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(54) Enzymatic delignification of lignocellulosic material.

(57) A single or plural stage process for the enzymatic delignification of lignocellulosic materials for use in the pulp and paper industry. Each stage comprises the step of incubating the lignocellulosic material with an effective amount of a ligninolytic enzyme preparation, in a reaction mixture comprising hydrogen peroxide at a low steady-state concentration. The ligninolytic enzyme preparation may comprise lignin peroxidases, Mn(II)-dependent peroxidases, or both these enzymes.

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ENZYMATIC DELIGNIFICATION OF LIGNOCELLULOSIC MATERIAL

Technical Field of the Invention

This invention relates to a single or plural stage process for the enzymatic delignification of lignocellulosic materials. More particularly, the invention is directed to an improved process for the enzymatic delignification of lignocellulosic materials for use in the pulp and paper industry.

In chemical pulping, lignocellulosic material is treated with harsh chemical oxidants which degrade lignin. Chemical pulping is most commonly achieved with the Kraft (sulfate) sulfite, soda, and modified sulfite processes which remove the bulk of the lignin matrix, freeing the cellulose fibers, and so cause the formation of pulp. For example, the Kraft (sulfate) process produces a pulp containing only 5-8% by weight of residual lignin, an approximately three to five fold reduction in the original lignin content. The above described pulping processes results in dark colored pulp due, almost exclusively, to residual lignin.

To produce a desirably bright paper product, the pulp must be brightened or bleached. The most commonly used bleaching processes employ chlorine or chlorine-containing compounds such as calcium hypochlorite, sodium hypochlorite and chlorine dioxide. These processes bleach pulp primarily by removing lignin. The first stages, usually chlorination and alkaline extraction, result in effective bleaching, however, they have severe drawbacks. First, the harsh nature of these treatments causes significant degradation of the cellulose fibers, and a decrease in fiber length. Thus, a bleaching process which minimizes the degradation of cellulose fibers would be preferable.

Another important drawback is that the highly corrosive effluent contains a large number of chlorinated lignin breakdown products, some of which are toxic, and potentially mutagenic and/or carcinogenic. This effluent presents a serious waste disposal problem. Another drawback is that the highly corrosive chlorides attack plant machinery and preclude effluent recycling. Finally, the corrosive nature of this effluent makes it hazardous to plant personnel. For these reasons, alternative bleaching processes which reduce the chlorine required for bleaching have been vigorously pursued by the industry.

To overcome various drawbacks of the previously mentioned bleaching processes, the development of non-polluting enzymatic bleaching processes has been explored. Several types of microorganisms secrete enzymes which modify or degrade lignin without attacking cellulose or hemicellulose. The most widely studied class of lignin degrading microorganisms is the white rot fungi. The best understood member of this class is Phanerochaete chrysosporium. However, direct use of these fungi to bleach pulp has drawbacks. Fungal delignification is very slow, requiring at least seven days for appreciable brightening. Another is that the fungi also secrete enzymes which degrade cellulose and hemicellulose, a most undesirable side effect. Finally, the process can only be carried out under the circumscribed conditions at which the fungi are viable. To overcome some of these obstacles, the use of enzyme preparations derived from fungal cultures has been explored.

White rot fungi are presumed to accomplish the complete degradation of lignin to carbon dioxide and water through the concerted action of many enzymes. The mechanisms that underly this complex process are just now becoming known.

Recently, several enzymes of the ligninolytic system of the white rot fungus Phanerochaete chrysosporium have been isolated and partially characterized. Members of a family of enzymes, termed lignin peroxidases ("LiP enzymes"), are commonly considered to be the enzymes most directly responsible for delignification. LiP enzymes require hydrogen peroxide for activity. Delignification activity of these enzymes is commonly measured in terms of their ability to oxidize veratryl alcohol (2,3-dimethoxybenzyl alcohol) to veratrylaldehyde (2,3-dimethoxybenzyl aldehyde). Veratryl alcohol has thus been used in the art as a lignin model compound, the oxidation of which is diagnostic of the presence of an enzyme which degrades lignin (also referred to as a "ligninase").

Another Phanerochaete chrysosporium enzyme recently suggested to participate in the delignification process is the Mn(II)-dependent peroxidase (also known as "NADH-oxidizing peroxidase"), hereafter referred to as "MnP". Like LiP enzymes, MnP requires hydrogen peroxide, but also requires Mn(II). MnP does not oxidize veratryl alcohol. However, it will oxidize several dyes, including 2,2'-azino-bis(3-ethyl-6-benzothiazolinesulfonate) ("ABTS") (J.K. Glenn and M.H. Gold, "Purification Of An Extracellular Mn(II)-Dependent Peroxidase From The Lignin-Degrading Basidiomycete, Phanerochaete chrysosporium", Arch. Biochem. Biophys., 242(2), pp. 329-41 (1985)) and phenol red. The oxidation of phenol red is a commonly used measure of MnP activity (M. Kuwahara et al., "Separation And Characterization of Two Extracellular H_2O_2-Dependent Oxidases From Ligninolitic Cultures Of Phanerochaete chrososporium", FEBS Lett., 162

(2), pp. 247-50 (1984)). The "true" function of MnP in the ligninolytic system is unknown. MnP has been hypothesized to participate in the ligninolytic process by generating the hydrogen peroxide required by the LiP enzymes. This hypothesis was prompted, in part, by the finding that hydrogen peroxide is produced as a by-product of the oxidation of NADH by MnP (Y. Asada et al., "An Extracellular NADH-Oxidizing Peroxidase Produced By A Lignin-Degrading Basidiomycete, Phanerochaete chrysosporium", J. Ferment. Technol., 65(4), pp. 483-87 (1987)).

Farrell et al. United States Patent 4,687,741 refers to (1) ligninolytic enzymes purified from Phanerochaete chrysosporium mutant strain SC26, (2) Phanerochaete chrysosporium mutant strain SC26, and (3) a process for degrading and modifying lignin in wood pulp using these purified enzymes. Farrell United States Patent 4,687,745 refers to a process for enhancing the strength properties and brightness stability of mechanical pulps using enzymes derived from Phanerochaete chrysosporium strain SC26. Farrell United States Patent 4,690,895 refers to a process for the bleaching of Kraft pulp using enzymes derived from Phanerochaete chrysosporium strain SC26. In contrast, the present invention discloses a novel process for the enzymatic delignification of lignocellulosic material which differs substantially from the processes referred to in the Farrell patents.

SUMMARY OF THE INVENTION

This invention concerns a practical method for the efficient delignification of lignocellulosic material with enzyme preparations derived from fungi.

An object of this invention is to providee a process which results in bleached lignocellulosic pulp of high viscosity, which is productive of paper displaying improved brightness and superior strength properties and wherein the effluents from each stage and washing step may be recycled for use in the washing of pulp from other stages.

An additional object of this invention is to provide a multi-stage bleaching process which includes one or more enzymatic delignification stages as well as one or more conventional bleaching stages.

In one embodiment, lignocellulosic material is treated with a ligninolytic enzyme preparation at appropriate reaction conditions in the presence of a low steady-state concentration of hydrogen peroxide. In a preferred embodiment, at least one of the following reagents will also be present: Mn(II); an alpha-hydroxy acid; a nonionic or zwitterionic detergent; and/or a substituted aromatic alcohol capable of serving as a substrate for ligninolytic enzymes.

In a preferred embodiment, following the enzymatic treatment, the lignocellulosic pulp is subjected to an alkaline extraction step, washed with water, and then subjected to a dilute acid extraction step. One step of enzymatic delignification, followed by washing and extraction steps constitutes a one stage enzymatic delignification process according to this invention. In another preferred embodiment, the lignocellulosic material is subjected to a plural stage delignification process, with each stage comprising these steps.

