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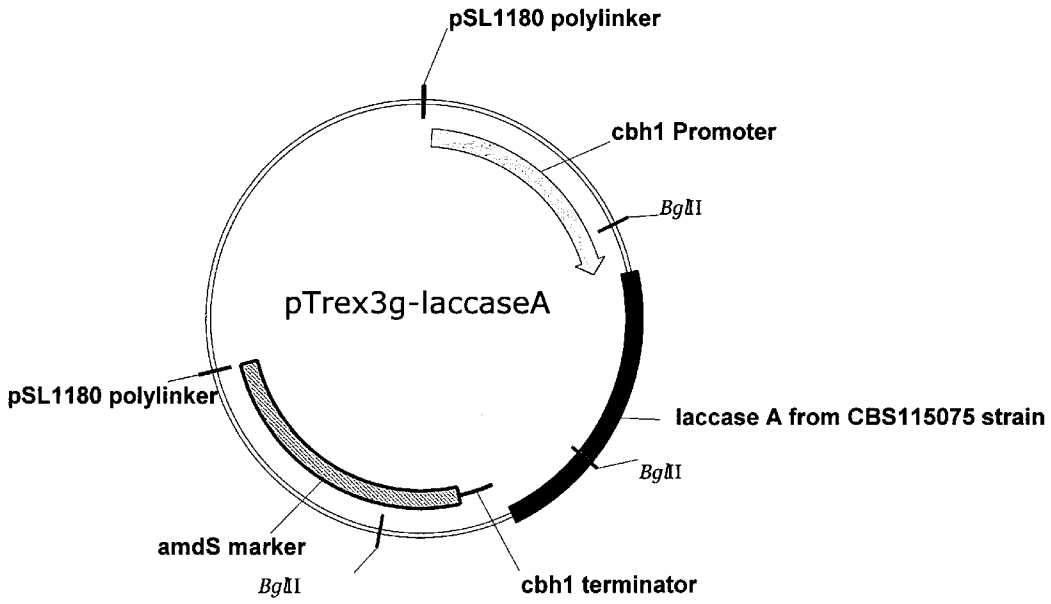
(54) **Title:** NOVEL LACCASES, COMPOSITIONS AND METHODS OF USE



(57) **Abstract:** Novel laccases, nucleic acid sequences encoding such laccases, and vectors and host cells for expressing the laccases are described. The novel laccase enzymes may be employed in conjunction with mediators to provide an improved method for bleaching denim fabrics.

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NOVEL LACCASES, COMPOSITIONS AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] The present application claims priority to U.S. Provisional Patent Application Serial No. 60/875,518, entitled "Novel Laccases, Compositions and Methods of Use", filed 18 December 2006 and U.S. Provisional Patent Application Serial No. 60/875,454, entitled "Laccase Mediators and Methods of Use", filed 18 December 2006.

FIELD OF THE INVENTION

[02] The present invention relates to laccases and nucleic acid sequences encoding the laccases, and to enzymatic methods for bleaching materials.

BACKGROUND OF THE INVENTION

[03] Laccases are copper-containing enzymes that are known to be good oxidizing agents in the presence of oxygen. Laccases are found in microbes, fungi, and higher organisms. Laccase enzymes are used for many applications, including pulp and textiles bleaching, treatment of pulp waste water, de-inking, industrial color removal, bleaching laundry detergents, oral care teeth whiteners, and as catalysts or facilitators for polymerization and oxidation reactions.

[04] Laccases can be utilized for a wide variety of applications in a number of industries, including the detergent industry, the paper and pulp industry, the textile industry and the food industry. In one application, phenol oxidizing enzymes are used as an aid in the removal of stains, such as food stains, from clothes during detergent washing.

[05] Most laccases exhibit pH optima in the acidic pH range while being inactive in neutral or alkaline pHs.

[06] Laccases are known to be produced by a wide variety of fungi, including species of the genii *Aspergillus, Neurospora, Podospora, Botrytis, Pleurotus, Fornes, Phlebia, Trametes, Polyporus, Stachybotrys, Rhizoctonia, Bipolaris, Curvularia, Amerosporium,* and *Lentinus.* However, there remains a need for laccases having different performance profiles in various applications.

[07] For many applications, the oxidizing efficiency of a laccase can be improved through the use of a mediator, also known as an enhancing agent. Systems that include a laccase and a mediator are known in the art as laccase-mediator systems (LMS). The same compounds can also be used to activate or initiate the action of laccase.

[08] There are several known mediators for use in a laccase-mediator system. These include HBT (l-hydroxybenzotriazole), ABTS [2,2'- azinobis(3- ethylbenzothiazoline-6-sulfinic acid)],

NHA (N-hydroxyacetanilide), NEIAA (N-acetyl-N-phenylhydroxylamine), HBTO (3-hydroxy 1,2,3-benzotriazin-4(3H)-one), and VIO (violuric acid). In addition, there are several compounds containing NH-OH or N-O that have been found to be useful as mediators.

[09] Functional groups and substituents have large effects on mediator efficiency. Even within the same class of compounds, a substituent can change the laccase specificity towards a substrate, thereby increasing or decreasing mediator efficiency greatly. In addition, a mediator may be effective for one particular application but unsuitable for another application. Another drawback for current mediators is their tendency to polymerize during use. Thus, there is a need to discover efficient mediators for specific applications. One such application is the bleaching of textiles, wherein it is also important that the mediators are not unduly expensive or hazardous. Other applications of the laccase-mediator system are given below.

[10] Thus, there is a need to identify additional mediators that activate laccase, and/or enhance the activity of enzymes that exhibit laccase activity.

SUMMARY OF THE INVENTION

[11] Described herein are novel laccases, nucleic acid sequences encoding such laccases, and vectors and host cells for expressing the laccases.

BRIEF DESCRIPTION OF THE FIGURES

[12] Figure 1 is a schematic of the *Trichoderma* expression plasmid, pTrex3g-laccaseA, used in Example 7. The laccase A gene may be replaced with other laccase genes described herein.

[13] Figure 2 is a schematic of the *Aspergillus* expression plasmid, pKB401, used in Example 8a. The laccase B gene may be replaced with other laccase genes described herein.

[14] Figure 3 is a schematic of the *Aspergillus* expression plasmid, pKB403, used in Example 8b. The laccase B gene fused to gene encoding the catalytic domain of glucoamylase. The laccase B gene may be replaced with other laccase genes described herein.

[15] Figure 4 is a schematic of the *Trichoderma* expression plasmid, pTrex4-laccaseB, used in Example 8d. The laccase B gene fused to gene encoding the catalytic domain of CBH1. The laccase B gene may be replaced with other laccase genes described herein.

[16] Figure 5 is a schematic of the *Streptomyces* expression plasmid (pKB251) for codon optimized laccase B gene, used in Example 9.

[17] Figure 6 is a schematic of the *Bacillus* expression plasmid (p2JMagk103lnk2E-laccase) for codon optimized laccase D gene fused to the gene encoding BCE103, used in Example 13.

[18] Figure 7 is a bar graph showing the results of bleaching soluble indigo using a *Thielavia* sp. laccase and a variety of mediators at 50 and 500 uM concentrations.

[19] Figure 8 is a bar graph showing the results of bleaching of soluble indigo using a *Thielavia*, *Myceliophthora* and *Cerrena* sp. laccase and a variety of mediators at pH 5.

[20] Figure 9 is a bar graph showing the results of bleaching of soluble indigo using a *Thielavia*, *Myceliophthora* and *Cerrena* sp. laccase and a variety of mediators at pH 7.

[21] Figure 10 is a total color difference graph for the recombinant laccase D and syringamide mediator as a function of mediator concentration and enzyme concentration at 60°C and pH 6.

[22] Figure 11 is a total color difference graph for the recombinant laccase D and syringonitrile mediator as a function of mediator concentration and enzyme concentration at 60°C and pH 6.

DETAILED DESCRIPTION OF THE INVENTION

[23] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, N.Y. (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

[24] The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

[25] All publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention.

I. Laccase and Laccase Related Enzymes

[26] In the context of this invention, laccases and laccase related enzymes contemplate any laccase enzyme comprised by the enzyme classification (EC 1.10.3.2). The laccase enzymes are known from microbial and plant origin. The microbial laccase enzyme may be derived from bacteria or fungi (including filamentous fungi and yeasts) and suitable examples include a laccase derivable from a strain of *Aspergillus, Neurospora,* e.g. *N. crassa. Podospora, Botrytis, Collybia, Cerrena, Stachybotrys, Panus, e.g., Panus rudis, Theilava, Fomes, Lentinus, Pleurotus, Trametes*, e.g. *T. villosa* and *T. versicolor, Rhizoctonia,* e.g. *R. solani, Coprinus,* e.g. *C. plicatilis* and *C. cinereus, Psatyrella, Myceliophthora*, e.g. *M. thermonhila, Schytalidium, Phlebia,* e.g., *P. radita* (WO 92/01046), or *Coriolus,* e.g. *C.hirsutus* (JP 2--238885), *Spongipellis sp., Polyporus, Ceriporiopsis subvermispora, Ganoderma tsunodae* and *Trichoderma.*

[27] The laccase or the laccase related enzyme may furthermore be produced by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said laccase as well as DNA sequences permitting the expression of the DNA sequence encoding the laccase, in a culture medium under conditions permitting the expression of the laccase enzyme, and recovering the laccase from the culture.

[28] The expression vector may be transformed into a suitable host cell, such as a fungal cell, preferred examples of which are species of *Aspergillus*, most preferably *Aspergillus oryzae* and *Aspergillus niger,* and species of *Fusarium,* most preferably *Fusarium venenatum.* Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238,023. The use of *Fusarium* as a host microorganism is described in WO 96/00787 and WO 97/08325.

[29] Alternatively, the host organism may be a bacterium, in particular strains of *Bacillus, Pseudomonas, Streptomyces,* or *E. coli.* The transformation of bacterial cells may be performed according to conventional methods, e.g., as described in T. Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982. The screening of appropriate DNA sequences and construction of vectors may also be carried out by standard procedures, cf. T. Maniatis et al., op. cit.

[30] The medium used to cultivate the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed enzyme may conveniently

be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

[31] In an embodiment, the expression host may be a *Trichoderma reesei* with the laccase coding region under the control of a CBH1 promoter and terminator. (See, *e.g.*, US Patent No. 5,861,271). The expression vector may be pTrex3g, as disclosed in US Patent Application No. 11/245,628 filed 07 October 2005 (Attorney Docket No. GC886).

[32] In this manner the following novel genes and laccases were prepared:

A. Cerrena laccase A1 gene from CBS115.075 strain (SEQ ID No. 1) having the sequence

```
ATGAGCTCAA AGCTACTTGC TCTTATCACT GTCGCTCTCG TCTTGCCACT    50
AGGCACCGAC GCCGGCATCG GTCCTGTTAC CGACTTGCGC ATCACCAACC   100
AGGATATCGC TCCAGATGGC TTCACCCGAC CAGCGGTACT AGCTGGGGGC   150
ACATTCCCTG GAGCACTTAT TACCGGTCAG AAGGTATGGG AGATCAACTT   200
GGTTGAATAG AGAAATAAAA GTGACAACAA ATCCTTATAG GGAGACAGCT   250
TCCAAATCAA TGTCATCGAC GAGCTTACCG ATGCCAGCAT GTTGACCCAG   300
ACATCCATTG TGAGTATAAT TTAGGTCCGC TCTTCTGGCT ATCCTTTCTA   350
ACTCTTACCG TCTAGCATTG GCACGGCTTC TTTCAGAAGG GATCTGCGTG   400
GGCCGATGGT CCTGCCTTCG TTACTCAATG CCCTATCGTC ACCGGAAATT   450
CCTTCCTGTA CGACTTTGAT GTTCCCGACC AACCTGGTAC TTTCTGGTAC   500
CATAGTCACT TGTCTACTCA ATATTGCGAT GGTCTTCGTG GCCCGTTCGT   550
TGTATACGAT CCAAAGGATC CTAATAAACG GTTGTACGAC ATTGACAATG   600
GTATGTGCAT CATCATAGAG ATATAATTCA TGCAGCTACT GACCGTGACT   650
GATGCTGCCA GATCATACGG TTATTACCCT GGCAGACTGG TACCACGTTC   700
TCGCAAGAAC TGTTGTCGGA GTCGCGTAAG TACAGTCTCA CTTATAGTGG   750
TCTTCTTACT CATTTTGACA TAGGACACCC GACGCAACCT TGATCAACGG   800
TTTGGGCCGT TCTCCAGACG GGCCAGCAGA TGCTGAGTTG GCTGTCATCA   850
ACGTTAAACG CGGCAAACGG TATGTTATTG AACTCCCGAT TTCTCCATAC   900
ACAGTGAAAT GACTGTCTGG TCTAGTTATC GATTTCGTCT GGTCTCCATC   950
TCATGTGACC CTAATTACAT CTTTTCTATC GACAACCATT CTATGACTGT  1000
CATCGAAGTC GATGGTGTCA ACACCCAATC CCTGACCGTC GATTCTATTC  1050
AAATCTTCGC AGGCCAACGA TACTCGTTCG TCGTAAGTCT CTTTGCACGA  1100
TTACTGCTTC TTTGTCCATT CTCTGACCTG TTTAAACAGC TCCATGCCAA  1150
CCGTCCTGAA AACAACTATT GGATCAGGGC CAAACCTAAT ATCGGTACGG  1200
ATACTACCAC AGACAACGGC ATGAACTCTG CCATTCTGCG ATACAACGGC  1250
GCACCTGTTG CGGAACCGCA AACTGTTCAA TCTCCCAGTC TCACCCCTTT  1300
GCTCGAACAG AACCTTCGCC CTCTCGTGTA CACTCCTGTG GTATGTTTCA  1350
AAGCGTTGTA ATTTGATTGT GGTCATTCTA ACGTTACTGC GTTTGCATAG  1400
CCTGGAAACC CTACGCCTGG CGGCGCCGAT ATTGTCCATA CTCTTGACTT  1450
GAGTTTTGTG CGGAGTCAAC ATTCGTAAAG ATAAGAGTGT TTCTAATTTC  1500
TTCAATAATA GGATGCTGGT CGCTTCAGTA TCAACGGTGC CTCGTTCCTT  1550
GATCCTACCG TCCCCGTTCT CCTGCAAATT CTCAGCGGCA CGCAGAATGC  1600
ACAAGATCTA CTCCCTCCTG GAAGTGTGAT TCCTCTCGAA TTAGGCAAGG  1650
TCGTCGAATT AGTCATACCT GCAGGTGTCG TCGGTGGACC TCATCCGTTC  1700
CATCTCCATG GGGTACGTAA CCCGAACTTA TAACAGTCTT GGACTTACCC  1750
GCTGACAAGT GCATAGCATA ACTTCTGGGT CGTGCGAAGT GCCGGAACCG  1800
ACCAGTACAA CTTTAACGAT GCCATTCTCC GAGACGTCGT CAGTATAGGA  1850
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GGAACCGGGG ATCAAGTCAC CATTCGTTTC GTGGTATGTT TCATTCTTGT  1900
GGATGTATGT GCTCTAGGAT ACTAACCGGC TTGCGCGTAT AGACCGATAA  1950
CCCCGGACCG TGGTTCCTCC ATTGCCATAT CGACTGGCAC TTGGAAGCGG  2000
GTCTCGCTAT CGTATTTGCA GAGGGAATTG AAAATACTGC TGCGTCTAAT  2050
TTAACCCCCC GTACGCGGTT TCCCTCACAT CCTGGAGCTA AGCAGCTTAC  2100
TAACATACAT TTGCAGAGGC TTGGGATGAG CTTTGCCCGA AGTATAACGC  2150
GCTCAGCGCA CAAAAGAAGG TTGCATCTAA GAAAGGCACT GCCATCTAAT  2200
TTTTGTAACA AACAAGGAGG GTCTCTTGTA CTTTTATTGG GATTTCTTTC  2250
TTGGGGTTTA TTGTTAAACT TGACTCTACT ATGTTTGGAA GACGAAAGGG  2300
GCTCGCGCAT TTATATACTA TCTCTCTTGG CATCACCTGC AGCTCAATCC  2350
TTCAACCACC TAA                                         2363
```

encoding the enzyme laccase A1, having the translated protein sequence (SEQ ID No. 2)

```
MSSKLLALIT VALVLPLGTD AGIGPVTDLR ITNQDIAPDG FTRPAVLAGG    50
TFPGALITGQ KGDSFQINVI DELTDASMLT QTSIHWHGFF QKGSAWADGP   100
AFVTQCPIVT GNSFLYDFDV PDQPGTFWYH SHLSTQYCDG LRGPFVVYDP   150
KDPNKRLYDI DNDHTVITLA DWYHVLARTV VGVATPDATL INGLGRSPDG   200
PADAELAVIN VKRGKRYRFR LVSISCDPNY IFSIDNHSMT VIEVDGVNTQ   250
SLTVDSIQIF AGQRYSFVLH ANRPENNYWI RAKPNIGTDT TTDSGMNSAI   300
LRYNGAPVAE PQTVQSPSLT PLLEQNLRPL VYTPVPGNPT PGGADIVHTL   350
DLSFDAGRFS INGASFLDPT VPVLLQILSG TQNAQDLLPP GSVIPLELGK   400
VVELVIPAGV VGGPHPFHLH GHNFWVVRSA GTDQYNFNDA ILRDVVSIGG   450
TGDQVTIRFV TDNPGPWFLH CHIDWHLEAG LAIVFAEGIE NTAASNLTPQ   500
AWDELCPKYN ALSAQKKLNP STT                               523
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B. Cerrena laccase A2 gene from CBS154.29 strain (SEQ ID No. 3)

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ATGAGCTCAA AGCTACTTGC TCTTATTACT GTCGCTCTCG TCTTGCCACT    50
AGGCACTGAC GCCGGCATCG GTCCTGTTAC CGACTTGCGC ATCACCAACC   100
AGGATATCGC TCCAGATGGC TTCACCCGAC CAGCTGTACT GGCTGGGGGC   150
ACATTCCCCG GAGCACTGAT TACCGGTCAG AAGGTATGGG AGATCGATTT   200
CGTTGAATAG AGAAATACAA CTGAAAACAA ATTCTTATAG GGAGACAGCT   250
TCCAAATCAA TGTCATCGAC GAGCTTACCG ATGCCAGCAT GTTGACCCAG   300
ACATCCATTG TGAGTATAAT ATGGGTCCGC TCTTCTAGCT ATCCTTTCTA   350
ACTCTTACCC TCTAGCATTG GCACGGCTTC TTTCAGAAGG GATCTGCGTG   400
GGCCGATGGT CCTGCCTTCG TTACTCAATG TCCTATCGTC ACCGGAAATT   450
CCTTCCTGTA CGACTTTGAT GTCCCCGACC AACCTGGTAC TTTCTGGTAC   500
CATAGTCACT TGTCTACTCA ATATTGCGAT GGTCTTCGGG GCCCGTTCGT   550
TGTATACGAT CCAAAGGATC CTAATAAACG GTTGTACGAC ATTGACAATG   600
GTATGTGCAT CATCATAAAA ATATAATTCA TGCAGCTACT GACCGCGACT   650
GATGCTGCCA GATCATACGG TTATTACCCT GGCAGACTGG TACCACGTTC   700
TCGCACGAAC TGTTGTCGGA GTCGCGTAAG TACAGTCTGA CTTATAGTGG   750
TCTTCTTACT CATTTTGACA TAGGACACCC GACGCAACCT TGATCAACGG   800
TTTGGGCCGT TCTCCAGACG GGCCAGCAGA TGCTGAGTTG GCTGTCATCA   850
ACGTTAAACG CGGCAAACGG TATGTCATTG AACTCCCGAT TTCTCCATTC   900
ACATTGAAAT GACTGTCTGG TCTAGTTATC GATTCCGTCT GGTCTCCATC   950
TCATGTGACC CTAATTACAT CTTTTCTATC GACAACCATT CTATGACTGT  1000
CATCGAAGTC GATGGTGTCA ACACCCAATC CCTGACCGTC GATTCTATCC  1050
AAATCTTCGC AGGCCAACGC TACTCGTTCG TCGTAAGTCT CTTTGAATGG  1100
TTGGTGCTTT TTCTGTCCAT TCTCTAACCT GTTTATACAG CTCCATGCCA  1150
ACCGTCCTGA AAACAACTAT TGGATCAGGG CCAAACCTAA TATCGGTACG  1200
GATACTACCA CAGACAACGG CATGAACTCT GCCATTCTGC GATACAACGG  1250
CGCACCTGTT GCGGAACCGC AAACTGTTCA ATCTCCCAGT CTCACCCCTT  1300
TGCTCGAACA GAACCTTCGC CCTCTCGTGT ACACTCCTGT GGTATGTTTC  1350
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AAAGCGTTGT AATTTGATTG TGGTCATTCT AACGTTACTG CCTTTGCACA  1400
GCCTGGAAAT CCTACGCCTG GCGGGGCCGA TATTGTCCAT ACTCTTGACT  1450
TGAGTTTTGT GCGGAGTCAA CATTCGTAAA GATAAGAGTG TTTCTAATTT  1500
CTTCAATAAT AGGATGCTGG TCGCTTCAGT ATCAACGGTG CCTCGTTCCT  1550
TGATCCTACC GTCCCTGTTC TCCTGCAAAT TCTCAGCGGC ACGCAGAATG  1600
CACAAGATCT ACTCCCTCCT GGAAGTGTGA TTCCTCTCGA ATTAGGCAAG  1650
GTCGTCGAAT TAGTCATACC TGCAGGTGTT GTCGGTGGAC CTCATCCGTT  1700
CCATCTCCAT GGGGTACGTA ACCCGAACTT ATAACAGTCT TGGACTTACC  1750
CGCTGACAAG TGTATAGCAT AACTTCTGGG TCGTGCGAAG TGCCGGAACC  1800
GACCAGTACA ACTTTAACGA TGCCATTCTC CGAGACGTCG TCAGTATAGG  1850
AGGAACCGAG GATCAAGTCA CCATTCGATT CGTGGTATAT ACTTCATTCT  1900
TGTGGATGTA TGTGCTCTAG GATACTAACT GGCTTGCGCG TATAGACCGA  1950
TAACCCCGGA CCGTGGTTCC TCCATTGCCA TATCGACTGG CACTTGGAAG  2000
CGGGTCTCGC TATCGTATTT GCAGAGGGAA TTGAAAATAC TGCTGCGTCT  2050
AATCCAACCC CCCGTATGCG GTTTCCCACA CATTCTGAAT CTAAGCAGCT  2100
TACTAATATA CATTTGCAGA GGCTTGGGAT GAGCTTTGCC CGAAGTATAA  2150
CGCGCTCAAC GCACAAAAGA AGGTTGCATC TAAGAAAGGC ACTGCCATCT  2200
AATCCTTGTA ACAAACAAGG AGGGTCTCTT GTACTTTTAT TGGGATTTAT  2250
TTCTTGGGGT TTATTGTTCA ACTTGATTCT ACTATGTTTG GAAGTAGCGA  2300
TTACGAAAGG GGCTTGCGCA TTTATATACC ATCTTTCTTG GCACCACCTG  2350
CAGCTCAATC CTTCAACCAC CTAA                              2374
```

