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(54) Title: MULTI-COMPONENT SYSTEM FOR THE ENZYME-CATALYSED OXIDATION OF SUBSTRATES AND
METHOD FOR CARRYING OUT ENZYME-CATALYSED OXIDATION

(57) **Abrégé/Abstract:**

The invention relates to a multi-component system for carrying out mediator-dependent enzymatic oxidation, comprising an oxidation catalyst, an oxidant and a mediator and characterised in that a) the oxidation catalyst is chosen from the group comprising manganese oxidases; b) the oxidant is selected from the group comprising oxygen and oxygen-containing compounds; c) the mediator is selected from the group comprising compounds containing Mn ions.



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(54) Title: MULTI-COMPONENT SYSTEM FOR THE ENZYME-CATALYSED OXIDATION OF SUBSTRATES AND METHOD FOR CARRYING OUT ENZYME-CATALYSED OXIDATION (54) Bezeichnung: MEHRKOMONENTENSYSYSTEM ZUR ENZYMKATALYSIERTEN OXIDATION VON SUBSTRATEN SOWIE VERFAHREN ZUR ENZYMKATALYSIERTEN OXIDATION (57) Abstract The invention relates to a multi-component system for carrying out mediator-dependent enzymatic oxidation, comprising an oxidation catalyst, an oxidant and a mediator and characterised in that a) the oxidation catalyst is chosen from the group comprising manganese oxidases; b) the oxidant is selected from the group comprising oxygen and oxygen-containing compounds; c) the mediator is selected from the group comprising compounds containing Mn ions. (57) Zusammenfassung Mehrkomponentensystem zur mediatorabhängigen enzymatischen Oxidation umfassend einen Oxidationskatalysator, ein Oxidationsmittel, und einen Mediator, dadurch gekennzeichnet, daß a) der Oxidationskatalysator aus der Gruppe der Manganoxidasen ausgewählt ist, b) das Oxidationsmittel aus der Gruppe Sauerstoff und sauerstoffhaltige Verbindungen ausgewählt ist, c) der Mediator aus der Gruppe der Verbindungen, die Mn-Ionen enthalten, ausgewählt ist.		

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Multicomponent system for enzyme-catalyzed oxidation of substrates and process for the enzyme-catalyzed oxidation

The present invention relates to a multicomponent system for mediator-dependent enzyme-catalyzed oxidation of substrates and a process for the enzyme-catalyzed oxidation.

Multicomponent systems for mediator-dependent enzyme-catalyzed oxidation of substrates and corresponding processes are known in principle. Mediators in this context are generally those compounds which can be oxidized by oxidoreductases, that is firstly also substrates of enzymatic oxidation catalysts. Mediators are particularly distinguished by the oxidized form of the mediator (the activated mediator, for example a mediator free radical or a mediator cation) having a sufficiently long life in order to diffuse from the oxidoreductase to the actual substrate of the oxidation system and to interact with this. In the interaction between activated mediator and substrate the substrate is oxidized by the mediator. By the oxidation of the substrate the mediator can either be regenerated (catalytic oxidation system) or inactivated (stoichiometric oxidation system). If the mediator is regenerated, it is available for a new catalysis

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cycle. The electrons transferred from the substrate to the oxidoreductase by the mediator are transferred to terminal electron acceptors such as oxygen or peroxide in direct enzymatic oxidation processes.

In the case of processes where peroxide serves (directly or released from precursors) as terminal electron acceptor, only peroxidases can be used as oxidoreductases. Although a number of such processes are known, they are burdened with numerous disadvantages:

Peroxidases are too expensive in manufacture in order to be used in industrial processes such as paper bleaching or in laundry detergents. In addition, the addition of peroxidases is a problem, since, in the concentrations necessary for the efficacy of the process, peroxide inactivates the peroxidase. Many of the compounds described as mediators for peroxidases cannot be used industrially because they are too intensively colored, are toxicologically or ecotoxicologically harmful, are not biodegradable or only poorly biodegradable and are not sufficiently available in sufficient amounts or cheap enough. Processes in which peroxide serves as terminal

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electron acceptor have therefore not been suitable to date for economic expedient industrial use.

In the case of processes in which oxygen acts as terminal electron acceptor for the electrons originating from the substrate to be oxidized, oxidases are used as oxidoreductases. Two groups of oxidases are described, which can be used in mediator-dependent enzymatic oxidation processes: laccases (Boubonnais & Paice (1990) FEBS LETTERS Vol 267 (1), p. 99 - 102, WO 94/29510) and tyrosinases (WO 94/29510). In contrast to the case with peroxidases, in which a prosthetic heme group acts as redox-active center, the electron transfer in the case of laccases and tyrosinases is catalyzed by four copper ions coordinating with four corresponding copper-binding domains of these oxidases ('blue copper' oxidases). A total of four electrons are sequentially taken up from mediators which are then transferred in a four-electron transfer to molecular oxygen. The oxygen is reduced to water, and the oxidase is regenerated for a new reaction cycle. A number of mediators which catalyze the electron transfer from the substrate to be oxidized to the oxidases

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are described, for example, in WO 94/29510, WO 97/06244, WO 96/10079 or WO 95/01426.

