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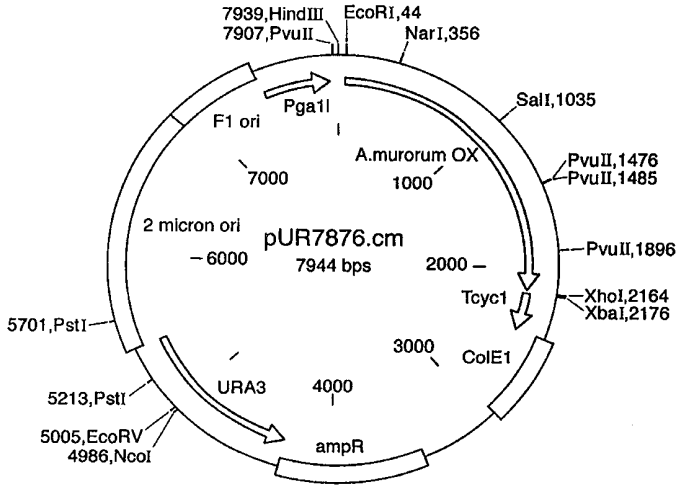

(57) Abstract

Purified phenol oxidizing enzymes obtainable from the fungus *Acremonium*, preferably from *Acremonium murorum* possess a pH optimum in the alkaline range and are useful in detergent compositions for bleaching fabrics. Also provided is a method for bleaching colored compounds which occur in stains present on fabrics, comprising the step of contacting the fabric with a detergent composition comprising the phenol oxidizing enzyme.

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PHENOL OXIDIZING ENZYMES

Field of the Invention

The present invention relates to novel phenol oxidizing enzymes, in particular, novel phenol oxidizing enzymes obtainable from strains of the genus *Acremonium*. The present invention also relates to enzymatic detergent compositions comprising said enzymes and to a method for stain bleaching during fabric washing.

Background of the Invention

Phenol oxidizing enzymes function by catalyzing redox reactions, i.e., the transfer of electrons from an electron donor (usually a phenolic compound) to molecular oxygen (which acts as an electron acceptor) which is reduced to H_2O. While being capable of using a wide variety of different phenolic compounds as electron donors, phenol oxidizing enzymes are very specific for molecular oxygen as the electron acceptor. Examples of phenol oxidizing enzymes are laccases (EC 1.10.3.2), bilirubin oxidases (EC 1.3.3.5), phenol oxidases (EC 1.14.18.1), catechol oxidases (EC 1.10.3.1) and ascorbate oxidases (EC 1.10.3.3).

Phenol oxidizing enzymes can be used for a wide variety of applications, including the detergent industry, the paper and pulp industry, the textile industry and the food industry. In the detergent industry, phenol oxidizing enzymes have been used for preventing the transfer of dyes in solution from one textile to another during detergent washing, an application commonly referred to as dye-transfer inhibition.

Phenol oxidizing enzymes are produced by a wide variety of fungi, including species of the genera *Aspergillus*, *Neurospora*, *Podospora*, *Botrytis*, *Pleurotus*, *Fomes*, *Phlebia*, *Trametes*, *Polyporus*, *Rhizoctonia* and *Lentinus*. Most phenol oxidizing enzymes exhibit pH optima in the acidic pH range while being inactive at neutral or alkaline pH. Thus, there remains a need to identify and isolate phenol oxidizing enzymes, and organisms

capable of naturally-producing phenol oxidizing enzymes, which present pH optima in the alkaline range for use in detergent washing methods and compositions.

Definition of the Invention

In a first aspect, the present invention relates to a purified phenol oxidizing enzyme obtainable from the fungus *Acremonium*, preferably from *Acremonium murorum* or *Acremonium obclavatum*.

In a second aspect, the present invention provides a detergent composition comprising a surfactant and a phenol oxidizing enzyme according to the invention. Preferably, the composition further comprises an enhancer.

According to a third aspect, the invention provides a method for bleaching colored compounds which occur in stains present on fabrics, comprising the step of contacting the fabric with a detergent composition of the invention, at a temperature between 10 and 60°C and wherein said composition has a pH of between 5.0 and 11.0.

The present invention also provides methods for preventing dye transfer in detergent and textile applications.

The present invention also encompasses recombinant host cells and expression vectors comprising an *Acremonium* phenol oxidizing enzyme of the present invention, as well as methods for purifying the phenol oxidizing enzyme from such host cells.

Detailed Description

Definitions

As used herein, the term "phenol oxidizing enzyme" refers to those enzymes which catalyze redox reactions and are specific for molecular oxygen as the electron acceptor.

As used herein, *Acremonium* refers to any *Acremonium* species which produces a phenol oxidizing enzyme capable of modifying colored compounds. The present invention encompasses derivatives of natural isolates of Acremonium, including progeny and mutants, provided that they are still capable still able to produce the phenol oxidizing enzyme capable of modifying colored compounds.

As used herein in referring to phenol oxidizing enzymes, the term "derived from" means that the phenol oxidizing enzymes originate from or are naturally-produced by the particular microbial strain mentioned. To exemplify, phenol oxidizing enzymes derived from *Acremonium* refer to those phenol oxidizing enzymes which are naturally-produced by *Acremonium*. The present invention encompasses phenol oxidizing enzymes identical to those produced by *Acremonium* species but which through the use of genetic engineering techniques are produced by non-*Acremonium* organisms transformed with a gene encoding said phenol oxidizing enzyme. The present invention also encompasses mutants, variants and derivatives of the phenol oxidizing enzymes of the present invention as long as the mutant, variant or derivative is able to retain at least one characteristic activity of the naturally occurring phenol oxidizing enzyme.

As used herein, the term "colored compound" refers to a substance that adds color to textiles, or to substances which result into the visual appearance of stains. Examples of substances which result in the visual appearance of stains are polyphenols, carotenoids, anthocyanins, tannins, Maillard reaction products, etc.

As used herein the phrase "modify the colored compound" or "modification of the colored compound" means that the compound is changed through oxidation such that either the color appears modified, i.e., the color visually appears to be decreased, lessened, decolored, bleached or removed. The present invention encompasses the modification of the color by any means including, for example, the complete removal of the colored compound from stain on a fabric by any means as well as a reduction of the color intensity or a change in the color of the compound.

In the context of the present invention, the term "mutants and variants", referring to phenol oxidizing enzymes, refers to phenol oxidizing enzymes which closely resemble the naturally occurring *Acremonium* phenol oxidizing enzymes, but are different in one or more amino acids, e.g. by substitution, deletion or insertion of one more amino acids. They will exhibit a high degree of homology (in terms of identity of residues) of at least

70%, preferably at least 80% or 90% or even 95% with the naturally occurring *Acremonium* phenol oxidizing enzymes.

Another way of defining "homology" is, that DNA encoding the variant or mutant phenol oxidizing enzyme will hybridize to the same probe as the DNA coding for the naturally occurring *Acremonium* phenol oxidizing enzyme, under certain specified conditions (i.e. presoaking in 5xSSC and prehybridizing for 1 hour at 40°C in a solution of 20% formamide, 5x Denhard't solution, 50 mM sodium phosphate, pH 6.8 and 50 µg of denaturated calf thymus DNA, followed by hybridization in the same solution supplemented with ATP for 18 hours at 40°C).

The term phenol oxidizing enzyme "derivative" as used herein refers to a portion or fragment of the full-length naturally occurring or variant phenol oxidizing enzyme amino acid sequence that retains at least one activity of the naturally occurring phenol oxidizing enzyme. As used herein, the term "mutants and variants", when referring to microbial strains, refers to cells that are changed from a natural isolated in some form, for example, having altered DNA nucleotide sequence of, for example, the structural gene coding for the phenol oxidizing enzyme; alterations to a natural isolate in order to enhance phenol oxidizing enzyme production; or other changes that effect phenol oxidizing enzyme expression.

The term "enhancer" refers to any compound that is able to modify the colored compound associated with dyes or pigments in association with a phenol oxidizing enzyme. The enhancer molecule increases the oxidative activity of the phenol oxidizing enzyme. The enhancing agent is typically an organic compound.

Phenol oxidizing enzymes

The present invention provides novel *Acremonium* phenol oxidizing enzymes which are capable of modifying colored compounds having different chemical structures, in particular at neutral or alkaline pH. Based on their compound modifying ability, phenol oxidizing enzymes of the present invention can be used, for example, for pulp and paper bleaching, for bleaching the color of stains on fabric and textile applications and for

decolourization of plant derived food ingredients. In another aspect of the present invention, the *Acremonium* enzyme is able to modify the colored compounds in the presence of a suitable enhancer.