encoding the enzyme laccase A2, having the translated protein sequence shown in (SEQ ID No. 4)

```
MSSKLLALIT VALVLPLGTD AGIGPVTDLR ITNQDIAPDG FTRPAVLAGG    50
TFPGALITGQ KGDSFQINVI DELTDASMLT QTSIHWHGFF QKGSAWADGP   100
AFVTQCPIVT GNSFLYDFDV PDQPGTFWYH SHLSTQYCDG LRGPFVVYDP   150
KDPNKRLYDI DNDHTVITLA DWYHVLARTV VGVATPDATL INGLGRSPDG   200
PADAELAVIN VKRGKRYRFR LVSISCDPNY IFSIDNHSMT VIEVDGVNTQ   250
SLTVDSIQIF AGQRYSFVLH ANRPENNYWI RAKPNIGTDT TTDNGMNSAI   300
LRYNGAPVAE PQTVQSPSLT PLLEQNLRPL VYTPVPGNPT PGGADIVHTL   350
DLSFDAGRFS INGASFLDPT VPVLLQILSG TQNAQDLLPP GSVIPLELGK   400
VVELVIPAGV VGGPHPFHLH GHNFWVVRSA GTDQYNFNDA ILRDVVSIGG   450
TEDQVTIRFV TDNPGPWFLH CHIDWHLEAG LAIVFAEGIE NTAASNPTPQ   500
AWDELCPKYN ALNAQKKLNP STT                               523
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C. Cerrena laccase B1 gene from CBS115.075 strain (SEQ ID No. 5)

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ATGTCTCTTC TTCGTAGCTT GACCTCCCTC ATCGTACTAG TCATTGGTGC    50
ATTTGCTGCA ATCGGTCCAG TCACTGACCT ACATATAGTG AACCAGAATC   100
TCGACCCAGA TGGTTTCAAC CGCCCCACTG TACTCGCAGG TGGTACTTTC   150
CCCGGTCCTC TGATTCGTGG TAACAAGGTA CGCTTCATAA CCGCCCTCCG   200
TAGACGTAGG CTTCGGCTGA CATGACCATC ATCTGTAGGG AGATAACTTT   250
AAAATTAATG TGATTGACGA CTTGACAGAG CACAGTATGC TCAAGGCTAC   300
GTCCATCGTA AGTCCCTGAT TAACGTTTCA CCTGGTCATA TCGCTCAACG   350
TCTCGAAGCA CTGGCATGGG TTCTTCCAGA AGGGAACCAA CTGGGCCGAT   400
GGCCCCGCCT TTGTCACCCA ATGTCCTATC ACATCAGGAA ACGCCTTCCT   450
GTATGATTTC AACGTTCCGG ACCAAGCTGG TACTTTCTGG TACCACAGCC   500
ATCTCTCTAC ACAGTATTGT GACGGTCTTC GTGGTGCCTT TGTCGTCTAT   550
GATCCTAATG ATCCCAACAA GCAACTCTAT GATGTTGATA ACGGCAAGTT   600
CCTTGCATAT TTCATTTCTA TCATATCCTC ACCTGTATTG GCACAGAAAG   650
CACCGTGATT ACCTTGGCTG ATTGGTATCA TGCCCTTGCT CAGACTGTCA   700
CTGGTGTCGC GTGAGTGACA AATGGCCCTC AATTGTTCAC ATATTTTCCT   750
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GATTATCATA TGATAGAGTA TCTGATGCAA CGTTGATCAA CGGATTGGGA   800
CGTTCGGCCA CCGGCCCCGC AAATGCCCCT CTGGCGGTCA TCAGTGTCGA   850
GCGGAATAAG AGGTCAGTTC CATAATTATG ATTATTTCCC GCGTTACTTC   900
CTAACAATTA TTTTTGTATC CCTCCACAGA TATCGTTTCC GATTGGTTTC   950
TATTTCTTGC GACCCTAACT TTATTTTCTC AATTGACCAC CACCCAATGA  1000
CCGTAATTGA GATGGACGGT GTTAATACCC AATCTATGAC CGTAGATTCG  1050
ATCCAAATAT TCGCAGGTCA ACGATATTCA TTTGTCGTAG GTTATTATAA  1100
ACTGCCCACC GATCATCTCT CACGTAACTG TTATAGATGC AAGCCAACCA  1150
ACCAGTTGGA AATTATTGGA TCCGCGCTAA ACCTAATGTT GGGAACACAA  1200
CTTTCCTTGG AGGCCTGAAC TCCGCTATAT TACGATATGT GGGAGCCCCT  1250
GACCAAGAAC CGACCACTGA CCAAACACCC AACTCTACAC CGCTCGTTGA  1300
GGCGAACCTA CGACCCCTCG TCTATACTCC TGTGGTATGT TGTTCTCGTT  1350
ACATATACCA AACCTAATAT GAAGACTGAA CGGATCTACT AGCCGGGACA  1400
GCCATTCCCT GGCGGTGCTG ATATCGTCAA GAACTTAGCT TTGGGTTTCG  1450
TACGTGTATT TCACTTCCCT TTTGGCAGTA ACTGAGGTGG AATGTATATA  1500
GAATGCCGGG CGTTTCACAA TCAATGGAGC GTCCCTCACA CCTCCTACAG  1550
TCCCTGTACT ACTCCAGATC CTCAGTGGTA CTCACAATGC ACAGGATCTT  1600
CTCCCAGCAG GAAGCGTGAT CGAACTTGAA CAGAATAAAG TTGTCGAAAT  1650
CGTTTTGCCC GCTGCGGGCG CCGTTGGCGG TCCTCATCCT TTTCACTTAC  1700
ATGGTGTAAG TATCAGACGT CCTCATGCCC ATATTGCTCC GAACCTTACA  1750
CACCTGATTT CAGCACAATT TCTGGGTGGT TCGTAGCGCC GGTCAAACCA  1800
CATACAATTT CAATGATGCT CCTATCCGTG ATGTTGTCAG TATTGGCGGT  1850
GCAAACGATC AAGTCACGAT CCGATTTGTG GTATGTATCT CGTGCCTTGC  1900
ATTCATTCCA CGAGTAATGA TCCTTACACT TCGGGTTCTC AGACCGATAA  1950
CCCTGGCCCA TGGTTCCTTC ACTGTCACAT TGACTGGCAT TTGGAGGCTG  2000
GGTTCGCTGT AGTCTTTGCG GAGGGAATCA ATGGTACTGC AGCTGCTAAT  2050
CCAGTCCCAG GTAAGACTCT CGCTGCTTTG CGTAATATCT ATGAATTTAA  2100
ATCATATCAA TTTGCAGCGG CTTGGAATCA ATTGTGCCCA TTGTATGATG  2150
CCTTGAGCCC AGGTGATACA TGA                               2173
```

encoding the enzyme laccase B1, having the translated protein sequence (SEQ ID No. 6)

```
MSLLRSLTSL IVLVIGAFAA IGPVTDLHIV NQNLDPDGFN RPTVLAGGTF    50
PGPLIRGNKG DNFKINVIDD LTEHSMLKAT SIHWHGFFQK GTNWADGPAF   100
VTQCPITSGN AFLYDFNVPD QAGTFWYHSH LSTQYCDGLR GAFVVYDPND   150
PNKQLYDVDN GNTVITLADW YHALAQTVTG VAVSDATLIN GLGRSATGPA   200
NAPLAVISVE RNKRYRFRLV SISCDPNFIF SIDHHPMTVI EMDGVNTQSM   250
TVDSIQIFAG QRYSFVMQAN QPVGNYWIRA KPNVGNTTFL GGLNSAILRY   300
VGAPDQEPTT DQTPNSTPLV EANLRPLVYT PVPGQPFPGG ADIVKNLALG   350
FNAGRFTING ASLTPPTVPV LLQILSGTHN AQDLLPAGSV IELEQNKVVE   400
IVLPAAGAVG GPHPFHLHGH NFWVVRSAGQ TTYNFNDAPI RDVVSIGGAN   450
DQVTIRFVTD NPGPWFLHCH IDWHLEAGFA VVFAEGINGT AAANPVPAAW   500
NQLCPLYDAL SPGDT                                       515
```

D. Cerrena laccase B2 gene from CBS154.29 strain (SEQ ID No. 7)

```
CACCGCGATG TCTCTTCTTC GTAGCTTGAC CTCCCTCATC GTACTAGCCA    50
CTGGTGCATT TGCTGCAATC GGTCCAGTCA CCGACCTACA TATAGTGAAC   100
CAGAATCTCG CCCCAGATGG TTTAAACCGC CCCACTGTAC TCGCAGGTGG   150
TACTTTCCCC GGTCCTCTGA TTCGTGGTAA CAAGGTACGC TTCATAACCG   200
CCCTCCGTAG ACGTAGGCTT CGGCTGACAT GACCATCATC TGTAGGGAGA   250
TAACTTTAAA ATTAATGTGA TTGACGACTT GACAGAACAC AGTATGCTCA   300
AGGCTACGTC CATTGTAAGT CCCTGATTAA CGTTTCACCT GGTCATATCG   350
CTCAACGTCT CGAAGCACTG GCATGGGTTC TTCCAGAAGG GAACCAACTG   400
GGCCGATGGC CCCGCCTTTG TCACCCAATG TCCTATCACA TCAGGAAACG   450
```

```
CCTTCTTGTA TGATTTCAAC GTTCCGGACC AAGCTGGTAC TTTCTGGTAC   500
CACAGCCATC TCTCYACACA GTATTGTGAC GGTCTTCGTG GTGCCTTTGT   550
CGTCTATGAT CCTAATGATC CCAACAAGCA ACTCTATGAT GTTGATAACG   600
GCAAGTCCCT TGCATATTTC AGTTCTATCA TATCCTCACC TGTATTGGCA   650
CAGAAAGCAC CGTGATTACC TTGGCTGATT GGTATCATGC CCTTGCTCAG   700
ACTGTCACTG GTGTCGCGTG AGTGACAAAT GGCCCTTAAT TGTTCACATA   750
TTTTCCTGAT TATCATATGA TAGAGTATCT GATGCAACGT TGATCAACGG   800
ATTGGGACGT TCGGCCACCG GCCCCGCAAA TGCCCCTCTG GCGGTCATCA   850
GTGTCGAGCG GAATAAGAGG TCAGTTCCAT AATTATGATT ATTTCCCGCG   900
TTACTTCCTA ACGATTATTT TTGTATCCCT CCACAGATAT CGTTTCCGAT   950
TGGTTTCTAT TTCTTGCGAC CCTAACTTTA TTTTCTCAAT TGACCACCAC  1000
CCAATGACCG TAATTGAGAT GGACGGTGTT AATACCCAAT CTATGACCGT  1050
AGATTCGATC CAAATATTCG CAGGTCAACG ATATTCATTT GTCGTAGGTT  1100
ATTATAAACT GCCCACCGAT CATCTCTCAC GTAACTGTTA TAGATGCAAG  1150
CCAACCAACC AGTTGGAAAT TATTGGATCC GYGCTAAACC TAATGTTGGG  1200
AACACAACTT TCCTTGGAGG CCTGAACTCC GCTATATTAC GATATGTGGG  1250
AGCCCCTGAC CAAGAACCGA CCACTGACCA AACACCCAAC TCTACACCGC  1300
TCGTCGAGGC GAACCTACGT CCCCTCGTCT ATACTCCTGT GGTATGTTGT  1350
TCTCGTTACA TATACCAAAC CTAATATGAG GACTGAACGG ATCTACTAGC  1400
CGGGACAGCC ATTCCCTGGC GGTGCTGATA TCGTCAAGAA CTTAGCTTTG  1450
GGTTTCGTAC GTGTATTTCA CTTCCCTTTT GGCAGTAACT GAGGTGGAAT  1500
GTATATAGAA TGCCGGGCGT TTCACAATCA ATGGAACATC CTTCACACCT  1550
CCTACAGTCC CTGTACTACT CCAGATCCTC AGTGGTACTC ACAATGCACA  1600
GGATCTTCTT CCAGCAGGAA GCGTGATCGA ACTTGAACAG AATAAAGTTG  1650
TCGAAATCGT TCTGCCCGCT GCGGGCGCCG TTGGCGGTCC TCATCCTTTC  1700
CACTTACATG GTGTAAGTAT CAGACGTCCT CATGCCTATA TTGCTCCGAA  1750
CCTTACACAC CTGATTTCAG CACAATTTCT GGGTGGTTCG TAGCGCCGGT  1800
CAAACCACAT ACAATTTCAA TGATGCTCCT ATCCGTGATG TTGTCAGTAT  1850
TGGCGGTGCA AACGATCAAG TCACGATCCG ATTTGTGGTA TGTATCTCGT  1900
GCCTTGCATT CATTCCACGA GTAATGATCC TTACACTTCG GGTTCTCAGA  1950
CCGATAACCC TGGCCCATGG TTCCTTCACT GTCACATTGA CTGGCATTTG  2000
GAGGCTGGGT TCGCTGTAGT CTTTGCGGAG GGAATCAATG GCACTGCAGC  2050
TGCTAATCCA GTCCCAGGTA AGACTCTCGC TGCTTTGCGT AATATCTATG  2100
AATTTAAAGC ATATCAATTT GCAGCGGCTT GGAATCAATT GTGCCCGTTG  2150
TATGATGCCT TGAGCCCAGG tGATACATGA TTACTCGTAG CTGTGCTTTC  2200
TTATACATAT TCTATGGGTA TATCGGAGTA GCTGTACTAT AGTATGTACT  2250
ATACTAGGTG GGATATGYTG ATGTTGATTT ATATAATTTT GTTTGAAGAG  2300
TGACTTTATC GACTTGGGAT TTAGCCGAGT ACATACTGAT CTCTCACTAC  2350
AGGCTTGTTT TGTCTTTGGG CGCTTACTCA ACAGTTGACT GTTTTTGCTA  2400
TTACGCATTG AACCGCATTC CGGTCYGACT CGTGTCCTCT ACTGTGACTT  2450
GTATTGGCAT TCTAGCACAT ATGTCTCTTA CCTATAGGAA CAATATGTCT  2500
CAACACTGTT CCAAAACCTG CGTAAACCAA ATATCGTCCA TCAGATCAGA  2550
TCATTAACAG TGCCGCACTA ACCTAATACA CTGGCARGGA CTGTGGAAAT  2600
CCCTATAAAT GACCTCTAGA CCGTGAGGTC ATTGCAAGGT CGCTCTCCTT  2650
GTCAAGATGA CCC                                          2663
```

encoding the enzyme laccase B2, having the translated protein sequence (SEQ ID No. 8)

```
MSLLRSLTSL IVLATGAFAA IGPVTDLHIV NQNLAPDGLN RPTVLAGGTF    50
PGPLIRGNKG DNFKINVIDD LTEHSMLKAT SIHWHGFFQK GTNWADGPAF   100
VTQCPITSGN AFLYDFNVPD QAGTFWYHSH LSTQYCDGLR GAFVVYDPND   150
PNKQLYDVDN GNTVITLADW YHALAQTVTG VAVSDATLIN GLGRSATGPA   200
NAPLAVISVE RNKRYRFRLV SISCDPNFIF SIDHHPMTVI EMDGVNTQSM   250
TVDSIQIFAG QRYSFVMQAN QPVGNYWIRA KPNVGNTTFL GGLNSAILRY   300
VGAPDQEPTT DQTPNSTPLV EANLRPLVYT PVPGQPFPGG ADIVKNLALG   350
```

```
FNAGRFTING TSFTPPTVPV LLQILSGTHN AQDLLPAGSV IELEQNKVVE   400
IVLPAAGAVG GPHPFHLHGH NFWVVRSAGQ TTYNFNDAPI RDVVSIGGAN   450
DQVTIRFVTD NPGPWFLHCH IDWHLEAGFA VVFAEGINGT AAANPVPAAW   500
NQLCPLYDAL SPGDT                                         515
```

E. Cerrena laccase B3 gene (partial) from ATCC20013 strain (SEQ ID No. 9)

```
GTGGGGGCGG ATCCCTAACT GTTTCGAATC GGCACCGAAG TATGCAGGTG    50
TGACGGAGAT GAGGCGTTTT TTCATCTTCC ACTGCAGTAT AAAATGTCTC   100
AGGTAACGTC CAGCTTTTTG TACCAGAGCT ACCTCCAAAT ACCTTTACTC   150
GCAAAGGTTT CGCGATGTCT CTTCTTCGTA GCTTGACCTC CCTCATCGTA   200
CTAGCCACTG GTGCATTTGC TGCAATCGGT CCAGTCACTG ACCTACATAT   250
AGTGAACCAG AATCTCGCCC CAGATGGTTT CAACCGCCCC ACTGTACTCG   300
CAGGTGGTAC TTTCCCCGGT CCTCTGATTC GTGGTAACAA GGTACGCTTC   350
ATAACCGCCC TCCGTAGACG TAGGCTTCGG CTGACATGAC CATCATCTGT   400
AGGGAGATAA CTTTAAAATT AATGTGATTG ACGACTTGAC AGAACACAGT   450
ATGCTCAAGG CCACGTCCAT TGTAAGTCCC TGATTAACGT TTCACCTGGT   500
CATATCGCTC AACGTCTCGA AGCACTGGCA TGGGTTCTTC CAGAAGGGAA   550
CCAACTGGGC CGATGGCCCC GCCTTTGTCA CCCAATGTCC TATCACATCA   600
GGAAACTCCT TCCTGTATGA TTTCAACGTT CCGGACCAAG CTGGTACTTT   650
CTGGTACCAC AGCCATCTCT CTACACAGTA TTGTGACGGT CTTCGTGGTG   700
CCTTTGTCGT CTATGATCCT AATGATCCCA ACAAGCAACT CTATGATGTT   750
GATAACGGCA AGTCCCTTGC ATATTTCATT TCTATCATAT CCTCACCTGT   800
ATTGGCACAG AAAGCACCGT GATTACCTTG GCTGATTGGT ATCATGCCCT   850
TGCTCAGACT GTCACTGGTG TCGCGTGAGT GACAAATGGC CCTCAATTGT   900
TCACATATTT TCCTGATTAT CATATGATAG AGTATCTGAT GCAACGTTGA   950
TCAACGGATT GGGACGTTCG GCCACCGGCC CCGCAAATGC CCCTCTGGCG  1000
GTCATCAGTG TCGAGCGGAA TAAGAGGTCA GTTCCATAAT TATGATTATT  1050
TCCCGCGTTA CTTCCTAACA ATTATTCTTG TATCCCTCCA CAGATATCGC  1100
TTCCGATTGG TGTCTATTTC TTGCGACCCT AACTTTATTT TCTCAATTGA  1150
TCACCACCCA ATGACCGTAA TTGAGATGGA CGGTGTTAAT ACCCAATCTA  1200
TGACCGTAGA TTCGATCCAA ATATTCGCAG GTCAACGATA TTCATTTGTC  1250
GTAGGTTATT ATAAACTGCC CACCGATCAT CTCTCACGTA ACTGTTATAG  1300
ATGCAAGCCA ACCAACCRGT TGGAAATTAT TGGATCC                1337
```

encoding the enzyme laccase B3, having the partial translated protein sequence (SEQ ID No. 10)

```
MSLLRSLTSL IVLATGAFAA IGPVTDLHIV NQNLAPDGFN RPTVLAGGTF    50
PGPLIRGNKG DNFKINVIDD LTEHSMLKAT SIHWHGFFQK GTNWADGPAF   100
VTQCPITSGN SFLYDFNVPD QAGTFWYHSH LSTQYCDGLR GAFVVYDPND   150
PNKQLYDVDN GKTVITLADW YHALAQTVTG VAVSDATLIN GLGRSATGPA   200
NAPLAVISVE RNKRYRFRLV SISCDPNFIF SIDHHPMTVI EMDGVNTQSM   250
TVDSIQIFAG QRYSFVMQAN QPVGNYWI                           278
```

F. Cerrena laccase C gene (partial) from CBS154.29 strain (SEQ ID No. 11)

```
TGCAATCGGA CCGGTBGCTG ACCTTCACAT TACGGACGAT ACCATTGCCC    50
CCGATGGTTT CTCTCGTCCT GCTGTTCTCG CTGGCGGGGG TTTCCCTGGC   100
CCTCTCATCA CCGGAAACAA GGTAATGCCT AATGGTTGCG TCTTTGTTGG   150
TGCTCTCATT CATCCACGAC ATTTTGTACC AGGGCGACGC CTTTAAACTC   200
AATGTCATCG ATGAACTAAC GGACGCATCC ATGCTGAAGY CGACTTCCAT   250
CGTAAGTCTC GCTGTATTGC TCCTTGAGCC ATTTCATTGA CTATAACTAC   300
AACCAGCACT GGCATGGATT CTTCCAAAAG GGTACTAATT GGGCAGATGG   350
TCCCGCTTTT GTGAACCAAT GCCCCATCAC CACGGGAAAC TCCTTCTTGT   400
ACGACTTCCA GGTTCCTGAT CAAGCTGGTA AGCATGAGAT TACACTAGGA   450
```

```
AAGTTTAATT TAATAACTAT TCAATCAGGA ACCTACTGGT ATCATAGTCA   500
TTTGTCTACG CAATACTGTG ATGGTCTCAG AGGTGCATTC GTTGTCTACG   550
ACCCTTCAGA TCCTCACAAG GATCTCTACG ACGTCGACGA CGGTGAGCTT   600
TGCTTTTTTC ATTGGTATCC ATTATCGCTC ACGTGTCATT ACTGCGCCAC   650
AGAAAGTACC GTCATCACTT TGGCTGATTG GTATCATACT TTGGCTCGTC   700
AGATTGTTGG CGTTGCGTGA GTAGTCTTGT ACCGACTGAA ACATATTCCA   750
GTTGCTGACT TCCCCACAGC ATTTCTGATA CTACCTTGAT AAACGGTTTG   800
GGCCGCAATA CCAATGGTCC GGCTGATGCT GCTCTTGCTG TGATCAATGT   850
TGACGCTGGC AAACGGTGTG TCCAGATTAC TATACTCCCC ATGACGTCTC   900
AATGCTGATG TGTACTACTT CCAGGTACCG TTTCCGTCTT GTTTCCATAT   950
CCTGTGACCC CAATTGGGTA TTCTCGATTG ACAACCATGA CTTTACGGTC  1000
ATTGAAGTCG ATGGTGTTAA CAGTCAACCT CTCAACGTCG ATTCTGTTCA  1050
GATCTTCGCC GGACAACGTT ACTCGTTCGT                        1080
```

encoding the enzyme laccase C, having the partial translated protein sequence (SEQ ID No. 12)

```
AIGPVADLHI TDDTIAPDGF SRPAVLAGGG FPGPLITGNK GDAFKLNVID    50
ELTDASMLKX TSIHWHGFFQ KGTNWADGPA FVNQCPITTG NSFLYDFQVP   100
DQAGTYWYHS HLSTQYCDGL RGAFVVYDPS DPHKDLYDVD DESTVITLAD   150
WYHTLARQIV GVAISDTTLI NGLGRNTNGP ADAALAVINV DAGKRYRFRL   200
VSISCDPNWV FSIDNHDFTV IEVDGVNSQP LNVDSVQIFA GQRYSF       246
```