DETAILED DESCRIPTION OF THE INVENTION

Preferred lignocellulosic materials for use in the process of this invention are such wood pulps prepared by the well known sulfite, sulfate or Kraft, soda and modified sulfite processes. The process of this invention will produce significant delignification of both softwood and hardwood pulps. This invention is particularly useful for the bleaching of hardwood Kraft pulp, where the kappa number of the untreated pulp is already so low that enzymatic delignification readily causes brightening. Significant brightening of softwood pulp produced by extensive Kraft processing (having a pre-bleaching kappa number of 8) was also observed. However, softwood pulps produced by standard Kraft processes have a very high lignin content, with a pre-bleaching kappa number of at least 20. The degree of delignification achieved in a one or two stage enzymatic delignification process with this high kappa number pulp has not yet been sufficient to result in significant brightening. However, the process according to this invention consisting of three or more enzymatic delignification stages has achieved sufficient delignification of southern softwood Kraft pulp to result in measurable brightening.

Partial delignification of the lignocellulosic material, achieved by the process of this invention, has the beneficial effect of reducing the amount of bleaching chemicals required to completely delignify and bleach this pulp. Thus, the level of chlorinated organics in the bleach plant waste effluent stream would be

reduced.

Also contemplated is the enzymatic delignification of mechanical pulps, thermomechanical pulps and chemithermomechanical pulps.

In addition, since the delignification process of this invention has a beneficial effect on pulp formation, the delignification and brightening of partially pulped lignocellulosics may give a pulp suitable for direct use in papermaking.

The lignocellulosic materials delignified according to the process of this invention may be washed with water, or otherwise treated, prior to enzymatic delignification. Chemical pulps may also be washed with dilute ethylenediamine tetra-acetic acid ("EDTA") (5 to 100 mM) or other chelating agentsand then washed extensively with water prior to enzymatic delignification. It may also be advantageous to wash the lignocellulosic material with dilute acid, for example 10 to 200 mM acetic acid, prior to the enzymatic delignification. Pulp consistency would normally range from 0.1 to 10.0% during these washing steps.

Ligninolytic enzyme preparations derived from white rot fungi, brown rot fungi, or dry rot fungi are useful for the purposes of this invention. Enzymes derived from mutant strains of these naturally occurring fungi are also useful, especially if the mutant was one selected for its increased production of the desired ligninolytic enzymes. Preferably, enzymes derived from a white rot fungus will be employed. Particularly preferred is the use of enzymes derived from Phanerochaete chrysosporium. Although any strain of this fungus may be used, examples of desirable strains include SC26 (Northern Regional Research Laboratory ("NRRL") #15978, ME-446 (American Type Culture Collection ("ATCC") #34541), and VKM-F-1767 (ATCC #24725).

Usually, the desired ligninolytic enzymes are secreted by the fungus into the extracellular culture medium. This medium is harvested and processed to achieve the desired degree of concentration and enzyme purification. Culturing conditions and time of harvesting will influence the total amount of ligninolytic activity recovered as well as the relative proportions of each of the various ligninolytic enzymes.

The harvested culture media may be used directly in the delignification process so long as it is relatively free of undesirable enzymes, such as cellulases or proteases. Preferably, though, the culture media is concentrated before use in the delignification reaction.

Any concentration method which preserves enzymatic activity may be used, such as lyophilization or precipitation with high salt, polyethylene glycol, acetone and alcohol. If any of these concentration procedures is used, the enzymes may be reconstituted in an appropriate buffer (e.g., sodium acetate, sodium tartarate, sodium dimethyl succinate or sodium succinate, at pH 3-5) to produce an unfractionated enzyme concentrate for use in the delignification reaction. Alternatively, the lyophilizate or precipitate may be added directly to the delignification reaction.

The preferred concentration method is by pressure dialysis of the extracellular culture media. A typical extracellular enzyme concentrate contains 0.5 -20.0 U/ml of veratryl alcohol oxidizing ("VAO") activity. One unit of VAO activity is defined, for the purposes of this application, as that amount of enzyme which is capable of oxidizing one micromole of veratryl alcohol per minute at 25° C, under the standard assay conditions described below.

VAO activity is quantified from the change in absorbance of the assay solution at 310 nm. Three prepared reagents are used: (A) 0.25 M sodium tartarate, adjusted to pH 3.0 with H_2SO_4; (B) 10.0 mM veratryl alcohol (Aldrich Chemical Co., Milwaukee, WI, #D13, 30U-O); and (C) 98 mM hydrogen peroxide (Fisher Scientific, Fairlawn, NJ, #H325-5) (prepared fresh daily). To perform a VAO activity assay: (a) 400 ul of reagent A are added to a quartz cuvette; (b) 200 ul of reagent B are added; (c) 390 ul of enzyme sample double-distilled water are added; (d) the contents of cuvette are mixed; (e) 10 ul reagent C are added; (f) the contents of the cuvette are mixed immediately; and (g) the absorbance at 310 nm is measured for 30 seconds at 25° C. Units of VAO activity per milliliter of a test sample (U/ml) are calculated as follows, using a value of 9300 M^{-1} cm^{-1} as the extinction coefficient for veratryl alcohol: (A/min.)(9300)-$^{-1}$(1000) (test sample volume (ml))$^{-1}$. For the purposes of this application, one unit of LiP equals one unit of VAO activity.

The A_{310}/min. is linear with respect to LiP concentration within the range of 0.1 to 0.5 A_{310}/min. Enzyme samples yielding rates outside this range are not used to calculate activity. Instead, the volume of test sample per cuvette is reduced or increased so that the measured rate falls within the linear range.

In some circumstances, it may be desirable to use partially purified subfractions of the harvested culture media. For example, use of a ligninase-containing subfraction which does not contain cellulases may be desired. Alternatively, it may be desirous to separate the LiP enzymes from any MnP enzymes so that these enzymatic activities may be independently used. Ligninolytic enzymes which have been purified to near homogeneity, and mixtures of these purified enzymes may be used.

An important, novel and unexpected feature of the present invention is that purified MnP of

Phanerochaete chrysosporium is effective in the delignification of wood pulp using the process of this invention. As used herein the term "MnP" applies to any manganese dependent peroxidase that is capable of modifying or degrading lignin, regardless of whether the enzyme is presently known in the art.

Recombinant ligninolytic enzymes, having either LiP or MnP activity, are also useful in the process of this invention.

The enzymatic delignification reaction can be conducted in any container of the desired size for which some provision has been made for the mixing, oxygenation and temperature regulation of the contents. In addition, convenient mechanisms for the introduction of non-gaseous reaction components and removal of reaction products must be provided. The order of addition of reaction components is not critical; however, it is preferred to add the enzymes last. The basic reaction mixture comprises the lignocellulosic material to be delignified, an active ligninolytic enzyme preparation, and hydrogen peroxide at a low steady-state concentration, all in a solution maintained at the appropriate pH. Preferably, the reaction mixture will also contain one or more of the following components: (a) a nonionic or zwitterionic detergent, (b) Mn(II), (c) an alpha-hydroxy acid, and/or (d) a substituted aromatic alcohol capable of serving as a substrate for ligninolytic enzymes. Most preferably, all of these components are present.

If the lignocellulosic material to be bleached is wood pulp, it should be present in the reaction mixture at a consistency (grams dry weight pulp per gram wet weight pulp) of 0.1 to 10% and preferably 0.5 to 4.0%.

If an enzyme preparation comprising at least one LiP and devoid of MnP activity is used, it should be present in the reaction mixture at a concentration of 0.05 to 10.0 U/ml of VAO activity, and preferably 0.4 to 2.0 U/ml. If an enzyme preparation comprising at least one MnP and devoid of LiP activity is used, it should be present in the reaction mixture at a concentration of 0.04 to 20.0 U/ml, and preferably 0.25 to 10.0 U/ml, of phenol red oxidizing ("PRO") activity.