The present invention also encompasses phenol oxidizing enzymes which are derived from species of the genus *Acremonium*, having at least one antigenic determinant in common with the phenol oxidizing enzyme obtainable from *Acremonium murorum* var. *murorum* having CBS accession number 157.72 and having an amino acid sequence substantially as in Sequence Listing #2. The present invention also encompasses mutants and variants of such enzymes, provided that they are still capable of modifying the colored compounds.

One method for measuring the presence of common antigenic determinants is with the Double Immunodiffusion tests (Ouchterlony technique) following the protocol set forth in, and under the conditions specified in, Clausen, J. (1988) Immunochemical Technique for the Identification and Estimation of Macromolecules (3rd revised edition) Burdon, R.H., and P.H. van Knippenberg, eds., page 281 (appendix 11, micro technique) and as interpreted following the protocol described in and under the conditions specified by Clausen, supra, chapter 6, p143-146. Another method for measuring the presence of common antigenic determinants is by Western blot (Current Protocols in Molecular Biology, Vol 2, John Wiley & Sons, Inc. Section 10.8: Immunoblotting and Immunodetection).

Phenol oxidizing enzyme activities

The phenol oxidizing enzymes of the present invention are capable of using a wide variety of different phenolic compounds as electron donors, while being very specific for molecular oxygen as the electron acceptor.

Suitable substrates for the measurement of phenol oxidizing enzyme activity include ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate), syringaldazine, guiacol and 2,6-dimethoxyphenol.

Applications of phenol oxidizing enzymes

The *Acremonium* phenol oxidizing enzymes of the present invention are capable of oxidizing a wide variety of colored compounds having different chemical structures, using oxygen as the electron acceptor. Accordingly, the phenol oxidizing enzymes of the present invention are used in applications where it is desirable to modify the coloured compounds, such as in cleaning, for removing the food stains on fabric; textiles; paper and pulp applications; and for decolourization of plant derived food ingredients. A particularly important property of the phenol oxidizing enzymes of the present invention is their high enzymatic activity, at about 5-40°C, in a broad range of pHs, including a broad range of neutral to alkaline pHs. In particular, their enzymatic activity is high in the pH range of from about 5.0 and 11.0, especially at pH values of about 7.0 to about 10.5 and at temperatures of about 20-35°C.

Coloured compounds

Several classes of substances one would like to oxidize can be envisaged. For detergents applications, coloured substances which may occur as stains on fabrics can be a target. Several types or classes of coloured substances which may occur in stains are considered, such as indicated below:

Porphyrin derived structures.

Porphyrin structures, often coordinated to a metal, form one class of coloured substances which occur in stains. Examples are heme or haematin in blood stain, chlorophyll as the green substance in plants, e.g. grass or spinage. Another example of a metal-free substance is bilirubin, a yellow breakdown product of heme.

Tannins, polyphenols

Tannins are polymerised forms of certain classes of polyphenols. Such polyphenols are catechins, leuantocyanins, etc. (P. Ribéreau-Gayon, Plant Phenolics, Ed. Oliver & Boyd, Edinburgh, 1972, pp.169-198). These substances can be conjugated

with simple phenols like e.g. gallic acids. These polyphenolic substances occur in tea stains, wine stains, banana stains, peach stains, etc. and are notoriously difficult to remove.

Carotenoids.

Carotenoids are the coloured substances which occur in tomato (lycopene, red), mango (-carotene, orange-yellow) (G.E. Bartley et al., The Plant Cell (1995), Vol 7, 1027-1038). They occur in food stains (tomato) which are also notoriously difficult to remove, especially on coloured fabrics, when the use of chemical bleaching agents is not advised.

Anthocyanins.

These substance are the highly coloured molecules which occur in many fruits and flowers (P. Ribéreau-Gayon, Plant Phenolics, Ed. Oliver & Boyd, Edinburgh, 1972, 135-169). Typical examples, relevant for stains, are berries, but also wine. Anthocyanins have a high diversity in glycosidation patterns.

Maillard reaction products

Upon heating of mixtures of carbohydrate molecules in the presence of protein/peptide structures, a typical yellow/brown coloured substance arises. These substances occur for example in cooking oil and are difficult to remove from fabrics.

Enhancers

A phenol oxidizing enzyme of the present invention can act to modify colored compounds in the presence or absence of enhancers depending upon the characteristics of the compound. If a compound is able to act as a direct substrate for the phenol oxidizing enzyme, the phenol oxidizing enzyme can modify the colored compound in the absence of an enhancer, although an enhancer may still be preferred for optimum phenol oxidizing enzyme activity. For other colored compounds unable to act as a direct substrate for the phenol oxidizing enzyme or not directly accessible to the phenol oxidizing enzyme, an enhancer is

required for optimum phenol oxidizing enzyme activity and modification of the color.

Enhancers are described in for example WO-A-94/12621, WO-A-94/12620, WO-A-94/12619, WO-A-94/29425, WO-A-95/01426, WO-A-96/06930, WO-A-96/10079, WO-A-97/11217, WO-A-97/06244. Suitable enhancers include but are not limited to phenothiazine-10-propionic acid (PTP), 10-methylphenothiazine (MPT), phenoxazine-10-propionic acid (POP), 10-methyl-phenoxazine (MPO), 10-ethylphenothiazine-4-carboxylic acid (EPC) acetosyringone, syringaldehyde, methylsyringate, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate (ABTS), or derivatives thereof. They can also be selected from the enhancers described in WO-A-98/56899, WO-A-99/13038 and our copending European Patent Application 99202023.0 (all Unilever).

Cultures

The present invention encompasses *Acremonium* strains and natural isolates, and derivatives of such strains and isolates, such as strains of the species *Acremonium murorum* and *Acremonium obclavatum*. The present invention also provides substantially biologically-pure cultures of novel strains of the genus *Acremonium*, and, in particular substantially biologically-pure cultures of the strain *Acremonium* from which phenol oxidizing enzymes can be purified.

Purification

The phenol oxidizing enzymes of the present invention may be produced by cultivation of phenol oxidizing enzyme-producing *Acremonium* strains (such as *Acremonium murorum* var. *murorum* having CBS accession number 157.72) under aerobic conditions in nutrient medium containing assimiable carbon and nitrogen together with other essential nutrient(s). The medium can be composed in accordance with principles well-known in the art.

During cultivation, the phenol oxidizing enzyme-producing strains secrete phenol oxidizing enzyme extracellularly. This permits the isolation and purification (recovery) of the phenol oxidizing enzyme to be achieved by, for example, separation of

cell mass from a culture broth (e.g. by filtration or centrifugation). The resulting cell-free culture broth can be used as such or, if desired, may first be concentrated (e.g. by evaporation or ultra-filtration). If desired, the phenol oxidizing enzyme can then be separated from the cell-free broth and purified to the desired degree by conventional methods, e.g. by column chromatography, or even crystallized.

Preferably, the phenol oxidizing enzymes of the present invention may be isolated and purified from the culture broth into which they are extracellularly secreted by concentration of the supernatant of the host culture, followed by ammonium sulfate fractionation and gel permeation chromatography.

The phenol oxidizing enzymes of the present invention may be formulated and utilized according to their intended application. In this respect, if being used in a detergent composition, the phenol oxidizing enzyme may be formulated, directly from the fermentation broth, as a coated solid using the procedure described in US-A-4,689,297. Furthermore, if desired, the phenol oxidizing enzyme may be formulated in a liquid form with a suitable carrier. The phenol oxidizing enzyme may also be immobilized, if desired, e.g. on a suitable carrier.

The present invention also encompasses expression vectors and recombinant host cells comprising a *Acremonium* phenol oxidizing enzyme of the present invention and the subsequent purification of the phenol oxidizing enzyme from the recombinant host cell.

Detergent Compositions

The *Acremonium* phenol oxidizing enzyme of the present invention may be a component of a detergent composition. This may comprise a surfactant and the enzyme, and it may comprise further detergent ingredients, such as other enzymes, for example, proteases, amylases, lipases, cutinases, cellulases or peroxidases; enhancers; detergent builder or complexing agents; stabilizing agents; fabric conditioners; bactericides; optical brighteners; or perfume. The detergent composition may be formulated as granulates, liquids or protected enzymes. Detergent

compositions are for example described in WO-A-95/01426 and WO-A-96/06930.

Surfactants

The detergent compositions of the invention contain, as essential ingredients, one or more surfactants which may be chosen from soap and non-soap anionic, cationic, nonionic, amphoteric and zwitterionic detergent-active compounds, and mixtures thereof. Many suitable detergent-active compounds are available and are fully described in the literature, for example, in "Surface-Active Agents and Detergents", Volumes I and II, by Schwartz, Perry and Berch. Preferred surfactants are synthetic anionic and nonionic compounds and soap.

