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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 October 2012 (11.10.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/138474 A1

(51) International Patent Classification:
C12N 9/02 (2006.01)

(21) International Application Number:
PCT/US2012/029786

(22) International Filing Date:
20 March 2012 (20.03.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/472,568 6 April 2011 (06.04.2011) US

(71) **Applicant** *(for all designated States except US)*: **DANISCO US INC.** [US/US]; 925 Page Mill Road, Palo Alto, California 94304 (US).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: **WANG, Huaming** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, California 94304 (US). **WARD, Michael** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, California 94304 (US).

(74) **Agent**: **BACSI, Steven G.**; Danisco US Inc., 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, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, 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, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, 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, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,

[Continued on next page]

(54) Title: LACCASE VARIANTS HAVING INCREASED EXPRESSION AND/OR ACTIVITY

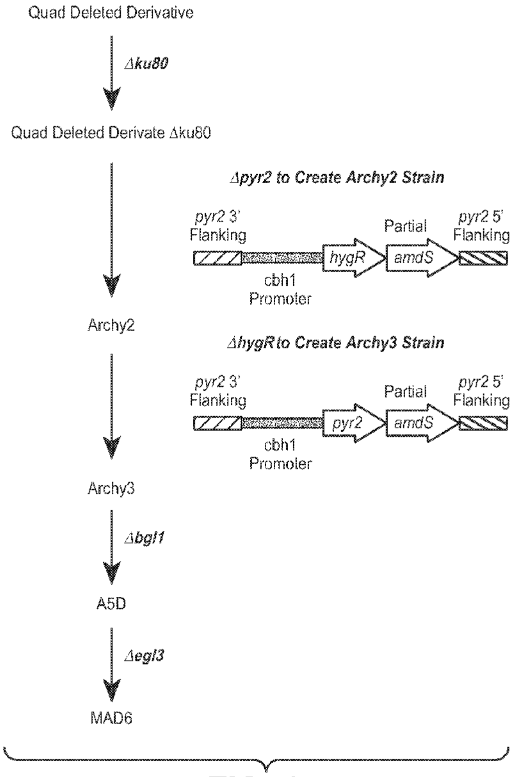


FIG. 1

(57) Abstract: The present compositions, methods, and systems, relating to variant laccase enzymes that demonstrate increased expression and/or activity compared to a reference parental laccase enzyme. The variant enzymes include mutations that affect glycosylation, surface charge, or surface hydrophobicity, resulting in improved enzyme expression and/or enzyme activity.

WO 2012/138474 A1

DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

LACCASE VARIANTS HAVING INCREASED EXPRESSION AND/OR ACTIVITY

PRIORITY

[1] The present application claims priority to U.S. Provisional Application Serial No. 61/472,568, filed on April 6, 2011, which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[2] The present compositions, methods, and systems, relating to variant laccase enzymes that demonstrate increased expression and/or activity compared to a reference parental laccase enzyme. The variant enzymes include mutations that affect glycosylation, surface charge, or surface hydrophobicity, resulting in improved enzyme expression and/or enzyme activity.

BACKGROUND

[3] Laccases are copper-containing phenol oxidizing 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 paper bleaching, treatment of pulp waste water, de-inking, industrial color removal, bleaching in laundry detergents, oral care teeth whiteners, and as catalysts or facilitators for polymerization and oxidation reactions.

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

[5] Laccases are known to be produced by a wide variety of fungi, including species of the genera *Aspergillus, Neurospora, Podospora, Botrytis, Pleurotus, Fornes, Phlebia, Trametes, Polyporus, Stachybotrys, Rhizoctonia, Bipolaris, Curvularia, Amerosporium, Lentinus*, *Myceliophtora*, *Coprinus*, *Thielavia*, *Cerrena*, *Streptomyces*, and *Melanocarpus.* For many applications, the oxidizing efficiency of a laccase can be improved through the use of a mediator, also known as an enhancing agent.

[6] Despite the availability of a wealth of microbial expression systems, laccases are difficult to express in culture at high levels. Laccases with high specific activity can be particularly difficult to express, in some cases at a level less than 1 g/L, presenting an impediment to their large scale production.

SUMMARY

[7] Described are compositions, methods, and systems, relating to variant laccase enzymes that demonstrate increased expression and/or activity compared to a reference parental laccase enzyme.

[8] In one aspect, a variant laccase enzyme derived from a parental laccase enzyme is provided, the variant laccase enzyme having: (a) a mutation at a position corresponding to position 68 of the amino acid sequence of SEQ ID NO: 11; (b) a mutation that alters the surface charge of the parental laccase enzyme; (c) a mutation that alters the surface hydrophobicity of the parental laccase enzyme; and/or (d) a mutation at an amino acid position corresponding to a non-conservative, hydrophobic amino acid residue located on the surface of the parental laccase enzyme; wherein the mutation is a substitution to a different amino acid residue compared to the parental laccase.

[9] In some embodiments, the variant laccase enzyme has a mutation at a position corresponding to position 68 of the amino acid sequence of SEQ ID NO: 11, wherein the mutation is a substitution of an aromatic amino acid residue to a non-aromatic amino acid residue. In some embodiments, the mutation is a substitution of an aromatic amino acid residue to an aliphatic amino acid residue. In some embodiments, the mutation is a substitution of an aromatic amino acid residue to A, V, L, or I. In some embodiments, the mutation is equivalent to F68L in SEQ ID NO: 11.

[10] In some embodiments, the variant laccase enzyme has a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at a position equivalent to position 130, 265, 287, 293, or 319, in SEQ ID NO: 11.

[11] In some embodiments, the variant laccase enzyme has a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at a position equivalent to position 130 in SEQ ID NO: 11.

[12] In some embodiments, the variant laccase enzyme has a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at: (a) an amino acid position equivalent to position 130 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from D, E, R, and K; (b) an amino acid position equivalent to position 265 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from R, H, and V; (c) an amino acid position equivalent to position 287 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from P, H, and G; (d) an amino acid position equivalent to position 293 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from N, T, and S; and/or (e) an amino acid position equivalent to position 319 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from W, T, and S.

[13] In some embodiments, the variant laccase enzyme has mutations equivalent to: (a) I265R/V287G, (b) I265R/V293T; (c) I265R/V319T; (d) I265R/V287G/V319T; (e) I265R/V287G/V293T/V319T; (f) I265R/V287P; (g) I265R/N335R; (h) I265R/N130E; (i) F68L/I265R; (j) F68L/I265R/V287G; (k) F68L/I265R/V293T; (l) F68L/I265R/V319T; (m) F68L/I265R/V287G/V319T; (n) F68L/I265R/V287G/V293T/V319T; (o) F68L/I265R/V287P; (p) F68L/I265R/N335R; or (q) F68L/I265R/N130E; in SEQ ID NO: 11.

[14] In some embodiments, the parental laccase is obtainable from a *Cerrena* species. In some embodiments, the parental laccase is obtainable from *Cerrena unicolor*. In some embodiments, the parental laccase is laccase D from *C. unicolor*.

[15] In some embodiments, the parental laccase has an amino acid sequence selected from the group consiting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, and SEQ ID NO: 28.

[16] In some embodiments, any of the variant laccase enzymes described herein have an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 11. In some embodiments, any of the variant laccase enzymes described herein has an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO: 11. In some embodiments, any of the variant laccase enzymes described herein has an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 11. In some embodiments, any of the variant laccase enzymes described herein has an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 11. In some embodiments, any of the variant laccase enzymes described herein has an amino acid sequence that is at least 96%, at least 97%, at least 98%, or even at least 99% identical to the amino acid sequence of SEQ ID NO: 11.

[17] In some embodiments, any of the variant laccase enzymes described herein further comprises a mutation that introduces a glycosylation site into the amino acid sequence of the parental laccase.

[18] In another aspect, a composition comprising one or more of any of the variant laccase enzymes described herein is provided. In some embodiments, the composition further comprises a chemical mediator. In some embodiments, the chemical mediator is a phenolic compound. In some embodiments, the chemical mediator is a phenolic compound is selected from the group consisting of syringonitrile, acetosyringone, and methyl syringate.

[19] In another aspect, a method of bleaching a surface is provided, comprising contacting the surface with a composition comprising one or more of any of the variant laccase enzymes described herein.

[20] These and other aspects and embodiments of present strains and methods will be apparent from the description, including the accompanying Figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[21] Figure 1 provides a schematic illustrating the derivation of the MAD6 host strain, from the quad-deleted derivative strain.

[22] Figure 2 provides a schematic of the *T. reesei* ku80 deletion cassette.

[23] Figure 3 provides a schematic of the pyr2 deletion cassette used to create the Archy2 strain.

[24] Figure 4 provides a schematic of the hygR deletion cassette used to create the Archy3 strain.

[25] Figure 5 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of seven different laccase glycosylation variants (mut1 to mut7).

[26] Figure 6 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of five different laccase negative charge variants (S1 to S5).