One unit of PRO activity is defined as that amount of enzyme which causes a change in the absorbance of the assay solution, at 530 nm, of one absorbance unit per minute, at 25° C under standard assay conditions. Three reagents are used in PRO activity assays. Reagent A consists of 0.11 g/L of the sodium salt of phenol red (Sigma Chemical Co., St. Louis, MO, #P-553Q), 1.1 g/L of ovalbumin, and 2.5 ml/L of 85% lactic acid, in 20 mM sodium succinate, pH 4.5. Reagent B is 10 mM manganese sulfate in 20 mM sodium succinate, pH 4.5. Reagent C is 9.8 mM hydrogen peroxide (Fisher Chemical Co.) (prepared fresh daily). To perform a PRO activity assay 900 ul of reagent A are added to a cuvette; 100 ul of enzyme sample/double-distilled water are added; 10 ul of reagent B are added; the contents of the cuvette are mixed immediately; the spectrophotometer is blanked at 530 nm on this cuvette; 10 ul reagent C are added; the contents of the cuvette are mixed immediately; and the absorbance at 530 nm is recorded for 30 seconds at 25° C. The units of PRO activity per milliliter of a test sample (U/ml) are calculated using the following equation: (Δ/min.)(test sample volume in assay (ml))$^{-1}$. For the purposes of this application, one unit of MnP equals one unit of PRO activity.

The A_{530} min. is linear with respect to MnP concentration within the range of 0.05 to 0.20 A_{530}/min. Enzyme samples yielding rates outside this range are not used to calculate activity. Instead, the volume of the test sample per cuvette is reduced or increased so that the measured rate falls within the linear range.

If an enzyme preparation comprising one or more LiP enzymes and one or more MnP enzymes is used, they should be present in the reaction mixture at the concentrations specified above.

An important feature of the present invention is the maintenance of a low steady-state concentration of hydrogen peroxide, 0.001 to 0.5 mM, preferably 0.005 to 0.1 mM in the reaction mixture throughout the delignification reaction. The hydrogen peroxide concentration may be maintained conveniently at the desired level by in situ enzymatic generation, for example through the action of glucose oxidase on glucose. Accordingly, 0.1 to 10.0 U/ml glucose oxidase oxidase and 0.1 to 20.0 mM glucose may be added to the reaction mixture, typically, 1.0 U/ml glucose and 0.1 to 10.0 mM glucose is used. Alternatively, hydrogen peroxide may be supplied via continuous metered addition. Metered addition would be the preferred method of maintaining hydrogen peroxide concentration in large-scale delignification reactions. The hydrogen peroxide concentration may also be roughly maintained by periodic addition.

The pH should be maintained at 2 to 8 throughout the delignification reaction, preferably within the range of about 3 to 5. The pH may be maintained by chemostating -- the metered or periodic addition of appropriate quantities of acid or base. Chemostating is the preferred method of pH maintenance in large-scale reactions. Alternatively, the pH may be maintained by use of a buffer in the reaction mixture. Any convenient buffer that is effective at the desired pH may be utilized. Examples of buffers appropriate at the preferred pH range include acetate, dimethyl succinate, tartarate and trans-aconitic acid. Buffer is usually added to the reaction mixture at a concentration of about 5 to 50 mM, and preferably at 15 to 25 mM.

Addition of a nonionic or zwitterionic detergent to the reaction mixture is preferred. Most usually, a

nonionic detergent is used. Examples of appropriate nonionic detergents include octyl glucoside, polyoxyethylene glycol, and compounds from the Triton and Tween series of detergents. Tween 80 is preferred. Detergent may be added at a concentration of 0.001 to 0.1% v/v, and preferably at 0.01 to 0.05% v/v.

If the ligninolytic enzyme preparation utilized comprises an MnP, Mn(II) is required in the reaction mixture. When the ligninolytic enzyme preparation used comprises at least one LiP and excludes MnP, the addition of Mn(II) is not essential. Mn(II) may conveniently be added as a salt, for example, as manganese sulfate or manganese acetate, normally manganese sulfate, at a concentration of 0.05 to 1.0 mM, and preferably 0.10 to 0.50 mM.

When Mn(II) is added to the reaction mixture, it is preferable to add at least one alpha-hydroxy acid as well. Appropriate alpha-hydroxy acids include malate, tatrate, citrate, lactate, phenyl-lactate, glycolate, 2-hydroxybutyrate, and salts thereof. Preferably, lactate is used. The alpha-hydroxy acid should be present in the reaction mixture at a concentration of 0.5 to 20.0 mM, and preferably at 1.0 to 10.0 mM.

If the ligninolytic enzyme preparation used comprises at least one LiP enzyme it is preferable to add to the reaction mixture 0.05 to 0.60 mM of a substituted aromatic alcohol capable of serving as a substrate for ligninolytic enzymes. Most preferably, veratryl alcohol is added.

Oxygen is required for enzymatic delignification. It is preferable to saturate the reaction mixture with oxygen before the addition of enzymes. Typically, after all reaction components have been added, the reaction vessel is briefly flushed with oxygen and then sealed to create an atmosphere enriched in oxygen.

The reaction mixture should be incubated at 15 to 50 °C for 0.25 to 18 hours, preferably at 30 to 50 °C for 2 to 8 hours. Most preferably, the delignification reaction is performed at 45 °C.

It is desirable to provide for mixing of the reaction mixture components during the delignification reaction.

A "one stage" enzymatic delignification process consists solely of the step of treating the lignocellulosic material with a ligninolytic enzyme preparation as described above. Preferably, however, a one stage process will comprise the further steps of extracting and washing the lignocellulosic material after the enzymatic delignification step.

In a preferred embodiment of this invention, the lignocellulosic material is subjected to an alkaline extraction step after the enzymatic delignification step. This alkaline extraction step involves the addition of an alkaline solution to the reaction mixture, followed by an optional incubation of up to two hours at 25 to 100 °C, at a 0.1 to 10% consistency of lignocellulosic material. Preferably, the incubation will be for about 0.1 hour at 25 °C, at a consistency of 0.3 to 1.0%. After incubation the mixture is usually filtered to separate the lignocellulosic material from the alkaline solution. The final concentration of base in this extraction step is preferably 0.1 to 1.0 N.

If reuse of the enzymes in the reaction mixture is desired, they must be separated from the lignocellulosic material prior to alkaline extraction, for instance by vacuum filtration, precipitation or sedimentation. Normally, this separation would be accomplished by filtration. After the enzymes have been removed. the alkaline solution is added to the lignocellulosic material.

In a preferred embodiment of this invention, the lignocellulosic material is washed with water following the alkaline extraction step by dispersion in water at a 0.1 to 10% consistency and then by filtration. Preferably, this wash would be repeated one to three times.

In the most preferred embodiment of this invention, the lignocellulosic material is subjected to an acid extraction step following the alkaline extraction step; or, if the lignocellulosic material is subjected to a water washing step after the alkaline extraction step, the acid extraction step is performed after the water washing step. The acid extraction step was found to produce particularly dramatic improvements in brightness of Kraft pulp which had been subjected to enzymatic delignification in the presence of Mn(II). The lignocellulosic material is collected, suspended in dilute acid, and incubated 0.1 to 10 minutes at 10 to 100 °C, and preferably about 0.1 minutes at 25 °C. The dilute acid solution preferably comprises 0.05 to 0.50 mM of acid. Appropriate acids include acetate, succinate, lactate, sulfurous, and other weak mineral acids. Acetic acid is preferred. The acid extraction step is performed with about 0.15 to 2.5 liters of dilute acid solution per gram dry weight of lignocellulosic material.

In a preferred embodiment of the present invention, the lignocellulosic material is subjected to a plural stage enzymatic delignification process in which each stage comprises the enzymatic delignification step described supra, and preferably further comprises an alkaline extraction step and a water washing step, as described supra. The final stage will preferably further comprise an acid extraction step.

A plural stage process achieves greater delignification of lignocellulosic material than a one stage process, given the same total units of a ligninolytic preparation applied to the same quantity of lignocellulosic material. Each stage in a two or three stage process apparently requires fewer units of ligninolytic activity than the previous stage to achieve the same degree of delignification. A one, two, or three stage

enzymatic delignification process may be preferable to a five stage process if the enzymatic delignification process is to be combined with conventional bleaching stages to produce a fully bleached product.

