



(86) Date de dépôt PCT/PCT Filing Date: 2007/12/12
(87) Date publication PCT/PCT Publication Date: 2008/06/26
(85) Entrée phase nationale/National Entry: 2009/06/12
(86) N° demande PCT/PCT Application No.: US 2007/025533
(87) N° publication PCT/PCT Publication No.: 2008/076322
(30) Priorités/Priorities: 2006/12/18 (US60/875,454);
2006/12/18 (US60/875,518)

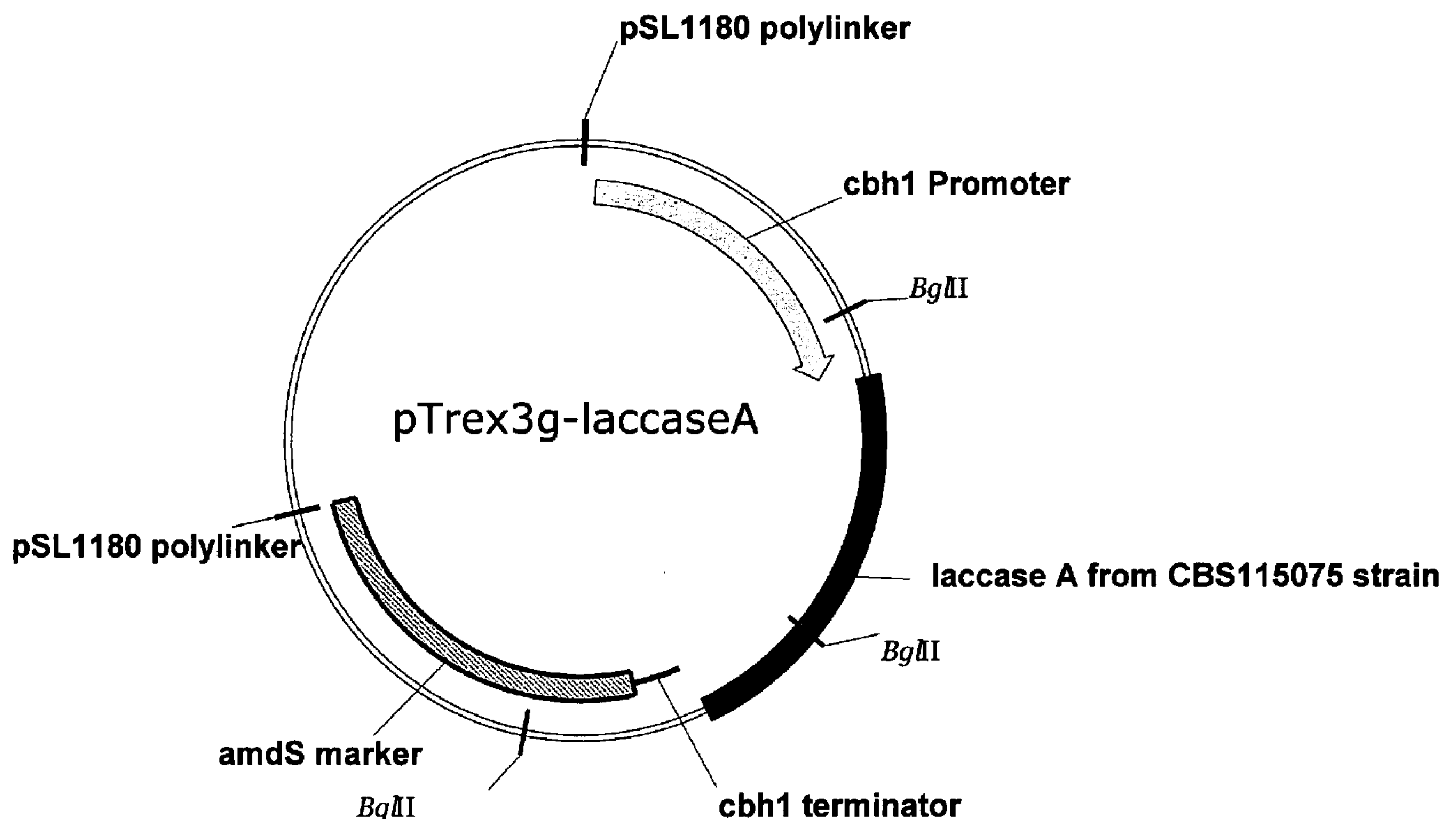
(51) Cl.Int./Int.Cl. *C12N 9/02* (2006.01),
C12N 15/53 (2006.01), *D06L 3/00* (2006.01),
D06P 5/00 (2006.01)

(71) Demandeur/Applicant:
DANISCO US, INC., GENENCOR DIVISION, US

(72) Inventeurs/Inventors:
MCAULIFFE, JOSEPH C., US;
WANG, HUAMING, US

(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : NOUVELLES COMPOSITIONS DE LACCASES, ET LEURS METHODES D'UTILISATION
(54) Title: NOVEL LACCASES, COMPOSITIONS AND METHODS OF USE



(57) Abrégé/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.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
26 June 2008 (26.06.2008)

PCT

(10) International Publication Number
WO 2008/076322 A3

(51) International Patent Classification:

C12N 9/02 (2006.01) *D06L 3/00* (2006.01)

C12N 15/53 (2006.01) *D06P 5/00* (2006.01)

(21) International Application Number:

PCT/US2007/025533

(22) International Filing Date:

12 December 2007 (12.12.2007)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/875,518 18 December 2006 (18.12.2006) US

60/875,454 18 December 2006 (18.12.2006) US

(71) Applicant (for all designated States except US):

DANISCO US, INC., GENENCOR DIVISION
[US/US]; 925 Page Mill Road, Palo Alto, California
94304 (US).

(72) Inventors; and

(75) **Inventors/Applicants (for US only): MCAULIFFE, Joseph, C.** [AU/US]; Danisco US, Inc., Genencor Division, 925 Page Mill Road, Palo Alto, California 94304 (US). **WANG, Huaming** [US/US]; Danisco US, Inc., Genencor Division, 925 Page Mill Road, Palo Alto, California 94304 (US).

(74) **Agent: BOYD, Victoria, L.**; Danisco US, Inc., Genencor Division, 925 Page Mill Road, Palo Alto, California 94304 (US).

(81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

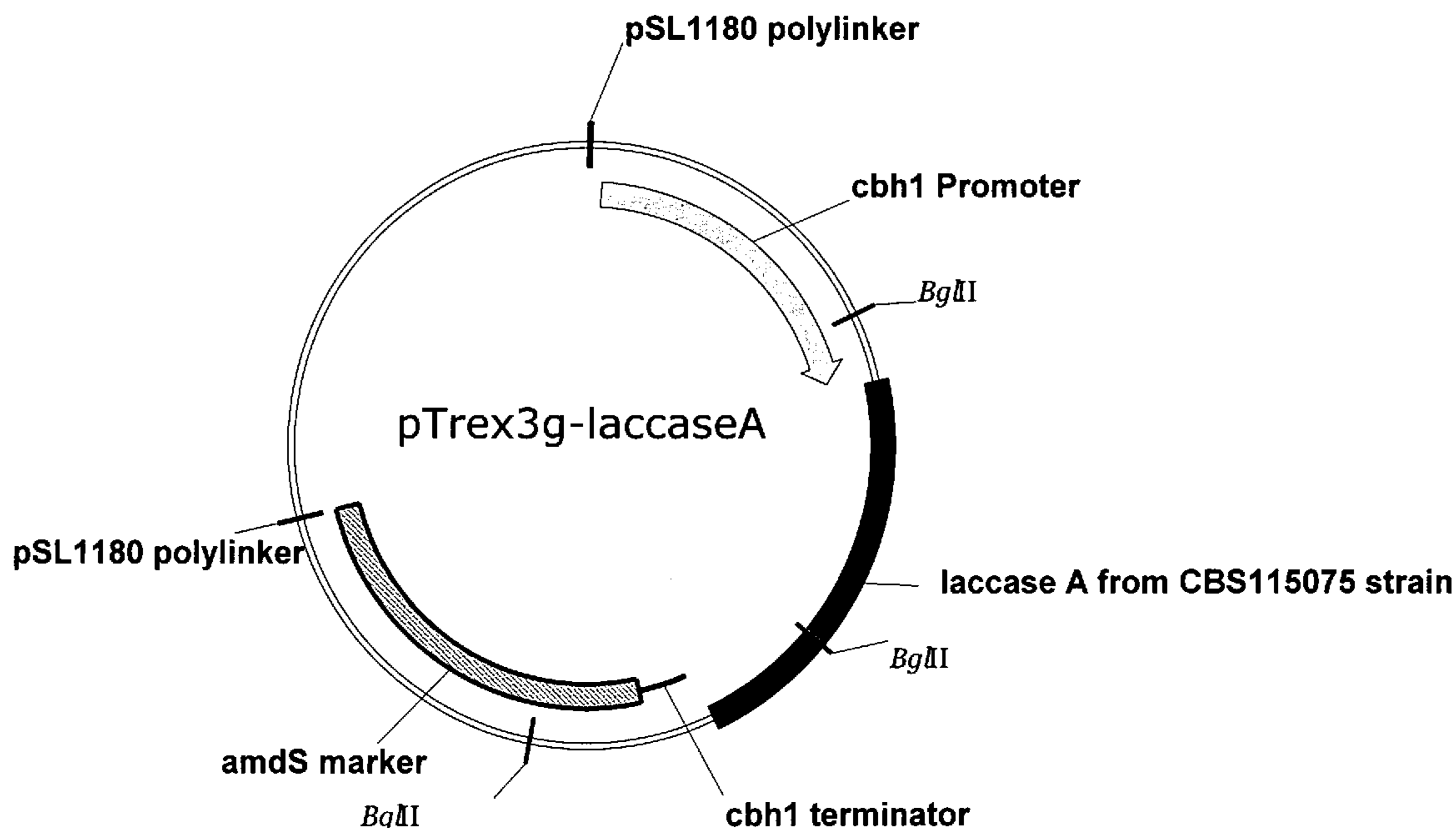
Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(88) Date of publication of the international search report:

21 August 2008

(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.

WO 2008/076322 A3

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 (1-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.

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.

10 [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

15 [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.

20 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.

25 [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.

30 [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
15	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
20	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
25	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
30	ACAGTGAAAT	GACTGTCTGG	TCTAGTTATC	GATTTTCGTCT	GGTCTCCATC	950
	TCATGTGACC	CTAATTACAT	CTTTTCTATC	GACAACCATT	CTATGACTGT	1000
	CATCGAAGTC	GATGGTGTCA	ACACCCAATC	CCTGACCGTC	GATTCTATTC	1050
	AAATCTTCGC	AGGCCAACGA	TACTCGTTTCG	TCGTAAGTCT	CTTTGCACGA	1100
	TTACTGCTTC	TTTGTCCATT	CTCTGACCTG	TTTAAACAGC	TCCATGCCAA	1150
35	CCGTCCTGAA	AACAACCTATT	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
40	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
45	TCGTCGAATT	AGTCATACCT	GCAGGTGTCTG	TCGGTGGACC	TCATCCGTTC	1700
	CATCTCCATG	GGGTACGTAA	CCCGAACTTA	TAACAGTCTT	GGACTTACCC	1750
	GCTGACAAGT	GCATAGCATA	ACTTCTGGGT	CGTGCGAAGT	GCCGGAACCG	1800
	ACCAGTACAA	CTTTAACGAT	GCCATTCTCC	GAGACGTCGT	CAGTATAGGA	1850

6

	GGAACCGGGG	ATCAAGTCAC	CATTCGTTTC	GTGGTATGTT	TCATTCTTGT	1900
	GGATGTATGT	GCTCTAGGAT	ACTAACCGGC	TTGCGCGTAT	AGACCGATAA	1950
	CCCCGGACCG	TGGTTCCTCC	ATTGCCATAT	CGACTGGCAC	TTGGAAGCGG	2000
	GTCTCGCTAT	CGTATTTGCA	GAGGGAATTG	AAAATACTGC	TGCGTCTAAT	2050
5	TTAACCCCCC	GTACGCGGTT	TCCCTCACAT	CCTGGAGCTA	AGCAGCTTAC	2100
	TAACATACAT	TTGCAGAGGC	TTGGGATGAG	CTTTGCCCCG	AGTATAACGC	2150
	GCTCAGCGCA	CAAAAGAAGG	TTGCATCTAA	GAAAGGCACT	GCCATCTAAT	2200
	TTTTGTAAAC	AACAAGGAGG	GTCTCTTGTA	CTTTTATTGG	GATTTCTTTC	2250
	TTGGGGTTTA	TTGTTAAACT	TGACTCTACT	ATGTTTGGAA	GACGAAAGGG	2300
10	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
15	TFPGALITGQ	KGDSFQINVI	DELTDASMLT	QTSIHWHGFF	QKGSAWADGP	100
	AFVTQCPIVT	GNSFLYDFDV	PDQPGTFWYH	SHLSTQYCDG	LRGPFVVYDP	150
	KDPNKRLYDI	DNDHTVITLA	DWYHVLARTV	VG VATPDATL	INGLGRSPDG	200
	PADAE LAVIN	VKRGKRYRFR	LVSISCDPNY	IFSIDNHSMT	VIEVDGVNTQ	250
	SLTVDSIQIF	AGQRYSFVLH	ANRPENNYWI	RAKPNIGTDT	TTDSGMNSAI	300
20	LRNGAPVAE	PQTVQSPSLT	PLLEQNLRLP	VYTPVPGNPT	PGGADIVHTL	350
	DLSFDAGRFS	INGASF LDPT	VPVLLQILSG	TQNAQDLLPP	GSVIPLELGK	400
	VVELVIPAGV	VGGPHPFHLH	GHNFVVV RSA	GTDQYNFNDA	ILRDVVSIGG	450
	TGDQVTIRFV	TDNPGPWFLH	CHIDWHLEAG	LAIVFAEGIE	NTAASNLT PQ	500
	AWDELCPKYN	ALSAQKKLNP	STT			523