The detergent composition may comprise both nonionic and/or anionic surfactant. Anionic surfactants are well-known to those skilled in the art. Examples include alkylbenzene sulphonates, particularly linear alkylbenzene sulphonates having an alkyl chain length of C_8-C_{15}; primary and secondary alkylsulphates, particularly C_8-C_{15} primary alkyl sulphates; alkyl ether sulphates; olefin sulphonates; alkyl xylene sulphonates; dialkyl sulphosuccinates; and fatty acid ester sulphonates. Sodium salts are generally preferred.

Nonionic surfactants that may be used include the primary and secondary alcohol ethoxylates, especially the C_8-C_{20} aliphatic alcohols ethoxylated with an average of from 1 to 20 moles of ethylene oxide per mole of alcohol, and more especially the C_{10}-C_{15} primary and secondary aliphatic alcohols ethoxylated with an average of from 1 to 10 moles of ethylene oxide per mole of alcohol. Non-ethoxylated nonionic surfactants include alkylpolyglycosides, glycerol monoethers, and polyhydroxyamides (glucamide).

The choice of the surfactant, and the amount present, will depend on the intended use of the detergent composition. In fabric washing compositions, different surfactant systems may be chosen, as is well known to the skilled formulator, for handwashing products and for products intended for use in different types of washing machine.

The total amount of surfactant present will also depend on the intended end use and may be as high as 60 wt%, for example, in a composition for washing fabrics by hand. In compositions for machine washing of fabrics, an amount of from 5 to 40 wt% is generally appropriate.

Detergent compositions suitable for use in most automatic fabric washing machines generally contain anionic non-soap surfactant, or nonionic surfactant, or combinations of the two in any ratio, optionally together with soap.

Detergency Builders

The detergent compositions of the invention will generally also contain one or more detergency builders. The total amount of detergency builder in the compositions will suitably range from 5 to 80 wt%, preferably from 10 to 60 wt%.

Inorganic builders that may be present include sodium carbonate, if desired in combination with a crystallisation seed for calcium carbonate, as disclosed in GB-A-1 437 950 (Unilever); crystalline and amorphous aluminosilicates, for example, zeolites as disclosed in GB-A-1 473 201 (Henkel), amorphous aluminosilicates as disclosed in GB-A-1 473 202 (Henkel) and mixed crystalline/amorphous aluminosilicates as disclosed in GB-A-1 470 250 (Procter & Gamble); and layered silicates as disclosed in EP-A-164 514 (Hoechst). Inorganic phosphate builders, for example, sodium orthophosphate, pyrophosphate and tripolyphosphate, may also be present used.

The detergent compositions of the invention preferably contain an alkali metal, preferably sodium, aluminosilicate builder. Sodium aluminosilicates may generally be incorporated in amounts of from 10 to 70% by weight (anhydrous basis), preferably from 25 to 50 wt%. The alkali metal aluminosilicate may be either crystalline or amorphous or mixtures thereof, having the general formula:

$$0.8\text{-}1.5\ Na_2O.\ Al_2O_3.\ 0.8\text{-}6\ SiO_2$$

These materials contain some bound water and are required to have a calcium ion exchange capacity of at least 50 mg CaO/g. The

preferred sodium aluminosilicates contain 1.5-3.5 SiO_2 units (in the formula above). Both the amorphous and the crystalline materials can be prepared readily by reaction between sodium silicate and sodium aluminate, as amply described in the literature.

Suitable crystalline sodium aluminosilicate ion-exchange detergency builders are described, for example, in GB-A-1 429 143 (Procter & Gamble). The preferred sodium aluminosilicates of this type are the well-known commercially available zeolites A and X, and mixtures thereof.

The zeolite may be the commercially available zeolite 4A now widely used in laundry detergent powders. However, according to a preferred embodiment of the invention, the zeolite builder incorporated in the compositions of the invention is maximum aluminium zeolite P (zeolite MAP) as described in EP-A-384 070 (Unilever). Zeolite MAP is defined as an alkali metal aluminosilicate of the zeolite P type having a silicon to aluminium ratio not exceeding 1.33, preferably within the range of from 0.90 to 1.33, and more preferably within the range of from 0.90 to 1.20. Especially preferred is zeolite MAP having a silicon to aluminium ratio not exceeding 1.07, more preferably about 1.00. The calcium binding capacity of zeolite MAP is generally at least 150 mg CaO per g of anhydrous material.

Organic builders that may be present include polycarboxylate polymers such as polyacrylates, acrylic/ maleic copolymers, and acrylic phosphinates; monomeric polycarboxylates such as citrates, gluconates, oxydisuccinates, glycerol mono-, di- and trisuccinates, carboxymethyloxysuccinates, carboxymethyloxymalonates, dipicolinates, hydroxyethyliminodiacetates, alkyl- and alkenylmalonates and succinates; and sulphonated fatty acid salts. This list is not intended to be exhaustive. Especially preferred organic builders are citrates, suitably used in amounts of from 5 to 30 wt%, preferably from 10 to 25 wt%; and acrylic polymers, more especially acrylic/maleic copolymers, suitably used in amounts of from 0.5 to 15 wt%, preferably from 1 to 10 wt%.

Builders, both inorganic and organic, are preferably present in alkali metal salt, especially sodium salt, form.

Bleach Components

Detergent compositions according to the invention may also suitably contain a bleach system. Fabric washing compositions may desirably contain peroxy bleach compounds, for example, inorganic persalts or organic peroxyacids, capable of yielding hydrogen peroxide in aqueous solution.

Suitable peroxy bleach compounds include organic peroxides such as urea peroxide, and inorganic persalts such as the alkali metal perborates, percarbonates, perphosphates, persilicates and persulphates. Preferred inorganic persalts are sodium perborate monohydrate and tetrahydrate, and sodium percarbonate. Especially preferred is sodium percarbonate having a protective coating against destabilisation by moisture. Sodium percarbonate having a protective coating comprising sodium metaborate and sodium silicate is disclosed in GB-A-2 123 044 (Kao).

The peroxy bleach compound is suitably present in an amount of from 5 to 35 wt%, preferably from 10 to 25 wt%. The peroxy bleach compound may be used in conjunction with a bleach activator (bleach precursor) to improve bleaching action at low wash temperatures. The bleach precursor is suitably present in an amount of from 1 to 8 wt%, preferably from 2 to 5 wt%. Preferred bleach precursors are peroxycarboxylic acid precursors, more especially peracetic acid precursors and peroxybenzoic acid precursors; and peroxycarbonic acid precursors. An especially preferred bleach precursor suitable for use in the present invention is N,N,N',N'-tetracetyl ethylenediamine (TAED). The novel quaternary ammonium and phosphonium bleach precursors disclosed in US-A-4 751 015 and US-A-4 818 426 (Lever Brothers Company) and EP-A-402 971 (Unilever) are also of great interest. Especially preferred are peroxycarbonic acid precursors, in particular cholyl-4-sulphophenyl carbonate. Also of interest are peroxybenzoic acid precursors, in particular, N,N,N-trimethylammonium toluoyloxy benzene sulphonate; and the cationic bleach precursors disclosed in EP-A-284 292 and EP-A-303 520 (Kao).

A bleach stabiliser (heavy metal sequestrant) may also be present. Suitable bleach stabilisers include ethylenediamine

tetraacetate (EDTA) and the polyphosphonates such as Dequest (Trade Mark), EDTMP.

An especially preferred bleach system comprises a peroxy bleach compound (preferably sodium percarbonate optionally together with a bleach activator), and a transition metal bleach catalyst as described in EP-A-458 397, EP-A-458 398 and EP-A-509 787 (Unilever).

Optional Further Enzymes

The detergent compositions of the present invention may additionally comprise one or more further enzymes which provide detergent performance, fabric care and/or sanitisation benefits. Said enzymes include enzymes selected from cellulases, hemicellulases, peroxidases, proteases, gluco-amylases, amylases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, polygalacturonase, keratanases, reductases, oxidases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases, β-glucanases, arabinosidases, hyaluronidases, chondroitinases, laccases or mixtures thereof. Also suitable are thioester hydrolase, phosphate monoester hydrolase, phosphate diester hydrolase, thioether hydrolase, a-amino-acyl- peptide hydrolase, peptidyl-amino acid hydrolase, acyl-amino acid hydrolase, dipeptide hydrolase, lysozyme, chitinase, dextranase, carboxyl esterase, pectin esterase, chlorophyllase, phosphatases, lipoxygenases, lipoxidase, mannanases, haloperoxidases, swollenin proteins, expansins, xyloglucanases, cytochrome, oxidoreductases, oxygenases, polyester hydrolytic enzymes, saccharide gum degrading enzyme, enzyme with galactanase activity, xylan degrading enzyme, isopeptidase, antibody, antibody fragment, antibody against an enzyme, cholesterol esterase, pectin degrading enzyme, pectin lyase, pectinesterase, pectolyase, xyloglycan endotransglycosylase

A preferred combination is a detergent composition having cocktail of conventional applicable enzymes like protease, amylase, lipase, cutinase and/or cellulase in conjunction with one or more plant cell wall degrading enzymes.