[27] Figure 7 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of four different laccase positive charge variants (S7 to S10).

[28] Figure 8 is a list of thirteen position 265 variants.

[29] Figure 9 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of 88 independent laccase variants obtained from a SEL1 library. The left-most line (ABST ≈ 400) is the wild type control.

[30] Figure 10 a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of six position 265 laccase variants that exhibit increased expression or activity.

[31] Figure 11 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of 88 independent laccase variants obtained from a SEL2 library. The left-most line (ABST ≈ 200) is the wild type control.

[32] Figure 12 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of four different laccase position 287 variants.

[33] Figure 13 is a list of thirteen position 319 variants.

[34] Figure 14 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of 65 independent laccase variants obtained from a SEL3 library. The left-most line (ABST ≈ 270) is the wild type control.

[35] Figure 15 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of four different laccase position 319 variants.

[36] Figure 16 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of sixteen independent laccase variants obtained from a SEL4 library. The left-most line (ABST ≈ 320) is the wild type control.

[37] Figure 17 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of five different laccase variants.

[38] Figure 18 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding one of six different laccase variants.

[39] Figure 19 is a graph showing laccase activity in filamentous fungi transformed with a vector encoding a wt (clones "42") laccase or an F68L/I265R variant laccase (clones "67") and grown in shake flasks.

[40] Figure 20 is an alignment of the amino acid sequences of a number of *Cerrena* laccases. Signal sequences are shown in italics.

[41] Figure 21 is an alignment of the amino acid sequences of a number of laccases from different organisms.

[42] Figure 22 is an amino acid sequence showing the relative location of a number of N-glycosylation mutations (bold), surface charge mutations (bold, underlined), and non-conservative hydrophobic residue mutations (bold, underlined) on a *Cerrena* laccase D amino acid sequence (SEQ ID NO: 11).

DETAILED DESCRIPTION

I. Overview

[43] Described are compositions, methods, and systems, relating to variant laccase enzymes that demonstrate increased expression and/or activity compared to a reference parental laccase enzyme. The variant enzymes include mutations that affect glycosylation, alter the surface charge, alter the surface hydrophobicity, or otherwise alter the biochemical properties of the variant laccase enzymes to improve enzyme expression and/or enzyme activity. Various features and embodiuments of the variants laccases, and applications, thereof, are to be described.

II. Definitions

[44] 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. Singleton *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, N.Y. (1991) provide a general dictionary of many of the terms used herein. The following terms are defined for additional clarity.

[45] As used herein, the term "enzyme" refers to a protein that catalyzes a chemical reaction. The catalytic function of an enzyme constitutes its "enzymatic activity" or "activity." An enzyme is typically classified according to the type of reaction it catalyzes, *e.g.*, oxidation of phenols, hydrolysis of peptide bonds, incorporation of nucleotides, etc.

[46] As used herein, the term "substrate" refers to a substance (*e.g.,* a chemical compound) on which an enzyme performs its catalytic activity to generate a product.

[47] As used herein, a "laccase" is a multi-copper containing oxidase (EC 1.10.3.2) that catalyzes the oxidation of phenols, polyphenols, and anilines by single-electron abstraction, with the concomitant reduction of oxygen to water in a four-electron transfer process.

[48] As used herein, "laccase activity" (or "laccase specific activity") is measured in units/gram (U/g), wherein one unit is defined as the amount of laccase activity required to oxidize 1 nmol of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate; ABTS) substrate per second under conditions of an assay based on the ability of laccase enzyme to oxidize ABTS into its corresponding stable cation radical, *i.e.*, $ABTS^+$. Unlike the initial form of ABTS, the radical form is dark green in color with increased absorbance at 420 nm. The amount of green color formation is proportional to the amount of laccase activity, and can be compared to a laccase standard curve to determine the absolute amount of laccase activty.

[49] As used herein, “expression,” in the context of increased laccase expression, refers to the production of active laccase enzyme molecules in cultured cells.

[50] As used herein, “variant” proteins encompass related and derivative proteins that differ from a parent/reference protein by a small number of amino acid substitutions, insertions, and/or deletions. In some embodiments, the number of different amino acid residues is any of about 1, 2, 3, 4, 5, 10, 20, 25, 30, 35, 40, 45, or 50. In some embodiments, variants differ by about 1 to about 10 amino acids residues. In some embodiments, variant proteins have at least about 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 99.5% amino acid sequence identity to a parent/reference protein.

[51] As used herein, the term “analogous sequence” refers to a polypeptide sequence within a protein that provides a similar function, tertiary structure, and/or conserved residues with respect to a sequence within a parent/reference protein. For example, in structural regions that contain an alpha helix or a beta sheet structure, replacement amino acid residues in an analogous sequence maintain the same structural feature. In some embodiments, analogous sequences result in a variant protein that exhibits a similar or improved function with respect to the parent protein from which the variant is derived.

[52] As used herein, a “homologous protein” or “homolog” refers to a protein (*e.g.*, a laccase enzyme) that has a similar function (*e.g.*, enzymatic activity) and/or structure as a reference protein (*e.g.*, a laccase enzyme from a different source). Homologs may be from evolutionarily related or unrelated species. In some embodiments, a homolog has a quaternary, tertiary and/or primary structure similar to that of a reference protein, thereby potentially allowing for replacement of a segment or fragment in the reference protein with an analogous segment or fragment from the homolog, with reduced disruptiveness of structure and/or function of the reference protein in comparison with replacement of the segment or fragment with a sequence from a non-homologous protein.

[53] As used herein, “wild-type,” “native,” and “naturally-occurring” proteins are those found in nature. The terms “wild-type sequence” refers to an amino acid or nucleic acid sequence that is found in nature or naturally occurring. In some embodiments, a wild-type sequence is the starting point of a protein engineering project, for example, production of variant proteins.

[54] As used herein, a “signal sequence” refers to a sequence of amino acids bound to the N-terminal portion of a protein, and which facilitates the secretion of the mature form of the protein from the cell. The mature form of the extracellular protein lacks the signal sequence which is cleaved off during the secretion process.

[55] As used herein, the term “derivative” refers to a protein that is derived from a parent/reference protein by addition of one or more amino acids to either or both the N- and C-terminal end(s), substitution of one or more amino acid residues at one or a number of different sites in the amino acid sequence, deletion of one or more amino acid residues at either or both ends of the protein or at one or more sites in the amino acid sequence, and/or insertion of one or more amino acids at one or more sites in the amino acid sequence. The preparation of a protein derivative is often achieved by modifying a DNA sequence which encodes for the native protein, transformation of that DNA sequence into a suitable host, and expression of the modified DNA sequence to form the derivative protein.

[56] As used herein, the terms “polypeptide, “protein,” and “peptide,” refer to a composition comprised of amino acids (*i.e.*, amino acid residues). The conventional one-letter or three-letter codes for amino acid residues are used. A polypeptide may be linear or branched, may comprise modified amino acids, and may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art.

[57] As used herein, a “conserved amino acid residue” refers to a residue that is the same at equivalent positions (based on an amino acid sequence alignment) of different laccase enzymes. In contrast, a “non-conserved amino acid residue” refers to a residue that is different at the equivalent positions (based on an amino acid sequence alignment) of different laccase enzymes. By way of example, where numerous laccases have an alanine at position X, alanine is a conserved residue at position X. Where different laccases have different amino acids at position Y, there are a number of non-conserved residues at position Y.

[58] As used herein, “equivalent” amino acid positions/residues are those that are structurally conserved among different laccase enzymes as determined by an amino acid sequence alignment. Such positions/residues can readily be determined using any one of a number of amino acid sequence alignment programs, and then determining which positions/residues "line-up" in different molecules. The language "equivalent to" and "corresponding to" are used interchangeably.

[59] As used herein, the term "textile" refers to fibers, yarns, fabrics, garments, and non-woven materials. The term encompasses textiles made from natural and synthetic (*e.g.*, manufactured) materials, as well as natural and synthetic blends. The term "textile" refers to both unprocessed and processed fibers, yarns, woven or knit fabrics, non-wovens, and garments. In some embodiments, a textile contains cellulose.

[60] As used herein, the term "fabric" refers to a manufactured assembly of fibers and/or yarns that has substantial surface area in relation to its thickness and sufficient cohesion to give the assembly useful mechanical strength.

[61] As used herein, the term "garment" refers to a clothing item made from one or more fabrics. Garments typically include fabrics that are already cut to size and sewn or stitched together. Garments may or may not include buttons, eyelets, straps, zippers, hook-and-loop closures, or other mechanical features, which can be attached before or after localized color modification.

[62] As used herein, the term "color modification" refers to a change in the chroma, saturation, intensity, luminance, and/or tint of a color associated with a fiber, yarn, fabric, garment, or non-woven material, collectively referred to as textile materials. Color modification encompasses chemical modification to a chromophore as well as chemical modification to the material to which a chromophore is attached. Examples of color modification include fading, bleaching, and altering tint. A particular color modification to indigo-dyed denim is fading to a "vintage look," which has a less intense blue/violet tint and more subdued grey appearance than the freshly-dyed denim.

