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54 **Bleaching wood pulp with enzymes.**

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JOURNAL OF BIOTECHNOLOGY, vol. 8, no. 2,
1988, AMSTERDAM/NL, pp. 97 - 112; JAN-
SHEKAR, H. et al.: "Cultivation of
Phanerochaete chrysosporium an produc-
tion of lignin peroxidases in submerged
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73 Proprietor: **SANDOZ LTD.**
Lichtstrasse 35
CH-4002 Basel (CH)

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73 Proprietor: **SANDOZ-PATENT-GMBH**
Humboldtstrasse 3
D-79539 Lörrach (DE)

84 Designated Contracting States:
DE

72 Inventor: **Gysin, Beat**
11 Lynton Gardens
Harrogate,
North Yorkshire HG1 4TE (GB)
Inventor: **Griessmann, Theophile**
22 rue de la Gare
F-68870 Bartenheim (FR)

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ABSTRACT BULLETIN OF THE INSTITUTE OF
PAPER CHEMISTRY, vol. 57, no.7, January
1987, APPLETON/US, p. 959; HUYNH, V.-B. et
al.: "Oxidation of lignin model compounds by
a Manganese-dependent enzyme from
Phanerochaete chrysosporium as compared
with chemically generated Mn(III)"

WORLD PATENTS INDEX LATEST Week 8744,
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AN87-310987

Description

This invention relates to a novel enzymatic composition for treating wood pulp, comprising at least one lignin-peroxidase derived from a fungus culture which is chemically modified so that it cannot be adsorbed onto the pulp. The invention further provides a process for bleaching wood pulp which comprises treating the pulp with such an enzymatic composition and it also provides a process for treating waste water with such a composition.

Wood is a complex material which is composed of cellulose, hemicellulose and lignin along with other minor components. The lignin is associated with and even covalently bound to a matrix of cellulose and hemicellulose. In paper making processes, lignin should be removed from the wood pulp since it reduces the strength, confers a brownish colour and imparts other undesirable characteristics to the finished product. Conventionally, wood chips are first treated with sodium sulphide (Na_2S) and sodium hydroxide (NaOH) to degrade the lignin substantially. This is called the sulphate or Kraft process. Alternatively other treatments may be of use e.g. the sulphite process. The pulps obtained therefrom are called "chemical pulps".

Chemical pulp e.g. Kraft pulp usually contains about 4-12% by weight of residual lignin which gives the pulp a characteristic brown colour. At this stage of delignification, the kappa number which reflects the lignin content of the pulp is usually from 10 to 45, more frequently from 12 to 30. To obtain a pulp of high brightness and brightness stability, the lignin content should be further reduced in one or more treatments or stages commonly referred to as bleaching. Many industrial bleaching processes already exist but almost all of them are divided into two main parts: A complementary delignification followed by a "true bleaching" for improving the brightness level. The complementary delignification typically starts with an oxygen stage or a chlorination-extraction step (C-E) stage or both. Chlorination and extraction are usually carried out in sequence, first forming chlorinated lignin compounds which are then solubilized in the subsequent extraction step. The objective is exclusively to delignify the pulp as very little brightening occurs at the C-E stage. A complementary process for brightening the lignin may further include the use of components other than chlorine such as chlorine containing chemicals e.g. hypochlorite and chlorine dioxide; or oxygen and hydrogen peroxide.

The effluents resulting from the complementary treatment (called E-1 effluents) contain a very large number of chlorinated organic compounds which are hazardous for the environment e.g. dioxines. Also, due to their highly corrosive nature, it is quite difficult to recycle the effluents. Thus, from the environmental point of view, it is clear that new techniques for bleaching which may reduce pollution are highly desirable.

In nature, there exist a number of microorganisms which delignify wood, and degrade and modify lignin. The enzymes involved in such a digestion belong to the classes of oxidases, peroxidases and hemicellulases. Thus, an enzymatic treatment may be usefully substituted for at least one of the chemical treatments involving chlorine compounds in pulp bleaching.