It is known to use these mediator-dependent enzymatic oxidation processes involving oxidases and oxygen for coal liquefaction (WO 94/29510), for wood pulp bleaching (WO 94/29510), for organic synthesis (Potthast et al. (1995) J. Org. Chem., Vol. 60, p. 4320 - 4321), as a bleaching system in laundry detergents and dishwashing detergents (WO 97/06244), for denim bleaching (WO 96/12846), for preventing dye transfer during washing (WO 98/23716) or for breaking down polycyclic aromatic hydrocarbons (Johannes et al. (1996) Appl. Microbiol. Biotechnol., Vol. 46, p. 313 - 317).

However, since the mediators have properties which stand in the way of industrial use, the enzymatic oxidation processes which use oxidases also have serious disadvantages:

- The mediator HBT which is described as particularly effective in WO 94/29510 is not biodegradable (Amann (1997) 9th Int. Symp. on Wood and Pulp. Chem., F4-1 - F4-5).

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- Many mediators such as HBT and violuric acid in their active form inactivate the oxidases used (Amann (1997) 9th Int. Symp. on Wood and Pulp. Chem., F4-1 - F4-5) and therefore require high enzyme usage in the respective process.

- A group of very active mediators is characterized by an N-O group and thus contains at least one N atom per mediator molecule (WO 94/29510). From this there result the problems known with the use of N-O-containing compounds with regard to toxicity and biodegradability. Biological wastewater purification systems in the papermaking pulp industry can, in addition, frequently not process additional N loads.

- Some of the mediators, for example ABTS, form intensively colored free-radicals and therefore lead to an unwanted discoloration of the substrate to be oxidized.

- Most of the mediators contain N or S atoms and are therefore relatively expensive to manufacture.

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These disadvantages have also limited to date wide industrial use of such systems for oxidizing substrates.

Archibald & Roy (Archibald & Roy (1992) Appl. Environ. Microbiol. Vol. 58, p. 1496 - 1499) described a process in which a redox cascade consisting of phenols and Mn^{2+}/Mn^{3+} acts as mediator between oxidase and the substrate to be oxidized. As terminal electron acceptor, the system uses oxygen. The oxidase used is a laccase. The primary substrate oxidized by the laccase is initially phenols such as m-hydroxybenzoic acid or m-cresol. In the presence of complexing agents such as pyrophosphate ions, secondary oxidation of Mn^{2+} to Mn^{3+} can then take place with the participation of the oxidized phenols. For this system, it could not be demonstrated that nonphenolic substrates could also be oxidized. Also, with a system in which only laccase, Mn^{2+} and a complexing agent was used for lignin oxidation, delignification could not be found. This mediator-dependent enzymatic oxidation system does not represent technically any significant improvement compared with the other mentioned systems, because, instead of the mediators, it uses a combination of phenols, complexing agents and Mn ions and the obligatory use of

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phenolic mediators hinders the use of the process for toxicological reasons. In addition, the long reaction times of > 15 h hinder practical use of this system.

The object of the present invention was to provide a multicomponent system for a mediator-dependent enzymatic oxidation of substrates which does not have the disadvantages of the known multicomponent systems and therefore makes possible simple and inexpensive conversion of the substrate to be oxidized.

The object is achieved by a multicomponent system comprising an oxidation catalyst, an oxidizing agent, and a mediator, characterized in that

a) the oxidation catalyst is selected from the group of manganese oxidases,

b) the oxidizing agent is selected from the group consisting of oxygen and oxygen compounds,

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c) the mediator is selected from the group of compounds which contain Mn ions.

Preferably, the inventive multicomponent system comprises a complexing agent which is selected from the group of complexing agents that can complex Mn ions.

For the purposes of the present invention, manganese oxidases are to be understood as those oxidases which oxidize Mn or Mn ions directly and which transfer the resulting electrons to oxygen. Preferably, such oxidases are able to oxidize Mn^{2+} to Mn^{3+} in the presence of oxygen and complexing agents. Preference is given to manganese oxidases which contain copper ions as the redox-active catalytic group.

Manganese oxidases can be isolated, for example, from known microorganisms. For this, such microorganisms are grown under conditions under which they form manganese oxidases. In the inventive composition, the manganese oxidases used can be in the simplest case enzymatically or chemically disrupted cell preparations of the complete manganese oxidase-containing microorganisms. However, it is also possible to

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use manganese oxidase-containing culture supernatants or isolated manganese oxidases.

Manganese oxidases are formed, for example, by *Leptothrix discophora*, *Bacillus SG-1* and *Pseudomonas* sp. (Nealson et al. (1989) Beveridge & Doyle (eds.) Metal ions and bacteria, John Wiley and sons, Inc., New York, pp. 383-411). The genes coding for manganese oxidases from *Leptothrix discophora* and *Bacillus SG-1* are cloned and sequenced (Corstjens et al. (1997) Geomicrobiol. Journal, Vol. 14, p. 91-108 and van Waasbergen et al. (1996) Journal of Bacteriology, Vol. 178 (12), p. 3517 - 3530). Both genes code for proteins which contain the copper-binding sequences known from 'blue copper' oxidases and their manganese oxidase activity is increased by adding copper.