G. Cerrena laccase D1 gene from CBS154.29 strain (SEQ ID No. 13)

```
GATTCTAATA GACCAGGCAT ACCAAGAGAT CTACAGGTTG ACAGACCATT    50
CTTCTAGGCG GCATTTATGC TGTAGCGTCA GAAATTATCT CTCCATTTGT   100
ATCCCACAGG TCCTGTAATA ACACGGAGAC AGTCCAAACT GGGATGCCTT   150
TTTTCTCAAC TATGGGCGCA CATAGTCTGG ACGATGGTAT ATAAGACGAT   200
GGTATGAGAC CCATGAAGTC AGAACACTTT TGCTCTCTGA CATTTCATGG   250
TTCACACTCT CGAGATGGGA TTGAACTCGG CTATTACATC GCTTGCTATC   300
TTAGCTCTGT CAGTCGGAAG CTATGCTGCA ATTGGGCCCG TGGCCGACAT   350
ACACATTGTC AACAAAGACC TTGCTCCAGA TGGCGTACAA CGTCCAACCG   400
TGCTTGCCGG AGGCACTTTT CCTGGGACGT TGATCACCGG TCAGAAAGTA   450
AGGGATATTA GTTTGCGTCA AAGAGCCAAC CAAAACTAAC CGTCCCGTAC   500
TATAGGGTGA CAACTTCCAG CTCAATGTCA TCGATGATCT TACCGACGAT   550
CGGATGTTGA CGCCAACTTC CATTGTGAGC CTATTATTGT ATGATTTATC   600
CGAATAGTTT CGCAGTCTGA TCATTGGATC TCTATCGCTA GCATTGGCAC   650
GGTTTCTTCC AGAAGGGAAC CGCTTGGGCC GACGGTCCCG CCTTCGTAAC   700
TCAGTGCCCT ATAATAGCAG ATAACTCTTT TCTGTATGAC TTCGACGTCC   750
CAGACCAAGC TGGTACTTTC TGGTATCATA GTCATCTATC CACTCAGTAC   800
TGTGACGGTT TACGTGGTGC CTTCGTTGTG TACGATCCTA ACGATCCTCA   850
CAAAGACCTA TACGATGTTG ATGACGGTGG GTTCCAAATA TTTGTTCTGC   900
AGACATTGTA TTGACGGTGT TCATTATAAT TTCAGAGAGC ACCGTGATTA   950
CCCTTGCGGA TTGGTACCAT GTTCTCGCCC AGACCGTTGT CGGCGCTGCG  1000
TGAGTAACAC ATACACGCGC TCCGGCACAC TGATACTAAT TTTTTTTTAT  1050
TGTAGCACTC CTGATTCTAC CTTGATCAAC GGGTTAGGCC GTTCACAGAC  1100
CGGACCCGCT GATGCTGAGC TGGCTGTTAT CAGCGTTGAA CATAACAAAC  1150
GGTATGTCAT CTCTACCCAG TATCTTCTCT CCTGCTCTAA TTCGCTGTTT  1200
CACCATAGAT ACCGTTTCCG TTTGGTTTCG ATTTCGTGCG ACCCCAACTT  1250
TACCTTCTCC GTTGATGGTC ATAATATGAC TGTCATCGAA GTCGATGGTG  1300
TCAACACACG ACCCCTGACC GTTGACTCTA TTCAAATCTT CGCCGGACAG  1350
AGGTATTCCT TTGTCGTAAG TTAATCGATA TATTCTCCTT ATTACCCCTG  1400
TGTAATTGAT GTCAATAGCT CAATGCTAAC CAACCCGAAG ACAATTACTG  1450
GATCCGTGCT ATGCCAAACA TCGGTAGAAA TACAACAACA CTGGACGGAA  1500
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AGAATGCCGC TATCCTTCGA TACAAGAATG CTTCTGTAGA AGAGCCCAAG  1550
ACCGTTGGGG GCCCCGCTCA ATCCCCGTTG AATGAAGCGG ACCTGCGTCC  1600
ACTCGTACCT GCTCCTGTGG TATGTCTTGT CGCGCTGTTC CATCGCTATT  1650
TCATATTAAC GTTTTGTTTT TGTCAAGCCT GGAAACGCTG TTCCAGGTGG  1700
CGCAGACATC AATCACAGGC TTAACTTAAC TTTCGTACGT ACACCTGGTT  1750
GAAACATTAT ATTTCCAGTC TAACCTCTCT TGTAGAGTAA CGGCCTCTTC  1800
AGCATCAACA ACGCCTCCTT CACTaATCCT TCGGTCCCCG CCTTATTACA  1850
AATTCTGAGC GGTGCTCAGA ACGCTCAAGA TTTACTTCCA ACGGGTAGTT  1900
ACATTGGCCT TGAACTAGGC AAGGTTGTGG AGCTCGTTAT ACCTCCTCTG  1950
GCAGTTGGAG GACCGCACCC TTTCCATCTT CATGGCGTAA GCATACCACA  2000
CTCCCGCAGC CAGAATGACG CAAACTAATC ATGATATGCA GCACAATTTC  2050
TGGGTCGTCC GTAGTGCAGG TAGCGATGAG TATAACTTTG ACGATGCTAT  2100
CCTCAGGGAC GTCGTRAGCA TTGGAGCGGG GACTGATGAA GTCACAATCC  2150
GTTTCGTGGT ATGTCTCACC CCTCGCATTT TGAGACGCAA GAGCTGATAT  2200
ATTTTAACAT AGACCGACAA TCCGGGCCCG TGGTTCCTCC ATTGCCATAT  2250
TGATTGGCAT TTGGAGGCAG GCCTTGCCAT CGTCTTCGCT GAGGGCATCA  2300
ATCAGACCGC TGCAGCCAAC CCAACACCCC GTACGTGACA CTGAGGGTTT  2350
CTTTATAGTG CTGGATTACT GAATCGAGAT TTCTCCACAG AAGCATGGGA  2400
TGAGCTTTGC CCCAAATATA ACGGGTTGAG TGCGAGCCAG AAGGTCAAGC  2450
CTAAGAAAGG AACTGCTATT TAAACGTGGT CCTAGACTAC GGGCATATAA  2500
GTATTCGGGT AGCGCGTGTG AGCAATGTTC CGATACACGT AGATTCATCA  2550
CCGGACACGC TGGGACAATT TGTGTATAAT GGCTAGTAAC GTATCTGAGT  2600
TCTGGTGTGT AGTTCAAAGA GACAGCCCTT CCTGAGACAG CCCTTCCTGA  2650
GACAGCCCTT CCTGAGACGT GACCTCCGTA GTCTGCACAC GATACTYCTA  2700
AATACGTATG GCAAGATGAC AAAGAGGAGG ATGTGAGTTA CTACGAACAG  2750
AAATAGTGCC CGGCCTCGGA GAGATGTTCT TGAATATGGG ACTGGGACCA  2800
ACATCCGGA                                               2809
```

encoding the enzyme laccase D1, having the translated protein sequence (SEQ ID No. 14)

```
MGLNSAITSL AILALSVGSY AAIGPVADIH IVNKDLAPDG VQRPTVLAGG   50
TFPGTLITGQ KGDNFQLNVI DDLTDDRMLT PTSIHWHGFF QKGTAWADGP  100
AFVTQCPIIA DNSFLYDFDV PDQAGTFWYH SHLSTQYCDG LRGAFVVYDP  150
NDPHKDLYDV DDGGTVITLA DWYHVLAQTV VGAATPDSTL INGLGRSQTG  200
PADAELAVIS VEHNKRYRFR LVSISCDPNF TFSVDGHNMT VIEVDGVNTR  250
PLTVDSIQIF AGQRYSFVLN ANQPEDNYWI RAMPNIGRNT TTLDGKNAAI  300
LRYKNASVEE PKTVGGPAQS PLNEADLRPL VPAPVPGNAV PGGADINHRL  350
NLTFSNGLFS INNASFTNPS VPALLQILSG AQNAQDLLPT GSYIGLELGK  400
VVELVIPPLA VGGPHPFHLH GHNFWVVRSA GSDEYNFDDA ILRDVVSIGA  450
GTDEVTIRFV TDNPGPWFLH CHIDWHLEAG LAIVFAEGIN QTAAANPTPQ  500
AWDELCPKYN GLSASQKVKP KKGTAI                            526
```

H. Cerrena laccase D2 gene from CBS115.075 strain (SEQ ID No. 15)

```
GATCTGGACG ATGGTATATA AGACGATGGT ATGAGACCCA TGAAGTCTGA   50
ACACTTTTGC TCTCTGACAT TTCATGGTTC ATACTCTCGA GATGGGATTG  100
AACTCGGCTA TTACATCGCT TGCTATCTTA GCTCTGTCAG TCGGAAGCTA  150
TGCTGCAATT GGGCCCGTGG CCGACATACA CATTGTCAAC AAAGACCTTG  200
CTCCAGATGG TGTACAACGT CCAACCGTGC TCGCCGGAGG CACTTTTCCT  250
GGGACGTTGA TCACCGGTCA GAAAGTAAGG AATATTAGTT TGCGTCAAAG  300
AGCCAACCAA AATTAACCGT CCCGTCCCAT AGGGTGACAA CTTCCAGCTC  350
AATGTCATTG ATGATCTTAC CGACGATCGG ATGTTGACAC CAACTTCCAT  400
TGTGAGCCTA TTATTGTATG ATTTATCCGT ATAGTTTCTC AGTCTGATCA  450
TTGGCTCTCT ATCGCTAGCA TTGGCACGGT TTCTTCCAGA AGGGAACCGC  500
```

```
TTGGGCCGAC GGTCCCGCCT TCGTAACTCA GTGCCCTATA ATAGCAGATA   550
ACTCTTTTCT GTATGACTTC GACGTCCCCG ACCAAGCTGG TACTTTCTGG   600
TATCATAGTC ATCTATCCAC TCAGTACTGT GACGGTTTAC GTGGTGCCTT   650
CGTTGTGTAC GATCCTAACG ATCCTCACAA AGACCTATAC GATGTTGATG   700
ACGGTGGGTT CCAAATACTT GACCAAGAAA CATTATATTG ATAGTATCCA   750
CTCTGATTTT CAGAGAGCAC CGTGATTACC CTTGCGGATT GGTACCATGT   800
TCTCGCCCAG ACCGTTGTCG GCGCTGCGTG AGTAACACAT ACACGCGCTC   850
CGGCACACTG ATACTAATTT TTTATTGTAG CACTCCTGAT TCTACCTTGA   900
TCAACGGGTT AGGCCGTTCA CAGACCGGAC CCGCTGATGC TGAGCTGGCT   950
GTTATCAGCG TTGAACATAA CAAACGGTAT GTCATCTCTA CCCATTATCT  1000
TCTCTCCTGC TTTAATTCGC TGTTTCACCA TAGATACCGA TTCCGTTTGG  1050
TTTCGATTTC GTGCGACCCC AACTTTACCT TCTCCGTTGA TGGTCATAAT  1100
ATGACTGTCA TCGAAGTCGA CGGTGTCAAC ACACGACCCC TGACCGTTGA  1150
CTCTATTCAA ATCTTCGCCG GACAGAGGTA TTCCTTTGTC GTAAGTTAAT  1200
CGATATATTC TCCCTATTAC CCCTGTGTAA TTGATGTCAA CAGCTCAATG  1250
CTAACCAACC CGACGACAAT TACTGGATCC GTGCTATGCC AAACATCGGT  1300
AGAAATACAA CAACACTGGA CGGAAAGAAT GCCGCTATCC TTCGATACAA  1350
GAATGCTTCT GTAGAAGAGC CCAAGACCGT TGGGGGCCCC GCTCAATCCC  1400
CGTTGAATGA AGCGGACCTG CGTCCACTCG TACCTGCTCC TGTGGTATGT  1450
CTTGTCGTGC TGTTCCATCG CTATTTCATA TTAACGTTTT GTTTTTGTCA  1500
AGCCTGGAAA CGCTGTTCCA GGTGGCGCAG ACATCAATCA CAGGCTTAAC  1550
TTAACTTTCG TACGTACACC TGGTTGAAAC ATTATATTTC CAGTCTAACC  1600
TCTTGTAGAG TAACGGCCTT TTCAGCATCA ACAACGCCTC CTTCACTAAT  1650
CCTTCGGTCC CCGCCTTATT ACAAATTCTG AGCGGTGCTC AGAACGCTCA  1700
AGATTTACTT CCAACGGGTA GTTACATTGG CCTTGAACTA GGCAAGGTTG  1750
TGGAGCTCGT TATACCTCCT CTGGCAGTTG GAGGACCGCA CCCTTTCCAT  1800
CTTCATGGCG TAAGCATACC ACACTCCCGC AGCCAGAATG ACGCAAACTA  1850
ATCATGATAT GCAGCACAAT TTCTGGGTCG TCCGTAGTGC AGGTAGCGAT  1900
GAGTATAACT TTGACGATGC TATCCTCAGG GACGTCGTGA GCATTGGAGC  1950
GGGGACTGAT GAAGTCACAA TCCGTTTCGT GGTATGTCTC ACCCCTCGCA  2000
TTTTGAGACG CAAGAGCTGA TATATTTTAA CATAGACCGA CAATCCGGGC  2050
CCGTGGTTCC TCCATTGCCA TATTGATTGG CATTTGGAGG CAGGCCTTGC  2100
CATCGTCTTC GCTGAGGGCA TCAATCAGAC CGCTGCAGCC AACCCAACAC  2150
CCCGTACGTG ACACTGAGGG TTTCTTTATA GTGCTGGATT ACTGAATCGA  2200
GATTTCTCCA CAGAAGCATG GGATGAGCTT TGCCCCAAAT ATAACGGGTT  2250
GAGTGCGAGC CAGAAGGTCA AGCCTAAGAA AGGAACTGCT ATTTAAACG   2299
```

encoding the enzyme laccase D2, having the translated protein sequence (SEQ ID No. 16)

```
MGLNSAITSL AILALSVGSY AAIGPVADIH IVNKDLAPDG VQRPTVLAGG    50
TFPGTLITGQ KGDNFQLNVI DDLTDDRMLT PTSIHWHGFF QKGTAWADGP   100
AFVTQCPIIA DNSFLYDFDV PDQAGTFWYH SHLSTQYCDG LRGAFVVYDP   150
NDPHKDLYDV DDGGTVITLA DWYHVLAQTV VGAATPDSTL INGLGRSQTG   200
PADAELAVIS VEHNKRYRFR LVSISCDPNF TFSVDGHNMT VIEVDGVNTR   250
PLTVDSIQIF AGQRYSFVLN ANQPDDNYWI RAMPNIGRNT TTLDGKNAAI   300
LRYKNASVEE PKTVGGPAQS PLNEADLRPL VPAPVPGNAV PGGADINHRL   350
NLTFSNGLFS INNASFTNPS VPALLQILSG AQNAQDLLPT GSYIGLELGK   400
VVELVIPPLA VGGPHPFHLH GHNFWVVRSA GSDEYNFDDA ILRDVVSIGA   450
GTDEVTIRFV TDNPGPWFLH CHIDWHLEAG LAIVFAEGIN QTAAANPTPQ   500
AWDELCPKYN GLSASQKVKP KKGTAI                             526
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I. Cerrena laccase E gene (partial) from CBS154.29 strain (SEQ ID No. 17)

```
TGCAATCGGA CCGGTGGCCG ACCTCAAGAT CGTAAACCGA GACATTGCAC    50
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CTGACGGTTT TATTCGTCCC GCCGTTCTCG CTGGAGGGTC GTTCCCTGGT   100
CCTCTCATTA CAGGGCAGAA AGTACGTTAC GCTATCTCGG TGCTTTGGCT   150
TAATTAAACT ATTTGACTTT GTGTTCTCTT AGGGGAACGA GTTCAAAATC   200
AATGTAGTCA ATCAACTGAC CGATGGTTCT ATGTTAAAAT CCACCTCAAT   250
CGTAAGCAGA ATGAGCCCTT TGCATCTCGT TTTATTGTTA ATGCGCCCAC   300
TATAGCATTG GCATGGATTC TTCCAGAAGG GAACAAACTG GGCAGACGGT   350
CCTGCGTTCG TGAACCAATG TCCAATCGCC ACGAACAATT CGTTCTTGTA   400
TCAGTTTACC TCACAGGAAC AGCCAGGTGA GTATGAGATG GAGTTCATCC   450
GAGCATGAAC TGATTTATTT GGAACCTAGG CACATTTTGG TACCATAGTC   500
ATCTTTCCAC ACAATACTGC GATGGTTTGC GAGGGCCACT CGTGGTGTAT   550
GACCCACAAG ACCCGCATGC TGTTCTCTAC GACGTCGACG ATGGTTCGTA   600
CTTCGCATAT CCACGCTCGC TTTCATACAA TGTAAACTTT GTTCCTCCAG   650
AAAGTACAAT CATCACGCTC GCGGATTGGT ATCATACCTT GGCTCGGCAA   700
GTGAAAGGCC CAGCGTAAGG CACTTTAGTG TTTCCTCATA GTCCAAGAAA   750
TTCTAACACG CCTTCTTCAT CAGGGTTCCT GGTACGACCT TGATCAACGG   800
GTTGGGGCGT CACAACAATG GTCCTCTAGA TGCTGAACTA GCGGTGATCA   850
GTGTTCAAGC CGGCAAACGG CAAGTTCAAT TCACACTTTT CACTCTGTAC   900
CTTCTTCCTG ACATTCTTTT CTTGTAGTTA CCGCTTCCGC CTGATTTCAA   950
TTTCATGCGA TCCCAACTAC GTATTCTCCA TTGATGGCCA TGATATGACT  1000
GTCATCGAAG TGGATAGTGT TAACAGTCAA CCTCTCAAGG TAGATTCTAT  1050
CCAAATATTT GCAGGTCAGA GATATTCGTT CGTGGTGAGT CAGATCAGGG  1100
CATATCCTTT TGTCGATACG TCATTGACCA TATAATGCTA CAAGCTGAAT  1150
GCCAACCAAC CAG                                         1163
```

encoding the enzyme laccase E, having the partial translated protein sequence (SEQ ID No. 18)