Lignin peroxidases (also called ligninases) and MnII-dependent peroxidase are enzymes of particular interest which are secreted by many microbial strains, especially filamentous fungi. *Phanerochaete chrysosporium* is a fungus which produces essentially both types of peroxidases. These enzymes are able to modify the lignin content of wood so that lignin is released from the hemicellulose matrix or made releasable upon washing or extraction.

However, the optimization of the experimental conditions in an enzymatic bleaching process has, however, not yet been achieved. This remains a major challenge since an enzymatic process must be able to compete with a chemical process on an industrial level.

Lignin peroxidases have been described up to now as enzymes which require the presence of H_2O_2 to be effective in degrading lignin with the optional presence of oxygen. In EP 345715 A1 it is claimed that this system works without the use of oxygen but in the presence of α -hydroxy acids and detergents. At the same time it is claimed that the peroxide needs to be produced *in situ* enzymatically. In DE 3636208 A1 it is claimed that certain oxidation and reducing agents have to be present and the redox potential has to be maintained at a certain level throughout the course of the reaction. The processes described in these two patents are commercially not feasible because of the high costs of the co-substrates needed. In a recent publication (Holzforschung 1989, 43(6), 375-384) it is shown that lignin peroxidases in the presence of hydrogen peroxide alone do not degrade lignin. In yet another publication (Enzyme Microbiol. Technol. 1985, 7(11), 564-566) it is shown that immobilized lignin peroxidases in combination with hydrogen peroxide alone do not delignify lignocellulosic material.

It has now been found that, surprisingly, very good results may be achieved in enzymatically delignifying wood pulp when treating the pulp with a lignin peroxidase when the enzymes are firstly chemically modified in such a way that they do not adsorb to the pulp.

Thus, the invention provides an enzymatic composition for treating wood pulp comprising at least one lignin peroxidase derived from a fungal culture, which lignin peroxidase is chemically modified so that it cannot be adsorbed onto pulp.

By "bleaching process" as used herein is meant a process for delignifying wood pulp or improving the
5 whiteness or brightness of wood pulp or both.

By "lignin" as used herein is meant not only natural, unmodified forms but also the forms as found in chemically treated pulps which are, in whole or in part, chemically modified by various agents such as those used in the Kraft, organosolv or sulphite pulping process and in the effluent of these processes.

The term "lignin-degrading enzyme" as used herein is meant to encompass peroxidases. Preferred
10 peroxidases are MnII-dependent peroxidases, and lignin peroxidases (also called ligninases). Lignin-degrading peroxidases are secreted by many microbial strains particularly filamentous fungi.

The term "lignin peroxidases" as used herein is meant to encompass the crude enzyme preparation produced by the fungus under ligninolytic conditions as well as the individual lignin peroxidase isoenzymes from natural or recombinant producers.

Of preferred use is the lignin peroxidase of a white-rot fungus e.g. *P. chrysosporium* either from its
15 native origin or in recombinant form. The recombinant form of a lignin peroxidase of *P. chrysosporium* may be obtained as described in PCT patent application No. WO-A-88/02023.

Strains of *P. chrysosporium* are publicly available and methods for culturing them in a N- or C-limited medium are already known. As an example, a suitable culture medium is the nitrogen-limited BIII/glucose
20 medium which contains 1.08×10^{-3} M ammonium tartrate, 1.47×10^{-2} M KH_2PO_4 , 2.03×10^{-3} M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.8×10^{-4} M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.96×10^{-6} M thiamine·HCl and $10 \text{ ml} \cdot \text{L}^{-1}$ of a trace element solution. The trace element solution contains 7.8×10^{-3} M nitrilo-acetic acid, 1.2×10^{-2} M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.7×10^{-2} M NaCl, 3.59×10^{-4} M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 7.75×10^{-4} M CoCl_2 , 9.0×10^{-4} M CaCl_2 , 3.48×10^{-4} M ZnSO_4 , 4×10^{-5} M $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2.1×10^{-5} M $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 1.6×10^{-4} M H_3BO_3 , 4.1×10^{-5} M
25 $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ and 2.9×10^{-3} M $\text{MnSO}_4 \cdot \text{H}_2\text{O}$.