[63] As used herein, the term "local color modification" refers to color modification, as defined, above, that is performed on only a portion of a fabric or garment. Unlike generalized textile color modification, which is typically performed in a bath, *i.e.*, in a submerged environment, local color modification is performed using a wetted but not submerged fabric or garment, typically on a table, work bench, or other hard surface, on a hanging or otherwise suspended fabric or garment, or using rollers or other processing equipment that do not subject the fabric or garment to a submerged environment, such that only a portion of the garment can be subjected to color modification without affecting the remainder of the fabric or garment.

[64] As used herein, "a portion of a fabric or garment" refers to anything less than the whole fabric or garment. Where specified, a portion of a fabric or garment may refer to an indicated structural or decorative feature a fabric or garment, such as a pant leg, a sleeve, a pocket, a belt loop, a cuff, a hem, and the like.

[65] As used herein, the term “bleaching” refers to the process of treating a textile material such as a fiber, yarn, fabric, garment or non-woven material to produce a lighter color. This term includes the production of a brighter and/or whiter textile, *e.g.*, in the context of a textile processing application, as well as lightening of the color of a stain, *e.g.*, in the context of a cleaning application.

[66] As used herein, the terms “size” and “sizing” refer to compounds used in the textile industry to improve weaving performance by increasing the abrasion resistance and strength of a yarn. Size is usually made of starch or starch-like compounds.

[67] As used herein, the terms “desize” and “desizing” refer to the process of eliminating/removing size (generally starch) from a textile, usually prior to applying special finishes, dyes or bleaches.

[68] As used herein, the term “desizing enzyme” refers to an enzyme used to remove size. Exemplary enzymes are amylases, cellulases, and mannanases.

[69] As used herein, the term “% identity” refers to the level of nucleic acid sequence identity between a nucleic acid sequence that encodes a laccase as described herein and another nucleic acid sequence, or the level of amino acid sequence identity between a laccase enzyme as described herein and another amino acid sequence. Alignments may be performed using a conventional sequence alignment program. Exemplary levels of nucleic acid and amino acid sequence identity include, but are not limited to, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, or more, sequence identity to a given sequence, *e.g.*, the coding sequence for a laccase or the amino acid sequence of a laccase, as described herein.

[70] Exemplary computer programs that 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.

[71] 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.)

[72] 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.

[73] As used herein, the terms "chemical mediator" and "mediator" are used interchangeably to refer to a chemical compound that functions as a redox mediator to shuttle electrons between an enzyme exhibiting oxidase activity (*e.g.*, a laccase) and a secondary substrate or electron donor. Such chemical mediators are also known in the art as "enhancers" and "accelerators."

[74] As used herein, the terms "secondary substrate" and "electron donor" are used interchangeably to refer to a dye, pigment (*e.g.*, indigo), chromophore (*e.g.*, polyphenolic, anthocyanin, or carotenoid), or other secondary substrate to and from which electrons can be shuttled by an enzyme exhibiting oxidase activity.

[75] The following abbreviations/acronyms have the following meanings unless otherwise specified:

EC	enzyme commission
EDTA	ethylenediaminetetraacetic acid
kDa	kiloDalton
MW	molecular weight
w/v	weight/volume
w/w	weight/weight
v/v	volume/volume
wt%	weight percent
°C	degrees Centigrade
H_2O	water
dH_2O or DI	deionized water
dIH_2O	deionized water, Milli-Q filtration
g or gm	gram
μg	microgram
mg	milligram
kg	kilogram
μL and μl	microliter
mL and ml	milliliter
mm	millimeter
μm	micrometer
M	molar
mM	millimolar
μM	micromolar

U	unit
sec and ″	second
min and ′	minute
hr	hour
eq.	equivalent
N	normal
RTU	ready-to-use
U	Unit
owg	on weight of goods
CIE	International Commission on Illumination

[76] Numeric ranges are inclusive of the numbers defining the range. The singular articles "a," "an," "the," and the like, include the plural referents unless otherwise clear from context. Unless otherwise specified, polypeptides are written in the standard N-terminal to C-terminal direction and polynucleotides are written in the standard 5′ to 3′ direction. It is to be understood that the particular methodologies, protocols, and reagents described, are not intended to be limiting, as equivalent methods and materials can be used in the practice or testing of the present compositions and methods. Although the description is divided into sections to assist the reader, section heading should not be construed as limiting and the description in one section may apply to another. All publications cited herein are expressly incorporated by reference.

III. Parental laccases

[77] A number of laccase enzymes from microbial and plant origin are known in the art. Exemplary laccases are derived or derivable from a strain of *Aspergillus*, *Neurospora* (*e.g.*, *N. crassa*), *Podospora*, *Botrytis*, *Collybia*, *Cerrena* (*e.g.*, *C. unicolor*), *Stachybotrys*, *Panus* (*e.g.*, *P. rudis*), *Thielavia*, *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 2238885)), *Spongipellis*, *Polyporus*, *Ceriporiopsis subvermispora*, *Ganoderma tsunodae*, and *Trichoderma*.

[78] The following laccases are described in U.S. Patent Publication No. 2008/0196173 and PCT Publication No. WO 2008/076322 (which are incorporated by reference) and are ideal for use use as described:

A. *Cerrena* laccase A1 from CBS115.075 strain (SEQ ID NO: 1):

```
MSSKLLALIT VALVLPLGTD AGIGPVTDLR ITNQDIAPDG FTRPAVLAGG        50
```

```
TFPGALITGQ KGDSFQINVI DELTDASMLT QTSIHWHGFF QKGSAWADGP    100
AFVTQCPIVT GNSFLYDFDV PDQPGTFWYH SHLSTQYCDG LRGPFVVYDP    150
KDPNKRLYDI DNDHTVITLA DWYHVLARTV VGVATPDATL INGLGRSPDG    200
PADAELAVIN VKRGKRYRFR LVSISCDPNY IFSIDNHSMT VIEVDGVNTQ    250
SLTVDSIQIF AGQRYSFVLH ANRPENNYWI RAKPNIGTDT TTDSGMNSAI    300
LRYNGAPVAE PQTVQSPSLT PLLEQNLRPL VYTPVPGNPT PGGADIVHTL    350
DLSFDAGRFS INGASFLDPT VPVLLQILSG TQNAQDLLPP GSVIPLELGK    400
VVELVIPAGV VGGPHPFHLH GHNFWVVRSA GTDQYNFNDA ILRDVVSIGG    450
TGDQVTIRFV TDNPGPWFLH CHIDWHLEAG LAIVFAEGIE NTAASNLTPQ    500
AWDELCPKYN ALSAQKKLNP STT                                 523
```

B. *Cerrena* laccase A2 from CBS154.29 strain (SEQ ID NO: 2):

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

C. *Cerrena* laccase B1 from CBS115.075 strain (SEQ ID NO: 3):

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

D. *Cerrena* laccase B2 from CBS154.29 strain (SEQ ID NO: 4):

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

E. *Cerrena* laccase B3 (partial) from ATCC20013 strain (SEQ ID NO: 5):

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

F. *Cerrena* laccase C (partial) from CBS154.29 strain (SEQ ID NO: 6):

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

G. *Cerrena* laccase D1 from CBS154.29 strain (SEQ ID NO: 7):

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

H. *Cerrena* laccase D2 from CBS115.075 strain (SEQ ID NO: 8):

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

I. *Cerrena* laccase E (partial) from CBS154.29 strain (SEQ ID NO: 9):

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

[79] In some embodiments, a laccase D enzyme having the following amino acid sequence (SEQ ID NO: 10; signal sequence in italics) may be used:

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

[80] The mature processed form of this polypeptide is as follows (SEQ ID NO: 11):

AIGPVADLHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQKGDNFQLNVIDDLTDDRMLTP
TSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGL
RGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTVVGAATPDSTLINGLGRSQTGP
ADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFA
GQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPAQSP
LNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFSINNASFTNPSVPALLQILSGA
QNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAI
LRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAGLAIVFAEGINQTAAANPTPQA
WDELCPKYNGLSASQKVKPKKGTAI

[81] Note that SEQ ID NO: 7 (*Cerrena* laccase D1), SEQ ID NO: 8 (*Cerrena* laccase D2), and SEQ ID NO: 10 (*Cerrena* laccase D) are nearly identical, except for position 8 (where SEQ ID NO: 7 and SEQ ID NO: 8 have Ile and SEQ ID NO: 10 has Leu) and position 254 (where SEQ ID NO: 7 and SEQ ID NO: 10 have Glu and SEQ ID NO: 8 has Asp), using the mature form of the laccase of SEQ ID NO: 10 (*i.e.*, SEQ ID NO: 11) for numbering (see, *e.g.*, Figure 20). These differences do not appear to substantially affect laccase expression or specific activity.