In addition to said manganese-oxidase-producing microorganisms, other microorganisms can be used as a source of manganese oxidases. Thus, for example, in the case of microorganisms where the manganese oxidase is accumulated in the spore, for example *Bacillus SG-1*, the isolated spores can also be used in the inventive process as enzyme source.

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In addition, manganese oxidases can be used which are produced by recombinant production processes. Recombinant production processes are taken to mean in this case all processes in which the genes coding for manganese oxidases are isolated from the natural producers and have then been introduced by known methods into suitable production strains, which can also be the original enzyme producers.

Manganese ions act as mediators in the inventive composition. These manganese ions can be added to the process in any oxidation state.

Preferably, manganese ions of oxidation state +2 or +3 are used.

Manganese of oxidation state +2 is preferably used in the form of manganese sulfate or manganese chloride.

Manganese of oxidation state +3 is preferably used in the form of soluble manganese complexes. Examples of such manganese complexes are manganese/formate, manganese/lactate, manganese/oxalate or manganese/ mannolate [sic].

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The mediator (for example the Mn^{3+} ion) takes up one electron from the substance to be oxidized. This oxidizes the substance, and the Mn ion itself is reduced (for example to Mn^{2+} ion). In this form the Mn ion transports the electron which is taken up to the manganese oxidase, releases it there and is oxidized back to the initial oxidation state (for example to the Mn^{3+} ion). Oxygen acts as oxidizing agent for the manganese oxidase and thus as terminal electron acceptor.

The oxygen can be produced directly in the reaction by means of known chemical or enzymatic oxygen generating systems. It can also be produced by electrical hydrolysis of water, or it can be used directly in the gaseous or liquid state.

Preferably, oxygen is used either directly in the gaseous state or in the form of liquid oxygen or as oxygen-containing gas mixture, for example air. Particularly preferably, oxygen is introduced as oxygen-containing gas mixture, for example air.

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Preferably, the Mn^{3+} , which is formed by direct oxidation of Mn^{2+} by manganese oxidases, acts in the presence of a complexing agent.

Complexing agents which are suitable are preferably compounds which complex Mn^{3+} and thus stabilize it.

Preferably, complexing agents are used which do not contain nitrogen, are readily biodegradable and are toxicologically harmless. Such complexing agents are, for example, formate, lactate, malonate or oxalate.

The inventive system has the following advantages compared with known systems:

- The problem of inactivation of the oxidoreductases, as occurs, for example, when peroxide is used as electron acceptor, does not occur in the case of the inventive composition, since oxygen acts as oxidizing agent and thus as terminal electron acceptor.

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- Oxygen can be added in a technically simple manner and in sufficient amounts.
- Manganese oxidases, as is known, do not require prosthetic groups, for example heme groups, for their activity, in contrast to peroxidases and also manganese peroxidases. Thus manganese oxidases are to be prepared in customary production systems on an industrial scale without problems.
- The inventive system does not require N-containing or S-containing mediators or colored or poorly biodegradable or toxic mediators. The only redox-active mediator acting is manganese ions, which alternate between two oxidation states, preferably the oxidation states 2^+ and 3^+ . An inactivation of this manganese mediator cannot take place by a chemical modification, for example, in contrast to the inactivation of known mediators.

The inventive composition may be used for oxidizing the most varied substrates. Preferably, it may be used to oxidize those substrates which can be oxidized by Mn ions.

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Particularly preferably, it may be used for oxidizing those substrates which can be oxidized by Mn^{3+} ions.

The inventive multicomponent system is suitable, for example, as a bleaching system in laundry detergents and dishwashing detergents, for wood pulp bleaching, for denim bleaching, for treating wastewater, for organic synthesis, for preventing dye transfer during washing or for breaking down polycyclic aromatic hydrocarbons. A further potential use is, for example, coal liquefaction.

Corresponding processes are described in the prior art for mediator-dependent enzymatic oxidation processes using oxidases and oxygen.

It is easy for a person skilled in the art to modify the respectively known processes with use of the components and process conditions specified in the present application and to use the inventive composition for said purposes.

The present invention further relates to a process for oxidizing a substrate, characterized in that a manganese

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oxidase, in the presence of oxygen and, if appropriate, a complexing agent for Mn ions, forms Mn^{3+} by direct Mn^{2+} oxidation, the Mn^{3+} ion oxidizes the substrate, itself being reduced to Mn^{2+} and, in turn, being available for direct oxidation by the manganese oxidase.

In the inventive process, the substrate is preferably used in the form of an aqueous solution, mixture or suspension.

In the inventive process, preferably, between 0.001 and 50 milligrams of active manganese oxidase per liter of reaction volume are used.