25

B. *Cerrena* laccase A2 gene from CBS154.29 strain (SEQ ID No. 3)

	ATGAGCTCAA	AGCTACTTGC	TCTTATTACT	GTCGCTCTCG	TCTTGCCACT	50
	AGGCACTGAC	GCCGGCATCG	GTCCTGTTAC	CGACTTGCGC	ATCACCAACC	100
	AGGATATCGC	TCCAGATGGC	TTCACCCGAC	CAGCTGTACT	GGCTGGGGGC	150
30	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
35	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
40	GATGCTGCCA	GATCATAACG	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
45	ACATTGAAAT	GACTGTCTGG	TCTAGTTATC	GATTCCGTCT	GGTCTCCATC	950
	TCATGTGACC	CTAATTACAT	CTTTTCTATC	GACAACCAT	CTATGACTGT	1000
	CATCGAAGTC	GATGGTGTCA	ACACCCAATC	CCTGACCGTC	GATTCTATCC	1050
	AAATCTTCGC	AGGCCAACGC	TACTCGTTTC	TCGTAAGTCT	CTTTGAATGG	1100
	TTGGTGCTTT	TTCTGTCCAT	TCTCTAACCT	GTTTATACAG	CTCCATGCCA	1150
50	ACCGTCCTGA	AAACAACAT	TGGATCAGGG	CCAAACCTAA	TATCGGTACG	1200
	GATACTACCA	CAGACAACGG	CATGAACCT	GCCATTCTGC	GATACAACGG	1250
	CGCACCTGTT	GCGGAACCGC	AAACTGTTCA	ATCTCCCAGT	CTCACCCCTT	1300
	TGCTCGAACA	GAACCTTCGC	CCTCTCGTGT	ACACTCCTGT	GGTATGTTTC	1350

	AAAGCGTTGT	AATTTGATTG	TGGTCATTCT	AACGTTACTG	CCTTTGCACA	1400
	GCCTGGAAAT	CCTACGCCTG	GCGGGGCCGA	TATTGTCCAT	ACTCTTGACT	1450
	TGAGTTTTGT	GCGGAGTCAA	CATTCGTAAA	GATAAGAGTG	TTTCTAATTT	1500
	CTTCAATAAT	AGGATGCTGG	TCGCTTCAGT	ATCAACGGTG	CCTCGTTCCT	1550
5	TGATCCTACC	GTCCCTGTTC	TCCTGCAAAT	TCTCAGCGGC	ACGCAGAATG	1600
	CACAAGATCT	ACTCCCTCCT	GGAAGTGTGA	TTCCTCTCGA	ATTAGGCAAG	1650
	GTCGTCGAAT	TAGTCATACC	TGCAGGTGTT	GTCGGTGGAC	CTCATCCGTT	1700
	CCATCTCCAT	GGGTACGTA	ACCCGAACCT	ATAACAGTCT	TGGACTTACC	1750
	CGCTGACAAG	TGTATAGCAT	AACTTCTGGG	TCGTGCGAAG	TGCCGGAACC	1800
10	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
15	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
20	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)

25	MSSKLLALIT	VALVLPLGTD	AGIGPVTDLR	ITNQDIAPDG	FTRPAVLAGG	50
	TFPGALITGQ	KGDSFQINVI	DELTDASMLT	QTSIHWHGFF	QKGSAWADGP	100
	AFVTQCPIVT	GNSFLYDFDV	PDQPGTFWYH	SHLSTQYCDG	LRGPFVVYDP	150
	KDPNKRLYDI	DNDHTVITLA	DWYHVLARTV	VGVATPDATL	INGLGRSPDG	200
	PADAE LAVIN	VKRGKRYRFR	LVSISCDPNY	IFSIDNHSMT	VIEVDGVNTQ	250
30	SLTVDSIQIF	AGQRYSFVLH	ANRPENNYWI	RAKPNIGTDT	TTDNGMNSAI	300
	LRYNGAPVAE	PQTVQSPSLT	PLLEQNLRLP	VYTPVPGNPT	PGGADIVHTL	350
	DLSFDAGRFS	INGASFLDPT	VPVLLQILSG	TQNAQDLLPP	GSVIPLELGK	400
	VVELVIPAGV	VGGPHPFHLH	GHNFVVVRS	GTDQYNFNDA	ILRDVVSIGG	450
	TEDQVTIRFV	TDNPGPWFLH	CHIDWHLEAG	LAIVFAEGIE	NTAASNPTPQ	500
35	AWDELCPKYN	ALNAQKKLNP	STT			523

C. *Cerrena* laccase B1 gene from CBS115.075 strain (SEQ ID No. 5)

	ATGTCTCTTC	TTCGTAGCTT	GACCTCCCTC	ATCGTACTAG	TCATTGGTGC	50
	ATTTGCTGCA	ATCGGTCCAG	TCACTGACCT	ACATATAGTG	AACCAGAATC	100
40	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
45	TCTCGAAGCA	CTGGCATGGG	TTCTTCCAGA	AGGGAACCAA	CTGGGCCGAT	400
	GGCCCCGCCT	TTGTCACCCA	ATGTCCTATC	ACATCAGGAA	ACGCCTTCCT	450
	GTATGATTTC	AACGTTCCGG	ACCAAGCTGG	TACTTTCTGG	TACCACAGCC	500
	ATCTCTCTAC	ACAGTATTGT	GACGGTCTTC	GTGGTGCTT	TGTCGTCTAT	550
	GATCCTAATG	ATCCCAACAA	GCAACTCTAT	GATGTTGATA	ACGGCAAGTT	600
50	CCTTG CATAT	TTCATTTCTA	TCATATCCTC	ACCTGTATTG	GCACAGAAAG	650
	CACCGTGATT	ACCTTGGCTG	ATTGGTATCA	TGCCCTTGCT	CAGACTGTCA	700
	CTGGTGTCGC	GTGAGTGACA	AATGGCCCTC	AATTGTTTAC	ATATTTTCCT	750

8

	GATTATCATA	TGATAGAGTA	TCTGATGCAA	CGTTGATCAA	CGGATTGGGA	800
	CGTTCGGCCA	CCGGCCCCGC	AAATGCCCCT	CTGGCGGTCA	TCAGTGTCGA	850
	GCGGAATAAG	AGGTCAGTTC	CATAATTATG	ATTATTTCCC	GCGTTACTTC	900
	CTAACAATTA	TTTTTGTATC	CCTCCACAGA	TATCGTTTCC	GATTGGTTTC	950
5	TATTTCTTGC	GACCCTAACT	TTATTTTCTC	AATTGACCAC	CACCCAATGA	1000
	CCGTAATTGA	GATGGACGGT	GTTAATACCC	AATCTATGAC	CGTAGATTCG	1050
	ATCCAAATAT	TCGCAGGTCA	ACGATATTCA	TTTGTCTAG	GTTATTATAA	1100
	ACTGCCCACC	GATCATCTCT	CACGTAAC TG	TTATAGATGC	AAGCCAACCA	1150
	ACCAGTTGGA	AATTATTGGA	TCCGCGCTAA	ACCTAATGTT	GGGAACACAA	1200
10	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	TTGGGTTCG	1450
15	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
20	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	TCGGGTTCCTC	AGACCGATAA	1950
25	CCCTGGCCCA	TGGTTCCTTC	ACTGTCACAT	TGACTGGCAT	TTGGAGGCTG	2000
	GGTTCGCTGT	AGTCTTTGCG	GAGGGAATCA	ATGGTACTGC	AGCTGCTAAT	2050
	CCAGTCCCAG	GTAAGACTCT	CGCTGCTTTG	CGTAATATCT	ATGAATTTAA	2100
	ATCATATCAA	TTTGCAGCGG	CTTGGAATCA	ATTGTGCCCA	TTGTATGATG	2150
30	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	AFLYDFNVDP	QAGTFWYHSH	LSTQYCDGLR	GAFVVYDPND	150
35	PNKQLYDVDN	GNTVITLADW	YHALAQTVTG	VAVSDATLIN	GLGRSATGPA	200
	NAPLAVISVE	RNKRYRFRLV	SISCDPNFIF	SIDHHPMTVI	EMDGVNTQSM	250
	TVDSIQIFAG	QRYSFVMQAN	QPVGNWYIRA	KPNVGNTTFL	GGLNSAILRY	300
	VGAPDQEPTT	DQTPNSTPLV	EANLRPLVYT	PVPGPFPFGG	ADIVKNLALG	350
	FNAGRFTING	ASLTPPTVPV	LLQILSGTHN	AQDLLPAGSV	IELEQNKVVE	400
40	IVLPAAGAVG	GPHPFHLHGH	NFWVVRSAQ	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)

45	CACCGCGATG	TCTCTTCTTC	GTAGCTTGAC	CTCCCTCATC	GTACTAGCCA	50
	CTGGTGCAAT	TGCTGCAATC	GGTCCAGTCA	CCGACCTACA	TATAGTGAAC	100
	CAGAATCTCG	CCCCAGATGG	TTTAAACCGC	CCCACTGTAC	TCGCAGGTGG	150
	TACTTTCCCC	GGTCCTCTGA	TTCGTGGTAA	CAAGGTACGC	TTCATAACCG	200
	CCCTCCGTAG	ACGTAGGCTT	CGGCTGACAT	GACCATCATC	TGTAGGGAGA	250
50	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
5	CAGAAAGCAC	CGTGATTACC	TTGGCTGATT	GGTATCATGC	CCTTGCTCAG	700
	ACTGTCACTG	GTGTCGCGTG	AGTGACAAAT	GGCCCTTAAT	TGTTCACATA	750
	TTTTCTTGAT	TATCATATGA	TAGAGTATCT	GATGCAACGT	TGATCAACGG	800
	ATTGGGACGT	TCGGCCACCG	GCCCCGCAA	TGCCCTCTG	GCGGTCATCA	850
	GTGTCGAGCG	GAATAAGAGG	TCAGTTCCAT	AATTATGATT	ATTTCCCGCG	900
10	TTACTTCCTA	ACGATTATTT	TTGTATCCCT	CCACAGATAT	CGTTTCCGAT	950
	TGGTTTCTAT	TTCTTGCGAC	CCTAACTTTA	TTTTCTCAAT	TGACCACCAC	1000
	CCAATGACCG	TAATTGAGAT	GGACGGTGTT	AATACCCAAT	CTATGACCGT	1050
	AGATTCGATC	CAAATATTCT	CAGGTCAACG	ATATTCATTT	GTCGTAGGTT	1100
	ATTATAAACT	GCCCACCGAT	CATCTCTCAC	GTAAGTGTTA	TAGATGCAAG	1150
15	CCAACCAACC	AGTTGGAAAT	TATTGGATCC	GYGCTAAACC	TAATGTTGGG	1200
	AACACAACCT	TCCTTGAGAG	CCTGAACCTC	GCTATATTAC	GATATGTGGG	1250
	AGCCCCTGAC	CAAGAACCGA	CCACTGACCA	AACACCCAAC	TCTACACCGC	1300
	TCGTCGAGGC	GAACCTACGT	CCCCTCGTCT	ATACTCCTGT	GGTATGTTGT	1350
	TCTCGTTACA	TATACCAAAC	CTAATATGAG	GACTGAACGG	ATCTACTAGC	1400
20	CGGGACAGCC	ATTCCTGGC	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
25	TCGAAATCGT	TCTGCCCCGT	GCGGGCGCCG	TTGGCGGTCC	TCATCCTTTC	1700
	CACTTACATG	GTGTAAGTAT	CAGACGTCCT	CATGCCTATA	TTGCTCCGAA	1750
	CCTTACACAC	CTGATTTTCA	CACAATTTCT	GGGTGGTTTC	TAGCGCCGGT	1800
	CAAACACAT	ACAATTTCAA	TGATGCTCCT	ATCCGTGATG	TTGTCAGTAT	1850
	TGGCGGTGCA	AACGATCAAG	TCACGATCCG	ATTTGTGGTA	TGTATCTCGT	1900
30	GCCTTGCAAT	CATTCCACGA	GTAATGATCC	TTACACTTCG	GGTTCTCAGA	1950
	CCGATAACCC	TGGCCCATGG	TTCCTTCACT	GTCACATTGA	CTGGCATTTC	2000
	GAGGCTGGGT	TCGCTGTAGT	CTTTGCGGAG	GGAATCAATG	GCACTGCAGC	2050
	TGCTAATCCA	GTCCCAGGTA	AGACTCTCGC	TGCTTTGCGT	AATATCTATG	2100
	AATTTAAAGC	ATATCAATTT	GCAGCGGCTT	GGAATCAATT	GTGCCCGTTG	2150
35	TATGATGCCT	TGAGCCCAGG	CGATACATGA	TTACTCGTAG	CTGTGCTTTC	2200
	TTATACATAT	TCTATGGGTA	TATCGGAGTA	GCTGTACTAT	AGTATGTACT	2250
	ATACTAGGTG	GGATATGYTG	ATGTTGATTT	ATATAATTTT	GTTTGAAGAG	2300
	TGACTTTATC	GACTTGGGAT	TTAGCCGAGT	ACATACTGAT	CTCTCACTAC	2350
	AGGCTTGTTT	TGTCCTTGGG	CGCTTACTCA	ACAGTTGACT	GTTTTTGCTA	2400
40	TTACGCATTG	AACCGCATTC	CGGTCYGACT	CGTGTCTCT	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
45	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
50	VTQCPITSGN	AFLYDFNVPD	QAGTFWYHSH	LSTQYCDGLR	GAFVVYDPND	150
	PNKQLYDVDN	GNTVITLADW	YHALAQTVTG	VAVSDATLIN	GLGRSATGPA	200
	NAPLAVISVE	RNKRYRFRLV	SISCDPNFIF	SIDHHPMTVI	EMDGVNTQSM	250
	TVDSIQIFAG	QRYSFVMQAN	QPVGNWYIRA	KPNVGNNTFL	GGLNSAILRY	300
	VGAPDQEPTT	DQTPNSTPLV	EANLRPLVYT	PVPGQPFPGG	ADIVKNLALG	350

