]	Basidiomycotina (Ascomycotina)	Lignin mineralization selective or non-selective delignification	*Phanerochaete* *Phlebia* *Trametes*
BRF[2] SRF[3]	Basidiomycotina Ascomycotina or Deuteromycotina	Lignin modification Limited lignin degradation	*Poria* *Polyporus* *Chaetomium* *Paecilomyces* *Fusarium*
Bacteria	Actinomycetes or Myxobacteroa	Limited lignin degradation	*Streptomyces* *Nocardia* *Pseudomonas*

[1]White-rot fungi; [2]Brown-rot fungi; [3]Soft-rot fungi

Different WRF possess different lignin-degrading systems, comprising extracellular enzymes like the lignin oxidative enzymes and the H_2O_2-generating enzymes, and low molecular weight cofactors (e.g. oxalate, veratryl alcohol, Mn^{2+}, etc.).

The main extracellular enzymes participating in lignin degradation are the heme-containing lignin peroxidase (ligninase, LiP, E.C. 1.11.1.14) and manganese peroxidase (MnP, E.C. 1.11.1.13) and the Cu-containing laccase (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2). A new group of ligninolytic heme-containing peroxidases, combining structural and functional properties of the LiPs and MnPs, are the versatile peroxidases (VPs). The enzymes involved in H_2O_2 production, such as glyoxal oxidase and aryl alcohol oxidase (EC 1.1.3.7), are also considered to belong to the ligninolytic system [4].

The yielding of free radical species as a result of peroxidases and laccases activity is of utmost importance from the industrial point of view. These radicals greatly increase the reactivity of the lignin molecules, leading to further polymerisations in a random non-enzymatic way to form 3-dimensional polymers of higher Mw and with a variety of new linkages. As a result, a wide variety of new materials with distinct properties can be obtained.

4 LIGNIN MODIFICATION BY A VERSATILE PEROXIDASE OF A NEW *BJERKANDERA* STRAIN

The above referred novel class of ligninolytic peroxidases — the versatile peroxidases (VPs), has high affinity for manganese and dyes, but is also able to oxidise 2,6-dimethoxyphenol (DMP) and veratryl alcohol (VA) in a manganese-independent reaction [5]. Unlike MnPs, that are only able to oxidise phenolic substrates in the presence manganese, VPs are able to oxidise such compounds both in manganese dependent and independent reactions.

VPs raised a great interest in the biotechnology field, because they do not seem to require any mediators for substrate oxidation. In fact, most studies to date pertaining to versatile enzymes have focused on characterisation of their oxidation ability towards several model compounds; however, little effort has been devoted to demonstrate the activity of the members of this enzyme group on what is thought to be one of its natural substrates, i.e. lignin.

A recent communication [6] has reported results of the first *in vitro* testing of a versatile peroxidase from *Bjerkandera* sp. strain BOS55, in biobleaching of an eucalyptus kraft pulp; those authors acknowledged a 6% reduction in the pulp kappa number, but no further assessement was carried out. The aformentioned study calls attention to the potential use of versatile peroxidases for applications in the pulp and paper industry, namelly for modification of lignins.

To date, VPs have only been isolated from *Pleurotus ostreatus, Pleurotus eryngii, Pleurotus pulmonarius, Bjerkandera adusta* and *Bjerkandera* sp. strain BOS55 [7, 8, 9, 10, 11, 12, 13, 14, 15, 16]. Their pH optima for oxidation of Mn(II) (i.e. pH 5.0) and aromatic compounds and dyes (i.e. pH 3.0) are quite different — but are similar to the optima of MnP and LiP activities. *Pleurotus eryngii* produces two VP isoenzymes (PS1 and MnPL1), which can be isolated from distinct culture media [8, 14]. PS1 presents similar activities on Mn(II), DMP and methoxy-*p*-hydroquinone, but lower than MnPL1 on VA, *p*-methoxybenzene and lignin model dimers — hence suggesting a lower redox potential [15]. The catalytic properties of *Pleurotus* and *Bjerkandera* sp. VPs are similar to each other[5].