The manganese oxidase is preferably introduced into the reaction mixture in granular form, as a solution, as a suspension or with a support material.

Particularly preferably, the manganese oxidase is introduced into the reaction mixture in the form of a suspension which comprises between 0.5 and 50 percent by weight of enzyme in a nonionic detergent.

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In the inventive process, preferably, manganese of the oxidation states Mn^{2+} or Mn^{3+} is used as redox mediator. Manganese ions of oxidation state 2+ or 3+ can be used directly in this oxidation state, or can be produced in the process from manganese ions of other oxidation states, such as Mn^{4+} or Mn^{7+} .

Manganese of oxidation state +3 is preferably added in the form of soluble manganese complexes such as manganese/formate, manganese/lactate, manganese/ oxalate or manganese/mannolate [sic].

Preferably, in the inventive process, manganese of oxidation state +2 is used. Particularly preferably, manganese sulfate or manganese chloride is used.

In the inventive process, manganese ions are used at concentrations between 0.005 mM to [sic] 50 mM. Preferably, manganese ions are used at concentrations between 0.05 mM and 5 mM, and particularly preferably at concentrations between 0.1 mM and 1 mM.

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Depending on the specific application, the required manganese may already be present in the substrate to be oxidized. One example of an inventive process in which generally no external manganese ions need to be added is wood pulp bleaching. Wood and the pulp obtained therefrom frequently already naturally contain a sufficiently high amount of manganese ions for the inventive oxidation process.

In the inventive process, oxygen is preferably used at a partial pressure of 0.05 - 5 bar. Particularly preferably, oxygen at a partial pressure from 0.1 to 2.5 bar is used. In particular preferably, oxygen at a partial pressure of from 0.2 to 1 bar is used.

In the inventive process, said complexing agents are preferably used at concentrations between 1 mM to [sic] 500 mM. Preferably, suitable complexing agents are used at concentrations between 5 mM and 100 mM, and particularly preferably at concentrations between 10 mM and 50 mM.

The inventive oxidation process can be used for oxidizing all compounds which can be oxidized by Mn^{3+} ions.

Inventive processes can be used, for example, for the oxidation of lignin in papermaking and wood pulp production, as oxidation system in laundry detergents and dishwashing detergents, for enzymatic dye bleaching, for enzymatic textile bleaching, as a system for preventing dye transfer during washing, for the specific oxidation of organic target molecules in organic synthesis, for oxidative wastewater treatment, for degrading chlorinated hydrocarbons, for cleaving polycyclic aromatic hydrocarbons and for liquefying coal.

The examples below serve for further illustration of the invention.

Example 1:

Isolation of manganese oxidase-producing microorganisms

1.a. Isolation of manganese-oxidizing microorganisms:

Manganese-oxidizing microorganisms, particularly fungi and bacteria, were isolated on indicator plates by the method of Krumbein & Altmann (Krumbein & Altmann (1973) Helgoländer wiss. Meeresunters., Vol. 25, pp. 347 - 356).

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In the presence of manganese ions of oxidation states Mn^{3+} - Mn^{7+} , Berbelin blue forms an intensive blue dye at pH 4 - 7. At an elevated concentration of Berbelin blue, in the presence of suitable Mn ions the blue oxidation product is formed up to pH 10.

For the plate screening, colorless Berbelin blue (N, N'-dimethylamino-p,p'-triphenylmethane-o''-sulfonic acid) was added to nutrient media. At concentrations of 10^{-4} - 10^{-5} g of Berbelin blue per 100 ml of nutrient media no inhibition of bacterial growth occurred.

To isolate heterotrophic manganese oxidizing bacteria, the following medium was used:

3.5 g/l Difco-Bacto-Peptone

0.8 g/l $MnSO_4 \cdot H_2O$

100 mg/l $FeSO_4 \cdot 7 H_2O$

15 g/l Difco-Bacto-Agar

750 ml of seawater

245 ml of distilled water

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10 ml of Berbelin blue stock solution (4 g/l)

pH 7.6

Bacteria-containing samples such as seawater samples, sediment samples, soil samples, soils from ore deposits, etc., were applied to such indicator plates at a suitable dilution or concentration such that, per indicator plate, between 100 and 300 individual colonies grew.

Bacteria which, after 5 to 10 days of incubation on such indicator plates, formed blue colonies (i.e. the manganese-oxidizing activity was cell-associated) or had blue haloes around the colonies (i.e. manganese-oxidizing enzymes were secreted into the culture supernatant), were analyzed further.