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

5

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
10	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
15	AGGGAGATAA	CTTTAAAATT	AATGTGATTG	ACGACTTGAC	AGAACACAGT	450
	ATGCTCAAGG	CCACGTCCAT	TGTAAGTCCC	TGATTAACGT	TTCACCTGGT	500
	CATATCGCTC	AACGTCTCGA	AGCACTGGCA	TGGGTTCTTC	CAGAAGGGAA	550
	CCAACGGGCG	CGATGGCCCC	GCCTTTGTCA	CCCAATGTCC	TATCACATCA	600
	GGAAACTCCT	TCCTGTATGA	TTTCAACGTT	CCGGACCAAG	CTGGTACTTT	650
20	CTGGTACCAC	AGCCATCTCT	CTACACAGTA	TTGTGACGGT	CTTCGTGGTG	700
	CCTTTGTCTG	CTATGATCCT	AATGATCCCA	ACAAGCAACT	CTATGATGTT	750
	GATAACGGCA	AGTCCCTTGC	ATATTTTCATT	TCTATCATAT	CCTCACCTGT	800
	ATTGGCACAG	AAAGCACCGT	GATTACCTTG	GCTGATTGGT	ATCATGCCCT	850
	TGCTCAGACT	GTCACCTGGT	TCGCGTGAGT	GACAAATGGC	CCTCAATTGT	900
25	TCACATATTT	TCCTGATTAT	CATATGATAG	AGTATCTGAT	GCAACGTTGA	950
	TCAACGGATT	GGGACGTTCC	GCCACCGGCC	CCGCAAATGC	CCCTCTGGCG	1000
	GTCATCAGTG	TCGAGCGGAA	TAAGAGGTCA	GTTCCATAAT	TATGATTATT	1050
	TCCCGCGTTA	CTTCCTAACA	ATTATTCTTG	TATCCCTCCA	CAGATATCGC	1100
	TTCCGATTGG	TGTCTATTTC	TTGCGACCC	AACTTTATTT	TCTCAATTGA	1150
30	TCACCACCCA	ATGACCGTAA	TTGAGATGGA	CGGTGTAAAT	ACCCAATCTA	1200
	TGACCGTAGA	TTGATCCAA	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)

35	MSLLRSLTSL	IVLATGAFAA	IGPVTDLHIV	NQNLAPDGFN	RPTVLAGGTF	50
	PGPLIRGNKG	DNFKINVIDD	LTEHSMLKAT	SIHWHGFFQK	GTNWADGPAF	100
	VTQCPITSGN	SFLYDFNVPD	QAGTFWYHSH	LSTQYCDGLR	GAFVVYDPND	150
	PNKQLYDVDN	GKTIVITLADW	YHALAQTVTG	VAVSDATLIN	GLGRSATGPA	200
	NAPLAVISVE	RNKRYRFRLV	SISCDPNFIF	SIDHHPMTVI	EMDGVNTQSM	250
40	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
45	CCTCTCATCA	CCGGAAACAA	GGTAATGCCT	AATGGTTGCG	TCTTTGTTGG	150
	TGCTCTCATT	CATCCACGAC	ATTTTGTACC	AGGGCGACGC	CTTTAAACTC	200
	AATGTCATCG	ATGAACATAAC	GGACGCATCC	ATGCTGAAGY	CGACTTCCAT	250
	CGTAAGTCTC	GCTGTATTGC	TCCTTGAGCC	ATTTCAATTGA	CTATAACTAC	300
	AACCAGCACT	GGCATGGATT	CTTCCAAAAG	GGTACTAATT	GGGCAGATGG	350
50	TCCCGCTTTT	GTGAACCAAT	GCCCCATCAC	CACGGGAAAC	TCCTTCTTGT	400
	ACGACTTCCA	GGTTCCTGAT	CAAGCTGGTA	AGCATGAGAT	TACACTAGGA	450

11

	AAGTTTAATT	TAATAACTAT	TCAATCAGGA	ACCTACTGGT	ATCATAGTCA	500
	TTTGTCTACG	CAATACTGTG	ATGGTCTCAG	AGGTGCATTC	GTTGTCTACG	550
	ACCCTTCAGA	TCCTCACAAG	GATCTCTACG	ACGTCGACGA	CGGTGAGCTT	600
	TGCTTTTTTC	ATTGGTATCC	ATTATCGCTC	ACGTGTCATT	ACTGCGCCAC	650
5	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
10	AATGCTGATG	TGTACTACTT	CCAGGTACCG	TTTCCGTCTT	GTTTCCATAT	950
	CCTGTGACCC	CAATTGGGTA	TTCTCGATTG	ACAACCATGA	CTTTACGGTC	1000
	ATTGAAGTCG	ATGGTGTAA	CAGTCAACCT	CTCAACGTCG	ATTCTGTTCA	1050
	GATCTTCGCC	GGACAACGTT	ACTCGTTCGT			1080

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

	AIGPVADLHI	TDDTIAPDGF	SRPAVLAGGG	FPGPLITGNK	GDAFKLNVID	50
	ELTDASMLKX	TSIHWHGFFQ	KGTNWADGPA	FVNQCPIITG	NSFLYDFQVP	100
	DQAGTYWYHS	HLSTQYCDGL	RGAFVVYDPS	DPHKDLYDVD	DESTVITLAD	150
	WYHTLARQIV	GVAISDTTLL	NGLGRNTNGP	ADAALAVINV	DAGKRYRFRL	200
20	VSISCDPNWV	FSIDNHDFTV	IEVDGVNSQP	LNVDVSVQIFA	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
25	ATCCCACAGG	TCCTGTAATA	ACACGGAGAC	AGTCCAAACT	GGGATGCCTT	150
	TTTTCTCAAC	TATGGGCGCA	CATAGTCTGG	ACGATGGTAT	ATAAGACGAT	200
	GGTATGAGAC	CCATGAAGTC	AGAACACTTT	TGCTCTCTGA	CATTTTCATGG	250
	TTCACACTCT	CGAGATGGGA	TTGAACTCGG	CTATTACATC	GCTTGCTATC	300
	TTAGCTCTGT	CAGTCGGAAG	CTATGCTGCA	ATTGGGCCCC	TGGCCGACAT	350
30	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
35	CGAATAGTTT	CGCAGTCTGA	TCATTGGATC	TCTATCGCTA	GCATTGGCAC	650
	GGTTTCTTCC	AGAAGGGAAC	CGCTTGGGCC	GACGGTCCCC	CCTTCGTAAC	700
	TCAGTGCCCT	ATAATAGCAG	ATAACTCTTT	TCTGTATGAC	TTCGACGTCC	750
	CAGACCAAGC	TGGTACTTTC	TGGTATCATA	GTCATCTATC	CACTCAGTAC	800
	TGTGACGGTT	TACGTGGTGC	CTTCGTTGTG	TACGATCCTA	ACGATCCTCA	850
40	CAAAGACCTA	TACGATGTTG	ATGACGGTGG	GTTCCAAATA	TTTGTTCTGC	900
	AGACATTGTA	TTGACGGTGT	TCATTATAAT	TTCAGAGAGC	ACCGTGATTA	950
	CCCTTGCGGA	TTGGTACCAT	GTTCTCGCCC	AGACCGTTGT	CGGCGCTGCG	1000
	TGAGTAACAC	ATACACGCGC	TCCGGCACAC	TGATACTAAT	TTTTTTTTTAT	1050
	TGTAGCACTC	CTGATTCTAC	CTTGATCAAC	GGGTTAGGCC	GTTCACAGAC	1100
45	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
50	AGGTATTCTT	TTGTCGTAAG	TTAATCGATA	TATTCTCCTT	ATTACCCCTG	1400
	TGTAATTGAT	GTCAATAGCT	CAATGCTAAC	CAACCCGAAG	ACAATTACTG	1450
	GATCCGTGCT	ATGCCAAACA	TCGGTAGAAA	TACAACAACA	CTGGACGGAA	1500

12

	AGAATGCCGC	TATCCTTCGA	TACAAGAATG	CTTCTGTAGA	AGAGCCCAAG	1550
	ACCGTTGGGG	GCCCCGCTCA	ATCCCCGTTG	AATGAAGCGG	ACCTGCGTCC	1600
	ACTCGTACCT	GCTCCTGTGG	TATGTCTTGT	CGCGCTGTTC	CATCGCTATT	1650
	TCATATTAAC	GTTTTGTTTT	TGTCAAGCCT	GGAAACGCTG	TTCCAGGTGG	1700
5	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
10	GCAGTTGGAG	GACCGCACCC	TTTCCATCTT	CATGGCGTAA	GCATACCACA	2000
	CTCCCGCAGC	CAGAATGACG	CAAAC TAATC	ATGATATGCA	GCACAATTTC	2050
	TGGGTCGTCC	GTAGTGCAGG	TAGCGATGAG	TATAACTTTG	ACGATGCTAT	2100
	CCTCAGGGAC	GTCGTRAGCA	TTGGAGCGGG	GACTGATGAA	GTCACAATCC	2150
	GTTTCGTGGT	ATGTCTCACC	CCTCGCATTT	TGAGACGCAA	GAGCTGATAT	2200
15	ATTTTAACAT	AGACCGACAA	TCCGGGCCCCG	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
20	CTAAGAAAGG	AACTGCTATT	TAAACGTGGT	CCTAGACTAC	GGGCATATAA	2500
	GTATTCGGGT	AGCGCGTGTG	AGCAATGTTT	CGATACACGT	AGATTCATCA	2550
	CCGGACACGC	TGGGACAATT	TGTGTATAAT	GGCTAGTAAC	GTATCTGAGT	2600
	TCTGGTGTGT	AGTTCAAAGA	GACAGCCCTT	CCTGAGACAG	CCCTTCCTGA	2650
	GACAGCCCTT	CCTGAGACGT	GACCTCCGTA	GTCTGCACAC	GATACTYCTA	2700
25	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)