[82] Additional laccases include but are not limited to those shown in the alignment in Figure 21, *i.e.*, *Panus rudis* (SEQ ID NO: 12), *Spongipellis* sp. (SEQ ID NO: 13), *Curiolus versicolor* CVL3 (SEQ ID NO: 14), *Curiolus versicolor* CVL G1 (SEQ ID NO: 15), *Lentinus* sp. (SEQ ID NO: 16), *Ceriporiopsis subvermispora* (SEQ ID NO: 17), *Cyathus bulleri* (SEQ ID NO: 18), *Pycnoporus sanguineus* (SEQ ID NO: 19), *Trametes villosa* (1) and (2) (SEQ ID NOs: 20 and 21, respectively), *Trametes* sp. LCC1 (SEQ ID NO: 22), *Trametes* sp. LCC4 (SEQ ID NO: 23),, *Ganoderma lucidum* (SEQ ID NO: 24), *Curiolus hirsutus* (SEQ ID NO: 25), *Basidiomycete* sp. PM1 (SEQ ID NO: 26), *Rigidoporus microporus* (SEQ ID NO: 27), and *Polyporus ciliatus* (SEQ ID NO: 28). A consensus (or majority) amino acid sequence is shown as SEQ ID NO: 29.

[83] A laccase may be produced by culturing a host cell transformed with a recombinant DNA vector that includes nucleotide sequences encoding the laccase. The DNA vector may further include nucleotide sequences permitting the expression of the laccase in a culture medium, and optionally allowing the recovery of the laccase from the culture.

[84] An expression vector containing a polynucleotide sequence encoding a laccase enzyme may be transformed into a suitable host cell. The host cell may be a fungal cell, such as a filamentous fungal cell, examples of which include but are not limited to species of *Trichoderma* [*e.g.*, *T. reesei* (previously classified as *T. longibrachiatum* and currently also known as *Hypocrea jecorina*], *T. viride*, *T. koningii*, and *T. harzianum*), *Aspergillus* (*e.g.*, *A. niger*, *A. nidulans*, *A. oryzae*, and *A. awamori*), *Penicillium*, *Humicola* (*e.g.*, *H. insolens* and *H. grisea*), *Fusarium* (*e.g.*, *F. graminum* and *F. venenatum*), *Neurospora*, *Hypocrea*, and *Mucor*. A host cell for expression of a laccase enzyme may also be from a species of *Cerrena* (*e.g.*, *C. unicolor*). Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall using techniques known in the art.

[85] Alternatively, the host organism may from a species of bacterium, such as *Bacillus* [*e.g.*, *B. subtilis*, *B. licheniformis*, *B. lentus*, *B.* (now *Geobacillus*) *stearothermophilus*, and *B. brevis*], *Pseudomonas, Streptomyces* (*e.g.*, *S. coelicolor*, *S. lividans*), or *E. coli*. The transformation of bacterial cells may be performed according to conventional methods, *e.g.*, as described in Maniatis, T. *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. supra*).

[86] The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells. In some embodiments, the expressed enzyme is secreted into the culture medium and may be recovered therefrom by well-known procedures. For example, laccases may be recovered from a culture medium as described in U.S. Patent Publication No. 2008/0196173. In some embodiments, the enzyme is expressed intracellularly and is recovered following disruption of the cell membrane.

[87] In particular embodiments, the expression host may be *Trichoderma reesei* with the laccase coding region under the control of a CBH1 promoter and terminator (see, *e.g.*, U.S. Patent No. 5,861,271). The expression vector may be, *e.g.*, pTrex3g, as disclosed in U.S.

Patent No. 7,413,887. In some embodiments, laccases are expressed as described in U.S. Patent Publication Nos. 2008/0196173 or 2009/0221030.

[88] In some embodiments, laccase enzymes suitable for use in the present compositions and methods are mature polypeptides that lack a signal sequence that may be used to direct secretion of a full-length polypeptide from a cell.

[89] A suitable mature polypeptide may have at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, or more, amino acid sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, and SEQ ID NO: 28. Preferably, such polypeptides have enzymatic laccase activity, as determined using the assays and procedures described, herein.

[90] In some embodiments, laccase enzymes suitable for use in the present compositions and methods are truncated with respect to a full-length or mature parent/reference sequence. Such truncated polypeptides may be generated by the proteolytic degradation of a full-length or mature polypeptide sequence or by engineering a polynucleotide to encode a truncated polypeptide. Exemplary polypeptides are truncated at the amino and/or carboxyl-terminus with respect to an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, and SEQ ID NO: 28. The truncation may be of a small number, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues, or of entire structural or functional domains. A suitable truncated polypeptide may have at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, or more, amino acid sequence identity to the corresponding portion of one or more of the above-references amino acid sequences.

Preferably, such polypeptides have enzymatic laccase activity, as determined using the assays and procedures described, herein.

IV. Variant laccases

[91] The present compositions, methods, and systems feature a variant laccase demonstrating increased expression and/or specific activity compared to the parental laccase from which it is derived, for example, the parental laccase of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, or SEQ ID NO: 28.

[92] In some embodiments, the variant laccase include mutations that introduce a glycosylation site into the laccase amino acid sequence. The glycosylation site is preferrably on the surface of the laccase enzyme. In some embodiments, the mutation introduces an N-glycosylation site [*i.e.*, the amino acid sequence Asn-Xaa-Thr/Ser (N-X-T/S), where X is any amino acid residue except proline] on the surface of the laccase enzyme. N-glycosylation sites may be introduced into an amino acid sequence by introducing an Asn residue in the correct context, by changing the context of an existing Asn residue, or both. In some cases, a single amino acid mutation is sufficient to introduce an N-glycosylation site. In other cases, two (or even three) amino acid mutations are required to introduce an N-glycosylation site. In some embodiments, an N-glycosylation site is introduced at one, two, three, or more positions equivalent to positions 12, 28, 47, 157, 317, 362, and 492 of the laccase amino acid sequence exemplified by SEQ ID NO: 11.

[93] In particular embodiments, an N-glycosylation site is introduced at a position equivalent to position 12 of SEQ ID NO: 11, *e.g.*, by changing the amino acid sequence NKD to NAT (or NAS, N-V/L/I-T/S) at positions equivalent to positions 12 to 14. In particular embodiments, an N-glycosylation site is introduced at a position equivalent to position 28 of SEQ ID NO: 11, *e.g.*, by changing the amino acid sequence GGT to NGT at positions equivalent to positions 28 to 30. In particular embodiments, an N-glycosylation site is introduced at a position equivalent to position 47 of SEQ ID NO: 11, *e.g.*, by changing the amino acid sequence NVI to NVT (or NVS) at positions equivalent to positions 47 to 49. In particular embodiments, an N-glycosylation site is introduced at a position equivalent to position 157 of SEQ ID NO: 11, *e.g.*, by changing the amino acid sequence QTV to NTT (or

NTS) at positions equivalent to positions 157 to 159. In particular embodiments, an N-glycosylation site is introduced at a position equivalent to position 317 of SEQ ID NO: 11, *e.g.*, by changing the amino acid sequence NAV to NAT (or NAS) at positions equivalent to positions 317 to 319. In particular embodiments, an N-glycosylation site is introduced at a position equivalent to position 362 of SEQ ID NO: 11, *e.g.*, by changing the amino acid sequence NAQ to NAS (or NAT) at residues 362 to 364. In particular embodiments, an N-glycosylation site is introduced at a position equivalent to position 492 of SEQ ID NO: 11, *e.g.*, by changing the amino acid sequence SAS to NAS at positions equivalent to positions 492 to 494. Ser and Thr are substitutable in the glycosylation site and are unlikely to affect laccase structure or function. Conservative substitutions in the "X" position are generally also substitutable.

[94] The amino acid sequences of exemplary glycosylation variants derived from *C. unicolor* laccase D (SEQ ID NO: 11) are shown, below.