1.b. Detection of manganese oxidases in Berbelin blue-oxidizing microorganisms

Microorganisms which were isolated as described in 1a can produce manganese oxidases, but the blue coloration could also have been caused by manganese peroxidases or by

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oxidation of Fe^{2+} . The specific detection of manganese oxidase activity was carried out as described by Boogerd & de Vrind (Boogerd & de Vrind (1987) J. Bacteriol., Vol. 169 (2), pp. 489 - 494):

First, the microorganisms under test were each grown for 10 days in 1 l of the medium used for isolation without agar or Berbelin blue addition. The cells were then removed by centrifugation (15 min, 1 000 x g, 4°C). In the case of cells which formed blue colonies on the indicator plates described in example 1a, the cell pellet was used for the gel electrophoresis described below. In the case of cells which formed blue haloes on indicator plates, the cell-free culture supernatant was concentrated by ultrafiltration to one fiftieth of its initial volume on a UF membrane having a retention capacity for particles > 10 000 D. The concentrate or the cell pellet was mixed with the same volume of a buffer (0.125 M Tris/Cl pH 6.8, 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue). 50 µl aliquots of such a mixture were electrophoretically separated on a 10% polyacrylamide gel. After the electrophoresis the gel was washed four times for 15 min with deionized water. The gel

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was then incubated for 2 h in 100 μM MnCl_2 in 10 mM Hepes pH 7.5. In samples which contained the manganese oxidases, during this procedure, at the point at which after the electrophoresis the manganese oxidases were localized in the gel, a brown precipitate of manganese oxide formed. The manganese oxide formation thus observed was caused in each case by manganese oxidases directly oxidizing Mn^{2+} , because no peroxide was present and no ions other than Mn^{2+} were present in the mixture.

Example 2:

Production and isolation of manganese oxidases

2.a. Manganese oxidase-containing spores from *Bacillus*

By the method described in example 1, *Bacillus* isolates were isolated which had incorporated manganese oxidase into their spores. For industrial uses, such manganese oxidase-containing spores were used directly or the manganese oxidase-containing spore coats were prepared and used. Such spores or spore coats were produced as follows:

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Growth:

To produce manganese oxidase-containing spores, a suitable *Bacillus* strain was grown aerobically at 25°C in the following medium:

2 g/l of peptone (Difco), 0.5 g/l of yeast extract (Difco), 10 µg/l of iron-EDTA, 100 µg/l of sterile-filtered $\text{MnCl}_2 \times 4 \text{ H}_2\text{O}$ in 50 mM Tris in 80% natural seawater, pH 7.0.

After a growth time of 10 days, > 95% of all cells had sporulated.

Spore preparation:

The spores were harvested by centrifugation (30 min 10 000 x g, 4°C), washed with deionized water and suspended in 10 mM Tris/Cl pH 7.0 (0.1 g/ml). For a preparation of the spore coat, this material was further processed as described below. If the spores were used directly in the inventive process, these were further treated as follows:

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50 µg/ml of lysozyme were added to the suspension, the mixture was incubated for 30 min at 37°C, then the spores were washed with 1M NaCl, 0.15 M NaCl, 0.1% SDS and five times with deionized water. Spores purified in this manner were stored at 4°C in deionized water until use in the inventive process.

Preparation of the spore coats:

The entire procedure was carried out at room temperature, unless stated otherwise. Except for the 1% SDS solution and the deionized water used for washing, all solutions contained 10 mM EDTA, pH 7.5 and PMSF (0.3 percent by weight). The purified spores were suspended as described above in 10 mM Tris pH 7.0 (0.1 g/ml) and the same volume of glass beads (diameter 10 - 50 µm) was added. The suspension was then treated for 15 min in 30 sec intervals at 0°C with an ultrasonic cell disrupter (Sonifier) at maximum amplitude. After the treatment the suspension was incubated on ice for 5 min; the supernatant was then taken off from the settling glass beads. The glass beads were then washed twice with a volume of 10 mM Tris pH 7.0 corresponding to their own

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volume. The first supernatant and the supernatants from the washing procedure were combined and centrifuged for 15 min at 15 000 g. The supernatant was then taken off, the precipitate was suspended in 10 mM Tris pH 7.5 and treated for 30 min at 37°C with lysozyme (100 µg/ml). The mixture was again centrifuged for 15 min at 15 000 g. The precipitate containing the spore coats was washed with: 1 M NaCl, 0.14 M NaCl, 1% SDS and 5 times with deionized water.

2.b. Manganese oxidase from *Leptothrix discophora*

Sheath-forming organisms such as *Leptothrix discophora* - isolates which produce a manganese oxidase, can be obtained by the method described in example 1. Such isolates can also be obtained from commercial strain collections (*Leptothrix discophora* ATCC 51168, *Leptothrix discophora* ATCC 51169). Those which are particularly suitable for producing manganese oxidase are derivatives of *Leptothrix discophora* which have lost the ability to form sheaths. Such derivatives arise spontaneously when sheath-forming strains are cultured continuously and over a relatively long time under laboratory conditions (Adams & Ghiorse (1986) Arch. Microbiol., Vol.

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145, pp. 126 - 135). Such derivatives which no longer form sheaths secrete manganese oxidases into the culture medium.