For use in the process of the invention, the lignin-degrading enzyme is chemically modified by covalent or non-covalent linkage to water-soluble or insoluble polymeric compounds which prevent the enzyme from being adsorbed onto pulp during the treatment. Suitable polymeric compounds are for example, polyethyl-
30 ene glycol (PEG), polypropylene glycol (PPG), polyacrylamides and polymeric sugars of various degrees of polymerization and composition like CM-cellulose, cellulose, agarose, alginate and chitosan. PEG is a preferred polymeric compound.

Alternatively, the enzyme may be deglycosylated so that the carbohydrate residues which are usually involved in the mechanism of adsorption are at least partially removed. Deglycosylation may be performed by known methods, for example, by treating a sample of lignin-degrading enzyme with an enzyme such as
35 an endoglycosidase capable of degrading carbohydrate residues on a glycoprotein.

The composition of the invention may be produced by chemical modification of a crude extract, filtrate, or supernatant obtained from a fungal culture, preferably after concentration. Alternatively, the enzymes may be purified from a fungal material before any chemical treatment. It is particularly advantageous to use lignin peroxidases from a species or strain which does not produce cellulases especially when the enzyme
40 is not purified. Of preferred use is the lignin peroxidase of a white-rot fungus, e.g. *P. chrysosporium* as indicated above.

The process of the invention may be applied to a wide variety of wood pulps the residual lignin content of which is to be reduced. Unbleached wood pulps which may be treated with the process of the invention are advantageously mechanical pulps, e.g. groundwood pulp, including the thermomechanical pulps such as
45 thermomechanical pulps (TMP), chemimechanical pulps (CMP), chemithermomechanical pulps (CTMP) and chemical pulps (CP) such as sulphite and Kraft pulps, these latter being preferred.

The process of this invention is preferably carried out in the absence of added peroxide and in the presence of added oxygen.

As a general rule, the enzyme concentration may range from 0.001 to 1000 VAO units/g pulp (a VAO
50 unit is determined by the conversion of veratryl alcohol to veratryl aldehyde at $310 \text{ nm} = 9.3 \mu\text{mol} \cdot \text{cm}^{-1}$ at 30°C , pH 3.5), preferably from 0.1 to 50 VAO units/g pulp, more preferably from 1 to 20 VAO units/g pulp. Optimal enzyme concentration depends upon the commercial origin and type of pulp.

Wood pulp is advantageously submitted to alkaline extraction before being enzymatically treated. The enzymatic treatment is advantageously carried out at a pulp consistency of from 0.1 % to 15 %, preferably
55 of from 1 % to 5 %. The pulp consistency is determined by a standard procedure as the dry weight of pulp after drying for 2 to 10 hours at about 105°C . To reach an optimal pulp consistency the unbleached wood pulp may be diluted with deionized water, fresh water or tap water during the bleaching process. However, for economical reasons, fresh water or tap water is preferred since it has been found that the characteristics

of the water do not influence the final results. By "fresh water" is meant water pumped directly e.g. from lakes, ponds or rivers.

It is preferable to wash the pulp with an alkaline solution before the enzymatic treatment or to perform the enzymatic treatment after an alkaline stage e.g. oxygen bleaching stage or E-stage.

5 The period of time necessary for treating the pulp may greatly vary with respect to the quality of the substrate and the nature of the enzyme modification from a few minutes to several hours. Optimal temperature and pH conditions should be adapted to the particular enzyme of use. However, temperature is generally in the range from 20 to 50 °C, preferably from 40 to 50 °C. The pH of the system is usually in the range of from 2 to 5, preferably from 3 to 4. The reaction time is usually 30 to 60 minutes.