Preferred proteolytic enzymes (proteases) are normally solid, catalytically active protein materials which degrade or alter protein types of stains when present as in fabric stains in a hydrolysis reaction. They may be of any suitable origin, such as vegetable, animal, bacterial or yeast origin.

Proteolytic enzymes or proteases of various qualities and origins and having activity in various pH ranges of from 4-12 are available and can be used in the instant invention. Examples of suitable proteolytic enzymes are the subtilisins, which are obtained from particular strains of B. subtilis and B. licheniformis, such as the commercially available subtilisins Savinase™, Alcalase™ and Durazyme™ as supplied by Novo Industri A/S, Copenhagen, Denmark or Purafect™ and Properase™ as supplied by Genencor International.

Suitable lipases are Lipolase™, Lipolase Ultra™ from Novo Nordisk, or Lipomax™ from Genencor. Suitable amylases are sold under the tradename Purafact Ox Am™ described in WO-A-94/18314, WO-A-96/05295 sold by Genencor; Termamyl™, Fungamyl™ and Duramyl™, all available from Novo Nordisk A/S and those described in WO-A-95/26397.

Preferred cellulolytic enzymes are sold under the tradename Carezyme™, Celluzyme™and/or Endolase™ by Novo Nordisk A/S.

A suitable peroxidase is sold as Guardzyme™ available from Novo Nordisk A/S.

The further enzymes are commonly employed in granular form in amounts of from about 0.1 to about 3.0 wt% of the detergent composition.

Other ingredients

The compositions of the invention may contain alkali metal, preferably sodium, carbonate, in order to increase detergency and ease processing. Sodium carbonate may suitably be present in amounts ranging from 1 to 60 wt%, preferably from 2 to 40 wt%. However, compositions containing little or no sodium carbonate are also within the scope of the invention.

Powder flow may be improved by the incorporation of a small amount of a powder structurant, for example, a fatty acid (or fatty acid soap), a sugar, an acrylate or acrylate/ maleate

polymer, or sodium silicate. One preferred powder structurant is fatty acid soap, suitably present in an amount of from 1 to 5 wt%.

The detergent compositions according to the present invention may also comprise from 0. 001% to 10%, more preferably from 0.01% to 2%, more preferably from 0.05% to 1% by weight of polymeric dye-transfer inhibiting agents. Said polymeric dye transfer inhibiting agents are normally incorporated into detergent compositions in order to inhibit the transfer of dyes from colored fabrics onto fabrics washed therewith. These polymers have the ability to complex or adsorb the fugitive dyes washed out of dyed fabrics before the dyes have the opportunity to become attached to other articles in the wash.

Especially suitable polymeric dye transfer inhibiting agents are polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinylpyrrolidone polymers, polyvinyloxazolidones and polyvinylimidazoles or mixtures thereof.

Soil release agents useful in compositions of the present invention are conventionally copolymers or terpolymers of terephthalic acid with ethylene glycol and/or propylene glycol units in various arrangements. Examples of such polymers are disclosed in US-A-4 116 885, US-A-4 711 730 and EP-A-272 033.

Other materials that may be present in detergent compositions of the invention include sodium silicate; anti-redeposition agents such as cellulosic polymers; inorganic salts such as sodium sulphate; lather control agents or lather boosters as appropriate; dyes; coloured speckles; perfumes; foam controllers; and fabric softening compounds.

Detergent compositions of the invention may be prepared by any suitable method. Particulate detergent compositions are suitably prepared by spray-drying a slurry of compatible heat-insensitive ingredients, and then spraying on or postdosing those ingredients unsuitable for processing via the slurry. The skilled detergent formulator will have no difficulty in deciding which ingredients should be included in the slurry and which should not.

Particulate detergent compositions of the invention preferably have a bulk density of at least 400 g/l, more preferably at least 500 g/l. Such powders may be prepared either by post-tower densification of spray-dried powder, or by wholly non-tower methods such as dry mixing and granulation; in both cases a high-speed mixer/granulator may advantageously be used. Processes using high-speed mixer/granulators are disclosed, for example, in EP-A-340 013, EP-A-367 339, EP-A-390 251 and EP-A-420 317 (Unilever).

Having thus described the phenol oxidizing enzymes of the present invention, the following examples are now presented for the purposes of illustration and are neither meant to be, nor should they be, read as being restrictive. Dilutions, quantities, etc. which are expressed herein in terms of percentages are, unless otherwise specified, percentages given in terms of per cent weight per volume (w/v). As used herein, dilutions, quantities, etc., which are expressed in terms of % (v/v), refer to percentage in terms of volume per volume. Temperatures referred to herein are given in degrees Celsius (°C).

IN THE DRAWINGS:

Figure 1 is the restriction map of plasmid **pUR7876;**

Figure 2 shows the construction route of plasmids **pUR7890, pUR7891** and **pUR7893.**

Figure 3 shows a 8-18% SDS gradient gel from supernatants of AWC-7983 transformants. The gel is stained with Coomassie Brilliant Blue. The phenol oxidase is indicated by an arrow. Lane 1: *AWC-7893-5p,* Lane 2: *AWC-7893-10A,* Lane 3: a medium sample of AWGLA, producing glucoamylase.

Figure 4 shows a 8-18% SDS gradient gel from supernatants of AWC-7983 transformants a gel stained with cyanidin, showing the decolorization of cyanidin at the position of the phenol oxidase. Lane 1: AWGLA, Lane 2: *AWC-7893-10A,* Lane 3: *AWC-7893-4A;* Lane 4: *AWC-7893-6P* and Lane 5: *AWC-7893-5P.*

MATERIALS AND METHODS

Bacterial, yeast and mould strains:

For standard bacterial cloning the *Escherichia coli* strains DH5α (genotype: F^-, *endA*1, *hsdR*17, (r_k^-, m_k^+), *supE*44, *thi*-1, lambda$^-$, *recA*1, *gyrA*96, *relA*1, Δ (*argF-lacIZYA*)U169, *deo*R, (phi80d(*lacZ*)DM15); Hanahan; J. Mol. Biol. 166 (1983) 557-580) and JM109 (genotype: F´ traD36 lacI^{q}Δ(lacZ)M15 proA$^+$B$^+$/e14$^-$(McrA$^-$) Δ(lac-proAB) thi gyrA96 (NaL^r) endA1 hsdR17($r_k^-m_k^+$) relA1 supE44 recA1; Yanisch-Perron, C., Viera, J. and Messing, J. (1985). Gene 33, 103-119) were used.

For cloning of cDNA libraries *E. coli* strain XL1-Blue MRF' (Δ(*mcrA*)183,Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *supE*44, *thi*-1, *recA*1, *gyrA*96, *relA*1, *lac* [F' *proAB lacI*^{q}Z*ΔM15* Tn*10* (Tet^r)]; Invitrogen) was used. The *Saccharomyces cerevisae* strain used was *S. cerevisiae* VW-K1 (*leu2*). Acremonium *murorum* var. *murorum* CBS 157.72 was obtained from the Centraal Bureau voor Schimmelcultures (CBS) in Baarn, the Netherlands. *Aspergillus awamori* #40 (described in WO-A-91/19782 p.13) is a derivative of *A. awamori* CBS 115.52.

Media

Fungal minimal medium (pH=6.5) contains 0.07 M $NaNO_3$, 0.011 M KH_2PO_4, 0.007 M KCl, 0.002 M $MgSO_4.7H_2O$, EGLI-trace elements 1x (composition 100x trace elements: per litre 60 g EDTA.$2H_2O$, 11 g $CaCl_2.2H_2O$, 7.5 g $FeSO_4.7H_2O$, 2.8 g $MnSO_4.H_2O$, 2.7 g $ZnSO_4.7H_2O$, 0.8 g $CuSO_4.5H_2O$, 0.9 g $CoCl_2.6H_2O$, 0.5 g $Na_2MoO_4.2H_2O$, 0.8 g H_3BO_3, and 0.5 g KI, pH 4.0 with NaOH). As carbon source was used either fructose or glucose as indicated below in the specification.

AW medium is identical to Fungal minimal medium, except that it additionally contains 0.1% Yeast extract.