30	MGLNSAITSL	AILALSVGSY	AAIGPVADIH	IVNKDLAPDG	VQRPTVLAGG	50
	TFPGTLITGQ	KGDNFQLNVI	DDLTD DRMLT	PTSIHWHGFF	QKGTAWADGP	100
	AFVTQCP IIA	DNSFLYDFDV	PDQAGTFWYH	SHLSTQYCDG	LRGAFVVYDP	150
	NDPHKDL YDV	DDGGTVITLA	DWYHVLAQTV	VGAATPDSTL	INGLGRSQTG	200
	PADAE LAVIS	VEHNKRYRFR	LVSISCDPNF	TFSVDGHNMT	VIEVDGVNTR	250
35	PLTVDSIQIF	AGQRYSFVLN	ANQPEDNYWI	RAMPNIGRNT	TTLDGKNAAI	300
	LRYKNASVEE	PKTVGGPAQS	PLNEADLRPL	VPAPVPGNAV	PGGADINHRL	350
	NLTFSNGLFS	INNASF TNPS	VPALLQILSG	AQNAQDLLPT	GSYIGLELGK	400
	VVELVIPPLA	VGGPHPFHLH	GHNFVVV RSA	GSDEYNFDDA	ILRDVVSIGA	450
	GTDEVTIRFV	TDNPGPWFLH	CHIDWHLEAG	LAIVFAEGIN	QTAAANPTPQ	500
40	AWDELCPKYN	GLSASQKV KP	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
45	AACTCGGCTA	TTACATCGCT	TGCTATCTTA	GCTCTGTCAG	TCGGAAGCTA	150
	TGCTGCAATT	GGGCCC GTGG	CCGACATACA	CATTGTCAAC	AAAGACCTTG	200
	CTCCAGATGG	TGTACAACGT	CCAACCGTGC	TCGCCGGAGG	CACTTTTCCT	250
	GGGACGTTGA	TCACCGGTCA	GAAAGTAAGG	AATATTAGTT	TGCGTCAAAG	300
	AGCCAACCAA	AATTAACCGT	CCCGTCCCAT	AGGGTGACAA	CTTCCAGCTC	350
50	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
5	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
10	GTTATCAGCG	TTGAACATAA	CAAACGGTAT	GTCATCTCTA	CCCATTATCT	1000
	TCTCTCCTGC	TTTAATTTCG	TGTTTCACCA	TAGATACCGA	TTCCGTTTGG	1050
	TTTCGATTTT	GTGCGACCCC	AACTTTACCT	TCTCCGTTGA	TGGTCATAAT	1100
	ATGACTGTCA	TCGAAGTCGA	CGGTGTCAAC	ACACGACCCC	TGACCGTTGA	1150
	CTCTATTCAA	ATCTTCGCCG	GACAGAGGTA	TTCCTTTGTC	GTAAGTTAAT	1200
15	CGATATATTC	TCCCTATTAC	CCCTGTGTAA	TTGATGTCAA	CAGCTCAATG	1250
	CTAACCAACC	CGACGACAAT	TACTGGATCC	GTGCTATGCC	AAACATCGGT	1300
	AGAAATACAA	CAACACTGGA	CGGAAAGAAT	GCCGCTATCC	TTGATACAA	1350
	GAATGCTTCT	GTAGAAGAGC	CCAAGACCGT	TGGGGGCCCC	GCTCAATCCC	1400
	CGTTGAATGA	AGCGGACCTG	CGTCCACTCG	TACCTGCTCC	TGTGGTATGT	1450
20	CTTGTCGTGC	TGTTCCATCG	CTATTTTATA	TTAACGTTTT	GTTTTTGTCA	1500
	AGCCTGGAAA	CGCTGTTCCA	GGTGGCGCAG	ACATCAATCA	CAGGCTTAAC	1550
	TTAACTTTTC	TACGTACACC	TGGTTGAAAC	ATTATATTTT	CAGTCTAACC	1600
	TCTTGTAGAG	TAACGGCCTT	TTCAGCATCA	ACAACGCCTC	CTTCACTAAT	1650
	CCTTCGGTCC	CCGCCTTATT	ACAAATTCTG	AGCGGTGCTC	AGAACGCTCA	1700
25	AGATTTACTT	CCAACGGGTA	GTTACATTGG	CCTTGAACTA	GGCAAGGTTG	1750
	TGGAGCTCGT	TATACCTCCT	CTGGCAGTTG	GAGGACCGCA	CCCTTTCCAT	1800
	CTTCATGGCG	TAAGCATAAC	ACACTCCCGC	AGCCAGAATG	ACGCAAACCTA	1850
	ATCATGATAT	GCAGCACAAT	TTCTGGGTCG	TCCGTAGTGC	AGGTAGCGAT	1900
	GAGTATAACT	TTGACGATGC	TATCCTCAGG	GACGTCGTGA	GCATTGGAGC	1950
30	GGGGACTGAT	GAAGTCACAA	TCCGTTTTCG	GGTATGTCTC	ACCCCTCGCA	2000
	TTTTGAGACG	CAAGAGCTGA	TATATTTTAA	CATAGACCGA	CAATCCGGGC	2050
	CCGTGGTTCC	TCCATTGCCA	TATTGATTGG	CATTTGGAGG	CAGGCCTTGC	2100
	CATCGTCTTC	GCTGAGGGCA	TCAATCAGAC	CGCTGCAGCC	AACCCAACAC	2150
	CCCGTACGTG	ACACTGAGGG	TTTCTTTTATA	GTGCTGGATT	ACTGAATCGA	2200
35	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
40	TFPGTLITGQ	KGDNFQLNVI	DDLTDRLMLT	PTSIHWHGFF	QKGTAWADGP	100
	AFVTQCPIIA	DNSFLYDFDV	PDQAGTFWYH	SHLSTQYCDG	LRGAFVVYDP	150
	NDPHKDLVDV	DDGGTVITLA	DWYHVLAQTV	VGAATPDSTL	INGLGRSQTG	200
	PADAEELAVIS	VEHNKRYRFR	LVSISCDPNF	TFSVDGHNMT	VIEVDGVNTR	250
	PLTVDSIQIF	AGQRYSFVLN	ANQPDDNYWI	RAMPNIGRNT	TTLDGKNAAI	300
45	LRYKNASVEE	PKTVGGPAQS	PLNEADLRPL	VPAPVPGNAV	PGGADINHRL	350
	NLTFSNGLFS	INNASFNTPS	VPALLQILSG	AQNAQDLLPT	GSYIGLELGK	400
	VVELVIPPLA	VGGPHPFHLH	GHNFVVVRS	GSDEYNFDDA	ILRDVVSIGA	450
	GTDEVTIRFV	TDNPGPWFLH	CHIDWHLEAG	LAIVFAEGIN	QTAAANPTPQ	500
50	AWDELCPKYN	GLSASQKVKP	KKGTAI			526

I. Cerrena laccase E gene (partial) from CBS154.29 strain (SEQ ID No. 17)

	TGCAATCGGA	CCGGTGGCCG	ACCTCAAGAT	CGTAAACCGA	GACATTGCAC	50
--	------------	------------	------------	------------	------------	----

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

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

	AIGPVADLKI	VNRDIAPDGF	IRPAVLAGGS	FPGPLITGQK	GNEFKINVVN	50
	QLTDGSMLKS	TSIHWGFFQ	KGTNWADGPA	FVNQCPIATN	NSFLYQFTSQ	100
	EQPGTFWYHS	HLSTQYCDGL	RGPLVVYDPQ	DPHAVLYDVD	DESTIITLAD	150
	WYHTLARQVK	GPAVPGTTLI	NGLGRHNNGP	LDAELAVISV	QAGKRQVQFT	200
30	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.

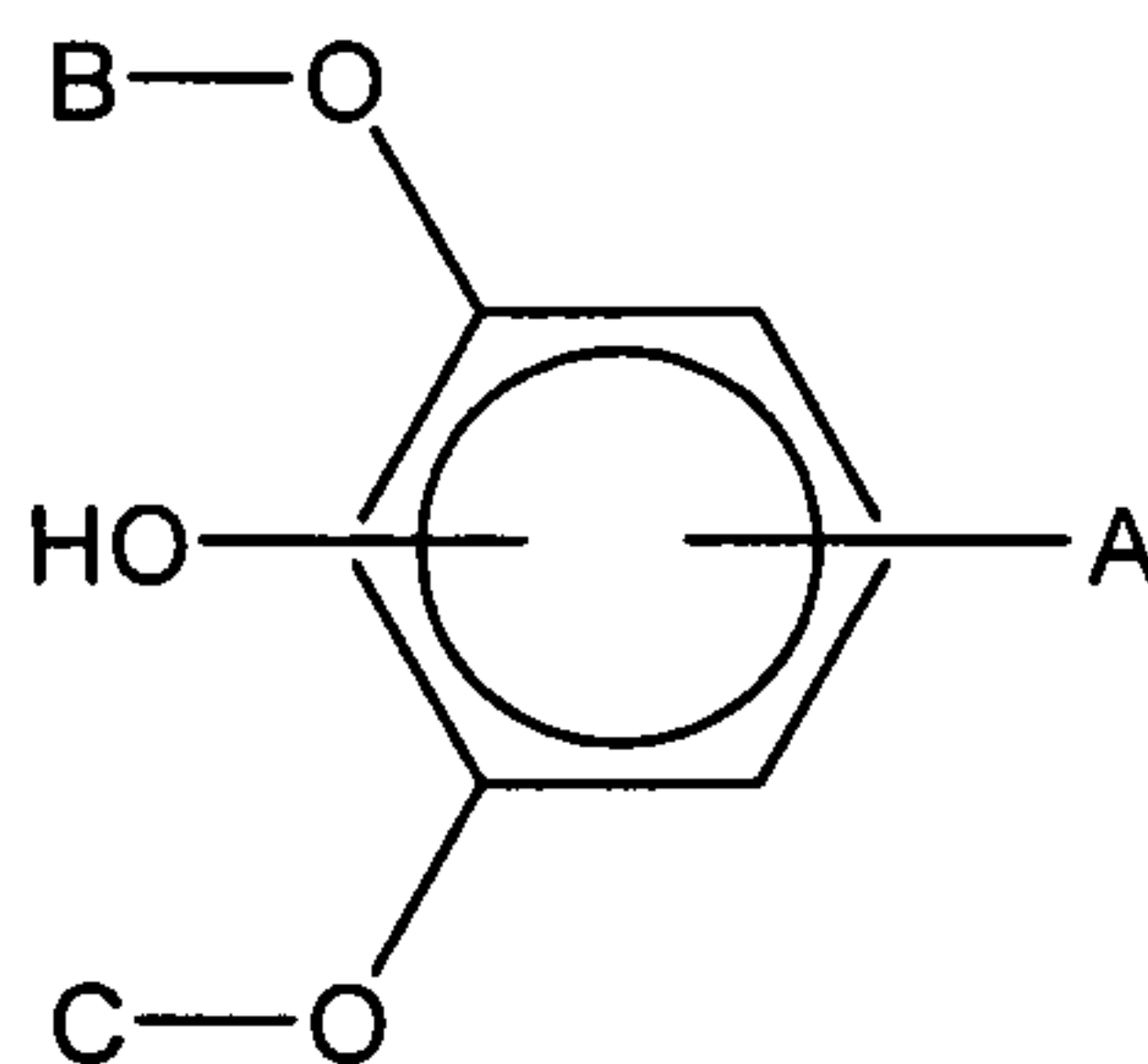
II. Mediators

[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 $-R$, $-D$, $-\text{CH}=\text{CH}-D$, $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-D$, $-\text{CH}=\text{N}-D$, $-\text{N}=\text{N}-D$, or $-\text{N}=\text{CH}-D$, in which D is selected from the group consisting of $-\text{CO}-E$, $-\text{SO}_2-E$, $-\text{CN}$, $-\text{NXY}$, and $-\text{N}^+\text{XYZ}$, in which E may be $-\text{H}$, $-\text{OH}$, $-\text{R}$, $-\text{OR}$, or $-\text{NXY}$, and X and Y and Z may be identical or different and selected from $-\text{H}$, $-\text{OH}$, $-\text{OR}$ and $-\text{R}$; R being a $\text{C}_1 - \text{C}_{16}$ alkyl, preferably a $\text{C}_1 - \text{C}_8$ 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 $\text{C}_m \text{H}_{2m+1}$; $1 \leq m \leq 5$.

[42] In an embodiment A in the above mentioned formula is $-\text{CN}$ or $-\text{CO}-E$, in which E may be $-\text{H}$, $-\text{OH}$, $-\text{R}$, $-\text{OR}$, or $-\text{NXY}$, where X and Y may be identical or different and selected from $-\text{H}$, $-\text{OH}$, $-\text{OR}$ and $-\text{R}$, R being a $\text{C}_1 - \text{C}_{16}$ alkyl, preferably a $\text{C}_1 - \text{C}_8$ 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 $\text{C}_m \text{H}_{2m+1}$; $1 \leq m \leq 5$.

[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 $\mu\text{mole per g denim}$, preferably 0.05-500 $\mu\text{mole per g denim}$, more preferably 0.5-100 $\mu\text{mole per g denim}$.

[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
5 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
10 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
15 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
20 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
25 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,
30 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

5 [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
10 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

15 [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
20 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.

25

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.
30 The primers were used in a Herculanase 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. Herculanase® 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

5 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
10 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
15 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
20 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

25 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
30 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.

5 [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.

10 [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
15 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.
20 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
25 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
30 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.

5 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
10 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
15 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
20 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
25 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®
30 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.

5 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 GCAGATCTGCGATGTCTTCTTCGTAGCTTGAC (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 transformed 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.