10 Following the enzymatic treatment, removal of the solubilized lignin from pulp may be carried out either by washing, filtration or by extraction, preferably by extraction. Suitable extractants include, for example, bases such as alkali metal hydroxides, dimethylformamide, dioxane, acetone and alcohol. A dilute aqueous sodium hydroxide extraction is generally preferred. A typical extraction step may be carried out at a pulp consistency from 1 to 20%, preferably from 1 to 5% at a temperature between 40 and 60 °C. The final pH is preferably from 10 to 11. Reaction time may be from 30 minutes to 3 hrs, preferably from 45 minutes to 2 hrs.

The extent of delignification of the pulp may be indicated by the Kappa number as measured in a standard method described in TAPPI Test Methods (Tappi, Atlanta, Ga.) Vol. 1, 1988 "Kappa number of pulp - T 236 cm 85". The Kappa number is the volume (in millilitres) of 0.1N potassium permanganate solution consumed by one gram of moisture-free pulp under the conditions specified in the above method. A lower Kappa number is desirable as it indicates that a smaller amount of lignin is present in the pulp.

Another similar process of particular interest involves also the treatment of aqueous waste water released from the pulping process of wood or from the bleaching process of wood pulp in order to further degrade the lignin component. A typical waste water which may be treated with a lignin peroxidase in the exclusive presence of oxygen as a co-substrate is the E1 effluent of the Kraft process.

The invention is further illustrated as follows:

Example 1

30 **Modification of Enzyme Preparation**

Crude lignin peroxidase from *Phanerochaete chrysosporium* was either produced according to published procedures (e.g. H. Janshekar, A. Fiechter; J. of Biotechnology 1988,8, 97-112) or purchased from Cultor Ltd., Helsinki; Finland.

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a) Modification with activated methoxypolyethylene glycol

1 ml of a crude lignin peroxidase preparation (activity: 110 VAO units; protein content 5 mg (Bradford et al., Anal. Biochem. 1976,72,248)) was diluted in 9 ml acetate buffer 50 mM. After adjusting the pH to 7.5, 1 g of cyanuric chloride activated methoxypolyethylene glycol (Sigma Nr. M-3277) was added. This solution was stirred over night at 4 °C. The enzymatic activity after the treatment was 75% of the original mixture. No further purification was carried out.

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b) Modification with ConA-Sepharose (Concanavalin A-Agarose)

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1 ml of a crude lignin peroxidase preparation (as in **a**) was mixed with 1 g of ConA-Sepharose (Pharmacia) in 10 ml acetate buffer (100 mM) at pH 7 overnight at 4 °C. The solid complex was then washed with 100 ml of the same buffer. The yield with respect to activity was 50%.

50 **c) Deglycosylation**

1 ml of a crude lignin peroxidase preparation (as in **a**) was diluted in 1 ml acetate buffer (100 mM, pH 5). Then 10 units of endoglycosidase F (Boehringer) are added to the preparation and the reaction is carried out at 37 °C for 2 hours. The yield with respect to activity was 30%.

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Example 2

Treatment of Wood Pulp

2.5 g of the appropriate pulp are extracted first with sodium hydroxide (2.5% of g dry pulp, 10% consistency) for one hour at 50 °C and then washed with tap water to neutrality. After addition of 100 ml of tap water the pH is lowered to 3.5 with hydrochloric acid. The mixture is then flushed with oxygen whilst stirring.

The enzyme preparation prepared as described in Example 1 is then added to the pulp suspension and the reaction is then performed for one hour at 40 °C. The reaction is terminated by filtration and a subsequent sodium hydroxide extraction as described above.