NKD to NAT at residues 12 to 14 (G*12, variant mut1; SEQ ID NO: 30):

AIGPVADLHIV**NAT**LAPDGVQRPTVLAGGTFPGTLITGQKGDNFQLNVIDDLTDDRMLTP
TSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGL
RGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTVVGAATPDSTLINGLGRSQTGP
ADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFA
GQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPAQSP
LNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFSINNASFTNPSVPALLQILSGA
QNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAI
LRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAGLAIVFAEGINQTAAANPTPQA
WDELCPKYNGLSASQKVKPKKGTAI

GGT to NGT at residues 28 to 30 (G*28; variant mut2; SEQ ID NO: 31):

AIGPVADLHIVNKDLAPDGVQRPTVLA**NGT**FPGTLITGQKGDNFQLNVIDDLTDDRMLTP
TSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGL
RGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTVVGAATPDSTLINGLGRSQTGP
ADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFA
GQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPAQSP
LNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFSINNASFTNPSVPALLQILSGA
QNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAI
LRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAGLAIVFAEGINQTAAANPTPQA
WDELCPKYNGLSASQKVKPKKGTAI

NVI to NVT at residues 47 to 49 (G*47; variant mut3; SEQ ID NO: 32):

AIGPVADLHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQKGDNFQL**NVT**DDLTDDRMLTP
TSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGL
RGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTVVGAATPDSTLINGLGRSQTGP

ADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFA
GQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPAQSP
LNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFSINNASFTNPSVPALLQILSGA
QNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAI
LRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAGLAIVFAEGINQTAAANPTPQA
WDELCPKYNGLSASQKVKPKKGTAI

QTV to NTT at residues 157 to 159 (N*157; variant mut4; SEQ ID NO: 33):

AIGPVADLHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQKGDNFQLNVIDDLTDDRMLTP
TSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGL
RGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLA**NTT**VGAATPDSTLINGLGRSQTGP
ADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFA
GQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPAQSP
LNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFSINNASFTNPSVPALLQILSGA
QNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAI
LRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAGLAIVFAEGINQTAAANPTPQA
WDELCPKYNGLSASQKVKPKKGTAI

NAV to NAT at residues 317 to 319 (N*317; variant mut5; SEQ ID NO: 34):

AIGPVADLHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQKGDNFQLNVIDDLTDDRMLTP
TSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGL
RGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTVVGAATPDSTLINGLGRSQTGP
ADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFA
GQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPAQSP
LNEADLRPLVPAPVPG**NAT**PGGADINHRLNLTFSNGLFSINNASFTNPSVPALLQILSGA
QNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAI
LRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAGLAIVFAEGINQTAAANPTPQA
WDELCPKYNGLSASQKVKPKKGTAI

NAQ to NAS at residues 362 to 364 (N*362; variant mut6; SEQ ID NO: 35):

AIGPVADLHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQKGDNFQLNVIDDLTDDRMLTP
TSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGL
RGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTVVGAATPDSTLINGLGRSQTGP
ADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFA
GQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPAQSP
LNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFSINNASFTNPSVPALLQILSGA
Q**NAS**DLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAI
LRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAGLAIVFAEGINQTAAANPTPQA
WDELCPKYNGLSASQKVKPKKGTAI

SAS to NAS at residues 492 to 494 (N*492; variant mut7; SEQ ID NO: 36):

AIGPVADLHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQKGDNFQLNVIDDLTDDRMLTP
TSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGL
RGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTVVGAATPDSTLINGLGRSQTGP
ADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFA
GQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPAQSP
LNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFSINNASFTNPSVPALLQILSGA

```
QNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAI
LRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAGLAIVFAEGINQTAAANPTPQA
WDELCPKYNGLNASQKVKPKKGTAI
```

[95] In some embodiments, the variant laccase include mutations that alter the surface charge of the laccase enzyme. In some embodiments, the variant includes a mutation at an amino acid position corresponding to position 130 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue. In some embodiments, the mutation introduces a negative charge at this position. The amino acid in the parental laccase may be N, which as shown in Figures 20 and 21 is the consensus amino acid residue at this position, based on an alignment of a number of laccases, but may be a different residue, such as K, Q, D, V, or A. In some embodiments, the substituting residue is D, E, R, or K. In some embodiments, the substituting residue is D or E. In particular embodiments, the mutation corresponds to N130E. In some embodiments, the variant includes a mutation at an amino acid position corresponding to position 335 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue. In some embodiments, the mutation introduces a negative charge at this position. The amino acid in the parental laccase may be N, or a different residue, such as A, P, S, or G. In particular embodiments, the substituting residue is D, E, R, or K. In particular embodiments, the substituting residue is R or K. In particular embodiments, the mutation corresponds to N335R.

[96] In some embodiments, the variant laccase includes a mutation at an amino acid position corresponding to a non-conservative, hydrophobic amino acid residue located on the surface of *Cerrena* laccase. In some embodiments, the variant includes a mutation at an amino acid position corresponding to position 265, 287, 293, or 319, in SEQ ID NO: 11.

[97] In some embodiments, the variant includes a mutation at an amino acid position corresponding to position 265 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue. In some embodiments, the amino acid in the parental laccase is a small aliphatic amino acid residue. In some embodiments, the amino acid in an aromatic amino acid residue. In some embodiments, the amino acid in the parental laccase is L, V, F, T, N, S, or P. In some embodiments, the amino acid in the parental laccase is I. In some embodiments, the substituting residue is R, H, V, K, I, or L. In particular embodiments, the mutation corresponds to I265R/H/V/K/I/L. In particular embodiments, the mutation corresponds to I265R, I265H, or I265V.

[98] In some embodiments, the variant includes a mutation at an amino acid position corresponding to position 287 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue. In some embodiments, the amino acid in the parental laccase is a small aliphatic amino acid residue. In some embodiments, the amino acid in the parental laccase is V, A, I, D, E, or P. In some embodiments, the amino acid in the parental laccase is V. In some embodiments, the substituting residue is P, H, or G. In particular embodiments, the mutation corresponds to V287P/H/G. In particular embodiments, the mutation corresponds to V287P, V287H, or V287G.

[99] In some embodiments, the variant includes a mutation at an amino acid position corresponding to position 293 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue. In some embodiments, the amino acid in the parental laccase is a small aliphatic amino acid residue. In some embodiments, the amino acid in the parental laccase is V, I, A, D, T, or N. In some embodiments, the amino acid in the parental laccase is V. In some embodiments, the substituting residue is N, T, or S. In particular embodiments, the mutation corresponds to V293N/T/S. In particular embodiments, the mutation corresponds to V293N or V293T.

[100] In some embodiments, the variant includes a mutation at an amino acid position corresponding to position 319 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue. In some embodiments, the amino acid in the parental laccase is a small aliphatic amino acid residue. In some embodiments, the amino acid in the parental laccase is V, G, F, T, N, or Q. In some embodiments, the amino acid in the parental laccase is V. In some embodiments, the substituting residue is W, T, or S. In particular embodiments, the mutation corresponds to V319W/T/S. In particular embodiments, the mutation corresponds to V319W or V319T.

[101] In some embodiments, the variant laccase includes a mutation at an amino acid position in or near the active site of the enzyme. In some embodiments, the variant includes a mutation at an amino acid position corresponding to position 68 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue. In some embodiments, the amino acid residue in the parental laccase is an aromatic amino acid residue, including or not limited to F, Y, W, or H. In some embodiments, the substitution is to a non-aromatic amino acid residue, *i.e.*, A, V, L, I, G, M, S, T, D, E, N, Q, R, K, C, or P. In some embodiments, the substitution is to an aliphatic amino acid residue, *i.e.*, A, V, L, I. In particular embodiments, the mutation corresponds to F68L.

[102] In some embodiments, the variant includes a plurality of mutations at amino acid positions corresponding to positions 68, 130, 265, 287, 293, 319, and/or 335 in SEQ ID NO: 11, which may be combined with a mutation that introduces an N-glycosylation site on the surface of the laccase enzyme. Figure 22 shows the location of all the aforementioned mutations with reference to SEQ ID NO: 11; wherein exemplary N-glycosylation mutations are shown in bold, and surface charge and non-conservative hydrophobic residue mutations are shown in bold and underlined. In some embodiments, the variant includes a plurality of mutations at amino acid positions corresponding to positions 287, 293, and or 319 in SEQ ID NO: 11.

In some embodiments, the variant includes a plurality of mutations corresponding to V287G, V287P, V293T, and V319T in SEQ ID NO: 11. Exemplary combinations of mutations are as follows: I265R/V287G, I265R/V293T, I265R/V319T, I265R/V287G/V319T, I265R/V287G/V293T/V319T, I265R/V287P, I265R/N335R, I265R/N130E, F68L/I265R, F68L/I265R/V287G, F68L/I265R/V293T, F68L/I265R/V319T, F68L/I265R/V287G/V319T, F68L/I265R/V287G/V293T/V319T, F68L/I265R/V287P, F68L/I265R/N335R, and F68L/I265R/N130E.

[103] Equivalents and variation on these mutations will be apparent to the skilled person in view of the present description.

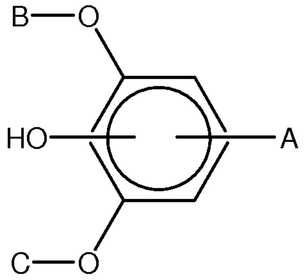
V. Mediators

[104] In some embodiments, the present laccase enzyme systems, compositions, and methods, further include one or more chemical mediator agents that enhance the activity of the laccase enzyme. A mediator (also called an enhancer or accelerator) is a chemical that acts as a redox mediator to effectively shuttle electrons between the enzyme exhibiting oxidase activity and a dye, pigment (*e.g.*, indigo), chromophore (*e.g.*, polyphenolic, anthocyanin, or carotenoid, for example, in a colored stain), or other secondary substrate or electron donor.