25 [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 inoculated 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 Herculanase 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)

	ACTAGTGTCG	CCGTTTACAA	ACGCGCAATC	GGTCCAGTCA	CTGACCTACA	50
	TATAGTGAAC	CAGAATCTCG	ACCCAGATGG	TTTCAACCGC	CCCACTGTAC	100
5	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
10	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	TGCATATTTT	ATTTCTATCA	TATCCTCACC	600
15	TGTATTGGCA	CAGAAAGCAC	CGTGATTACC	TTGGCTGATT	GGTATCATGC	650
	CCTTGCTCAG	ACTGTCACTG	GTGTCGCGTG	AGTGACAAAT	GGCCCTCAAT	700
	TGTTTCACATA	TTTTCTGAT	TATCATATGA	TAGAGTATCT	GATGCAACGT	750
	TGATCAACGG	ATTGGGACGT	TCGGCCACCG	GCCCCGCAA	TGCCCCCTCTG	800
	GCGGTCATCA	GTGTCGAGCG	GAATAAGAGG	TCAGTTCCAT	AATTATGATT	850
20	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	GTAACGTGTA	1100
25	TAGATGCAAG	CCAACCAACC	AGTTGGAAAT	TATTGGATCC	GCGCTAAACC	1150
	TAATGTTGGG	AACACAACCT	TCCTTGAGG	CCTGAACTCC	GCTATATTAC	1200
	GATATGTGGG	AGCCCCTGAC	CAAGAACCGA	CCACTGACCA	AACACCCAAC	1250
	TCTACACCGC	TCGTTGAGGC	GAACCTACGA	CCCCTCGTCT	ATACTCCTGT	1300
	GGTATGTTGT	TCTCGTTACA	TATACCAAAC	CTAATATGAA	GACTGAACGG	1350
30	ATCTACTAGC	CGGGACAGCC	ATTCCCTGGC	GGTGCTGATA	TCGTCAAGAA	1400
	CTTAGCTTTG	GGTTTCGTAC	GTGTATTTCA	CTTCCCTTTT	GGCAGTAACT	1450
	GAGGTGGAAT	GTATATAGAA	TGCCGGGCGT	TTCAACAATCA	ATGGAGCGTC	1500
	CCTCACACCT	CCTACAGTCC	CTGTACTACT	CCAGATCCTC	AGTGGTACTC	1550
	ACAATGCACA	GGATCTTCTC	CCAGCAGGAA	GCGTGATCGA	ACTTGAACAG	1600
35	AATAAAGTTG	TCGAAATCGT	TTTGCCCGCT	GCGGGCGCCG	TTGGCGGTCC	1650
	TCATCCTTTT	CACTTACATG	GTGTAAGTAT	CAGACGTCCT	CATGCCCATA	1700
	TTGCTCCGAA	CCTTACACAC	CTGATTTTCA	CACAATTTCT	GGGTGGTTCG	1750
	TAGCGCCGGT	CAAACCACAT	ACAATTTCAA	TGATGCTCCT	ATCCGTGATG	1800
	TTGTCAGTAT	TGGCGGTGCA	AACGATCAAG	TCACGATCCG	ATTTGTGGTA	1850
40	TGTATCTCGT	GCCTTGCAAT	CATTCCACGA	GTAATGATCC	TTACACTTCG	1900
	GGTTCTCAGA	CCGATAACCC	TGGCCCATGG	TTCTTTCAC	GTCACATTGA	1950
	CTGGCATTG	GAGGCTGGGT	TCGCTGTAGT	CTTTGCGGAG	GGAATCAATG	2000
	GTAATGTCAGC	TGCTAATCCA	GTCCCAGGTA	AGACTCTCGC	TGCTTTGCGT	2050
	AATATCTATG	AATTTAAATC	ATATCAATTT	GCAGCGGCTT	GGAATCAATT	2100
45	GTGCCCATTG	TATGATGCCT	TGAGCCCAGG	TGATACATGA	TTACAAGGGT	2150
	GGGCGCGCC					2159

was purified using the QIAquick spin column and digested with restriction enzymes SpeI and

Ascl. This fragment (SEQ ID No. 66) was then cloned into pTrex4 vector which was also

digested with SpeI and Ascl 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
5 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
10 *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
CTCTGATCAAGGTCATCAGGTGTCGCCCCGGGGACAGG (SEQ ID No. 76) were used in the
Herculase PCR reaction containing the optimized DNA template (See Example 2b). The PCR
15 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 digested 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
20 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.

25

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):

30	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
35	TTCCTGTACG	ATTTCAACGT	TCCGGACCAA	GCTGGTACTT	TCTGGTACCA	350
	CAGCCATCTC	TCTACACAGT	ACTGTGACGG	TCTTCGTGGT	GCCTTTGTCG	400
	TCTACGATCC	TAATGATCCC	AACAAGCAAC	TCTACGATGT	TGATAACGGC	450

	AACACCGTGA	TTACCTTGGC	TGATTGGTAC	CATGCCCTTG	CTCAGACTGT	500
	CACTGGTGTC	GCAGTCTCTG	ATGCAACGTT	GATCAACGGA	TTGGGACGTT	550
	CGGCCACCGG	CCCCGCAAAT	GCCCCTCTGG	CGGTCATCAG	CGTCGAGCGC	600
	AATAAGCGCT	ATCGTTTCCG	ATTGGTTTCT	ATTTCTTGCG	ACCCTAACTT	650
5	TATTTTCTCA	ATTGACCACC	ACCCCATGAC	CGTCATTGAG	ATGGACGGTG	700
	TTAATACCCA	ATCTATGACC	GTAGATTCTG	TCCAAATCTT	CGCAGGTCAA	750
	CGATACTCAT	TTGTCATGCA	AGCCAACCAA	CCAGTTGGAA	ATTACTGGAT	800
	CCGCGCTAAA	CCTAATGTTG	GCAACACAAC	TTTCCTTGGA	GGCCTGAACT	850
	CCGCTATCTT	GCGATACGTG	GGAGCCCCTG	ACCAAGAACC	GACCACTGAC	900
10	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	GAACCTGAAC	1150
15	AGAATAAAGT	TGTCGAAATC	GTTTTGCCCG	CTGCGGGCGC	CGTTGGCGGT	1200
	CCTCATCCTT	TTCACTTGCA	TGGTCACAAT	TTCTGGGTGG	TCGTAGCGC	1250
	CGGTCAAACC	ACATACAATT	TCAATGATGC	TCCTATCCGT	GATGTTGTCA	1300
	GCATTGGCGG	TGCAAACGAT	CAAGTCACGA	TCCGATTGTG	GACCGATAAC	1350
	CCTGGCCCAT	GGTTCCTTCA	CTGTACACAT	GACTGGCATT	TGGAGGCTGG	1400
20	ATTCGCTGTC	GTCTTTGCGG	AGGGAATCAA	TGGTACTGCA	GCTGCTAATC	1450
	CCGTCCC GGC	GGCTTGGAAT	CAATTGTGCC	CGTTGTACGA	TGCCTTGAGC	1500
	CCGGGTGATA	CATGAGGCGC	GCC			1523

encoding the laccase B gene was synthesized by McLab Inc. (Molecular Cloning Laboratories,
 25 384 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
 30 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.

35

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 Herculanase PCR reaction
 40 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 (GGACTAGTGTCGCCGTTTACAAACGCGCAATTGGGCCCCGTGGCCGAC, SEQ ID No. 68) and (AAGGCGCGCCTTAAATAGCAGTTCCTTTCTTAG, SEQ ID No. 69) were designed and obtained from Invitrogen. The primers were used in the Herculanase 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	CCAAGTGTTC	100
TGGCCGGTGG	AACTTTTCCG	GGCACGCTGA	TTACCGGTCA	AAAGGGCGAC	150
AACTTCCAGC	TGAACGTGAT	TGATGACCTG	ACCGACGATC	GATGTTGAC	200
CCCTACTTCG	ATCCATTGGC	ATGGTTTCTT	CCAGAAGGGA	ACCGCCTGGG	250
CCGACGGTCC	GGCTTTCGTT	ACACAGTGCC	CTATTATCGC	AGACAACTCC	300

	TTCCTCTACG	ATTTCGACGT	TCCCGACCAG	GCGGGCACCT	TCTGGTACCA	350
	CTCACACTTG	TCTACACAGT	ACTGCGACGG	TCTGCGCGGT	GCCTTCGTTG	400
	TTTACGACCC	CAACGACCCT	CACAAGGACC	TTTATGATGT	CGATGACGGT	450
	GGCACAGTTA	TCACATTGGC	TGACTGGTAT	CACGTCCTCG	CTCAGACCGT	500
5	TGTCGGAGCT	GCTACACCCG	ACTCTACGCT	GATTAAACGGC	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
10	CGATACTCCT	TTGTGCTGAA	TGCCAATCAG	CCCGAGGATA	ACTACTGGAT	800
	CCGCGCTATG	CCTAACATCG	GACGAAACAC	CACTACCCTT	GATGGCAAGA	850
	ATGCCGCTAT	CCTGCGATAC	AAGAACGCCA	GCGTTGAGGA	GCCCAAACC	900
	GTCGGAGGAC	CCGCGCAGAG	CCCATTGAAC	GAGGCCGACC	TGCGACCTCT	950
	GGTGCCCGCT	CCTGTCCCTG	GCAACGCAGT	TCCTGGTGGT	GCGGACATCA	1000
15	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
20	TGGTTCTGAC	GAGTATAATT	TCGACGATGC	AATTTTGCGC	GACGTGGTCA	1300
	GCATTGGCGC	GGGAAC TGAC	GAGGTTACTA	TCCGTTTTGT	CACTGATAAC	1350
	CCAGGCCCTT	GGTTCCTCCA	TTGCCACATC	GACTGGCACC	TCGAAGCCGG	1400
	CCTCGCCATT	GTTTTCGCCG	AAGGCATCAA	TCAAACCGCA	GCCGCCAACC	1450
	CGACTCCACA	GGCCTGGGAC	GAAGTCTGCC	CCAAGTATAA	CGGACTCTCC	1500
25	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
40	ATCTTGCAAC	TGACGGCGTT	CAACGTCCAA	CTGTACTTGC	TGGTGGAACA	100
	TTCCCTGGTA	CACTTATTAC	TGGTCAAAAA	GGTGACAAC	TCCAATTAAA	150
	CGTAATTGAC	GATCTTACAG	ATGACCGTAT	GCTTACACCG	ACTTCAATTC	200
	ACTGGCACGG	TTTCTTTCAA	AAAGGAACAG	CATGGGCTGA	TGGTCCTGCA	250
	TTCGTTACAC	AATGTCCAAT	CATTGCTGAT	AACTCTTTCC	TTTACGATTT	300
45	TGACGTTTCT	GATCAAGCTG	GTACATTCTG	GTATCACTCA	CACTTATCCA	350
	CACAATACTG	CGATGGACTT	CGCGGAGCTT	TCGTAGTTTA	CGACCCAAAC	400

	GATCCTCATA	AAGACCTTTA	CGATGTAGAT	GATGGTGGAA	CAGTTATCAC	450
	ATTAGCTGAT	TGGTACCATG	TACTTGCTCA	AACAGTTGTA	GGTGCAGCTA	500
	CACCAGATTC	AACACTTATC	AATGGATTAG	GACGTTCTCA	AACTGGTCCT	550
	GCTGACGCAG	AACTTGCTGT	AATCTCTGTT	GAACATAACA	AACGTTACAG	600
5	ATTCCGTCTT	GTTAGCATTT	CTTGCGATCC	AAACTTCACA	TTTTCAGTTG	650
	ACGGACATAA	CATGACAGTT	ATCGAAGTAG	ATGGTGTAAG	CACACGTCCA	700
	CTTACTGTAG	ACTCTATCCA	AATCTTCGCA	GGACAACGTT	ACTCATTCGT	750
	ATTAAACGCA	AATCAACCAG	AAGATAACTA	CTGGATTTCG	GCAATGCCAA	800
	ACATCGGACG	TAACACTACA	ACTCTTGACG	GCAAAAACGC	AGCTATTCTT	850
10	CGTTACAAAA	ACGCTTCTGT	TGAAGAACCT	AAAACAGTTG	GTGGACCAGC	900
	ACAATCACCA	CTTAACGAAG	CTGACTTACG	TCCACTGGTT	CCAGCACCTG	950
	TACCTGGAAA	CGCTGTACCA	GGAGGTGCTG	ATATTAATCA	TAGACTTAAC	1000
	CTTACTTTCT	CTAACGGTCT	GTTCTCAATC	AACAACGCTT	CATTCACAAA	1050
	TCCTTCAGTT	CCAGCACTTT	TACAAATTCT	TAGCGGTGCA	CAAAATGCTC	1100
15	AGGATCTTTT	ACCAACTGGA	TCTTACATTG	GTCTTGAAC	GGGTAAAGTA	1150
	GTTGAATTAG	TAATTCCTCC	GCTTGCTGTA	GGTGGACCAC	ATCCTTTCCA	1200
	TCTTCACGGT	CATAACTTCT	GGGTGCTACG	TTCTGCTGGT	TCAGATGAAT	1250
	ACAACCTCGA	TGACGCAATT	CTTCGTGATG	TTGTATCTAT	TGGTGCTGGA	1300
	ACAGATGAAG	TAACATTCG	TTTCGTAACA	GATAACCCTG	GTCCTTGGTT	1350
20	CTTACATTGT	CATATCGATT	GGCATCTTGA	AGCTGGACTT	GCTATTGTTT	1400
	TCGCTGAAGG	AATCAATCAA	ACAGCTGCAG	CTAACCCAAC	ACCTCAAGCA	1450
	TGGGACGAAT	TATGTCCAAA	ATACAACGCA	CTTCTCCAG	GAGATACTTA	1500
	AAAGCTT					1507

encoding the laccase D gene (based on the gene from CBS 115.075) was synthesized by
 25 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
 30 (*degU^{Hy32}*, *oppA*, *DspoII^E*, *DaprE*, *DnprE*, *Depr*, *DispA*, *Dbpr*, *Dvpr*, *DwprA*, *Dmpr-ybfJ*,
DnprB, *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
 35 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.

40 [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 deionized 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	
	ΔA_{600}	Std Dev	ΔA_{600}	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

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

[107] Laccases derived from *Myceliophthora* (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.

10 [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).