Extraction of total RNA:

Total RNA was prepared by extraction with Trizol (Life Technologies, nr 15596-018). Frozen mycelium was ground in liquid N_2 to a fine powder with a mortar and a pestle and mixed with 10 ml Trizol. After incubation for 10 min. at room temperature, 2 ml chloroform was added. The mixture was stirred and centrifuged for 10 min at 5000 rpm to pellet the cell debris. The supernatant was transferred to a new tube and the RNA was precipitated by addition of 0.7 volumes isopropanol and subsequent centrifugation for 15 min. at 5,000 rpm. The RNA pellet was resuspended in 3 ml RNA extraction buffer (100 g Guanidinium isothiocyanate, 100 ml H_2O, 10.6 ml 1M Tris pH 7.6, 10.6 ml 0.2 M EDTA pH 8.0). Then 3 ml phenol/chloroform mix was added, stirred and the mixture was centrifuged for 10 min. at 5000 rpm. The supernatant was transferred to a new tube and the RNA was precipitated with 1/10 volume 3M NaAc pH5.6, 0.7 volume isopropanol, followed by centrifugation for 15 min, 5000rpm. The RNA pellet was resuspended in 3 ml H_2O and again precipitated as described above. This step was repeated once, and finally the RNA pellet was rinsed with 70% ethanol and resuspended in 250 µl DEPC-treated H_2O. The RNA concentration was determined by measuring $OD_{260/280}$.

Isolation of poly(A)$^+$ RNA:

Purification of poly(A)$^+$ mRNA from total RNA was carried out with the Oligotex™ mRNA Kit from Qiagen. One mg of total RNA was mixed in a total volume of 1 ml with 500 µl 2x binding buffer (20 mM Tris.HCl pH7.5, 1000 mM NaCl, 2 mM EDTA, 0.2% SDS) and 55 µl Oligotex suspension. Secondary structures were disrupted by incubation at 65°C for 3 min. Then, the mixture was transfered to a 37°C waterbath and incubated for 10 min, followed by 2 min. centrifugation in an eppendorf centrifuge. The pellet was washed twice with 1 ml wash buffer OW2 (10 mM Tris.HCl pH7.5, 150 mM NaCl, 1 mM EDTA). Finally, mRNA was eluted in 20-50 µl of preheated (70°C) elution buffer (5 mM Tris.HCl pH7.5). Quantitation of mRNA was carried out by measuring the absorbance at 260 nm in a spectrophotometer.

cDNA synthesis:

First strand cDNA synthesis:

Double stranded cDNA was synthesized from 5 µg of poly(A)+ RNA using the Stratagene cDNA synthesis kit. 75-1000 Units reverse transcriptase Superscript II (Gibco-BRL) was used instead of Moloney murine leukemia virus reverse transcriptase. In an RNase-free microcentrifuge tube, the following reagents were added: 5.0 µl 10x first-strand buffer, 3.0 µl first-strand methyl nucleotide mixture, 2.0 µl linker-primer (5' GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTT 3'), 1.0 µl RNase Block Ribonuclease Inhibitor, x µl (5 µg) of poly(A)+ RNA. DEPC-treated water was added to a final volume of 50 µl. Template and primer were annealed for 10 minutes at room temperature and x µl (75-1000 Units) reverse transcriptase Superscript II was added. The reaction was incubated 1 hour at 37°C.

Second strand cDNA synthesis:

To the 50 µl of the first-strand reaction the following components were added: 20.0 µl 10x second-strand buffer, 6.0 µl second-strand nucleotide mixture, 113.9 µl water, 2.0 µl *E. coli* RNase H (3.0 Units) and 11.1 µl *E. coli* DNA polymerase. cDNA synthesis was carried out for 2.5 hours at 16°C.

The cDNA termini were blunted by adding 23 µl of blunting dNTP mix and 2.0 µl *Pfu* DNA polymerase, followed by incubation at 72°C for 30 minutes. Then, 200 µl phenol-chloroform (1:1 (v/v)) was added, the contents were mixed, and centrifuged. The upper aqueous layer was extracted again with an equal volume of chloroform, the mixture was centrifuged and finally, the cDNA, present in the aqueous layer, was precipitated overnight by addition of 20 µl 3 M sodium acetate and 400 µl of 100 % (v/v) ethanol. The cDNA was pelleted by centrifugation (1 hour at 4°C), washed with 70% (v/v) ethanol and resuspended in 9.0 µl of *EcoRI* adapters (annealed oligonucleotides 5'-AATTCGGCACGAG-3' and 5'-CTCGTGCCG-3'), 1.0 µl 10x ligase buffer, 1.0 µl 10 mM ATP and 1.0 µl T4 DNA ligase (4 Units). This mixture was incubated overnight at 8°C.

The ligase was inactivated by incubation at 70°C (30 min) and the following components were added to phosphorylate the cDNA ends (30 minutes at 37°C): 1.0 µl 10x ligase buffer, 2.0 µl 10 mM ATP, 6.0 µl water and 1.0 µl T4 polynucleotide kinase (10 Units). The kinase was inactivated at 70°C (30 min). Then, the cDNA was digested with *XhoI* by addition of 28 µl of *XhoI* buffer supplement and 3.0 µl of *XhoI* (120 Units) and the mixture was incubated at 37°C for 1.5 hours and finally size-fractionated with a Sephacryl S-500 column which was equilibrated with STE (0.1 M NaCl, 20 mM Tris.HCl pH7.5, 10 mM EDTA). To the cDNA sample 5 µl of 10x STE buffer was added, and the cDNA was loaded onto the Sephacryl S-500 column. The column was centrifuged for 2 minutes at 400g and the cDNA fraction was collected. Then, 60 µl of STE was loaded onto the column and again the fraction was collected after centrifugation. This step was repeated once. Finally, the three fractions were pooled to yield approximately 180 µl of cDNA. The cDNA was extracted once with phenol-chloroform (1:1 (v/v)) and once with chloroform and finally precipitated with 2.5 volumes of 100% ethanol. The cDNA pellet was dissolved in 20 µl of water.

Construction of cDNA libraries:

For the construction of a cDNA library in *Escherichia coli* the cDNA was cloned in the commercially available plasmid pYES2.0 (Invitrogen). This vector was digested with *EcoRI* and *XhoI*, size fractionated on an agarose gel, and a 5.9 kb fragment was purified using the QiaEx II fragment isolation kit. For large scale ligations approximately 200 ng cDNA was ligated to 1.5 µg of *EcoRI / XhoI* digested pYES2.0, in a total volume of 7.5 µl with 1 unit T4 DNA ligase for 5 hours at room temperature. 2.5 µl aliquots were used to transform 50 µl of electrocompetent *E. coli* XL1-Blue MRF' cells (Stratagene; conditions 1700 V, 200 Ω and 25µF). After addition of 1 ml SOC to each mix, the cells were grown for 1 hr at 37°C, plated on LB + ampicillin plates (100 µg/ml) and grown at 37°C for another 16 hours. Dilutions were plated to calculate the titer of the library. To each plate 3 ml LB was added, the bacteria were scraped off, pooled and stored in small aliquots. Large scale DNA was prepared from 200-500 ml

cultures of LB inoculated with an aliquot of transformants, propagated overnight.

Transformation of *Saccharomyces cerevisiae*:

Transformations of *S.cerevisae* VW-K1 were carried out according to Gietz, R.D. and R.H. Schiestl (1996) Transforming Yeast with DNA, Methods in Molecular and Cellular Biology (In Press). 5 ml YPD was inoculated with a yeast colony from a plate and grown overnight at 30°C. Dilutions of 1/1,000, 1/10,000 and 1/100,000 were made in 50 ml YPD, and grow again overnight at 30°C. The cells were harvested at an OD_{660} of 1.0-2.0, by centrifugation 10 min at 5,000 rpm in Falcon tubes, washed in water and once in 1.0 ml 100 mM LiAc. Finally, the cells were resuspended to a final volume of 300 µl (2 x 10E9 cells/ml) 100 mM LiAc. 50 µl cells were transferred into labelled tubes, the cells were pelleted, and the LiAc was removed. Then the following ingredients were added:

240 µl PEG 4000 (50% w/v), 36 µl 1.0 M LiAc, 25 µl single strand-DNA (2.0 mg/ml), 50 µl water and plasmid DNA (0.1 - 10 µg). This mixture was incubated at 30°C for 30 min, followed by a heat shock in a water bath at 42°C for 20-25 min. Then, the cells were pelleted, the transformation mix was removed and 250 µl of sterile water was added. The cells were resuspended and plated on selective medium. Plates were incubated for 2 - 4 days to recover transformants.

Transformation of *Aspergillus awamori*

Preparation of protoplasts:

Conidia were obtained by growing the *A. awamori* strain at 30°C on a nitrocellulose filter (Hybond-N, Amersham) placed on a PDA (Potato Dextrose Agar) plate for several days and subsequently washing the filters with physiological salt solution.