```
AIGPVADLKI VNRDIAPDGF IRPAVLAGGS FPGPLITGQK GNEFKINVVN    50
QLTDGSMLKS TSIHWHGFFQ KGTNWADGPA FVNQCPIATN NSFLYQFTSQ   100
EQPGTFWYHS HLSTQYCDGL RGPLVVYDPQ DPHAVLYDVD DESTIITLAD   150
WYHTLARQVK GPAVPGTTLI NGLGRHNNGP LDAELAVISV QAGKRQVQFT   200
LFTLYRFRLI SISCDPNYVF SIDGHDMTVI EVDSVNSQPL KVDSIQIFAG   250
QRYSFVLNAN QP                                          262
```

[33] The term "% identity" herein and refers to the level of nucleic acid or amino acid sequence identity between the nucleic acid sequence that encodes a laccase described herein or the laccase amino acid sequence, when aligned using a sequence alignment program.

[34] For example, as used herein, 80% sequence identity is determined by an algorithm, and accordingly a homologue of a given sequence has greater than 80% sequence identity over a length of the given sequence. Exemplary levels of sequence identity include, but are not limited to, 80, 85, 90, 95, 98% or more sequence identity to a given sequence, *e.g.*, the coding sequence for a laccase, as described herein.

[35] Exemplary computer programs which can be used to determine identity between two sequences include, but are not limited to, the suite of BLAST programs, *e.g.*, BLASTN, BLASTX, and TBLASTX, BLASTP and TBLASTN, publicly available on the Internet at www.ncbi.nlm.nih.gov/BLAST. See also, Altschul, *et al.*, 1990 and Altschul, *et al.*, 1997.

[36] Sequence searches are typically carried out using the BLASTN program when evaluating a given nucleic acid sequence relative to nucleic acid sequences in the GenBank DNA Sequences and other public databases. The BLASTX program is preferred for searching nucleic acid sequences that have been translated in all reading frames against amino acid sequences in the GenBank Protein Sequences and other public databases. Both BLASTN and BLASTX are run using default parameters of an open gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix. (See, *e.g.*, Altschul, *et al.*, 1997.)

[37] An alignment of selected sequences in order to determine "% identity" between two or more sequences, may be performed using, for example, the CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

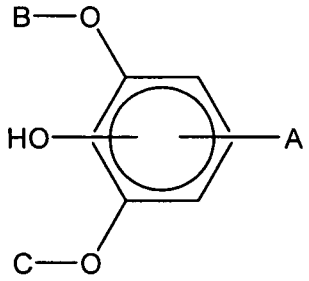
II. Mediators

[38] In an embodiment, the enzymatic oxidation system further comprises one or more chemical mediator agents which enhance the activity of the laccase enzyme. The term "chemical mediator" (or "mediator" may be used interchangeably herein) is defined herein as a chemical compound which acts as a redox mediator to effectively shuttle electrons between the enzyme exhibiting oxidase activity and the dye. Chemical mediators are also known as enhancers and accelerators in the art.

[39] The chemical mediator may be a phenolic compound, for example, methyl syringate, and related compounds, as described in WO 95/01426 and 96/12845. The chemical mediator may also be an N-hydroxy compound, an N-oxime compound, or an N-oxide compound, for example, N-hydroxybenzotriazole, violuric acid, or N-hydroxyacetanilide. The chemical mediator may also be a phenoxazine/phenothiazine compound, for example, phenothiazine-10-propionate. The chemical mediator may further be 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Other chemical mediators are well known in the art. For example, the compounds disclosed in WO 95/01426 are known to enhance the activity of a laccase. In particular embodiments, the mediator may be acetosyringone, methyl syringate, ethyl syringate, propyl syringate, butyl syringate, hexyl syringate, or octyl syringate.

[40] Preferably, the mediator is 4-cyano-2,6-dimethoxyphenol, 4-carboxamido-2,6-dimethoxyphenol or an *N*-substituted derivative thereof such as, for example, 4-(*N*-methyl carboxamido)-2,6-dimethoxyphenol, 4-[*N*-(2-hydroxyethyl) carboxamido]-2,6-dimethoxyphenol, or 4-(*N,N*-dimethyl carboxamido)-2,6-dimethoxyphenol.

[41] The mediator used in the present invention may be described by the following formula:



in which formula A is a group such as –R, –D, –CH=CH–D, –CH=CH-CH=CH–D, -CH=N–D, –N=N–D, or –N=CH–D, in which D is selected from the group consisting of -CO–E, $–SO_2–E$, -CN, –NXY, and $–N^+XYZ$, in which E may be –H, –OH, –R, –OR, or -NXY, and X and Y and Z may be identical or different and selected from –H, -OH, -OR and –R; R being a C_1 - C_{16} alkyl, preferably a C_1 -C_8 alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from $C_m H_{2m+1}$; $1 \leq m \leq 5$.

[42] In an embodiment A in the above mentioned formula is –CN or –CO–E, in which E may be –H, –OH, –R, –OR, or -NXY, where X and Y may be identical or different and selected from –H, -OH, -OR and –R, R being a C_1 -C_{16} alkyl, preferably a C_1 -C_8 alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from $C_m H_{2m+1}$; $1 \leq m \leq 5$.

[43] In the above mentioned formula A may be placed meta to the hydroxy group instead of being placed in the para-position as shown.

[44] In particular embodiments, the mediator may be acetosyringone, methylsyringate, ethylsyringate, propylsyringate, butylsyringate, hexylsyringate, or octylsyringate. Preferably, the mediator is 4-cyano-2,6-dimethoxyphenol, 4-carboxamido-2,6-dimethoxyphenol or a *N*-substituted derivative thereof such as 4-(*N*-methyl carboxamido)-2,6-dimethoxyphenol, 4-[*N*-(2-hydroxyethyl) carboxamido]-2,6-dimethoxyphenol, or 4-(*N,N*-dimethyl carboxamido)-2,6-dimethoxyphenol.

[45] The mediator of the invention may be present in concentrations of from 0.005-1000 μmole per g denim, preferably 0.05-500 μmole per g denim, more preferably 0.5-100 μmole per g denim.

[46] The mediators may be prepared by methods known to the skilled artisan, such as those disclosed in WO 97/11217, WO 96/12845 and US 5752980.

III. Utility

[47] Industrial applications of laccases include bleaching of pulp and paper and textile bleaching, for example, of indigo-dyed denim fabrics. Laccases have also been found to be useful for hair dyeing (see, e.g., WO 95/33836 and WO 95/33837). European Patent No. 0504005 discloses that laccases can be used for dyeing wool.

[48] The laccases described herein find use in the dyeing and bleaching of textiles, fibers, yarns and the like. The laccases also find use in the treatment of waste water, the delignification of pulp, the depolymerization of high molecular weight aggregates, deinking waste paper, the polymerization of aromatic compounds, radical mediated polymerization and cross-linking reactions (*e.g.*, paints, coatings, biomaterials), and the activation of dyes and to couple organic compounds. The laccases may be used in a cleaning composition or component thereof, or in a detergent.

[49] As described herein, the laccases are capable of oxidizing a wide variety of colored compounds having different chemical structures, using oxygen as the electron acceptor. Accordingly, the laccases presented herein can be used in applications where it is desirable to modify the color associated with colored compounds, such as in cleaning, e.g., for removing the food stains on fabric. In certain situations, a mediator or enhancer can be used to obtain desirable effects.

[50] The laccases presented herein can be used in the field of textiles. For example, the laccases described herein can be used in the treatment, processing, finishing, polishing, or production of fibers, or other fabrics or articles of manufacture. The enzymes herein can be useful, for example, in denim treatment (bleaching work-up processes); in de-coloring indigo waste; in fabric dyeing; in textile bleaching processes; in fiber modification; in achieving enhanced fiber or fabric properties; etc.

[51] The laccases described herein can be used in the leather industry. For example, the laccases can be used in the processing of animal hides including but not limited to de-hairing, liming, bating and/or tanning of hides.

[52] Also disclosed herein is a process for the removal of lignin from lignocellulose-containing material, the bleaching of lignocellulose-containing material (i.e. the enzymatic de-

inking of recycled paper) and/or the treatment of waste water arising from the manufacture of paper or cellulose. The process uses laccase enzymes obtained from *Cerrena sp.*, at the same time adding or metering in non-aromatic redox agents plus phenolic and/or non-phenolic aromatic redox compounds, the phenolic and non-phenolic units of the lignin either being oxidized directly by the action of these phenolic and/or non-phenolic aromatic compounds, or the lignin being oxidized by other phenolic and/or non-phenolic compounds produced by the oxidizing action of these compounds.

[53] The laccases described herein can be used in the field of pulp and paper. For example, the laccases can be used in the manufacture of paper pulps and fluff pulps from raw materials such as wood, bamboo, and cereal rice straw; the manufacture of paper and boards for printing and writing, packaging, sanitary and other technical uses; recycling of cellulose fiber for the purpose of making paper and boards; and the treatment of waste products generated by and treated at pulp or paper mills and other facilities specifically dedicated to the manufacture of paper, pulp, or fluff. The enzymes presented herein can be useful, for example, in wood processing; in pulp bleaching; in wood fiber modification; in bio-glue (lignin activation) for MDF manufacturing; for enhanced paper properties; in ink removal; in paper dyeing; in adhesives (e.g. lignin based glue for particle- or fiber boards); etc.

[54] The laccases described herein can be used in the field of feed. For example, the laccases presented herein can be used as a feed additive alone or as part of a feed additive with the aim to increase the nutritional value of feed for any kind of animals such as chicken, cows, pigs, fish and pets; and/or as a processing aid to process plant materials and food industry by products with the aim to produce materials/products suitable as feed raw materials.

[55] The laccases described herein can be used in the field of contact lens cleaning. For example, the laccases can be used in the cleaning, storage, disinfecting, and/or preservation of contact lens.

[56] The laccases described herein can be used in the field of starch. For example, the laccases can be used in the processing of a substrate including starch and/or grain to glucose (dextrose) syrup, fructose syrup or any other syrup, alcohol (potable or fuel) or sugar. Such starch processing may include processing steps such as liquefaction, saccharification, isomerization, and de-branching of a substrate.

[57] The laccases described herein can be used in the field of food. For example, the laccases can be used in the preparation, processing, or as an active ingredient in foods such as yellow fat, tea based beverages, culinary products, bakery, and frozen foods for human consumption. The

laccases can be used, for example, as a bread improver, in food preservation, as an oxygen scavenger, etc.

[58] The laccases described herein can be used in the field of personal care. For example, the laccases can be used in the preparation of personal products for humans such as fragrances, and products for skin care, hair care, oral hygiene, personal washing and deodorant and/or antiperspirants, for humans. The enzymes presented herein can be useful, for example, in hair dyeing and/or bleaching, nails dyeing and/or bleaching; skin dyeing and/or bleaching; surface modification (e.g., as coupling reagent); as an anti-microbial agent; in odor removal; teeth whitening; etc.

[59] The laccases described herein can be used in the field of cleaning. For example, the laccases can be used in the cleaning, treatment or care of laundry items such as clothing or fabric; in the cleaning of household hard surfaces; in dishcare, including machine dishwashing applications; and in soap bars and liquids and/or synthetic surfactant bars and liquids. The enzymes presented herein can be useful, for example, in stain removal/de-colorization, and/or in the removal of odors, and/or in sanitization, etc.

[60] The laccases described herein can be used in the field of waste-water treatment. For example, the laccases can be used in decolorization of colored compounds; in detoxification of phenolic components; for anti-microbial activity (e.g., in water recycling); in bio-remediation; etc.

[61] The laccases described herein can be used in the field of bio-materials. For example, the laccases can be used as bio-catalysts for various organic reactions; and/or in connection with biopolymers; in connection with packaging; in connection with adhesives; in surface modification (activation and coupling agent); in production of primary alcohols; in connection with biosensors and/or organic syntheses; etc.

[62] The laccases described herein can be used in the field of anti-microbials. For example, the laccases can be used as an anti-microbial agent in cleaning compositions, or for reducing or eliminating the microbial load of various foods (e.g., meats) or feed.

[63] The laccase mediators can be used as sanitization and antimicrobial agents (*e.g.*, wood protection, detergents). The mediators may be used independently of the enzymes or in conjunction with the enzymes.

[64] As used herein, "cleaning compositions" and "cleaning formulations" refer to compositions that find use in the removal of undesired compounds from items to be cleaned, such as fabric, etc. The term encompasses any materials/compounds selected for the particular

type of cleaning composition desired and the form of the product (*e.g.*, liquid, gel, granule, or spray composition), as long as the composition is compatible with the laccase and other enzyme(s) used in the composition. The specific selection of cleaning composition materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use.

[65] The terms further refer to any composition that is suited for cleaning and/or bleaching any object and/or surface. It is intended that the terms include, but are not limited to detergent compositions (*e.g.*, liquid and/or solid laundry detergents and fine fabric detergents; hard surface cleaning formulations, such as for glass, wood, ceramic and metal counter tops and windows; carpet cleaners; oven cleaners; and textile and laundry pre-spotters, as well as dish detergents).

[66] Indeed, the term "cleaning composition" as used herein, includes unless otherwise indicated, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid (HDL) types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, car or carpet shampoos, bathroom cleaners; hair shampoos and hair-rinses; shower gels and foam baths and metal cleaners; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types.

[67] As used herein, the terms "detergent composition" and "detergent formulation" are used in reference to mixtures which are intended for use in a wash medium for the cleaning of soiled objects. In some embodiments, the term is used in reference to laundering fabrics and/or garments (*e.g.*, "laundry detergents"). In alternative embodiments, the term refers to other detergents, such as those used to clean dishes, cutlery, etc. (*e.g.*, "dishwashing detergents"). It is not intended that the presently contemplated compositions be limited to any particular detergent formulation or composition. Indeed, it is intended that in addition to laccase, the term encompasses detergents that contain surfactants, transferase(s), hydrolytic enzymes, builders, bleaching agents, bleach activators, bluing agents and fluorescent dyes, caking inhibitors, masking agents, enzyme activators, antioxidants, and solubilizers.

[68] As used herein the term "hard surface cleaning composition," refers to detergent compositions for cleaning hard surfaces such as floors, walls, tile, stainless steel vessels (*e.g.*, fermentation tanks), bath and kitchen fixtures, and the like. Such compositions are provided in any form, including but not limited to solids, liquids, emulsions, etc.

EXAMPLES

Example 1. Amino Acid Sequence Analysis of *Cerrena unicolor* laccase

[69] Four Peptide sequences were obtained using a commercially available laccase: AIGPVADLHI (SEQ ID No. 19), MLTPTSI (SEQ ID No. 20), TVGGPA (SEQ ID No. 21) and YSFVLNANQP (SEQ ID No. 22). The commercially available laccase was purified. N-terminal sequencing resulted in SEQ ID No. 19. Proteolytic digestion with trypsin of the purified sample was performed. Fragments were separated by gel electrophoresis with 3 bands selected and collected manually. Peptide sequencing was performed for each band and resulted in SEQ ID Nos. 20, 21 and 22.

Example 2

a. Cloning of *Cerrena unicolor* laccase A gene from ATCC20013 strain

[70] To clone the laccase A gene from ATCC 20013 strain, two primers were designed and obtained from Invitrogen: TTCGCAGGTCAACGATATTC (SEQ ID No. 35) based on DNA sequence of the laccase B gene obtained from ATCC20013 strain (see example 3a) and GTTAGGTGGTTGAAGGATTG (SEQ ID No. 36) based on laccase A gene obtained from CBS115.075 strain (see example 2c). The primers were used in a highT PCR reaction containing genomic DNA obtained from ATCC 20013 strain as template (see example 3). The PCR fragment was purified using a QIAquick spin column from Qiagen and cloned into pTOPO plasmid using TOPO cloning kit (Invitrogen). Twenty-two clones were amplified using Ready-To-Go PCR beads (GE Healthcare) and three PCR fragments (2-1, 2-3 and 2-6) were sequenced. 1316 bps DNA sequence of the laccase A gene from ATCC20013 is listed as SEQ ID No 37.

b. Cloning of *Cerrena unicolor* laccase A gene from CBS154.29 strain

[71] To clone the laccase A gene from CBS154.29 strain, two primer was designed and obtained from Invitrogen: CACCAGCATGAGCTCAAAGCTAC (SEQ ID No. 45) based on laccase A gene obtained from CBS115.075 strain (see example 2c) and primer of the SEQ ID No. 36. The primers were used in a Herculase PCR reaction containing genomic DNA template obtained from CBS154.29 strain, dNTPs, primer and 4% DMSO in 1x buffer. The PCR mixture was heated to 98°C for 4 minutes to denature the DNA template. Herculase® II enzyme (Stratagene) was added to the tube and PCR reaction was performed in 30 cycles of 98°C for 30 seconds,

50°C for 30 seconds and 72°C for 2 minute. The final extension at 72°C was done for 5 minutes and the reaction was chilled to 4°C. The PCR fragment was purified using the QIAquick spin column and cloned into pENTR/D-TOPO vector (Invitrogen). Fifteen clones were amplified using Ready-To-Go PCR beads and plasmids were isolated from two clones (pENTR15-24 and pENTR15-30) and the DNA templates were sequenced. 2374 bps DNA sequence of the laccase A gene from CBS154.29 was obtained. The DNA sequence is listed as SEQ ID No. 3 and the translated protein sequence is listed as SEQ ID No. 4.

c. Cloning of *Cerrena unicolor* laccase A gene from CBS115.075 strain

[72] The primer CAATCTATGACCGTAGATTC (SEQ ID No. 39) based on the laccase B gene from ATCC20013 strain (see example 3a) and primer NNNNNNNNNNCGATCG (SEQ ID No. 38) where N represents a mixture of all four nucleotides (A, T, C and G) were used in lowT PCR reaction (see example 3a). Genomic DNA was extracted from *Cerrena unicolor* strain (CBS115.075) and was used as template in the first round of lowT PCR reaction. The PCR fragments were purified with a QIAquick spin column and used as template in the second round of lowT PCR reaction with primers of SEQ ID No.35 based on the laccase B gene from ATCC20013 strain (see example 3a) and primer of the SEQ ID No. 38. The PCR fragments were cloned into pTOPO plasmid using TOPO cloning kit. Sixteen clones were amplified using Ready-To-Go PCR beads and three cloned PCR fragments (B2#1, B2#4 and B2#11) were sequenced.

[73] To clone the 3' end of laccase A gene, the primer ACCGTGGTTCCTCCATTGCC (SEQ ID No.40) and primer of SEQ ID No. 31 were used in the lowT PCR reaction with the genomic DNA extracted from *Cerrena unicolor* strain (CBS115.075) as template in the first round of lowT PCR reaction. The PCR fragments were purified with a QIAquick spin column and used as template in the second round of lowT PCR reaction with primers GACTGGCACTTGGAAGCGGG (SEQ ID No.41) and primer of SEQ ID No. 31. The PCR fragments were cloned into pTOPO plasmid using TOPO cloning kit. Twenty-two clones were amplified using Ready-To-Go PCR beads and one cloned PCR fragment (D2#2) was sequenced.

[74] To clone the 5' end of the laccase A gene, a primer, GGACCAAGCTGGTACTTTC (SEQ ID No.42), was designed based on the laccase B gene sequence. It was used to amplify a DNA fragment with primer of SEQ ID No. 36. The genomic DNA extracted from *Cerrena unicolor* strain (CBS115.075) was used as the PCR template. The 1.7 kb PCR fragment was obtained, purified with a QIAquick spin column and cloned into pTOPO plasmid using TOPO cloning kit.

Twenty-two clones were analyzed using Ready-To-Go PCR beads. Plasmid DNA from clone (C5#20) was sequenced. To further clone the 5' of laccase A gene, the primer CGTGGTACCAGTCTGCCAGGG (SEQ ID No.43) and primer of SEQ ID No. 31 were used in the lowT PCR reaction with the genomic DNA extracted from *Cerrena unicolor* CBS115.075 strain as template. From the first round of lowT PCR reaction, the PCR fragment was purified with a QIAquick spin column and used as template in the second round of lowT PCR reaction with primers GGCAGCATCAGTCACGGTCAG (SEQ ID No.44) and primer of SEQ ID No. 31. The PCR fragment (a3) was amplified again and used as template in a third round of lowT PCR reaction with primers GGCAGCATCAGTCACGGTCAG (SEQ ID No.44) and primer of SEQ ID No. 31. The PCT fragment (a3-2) was cloned into pTOPO plasmid using TOPO cloning kit. Eleven clones were amplified using Ready-To-Go PCR beads and two cloned PCR fragments (a3-2#10 and a3-2#11) were sequenced. The DNA sequence of the laccase A gene from CBS 115.075 strain including the sequence of 5' and 3' of the coding region is listed as SEQ ID No.1 and the translated protein sequence is listed as SEQ ID No.2.

Example 3

a. Cloning and sequencing of the *Cerrena unicolor* laccase B gene from ATCC20013 strain

[75] To clone the DNA fragment encoding the *Cerrena* laccase gene, four degenerated primers were designed based on the peptide sequence AIGPVADLHI (SEQ ID No. 19) and obtained from Invitrogen. They are named as

primerA GCAATCGGACCNGTNGCAGA (SEQ ID No. 23);

primerB GCAATCGGACCNGTNGCTGA (SEQ ID No. 24);

primerC GCAATCGGACCNGTNGCGGA (SEQ ID No. 25) and

primerD GCAATCGGACCNGTNGCCGA (SEQ ID No. 26).

[76] Two degenerated primers were designed based on the peptide sequence YSFVLNANQP (SEQ ID No. 22) and obtained from Invitrogen. They are named as

primerE GGTTGATTTGCATTNAGNAC (SEQ ID No. 27) and

primerF GGTTGATTTGCGTTNAGNAC (SEQ ID No. 28)

where N represents a mixture of all four nucleotides (A, T, C and G). The genomic DNA was extracted from ATCC20013 strain and used as template in the lowT PCR reaction contain following combination of primers: PCR reaction 1 contains no DNA and no primer; PCR reaction 2 contains primerA and primerE; PCR reaction 3 contains primerB and primerE; PCR reaction 4 contains primerC and primerE; PCR reaction 5 contains primerD and primerE; PCR

reaction 6 contains primerA and primerF; PCR reaction 7 contains primerB and primerF; PCR reaction 8 contains primerC and primerF and PCR reaction 9 contains primerD and primerF. The PCR reaction mixture contained DNA template, primers, 1x buffer, 0.2 mM dNTP and 1 unit of Taq DNA polymerase. The PCR reaction was performed in 30 cycles of 95°C for 1 minute, 45°C for 1 minute and 68°C for 1 minute. The final extension at 72°C was done for 7 minutes and the reaction was chilled to 4°C. The PCR fragments from reaction 4, 5 and 8 were cut out of a 1.2% agarose gel and pooled. The PCR fragments were extracted from gel with a Qiagen spin column and cloned into pTOPO plasmid using TOPO cloning kit. Thirty-two cloned PCR fragments were selected and sequenced using Ready-To-Go PCR beads and DNA sequence of clone #A30 was identified as laccase B gene.

[77] To clone the 5' end of laccase gene, a primer was designed and obtained from Invitrogen: GGACGTGGCCTTGAGCATAC (SEQ ID No. 29). It was used in first round of lowT PCR reaction with a degenerated oligo NNNNNNNNNNGGATCC (SEQ ID No. 31) where N represents a mixture of all four nucleotides (A, T, C and G). The PCR product was purified using a QIAquick spin column and used as template in a second lowT PCR reaction containing a primer TCTGTCAAGTCGTCAATCAC (SEQ ID No. 30) and primer of SEQ ID No. 31. The PCR fragment was purified using a QIAquick spin column and diluted 1:10 and 1:100 and used as template in the first round of highT PCR reaction performed in 30 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute with two primers (SEQ ID No.30 and SEQ ID No. 31). The final extension at 72°C was done for 7 minutes and the reaction was chilled to 4°C. The PCR fragment was purified with a QIAquick spin column and used in the second round of highT PCR reaction with primers of TTACCACGAATCAGAGGACC (SEQ ID No. 32) and SEQ ID No. 31. The PCR fragment (D13) was sequenced.

[78] To clone the 3' end of the laccase B gene, a primer was designed and obtained from Invitrogen: CCTCACCTGTATTGGCACAG (SEQ ID No. 33) and used with primer of SEQ ID No. 31 in a first round of lowT PCR reaction. The PCR fragment was purified in a QIAquick spin column and used as template in second round of lowT PCR reaction with primer TTGGTATCATGCCCTTGCTC (SEQ ID No. 34) and primer of SEQ ID No. 31. The PCR fragment was cloned into a pTOPO plasmid using TOPO cloning kit. Sixteen clones were amplified using Ready-To-Go PCR beads and four cloned PCR fragments (C3, C4, C5 and C7) were sequenced.