This invention also contemplates multi-stage bleaching processes of which one or more enzymatic delignification stages are but one component. One or more enzymatic delignification stages would be combined in series with one or more conventional bleaching stages. Examples of effective supplementary bleaching steps would be stages utilizing chlorine dioxide, chlorine, hypochlorite, oxygen, ozone, hydrogen peroxide, other weak oxidants and electroreductive bleaching. Even though additional chlorine-based bleaching stages are employed in this embodiment, the total amount of chlorinated organics in the bleach plant effluent will be significantly reduced by the inclusion of one or more enzymatic delignification stages.

In order that the present invention may be more fully understood, the following examples of the process of this invention are set forth. These examples are for purposes of illustration only and this invention should not be considered to be limited by any recitation used herein.

EXAMPLE 1

Preparation Of Unfractionated Ligninolytic Enzyume Concentrate

Ligninolytic enzyme concentrates were prepared from two strains of Phanerochaete chrysosporium: SC26 (NRRL 15978) and VKM-F-1767 (ATTC 24725).

Culture strain VKM-F-1767 was initiated from a spore inoculum prepared as follows: (1) propagating the cultures on agar at 24° C for 14 to 28 days, in plates containing a spore induction medium ("Medium N" described in H. J. Vogel, "Distribution Of Lysine Pathways Among Fungi: Evolutionary Implications", Am. Nat., XCVIII(903), pp. 435-46 (1964)), prepared with a 1/500 dilution of the Trace Element Solution, a 1/100 dilution of the Biotin Solution, without chloroform and supplemented with 2% w/v malt extract and 3% w/v yeast extract); (2) after sufficient spore development (as evidenced by the billowy appearance of the plates), washing the agar surface of each 100 x 15 mm plate with about 3 to 10 ml sterile water to liberate the spores; and (3) passing the wash water, containing spores, through a sterile funnel packed with sterile glass wool to remove contaminating fungal mycelia. The absorbance of the resulting spore suspension at 650 nm was then determined, an absorbance of 1 at 650 nm being approximately equivalent to 5 x 10^6 spores/ml. These spore preparations were stored at 4° C until use.

To initiate VKM-F-1767 cultures, Growth Medium A was inoculated with the appropriate spore suspension to a final concentration of at least 0.5 x 10^5 spores/ml, and usually to 2.5 x 10^5 spores/ml. Growth Medium A is the medium described in T. K. Kirk et al., "Influence Of Culture Parameters On Lignin Metabolism By Phanerochaete chrysosporium", Arch. Microbiol., 117, pp. 277-85 (1978), with the "Minerals" being added at a seven-fold higher concentration, and supplemented with the following reagents at the concentrations indicated: 1 mM ammonium tartarate; 0.1% v/v Tween 80; 1.8 µM manganese sulfate; 20 mM sodium acetate, pH 4.5; 6 mM benzyl alcohol; and 2% w/v glucose (as a carbon source).

Strain SC26 was initiated from starter cultures prepared by direct transfer of fungal mycelia from agar plates to 10 ml of Growth Medium A. Starter cultures were alternatively prepared by blending an existing fungal culture in a sterile Waring blender. These starter cultures were incubated at 37° C for at least three days before use.

To initiate an SC26 culture, Growth Medium A was inoculated with 1% by volume, and usually with 10% by volume, of a starter culture.

All strains of fungi were routinely cultured in volumes of 0.1 to 1.0 liter, in 2-liter sterile containers (either Erlenmeyer flasks or roller bottles). After inoculation, the cultures were purged with oxygen and incubated at 37° C, on a rotary shaker at 50 rpm (if in an Erlenmeyer flask), or on a roller bottle incubator at 40 rpm. Mixing was commenced immediately after inoculation.

The extracellular media of the cultures was monitored for VAO activity starting on the fourth day after inoculation. When at least about 0.05 U/ml of VAO activity was detected, the culture media was harvested and replaced with Growth Medium B. Growth Medium B is a low-carbon version of Growth Medium A, differing from Growth Medium A in that it contains 3.6 mM ammonium tartarate and 0.2 % w/v glucose. If an individual culture did not attain the desired level of enzymatic activity within 15 days, the media was changed to Growth Medium A to boost enzymatic activity.

When the extracellular culture media achieved the desired level of VAO activity, the fungal mycelia

were removed by pouring the culture through a funnel containing glass wool. Ligninolytic enzyme concentrate was then prepared from this active media by pressure dialysis using an Amicon Model 8400 ultrafiltration cell outfitted with a PM 10 dialysis membrane. Ultrafiltration was performed under 20 psi of nitrogen. When the volume of the media was reduced to approximately 5 to 10% of its original volume, the concentrate was poured off. Concentrate was stored at -70° C until use. This concentrated ligninolytic enzyme preparation typically contained 0.1 to 1.0 U/ml VAO activity.

EXAMPLE 2

Delignification And Brighteneing Of Northern Hardwood Kraft Pulp By An SC26 Enzyme Concentrate Fraction Containing PRO Activity But Substantially Devoid Of VAO Activity

Unfractionated enzyme concentrate of Phanerochaete chrysosporium strain SC26 was fractionated by anion exchange chromatography. The fractions were assayed for VAO activity and ABTS oxidizing ("ABTSO") activity (ABTS is a peroxidase substrate that is largely resistant to LiP enzymes). The fractions' absorbance at 280 nm and 407 nm was also measured. Fractions were selected on the basis of enzymatic activity and combined to produce three pools. The fractions combined to form pool I contained ABTSO activity but no detectable VAO activity. The fractions combined to form pool III contained VAO activity, but were substantially devoid of ABTSO activity. Fractions combined to form pool II exhibited both types of activity. The three pools were then assayed for their VAO, PRO, and ABTSO activity and for their absorbance at 280 nm and 407 nm. Finally, the ability of these pools to delignify and brighten hardwood Kraft pulp was tested. The surprising result was that pool I, which is totally devoid of measurable VAO activity, and thus presumably does not contain any active LiP, was very effective in pulp brightening. All steps were performed at room temperature except where otherwise noted.

Fractionation Of SC26 Enzyme Concentrate

Unfractionated enzyme concentrate was prepared from Phanerochaete chrysosporium strain SC26 as described in Example 1, with the exception that Growth Medium B was modified to replace the glucose with 0.2% w v glycerol. A total of 1.3 L of culture supernatant (pooled from three cultures) was concentrated by pressure dialysis as described in Example 1.

The SC26 enzyme concentrate was run through a 1.5 x 6 cm column packed with Amberlite XAD-2 resin (Mallinckrodt, Inc., Paris, KY, #3409) which had been pre-equilibrated with double-distilled water. The XAD-2 column was then washed with double-distilled water, and this eluate was pooled with the flowthrough. The combined XAD-2 pool had a volume of 65 ml. It was further characterized as follows: A_{280} = 2.5; A_{407} = 0.5; VAO activity = 0.81 U/ml; and ABTSO activity = 9.0 U/ml.

The entire XAD-2 pool was loaded onto a 6 x 10 cm DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) column pre-equilibrated with double-distilled water. After loading, the column was washed with 100 ml of double-distilled water. The column was then eluted at a flowrate of about 100 -200 ml/hr with a 770-ml linear gradient of 0.1 to 0.5 M sodium chloride in 5 mM sodium tartarate, pH 4.8. Fractions (300 drops/fraction) were collected from the time the gradient was started.

Selection Of Column Fractions

Every other fraction was assayed for VAO and ABTSO activity, as well as for absorbance at 280 nm.