The example below describes the production and isolation of manganese oxidases into, and out of, the culture supernatant of derivatives of *Leptothrix discophora* which can no longer form sheaths and are deposited in accordance with the Budapest Convention at the DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, D-38124 Brunswick) under the number DSMZ 12667:

Culture:

Leptothrix discophora, to produce manganese oxidase, was cultured in a medium which contained the following constituents:

0.25 g/l Difco-Peptone
0.25 g/l Difco yeast extract
0.25 g/l glucose
0.6 g/l $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$
0.07 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

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0.015 g/l $\text{MgSO}_4 \cdot \text{H}_2\text{O}$

per liter of deionized water.

Before autoclaving the medium, the pH was set to pH 7.6 with 1 M NaOH.

To produce manganese oxidase, 45 l of the medium described were inoculated with two liters of a *Leptothrix discophora* preliminary culture in the same medium. Culture was performed with aeration (0.2 volumes of air per unit volume medium and minute) at 26°C for 40 hours.

Enzyme preparation from culture supernatant:

After 40 hours culture was ended. The cells of *Leptothrix discophora* were separated from the culture supernatant by centrifugation. The cell-free, manganese oxidase-containing culture supernatant was concentrated to 0.5 l by ultrafiltration on a UF membrane having a retention capacity for particles > 10 000 D. The concentrated culture supernatant was used as manganese oxidase source for the examples described below.

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Example 3:

Quantitative determination of the activity of manganese oxidases using N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)

Mn³⁺ ions are able to oxidize the colorless compound TMPD. The oxidation product 'Wuster blue' has an intensive blue color, and the increase in this color can be followed photometrically at a wavelength of 610 nm.

Procedure:

A TMPD stock solution of 2.1 mM TMPD in distilled water was prepared freshly before each measurement. The enzyme preparation to be assayed was diluted in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH7.5) so that in the assay of activity described below, an OD 610 between 0.5 and 1.5 was measured after 10 minutes of assay time.

The manganese source used was a solution of 10 mM MnSO₄ in distilled water.

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To determine the manganese oxidase activity in enzyme samples, in parallel, 3 ml aliquots of the enzyme dilutions in HEPES (see above) were mixed with 0.3 ml of TMPD stock solution. To one of each of the parallel assay solutions, at a time point 0 min, 10 μ l of the MnSO_4 stock solution were added. Both samples were mixed and incubated for 10 min at 40°C. After 10 min the samples were briefly centrifuged (15 sec; 5 000 rpm), then the OD610 was determined in 1 ml of each of the MnSO_4 -containing supernatants. The reference used was the comparison sample without MnSO_4 . An increase in absorption at 610 nm by 1.0 is equivalent to the formation of 100 μ M of 'Wuster blue'. One unit (1U) of manganese oxidase was defined as the amount of enzyme which leads to the formation of 1 μ M of 'Wuster blue' from TMPD in 1 min.

For the subsequent assay, in each case concentrated manganese oxidase-containing culture supernatants from example 2 b and 50 μ l of the spore coat suspension from example 2 a were used. The following results were obtained:

Enzyme from	OD610
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Leptothrix	1.03
discophora	
Bacillus	0.85

Example 4:

Oxidation of the homogeneously available substrate veratryl alcohol as a function of varying manganese concentrations and complexing agents

Procedure:

A stock solution of 0.25 g/ml of the substrate veratryl alcohol (3,4-dimethoxybenzyl alcohol (Aldrich)) in ethanol was prepared.

The oxidation reaction was carried out at 45°C with gentle stirring. For this MnSO_4 was added (0.00, 0.05, 0.50 mM MnSO_4) was added to 22.23 ml in each case of a solution containing a complexing agent (50 mM formate, lactate, malate or oxalate; in each case pH 7.5). Then, 0.268 ml of the veratryl alcohol stock solution was added in each case. The mixture was equilibrated at 45°C for 10 min, then the reaction was started by adding 2.5 ml of enzyme solution

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(manganese oxidase from *Leptothrix discophora*, 10 U/ml). After 8 h the reaction was studied by HPLC analysis for the formation of veratryl aldehyde. For this, 0.4 ml of an H_2SO_4 solution (0.5 mol/l) was added to 0.8 ml of assay solution. 20 μl of this sample were applied to a LiChrospher 60 RP Select B separation column (Merck 50940) and eluted with a mixture of $\text{H}_2\text{O}/\text{MeOH}$ (65:35). The flow rate was 1 ml/min. The products in the eluate were detected by a UV detector at 275 nm.