15 **Figures 8 and 9** shows the results of soluble indigo bleaching at pH values of 5 and 7 using three laccases derived from *Myceliophthora*, *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*, *Myceliophthora* and *Cerrena* sp. at pH 5, at a mediator concentration of 250 uM.

Mediator	Laccase					
	Thielavia		Myceliophthora		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*, *Myceliophthora* and *Cerrena* sp. at pH 7, at a mediator concentration of 250 μ M.

Mediator	Laccase					
	Thielavia		Myceliophthora		Cerrena	
	ΔA_{600}	Std Dev	ΔA_{600}	Std Dev	ΔA_{600}	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 bleachingMediators

[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.

5 [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

15 [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

$$20 \quad \Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$$

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

25 [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.

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

- 10 [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.

15 **Example 19 - Effect of recombinant laccase enzyme and mediator concentration on bleaching performance (Lauder-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 (μ l/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

5

[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.

10

Table 8

LACCASE + SA at 60°C pH 6		
Laccase (μ l/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

5

[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.

10 **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:

20

<i>Cerreña unicolor</i> 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

5 [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.

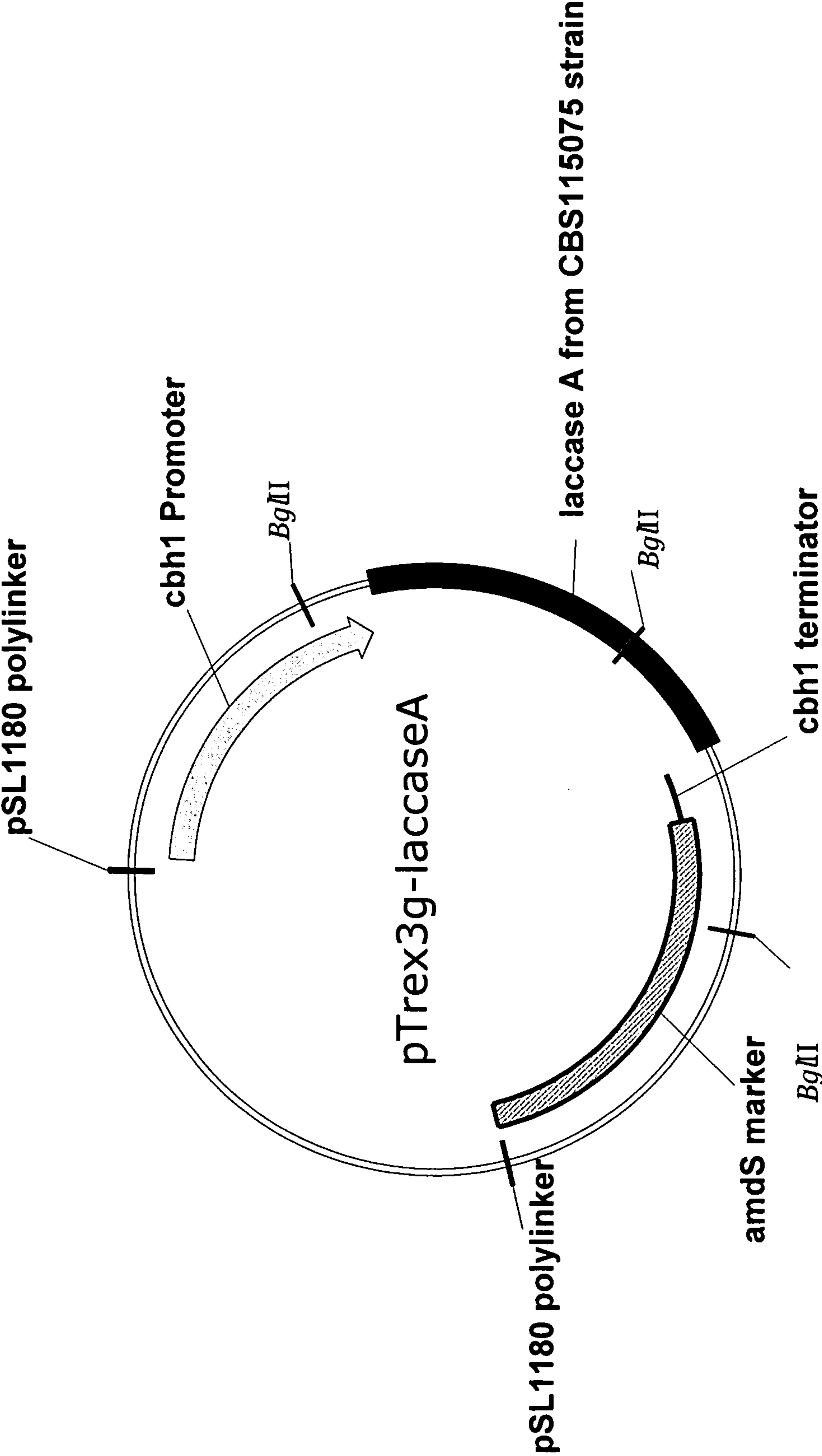
10

[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
15 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.
- 5 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.
- 10 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
15 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
20 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.
- 25 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.