One unit of ABTSO activity was defined as that amount of enzyme which caused a change in the absorbance of the assay solution, at 415 nm, of one absorbance unit per minute, at 25° C under standard assay conditions. Three reagents were used in ABTSO assays. Reagent A consisted of 0.045 g/L of ABTS (Boehringer Mannheim, West Germany, #102946), 10 ml/L of 60% sodium lactate, 3.4 g/L bovine serum albumin or gelatin, in 50 mM sodium succinate, pH 4.5. Reagent B was 10 mM manganese sulfate in 50 mM sodium succinate, pH 4.5. Reagent C was 0.0171% hydrogen peroxide in 50 mM sodium succinate, pH 4.5 (prepared fresh daily). To perform an ABTSO assay : 900 µl of reagent A were added to a quartz

cuvette;
100 µl of test sample were added; 10 µl of reagent B were added; the contents of the cuvette were mixed immediately using a cable tie; the spectrophotometer was blanked at 415 nm on this cuvette; 10 µl of reagent C were added; the contents of the cuvette were mixed immediately with a cable tie;and the absorbance at 415 nm was recorded for 30 seconds at 25 ° C. The units of ABTSO activity per milliliter of test sample (U/ml) were then calculated using the following equation:
$(\Delta A/\text{min.})(\text{test sample volume in assay (ml)})^{-1}$.

A broad, complex peak of ABTSO activity spanned approximately fractions 18 through 60. There were three discrete peaks of VAO activity spanning approximately fractions 35 to 50, 70 to 80, and 80 to 92. Column fractions were selected and combined to form three eluate pools, which were stored at -70 ° C until use. These pools were assayed for VAO, ABTSO, and PRO activity, as well as for their absorbance at 280 and 407 nM. The column fractions combined to form the pools, as well as the characteristics of these pools are set forth in Table I.

TABLE I

Pool	I	II	III
Fractions	18-34	36-48	72-77 & 82-89
ABTSO Activity (U/ml)	3.7	2.4	0.05
PRO Activity (U/ml)	2.9	0.2	0.03
VAO Activity* (U/ml)	0.00 ± 0.00	0.16 ± 0.02	0.42 ± 0.10
A_{280}	0.43	0.15	0.20
A_{407}	0.04	0.05	0.11

* The values for VAO activity represent the means of two independent determinations; standard deviations are indicated. Pool I exhibited no VAO activity in either determination.

Preparation of Pulp

Thirty grams of wet weight northern hardwood Kraft pulp was suspended in 2 L of double-distilled water and whirled in a Waring blender (on high) for 15 seconds. The pulp was collected by vacuum filtration, resuspended in 1 L of 5 mM EDTA and collected again by vacuum filtration. The pulp was then resuspended in 1 L of 0.17 N acetic acid and collected by vacuum filtration. Finally, the pulp was resuspended in 2 L of double-distilled water, collected by vacuum filtration, and then stored at 4 ° C until use. This damp "washed pulp" had a consistency of 24% (0.24 g dry weight pulp per gram wet weight washed pulp).

Enzyme Reactions: Experiment A

A master mix containing the following reaction components was prepared : 175 ml of double-distilled water; 20 ml of 0.2 M sodium acetate, pH 5.0; 0.5 ml of 10% v/v Tween 80; 1.0 ml of 2.0 M lactate; 1.0 ml of 0.3 M glucose; 0.2 ml of 0.1 M manganese sulfate; 0.8 ml of 0.1 M veratryl alcohol; and 0.5 ml of NADH (2 mg/ml). Oxygen was bubbled through this master mix for three minutes. The reaction mix was then evenly distributed into ten 50-ml conical-bottom polypropylene centrifuge tubes (~20 ml per tube) and 0.5 g wet weight washed pulp (0.12 g dry weight) were added to every tube. Next, enzyme concentrate fractions were added as follows:

Reaction	Enzyme Pool	Volume (ml)
1	-	0
2	I	1
3	I	2
4	I	4
5	I	10
6	I	20
7	II	2
8	II	5
9	III	2
10	III	5

Finally, 20µl of glucose oxidase (Sigma Chemical Co., #G 6500, 1.0 U/µl) was added to each tube. Each tube was then flushed briefly with oxygen, capped, and incubated at 37° C for 18 hr, horizontally on a rotary shaker at 60 rpm.

After incubation, 0.5 N sodium hydroxide was added to each tube, to a final volume of approximately 50 ml. The contents of each tube were added to individual scintered glass filter funnels equipped with Whatman 3M filters. The empty reaction tubes were then each washed with approximately 30 ml of 0.5 N sodium hydroxide, and these washes were added to the appropriate filter funnel . The pulp in each filter funnel was collected by vacuum filtration. About 250 ml of double-distilled water was added to each filter funnel, with stirring, and the pulp was collected. Next, about 250 ml of 0.17 N acetic acid was added to each filter funnel, with stirring, and the pulp was collected again. The pulp was allowed to air-dry for three hours . and brightness (% G.E.) was determined using a Technidyne Corporation Model S4 Brightimeter. A summary of the results of these reactions is presented in Table II.

TABLE II

Reaction	VAO Activity		PRO Activity		Brightness
	(U/ml)	(U/g pulp)*	(U/ml)	(U/g pulp)*	(% G.E.)
1	0.0	0.0	0.0	0.0	39.2
2	0.0	0.0	0.14	24.2	44.0
3	0.0	0.0	0.26	48.3	47.8
4	0.0	0.0	0.48	96.6	50.5
5	0.0	0.0	0.97	241.7	53.2
6	0.0	0.0	1.45	483.3	53.4
7	0.015	2.7	0.02	3.3	46.0
8	0.032	6.7	0.04	8.3	46.3
9	0.038	7.0	0.003	0.5	52.2
10	0.084	17.5	0.006	1.3	52.7

* Units per gram of dry weight pulp in the reaction mixture.

Enzyme Reactions: Experiment B

The following reagents were added to each of three 250-ml polycarbonate Erlenmeyer flasks equipped with screw caps: 125 ml of double-distilled water; 20 ml of 0.2 M sodium acetate, pH 5.0; 0.5 ml of 10% v/v Tween 80; 1.0 ml of 2.0 M lactate; 1.0 ml of 0.3 M glucose; 0.2 ml of 0.1 M manganese sulfate; 0.8 ml of 0.1 M veratryl alcohol; and 0.5 ml of NADH (2 mg/ml). Oxygen was bubbled through the reaction mixture in each flask for three minutes. Next, 5 grams of wet weight washed pulp (1.2 g dry weight) was added to each flask. Fifty milliliters of double-distilled water were added to reaction A. Fifty milliliters of pool I were added to reaction B. Fifty milliliters of pool III were added to reaction C. Finally, 0.2 ml of glucose oxidase

(1.0 U·µl) were added to each flask.

Then each flask was flushed with oxygen, capped, and incubated at 37° C for 18 hr on a rotary shaker at 60 rpm.

After incubation, the pulp from each reaction was collected by vacuum filtration in scintered glass filter funnels equipped with Whatman 3M filters. Each pulp pad was resuspended, with stirring, in approximately 200 ml of 0.5 N sodium hydroxide, and then collected by filtration. This sodium hydroxide wash was then repeated. Finally, the pulps were resuspended, with stirring, in about 250 ml of 0.17 N acetic acid, and collected by filtration. The pulp pads were air dried at least four hours before measuring lignin content (kappa number) and brightness (% G.E.) Microkappa number was determined essentially as described in V. Berzins, "A Rapid Procedure For The Determination Of Kappa Number", Tappi, 48(1), pp. 15-18 (1965). The results of these analyses are presented in Table III.

TABLE III

Reaction	A	B	C
VAO Activity			
(U/ml)	0.0	0.0	0.11
(U/g pulp)*	0.0	0.0	17.5
PRO Activity			
(U/ml)	0.0	0.73	0.01
(U/g pulp)*	0.0	120.8	1.3
Brightness			
(% G.E.)	41.1	54.5	51.0
Lignin Content			
(µkappa No.)**	7.9 ± 0.5	6.2 ± 1.2	7.0 ± 0.5

* Units per gram of dry weight pulp in each reaction.

