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(54) **Bleaching wood pulp with enzymes.**

(57) Wood pulp may be delignified enzymatically with very good results when treating it with a lignin peroxidase in the absence of a peroxide and when the enzyme is firstly chemically modified in such a way that it does not adsorb to the pulp.

EP 0 418 201 A2

BLEACHING WOOD PULP WITH ENZYMES

This invention relates to a novel enzymatic process for bleaching wood pulp in which a crude lignin peroxidase is used in the presence of oxygen rather than hydrogen peroxide as co-substrate to reduce the lignin content of wood pulp. The lignin peroxidase can be used in a modified form.

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 vith 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 fro 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 in the absence of a peroxide and when the enzymes are firstly chemically modified in such a way that they do not adsorb to the pulp.

Thus, the invention provides a process for bleaching wood pulp which comprises treating the pulp with at least one lignin peroxidase in the substantial absence of added peroxide and in the presence of oxygen.

In addition, the invention provides an enzymatic composition (enzyme preparation) comprising at least one lignin peroxidase derived from a fungal culture which 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 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.

By "substantial absence of added peroxide" is meant the absence of a substantial amount of added peroxide which would be effective in inducing degradation of lignin. Thus, the scope of the invention intends to encompass a process in which a peroxide is added to the reaction medium in an uneffective amount.

The term "lignin-degrading enzyme" as used herein is meant to encompass any enzyme which modifies the lignin or hemicellulose component of wood so that lignin is released from the hemicellulose matrix or made releasable upon washing or extraction. Suitable lignin-degrading enzymes are hemicellulases, oxidases and peroxidases, the latter being particularly preferred. Examples of hemicellulases are mannanases, xylanases, galactomannanases and arabinosidases, while laccases fall under the group of oxidases. Preferred peroxidases are MnII-dependent peroxidases, and lignin peroxidases (also called ligninases). Lignin-degrading enzymes 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 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. 88/2023.

For use in the preferred process of the invention the enzymatic composition may be in a substantially purified form. It is, however, preferred that the enzymatic composition be a crude extract, a filtrate or a supernatant of a culture of a white-rot fungus, e.g. P. chrysosporium.

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 medium which contains 1.08 x 10^{-3} M ammonium tartrate, 1.47 x 10^{-2} M KH_2PO_4, 2.03 x 10_{-3} M $MgSO_4.7H_2O$, 6.8 x 10^{-4} M $CaCl_2 \cdot 2H_2O$, 2.96 x 10^{-6} M thiamine•HCl and 10 ml•L^{-1} of a trace element solution. The trace element solution contains 7.8 x 10^{-3} M nitrilo-acetic acid, 1.2 x 10^{-2} M $MgSO_4 \cdot 7HO_2$, 1.7 x 10^{-2} M NaCl, 3.59 x 10^{-4} M $FeSO_4 \cdot 7H_2O$, 7.75 x 10^{-4} M $CoCl_2$, 9.0 x 10^{-4} M $CaCl_2$, 3.48 x 10^{-4} M $ZnSO_4$, 4 x 10^{-5} M $CuSO_4 \cdot 5H_2O$, 2.1 x 10^{-5} M $AlK(SO_4)_2 \cdot 12H_2O$, 1.6 x 10^{-4} M H_3BO_3, 4.1 x 10^{-5} M $NaMoO_4 \cdot 2H_2O$ and 2.9 x 10^{-3} M $MnSO_4 \cdot H_2O$.

For use in the process of the invention, the lignin-degrading enzyme may be 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, polyethylene glycol (PEG), po3ypropylene 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 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 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. bleached wood pulps which may be treated vith the process of the invention are advantageously mechanical pulps, e.g. groundwood pulp, including the thermomechanical pulps such as thermomechanical pulps (TMP), chemimechanical pulps (CMP), chemithermomechanical pulps (CTMP) and chemical pulps (CP) such as sulphite and Kraft pulps, these latter being preferred.

As a general rule, the enzyme concentration may range from 0.001 to 1000 VAO units/g pulp (a VAO unit is determined by the conversion of veratrylalcohol to veratrylaldehyde at 310 nm 9.3 μmol.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 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 vater, fresh water or tap water during the bleaching process. Hovever, 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.

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 fern 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.

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

Treatment of wood pulp with recombinant lignin peroxidase (ligninase)

1) Production of recombinant ligninase

The recombinant apo-ligninase of P. chrysosporium is recovered from the pellet fraction of a culture lysate of E. coli (pBSR3) NRRL-18068 by extraction in 4Murea 50 mM sodium acetate 10mM dithiothreitol (DTT). The supernatant extract is then separated from the pellet by appropriate centrifugation and applied on a DEAE-Sepharose anion exchange column. A gradient of 0 to 1M NaCl is run in the extraction buffer. Fractions are collected and analysed for their immunoreactivity with an anti-ligninase antibody. The most strongly immunoreactive fractions are pooled and applied to a sizing column (S-300 Sephacryl; Pharmacia) in 4M urea 50mM KH_2PO_4 4mM DTT pH 7. Again, the fractions are checked for their immunoreactivity and the most strongly reactive fractions are pooled and dialysed against Tris-HCl pH 8 1mM DTT 20% (v/v) glycerol.