Figure 1



2/10

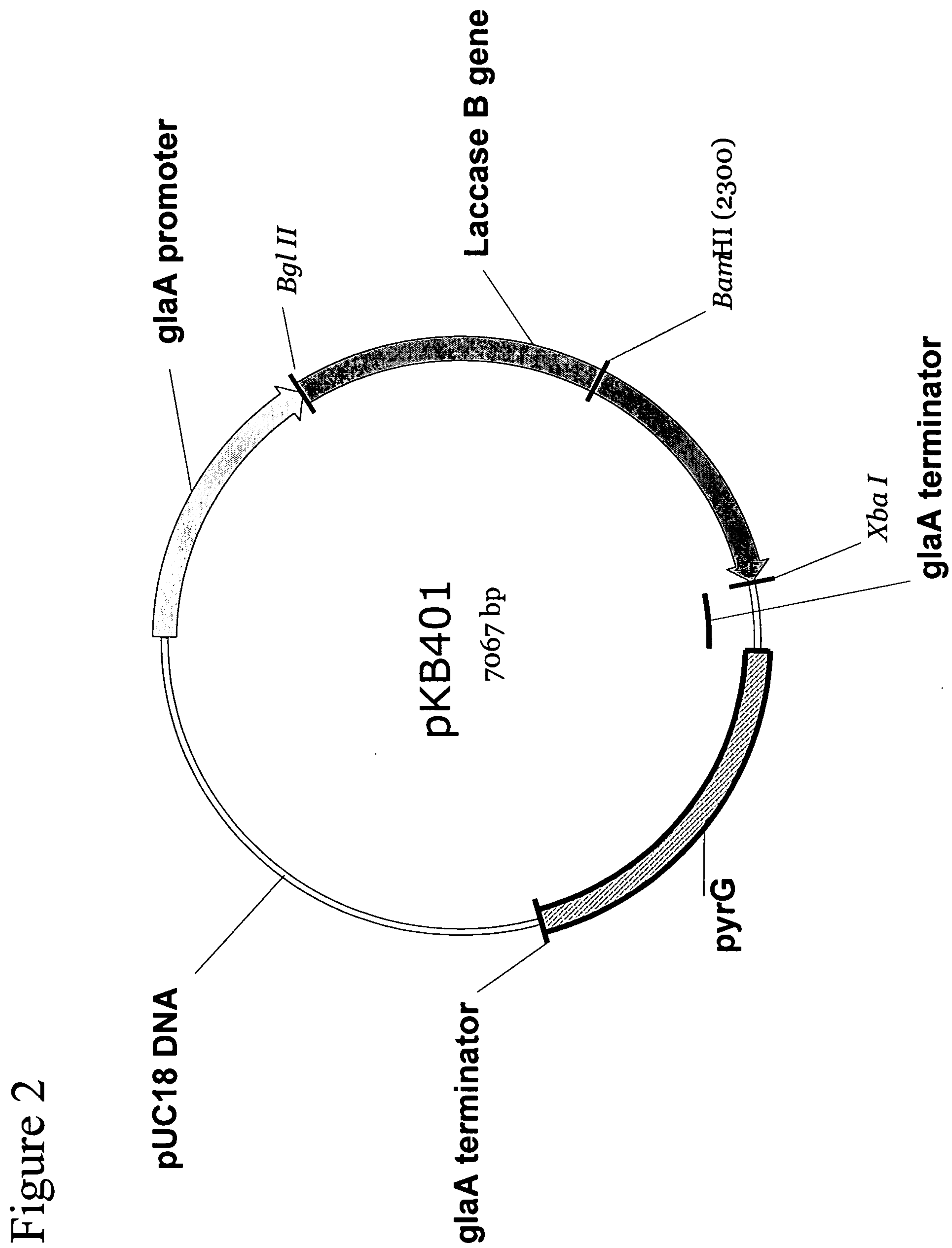


Figure 3

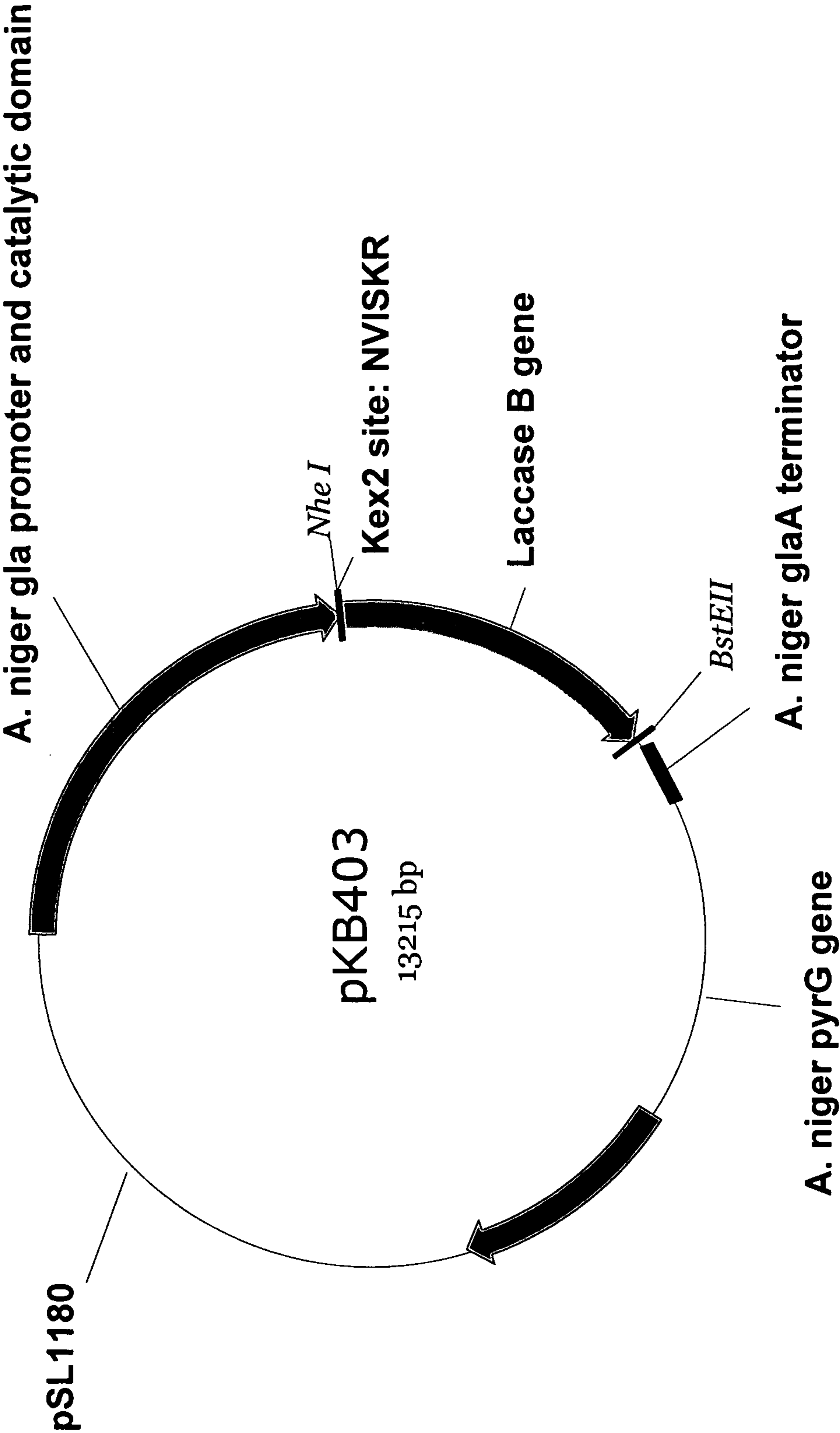
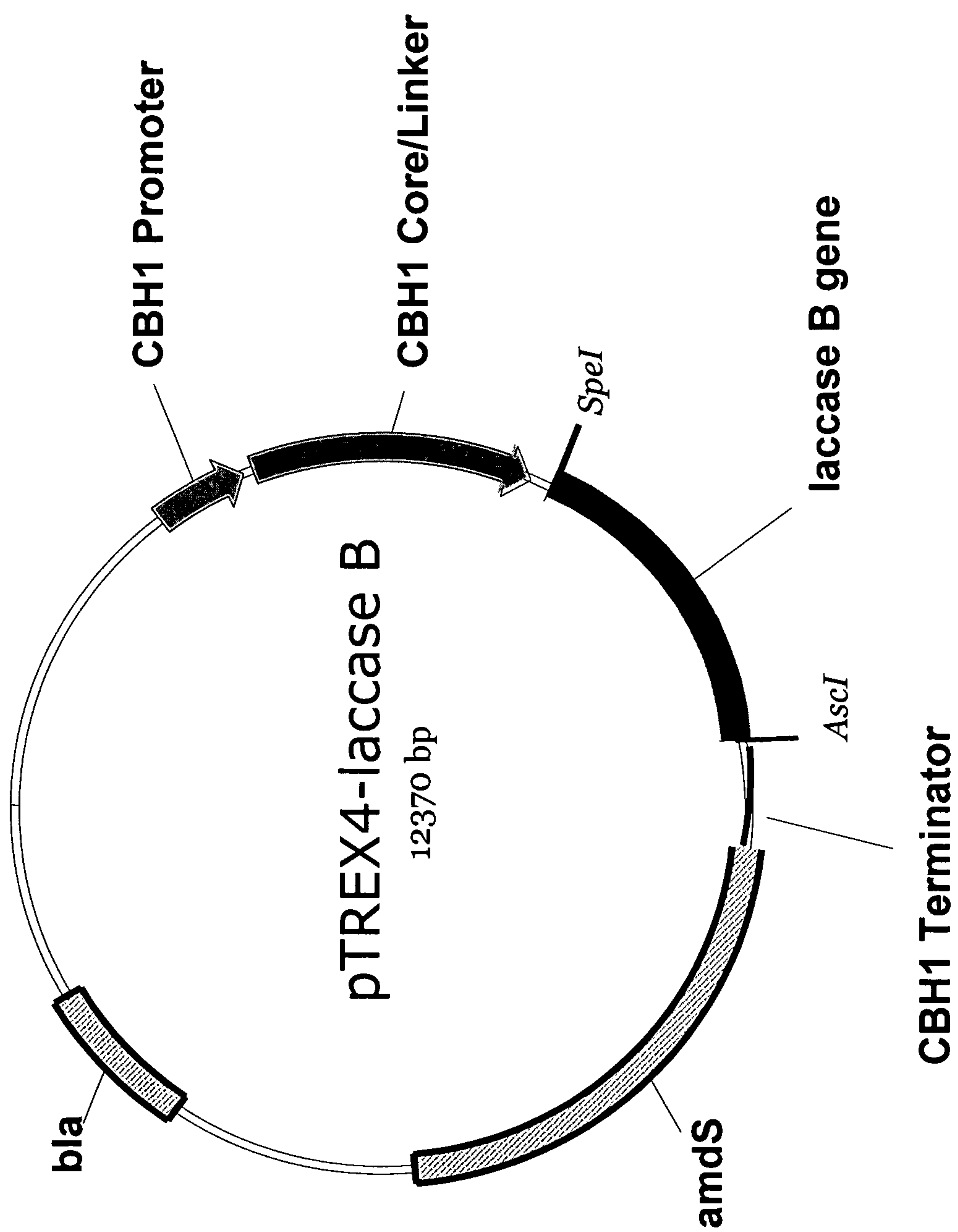
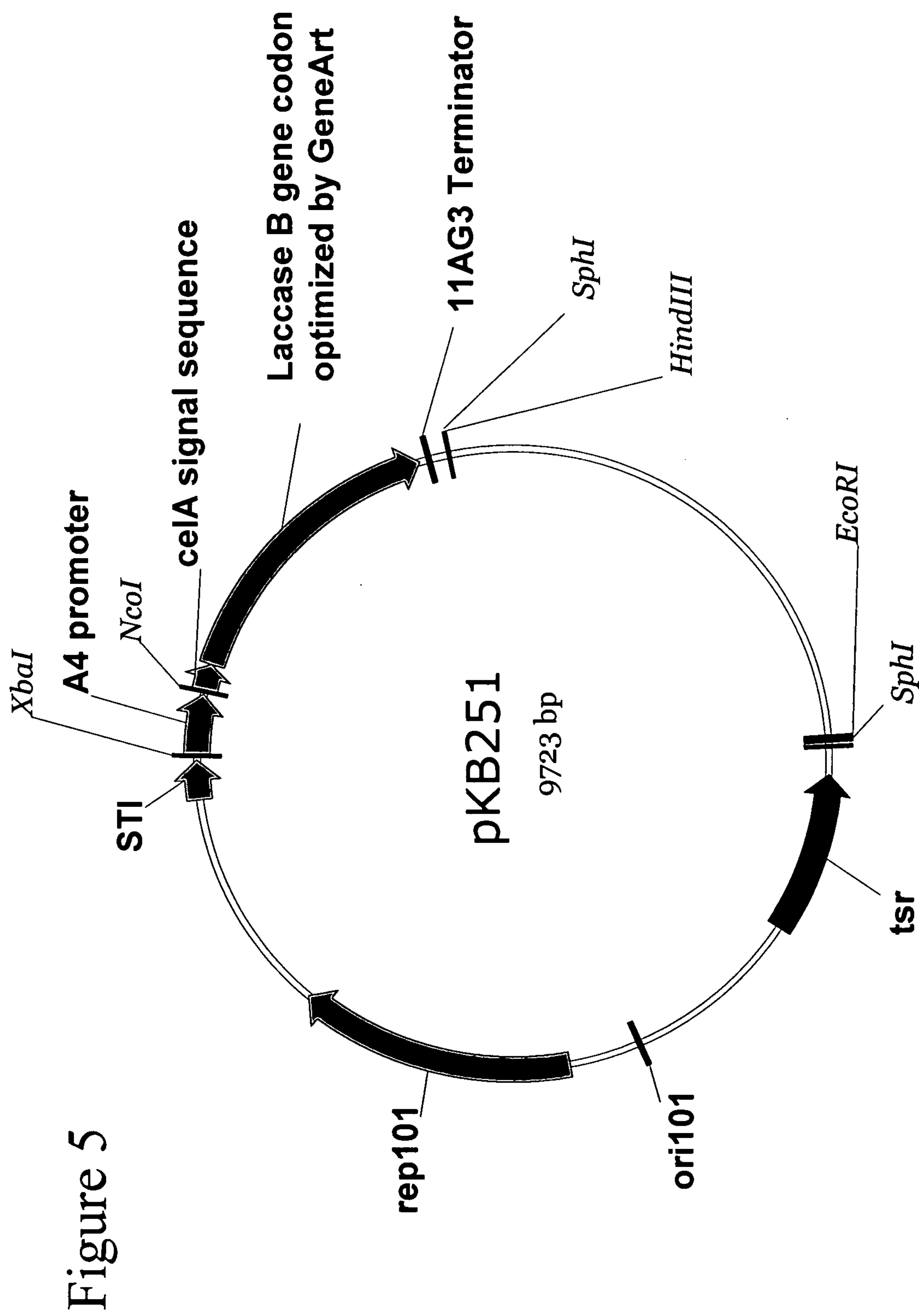


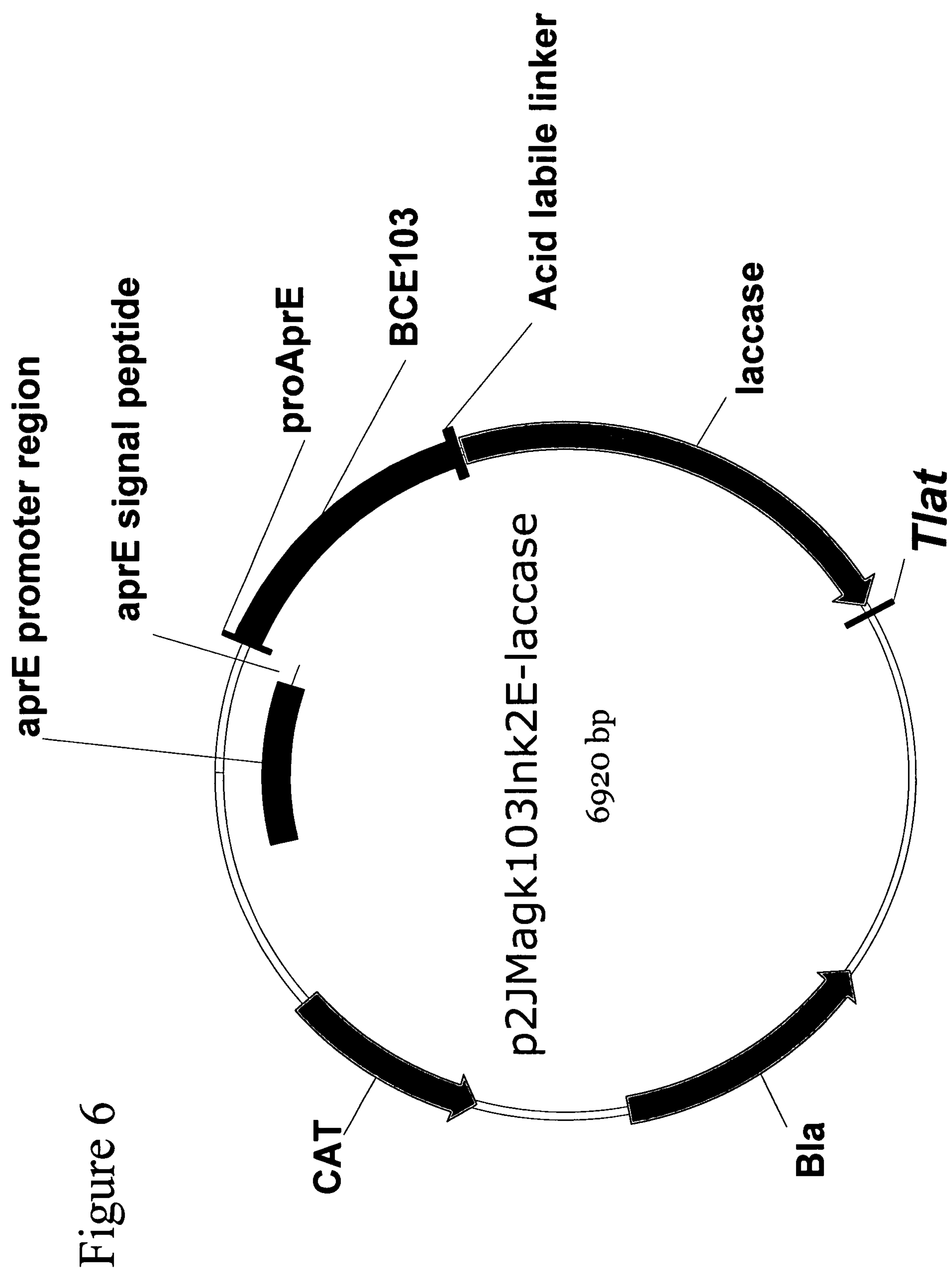
Figure 4



5/10



6/10



7/10

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

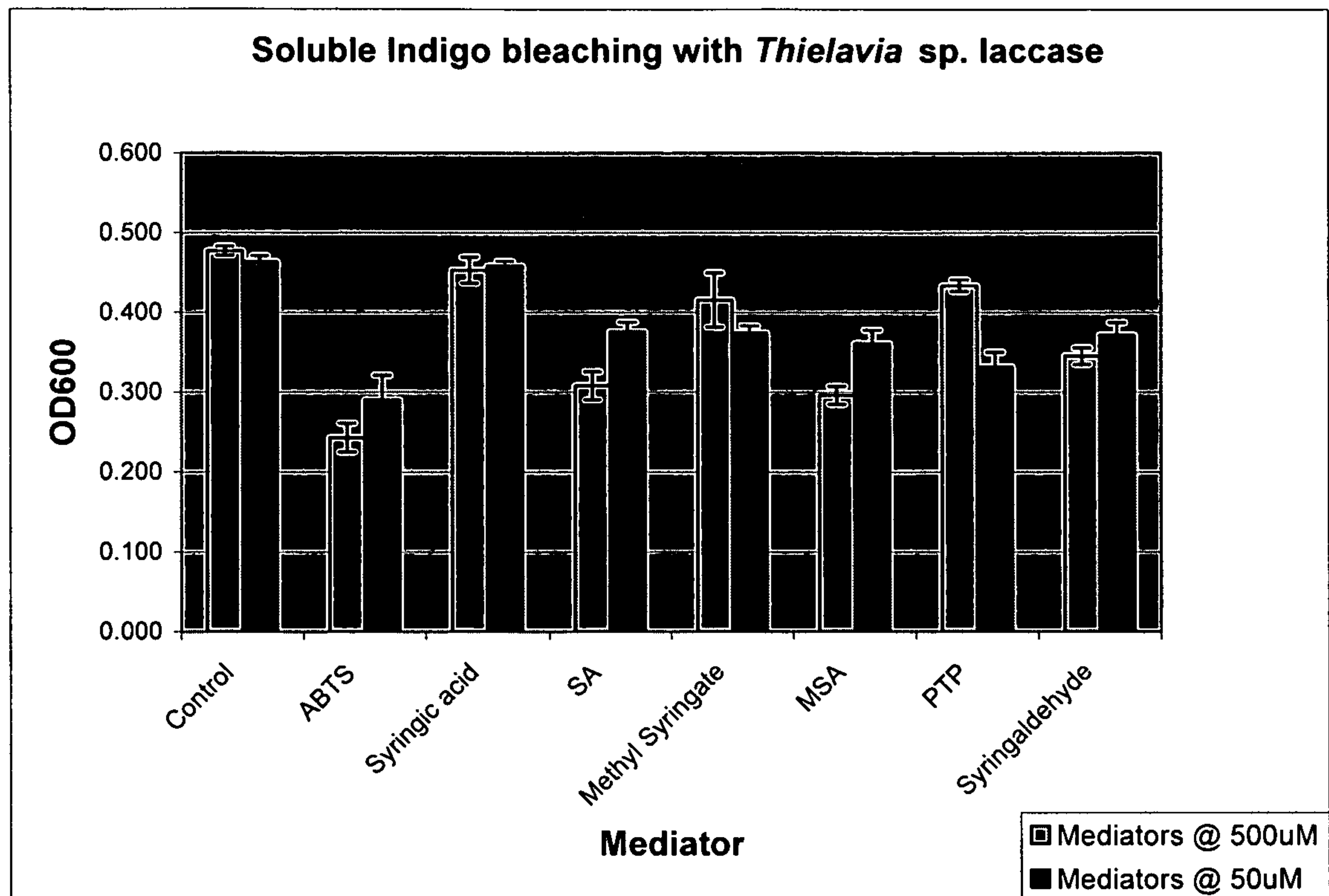


Figure 8. Bleaching of soluble indigo using a *Thielavia*, *Myceliophthora* and *Cerrena* sp. laccase and a variety of mediators at pH 5.