Protoplasts of *A. awamori* were prepared as follows: a shake flask containing 200 ml of fungal minimal medium including 0.5% yeast extract and 1% glucose was inoculated with 10^6 conidia/ml of *A. awamori* and incubated for 18 hours at 30°C in a shaker at 200 rpm. Mycelium was harvested through sterile Miracloth^R and washed

with ice-cold 0.6 M $MgSO_4$. The mycelium was resuspended in OM medium (per litre: 500 ml 2.4 M $MgSO_4$, 480 ml H_2O, 16.8 ml 0.5 M Na_2HPO_4, 3.2 ml 0.5 M NaH_2PO_4, pH 5.8-5.9) at 5 ml/g mycelium. Subsequently, 5 mg Novozym 234^R and 6 mg BSA were added per g mycelium. Protoplasting was allowed to proceed for 1-2 hours at 30°C in a shaker at 80-100 rpm. The formation of protoplasts was checked using a light microscope. Protoplasts were filtered through sterile Miracloth^R and the sample was divided in 30 ml aliquots in falcon tubes. STC (1.2 M sorbitol, 10 mM Tris/HCl pH 7.5, 50 mM $CaCl_2.2H_2O$) was added to bring the volume up to 50 ml and the protoplasts were harvested by centrifugation at 2000 rpm for 10 minutes at 4°C. The protoplasts were washed again in 50 ml STC and resuspended in STC at a concentration of approximately 10^8 protoplasts/ml.

PEG transformations:

Ten µg of a plasmid DNA was added to an aliquot of 100 µl (10^7) protoplasts, mixed and incubated for 25 minutes on ice. PEG was added in two 200 µl aliquots and an 850 µl aliquot, and the mixture was incubated at room temperature for 20 minutes. Finally, the mixture was washed with 10 ml of STC, harvested by centrifugation at 2,000 rpm for 10 minutes at room temperature and the sample was plated on a minimal medium plate for selection of transformants.

Isolation of plasmid DNA from yeast:

Plasmid DNA was isolated as described by Hoffman, C.S. and Winston, F. (A ten minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene, 57 (1987) 267-272). A 2 ml yeast culture was grown in selective medium for 48 hours and spun down in an eppendorf tube 10 min. at 13,000 rpm. The supernatant was discarded and the pellet resuspended in the remaining liquid by vortexing. To this suspension 200 µl lysismix (2% TritonX, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8), 200 µl Phenol-Chloroform-Isoamylalcohol (25:24:1) and 0.3 g glassbeads (425-600 µm diameter) was added. The mixture was vortexed for 2 min.,

centrifuged for 10 min, and the supernatant was retained. One µl of this supernatant was then used to transform electrocompetent *E. coli* DH5α cells. The DNA was isolated from the bacteria for further analysis.

DNA sequence analysis:

DNA sequence analysis was carried out on a Pharmacia LKB ALF fluorescent sequencer.

Plasmid constructions:

Standard recombinant DNA techniques were used for cloning (Sambrook et al.; Molecular cloning - A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989)).

Protein analysis:

For analysis of the secreted phenol oxidase the extracellular medium was separated from the mycelium by filtration through Miracloth. Concentration of the proteins in the medium was carried out by ammoniumsulphate precipitation (60 g/100 ml). The precipitate was kept at 4°C for 16 hr and pelleted by centrifugation for 45 min. at 12,000 rpm. The protein pellet was dissolved in 2.5 ml 30 mM MES pH 6.0 to which PMSF was added as proteinase inhibitor and subsequently desalted using a PD10 column. Proteins were eluted with 3.5 ml 30 mM MES pH6.0. The enzyme was analyzed by means of polyacrylamide gelelectrophoresis and enzyme activity assays.

The invention will now be further illustrated by the following examples, without being restricted to these specific embodiments.

EXAMPLE 1

Production and secretion of a phenol oxidase by the mould *Acremonium murorum*.

Acremonium murorum was inoculated from a PDA plate, which had been incubated for approximately 1 week at 25°C, in 10 ml Potato Dextrose Broth. The culture was grown for three days at 25°C in a

rotary shaker (250 rpm), then transferred to 100 ml Minimal Medium including 0.5% yeast extract and 2% glucose, and grown for another three days at 25°C. The medium was separated from the mycelium by filtration through Miracloth. Proteins in the medium were concentrated by ammonium sulphate precipitation as described in Materials and Methods under **Protein analysis** and analyzed on a polyacrylamide gel and by enzymatic assays. Both methods of analysis indicated that a phenol oxidising enzyme was produced and secreted into the medium by *Acremonium murorum.*

EXAMPLE 2

Isolation, cloning and characterization of a gene encoding a phenol oxidase from *Acremonium murorum*.

An *Acremonium murorum* library in *S. cerevisiae* VW-K1 consisting of approximately 50,000 colonies was plated on medium containing 4% galactose (w/v), 0.5% glucose (w/v), 0.67% YNB (w/v), 0.1 M sodiumphosphate buffer pH7.2, appropriate amino acids and 120 mg/l cyanidin to yield approximately 3,000 colonies per plate, and incubated at 30°C. After approximately one week of growth one transformant was obtained that produced a clearing zone (halo) around the colony on the cyanidin background. This transformant was purified and plasmid DNA was isolated. The plasmid DNA, designated **pUR7876** (Figure 1), was used to retransform *S. cerevisiae*, which again resulted in halo producing colonies. To determine the DNA sequence of the cDNA insert in pUR7876, a number of internal fragments were subcloned in pUC19. The sequence was determined with the fluorescent M13 universal and reverse primers, using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP Kit (Amersham). Sequence ID No #1 shows the sequence of the EcoRI/XhoI cDNA insert. This insert has a length of 2120 nucleotides, including 5' and 3' non-translated sequences and a poly(A) tail, and comprises an ORF of 1806 nucleotides, encoding an phenol oxidase of 602 amino acids (see Sequence ID No #2).

EXAMPLE 3

Production and secretion of an *Acremonium murorum* phenol oxidase by the mould *Aspergillus awamori*.

To further increase the production levels of the *A. murorum* phenol oxidase, *A. awamori* was used as an alternative expression system. For achievement of the highest expression levels, expression cassettes encoding a fusion protein were constructed. These fusion proteins consist of the *A. niger* glucoamylase preprosequence, the first 514 amino acids of the mature glucoamylase protein and the *A. murorum* phenol oxidising enzyme. In the cassette the glucoamylase and the *A. murorum* anthocyanidin oxidase are separated by the peptide sequence Asn-Val-Ile-Ser-Lys-Arg which comprises a KEX2-type recognition site (Lys-Arg). The expression signals (promoter and terminator) are derived from the *A. awamori* endoxylanase gene as described in WO-A-93/12237 (Unilever).

Construction of the expression vector containing a fusion of *A. niger* glucoamylase with the *A. murorum* phenol oxidase.

In plasmid **pUR7876**, the 3' end of the *A. murorum* gene was adapted for a correct fusion with the *exlA* terminator by introduction of an *AflII* site. Therefore, pUR7876 was digested with *XhoI* and *XbaI* and ligated with a synthetic adapter, consisting of two annealed oligonucleotides with the sequences: (I) 5' TCGAGCTTAAGT 3' and (II) 5' CTAGACTTAAGC 3', resulting in the vector **pUR7880** (Fig. 2). For fusion with the glucoamylase gene and the KEX2 site, the 5' sequence of the *A. murorum* gene in pUR7880 was modified by replacing an *EcoRV/NarI* fragment by a 170 bp PCR fragment which contained a part of the KEX2 recognition site starting at an *EcoRV* site and the 5' part of the *A. murorum* gene up to the *NarI* site. The PCR fragment was obtained in a PCR reaction on plasmid pUR7876 using the primers Acr-06-RG (5'-GAGAGAGATATCCAAGCGCATGCCCAAGTTCGAGCTGGACATTCCTGAGG-3') and Acr-02-RG (5'-GCTTGATCTCGATCTCATAGTAGT-3'). The PCR reaction was carried out with the following parameters: 5 min. 94°C, 25 cycli of 1 min. 94°C, 1 min. 60°C, 1 min. 72°C, and 5 min. 72°C. The 200 bp PCR fragment was subsequently digested with *EcoRV* and *NarI* to give a 170 bp fragment, which was purified from a 2% agarose

gel and ligated to pUR7880 digested with *EcoRV* and *NarI*, giving plasmid **pUR7890** (Fig. 2). From pUR7890, **pUR7891** (Fig. 2) was constructed by cloning the *A. murorum* gene, present on a 2 kb *EcoRV/AflII* fragment, in the *Aspergillus* expression vector pAWGLA2, which was also digested with *EcoRV* and *AflII*. Vector pUR7891 contains an in-frame fusion of glucoamylase with the *A. murorum* gene, separated by a KEX2-like site. Finally, pUR7891 was digested with *NotI* and provided with a double selection marker which was isolated as a 7 kb *NotI* fragment and comprises the *A. nidulans amdS* gene and the *A. awamori pyrG* gene. The resulting vector was called **pUR7893** (Fig. 2).