** The values for microkappa number represent the means of two independent determinations; standard deviations are indicated.

EXAMPLE 3

Delignification And Brightening Of Northern Hardwood Kraft Pulp By A Plural Stage Treatment With VKM-F-1767 Unfractionated Enzyme Concentrate

Northern hardwood Kraft pulp was subjected to a one, two, or three stage treatment with Phanerochaete chrysosporium VKM-F-1767 unfractionated enzyme concentrate. Each stage before the final stage of a two or three stage treatment consisted of three sequential steps: (1) incubating the pulp with the enzyme concentrate; (2) extracting the pulp with alkali; and (3) washing the pulp with water. The final stage of a plural stage treatment, or the single stage of a one stage treatment, comprised the fourth additional step of extracting the pulp with dilute acid. Three different concentrations of enzyme concentrate were tested at each stage. All but one reaction condition was run in duplicate. Control reactions lacking enzyme concentrate were run in triplicate for analysis at each stage.

Preparation of Pulp

Northern hardwood Kraft pulp was prepared for enzyme treatment by "washing" with distilled water.

Pulp (100-200 g wet weight) was dispersed in approximately 1 L distilled water using a British Sheet Disintegrator, for four to five minutes. The pulp was collected by vacuum filtration in a Buchner funnel over Whatman 3M filter paper; washed with approximately 30 L of distilled water per 100 g wet weight pulp in aliquots, and the water removed by vacuum filtration and another aliquot of water was added. This process was repeated until the pulp had been washed with the desired volume of water.

The damp "washed" pulp had a consistency of 23.4% (0.234 gram dry weight pulp per gram wet weight pulp), a brightness of 38% G.E., a μkappa number of 13.3 and a viscosity of 28.0 cp.

Preparation of Enzyme Concentrate

The VKM-F-1767 unfractionated enzyme concentrates were prepared as described in Example 1, using roller bottles and Growth Medium A, and stored at -70° C until used. The same concentrate preparation was used for all stage one reactions. A second concentrate preparation was used in stage two and stage three reactions; this concentrate was stored at 4° C between these stages. The VAO and PRO activities of the enzyme concentrates were measured immediately prior to each stage, according to the protocols already described. These activities were:

Stage	VAO Activity (U ml)	PRO Activity (U/ml)
1	8.7	13.4
2	3.0	12.1
3	1.9	12.0

Stage One

A reaction mix containing all reaction components except pulp, enzyme concentrate and glucose oxidase was prepared. A sodium acetate, pH 4.5 stock solution was used and the reaction mix was adjusted after preparation to pH 4.5 with 1.0 N sodium hydroxide. Reaction mix was added to each of 17 reaction tubes (50-ml conical bottom polypropylene centrifuge tubes) and 9 control reaction tubes, to give final reaction conditions (excluding any reaction components that may be contributed by the enzyme concentrate) of: 20 mM sodium acetate, pH 4.5; 0.025% Tween 80; 10 mM glucose; 0.45 mM veratryl alcohol; 10 mM lactic acid; 0.1 mM manganese sulfate. Oxygen was bubbled through the reaction mix in each tube for three minutes. Next, 1.7 g washed pulp (0.4 g dry weight pulp) was added to each tube, and the tubes were gently vortexed to disperse the pulp. Twenty microliters of glucose oxidase (1.0 U/μl, Sigma Chemical Co. #G 6500) was added to each tube. Enzyme concentrate and/or double-distilled water was added to give a final reaction volume of 20.0 ml in each tube: five tubes received 5.0 units LiP and 7.7 units MnP (samples 4-5, 13 and 21-22); six tubes received 10 units LiP and 15.4 units MnP (samples 6-7, 14-15 and 23-24); six tubes received 20 units LiP and 30.8 units MnP (samples 8-9, 16-17 and 25-26); and nine tubes served as controls (samples 1-3, 10-12 and 18-20), receiving only double-distilled water with no enzyme concentrate (see Table IV). Finally, the reaction tubes were purged briefly with oxygen, capped, and inverted gently several times to mix.

The reaction tubes were incubated overnight at 37° C in a G24 Environmental Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ), on a horizontal position during the incubation at approximately 125 rpm.

Then the pulps from each reaction were added to separate scintered glass funnels equipped with Whatman 3M filters. Next, 80 ml of 0.5 N sodium hydroxide was added to each filter funnel and the funnel contents were stirred and the pulps collected by vacuum filtration. The pulp in each filter funnel was resuspended in approxiately 250 ml of distilled water and collected again by vacuum filtration. This water wash was repeated two times.

After the last water wash, the pulps from samples 1 to 9 were each resuspended in approximately 250 ml of 0.17 N acetic acid, and then collected by filtration. These pulp pads were air dried at least 12 hours before measuring the brightness (% G.E.), lignin content (μkappa number), and viscosity (cp) of the pulps. Percent G.E. and μkappa number were determined as described in Example 2. Viscosity was determined

essentially as described in "Viscosity of Pulp: Capillary Viscometer Method", TAPPI, Test Method No. T230-os-76, Atlanta, GA (1976). Table V displays the results of these analyses.

Stage Two

The pulps from samples 10 to 26 were again placed in separate reaction tubes containing pH- adjusted, oxygenated reaction mix as described above. The tubes were gently vortexed to disperse the pulps, and 20 µl of glucose oxidase were added to each reaction tube. Enzyme concentrate and/or double-distilled water was added as follows, giving a final reaction volume of 20.0 ml in each tube: three tubes received 2.0 units LiP and 8.1 units MnP (samples 13 and 21-22); four tubes received 4.0 units LiP and 16.1 units MnP (samples 14-25 and 23-24); four tubes received 8.0 units LiP and 32.3 units MnP (samples 16-17 and 25-26); and six tubes served as controls (samples 10-12 and 18-20), receiving no enzyme concentrate (see Table IV). Finally, the reaction tubes were purged briefly with oxygen, capped, and inverted gently several times to mix.

The tubes were incubated as described above for stage one. The pulps were then removed from the tubes and separately washed once with sodium hydroxide and three times with water, as described above. After the last water wash, the pulps from samples 10 to 17 were washed with 0.17 N acetic acid, as described above. The resulting pulp pads were air dried at least 12 hours before measuring the brightness (% G.E.), lignin content (µkappa number), and viscosity (cp) of the pulps, as described above.

Stage Three

The pulps from samples 18 to 26 were again placed in separate reaction tubes containing pH-adjusted, oxygenated reaction mix as described above. The tubes were gently vortexed to disperse the pulps, and 20 µl of glucose oxidase were added to each reaction tube. Enzyme concentrate and/or double-distilled water was added as follows, giving a final reaction volume of 20.0 ml in each tube: two tubes received 1.0 unit LiP and 6.3 units MnP (samples 21-22); two tubes received 2.0 units LiP and 12.6 units MnP (samples 23-24); two tubes received 4.0 units LiP and 25.3 units MnP (samples 25-26); and three tubes served as controls (samples 18-20), receiving no enzyme concentrate (see Table IV). Finally, the reaction tubes were purged briefly with oxygen, capped, and inverted gently several times to mix.

The tubes were incubated as described above for stage one. The pulps were then separately washed once with sodium hydroxide and three times with water, as described above. After the last water wash, the pulps from samples 18 to 26 were washed with 0.17 N acetic acid. These pulp pads were air dried at least 12 hours before measuring the brightness (% G.E.), lignin content (µkappa number), and viscosity (cp) of the pulps, as described above.

Results

Table IV displays the units of LiP and MnP added per pulp sample at each stage. Units of LiP represent units of VAO activity. Units of MnP represent units of PRO activity. Units per gram dry weight pulp and units per reaction volume may be calculated from the values in Table IV since every reaction contained 0.4 g dry weight pulp in a volume of 20 ml.