The table below shows in % the amount of veratryl alcohol oxidized to veratryl aldehyde in 8 h under the conditions described in the process of the invention:

Complexing agent	MnSO_4 [mM]	Without Mn oxidase [% v. aldehyde]	With Mn oxidase from <i>L. discophora</i> [% v. aldehyde]
50 mM formate	0	0.00	0.00
50 mM formate	0.05	0.01	7.24
50 mM formate	0.5	0.00	12.46
50 mM lactate	0	0.00	0.00
50 mM lactate	0.05	0.00	3.32
50 mM lactate	0.5	0.02	2.79

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50 mM malonate	0	0.00	0.00
50 mM malonate	0.05	0.03	14.23
50 mM malonate	0.5	0.02	22.96
50 mM oxalate	0	0.00	0.00
50 mM oxalate	0.05	0.06	17.30
50 mM oxalate	0.5	0.07	21.44

Example 5:**Wood pulp bleaching**

Oxygen-delignified softwood kraft pulp having a lignin content of kappa 16.5 was washed and brought to a pulp density of 5% with 30 mM Na oxalate buffer (pH 7.5). M_gSO_4 (0.5 mM) was then added. The suspension was homogenized for 60 sec using a suitable stirrer. Then, in two parallel solutions, 5 U of manganese oxidase (Leptothrix, Bacillus) were added in each case per g of pulp. A third solution without enzyme served as control. The solutions were homogenized again for 60 sec and introduced into a controlled-temperature steel autoclave which permitted gas introduction. The autoclave was sealed, an oxygen pressure of 3 bar was applied and the autoclave was incubated at 45°C for 4 h. The reaction solution was then discharged. The pulp was washed with 10 volumes of flowing water at 50°C, then lignin

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fragments were extracted under alkaline conditions from the pulp using an aqueous NaOH solution at 70°C. 20 g of NaOH were used per kg of pulp for the extraction. After the extraction the pulp was again washed with 10 volumes of flowing water at 50°C. The lignin content of the pulp samples was finally determined via the kappa value according to standardized test method SCAN-C 1:77 of the 'Scandinavian Pulp and Paper Board':

Enzyme	Kappa value	Delignification in %
Control without enzyme	15.2	7.9
Leptothrix discophora	13.4	18.8
Bacillus spore coats	10.7	35.1

Compared with the control sample, when the inventive oxidation process is used, an additional delignification between 10.9 and 27.2% was achieved.

Example 6:

Suppression of dye transfer

The experiments to demonstrate the suppression of dye transfer by inventive oxidation systems were carried out in

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heatable glass beakers (100 ml in volume). The control used was a solution in which 0.25 g of a commercial detergent had been dissolved in 50 ml of water. The experiments with manganese oxidase were carried out in 50 ml of a 30 mM Na malonate buffer (pH 7.5) containing 0.5 mM MnSO_4 . The 50 ml assay solutions were first heated to 45°C, then 0.5 U/ml of manganese oxidase was added where appropriate in each case to the buffer solutions. The cotton textile samples (5 g in each case) were added to the assay solutions, then 10 ml of a solution of 15 μM Cibacron Marine C-B (Ciba), preheated to 45°C, were added as test dye to each assay solution. After incubation for one hour at 45°C, the textile samples were removed from the assay solutions and each washed with 1 l of flowing water at 50°C. The textile samples were then washed and dried. The brightness of the initial samples and the treated samples was then determined spectroscopically:

In the table the brightnesses of the treated cotton samples is compared with an untreated sample:

Enzyme	Malonate buffer / MnCl_2	Water/ detergent
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none	73.3%	89%
Leptothrix discophora	89.2%	-
Bacillus	92%	-

The values shown in the table indicate that the inventive oxidation system is suitable for preventing transfer of the added dye to the cotton samples. The effect is comparable in quality to the effect achievable with commercial detergents.

Example 7:**Stain bleaching (laundry detergent application)**

In order to demonstrate the stain-bleaching action of the inventive process, washing experiments were carried out. For this, standardized soiling was produced by applying dropwise 0.3 ml of an aqueous solution of 100 ppm of Evan's blue (obtainable from Wako Pure Chemical Industry Co., Osaka) in each case to white cotton samples (7.5 cm x 7.5 cm). These textile samples were then treated according to the invention with manganese oxidase, MnSO_4 and suitable complexing agent in the presence of oxygen in heatable glass beakers (100 ml volume) with stirring (magnetic stirrer). The control used

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was assay solutions in which a) only malonate buffer was used, or b) no MnSO_4 was added, or c) no manganese oxidase was added:

The experiments were carried out in 50 ml of a 30 mM Na malonate buffer (pH 7.5) with or without 0.5 mM MnSO_4 . The 50 ml test solutions were first heated to 45°C, then 1 U/ml of manganese oxidase from *Bacillus* or from *Leptothrix* was added to each of the test solutions which were to contain manganese oxidase. The soiled cotton samples were added to the test solutions. After incubation for one hour at 45°C, the textile samples were removed from the test solutions and washed in each case with 1 l of flowing water at 50°C. The textile samples were then dried.

To determine the differences in color of the samples compared with the control sample without manganese oxidase and MnSO_4 , the Y, y and x values of the air-dried cotton samples were measured using a color difference meter (CR-200, Minolta). To assess the bleaching effects of the inventive processes, the Z value ($Z = (1-x-y)Y/y$) was determined from the resulting values.

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The table shows the whitenesses of the measured samples compared with the whiteness of the control without enzyme and without MnSO_4 :

	+ MnSO_4	- MnSO_4
Bacillus	2.7	-0.3
Leptothrix	1.6	0.1
no enzyme	0.2	0.0

It can be seen from the table that the whiteness of samples containing manganese oxidases and MnSO_4 was increased by between 1.6 and 2.7 points compared with samples without enzyme and without MnSO_4 .