The degree of delignification is measured by determination of the Kappa number. The lignin is also analytically detectable in the combined filtrate/alkaline extract e.g. by gel filtration high performance liquid chromatography using UV/Vis spectroscopy for detection.

Better results in delignifying the pulp are obtained with a modified enzyme preparation than with a non-modified enzyme preparation although the enzymatic activity of the modified preparation per g of pulp was 10 times lower than of the non-modified preparation (Table 1).

Better results are obtained when oxygen alone is used than when hydrogen peroxide alone is used (Table 2).

Delignification of hardwood kraft pulp, softwood kraft pulp, mixed mechanical pulp and softwood sulfite pulp can be achieved (Table 3).

Table 1

Delignification of Hardwood Kraft Pulp	
TYPE OF ENZYME PREPARATION	DELIGNIFICATION (% Kappa Number Decrease)
no enzyme	0
PEG alone	2
ConA-Sepharose alone	1
(a)PEG modified BSA, Heme	1
(b)PEG modified, heat denatured	2
(b)PEG modified	15
(b)ConA modified	16
(c)recombinant	18
(c)non-modified	6
All reaction mixtures were flushed with oxygen before and during the course of the reaction (1 hour).	

a: PEG modified bovine serum albumine prepared in the same way as the modified enzyme preparation, bovine Heme (Sigma Nr. H-2250), 10 µg/ml.

b: 5 VAO-units/g pulp.

c: 50 VAO-units/g pulp.

Table 2

5	Effect of Hydrogen Peroxide vs. Oxygen alone		
	TYPE OF ENZYME PREPARATION	DELIGNIFICATION (% Kappa Number Decrease)	
		H ₂ O ₂	O ₂
10	no enzyme	0	0
	non-modified 50 units/g	0	6
	recombinant 50 units/g	0	18
	PEG-modified 5 units/g	0	15
	ConA-modified 5 units/g	0	16
15	Hydrogen peroxide concentration was 100 µMol/litre; oxygen was as in Table 1.		

Table 3

20	Delignification of Different Pulp Types	
	PULP TYPE	DELIGNIFICATION (% Kappa Number Decrease)
	hardwood kraft	15
25	softwood kraft	8
	mixed mechanical	4
	softwood sulfite	7
	Enzyme concentration was 5 VAO-units/g pulp; A PEG-modified enzyme preparation was used.	

Example 3

Treatment of Wood Pulp

Example 2 is repeated using 50 VAO units/g pulp of the enzymatic mixture as prepared in Example 1 c). When added at the same concentration, the enzymatic mixture treated with endoglycosidase F is more effective in delignifying the pulp than a non-modified mixture.

Example 4

Modification of Lignin

200 µg Organosolv lignin (87/64003; Organocell, Munich BRD) from a 2% stock solution in dioxan in 1 ml of sodium tartrate buffer (100 mM) pH 3.5 were incubated with 1 VAO unit of ConA-Sepharose modified enzyme preparation at 40 °C for one hour whilst flushed with oxygen. After that time the pH was adjusted to 10.5 with sodium hydroxide and the sample was filtered through a 0.45 µm filter to remove the enzyme/ConA complex. A sample treated in the same way with ConA-Sepharose but no enzyme was prepared at the same time.

The reaction products were analysed by gel permeation high performance liquid chromatography (HPLC) on two serially connected TSK (GMP W&L, 7.8 x 300 mm) columns (Toya Soda, Japan). The flow rate was 1 ml/min. and sodium carbonate (10 mM, pH 10.5) with 0.05% polyethylene glycol (PEG 6000) was used as eluent. Absorption at 250, 310 and 360 nm was recorded using a diode array UV-detector.

The enzyme treated lignin was extensively modified. Substantial brightening of the lignin suspension was observed after the enzyme treatment. The UV absorption spectra at 250, 310 and 360 nm of the individual lignin components after separation by gel permeation chromatography was extensively altered.