[105] In some embodiments the chemical mediator is a phenolic compound, for example, methyl syringate, or a related compound, as described in, *e.g.*, PCT Application Nos. WO 95/01426 and WO 96/12845. The 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 mediator may also be a phenoxazine/phenothiazine compound, for example, phenothiazine-10-propionate. The 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 PCT Application No. WO 95/01426, which are known to enhance the activity of a laccase. The mediator may also be acetosyringone, methyl syringate, ethyl syringate, propyl syringate, butyl syringate, hexyl syringate, or octyl syringate.

[106] In some embodiments, 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.

[107] In some embodiments, the mediator is described by the following formula:



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

[108] In some embodiments, A in the above mentioned formula is –CN or –CO–E, wherein E may be –H, –OH, –R, –OR, or -NXY, where X and Y are independently selected from –H, -OH, -OR, and –R, where R is a C_1 -C_{16} alkyl, preferably a C_1 -C_8 alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C are independently selected from $C_m H_{2m+1}$; $1 \leq m \leq 5$. In some embodiments, the mediator is 4-hydroxy-3,5-dimethoxybenzonitrile (also referred to as "syringonitrile" or "SN").

[109] Note that in the above mentioned formula, A may be placed *meta* to the hydroxy group, instead of being placed in the *para* position as shown.

[110] For applications such as textile processing, the mediator may be present in a concentration of about 0.005 to about 1,000 μmole per g denim, about 0.05 to about 500 μmole per g denim, about 0.1 to about 100 μmole per g denim, about 1 to about 50 μmole per g denim, or about 2 to about 20 μmole per g denim.

[111] The mediators may be prepared by methods known to the skilled artisan, such as those disclosed in PCT Application Nos. WO 97/11217 and WO 96/12845 and U.S. Patent No. 5,752,980. Other suitable mediators are described in, *e.g.*, U.S. Patent Publication No. 2008/0189871.

VI. Utility

[112] Industrial applications of laccases include bleaching and delignification of pulp and paper, deinking waste paper, textile color modification, decolorizing dyes, waste water treatment, depolymerization of high molecular weight aggregates, polymerization of aromatic compounds, radical mediated polymerization and cross-linking reactions (*e.g.*, paints, coatings, biomaterials), activation of dyes, coupling of organic compounds, processing animal hides (*e.g.*, de-hairing, liming, bating and/or tanning), keratinous fiber dyeing (*e.g.*, hair and wool), in food or feed preparation or processing, or as an active ingredient in food or feed, and use in cleaning compositions, including detergent compositions, and generally for cleaning, disinfecting, decontaminating, and sanitizing. Additional uses are described in, *e.g.*, U.S. Patent Publication No. 2008/0196173 and PCT Publication No. WO 2008/076322, which are incorporated by reference.

[113] Particular textile applications include but are not limited to the treatment, processing, finishing, polishing, or production of fibers, yarns, fabrics, or garments, bleaching work-up processes, decolorizing of dye wastes, color modification (including by limited to bleaching) of dyed textiles, and the like. Color modification may be general (*i.e.*, applied to an entire fabric or garment) or local (*i.e.*, applied to only a portion of a fabric or textile). In some cases, it may be desirable to perform color modification simultaneously or sequencially with other enzymatic processing steps, such as abrading (*e.g.*, using cellulases). Numerous uses are described in, *e.g.*, U.S. Patent Publication No. 2008/0196173 and PCT Publication No. WO 2008/076322, which are incorporated by reference.

[114] The present laccase enzymes can be used to decolorize any dye that can be decolorized using a laccase enzyme. Examples of such dyes include, but are not limited to, azo, monoazo, disazo, nitro, xanthene, quinoline, anthroquinone, triarylmethane, paraazoanyline, azineoxazine, stilbene, aniline, and phtalocyanine dyes, or mixtures thereof. In some embodiments, the dye is an azo dye (*e.g.*, Reactive Black 5 (2,7-naphthalenedisulfonic acid, 4-amino-5- hydroxy-3,6-bis((4-((2-(sulfooxy)ethyl)sulfonyl)phenyl)azo)-tetrasodium salt), Reactive Violet 5, methyl yellow, congo red). In some embodiments, the dye is an anthraquinone dye (*e.g.*, remazol blue), indigo (indigo carmine), or a triarylmethane/paraazoanyline dye (*e.g.*, crystal violet, malachite green). In various embodiments, the dye is a reactive, direct, disperse, or pigment dye. In some embodiments, the dye is comprised within an ink. In some embodiments, the dye is indigo and/or a sulfur-based dye. In some embodiments, the textile is denim dyed with indigo and/or a sulfur-based dye. In a particular embodiment, the textile is dyed with indigo, and the laccase enzyme and mediator are used to oxidize the indigo to isatin.

[115] Various aspects and embodiments of the present compositions and methods are further described in the following numbered paragraphs:

1. A variant laccase enzyme derived from a parental laccase enzyme is provided, the variant laccase enzyme having:

(a) a mutation at a position corresponding to position 68 of the amino acid sequence of SEQ ID NO: 11;

(b) a mutation that alters the surface charge of the parental laccase enzyme;

(c) a mutation that alters the surface hydrophobicity of the parental laccase enzyme; or

(d) a mutation at an amino acid position corresponding to a non-conservative, hydrophobic amino acid residue located on the surface of the parental laccase enzyme;

wherein the mutation is a substitution to a different amino acid residue compared to the parental laccase.

2. In some embodiments the variant laccase enzyme of paragraph 1 has a mutation at a position corresponding to position 68 of the amino acid sequence of SEQ ID NO: 11, wherein the mutation is a substitution of an aromatic amino acid residue to a non-aromatic amino acid residue.

3. In some embodiments, the variant laccase enzyme of paragraph 2 has a substitution of an aromatic amino acid residue to an aliphatic amino acid residue.

4. In some embodiments, the variant laccase enzyme of paragraph 3 has a substitution of an aromatic amino acid residue to A, V, L, or I.

5. In some embodiments, the variant laccase enzyme of paragraph 4 has a mutation equivalent to F68L in SEQ ID NO: 11.

6. In some embodiments, the variant laccase enzyme of paragraph 1 has a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at a position equivalent to position 130, 265, 287, 293, or 319, in SEQ ID NO: 11.

7. In some embodiments, the variant laccase enzyme of paragraph 1 has a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at a position equivalent to position 130 in SEQ ID NO: 11.

8. In some embodiments, the variant laccase enzyme of paragraph 1 has a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at:

(a) an amino acid position equivalent to position 130 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from D, E, R, and K;

(b) an amino acid position equivalent to position 265 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from R, H, and V;

(c) an amino acid position equivalent to position 287 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from P, H, and G;

(d) an amino acid position equivalent to position 293 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from N, T, and S; or

(e) an amino acid position equivalent to position 319 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from W, T, and S.

9. In some embodiments, the variant laccase enzyme of paragraph 1 has mutations equivalent to:

(a) I265R/V287G,
(b) I265R/V293T;
(c) I265R/V319T;
(d) I265R/V287G/V319T;
(e) I265R/V287G/V293T/V319T;
(f) I265R/V287P;
(g) I265R/N335R;
(h) I265R/N130E;
(i) F68L/I265R;
(j) F68L/I265R/V287G;
(k) F68L/I265R/V293T;
(l) F68L/I265R/V319T;
(m) F68L/I265R/V287G/V319T;

(n) F68L/I265R/V287G/V293T/V319T;
(o) F68L/I265R/V287P;
(p) F68L/I265R/N335R; or
(q) F68L/I265R/N130E;
in SEQ ID NO: 11.

10. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs is derived from a parental laccase is obtainable from a *Cerrena* species.

11. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs is derived from a parental laccase is obtainable from *Cerrena unicolor*.

12. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs is derived from laccase D from *C. unicolor*.

13. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs is derived from a parental laccase having an amino acid sequence selected from the group consiting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, and SEQ ID NO: 28.

14. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs has an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 11.

15. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs has an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO: 11.

16. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs has an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 11.

17. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs has an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 11.

18. In some embodiments, the variant laccase enzyme of any of the preceding paragraphs further comprises a mutation that introduces a glycosylation site into the amino acid sequence of the parental laccase.

19. A composition comprising the variant laccase of any of the preceding paragraphs is provided.

20. In some embodiments, the composition of paragraph 19 further comprises a chemical mediator.

21. In some embodiments, the composition of paragraph 20 comprises a phenolic compound chemical mediator.

22. In some embodiments of the composition of paragraph 21, the chemical mediator is a phenolic compound is selected from the group consisting of syringonitrile, acetosyringone, and methyl syringate.

23. A method of bleaching a surface comprising contacting the surface with a composition of any of the preceding paragraphs is provided.