Table V displays the results of this experiment -- brightness, lignin content and viscosity of the treated pulps. Replicates are grouped together. Samples 1 to 9 were assayed after one stage. Samples 10 to 17 were assayed after two stages. Samples 18 to 26 were assayed after three stages. Table V also displays the cumulative units of LiP and MnP applied to each pulp sample. For example, the value given for cumulative units of LiP for sample 26 (32.0) represents the sum of the units of LiP applied in stages one, two and three (20.0 + 8.0 + 4.0).

One stage treatment of hardwood Kraft pulp with VKM-F-1767 enzyme concentrate resulted in significant delignification and brightening of the pulp, compared with pulps from control samples that did not receive enzyme. The two stage treatments produced pulp with a lower lignin content and greater brightness than the one stage treatments. The three stage treatments worked even better than the two stage treatments. Decreased lignin content and increased brightness were obtained with acceptable decreases in viscosity.

TABLE IV

Units LiP And MnP Added Per Reaction						
Sample	STAGE ONE					
	LiP	MnP				
1	0.0	0.0				
2	0.0	0.0				
3	0.0	0.0				
4	5.0	7.7				
5	5.0	7.7				
6	10.0	15.4				
7	10.0	15.4				
8	20.0	30.8				
9	20.0	30.8				
			STAGE TWO			
			LiP	MnP		
10	0.0	0.0	0.0	0.0		
11	0.0	0.0	0.0	0.0		
12	0.0	0.0	0.0	0.0		
13	5.0	7.7	2.0	8.1		
14	10.0	15.4	4.0	16.1		
15	10.0	15.4	4.0	16.1		
16	20.0	30.8	8.0	32.3		
17	20.0	30.8	8.0	32.3		
					STAGE THREE	
					LiP	MnP
18	0.0	0.0	0.0	0.0	0.0	0.0
19	0.0	0.0	0.0	0.0	0.0	0.0
20	0.0	0.0	0.0	0.0	0.0	0.0
21	5.0	7.7	2.0	8.1	1.0	6.3
22	5.0	7.7	2.0	8.1	1.0	6.3
23	10.0	15.4	4.0	16.1	2.0	12.6
24	10.0	15.4	4.0	16.1	2.0	12.6
25	20.0	30.8	8.0	32.3	4.0	25.3
26	20.0	30.8	8.0	32.3	4.0	25.3

TABLE V

Sample	Stage	Cumulative Units Enzyme Added		Brightness (% G.E.)	Lignin Content (μkappa No.)	Viscosity (cp)
		LiP	MnP			
1	1	0.0	0.0	46	12.2	27.3
2	1	0.0	0.0	48	10.6	28.2
3	1	0.0	0.0	48	11.1	27.6
4	1	5.0	7.7	46	11.2	26.7
5	1	5.0	7.7	52	8.9	24.9
6	1	10.0	15.4	53	7.9	24.9
7	1	10.0	15.4	53	8.5	24.7
8	1	20.0	30.8	54	8.0	24.5
9	1	20.0	30.8	55	5.2	24.6
10	2	0.0	0.0	49	10.7	26.4
11	2	0.0	0.0	49	11.7	26.9
12	2	0.0	0.0	49	11.2	25.2
13	2	7.0	15.8	68	8.8	20.4
14	2	14.0	31.5	67	6.1	20.4
15	2	14.0	31.5	66	6.1	20.2
16	2	28.0	63.1	66	4.5	19.8
17	2	28.0	63.1	67	5.2	20.3
18	3	0.0	0.0	51	10.4	27.4
19	3	0.0	0.0	50	25.5	
20	3	0.0	0.0	51	10.0	25.5
21	3	8.0	22.1	75	4.3	20.1
22	3	8.0	22.1	76	4.3	20.0
23	3	16.0	44.1	76	4.3	18.7
24	3	16.0	44.1	76	4.3	18.8
25	3	32.0	88.4	76	4.7	23.3
26	3	32.0	88.4	76	4.0	18.3

EXAMPLE 4

Delignification Of Southern Softwood Kraft Pulp By A One, Two, Or Three Stage Treatment With VKM-F-1767 Unfractionated Enzyme Concentrate

Southern softwood Kraft pulp was subjected to a one, two, or three stage treatment with Phanerochaete chrysosporium-VKM-F-1767 unfractionated enzyme concentrate. The experiment was conducted as described in Example 3, with the modifications noted below.

Preparation of Pulp

The damp "washed" Southern softwood Kraft pulp had a consistency of 25.4%, a brightness of 25% G.E. and a μkappa number of 25.0.

Preparation of Enzyme Concentrate

The VKM-F-1767 unfractionated enzyme concentrate was prepared as described in Example 1, using roller bottles and Growth Medium A and was stored at -70 ° C until used. The same concentrate preparation was used for all reactions. The enzyme concentrate was thawed and its VAO activity measured immediately prior to its addition to the stage one reactions; this value is listed below. The listed value for PRO activity of the enzyme concentrate used in the stage one reactions represents the average of the PRO activity of the concentrate before freezing and the PRO activity of the concentrate as measured before stage two. After removing aliquots for use in the stage one reactions, the enzyme concentrate was stored overnight at 4 ° C. The enzyme concentrate was assayed again the next day, for both VAO and PRO activities, prior to its addition to the stage two reactions. The enzyme concentrate was again stored at 4 ° C until stage three. In this experiment, stage three was initiated on the same day as stage two. Because the lapse of time between the second and third stages was relatively brief, the concentrate was not assayed a third time immediately prior to the third stage. Therefore, the units of LiP and MnP listed in Table VII for the stage three reactions were calculated using values for VAO and PRO activities from the assays performed immediately prior to stage two. The values for the VAO AND PRO activities of the enzyme concentrate, used for calculation of enzyme units applied at each stage were:

Stage	VAO Activity (U/ml)	PRO Activity (U/ml)
1	12.0	23.8
2	11.2	22.5
3	11.2	22.5

Stage One

A 10x reaction mix of the following composition was prepared: 200 mM sodium acetate, pH 4.5; 0.5% Tween 80; 4.0 mM veratryl alcohol; 100 mM lactate; 1.0 mM manganese sulfate. This reaction mix was adjusted to pH 4.5 with sodium hydroxide. Reaction mix was added (2.0 ml per tube) to each of 11 reaction tubes and 6 control reaction tubes. Next, 1.6 g washed pulp (0.4 g dry weight pulp) were added to each tube. Double-distilled water was then added to each tube, in the volume necessary to give a final reaction volume of 20 ml per tube when all reaction components had been added. Glucose was added to each tube to give a final reaction concentration of 10.0 mM, excluding any glucose that may be contributed by the enzyme concentrate. The tubes were then vortexed to mix. Oxygen was bubbled through the contents of the tubes for three minutes. Enzyme concentrate was then added; Table VI displays the units of LiP and MnP added to each reaction tube. Finally, 20 µl of glucose oxidase (1.0 U/µl) were added to each tube. The final reaction volume in each tube was 20 ml. After the addition of glucose oxidase, the tubes were capped and vortexed gently to mix the contents and disperse the pulp.

The samples were incubated overnight. After incubation, the pulps from each reaction were washed with sodium hydroxide and then washed twice with water. Then the pulps from samples 1 to 6 were washed with acetic acid, allowed to air dry overnight, and assayed for brightness, lignin content and viscosity. Table VII displays the results of these analysis.

Stage Two

The pulps from samples 7 to 17 were subjected to another water wash with approximately 250 ml of distilled water. After this last wash, the pulps from these samples were again placed in reaction tubes and subjected to another incubation with enzyme concentrate, identically as described above for stage one. Table VI displays the units of LiP and MnP added to the stage two reactions.

The stage two reactions were incubated for five hours, as described in Example 3. The pulps from each reaction were then washed once with sodium hydroxide and twice with water, as described for stage one. Then the pulps from samples 7 to 11 were washed with acetic acid, allowed to air dry overnight, and assayed for brightness, lignin content and viscosity. Table VII displays the results of these analyses. Table VII displays the results of these analyses.