[79] 1337 bps DNA fragment was obtained. The DNA sequence is listed as SEQ ID No. 9 and translated protein sequence is listed as SEQ ID No. 10.

b. Cloning of *Cerrena unicolor* laccase B gene from CBS154.29 strain

[80] Two primers were designed and obtained from Invitrogen:

CACCGCGATGTCTCTTCTTCGTAG (SEQ ID No. 46) and

TGRAGRTGGAASGGATGWGGTCC (SEQ ID No. 47)

where R represent mixture of nucleotides A and G, S represent mixture of nucleotides C and G, and W represent mixture of nucleotides A and T. The two primers were used in the highT PCR reaction. The PCR fragment (A3) was purified using a QIAquick spin column. The PCR fragment was cloned into pTOPO plasmid using TOPO cloning kit. Sixteen clones were amplified using Ready-To-Go PCR beads and two PCR fragments (A3#1 and A3#5) were sequenced.

[81] To clone the 3' end of the laccase B gene from CBS154.29 strain, a primer was designed and obtained from Invitrogen: GTCCCTGTACTACTCCAGATCC (SEQ ID No. 48) and used with a primer having SEQ ID No. 31 in first round of lowT PCR reaction. The PCR fragment was purified in a QIAquick spin column and used as template in second round of lowT PCR reaction with primer CCAGCAGGAAGCGTGATCGAAC (SEQ ID No. 49) and primer of SEQ ID No. 31. The PCR fragment was cloned into pTOPO plasmid using TOPO cloning kit. Sixteen clones were amplified using Ready-To-Go PCR beads and three PCR fragments (7#6, 7#7 and 7#8) were sequenced. 2663 bps of the laccase B DNA sequence of the CBS154.29 strain is listed as SEQ ID No. 7 and translated protein sequence is listed as SEQ ID No. 8.

c. Cloning of *Cerrena unicolor* laccase B gene from CBS115.075 strain

[82] A primer was designed and obtained from Invitrogen:

GTAATCATGTATCACCTGGGCTCAAGG (SEQ ID No. 50). The primer was used in the Herculase PCR reaction (see Example 2b) with primer of SEQ ID No. 46. The PCR fragment was purified using a QIAquick spin column. The PCR fragment was cloned into pTOPO plasmid using TOPO cloning kit. Seventeen clones were analyzed using Ready-To-Go PCR beads and the PCR fragments from four clones (#1, #2, #4 and #5) were sequenced. The plasmid DNA was prepared from two clones (pENTR-laccaseB CBS115075#1 and pENTR-laccaseB CBS115075#3) and both plasmids were sequenced. 2173 bps of the laccase B DNA sequence of the CBS115.075 strain is listed as SEQ ID No. 5 and translated protein sequence is listed as SEQ ID No. 6.

Example 4. Cloning of the *Cerrena unicolor* laccase C gene from CBS154.29 strain

[83] A primer ACGAACGAGTANCGTTGNCC (SEQ ID No. 51), where N represents a mixture of all four nucleotides (*i.e.*, A, T, C and G), was designed based on the translated peptide sequence GQRYSFV (SEQ ID No. 52). This peptide is conserved between the laccase A gene and the laccase B gene (see Examples 2 and 3). The primer was obtained from Invitrogen and was used in the lowT reaction with primer of the SEQ ID No.24. The PCR fragment was purified using a QIAquick spin column. The PCR fragment was cloned into pTOPO plasmid using TOPO cloning kit. Thirty-three clones were analyzed using Ready-To-Go PCR beads and the PCR fragments from four clones (#12, #5a, #19a and #21a) were sequenced. 1080 bps of the laccase C gene sequence from the CBS154.29 strain is listed as SEQ ID No. 11 and translated protein sequence is listed as SEQ ID No. 12.

Example 5

a. Cloning of *Cerrena unicolor* laccase D gene from CBS115.075 strain

[84] To clone the 5' end of the laccase D gene from CBS115.075 strain, a primer was designed based on laccase D gene from CBS154.29 strain (see Example 5b) (AACACGGAGACAGTCCAAAC, SEQ ID No. 62). It was used in the highT PCR reaction with primer of SEQ ID No. 56. The PCR fragment was purified using a QIAquick spin column and sequenced.

[85] To clone the laccase D gene from CBS115.075 strain, two primers (CACCTCTCGAGATGGGATTGAAC, SEQ ID No. 63 and CGTTTAAATAGCAGTTCCTTTC, SEQ ID No. 64) were designed based on the laccase D gene from CBS154.29 strain (see example 5b). The primers were used in a Herculase PCR reaction (see example 2b) with DNA template of the genomic DNA from CBS115.075 strain. The PCR fragment was purified using the QIAquick spin column and cloned into pENTR/D-TOPO vector. Sixteen clones were amplified using Ready-To-Go PCR beads and the PCR fragments generated from four clones were sequenced. The plasmids were isolated from clone #2 (pENTRE-laccaseD#2) and it was sequenced. 2809 bps DNA sequence of the laccase D gene from CBS115.075 was obtained. The DNA sequence is listed as SEQ ID No. 15 and the translated protein sequence is listed as SEQ ID No. 16.

b. Cloning of *Cerrena unicolor* laccase D gene from CBS154.29 strain

[86] A primer, CTGGTTGGTTNGCATTNAG (SEQ ID No. 53), was designed based on the peptide sequence LNANQP (SEQ ID No. 54). The primer was obtained from Invitrogen and used

in the lowT PCR reaction with primer of the SEQ ID No.26. The PCR fragment was purified using a QIAquick spin column and was cloned into pTOPO plasmid using TOPO cloning kit. Eighteen clones were analyzed using Ready-To-Go PCR beads and PCR fragment from a clone was sequenced.

[87] To clone the 3' end of the laccase D gene, a primer (CACACGACCCCTGACCGTTG, SEQ ID No. 55) was designed. The primer was used in the lowT PCR reaction with primer of the SEQ ID No.31. The PCR fragment was purified using a QIAquick spin column and was cloned into pTOPO plasmid using TOPO cloning kit. Twenty-four clones were analyzed using Ready-To-Go PCR beads and PCR fragment(s) from a clone were sequenced.

[88] To clone more of the 3' and the 5' ends of the laccase D gene, inverse PCR was used. 0.4 ug of the genomic DNA from the *Cerrena* CBS154.29 strain was digested with EcoRV restriction enzyme at 37°C for 1.5 hours. Digested genomic DNA fragments were precipitated with ethanol. The linear DNA fragments were ligated with T4 DNA ligase in 100 ul volume for more than 5 hours. The ligated DNA fragments were heated to 100°C for 3 minutes and were used as the DNA template in a first round of the highT PCR reaction using two primers (TGACCGGTGATCAACGTCCC, SEQ ID No. 56, and GGCGCAGACATCAATCACAG, SEQ ID No. 57). The PCR fragments were purified using a QIAquick spin column and were used as a DNA template in the second round of the highT PCR reaction using two primers (TCTTCAGCATCAACAACGCC, SEQ ID No. 58 and TCCGGCAAGCACGGTTGG, SEQ ID No. 59). The PCR fragments from second round of PCR reaction were purified using a QIAquick spin column and were sequenced.

[89] To clone more of the 3' end of laccase D gene from CBS154.29 strain, inverse PCR was used. 0.4 ug of the genomic DNA from the *Cerrena* CBS154.29 strain was digested with SmaI restriction enzyme at 37°C for 1.5 hours. Digested genomic DNA fragments were precipitated with ethanol. The linear DNA fragments were ligated with T4 DNA ligase in 100 ul volume for more than 5 hours. The ligated DNA fragments were heated to 100°C for 3 minutes and were used as the DNA template in a first round of highT PCR reaction with primer TCGTCTTCGCTGAGGGCATC, SEQ ID No. 60, and primer of SEQ ID No. 56. The PCR fragments were purified using a QIAquick spin column and were used as DNA template in the second round of the highT PCR reaction using primer (CAGACCGCTGCAGCCAACCC, SEQ ID No. 61) and primer of SEQ ID No. 59. The PCR fragments from the second round of PCR reaction were purified using a QIAquick spin column and cloned into pTOPO plasmid using TOPO cloning kit. Twenty-one clones were analyzed using Ready-To-Go PCR beads and PCR

fragment from clones #Ce11 and #Ce14 were sequenced. 2809 bps of the laccase D gene sequence from the CBS154.29.49 strain is listed as SEQ ID No. 13 and the translated protein sequence is listed as SEQ ID No. 14.

Example 6. Cloning of *Cerrena unicolor* laccase E gene from CBS154.29 strain

[90] The primer of SEQ ID No. 53 was used in the lowT PCR reaction with primer of the SEQ ID No.26 (see Example 5b). The PCR fragment was purified using a QIAquick spin column and was cloned into pTOPO plasmid using TOPO cloning kit. Eighteen clones were analyzed using Ready-To-Go PCR beads and the PCR fragment from clone #Ae17 was sequenced. 1163 bps of the laccase E gene sequence from the CBS154.29.49 strain is listed as SEQ ID No. 17 and the translated protein sequence is listed as SEQ ID No. 18.

Example 7. Expression of laccase A gene in *Trichoderma*

[91] To construct the expression plasmid for the laccase A gene of the CBS strain 115.075, two primers (SEQ ID No. 45 and SEQ ID No. 36) were used in the Herculase PCR reaction containing genomic DNA template obtained from 115.075 strain, dNTPs, and 4% DMSO in 1x buffer. The PCR mixture was heated to 98°C for 4 minutes to denature the DNA template. Herculase® II enzyme (Stratagene) was added to the tube and PCR reaction was performed in 30 cycles of 98°C for 30 seconds, 50°C for 30 seconds and 72°C for 2 minute. The final extension at 72°C was done for 5 minutes and the reaction was chilled to 4°C. The PCR fragment was purified using the QIAquick spin column and cloned into pENTR/D-TOPO vector. Fifteen clones were amplified using Ready-To-Go PCR beads and plasmid DNA was isolated from pENTR-laccaseA-CBS115.075#11 clone. The laccase A gene portion was sequenced to confirm fidelity of the PCR amplification of the laccase A gene. The plasmid of pENTR-laccaseA-CBS115.075#11 (50 ng) was converted to the expression plasmid pTrex3g-laccaseA (Figure 1) in a 10 ul LB clonase II reaction (Invitrogen) containing 6.5 ul of TE, 1 ul of pTrex3g vector (0.1mg/ml) and 2 ul of ClonaseII. The expression plasmid was confirmed by DNA sequencing and transformed biolistically into a *Trichoderma* strain. Transformation of the *Trichoderma* strain by the biolistic transformation method was accomplished using a Biolistic® PDS-1000/he Particle Delivery System from Bio-Rad (Hercules, CA) following the manufacturers instructions (see WO 05/001036 and US 2006/0003408). Sixty-six transformants were selected and were transferred to new plates. A total of 15 stable transformants were grown in 30 ml of the Proflo media for 2 days at 30°C. Five mls of 2 days

old culture from Proflo media were transferred to 50 mls of defined media containing 1mM copper. The cultures were grown for 5 days at 28°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

Example 8

a. Expression of laccase B gene in *Aspergillus*

[92] To construct the expression plasmid for the laccase B gene of the CBS strain 115.075, two primers GCAGATCTGCGATGTCTCTTCTTCGTAGCTTGAC (SEQ ID No. 72) and GAGGTCACCTCTAGATCATGTATCACCTGGGCTCAAGGCATC (SEQ ID No. 73) were used in the Herculase PCR reaction containing genomic DNA template obtained from 115.075 strain (see Example 2b). The PCR fragment was purified using the QIAquick spin column and digested with restriction enzyme BglII and XbaI. The DNA fragment was purified again with the QIAquick spin column and was cloned into BglII and XbaI digested pGAPT vector. Fidelity of the plasmid was confirmed by DNA sequencing. The resulting plasmid pKB401 (Figure 2) was tranformed into *A. niger* 2445 for checking expression of laccase B gene. Thirty-four transformants were selected and were transferred onto MM plates and grew for 4 days at 30°C. A small plug of single colony including spores and mycelium was innoculated on to a CMA plate and grew for 4 days at 30°C. A plug of CMA plate containing confluent spores and mycelium was transferred into to 30 mls of Promosoy special broth (pH6.2) containing 1mM copper. The cultures were grown for 5 days at 30°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

b. Expression of laccase B gene in *Aspergillus* as fusion to catalytic domain of the glucoamylase.

[93] To construct the fusion expression plasmid for the laccase B gene of the CBS strain 115.075, two primers TTGCTAGCAACGTGATCTCCAAGCGTGCAATCGGTCCAGTCACTGACCTAC (51mer, SEQ ID No. 74) and primer of SEQ ID No. 73 were used in the Herculase PCR reaction containing genomic DNA template obtained from CBS115.075 strain (see Example 2b). The PCR fragment was purified using the QIAquick spin column and digested with NheI and BstEII and was purified again with the QIAquick spin column. This purified fragment was cloned into NheI and BstEI digested vector pGAMpR2-GV (see US Patent application US20050153399). The resulting plasmid pKB403 (Figure 3) was confirmed by sequencing analysis and was transformed into *A. niger* 2445. Twenty-eight transformants were selected and were transferred

onto MM plates and grew for 4 days at 30°C. A small plug of single colony including the spores and mycelium were innoculated onto CMA plate and grew for 4 days at 30°C. A plug of CMA plate containing confluent spores and mycelia was transferred into to 30 mls of Promosoy special broth (pH6.2) (see US Patent application US20050153399) containing 1mM copper. The cultures were grown for 5 days at 30°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

c. Expression of laccase B gene in *Trichoderma*

[94] To construct expression plasmid for the laccase B gene of the CBS 115.075 strain (see Example 2b). A primer was designed and obtained from Invitrogen: GTAATCATGTATCACCTGGGCTCAAGG (SEQ ID No. 50). The primer was used in the Herculase PCR reaction (see Example 2b) with primer of SEQ ID No. 46. The PCR fragment was purified using the QIAquick spin column and cloned into pENTR/D-TOPO vector (Invitrogen). Seventeen clones were amplified using Ready-To-Go PCR beads and plasmid DNA was isolated from pENTR-CBS115.075#1 clone (see Example 3c). The laccase B gene portion was sequenced to confirm fidelity of the PCR amplification. The plasmid of pENTR-laccaseB-CBS115.075#1 (50 ng) was converted to expression plasmid pTrex3g-laccaseB (see Figure 1 with the laccase A gene replaced with the laccase B gene) in a 10 ul LB clonase II reaction (Invitrogen) containing 6.5 ul of TE, 1 ul of pTrex3g vector (0.1mg/ml) and 2 ul of ClonaseII. The expression plasmid was confirmed by DNA sequencing and transformed biolistically into a *Trichoderma* strain. Sixty transformants were selected and were transferred to new plates. A total of 20 stable transformants were grown in 30 ml of the Proflo media for 2 days at 30°C. Three mls of 2 day old culture from Proflo media were transferred to 30 mls of defined media (see US Patent Application 20050153399) containing 1mM copper. The cultures were grown for 4 days at 28°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

d. Expression of the laccase B gene in *Trichoderma* as CBH1 fusion

[95] To construct the expression plasmid for the laccase B gene of the CBS strain 115.075, a primer was designed and obtained from Invitrogen (GGACTAGTGTCGCCGTTTACAAACGCGCAATCGGTCCAGTCACTGACC, SEQ ID No. 65). The primer was used in combination with the reverse primer (obtained from New England Biolab) in

the Herculase PCR reaction containing pENTR-laccaseB CBS115075#1 (see example 3c) as the DNA template. The PCR fragment (SEQ ID No. 66)

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ACTAGTGTCG CCGTTTACAA ACGCGCAATC GGTCCAGTCA CTGACCTACA    50
TATAGTGAAC CAGAATCTCG ACCCAGATGG TTTCAACCGC CCCACTGTAC   100
TCGCAGGTGG TACTTTCCCC GGTCCTCTGA TTCGTGGTAA CAAGGTACGC   150
TTCATAACCG CCCTCCGTAG ACGTAGGCTT CGGCTGACAT GACCATCATC   200
TGTAGGGAGA TAACTTTAAA ATTAATGTGA TTGACGACTT GACAGAGCAC   250
AGTATGCTCA AGGCTACGTC CATCGTAAGT CCCTGATTAA CGTTTCACCT   300
GGTCATATCG CTCAACGTCT CGAAGCACTG GCATGGGTTC TTCCAGAAGG   350
GAACCAACTG GGCCGATGGC CCCGCCTTTG TCACCCAATG TCCTATCACA   400
TCAGGAAACG CCTTCCTGTA TGATTTCAAC GTTCCGGACC AAGCTGGTAC   450
TTTCTGGTAC CACAGCCATC TCTCTACACA GTATTGTGAC GGTCTTCGTG   500
GTGCCTTTGT CGTCTATGAT CCTAATGATC CCAACAAGCA ACTCTATGAT   550
GTTGATAACG GCAAGTTCCT TGCATATTTC ATTTCTATCA TATCCTCACC   600
TGTATTGGCA CAGAAAGCAC CGTGATTACC TTGGCTGATT GGTATCATGC   650
CCTTGCTCAG ACTGTCACTG GTGTCGCGTG AGTGACAAAT GGCCCTCAAT   700
TGTTCACATA TTTTCCTGAT TATCATATGA TAGAGTATCT GATGCAACGT   750
TGATCAACGG ATTGGGACGT TCGGCCACCG GCCCCGCAAA TGCCCCTCTG   800
GCGGTCATCA GTGTCGAGCG GAATAAGAGG TCAGTTCCAT AATTATGATT   850
ATTTCCCGCG TTACTTCCTA ACAATTATTT TTGTATCCCT CCACAGATAT   900
CGTTTCCGAT TGGTTTCTAT TTCTTGCGAC CCTAACTTTA TTTTCTCAAT   950
TGACCACCAC CCAATGACCG TAATTGAGAT GGACGGTGTT AATACCCAAT  1000
CTATGACCGT AGATTCGATC CAAATATTCG CAGGTCAACG ATATTCATTT  1050
GTCGTAGGTT ATTATAAACT GCCCACCGAT CATCTCTCAC GTAACTGTTA  1100
TAGATGCAAG CCAACCAACC AGTTGGAAAT TATTGGATCC GCGCTAAACC  1150
TAATGTTGGG AACACAACTT TCCTTGGAGG CCTGAACTCC GCTATATTAC  1200
GATATGTGGG AGCCCCTGAC CAAGAACCGA CCACTGACCA AACACCCAAC  1250
TCTACACCGC TCGTTGAGGC GAACCTACGA CCCCTCGTCT ATACTCCTGT  1300
GGTATGTTGT TCTCGTTACA TATACCAAAC CTAATATGAA GACTGAACGG  1350
ATCTACTAGC CGGGACAGCC ATTCCCTGGC GGTGCTGATA TCGTCAAGAA  1400
CTTAGCTTTG GGTTTCGTAC GTGTATTTCA CTTCCCTTTT GGCAGTAACT  1450
GAGGTGGAAT GTATATAGAA TGCCGGGCGT TTCACAATCA ATGGAGCGTC  1500
CCTCACACCT CCTACAGTCC CTGTACTACT CCAGATCCTC AGTGGTACTC  1550
ACAATGCACA GGATCTTCTC CCAGCAGGAA GCGTGATCGA ACTTGAACAG  1600
AATAAAGTTG TCGAAATCGT TTTGCCCGCT GCGGGCGCCG TTGGCGGTCC  1650
TCATCCTTTT CACTTACATG GTGTAAGTAT CAGACGTCCT CATGCCCATA  1700
TTGCTCCGAA CCTTACACAC CTGATTTCAG CACAATTTCT GGGTGGTTCG  1750
TAGCGCCGGT CAAACCACAT ACAATTTCAA TGATGCTCCT ATCCGTGATG  1800
TTGTCAGTAT TGGCGGTGCA AACGATCAAG TCACGATCCG ATTTGTGGTA  1850
TGTATCTCGT GCCTTGCATT CATTCCACGA GTAATGATCC TTACACTTCG  1900
GGTTCTCAGA CCGATAACCC TGGCCCATGG TTCCTTCACT GTCACATTGA  1950
CTGGCATTTG GAGGCTGGGT TCGCTGTAGT CTTTGCGGAG GGAATCAATG  2000
GTACTGCAGC TGCTAATCCA GTCCCAGGTA AGACTCTCGC TGCTTTGCGT  2050
AATATCTATG AATTTAAATC ATATCAATTT GCAGCGGCTT GGAATCAATT  2100
GTGCCCATTG TATGATGCCT TGAGCCCAGG TGATACATGA TTACAAGGGT  2150
GGGCGCGCC                                              2159
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was purified using the QIAquick spin column and digested with restriction enzymes SpeI and AscI. This fragment (SEQ ID No. 66) was then cloned into pTrex4 vector which was also digested with SpeI and AscI to create the expression plasmid (pTrex4-laccaseB, Figure 4). The fidelity of the expression plasmid was confirmed by DNA sequencing and transformed

biolistically into a *Trichoderma* strain. More than 100 transformants were generated and sixty transformants were transferred to new plates. A total of 20 stable transformants were grown in 30 ml of the Proflo media for 2 days at 30°C. Five mls of 2 days old culture from Proflo media were transferred to 50 mls of defined media containing 1mM copper. The cultures were grown for 4 days at 28°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

Example 9

a. Expression of laccase B gene of the CBS strain 115.075 in *Streptomyces*

[96] The laccase B protein sequence was used for codon optimization according to *Streptomyces lividans* codon usage. To construct the expression plasmid for the synthesized laccase B gene of the CBS 115.075 strain in *Streptomyces*, two primers ACGCAGCCTGAACTAGTTGCGATCCTCTAGAG (SEQ ID No. 75) and CTCTGATCAAGGTCATCAGGTGTCGCCCGGGGACAGG (SEQ ID No. 76) were used in the Herculase PCR reaction containing the optimized DNA template (See Example 2b). The PCR fragment was purified using the QIAquick spin column and was digested with XbaI and BclI. The digested fragment was purified by the QIAquick spin column and was cloned into XbaI and BamHI disgested pKB105 (see US 20060154843). The correctness of the resulting plasmid pKB251 (Figure 5) was confirmed by DNA sequencing. The DNA of plasmid pKB251 was transformed into *Streptomyces lividans* g3s3 strain (see US 20060154843). Twelve thiostrepton resistant transformants were picked and transferred into seed shake flask (20 ml of TSG medium containing 50 ug/ml of thiostrepton in DMSO), grown for 2 days at 30°C. Three mls of 2 days old culture from seed shake flask were transferred to 30 mls of *Streptomyces* modified production medium II containing 1mM copper. The cultures were grown for 4 days at 30°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

Example 10 - Expression of the laccase B gene in *Trichoderma* as CBH1 fusion using codon optimized synthetic gene

[97] The optimized synthetic laccase B gene (SEQ ID NO:67):

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ACTAGTGTCG CCGTTTACAA ACGCGCAATC GGTCCCGTCA CTGACCTGCA    50
TATTGTGAAC CAGAATCTCG ACCCCGATGG TTTCAACCGC CCCACTGTCC   100
TCGCAGGTGG TACTTTCCCC GGTCCTCTGA TTCGTGGTAA CAAGGGAGAT   150
AACTTTAAAA TTAATGTGAT TGACGACTTG ACAGAGCACA GCATGCTCAA   200
GGCTACGTCC ATCCACTGGC ATGGCTTCTT CCAGAAGGGA ACCAACTGGG   250
CCGATGGCCC CGCCTTTGTC ACCCAATGTC CTATCACATC AGGAAACGCC   300
TTCCTGTACG ATTTCAACGT TCCGGACCAA GCTGGTACTT TCTGGTACCA   350
CAGCCATCTC TCTACACAGT ACTGTGACGG TCTTCGTGGT GCCTTTGTCG   400
TCTACGATCC TAATGATCCC AACAAGCAAC TCTACGATGT TGATAACGGC   450
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AACACCGTGA TTACCTTGGC TGATTGGTAC CATGCCCTTG CTCAGACTGT   500
CACTGGTGTC GCAGTCTCTG ATGCAACGTT GATCAACGGA TTGGGACGTT   550
CGGCCACCGG CCCCGCAAAT GCCCCTCTGG CGGTCATCAG CGTCGAGCGC   600
AATAAGCGCT ATCGTTTCCG ATTGGTTTCT ATTTCTTGCG ACCCTAACTT   650
TATTTTCTCA ATTGACCACC ACCCCATGAC CGTCATTGAG ATGGACGGTG   700
TTAATACCCA ATCTATGACC GTAGATTCGA TCCAAATCTT CGCAGGTCAA   750
CGATACTCAT TTGTCATGCA AGCCAACCAA CCAGTTGGAA ATTACTGGAT   800
CCGCGCTAAA CCTAATGTTG GCAACACAAC TTTCCTTGGA GGCCTGAACT   850
CCGCTATCTT GCGATACGTG GGAGCCCCTG ACCAAGAACC GACCACTGAC   900
CAAACACCCA ACTCTACACC GCTCGTTGAG GCGAACCTGC GACCCCTCGT   950
CTACACTCCT GTGCCGGGAC AGCCATTCCC TGGCGGTGCT GATATCGTCA  1000
AGAACTTGGC TTTGGGTTTC AATGCCGGGC GTTTCACAAT CAATGGAGCG  1050
TCCCTCACAC CTCCTACAGT CCCTGTCCTG CTCCAGATCC TCAGCGGTAC  1100
TCACAATGCA CAGGATCTTC TCCCGGCAGG AAGCGTGATC GAACTTGAAC  1150
AGAATAAAGT TGTCGAAATC GTTTTGCCCG CTGCGGGCGC CGTTGGCGGT  1200
CCTCATCCTT TTCACTTGCA TGGTCACAAT TTCTGGGTGG TTCGTAGCGC  1250
CGGTCAAACC ACATACAATT TCAATGATGC TCCTATCCGT GATGTTGTCA  1300
GCATTGGCGG TGCAAACGAT CAAGTCACGA TCCGATTTGT GACCGATAAC  1350
CCTGGCCCAT GGTTCCTTCA CTGTCACATT GACTGGCATT TGGAGGCTGG  1400
ATTCGCTGTC GTCTTTGCGG AGGGAATCAA TGGTACTGCA GCTGCTAATC  1450
CCGTCCCGGC GGCTTGGAAT CAATTGTGCC CGTTGTACGA TGCCTTGAGC  1500
CCGGGTGATA CATGAGGCGC GCC                                1523
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encoding the laccase B gene was synthesized by McLab Inc. (Molecular Cloning Laboratories, 384 Oyster Point Blvd, Suite15, South San Francisco, CA94080). The synthetic plasmid DNA was digested with restriction enzymes SpeI and AscI and the 1.5 kb DNA fragment was isolated from gel and cloned into pTrex4 vector which was also digested with SpeI and AscI to create the expression plasmid (pTrex4-laccaseBopt), which is similar to the expression plasmid shown in Figure 4 except that the codon optimized laccase B gene replaced the (non-optimized) laccase B gene. The plasmid was transformed biolistically into a *Trichoderma* strain. More than 30 transformants were generated and were transferred to new plates. A total of 20 stable transformants were selected and mycelia were transferred to 30 mls of defined media containing 1mM copper. The cultures were grown for 4 days at 28°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

Example 11

a. Expression of laccase D gene in *Trichoderma*

[98] To construct the expression plasmid for the laccase D gene of the CBS 115.075 strain, two primers (SEQ ID No. 63 and SEQ ID No. 64) were used in the Herculase PCR reaction containing genomic DNA template obtained from CBS 115.075 strain (see Example 2b). The PCR fragment was purified using the QIAquick spin column and cloned into pENTR/D-TOPO vector. Sixteen clones were amplified using Ready-To-Go PCR beads and four plasmid DNAs

were sequenced. The pENTR-laccaseD CBS115.075#2 clone was selected. The pENTR-laccaseD CBS115.075#2 plasmid (50 ng) was converted to expression plasmid pTrex3g-laccaseD, which is similar to the expression plasmid shown in Figure 1 except that the codon optimized laccase D gene replaced the laccase A gene, in a 10 ul LB clonase II reaction containing 6.5 ul of TE, 1 ul of pTrex3g vector (0.1mg/ml) and 2 ul of ClonaseII. The expression plasmid was confirmed again by DNA sequencing and transformed biolistically into a *Trichoderma* strain. Forty-five transformants were selected and were transferred to new plates. Mycelia from 28 stable transformants were transferred to 30 mls of defined media containing 0.5mM copper. The cultures were grown for 4 days at 28°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

b. Expression of the laccase D gene in *Trichoderma* as CBH1 fusion

[99] To construct the expression plasmid for the laccase D gene of the CBS 115.075 strain, two primers (GGACTAGTGTCGCCGTTTACAAACGCGCAATTGGGCCCGTGGCCGAC, SEQ ID No. 68) and (AAGGCGCGCCTTAAATAGCAGTTCCTTTCTTAG, SEQ ID No. 69) were designed and obtained from Invitrogen. The primers were used in the Herculase PCR reaction containing genomic DNA of the CBS115.075 strain as the DNA template. The PCR fragment was purified using the QIAquick spin column and digested with restriction enzymes SpeI and AscI and cloned into pTrex4 vector (see US Patent Application 10/590,956; WO 05/093050) which was also digested with SpeI and AscI to create the expression plasmid (pTrex4-laccaseD). The fidelity of the expression plasmid was confirmed by DNA sequencing and transformed biolistically into *Trichoderma* strain. More than 300 transformants were generated and sixty transformants were transferred to new plates. Mycelia of 25 stable transformants were transferred to 30 mls of defined media containing 0.5 mM copper. The cultures were grown for 4 days at 28°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

Example 12

Expression of the laccase D gene in Trichoderma as CBH1 fusion using codon optimized synthetic gene.

[100] DNA (SEQ ID NO:70):