During a selection program carried out at our laboratory aimed at isolating new potential lignin degraders, 106 fungal strains were isolated from several lignocellulosic materials, typified and tested for their ability to decolorize RBBR and Poly R 478 dyes. One of the most promising strains, named as B33/3, was tested and consequently identified to be a member of *Bjerkandera* genus. Further investigation provided a detailed characterization of B33/3 ligninolytic enzymes, leading to the description of a novel VP in genus *Bjerkandera* [16, 17]. Phenetic and phylogenetic studies carried out led to evidence of a new species in genus *Bjerkandera* and purposed as *Bjerkandera paranensis* sp. nov. [18].

Sulphur-free lignin obtained from a soda straw pulping process (Granit SA, Lausanne, Switzerland) was used as substrate for the biotransformation studies. Lignin in solution was made to react with the versatile peroxidase (RBP) from *Bjerkandera paranensis* sp. nov., in the absence of any external mediators. Gel filtration was used to follow the evolution in molecular mass distribution of lignin [19].

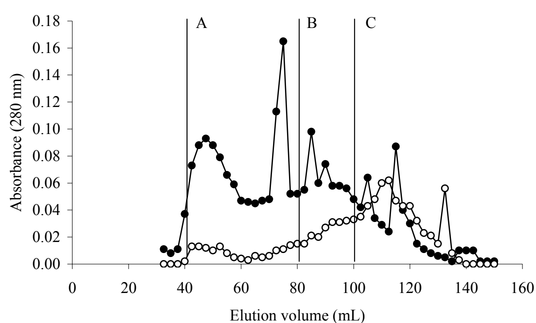
The reaction of lignin, as catalysed by RBP enzyme, was quantified *via* decrease in the true colour of samples

(20-fold diluted in 100 mM NaOH) — which was determined at 455 nm, using a DR/1020 colorimeter (Hach, Loveland, OH, USA) and according to manufacturer's instructions. The apparent reaction yield was calculated based on decolourisation of reaction samples, with reference to control samples. Reaction samples were selected following the experimental design methodology described elsewhere [20].

The molecular mass distribution of the soluble fragments of lignin (which is correlated with the true colour of the sample) was determined by gel filtration chromatography in a 40×2.5 cm Sephadex G-100 column (Sigma-Aldrich, St. Louis, MO, USA); elution proceeded at 0.5 $mL.min^{-1}$, using 100 mM NaOH (pH 13) as eluant; the effluent was monitored spectrophotometrically at 280 nm. The gel filtration system was calibrated with proteins of known molecular mass (Bio-Rad, Hercules, CA, USA). Aliquotes (1 mL) of 2-fold dilutions of filtered samples were injected directly into the column.

The results of gel filtration chromatography of the lignin solution, obtained from the set of experimental conditions that yield the higher decolourisation extent, as well as a control sample, are presented in Fig. 1.

Figure 1 Gel permeation chromatogram of samples obtained from the control (pH 4.0) (●) and from run 6 (○), for decolourisation reactions initiated with peroxidase/H_2O_2 systems. A — excluded lignin fragments: above 158 kDa; B — separated lignin fragments: between 158 and 44 kDa; C — separated lignin fragments: below 44 kDa.



From the analysis of the chromatogram it is possible to distinguish a notorious change in the molecular weight distribution of soluble lignin; a strong indication that these changes are dependent of enzymatic reactions (in optimal conditions).

5 ADDED-VALUE APPLICATIONS OF ENZYME MODIFIED LIGNINS

So far, only a small portion of the available technical lignins are used for the industrial production of added-value products: oil well and concrete additives, driling muds, viscosity control agents, dyestuff dispersants, agricultural chemicals, animal feed, other industrial binders, and even as heavy metal adsorbents in water purification. Progress has recently been made using industrial lignins as feedstock for novel chemicals production.

However, there is a considerable variety of new and eco-friendly products that have recently been produced through enzyme-based technologies and using industrial lignins as raw materials, namely for the production of lignin based copolymers by grafting, binders for wood composites, chelating agents, compositions for treating porous materials, coatings and paintings.

The evolution of the molecular mass distribution of the lignin fragments generated by the versatile peroxidase of *Bjerkandera paranensis* here described, indicate that this enzyme is able to interact directly with lignin in the absence of other mediators. The identification of these products and its use as natural binders, among others applications, is understudy. Scale-up of these new biotechnological applications is still limited. It is necessary to clarify the exact mechanisms of the enzymatic synthesis of these products, which will allow to increase production yields, to improve the existing processes for scale-up and to enhance product qualities.

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