Stage Three

The pulps from samples 12 and 17 were subjected to another water wash with approximately 250 ml of distilled water. Then the pulps from samples 12 to 17 were again placed in reaction tubes and subjected to another incubation with enzyme concentrate, as described above for stage one. Table VI displays the units of LiP and MnP added to the stage three reactions.

The stage three reactions were incubated overnight. The pulps from each reaction were then washed with sodium hydroxide and water, as described for stage one. Then the pulps from samples 12 to 17 were washed with acetic acid, allowed to air dry overnight, and assayed for brightness, lignin content and viscosity. Table VII displays the results of these analyses.

Results

Table VI displays the units of LiP and MnP added per pulp sample at each stage. As in Example 3, units of LiP or MnP per gram of dry weight pulp and units per volume of reaction mixture may be calculated from the values in Table VI since every reaction contained 0.4 g dry weight pulp in a volume of 20 ml.

Table VII displays the results of this experiment -- brightness, lignin content and viscosity of the treated pulps. Replicates are grouped together. Samples 1 to 6 were assayed after one stage. Samples 7 to 11 were assayed after two stages. Samples 12 to 17 were assayed after three stages. Table VI also displays the cumulative units of LiP and MnP applied to each pulp sample.

One stage treatment of southern softwood Kraft pulp with VKM-F-1767 enzyme concentrate resulted in significant delignification of the pulp. The two stage treatments produced pulps with still lower lignin content. The three stage treatments worked even better than the two stage treatments. Although significant delignification was achieved with one stage and two stage treatments, the pulps were not brightened, probably because the lignin content had not been decreased sufficiently to cause brightening. However, after a three stage treatment, the lignin content was decreased sufficiently and brightening was observed. Pulp viscosity was not reduced by the enzymatic treatments.

TABLE VI

Units LiP And MnP Added Per Reaction						
Sample	STAGE ONE					
	LiP	MnP				
1	0.0	0.0				
2	0.0	0.0				
3	9.6	19.0				
4	9.6	19.0				
5	19.2	38.1				
6	19.2	38.1				
			STAGE TWO			
			LiP	MnP		
7	0.0	0.0	0.0	0.0		
8	0.0	0.0	0.0	0.0		
9	9.6	19.0	4.8	9.6		
10	9.6	19.0	4.8	9.6		
11	19.2	38.1	9.6	19.3		
					STAGE THREE	
					LiP	MnP
12	0.0	0.0	0.0	0.0	0.0	0.0
13	0.0	0.0	0.0	0.0	0.0	0.0
14	9.6	19.0	4.8	9.6	0.6	1.2
15	9.6	19.0	4.8	9.6	0.6	1.2
16	19.2	38.1	9.6	19.3	1.3	2.6
17	19.2	38.1	9.6	19.3	1.3	2.6

TABLE VII

Sample	Stage	Cumulative Units Enzyme Added		Brightness (% G.E.)	Lignin Content (μkappa No.)	Viscosity (cp)
		LiP	MnP			
1	1	0.0	0.0	36	23.5	
2	1	0.0	0.0	36	22.4	14.3
3	1	9.6	19.0	28	19.9	21.4
4	1	9.6	19.0	27	20.1	19.4
5	1	19.2	38.1	28	17.9	18.3
6	1	19.2	38.1	28	19.4	19.5
7	1	0.0	0.0	35	23.0	7.7
8	1	0.0	0.0	34	22.3	12.8
9	1	14.4	28.6	31	16.0	14.3
10	2	14.4	28.6	30	15.0	
11	2	28.8	57.4	30	16.4	15.9
12	2	0.0	0.0	33	20.1	
13	2	0.0	0.0	35	20.4	11.9
14	2	15.0	29.8	35	12.4	
15	2	15.0	29.8	40	10.2	14.2
16	2	30.1	60.0	40	10.1	13.9
17	2	30.1	60.0	43	8.7	14.3
Untreated washed pulp				25	25.0	

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Claims

1. A process for delignifying lignocellulosic material, comprising one or more enzymatic delignification stages characterized by the fact that each stage comprises the step of incubating the lignocellulosic material with an effective amount of a ligninolytic enzyme preparation, in a reaction mixture comprising hydrogen peroxide, at a steady-state concentration of about 0.001 to 0.5mM throughout the delignification reaction, and

(a) 0 up to 1.0 mM Mn (II);

(b) 0 up to 20 mM of an α-hydroxy acid;

(c) 0 up to 0.6 mM of a substituted aromatic alcohol capable of serving as a substrate for said ligninolytic enzyme; and

(d) 0 up to 0.1% of a detergent selected from the group consisting of nonionic and zwitterionic detergents.

2. The process according to claim 1 characterized by the fact that the substituted aromatic alcohol is in the amount of 0.005 to 0.5 mM.

3. The process according to claim 1 or 2, characterized by the fact that the lignocellulosic material is a wood pulp.

4. The process according to claims 1, 2 or 3 characterized by the fact that the hydrogen peroxide concentration is maintained at the steady-state level by the in situ enzymatic generation of hydrogen peroxide or by the metered or periodic addition of hydrogen peroxide.

5. The process according to anyone of the preceding claims characterized by the fact that the α-hydroxy acid is a lactate, the substituted aromatic alcohol is veratryl alcohol.

6. The process according to any one of the preceding claims characterized by the fact that the ligninolytic enzyme preparation is derived from a white rot fungus.

7. The process according to claim 6 characterized by the fact that the white rot fungus is a strain of Phanerochaete chrysosporium.

8. The process according to claim 7 characterized by the fact that the strain of Phanerochaete chrysosporium is a member selected from the group consisting of: SC26 (NRRL 15987), having the identifying characteristics of NRRL 15987; VKM-F-1767 (ATCC 24725), having the identifying characteristics of ATCC 24725; and ME-446 (ATCC 34531), having the identifying characteristics of ATCC 34531).

9. The process according to claim 6 characterized by the fact that the ligninolytic enzyme preparation is an unfractionated enzyme concentrate consisting of concentrated extracellular culture media of the white rot fungus.

10. The process according to claim 1 characterized by the fact that the ligninolytic enzyme preparation comprises at least one lignin peroxidase.

11. The process according to claim 10 characterized by the fact that the ligninolytic enzyme preparation comprises at least one Mn(II)-dependent peroxidase.

12. The process according to claim 1 characterized by the fact that the ligninolytic enzyme preparation consists essentially of one or more lignin peroxidases.

13. The process according to claim 1 characterized by the fact that the ligninolytic enzyme preparation consists essentially of one of more Mn(II)-dependent peroxidases.

14. The process according to claim 1 or 2 characterized by the fact that each enzymatic delignification stage comprises the further steps of:

(a) extracting the lignocellulosic material with alkali after the step of incubating with the ligninolytic enzyme preparation, and then

(b) extensively washing the lignocellulosic material with water.

15. The process according to claim 14 characterized by extracting the lignocellulosic material with a dilute acid solution after the step of extracting with alkali.

16. The process according to claim 1 comprising at least one conventional bleaching stage in addition to said one or more enzymatic delignification stages.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 89 11 0176

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	DE-A-3 636 208 (H.P. CALL) * Whole document *	1,3,6,7	D 21 C 3/00
X,D	US-A-4 687 741 (R.L. FARRELL et al.) * Whole document *	1,3,6-9	
X	ABSTRACT BULLETIN OF THE INSTITUTE OF PAPER CHEMISTRY, vol. 57, no. 7, January 1987, page 959, abstract no. 8578, Appleton, WI, US; V.-B. HUYNH et al.: "Oxidation of lignin model compounds by a manganese-dependent enzyme from phanerochaete chrysosporium as compared to chemically generated Mn(III)" * Whole document *	1,7,10-13	
A,D	US-A-4 687 745 (R.L. FARRELL et al.)		
A,D	US-A-4 690 895 (R.L. FARRELL et al.)		
			TECHNICAL FIELDS SEARCHED (Int. Cl.4) D 21 C

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	15-09-1989	SONGY O.M-L.A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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