```
ACTAGTGTCG CCGTTTACAA ACGCGCTATT GGACCAGTTG CTGATCTGCA    50
CATCGTTAAC AAGGATTTGG CCCCAGACGG CGTCCAGCGC CCAACTGTTC   100
TGGCCGGTGG AACTTTTCCG GGCACGCTGA TTACCGGTCA AAAGGGCGAC   150
AACTTCCAGC TGAACGTGAT TGATGACCTG ACCGACGATC GCATGTTGAC   200
CCCTACTTCG ATCCATTGGC ATGGTTTCTT CCAGAAGGGA ACCGCCTGGG   250
CCGACGGTCC GGCTTTCGTT ACACAGTGCC CTATTATCGC AGACAACTCC   300
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TTCCTCTACG ATTTCGACGT TCCCGACCAG GCGGGCACCT TCTGGTACCA   350
CTCACACTTG TCTACACAGT ACTGCGACGG TCTGCGCGGT GCCTTCGTTG   400
TTTACGACCC CAACGACCCT CACAAGGACC TTTATGATGT CGATGACGGT   450
GGCACAGTTA TCACATTGGC TGACTGGTAT CACGTCCTCG CTCAGACCGT   500
TGTCGGAGCT GCTACACCCG ACTCTACGCT GATTAACGGC TTGGGACGCA   550
GCCAGACTGG CCCCGCCGAC GCTGAGCTGG CCGTTATCTC TGTTGAACAC   600
AACAAGAGAT ACCGTTTCAG ACTCGTCTCC ATCTCGTGCG ATCCCAACTT   650
CACTTTTAGC GTCGACGGTC ACAACATGAC GGTTATCGAG GTTGATGGCG   700
TGAATACCCG CCCTCTCACC GTCGATTCCA TTCAAATTTT CGCCGGCCAG   750
CGATACTCCT TTGTGCTGAA TGCCAATCAG CCCGAGGATA ACTACTGGAT   800
CCGCGCTATG CCTAACATCG GACGAAACAC CACTACCCTT GATGGCAAGA   850
ATGCCGCTAT CCTGCGATAC AAGAACGCCA GCGTTGAGGA GCCCAAAACC   900
GTCGGAGGAC CCGCGCAGAG CCCATTGAAC GAGGCCGACC TGCGACCTCT   950
GGTGCCCGCT CCTGTCCCTG GCAACGCAGT TCCTGGTGGT GCGGACATCA  1000
ACCACCGCCT GAACCTGACA TTCAGCAACG GCCTCTTCTC TATCAATAAC  1050
GCATCATTTA CAAACCCCAG CGTCCCTGCC TTGTTGCAGA TTCTTTCCGG  1100
CGCACAAAAC GCTCAGGATC TGCTTCCCAC CGGTTCTTAT ATCGGCTTGG  1150
AGTTGGGCAA GGTCGTTGAA CTCGTGATCC CTCCCTTGGC CGTTGGTGGC  1200
CCCCATCCAT TCCACTTGCA CGGCCACAAC TTTTGGGTCG TCCGAAGCGC  1250
TGGTTCTGAC GAGTATAATT TCGACGATGC AATTTTGCGC GACGTGGTCA  1300
GCATTGGCGC GGGAACTGAC GAGGTTACTA TCCGTTTTGT CACTGATAAC  1350
CCAGGCCCTT GGTTCCTCCA TTGCCACATC GACTGGCACC TCGAAGCCGG  1400
CCTCGCCATT GTTTTCGCCG AAGGCATCAA TCAAACCGCA GCCGCCAACC  1450
CGACTCCACA GGCCTGGGAC GAACTCTGCC CCAAGTATAA CGGACTCTCC  1500
GCTTCCCAGA AAGTGAAGCC CAAGAAGGGA ACAGCCATCT AAGGCGCGCC  1550
```

encoding the laccase D gene (based on the gene from CBS 115.075) was synthesized by DNA2.0 Inc. (1455 Adams Drive, Menlo Park, CA94025). The synthetic plasmid DNA was digested with restriction enzymes SpeI and AscI and The 1.5 kb DNA fragment was isolated from gel and cloned into pTrex4 vector which was also digested with SpeI and AscI to create the expression plasmid (pTrex4-laccaseDopt). The plasmid was transformed biolistically into a *Trichoderma* strain. Forty transformants were transferred to new plates. A total of 24 stable transformants were selected and mycelia were transferred to 30 mls of defined media containing 0.5 mM copper. The cultures were grown for 4 days at 28°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

Example 13. Expression of the laccase D gene in *Bacillus* as BCE103 fusion using codon optimized synthetic gene.

[101] DNA (SEQ ID NO:71):