Example 8:

Organic synthesis

The examples shown below demonstrate that an inventive process is suitable for the targeted synthesis of organic substances.

a) Oxidation of alcohol groups to aldehydes

269 mg (1.6 mmol) of 3,4-dimethoxybenzyl alcohol in 1 ml of ethanol were added at 45°C to 22 ml of a 30 mM malonate

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solution pH 7.5 containing 0.5 mM MnSO_4 . After incubation for 10 min, the manganese oxidase (40 U) was added. After a reaction time of 24 h, the reaction solution was extracted with chloroform and studied by NMR spectroscopy. In the case of manganese oxidase from *Leptothrix*, the yield was 32% 3,4-dimethoxybenzaldehyde, and in the case of manganese oxidase from *Bacillus*, the yield was 27% 3,4-dimethoxybenzaldehyde.

b) Oxidation of alcohols to ketones

196 mg (1.6 mmol) of 1-phenylethanol were added at 45°C to 22 ml of a 30 mM malonate solution pH 7.5 containing 0.5 MnSO_4 . After incubation for 10 min, the manganese oxidase (40 U) was added. After a reaction time of 24 h, the reaction solution was studied by HPLC. In the case of manganese oxidase from *Leptothrix*, the yield was 17% acetophenone, and in the case of manganese oxidase from *Bacillus*, the yield was 19% acetophenone.

In both examples, the inventive oxidation process was successfully used for the synthesis of defined substances. The substrates used are intended here only to demonstrate the oxidation capacity of the process in an exemplary manner and not to restrict the region of synthesizable products.

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Example 9:**Denim bleaching**

Pieces of dyed denim fabric cut into squares (9 g / 160 cm²) were incubated together with manganese oxidase, 0.2 mM MnSO₄ and 15 mM complexing agent (oxalate) in a closed 500 ml beaker in a total liquid volume of 11.5 ml at 45°C. The pH of the test solutions was 7.0 (15 mM oxalate). 10 U of manganese oxidase were added per gram of textile. After incubation for 4 hours, the textile pieces were washed under flowing water until the wash water was colorless. The textile pieces were dried in a sheet dryer, then pressed and optically assessed using a suitable spectrophotometer.

The degree of bleaching was determined using a CM 3700d spectrophotometer (Minolta) in accordance with the manufacturer's instructions. Measurements were made without gloss and without UV. The brightness of the samples was determined as the percentage of total reflection compared with a whiteness standard (R 457). L* is the measure of brightness (white = 100; black = 0).

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The values of the treated textiles were compared with the values of untreated reference textiles. The change in brightness (ΔL^*) of the samples compared with untreated controls was calculated using the software PP2000 (Opticontrol).

	ΔL^*
Control	0
Leptothrix	23.14
Bacillus	28.72

A change in brightness (ΔL^*) of 5 is already visible to the naked eye, that is to say significant bleaching action was achieved with both manganese oxidases.

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Patent claims:

1. A multicomponent system for mediator-dependent enzymatic oxidation comprising an oxidation catalyst, an oxidizing agent, and a mediator, characterized in that
 - a) the oxidation catalyst is selected from the group of manganese oxidases,
 - b) the oxidizing agent is selected from the group consisting of oxygen and oxygen compounds,
 - c) the mediator is selected from the group of compounds which contain Mn ions.
2. The multicomponent system as claimed in claim 1, characterized in that it additionally comprises a complexing agent selected from the group of complexing agents that can complex Mn ions.
3. The multicomponent system as claimed in claim 1 or 2, characterized in that the mediator is Mn^{2+} or Mn^{3+} ions.
4. The multicomponent system as claimed in claim 2 or 3, characterized in that the complexing agent does not contain nitrogen, is readily biodegradable and is toxicologically harmless.

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5. The multicomponent system as claimed in one of claims 1 to 4, characterized in that the oxygen is present either directly in the gaseous form or in the form of liquid oxygen or as oxygen-containing gas mixture.

6. A process for oxidizing a substrate, characterized in that a manganese oxidase, in the presence of oxygen and, if appropriate, a complexing agent for Mn ions, forms Mn^{3+} by direct Mn^{2+} oxidation, and in that the Mn^{3+} ion oxidizes the substrate, itself being reduced to Mn^{2+} and, in turn, being available for direct oxidation by the manganese oxidase.

7. The process as claimed in claim 6, characterized in that the substrate is used in the form of an aqueous solution, mixture or suspension.

8. The process as claimed in claim 6 or 7, characterized in that manganese ions are used at concentrations between 0.005 mM to [sic] 50 mM.

9. The process as claimed in claim 6, 7 or 8, characterized in that oxygen having a partial pressure of 0.05 - 5 bar is used.

10. The process as claimed in one of claims 6 to 9, characterized in that the complexing agents are used at concentrations between 1 mM to [sic] 500 mM.