Strain *A. awamori* AWC4.20 was transformed with pUR7893 as described in the Methods section. Transformants, either obtained from selection for the presence of a wildtype *pyrG* gene or an *amdS* gene, were purified twice on *Aspergillus* minimal medium containing 1% glucose. Conidia were obtained by growing mycelium on potato dextrose agar plates for 5-7 days at 30°C.

AWC-7893 transformants were screened for extracellular phenol oxidase production in a cyanidin plate assay. Four transformants that produced a clearing zone around the fungal colony were chosen for further characterization in submerged cultivations. Shake flasks containing 200 ml AW-medium with 1% sucrose were inoculated with $3x10^8$ spores and precultured during 24 hours, at 30°C and 250 rpm. The mycelium was filtered through miracloth, washed with AW-medium and added to AW-medium (final volume 100 ml) containing 5 % D-xylose. Medium samples were taken 40 hours after induction and applied onto a 8-18% SDS-PAGE gradient gel. Figure 3 shows the gel after staining with Coomassie Brilliant Blue. The phenol oxidase is indicated by an arrow. The gel showed that the transformants *AWC-7893-5p* and *AWC-7893-10A* produced high levels of the recombinant phenol oxidase. As a control, a medium sample of AWGLA, producing glucoamylase, was also applied onto the gel. Figure 4 shows a gel stained with cyanidin, showing the decolorization of cyanidin at the position of the phenol oxidase.

EXAMPLE 4

Purification of the Enzyme.

To purify the enzyme of interest from fermentation supernatant, hydrofobic interaction chromatography (HIC Phenyl Sepharose 6 Fast Flow) is used. The column was equilibrated with 50 mM sodium-phosphate + 30% (1.3M) ammonium-sulphate pH6.0. To the enzyme sample 1.3 M ammonium sulphate was added. Bound proteins were eluted with a lineair decreasing salt gradient. Enzyme activity was measured at pH 6.0 using 2 mM ABTS was measured of the different fractions, those containing phenol oxidizing enzyme were pooled and dialysed against 20mM sodium-phoshate pH 8.0. The enzyme was stored in -20C°.

EXAMPLE 5

Bleaching of Tomato Stains.

The potential of the enzymatic system to bleach stains was assessed by washing cotton swatches soiled with tomato paste. The experiments were performed in 15 ml phosphate buffer with 0,6 g/l LAS added, pH 7, and the enzyme dosed at 0, 1, 2 and 4 mg/l wash solution. Phenothiazine-10-propionate was added as an enhancer of the enzyme activity. This enhancer was added at concentrations of 250 µM. The swatches were washed during 30 minutes, at 30°C. Afterwards, they were tumble dried and the reflectance spectra were measured using a Minolta spectrometer. The data thereby obtained were transferred to the CIELAB L*a*b* colour space parameters. In this colour space, L* indicates lightness and a* and b* are the chromaticity coordinates.

The colour differences between the control swatch, without addition of the enzymatic bleach system, and the swatch washed in the presence of the enzyme and/or phenothiazine-10-propionate, was expressed as ΔE, calculated from the following equation:

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

The colour difference (ΔE) obtained by the above method are given in the table below.

Wash condition	ΔE
1 mg/l enzyme, 250 μM PTP, pH 7	2.3
2 mg/l enzyme, 250 μM PTP, pH 7	3.3
4 mg/l enzyme, 250 μM PTP, pH 7	3.7

As can be seen from the ΔE values, the bleaching of the tomato stain is improved in the presence of the *Acremonium murorum* phenol oxidising enzyme preparation.

CLAIMS

1. A purified phenol oxidizing enzyme obtainable from the fungus *Acremonium*.

2. Phenol oxidizing enzyme according to Claim 1, obtainable from the fungus *Acremonium murorum*.

3. Phenol oxidizing enzyme according to Claim 1, obtainable from the fungus *Acremonium obclavatum*.

4. Phenol oxidizing enzyme according to Claim 2, wherein the fungus is *Acremonium murorum* var. *murorum* having CBS accession number 157.72

5. Phenol oxidizing enzyme according to Claim 2, having an amino acid sequence substantially as in Sequence Listing #2.

6. Phenol oxidizing enzyme having at least one antigentic determinant in common with the phenol oxidizing enzyme obtainable from *Acremonium murorum* var. *murorum* having CBS accession number 157.72 and having an amino acid sequence substantially as in Sequence Listing #2.

7. The phenol oxidizing enzyme of Claim 1 having an apparent molecular weight of approximately 60 kD as determined by SDS-PAGE.

8. Phenol oxidizing enzyme according to any one of the preceding claims, further characterized by having a pH optimum of from 5.0 to 7.0, as determined by incubation for 2 minutes at 20°C with ABTS as substrate.

9. Phenol oxidizing enzyme according to any one of the preceding claims, further characterized by having a pH optimum of from 6.0 to 7.5, as determined by incubation for 2 minutes at 20°C with syringaldizin as substrate.

10. Phenol oxidizing enzyme according to any one of the preceding claims, further characterized by having a pH optimum of from 7.0 to 9.0, as determined by incubation for 2 minutes at 20°C with 2,6-dimethoxyphenol as substrate.

11. Detergent composition comprising a surfactant and a phenol oxidizing enzyme according to any one of the preceding claims.

12. Detergent composition according to Claim 11, further comprising an enhancer.

13. Detergent composition according to Claim 12, wherein the enhancer is selected from the group consisting of phenothiazine-10-propionic acid (PTP), 10-methylphenothiazine (MPT), phenoxazine-10-propionic acid (POP), 10-methylphenoxazine (MPO), 10-ethylphenothiazine-4-carboxylic acid (EPC) acetosyringone, syringaldehyde, methylsyringate, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate (ABTS)

14. A method for bleaching colored compounds which occur in stains present on fabrics, comprising the step of contacting the fabric with a detergent composition according to any one of claims 11-13, at a temperature between 10 and 60°C and wherein said composition has a pH of between 5.0 and 11.0, preferably between 7 and 10.5.

15. Method according to Claim 14, wherein the pH is between 8.0 and 10.5.

16. Method according to Claim 14, wherein the temperature is between 20 and 40°C.

****

Fig.1.