```
GGATCCTGAA GCTATCGGTC CGGTTGCAGA TTTACACATC GTAAACAAAG    50
ATCTTGCACC TGACGGCGTT CAACGTCCAA CTGTACTTGC TGGTGGAACA   100
TTCCCTGGTA CACTTATTAC TGGTCAAAAA GGTGACAACT TCCAATTAAA   150
CGTAATTGAC GATCTTACAG ATGACCGTAT GCTTACACCG ACTTCAATTC   200
ACTGGCACGG TTTCTTTCAA AAAGGAACAG CATGGGCTGA TGGTCCTGCA   250
TTCGTTACAC AATGTCCAAT CATTGCTGAT AACTCTTTCC TTTACGATTT   300
TGACGTTCCT GATCAAGCTG GTACATTCTG GTATCACTCA CACTTATCCA   350
CACAATACTG CGATGGACTT CGCGGAGCTT TCGTAGTTTA CGACCCAAAC   400
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GATCCTCATA AAGACCTTTA CGATGTAGAT GATGGTGGAA CAGTTATCAC   450
ATTAGCTGAT TGGTACCATG TACTTGCTCA AACAGTTGTA GGTGCAGCTA   500
CACCAGATTC AACACTTATC AATGGATTAG GACGTTCTCA AACTGGTCCT   550
GCTGACGCAG AACTTGCTGT AATCTCTGTT GAACATAACA AACGTTACAG   600
ATTCCGTCTT GTTAGCATTT CTTGCGATCC AAACTTCACA TTTTCAGTTG   650
ACGGACATAA CATGACAGTT ATCGAAGTAG ATGGTGTAAA CACACGTCCA   700
CTTACTGTAG ACTCTATCCA AATCTTCGCA GGACAACGTT ACTCATTCGT   750
ATTAAACGCA AATCAACCAG AAGATAACTA CTGGATTCGT GCAATGCCAA   800
ACATCGGACG TAACACTACA ACTCTTGACG GCAAAAACGC AGCTATTCTT   850
CGTTACAAAA ACGCTTCTGT TGAAGAACCT AAAACAGTTG GTGGACCAGC   900
ACAATCACCA CTTAACGAAG CTGACTTACG TCCACTGGTT CCAGCACCTG   950
TACCTGGAAA CGCTGTACCA GGAGGTGCTG ATATTAATCA TAGACTTAAC   100
CTTACTTTCT CTAACGGTCT GTTCTCAATC AACAACGCTT CATTCACAAA  1050
TCCTTCAGTT CCAGCACTTT TACAAATTCT TAGCGGTGCA CAAAATGCTC  1100
AGGATCTTTT ACCAACTGGA TCTTACATTG GTCTTGAACT GGGTAAAGTA  1150
GTTGAATTAG TAATTCCTCC GCTTGCTGTA GGTGGACCAC ATCCTTTCCA  1200
TCTTCACGGT CATAACTTCT GGGTTGTACG TTCTGCTGGT TCAGATGAAT  1250
ACAACTTCGA TGACGCAATT CTTCGTGATG TTGTATCTAT TGGTGCTGGA  1300
ACAGATGAAG TAACTATTCG TTTCGTAACA GATAACCCTG GTCCTTGGTT  1350
CTTACATTGT CATATCGATT GGCATCTTGA AGCTGGACTT GCTATTGTTT  1400
TCGCTGAAGG AATCAATCAA ACAGCTGCAG CTAACCCAAC ACCTCAAGCA  1450
TGGGACGAAT TATGTCCAAA ATACAACGCA CTTTCTCCAG GAGATACTTA  1500
AAAGCTT                                                 1507
```

encoding the laccase D gene (based on the gene from CBS 115.075) was synthesized by DNA2.0 Inc. (1455 Adams Drive, Menlo Park, CA94025). The synthetic plasmid DNA was digested with restriction enzymes BamHI and HindIII and the 1.5 kb DNA fragment was isolated from a gel and ligated into the p2JMagk103lnk2 vector (see US20050202535A1) digested with the same two restriction enzymes to create the expression plasmid p2JMagk103lnk2E-laccase (Figure 6). The plasmid was transformed into a B. subtilis strain (*degU^{Hy}32*, *oppA*, D*spoIIE*, D*aprE*, D*nprE*, D*epr*, D*ispA*, D*bpr*, D*vpr*, D*wprA*, D*mpr-ybfJ*, D*nprB*, *amyE*::*xylRPxylAcomK-ermC*) (see US20050202535A1). Two transformants were selected on Luria Broth agar plates with 5 mg/ml chloramphenicol, and then to select for clones with higher gene copy numbers, colonies were serially streaked on Luria Broth agar plates with 25 mg/ml chloramphenicol until rapid colony growth was obtained. The amplified transformants were inoculated into 30 ml MBD medium (see US20050202535A1) containing 0.5 mM copper. The cultures were grown for 60 h at 37°C. Culture broths were centrifuged and supernatants were used for ABTS assay.

Example 14. Bleaching of solubilized indigo with different laccases.

[102] An assay for the bleaching of the solubilized indigo substrate by laccase/mediator combinations was performed in a 96-well microtitre plate as follows

[103] A saturated solution of indigo in *N*-methylpyrrolidone (NMP) was prepared by stirring indigo (30 mg) in NMP (10 ml) at room temperature for 5 hours. The NMP solution was diluted 10-fold into an aqueous buffer solution resulting in a blue solution. For example, dilution into 50 mM sodium acetate buffer at pH 5, or 50 mM sodium phosphate buffer at pH 7. Solutions were shaken well immediately before use.

[104] The assay for the bleaching of the solubilized indigo substrate was performed in a 96-well microtitre plate whereby each well received the soluble indigo solution in 50 mM sodium acetate buffer at pH 5 (180 uL), laccase (10 ppm enzyme) and mediator solution (from a 20 mM stock solution in methanol). The total volume of each well was adjusted to 200 uL with deionzed water. A control containing laccase only was run in duplicate. The plate was sealed and incubated at 50°C for 2 hours at 800 rpm on a heated agitator (Thermomixer, Eppendorf). Following this period, the plates were unsealed and a solution of ascorbic acid (20 uL of a 10% aqueous solution) added to each well in order to reduce the oxidized forms of the mediators. The extent of indigo bleaching was then assessed by determining the absorbance for each well at 600 nm using a microtitre plate reader. The lower the absorbance reading, the greater the extent of indigo bleaching.

[105] Figure 7 shows the results for a *Thielavia* sp. laccase (Ecostone LCC10, AB enzymes, Darmstadt, Germany). The mediators used were 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), syringic acid, 4-carboxamido-2,6-dimethoxyphenol (SA), methyl syringate (MS), 4-(*N*-methyl carboxamido)-2,6-dimethoxyphenol (MSA), 10-(carboxypropyl)-phenothiazine (PTP) and syringaldehyde. The changes in absorbance at 600 nm relative to control are listed in Table 1 where the greatest change in absorbance corresponds to the largest extent of indigo bleaching.

[106] At a mediator concentration of 500 uM, the most effective mediator for indigo bleaching was ABTS, followed by the *N*-methyl amide (MSA) and the unsubstituted amide, 4-carboxamido-2,6-dimethoxyphenol (SA). At the lower mediator concentration of 50 uM, ABTS was still the most effective mediator, with the remaining mediators being more or less equivalent. The exception was syringic acid, which bleached soluble indigo no more effectively than the control condition.

Table 1. Change in absorbance at 600 nm following bleaching of soluble indigo using a *Thielavia* sp. laccase and a variety of mediators at 500 and 50 uM concentrations (n = 2).

Mediator	500mM Concentration		50mM Concentration	
	ΔA600	Std Dev	ΔA600	Std Dev
Control	0	0.008	0	0.010
ABTS	0.235	0.019	0.174	0.032
Syringic acid	0.024	0.017	0.005	0.009
SA	0.170	0.018	0.088	0.014
Methyl Syringate	0.062	0.035	0.090	0.012
MSA	0.181	0.013	0.103	0.018
PTP	0.044	0.009	0.132	0.020
Syringaldehyde	0.132	0.012	0.092	0.017

Example 15. Soluble indigo bleaching assay with different laccases at two pH values

[107] Laccases derived from *Myceliophtora* (Denilite® II, Novozymes, Bagsvaerd, Denmark), *Thielavia* (Ecostone LCC10, AB enzymes, Darmstadt, Germany) and *Cerrena* sp. were assessed for their ability to bleach solubilized indigo in conjunction with low molecular weight mediators at two pH values.

[108] Bleaching of solubilized indigo in 96-well microtitre plates was performed as described in Example 14, using 3 different laccases at pH values of 5 and 7. The mediators used were sinapinic acid, 4-carboxamido-2,6-dimethoxyphenol (SA), methyl 4-acetyl syringate (AMS), methyl syringate (MS) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS). **Figures 8 and 9** shows the results of soluble indigo bleaching at pH values of 5 and 7 using three laccases derived from *Myceliophtora*, *Thielavia* and *Cerrena* sp. respectively. These data are tabulated in Tables 2 and 3.

Table 2. Change in absorbance at 600 nm relative to a control following bleaching of soluble indigo using laccases from *Thielavia*, *Myceliophtora* and *Cerrena* sp. at pH 5, at a mediator concentration of 250 uM.

Mediator	Laccase					
	Thielavia		Myceliophtora		Cerrena	
	ΔA_{600}	Std Dev	ΔA_{600}	Std Dev	ΔA_{600}	Std Dev
Control 1	0	0.016	0	0.010	0	0.005
Sinapinic acid	0.068	0.019	0.157	0.020	0.240	0.007
SA	0.170	0.011	0.254	0.013	0.142	0.005
AMS	0.100	0.012	0.117	0.007	0.028	0.003
MS (AB)	0.048	0.011	0.057	0.007	0.005	0.011
MS (Denilite)	0.050	0.013	0.061	0.007	0.043	0.013
ABTS	0.234	0.012	0.267	0.008	0.329	0.031
Control 2	-0.007	0.017	-0.011	0.007	-0.006	0.005

Table 3. Change in absorbance at 600 nm relative to a control following bleaching of soluble indigo using laccases from *Thielavia*, *Myceliophtora* and *Cerrena* sp. at pH 7, at a mediator concentration of 250 uM.

Mediator	Laccase					
	Thielavia		Myceliophtora		Cerrena	
	ΔA600	Std Dev	ΔA600	Std Dev	ΔA600	Std Dev
Control 1	0	0.008	0	0.001	0	0.006
Sinapinic acid	0.112	0.015	0.204	0.020	0.257	0.005
SA	0.162	0.006	0.220	0.009	0.128	0.010
AMS	0.087	0.006	0.078	0.005	0.077	0.007
MS (AB)	0.053	0.010	0.076	0.006	0.000	0.006
MS (Denilite)	0.069	0.017	0.086	0.001	0.008	0.018
ABTS	0.145	0.006	0.155	0.014	0.215	0.056
Control 2	0.007	0.006	-0.004	0.001	0	0.005

Example 16. Purification and Determination of Specific Activity

[109] The laccase D optimized gene (SEQ ID NO:70) was expressed using the expression system described in co-pending application US 60/984,430 (Attorney Docket No. GC993P entitled "Signal Sequences and co-expressed chaperones for improved heterologous protein production in a host cell" filed 1 November 2007) in 14 liter fermenters. Fermentation broth from was harvested at 184 hours and concentrated by ultra filtration (UFC 20070245). The concentrate was diafiltered into 25mM sodium acetate, pH4.0 buffer. Then 500 ml of the diafiltered UFC sample was loaded on to an ion exchange column containing Poros HS-20 resin (Applied Biosystems, 20 X 275mm column) equilibrated with 25mM sodium acetate buffer, pH 4.0. The column was washed with 10 column volumes of 25mM sodium acetate buffer, pH 4.0. The laccase D protein was eluted from the column using a salt gradient (12 column volumes) from 40mm to 80mM sodium chloride in 25mM sodium acetate buffer, pH 4.0. Fractions containing laccase activity were pooled and further concentrated using an Amicon 400mL stir cell with a 10K membrane. Total protein was measure by SDS protein gel using BSA as standard as 4mg/ml (>90% pure). The laccase sample was diluted 10,000 fold with water and stored at RT for 18 hours and at 4°C for more than 24 hours. ABTS activity was measured as 8570 units/ml. The specific activity of the recombinant laccase D is then calculated by dividing 8570 units/ml by 4 mg/ml resulting in 2140 units/mg of protein which is 100 times more activity than the Stachybotrys laccase (16 u/mg), see Mander et al, Appl. Environ. Microbiol. (2006) 72:5020-5026). Thus, this enzyme results in lower copper discharge into the environment than other laccases, e.g., Stachybotrys laccase, by virtue of the high specific activity.

Example 17. Procedure for denim bleaching

Mediators

[110] 4-hydroxy-3,5-dimethoxybenzamide (syringamide, SA) was purchased from Punjab Chemicals & Crop Protection Limited (Mumbai, India). 4-hydroxy-3,5-dimethoxybenzonitrile (syringonitrile, SN) was acquired from StereoChemical, Inc., (Newark, DE) or Punjab Chemicals & Crop Protection Limited (Mumbai, India).

Enzyme

[111] Laccase enzyme, derived from *Cerrena unicolor* (Example 16, 8570 U/ml, 4 mg protein /ml) was used in the experiments.

Procedure

[112] The enzyme incubations were done in an ATLAS LP 2 Launder-O-meter at different conditions in relation to pH, temperature, enzyme concentration and mediator concentration.