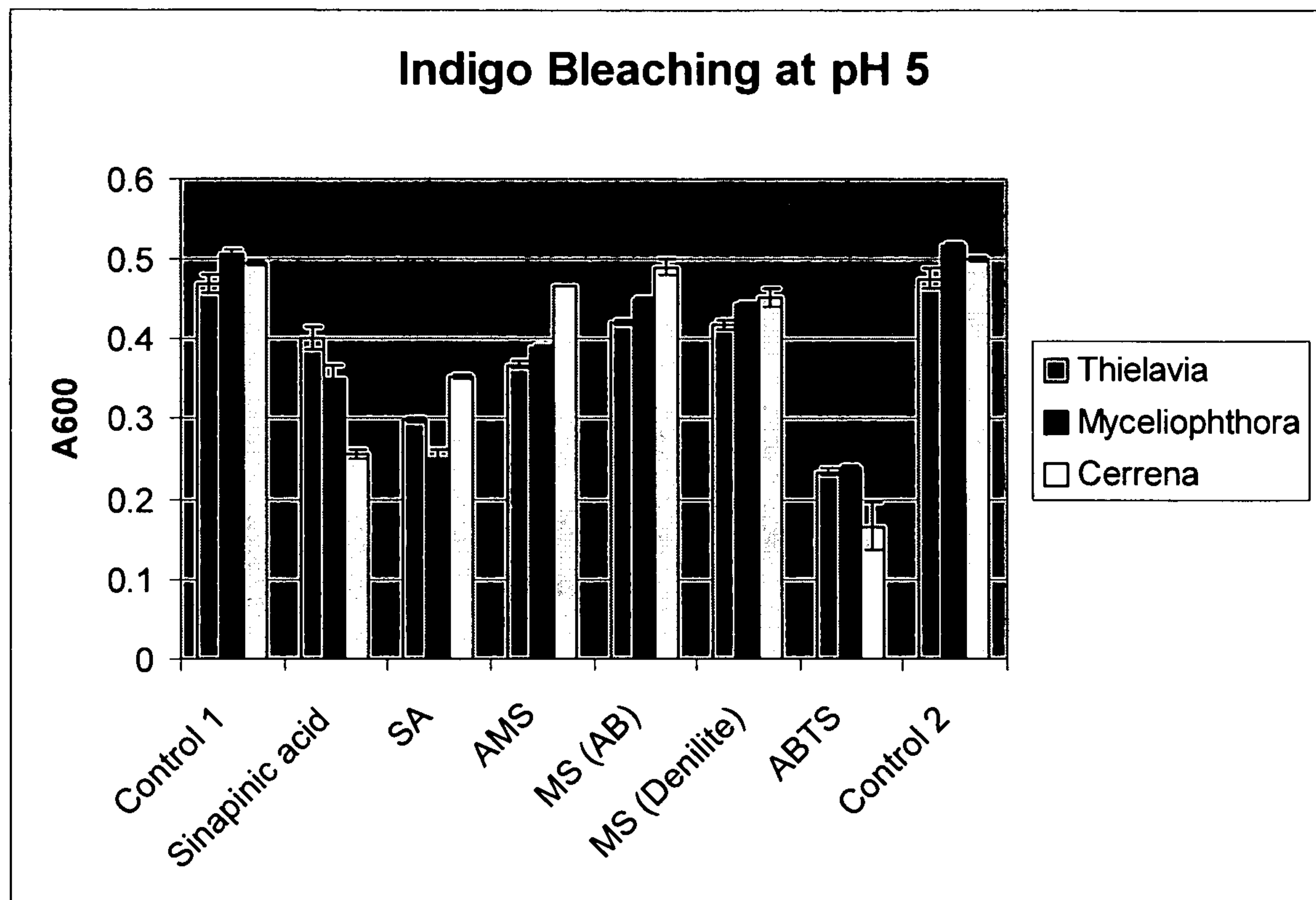
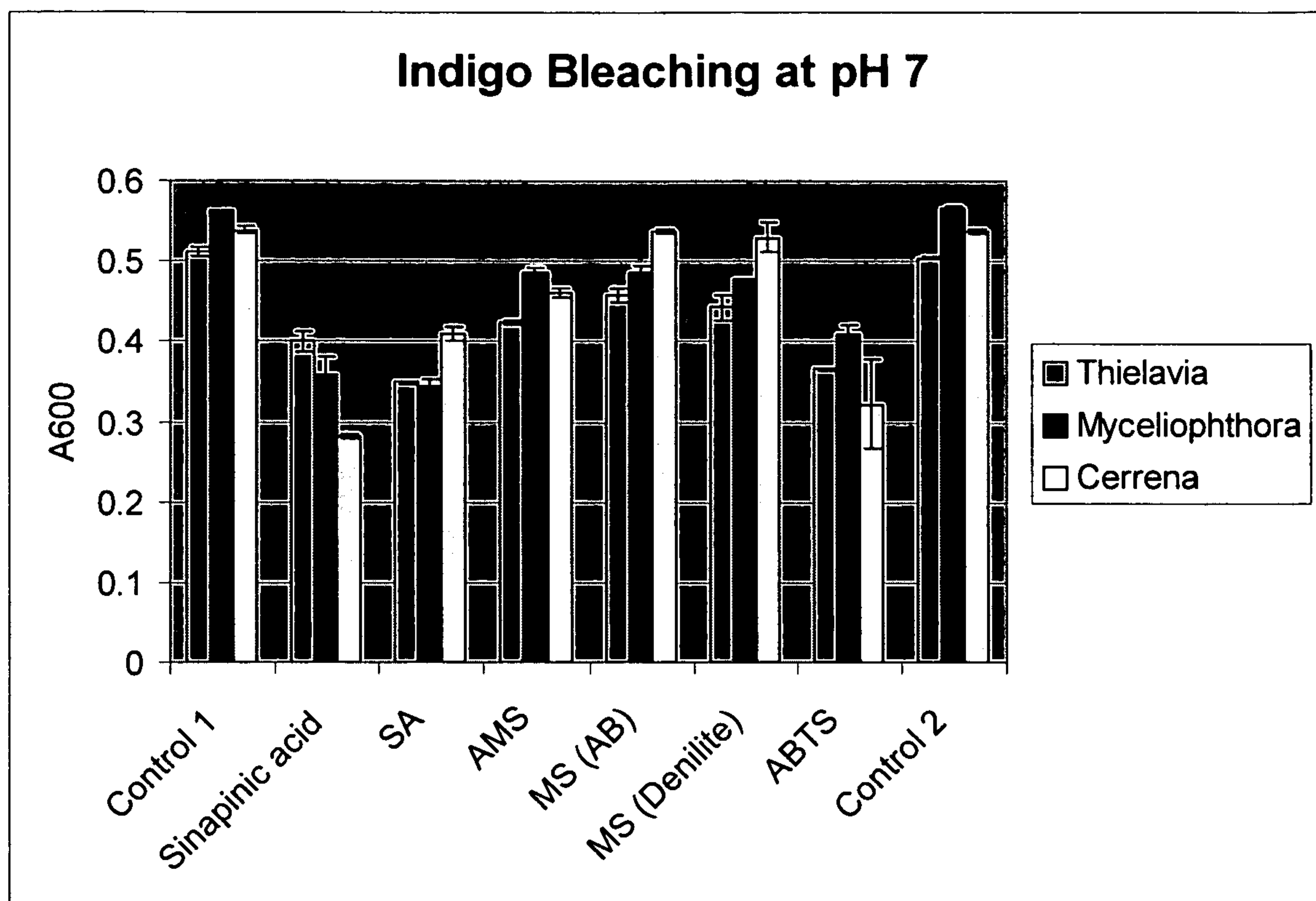


Figure 9. Bleaching of soluble indigo using a *Thielavia*, *Myceliophthora* and *Cerrena* sp. laccase and a variety of mediators at pH 7.



10/10

Figure 10

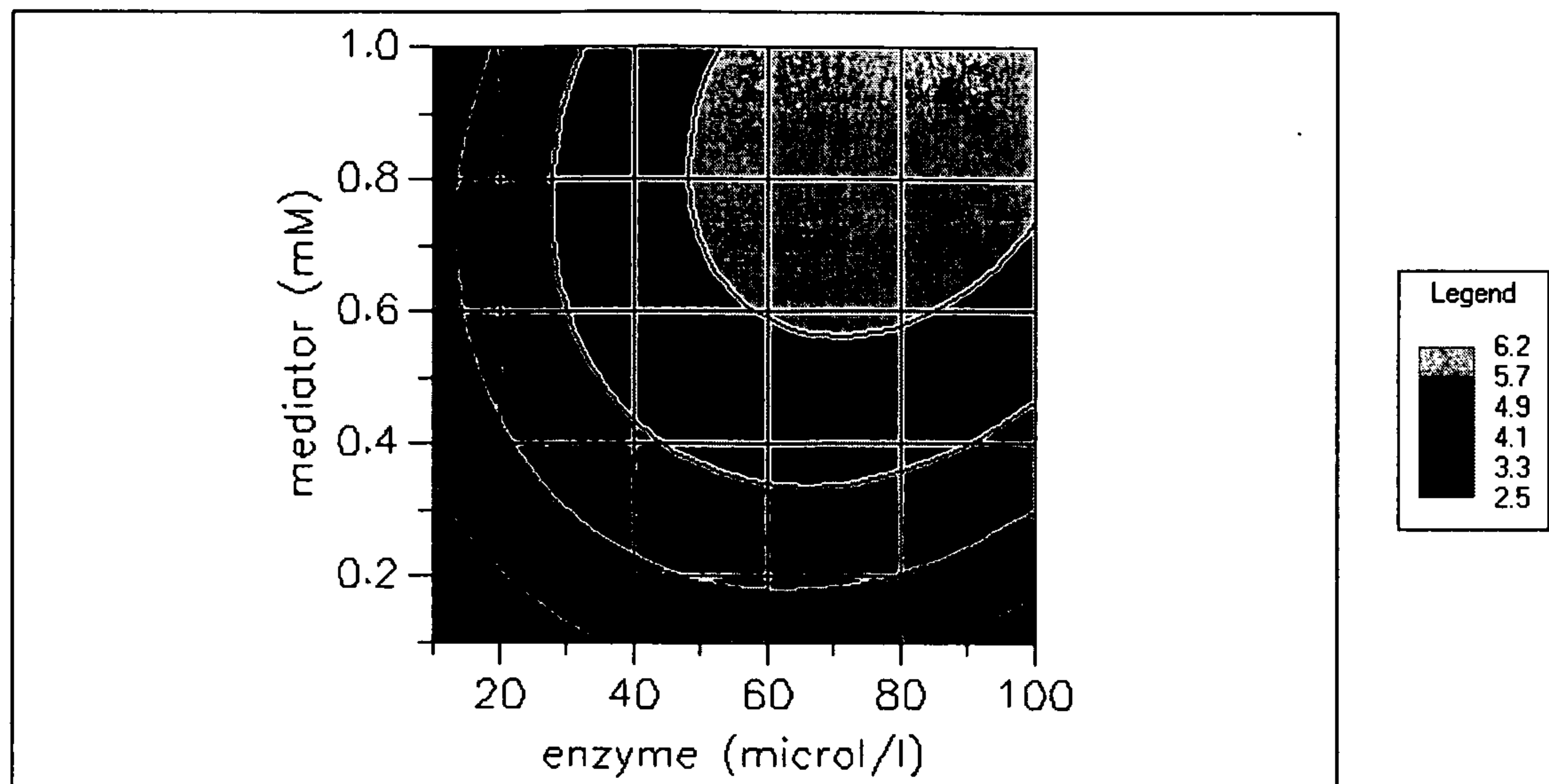


Figure 11