Protoheme IX (Sigma) dissolved in 0.1N KOH is added to the dialysed solution. This is then dialysed against 50mM Tris HCl pH 8. 1mM reduced glutathione 100µM oxidized glutathione overnight at 4 °C. Finally the sample is dialysed against 10mM sodium acetate pH 6.

2) Bleaching process with the recombinant ligninase

2.5 g of Kraft pulp obtained from hardwood are extracted first with 2.5% sodium hydroxide for one hour at 50° C and then washed with tap water to neutrality. The consistency of the pulp is adjusted to 2.5% (approximately corresponding to 2.5 g pulp diluted in 100 ml tap water) and the pH is lowered to pH 3.5 with hydrochloric acid. The mixture is then flushed with oxygen whilst stirring.

50 VAO units/g pulp of the reconstituted ligninase are added to the mixture and the reaction is performed for one hour at 40° C. A control sample with heat denatured enzyme is also prepared as well as a sample without enzyme.

The reaction is stopped by washing with tap water and the Kappa number of the enzymatically treated preparation and the control sample is measured. Surprisingly, the preparation treated with the recombinant ligninase in the exclusive presence of oxygen as co-substrate exhibits a lower Kappa number in comparison with the control sample, revealing that a significant delignification has been achieved.

Example 2

Treatment of wood pulp with a filtrate from a culture of P. chrysosporium

A concentrated ligninolytic enzyme mixture essentially containing ligninases and Mn-dependent peroxidases is obtained by ultrafiltration (MW cut off 10,000) of a culture of P. chrysosporium ATCC 24 725 produced by the method of Linko, Enzyme Microb. Technol. 1988, 10 , 410-417. Such a mixture has an enzymatic activity of 125 VAO units/ml. The protein content of the mixture is 5 mg/ml as determined by the method of Bradford et al, Anal. Biochem. (1976) 72 : 248.

7.5 g of Kraft pulp obtained from hardwood are extracted first with 2.5% sodium hydroxide for one hour at 50° C and then washed with tap water to neutrality. The consistency of the pulp is adjusted to 2.5% (approximately corresponding to 2.5 g pulp diluted in 100 ml tap water) and the pH is lowered to pH 3.5 with hydrochloric acid.

The preparation is divided into three samples. One is supplemented with 100µM H_2O_2, another one is supplemented with 100µM H_2O_2 and flushed with oxygen, yet another one is only flushed with oxygen.

50 VAO units/g pulp of the enzymatic mixture are added to each sample and the reaction is performed for one hour at 40° C whilst stirring. The reaction is stopped by washing with tap water and the Kappa number of the samples is measured.

Surprisingly, better results in delignifying the pulp are obtained in the exclusive presence of oxygen as a co-substrate than in the presence of hydrogen peroxide supplemented or not with oxygen.

Example 3

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.

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.

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

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%.

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%.

Example 4

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 boor 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 3 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 Draft 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

Effect of Hydrogen Peroxide vs. Oxygen alone		
TYPE OF ENZYME PREPARATION	DELIGNIFICATION (% Kappa Number Decrease)	
	H_2O_2	O_2
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
Hydrogen peroxide concentration was 100 µMol/litre; oxygen was as in Table 1.		

Table 3

Delignification of Different Pulp Types	
PULP TYPE	DELIGNIFICATION (% Kappa Number Decrease)
hardwood kraft	15
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 5

Treatment of Wood Pulp

Example 4 is repeated using 50 VAO units/g pulp of the enzymatic mixture as prepared in Example 3 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 6

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 absorption spectra at 250, 310 and 360 nm of the individual lignin components after separation by gel permeation chromatography was extensively altered.

Claims

1. A process for bleaching wood pulp which comprises treating the pulp with at least one lignin peroxidase in the substantial absence of added peroxide and in the presence of added oxygen.
2. A process according to claim 1 in which the enzymatic composition is selected from a crude extract, a filtrate or a supernatant of Phanerochaete chrysosporium .
3. 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 at least one lignin peroxidase in the absence of a peroxide and in the presence of oxygen.
4. An 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.
5. A composition according to claim 4 in which the lignin peroxidase is associated with a water-soluble or

insoluble polymeric compound by covalent or non-covalent linkage.

6. A composition according to claim 4 comprising a lignin peroxidase which is at least partially deglycosylated.

7. A process for bleaching wood pulp which comprises treating the pulp with a composition according to any one of claims 4 to 6.

8. A process according to claim 7 in the substantial absence of added peroxide and in the presence of added oxygen.

9. 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 4 to 6 in the absence of a peroxide and in the presence of oxygen.

10. Delignified wood pulp treated by a process according to any one of claims 1, 2, 7 or 8.