[113] Reactions were carried out in 500 ml stainless steel reaction vessels containing 100 ml of liquid. To each vessel five (7 x 7 cm) stonewashed denim swatches (ACG denim style 80270) and 6 steel balls of 6 mm diameter were added. The reactions vessels were closed and entered into the launder-O-meter that was pre-heated to the desired temperature. The incubation was carried out for 30 minutes after which the swatches were washed with 'running' tap water, spin dried in an AEG IPX4 centrifuge and dried with an Elna Press Electronic iron at program cotton and evaluated.

Stonewashing of denim

[114] Denim, 12 legs weighing approximately 3 kg, was desized in a Unimac UF 50 washing machine under the following conditions:

- Desizing for 15 minutes at 10:1 liquor ratio 50 °C with 0.5 g/l (15 g) of Optisize 160 amylase (Genencor) and 0.5 g/l (15 g) of a non-ionic surfactant (e.g. Rucogen BFA, (Rudolf Chemie) or Ultravon GPN, (Huntsman))
- 2 cold rinses for 5 minutes at 30:1 liquor ratio.

[115] Following desizing the denim was stonewashed in a Unimac UF 50 washing machine under the following conditions:

- Cold rinse for 5 minutes at 10:1 liquor ratio

- Stonewashing for 60 minutes at 10:1 liquor ratio 55 °C with 1 kg of pumice stone, citrate buffer (30 g tri-sodium citrate dihydrate and 30 g citric acid monohydrate) and 35 g IndiAge 2XL cellulase (Genencor).
- 2 cold rinses for 5 minutes at 30:1 liquor ratio.

[116] The denim was dried in a Miele Novotronic T494C household fabric dryer. From the denim legs, swatches of 7 x 7 cm were cut.

Evaluation of denim swatches

[117] The color of the five denim swatches is measured with a Minolta Chromameter CR 310 in the CIE Lab color space with a D 65 light source. Measurements were done before and after laccase treatment and the results of the five swatches were averaged. The total color difference (TCD) is calculated. The total color difference can be calculated with the formula: $TCD = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$.

Evaluation of denim legs

[118] Denim legs were evaluated with a Minolta Chromameter CR 310 in the CIE Lab color space with a D 65 light source. Measurements were done only after laccase treatment. For each denim leg 8 measurements are taken and the result of the 12 legs (96 measurements) was averaged. The total color difference (ΔE) is calculated from the difference between the initial and final CIE L*a*b* values according to the formula

$$\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$$

Example 18 - Effect of temperature on the recombinant laccase D bleaching performance (Unimac)

[119] Laccase bleaching of stonewashed denim: Denim, 12 legs approximately 3 kg, was desized and stonewashed as described in example 17. After stonewashing a laccase treatment was done in a Unimac UF 50 washing machine according to the following process:

- 30 minutes at 10:1 liquor ratio,
- pH 6 (21 g monosodium phosphate and 5 g adipic acid, laccase D laccase) or pH 4.8 (8.6 g monosodium phosphate and 16.8 g of adipic acid, Novoprime Base 268 laccase)
- laccase (laccase D or Novoprime Base 268)
- mediator (syringamide (SA) and syringonitrile (SN))

- After laccase treatment the denim use rinsed twice in cold water for 5 minutes at 30 : 1 liquor ratio.

[120] The laccase experiments were carried out and the results are presented in Tables 4 and 5.

Table 4

Laccase D concentration	Mediator	Mediator concentration	Temperature (°C)	Bleaching level (CIE L)
0.05 g/l / 0.4 U/ml	SA	0.33 mM	60	35.6
0.05 g/l / 0.4 U/ml	SN	0.47 mM	60	35.9
0.05 g/l / 0.4 U/ml	SA	0.33 mM	40	35.6
0.05 g/l / 0.4 U/ml	SN	0.47 mM	40	35.7

Table 5

Novoprime base 268 concentration	Mediator concentration	Temperature (°C)	Bleaching level (CIE L)
0.05 g/l	0.023g/l	60	35.9
0.05 g/l	0.023g/l	40	33.7

[121] The recombinant laccase D has better performance at lower temperatures than currently available commercial laccases. The laccase D (in the presence of mediator) provides a bleaching effect at temperatures below 60°C, preferably between 40°C and 60°C. Thus, the laccase may provide an energy benefit to the textile processor.

Example 19 - Effect of recombinant laccase enzyme and mediator concentration on bleaching performance (Launder-O-meter)

[122] The effect of laccase and mediator concentration was evaluated running the experiments in the table below at pH 6 (50 mM monosodium phosphate buffer pH adjusted with sodium hydroxide 4N solution) and a temperature of 60°C.

[123] The experiments were done with syringamide (SA) - and syringonitrile (SN) mediator.

[124] 100 ml buffer was added to a beaker with five swatches, 7 x 7 cm. The total weight 12 g, (denim:liquor ratio=1:8). Laccase and mediator concentrations were used as indicated in the tables below.

Table 6

Laccase enzyme concentration (µl/l)	Activity correspondence (Laccase unit / g denim)
10	0.67
33	2.17
55	3.67
78	5.17
100	6.67

Table 7

Mediator Concentration (mM)
0.10
0.33
0.55
0.78
1.00

[125] The amounts of syringamide or syringonitrile mediator as indicated in the tables below were added to each beaker as a dilution of a 275 mM SA - or - SN stock solution in 98 % methanol. The laccase was added to each beaker as indicated in the tables below, as dilution of a 400 units/ml laccase stock solution. The beakers were closed and processed at 60°C as described in the example 17. The swatches were evaluated as described in example 17.

Table 8

LACCASE + SA at 60°C pH 6		
Laccase (µl/l)	Mediator syringamide (mM)	TCD
100	1.00	5.6
100	1.00	6.0
100	0.10	2.9
78	0.33	4.4
55	1.00	6.2
55	0.55	5.3
33	0.78	5.5
33	0.33	4.6
10	1.00	3.2
10	0.10	2.5
55	0.55	5.8
100	0.55	5.3
78	0.78	5.9
100	0.10	3.2
55	0.10	3.1
10	0.55	3.6

TCD = total color difference

Table 9

LACCASE + SN at 60°C pH 6		
Laccase (µl/l)	**Mediator syringonitrile (mM)**	**TCD**
100	1.00	7.6
100	1.00	8.1
100	0.10	4.1
78	0.33	5.6
55	1.00	7.0
55	0.55	6.0
33	0.78	5.5
33	0.33	4.4
10	1.00	3.8
10	0.10	2.7
55	0.55	6.3
100	0.55	7.1
78	0.78	7.1
100	0.10	4.0
55	0.10	3.5
10	0.55	3.4

TCD = total color difference

[126] The above Tables and Figures 10 and 11 show that you need both enzyme and mediator to get bleaching. Also it shows there is some flexibility in the enzyme / mediator ratio in achieving a certain bleaching level.

Example 20 – Recombinant Laccase D dose response effect on the bleaching performance (Unimac)

[127] Laccase bleaching of stonewashed denim - Denim, 12 legs weighing approximately 3 kg, was desized and stonewashed as described in Example 17. After stonewashing, a laccase treatment was done according to the following process: 30 minutes at 10:1 liquor ratio and pH 6 (21 g monosodium phosphate and 5 g adipic acid) and 60 °C with laccase and mediator. After laccase treatment the denim use rinsed twice in cold water for 5 minutes at 30 : 1 liquor ratio.

[128] The following experiments were carried out.

- Syringamide 0.33mM:

***Cerrena unicolor* laccase concentration (g/l)**	**Bleaching level (CIE L)**
0.010	34.6
0.05	36.2
0.25	36.2

- Syringonitrile 0.39 mM:

Laccase D concentration (g/l)	Bleaching level (CIE L)
0.25	37.7
0.4	39.5
0.53	38.8

[129] The results are shown in the above tables. This shows that with recombinant laccase D and the amide mediator the bleaching level flattens quite quickly. With an enzyme concentration of 0.05 and 0.25 the same bleaching level is obtained. For the recombinant laccase D and the nitrile mediator the bleaching level increases up to 0.4 g/l, where there appears to be an optimum.

[130] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety.

Claims

1. A laccase selected from the group consisting of SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18 and a laccase having an identity of at least 90% to any of SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16 or 18.
2. A nucleic acid sequence encoding a laccase wherein said laccase is selected from the group consisting of SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16, 18 and a laccase having an identity of at least 90% to any of SEQ ID NOS. 2, 4, 6, 8, 10, 12, 14, 16 or 18.
3. A nucleic acid sequence encoding a laccase wherein said nucleic acid sequence is selected from the group consisting of SEQ ID NOS. 1, 3, 5, 7, 9, 11, 13, 15 and 17.
4. An expression vector comprising a nucleic acid sequence of claim 2.
5. An expression vector comprising a nucleic acid sequence of claim 3.
6. A host cell comprising a vector of claim 4.
7. A host cell comprising a vector of claim 5.
8. A process of bleaching dyes in a solution, which method comprises treating the dyes in the solution with a laccase of claim 1 and an effective mediator.
9. A process according to claim 8, in which the mediator is selected from the group consisting of acetosyringone, syringaldehyde, syringamide, methyl syringamide, 2-hydroxyethyl syringamide, methyl syringate, syringonitrile, dimethylsyringamide, and syringic acid.
10. In a process for bleaching fabrics using laccases, the improvement comprising the use of a laccase of claim 1.
11. A process of claim 10 further comprising a mediator selected from the group consisting of acetosyringone, syringaldehyde, syringamide, methyl syringamide, 2-hydroxyethyl syringamide, methyl syringate, syringonitrile, dimethylsyringamide, and syringic acid.
12. A process according to claim 10, wherein the fabric is dyed with a vat dye.
13. A process according to claim 10, wherein the fabric is a cellulosic fabric, a mixture of cellulosic fibers, or a mixture of cellulosic and synthetic fibers.
14. A process according to claim 10, wherein the fabric is denim.

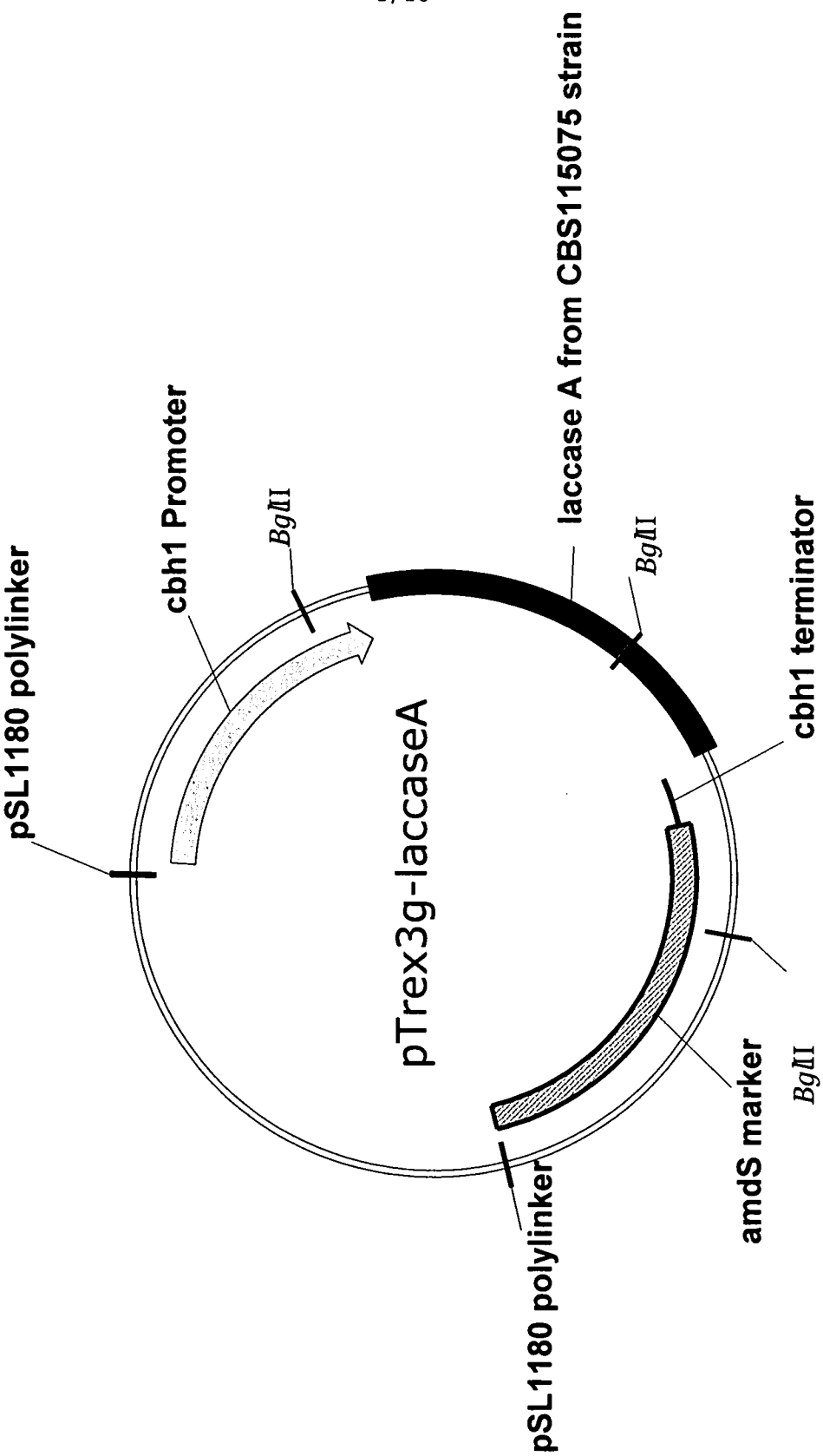
pSL1180 polylinker
cbh1 Promoter
BglII
laccase A from CBS115075 strain
BglII
cbh1 terminator
pTrex3g-laccaseA
amdS marker
BglII
pSL1180 polylinker

Figure 1

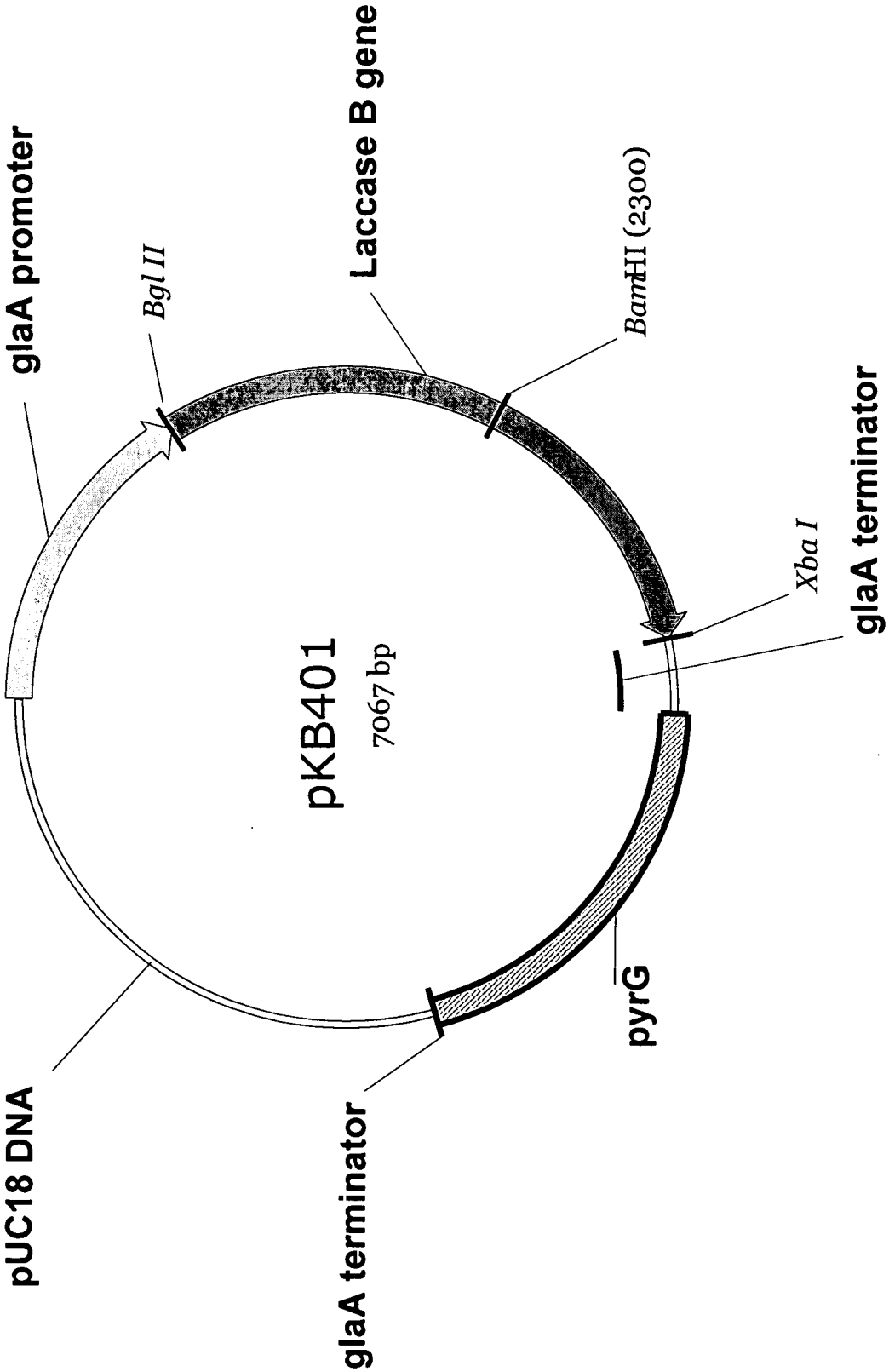
glaA promoter
Bgl II
Laccase B gene
BamHI (2300)
Xba I
glaA terminator
pKB401
7067 bp
pyrG
glaA terminator
pUC18 DNA

Figure 2

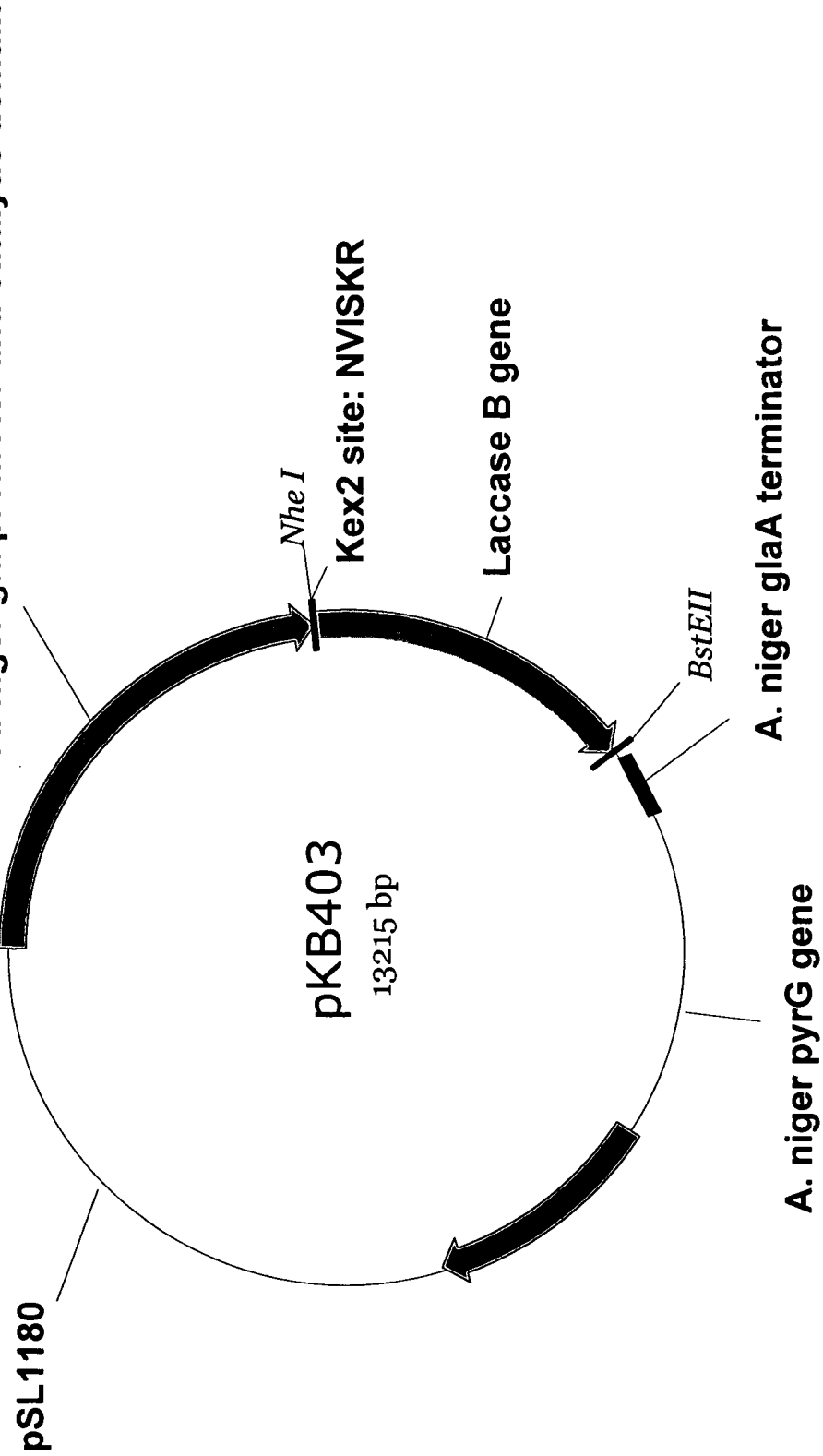
A. niger gla promoter and catalytic domain
Nhe I
Kex2 site: NVISKR
Laccase B gene
BstEII
A. niger glaA terminator
pKB403
13215 bp
pSL1180
A. niger pyrG gene

Figure 3

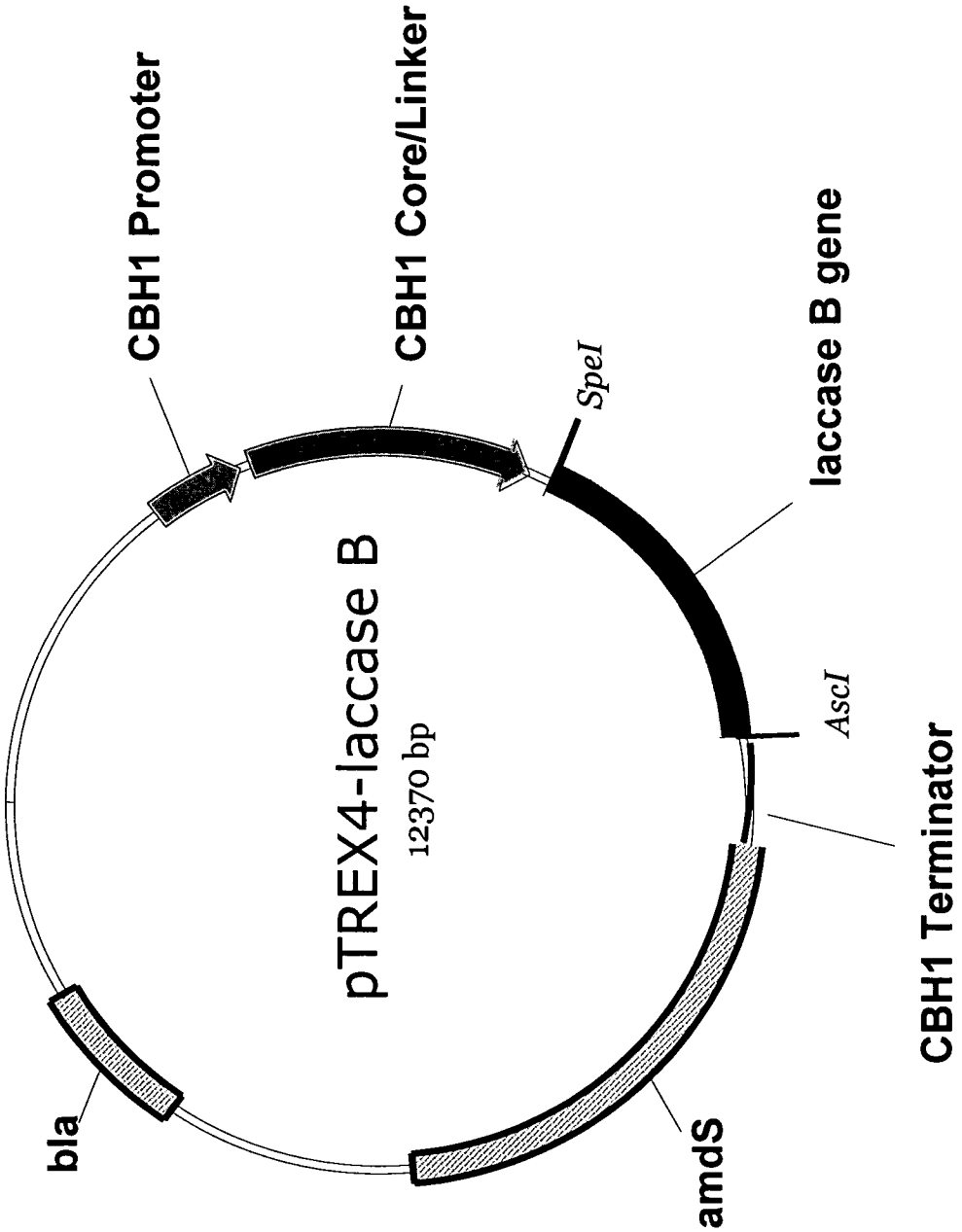
CBH1 Promoter
CBH1 Core/Linker
SpeI
laccase B gene
AscI
CBH1 Terminator
amdS
bla
pTREX4-laccase B
12370 bp

Figure 4

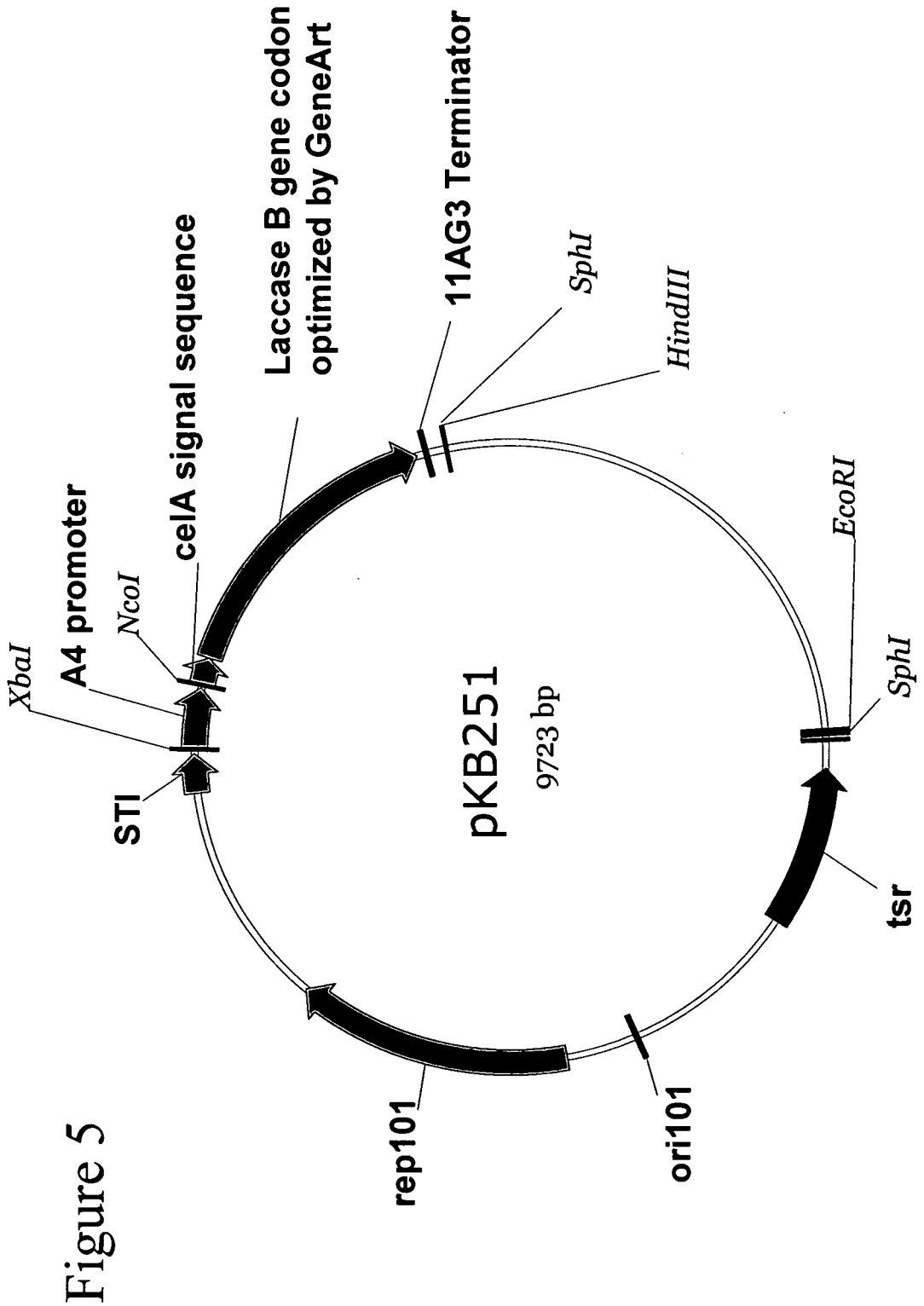
pKB251
9723 bp
XbaI
A4 promoter
NcoI
celA signal sequence
Laccase B gene codon optimized by GeneArt
11AG3 Terminator
SphI
HindIII
EcoRI
SphI
tsr
ori101
rep101
STI

Figure 5

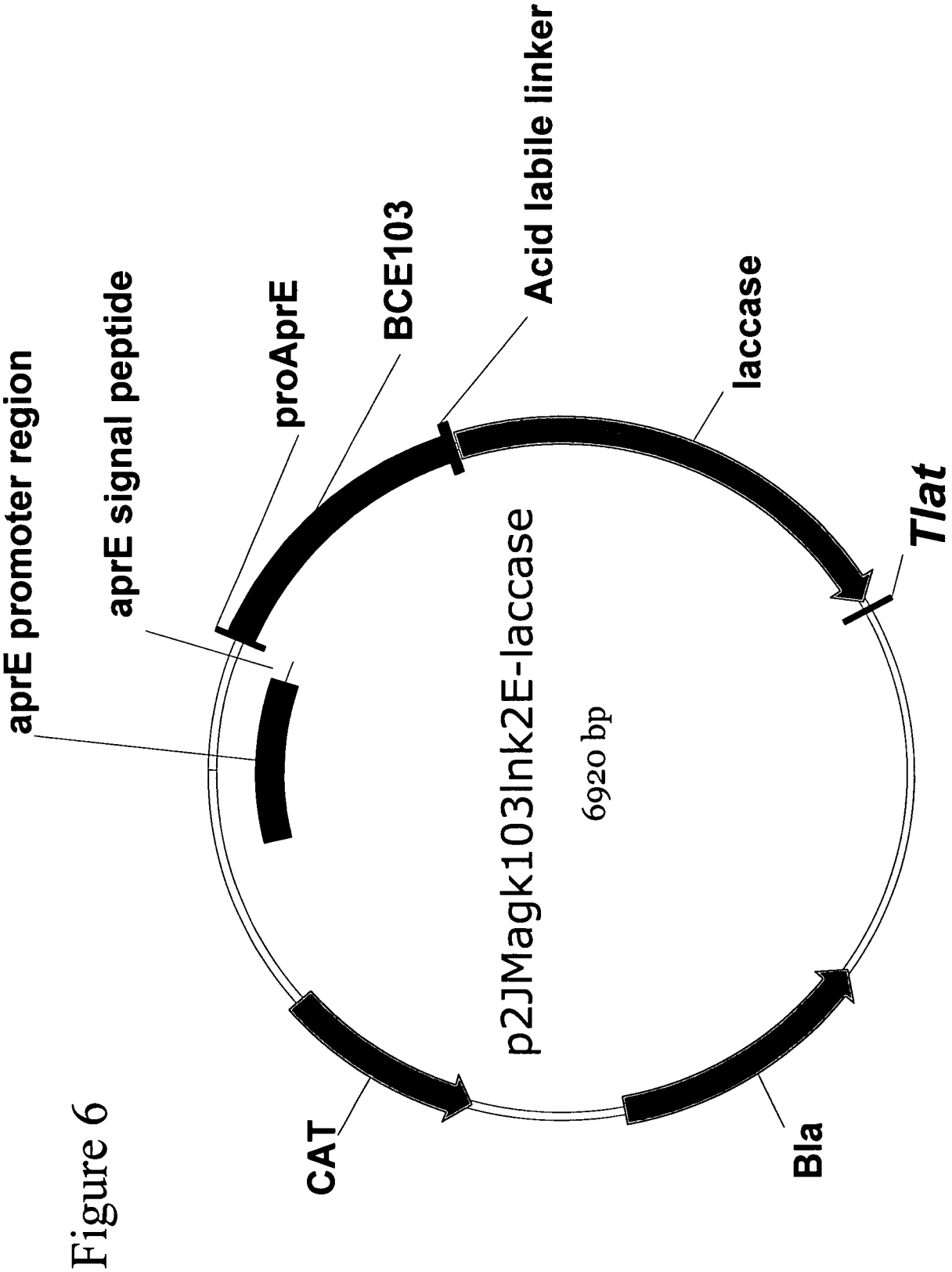
aprE promoter region
aprE signal peptide
proAprE
BCE103
Acid labile linker
laccase
Tlat
p2JMagk103lnk2E-laccase
6920 bp
CAT
Bla

Figure 6

Figure 7. Bleaching of soluble indigo using a *Thielavia* sp. laccase and a variety of mediators at 50 and 500 uM concentrations.

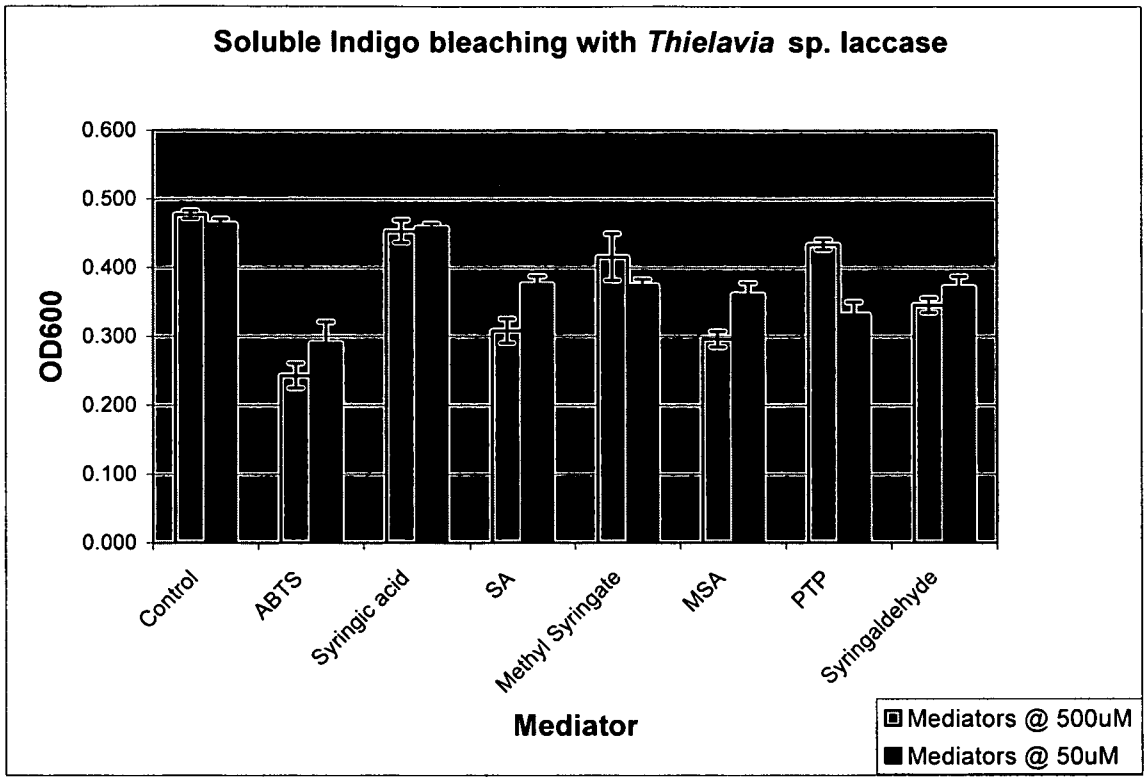

Figure 8. Bleaching of soluble indigo using a *Thielavia*, *Myceliophthora* and *Cerrena* sp. laccase and a variety of mediators at pH 5.

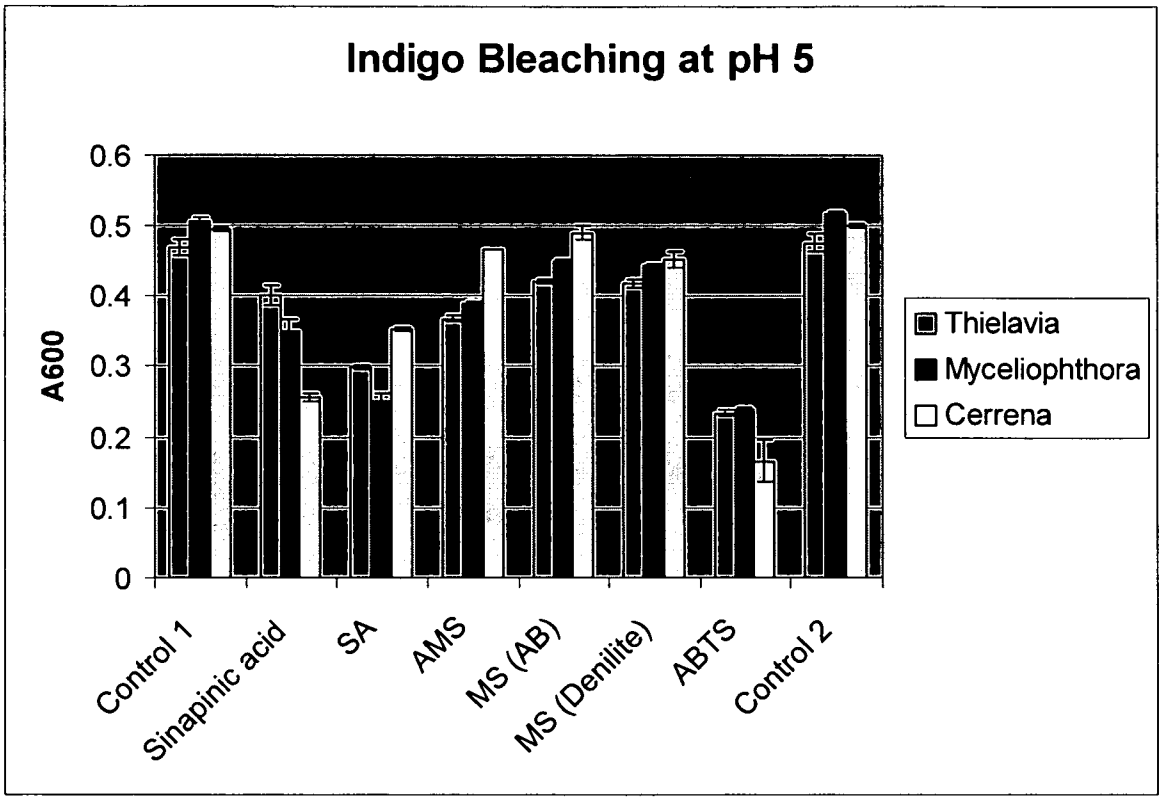

Figure 9. Bleaching of soluble indigo using a *Thielavia*, *Myceliophthora* and *Cerrena* sp. laccase and a variety of mediators at pH 7.

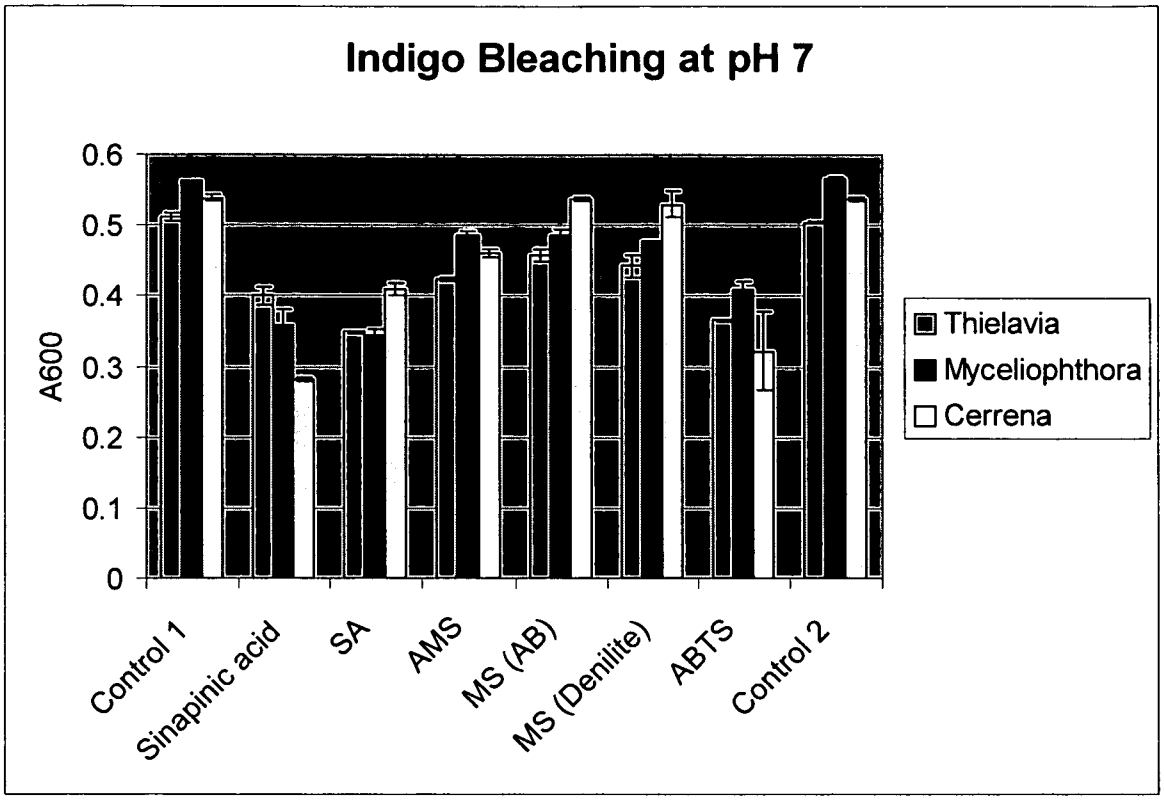

Figure 10

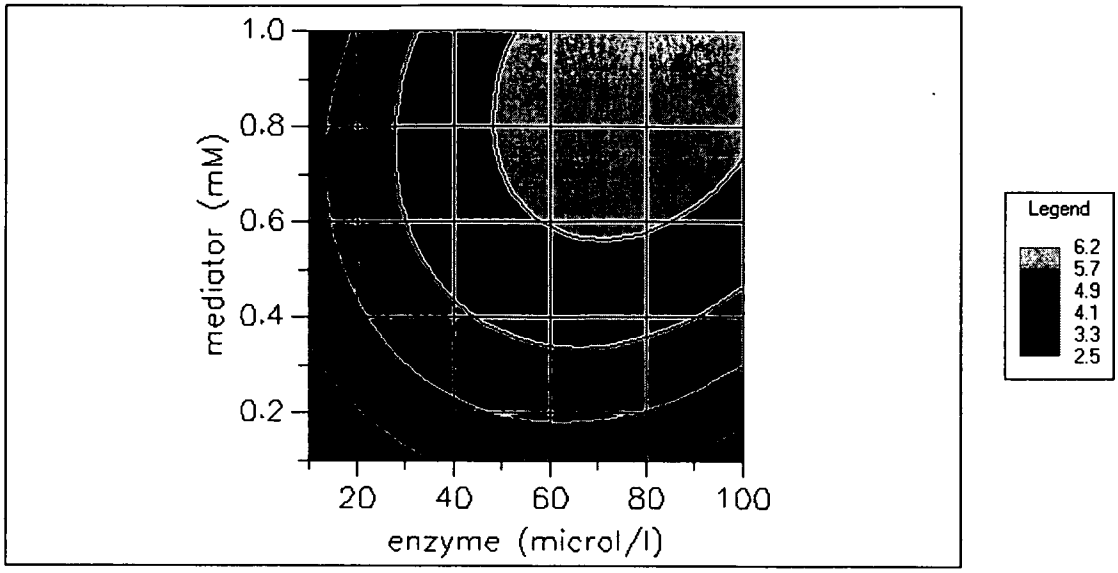

Figure 11