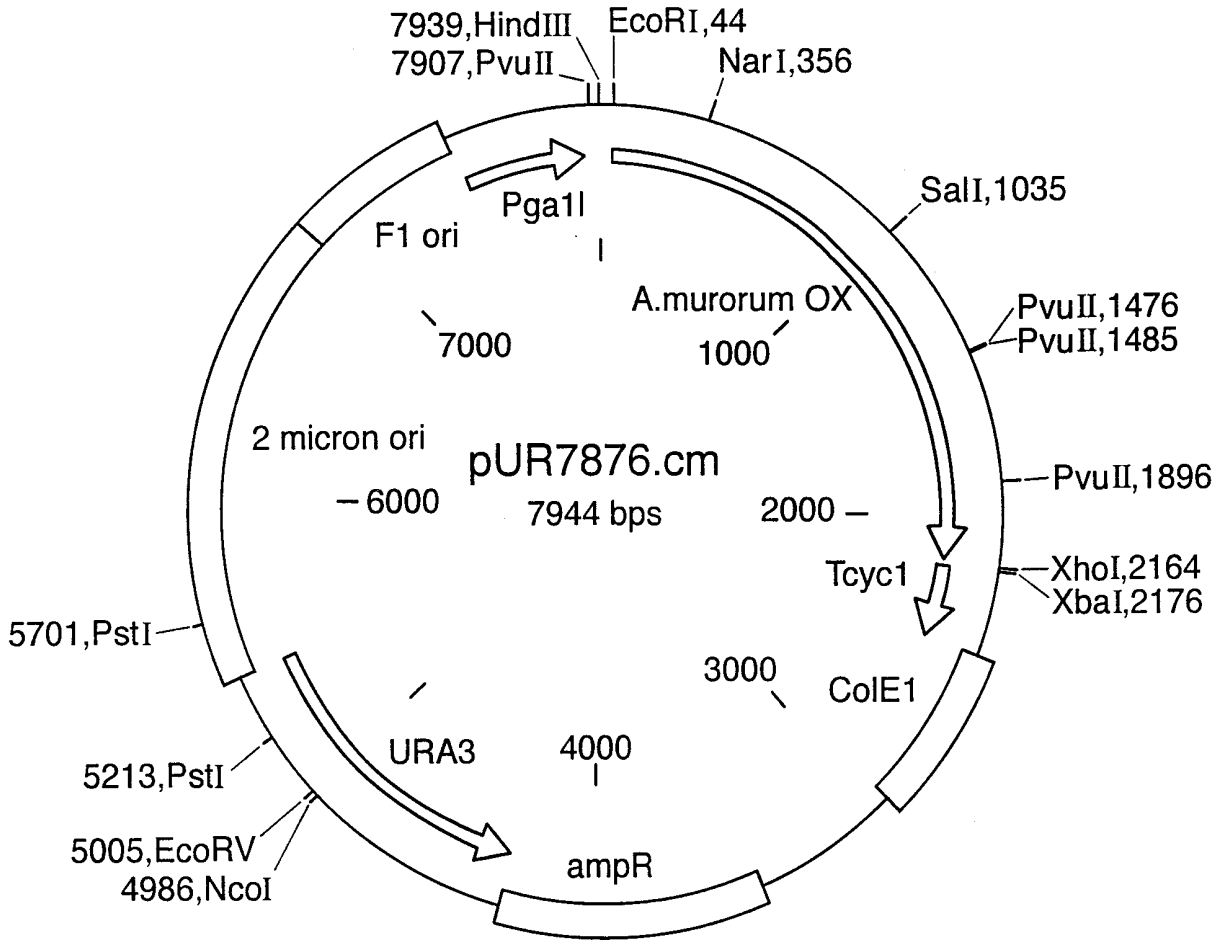
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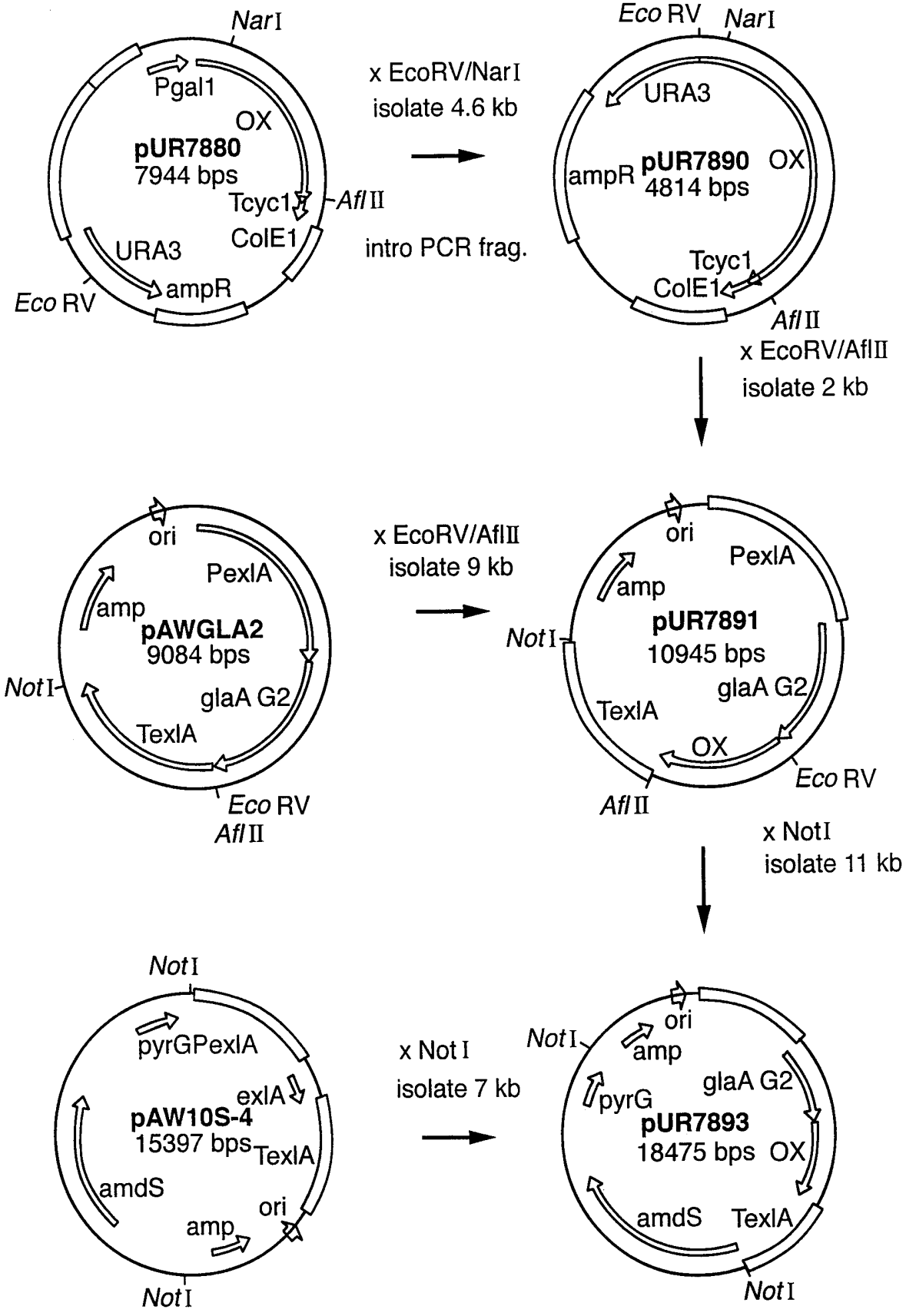
Fig.2.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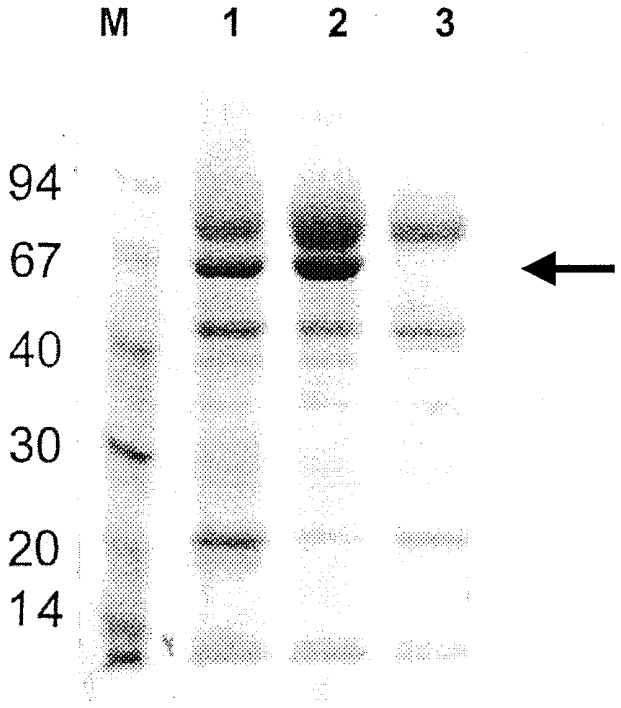

Figure 3

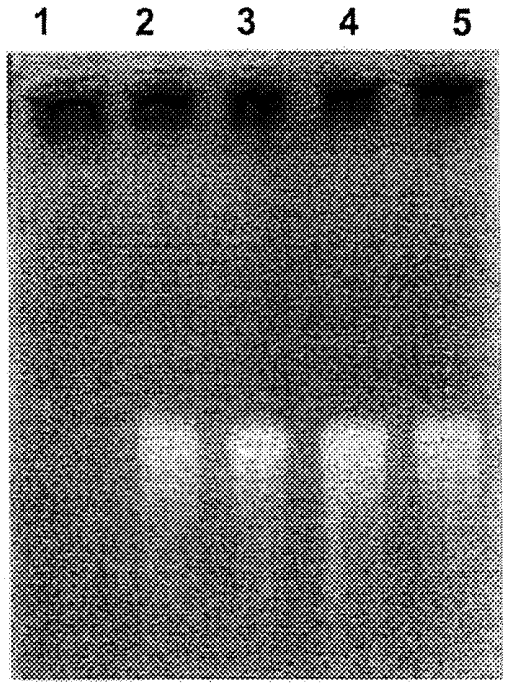

Figure 4

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

Int[illegible]ational Application No
PCT/EP 99/04922

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 05 199882 A (AMANO PHARMACEUT CO LTD) 10 August 1993 (1993-08-10) *SEQ ID NO 13 (page 29 - page 32)* *page 11, column 20* ---	1-10
X	EP 0 852 260 A (NOVONORDISK AS) 8 July 1998 (1998-07-08) *See abstract* *pages 3 and 6-8* ---	1-11, 14-16
X	US 5 180 672 A (ITOH HOMARE ET AL) 19 January 1993 (1993-01-19) *See abstract* -----	1-6,8-10

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☒ Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 November 1999	09/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mata Vicente, T.

INTERNATIONAL SEARCH REPORT

information on patent family members

Int ational Application No
PCT/EP 99/04922

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 9700948 A	09-01-1997
US 5180672 A	19-01-1993	JP 2512578 B	03-07-1996
		JP 3236766 A	22-10-1991
		DE 69123162 D	02-01-1997
		DE 69123162 T	19-06-1997
		EP 0442781 A	21-08-1991