Claims

1. An enzymatic composition for treating wood pulp comprising at least one ligninperoxidase derived from a fungus culture, which lignin peroxidase is chemically modified so that it cannot be adsorbed onto the pulp.
2. A composition according to claim 1 in which the lignin peroxidase is associated with a water-soluble or insoluble polymeric compound by covalent or non-covalent linkage.
3. A composition according to claim 1 comprising ligning peroxidase which is at least partially deglycosylated.
4. A composition according to any of claims 1 to 3 selected from a crude extract, a filtrate or a supernatant of Phanerochaete chrysosporium.
5. A process for bleaching wood pulp which comprises treating the pulp with a composition according to any one of claims 1 to 4.
6. A process according to claim 5 which is carried out in the absence of added peroxide and in the presence of added oxygen.
7. A process for treating waste water released from the pulping treatment of wood or from the bleaching treatment of wood pulp which comprises treating the waste water with a composition according to any one of claims 1 to 4 in the absence of a peroxide and in the presence of oxygen.

Patentansprüche

1. Enzymatische Zusammensetzung zur Behandlung von Zellstoff, welche zumindest eine Lignin-Peroxidase, die von einer Pilzkultur stammt, umfasst und welche chemisch modifiziert ist, so dass sie nicht an den Zellstoff adsorbiert werden kann.
2. Zusammensetzung nach Anspruch 1, worin die Lignin-Peroxidase mit einer wasserlöslichen oder unlöslichen polymeren Verbindung durch kovalente oder nichtkovalente Bindung assoziiert ist.
3. Zusammensetzung nach Anspruch 1, umfassend eine Lignin-Peroxidase, die zumindest teilweise deglycosiliert ist.
4. Zusammensetzung nach einem der Ansprüche 1 bis 3 ausgewählt aus einem Rohextrakt, einem Filtrat oder einem Überstand von Phanerochaete chrysosporium.
5. Verfahren zum Bleichen von Zellstoff, welches die Behandlung von Zellstoff mit einer Zusammensetzung nach einem der Ansprüche 1 bis 4 umfasst.
6. Verfahren nach Anspruch 5, welches in Abwesenheit von hinzugefügtem Peroxid und in Gegenwart von Sauerstoff durchgeführt wird.
7. Verfahren zur Behandlung von Abwasser, das vom Zellaufschlussverfahren von Holz oder von der Bleichbehandlung von Zellstoff freigesetzt wird, welches die Behandlung des Abwassers mit einer Zusammensetzung gemäss einem der Ansprüche 1 bis 4 in Abwesenheit von einem Peroxid und in Gegenwart von Sauerstoff umfasst.

Revendications

1. Une composition enzymatique pour le traitement de la pâte de bois comprenant au moins une lignineperoxidase dérivée d'une culture de champignons, laquelle lignineperoxidase est modifiée chimiquement afin de ne pas pouvoir être adsorbée dans la pâte.

2. Une composition selon la revendication 1, dans laquelle la lignineperoxidase est associée avec un composé polymère soluble ou insoluble dans l'eau par liaison covalente ou non.
3. Une composition selon la revendication 1, comprenant une lignineperoxidase qui est au moins
5 partiellement déglycosylée.
4. Une composition selon l'une quelconque des revendication 1 à 3, choisie parmi un extrait brut, un filtrat ou un surnageant de *Phanerochaete chrysosporium*.
- 10 5. Un procédé de blanchiment de la pâte de bois qui comprend le traitement de la pâte par une composition selon l'une quelconque des revendications 1 à 4.
6. Un procédé selon la revendication 5, qui est effectué en l'absence de peroxyde ajouté et en présence
15 d'oxygène ajouté.
7. Un procédé de traitement des eaux résiduelles provenant du traitement de la pâte de bois ou du blanchiment de la pâte de bois, qui comprend le traitement des eaux résiduelles par une composition selon l'une quelconque des revendications 1 à 4, en l'absence de peroxyde et en présence d'oxygène.

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