[116] The following Examples are provided to further illustrate the present compositions, methods, and systems, and should not be construed as limiting.

EXAMPLES

Example 1: Creation of *Trichoderma reesei* Screening Strains

[117] Improved screening strains were created to increase the consistency of CBH2 variant expression in the presence of factors unrelated to the amino acid sequences of the enzyme variants. In particular, *T. reesei* screening strains were developed in combination with a targeting vector to force integration of *cbh2* variant genes (*e.g.*, coding region in operable combination with a regulatory sequence). The new strains prepared during development of the present disclosure, combine several mutations that are advantageous for screening variant libraries. A schematic of the genetic engineering steps is shown in Figure 1.

[118] Deletion of *ku80* from the *T. reesei* quad deleted derivative strain. A single orthologue of MUS52, the *N. crassa* orthologue of the human KU80, was identified by TBLASTN search in the genome sequence of *H. jecorina* QM6a (*T. reesei*) and was consequently named *T.reesei ku80.* protein id 58213; http://genome.jgi-psf.org/Trire2/Trire2.home.html The nucleotide sequence of the *T.reesei ku80* gene is provided as SEQ ID NO: 37:

[119] ATGGCGGACAAGGAAGCAACCGTCTTCATCATCGACCTCGGCGCGTCCATG
GCAGCTGTCAATGGGGGTCGAGAAGAATCCGACCTTGATTGGAGCATGAGCTACG
TCTGGGACAAGATCAGCAACGTCGTGGCCTCGAATCGCAAGACGCTGTGCGTTGG
CGTCGTGGGGTTCAGAACCGACGAGACAAACCACACGCTGAGCGAGGATGGGTA
CGAGAACATCTCCATATTGCAGCCCCTGGGGCCGATGAGCATGTCCAGCCTCAAG

GCTCTTCAGCCCAAGGTGAAGCCGAGCAGGACGGTGGAAGGCGATGCCATCTCG
GCGATTGTCATTGCCGTCGACATGATTGACAAGTACACGAAGAAGAACAAATGGA
AGCGGCAGATTGTTCTCATTACCGACGGCCAAGGCGAGATTGATCCAGATGATATT
GGCGACATTGCTAGAAAGATGCGCGACTCGAATATTGAATTGACAGTCTTGTGAGT
TGGCGAGACCGTTTGGCGGACGGTAATGGTGCTGACGGTGATGCAAGGGGCGTC
GACTTTGATGCTCCCGATTACGGCTTCAAAGAGGAGGACAAACCTTCAGTCAAGG
TACTCCATATGTTCACTTCTTTTCTTTTTCTTCTTTATTTTCTTTTCTTTTGAAGCTTT
CATTAACCTCTTCGTTAGAAGCAAAACGAAGAGACCCTAAAAAAGCTCGTGGATG
GCTGTGGCGACGACTCAAGGTTCGCCTCCATGGTCGAGGCCATTGACGACTTGAA
TGAGCCACGAGCAAAGTCGGTCAAGCCTTACAAAACGTACGAAGGTCTCTTGAC
CTTGGGAGATCCGAAAAACGCTCCCGCAGTGGTGGAAATCCGCGTCGAGAGATAC
TTCAAGACCCATCTAGCCAGGCCACCTGCCGCCAGCACCGTGGTGGTCAAGGAGG
AGCAAGCTGGGCCGTCTCAGGCAGACGAGGACGAACAGATGGACGGAGCGGAA
CTTACAGCTGTGAGGCAGGCCAGGACATACAAGGTCAATGATCCAGATGCCCCTG
GCGGTAAGCGTGACGTTGAGTTTGAGTCTCTGGCCAAAGGGTACGAGTACGGCAG
GACGGCAGTCCACATCAGCGAGTCTGATCAAAACGTCACCAAGCTCGCGACAGA
AAAGAGCTTCAAGATCATCGGCTTCGTCCAGAAAGAAAAGGTATTGGCTTGGCTC
TCAGCATTTGACCCGTTGCTCTTGGCTAACCCTTGTTTAGTATGAAATGCTCCTTAA
TCTTGGCGAAACCTGCGTTACCGTTGCATCCAAGTACGATGAAAAGTCTGAGCTG
GCTTTTAGCTCTCTGGTGTGGGCGCTCTCGGAGCTCGACGCCTACGCCGTGGCCC
GCCTAGTAACTAAGGACCAAAAGGACCCCATGCTGGTGTTACTGATGCCGTATATG
GAGCCTGATTATGTTTGTCTCTATGATGTGCCTCTGCCTTTCGCAGAGGACATCAG
GACGTACCAGTTTCCTCCCTTGGACAGAGTCGTTACCGTCAGTGGCCAAACGCTC
ACCAACCATCGCCTATTGCCATCCGACGAGCTCAACCAAGCGATGAGCGACTACG
TAGATGCCATGGACATTTCAAGTTATGGTATCGATGAAGATGGGTGAGTATAGAAG
ATGATTGTTCAAATCTTTCACTTCTAAGCATTGCTTCTGATCTAGGCAACCGGCTGA
ATATGCCACCATCGATGAGTTATACAACCCTGCGATACATCGCATAGGCCATGCGAT
CAAACAACGAGCGATCCACCCAGAGAAACCCGTGCCCGAGATCCCCCCAGTCTT
GCTTAGATTCGCAGCACCCCCGACAGAACTCGTCGAGACTGTGCAGCCTCATATC
GATGCACTGATTCACGCTGCAGACGTGAAGAAAGGTACTGATTCCATTACATATGC
TTCTCTGCACACTGATGTTTGATTTGTGCTAACGCCCCCCTTAGTGCCGCCCAAGG
CCAAGGGCAAGCGCCAAAGAGAAACAGTTAAACCCATCTCGGGACTGGATGTGG
ATGCCCTTCTGGGAGAAGAGCAGAAAGGTTCCATTAGTCCGGAGAATGCCATTCC
GGACTTCAAACGAGCCCTCAACTCGTCCGAAGAAGTCGAGCAGATTGCCGACGC
CACAAAACAAATGGGGGCCATTGTGCGGTCTCTCATTACGGACAGCTTCGGGGAT
AGCAAATATGCCCAGGCAATGGAAGGCATTGGTGCGATGCGTGAGGAGCTGATCA
ACCTGGAAGAGCCTGGCCTGTACAACGACTTTGTGCGCGACTTGAAGAAAAGTTT
GCTATCTGGAGCCTTGGGTGGTGACAGGCGAGATTTCTGGTTCAAGATGAGGTGG
GCGAAGCTGGGCCTGATTGACAAGAAACAGTCGGAGGTGTCTTCGGTCACTCTTG
AGGAGGCGGACGAGGTGAGTGGTGCAGCATGCTGTCGGATTATACGGACGTTGTT
TGCTAACTTGTGGGATAGTTTTACAAGTCGAGGTGAGGTATCTACGTTGACCAAGA
ATGGGACCATGTATATGAGCGGTGTAACAACAGAATCCTGTGCTTTGAGCATTGTA
TGA

[120] The *T. reesei ku80* gene was deleted from the quad deleted derivative strain using standard methods of the art (WO 2005/001036). Briefly, a *ku80* deletion cassette was utilized that employed a selectable marker flanked between 1.3 kb of 5′ *ku80* sequence and 2.3 kb of 3′ *ku80* sequence, as schematically shown in Figure 2. The variant *T. reesei als,* which confers resistance to the herbicide chlorimuron ethyl, was used as selectable marker as described in WO 2008/039370. The nucleotide sequence of the *ku80* knockout cassette is 7685 base pairs in length: bases 1-1271 correspond to the 5' *ku80* homologous region; bases 1280-7685 correspond to the *als*-chlorimuron ethyl resistant variant (A190D); and bases 5381-7685 correspond to the 3' *ku80* homologous region. The nucleotide sequence of the *ku80* knockout cassette is provided as SEQ ID NO: 38:

GGCCGCCTCAACACCCACACTCGAGGCACACGAGTTCATCGGCGGCTTCCCCCAC
AAGCTCTCGGCCAACCTGCTACCGGCTCTCTCGCGAGACTTCCCAAAGCCTACAA
ACGAGGTCGACGTCAAGGAGGCCCTCGAGCGCCAGCCCGGCAGATGGAGCCTCC
AGGGCCAGATCAAGGCCAACAACATGAGAGCCCAGAGCGCCGCACTCCGGCTCG
ACGACAAGGAGGGCAAGGCGAGAGCCTTTGAGGAGGCCAAGCGCGAGCTACTG
GCGTATCACCACAGCGCCCTGCGGAAGCCTTCCGGCGCAAGATAATGAGCTTGAT
CGCAATGACGAGTTCACGTACGCTTTGCCATATTGTTGTTGCTTTTTGTTTGGTCCT
ACATGTACGGCGCATTGGTTGGGAGGATATACCCACGGAGAGTGTCCGAGTGGCT
TCTGGGATTTAGAGCGTCATTAGCAGGATAGAGATGGTTGGCCAGGGGAATGGAA
TTGACTTTTCACTACAAGGAACTTGTTCACTCTGGTGTTGATTCCCATTGCGTGAC
TGGTAGTAGGGAGGAATGCTTTTACTTTGTGCCACTAGACCGCAGAGAAGGGTTG
GTTGCAAGCGGGGTCCGTGTATACCGACCAAGAGTGATGGGCATACAGCAACGTT
TCTGAACGACTTCATTTTGTCCGAGTCTACTGGATGCGAGATGCCAGCGTGAAGCC
GTACGCCACCAGGGCGACGAACTCGACAAGGTTGACGAGGGAGGAGATGCCGTG
CAGCATGCCAAACTTCTTGTTGAGGGCACGCATCTCATCCGACTGTGCATCCTTGT
CATACCACTCCTTTCCGTCTCGCTTGGCTGGTGGGAGGGTTCAACAAATCCATCGT
CAGCCATCCGGGGTCTCAAATCAATGGCGTGCATGCGGAGTCGGGCTTGAGGCTA
ACCTTGTCCATGGCGGTCCTTCATGGTCTTGACAGTGGCGGGAAGCAGCACGGCG
AGGTTGACGAGGCCGCTGACGAACATGGTTGCGATGGGCACCAAGGAGCTCCAC
TTGTTGGGAGCGTCGACGAGGCCGCCGATGCCGCCCTTGATGCCCAAGAGGGCGT
TTCCGGGGAACGTGAGGGCGAGCAGCGCGGGGATGGCCGTCTGCATGCCAAAGT
AGATGGGGAACAGCTTGCTCTGGATGGCGGAGAAGGAGGGCCGGCTGACGGTGC
GGAACATGACGATGCCGTTGACGAAGGACTGCAGTAGCGTAGTGTGATGGTAAGC
AGCTGGCCGGCGCGCCTGAGACAATGGCCGGCAATGGTAAAAAGGACCAAGATG
TACTAGGTAGTTGCAATGTGGCTTATTACCTACCTACTACCTGGTAGGCACCTACTA
GGTACTTGGGTAGACGGACAATGAAATTTGAAGTCGGGGTTGCAGGAAAGCAGG
GCGCTGGACACATTGTGCTTCAGGCGGTACCCGTCGTCATCGTCAGCCAATGTCG
AGGCCCGGCAGCCCGAGGAGCGAGACAACCTTGGCCGGAGGAGCCCGCAGGTAC
CTGCCAAAGCGCGGCTGGTACCTCTCAACCCTCTCAGGCCTGTTGGATGCCCTATG

ACATGCCCTGGGGGATGCAGCTGTTGCCCCGGCCCCGCACTTTCGGGTGACCGCGAGGCTGCTGATTGGCTGGTTGCCACGGGCTGGGCGGTCCCTGAAGTTGTTGCCATCTGAACTCTGTCGGCGCTGGCGTCGGCTGCGCCCAATGGGAGGCGAGACAACTCAGGGTACTAGAATCACTGACAGAAGAAGAGAATCGAAAGTAGGTAGACAGCCAATTCGTTGCATGGCAGGCAACCGCACAGGAGAAAAATTGACTACCCCACAATCAGGCACAGTAAGTAGGGCACAGTACGTATGTACAGACAAGGCGCAAGCGATACTGCGCGACCCGGTACCTCGCCGGCTTGACACGTGCGACAGGCTACTTTACTAGTATTCGCAGCGGCGGGTCGCGCATTATTACATGTACTGTGCCGCCATTTGATGACTGGGCTGCTGCAGTATTAGTAGATCTGCCCGGCATCGCCCTTCCATGGGCGCGACCCGGGACTGGACCCTCTGACTCTACCTACATGTACCTAGGCCGGGCCGGGCTTGGTGACTTTTGTCCGATCAGGTCGTTCGCCTGGCTACCTATTATTTCTCTTTCTTCTTCTCCATCCTGCTTCTGGCCTTGCAATTCTTCTTCGCCACTCCTCCCTCTTCCCCCCGCGATACCCTTGAATTCGTCAGAGAGGAAAAGACGAGAAAAAAAAGGGCAGCAGAGACGTCGGTCTGGCTCACGTGCTGCATCTCTGCGCACTCTCATTTTTTTATTGTCCGACCCCTCCCTCAACCTTCTCCTTCGTTGACAGGCTAAGCCTTGCTTCGACGCTCTCTCTTTGAATTTTTCTACTTCTACCTTCTTTTCTTGCGTGTTACCCACCATAGCTCGATTCACGATGCTCCGAAGTCGCCAAGTCACAGCCAGGGCCGTCCGGGCTCTGGGCCAGGCGCGCGCCTTTACCTCGACGACCAAGCCTGTCATGATCCAGAGCAGCCAGAGGAAACAGGCCAACGCCAGCGCTGCTCCGTAAGTCGCCCATTGCCATTGCATCTTCTGTTTGATATATACTTCCTGCTGCTTGCGTGGCGTCGTCTCTCGGTTATGCGTGTCAAGGACCAGGTGTGTTCGCATCGTGGTTTTCCAGCGCCGATTACCGGGGGACGAATTTTTGGCTGCTCAACTCGCGCGCGCGCATTCTGATTCTTCGTTTTCAATCTTGAGCGACAACTGGCTAACATAATGGCCATTGGCAATTGCTTCACACAGACAAGTCCGCCCTGTACCGAGCCCTGCTTTCAACGCTGAAGACAAAGACCGCAGCCATGTGCAGCCTCTGGTCAACCCGTCGAAGCCCGACATGGATGAATCGTATGTCCACGTCCCCTCGTCCCGCCCTACAAAATGAACACGATTACACCAGAATTTTTGCAACAATCGACACTTCTATAACAGACCAATTGAGCTTTGTTCTGACCAATCATGTTGCTCTAGATTCATTGGCAAAACCGGAGGCGAAATCTTCCACGAGATGATGCTGCGACAGGGTGTCAAGCACATTTGTAGGTTCCGATGCCGGCCGCCCACACGGGCTCCATCCTTGCTCCATCTCTCCAGCTAGGCAAATCTCGCTAACCTTGAGTCACCATCCAGTCGGATACCCTGGCGGCGCTATCCTGCCCGTCTTCGACGCCATCTACAACTCAAAACACTTCGACTTCATCCTGCCCCGTCATGAGCAGGGAGCTGGCCATATGGCCGAGGGCTATGCCCGTGCCTCGGGCAAACCCGGTGTCGTCCTGGTGACTTCCGGCCCCGGTGCTACCAATGTCATCACGCCCATGCAGGATGCCCTGTCGGACGGAACGCCCTTGGTCGTCTTCTGCGGCCAGGTCCCCACCACGGCC

ATCGGCAGCGATGACTTCCAAGAGGCCGACGTCGTGGGCATCTCGCGGGCCTGCA
CCAAGTGGAACGTCATGGTCAAGAGCGTTGCTGAGCTGCCGCGGAGAATCAACG
AGGCCTTTGAGATTGCCACCAGCGGCCGCCCTGGCCCCGTCCTCGTCGACCTGCC
CAAGGATGTCACGGCTGGTATCCTGAGGAGAGCCATCCCTACGGAGACTGCTCTG
CCGTCTCTGCCCAGTGCCGCCTCCCGCGCCGCCATGGAGCTGAGCTCCAAGCAGC
TCAACGCCTCCATCAAGCGTGCCGCCGACCTCATCAACATCGCCAAGAAGCCCGT
CATCTACGCCGGTCAGGGTGTCATCCAGTCCGAGGGCGGCGTTGAGCTCCTGAAG
CAGCTGGCGGACAAGGCCTCCATCCCCGTCACCACCACCCTCCATGGCCTGGGTG
CCTTTGATGAGCTGGACGAGAAGTCGCTGCACATGCTGGGCATGCACGGCTCGGC
GTATGCCAACATGGCCATGCAGCAGGCCGACCTCATCATCGCCCTCGGCAGCCGAT
TCGACGACCGTGTTACTCTGAATGTCTCCAAATTTGCGCCTGCAGCCAGGCAAGC
TGCTGCCGAGGGCCGCGGCGGCATCATTCACTTTGAGATCATGCCCAAGAACATC
AACAAGGTCATCCAGGCGACCGAGGCCGTCGAGGGCGACGTCGCCACCAACCTG
AAGCACCTCATTCCCCAGATTGCCGAAAAGTCCATGGCGGACCGAGGAGAGTGGT
TCGGCCTCATCAATGAGTGGAAGAAGAAGTGGCCCCTGTCAAACTACCAGCGCGC
GGAGCGGGCTGGCCTCATCAAGCCGCAGACGGTCATGGAGGAGATTAGCAACCT
GACGGCCAACCGAAAGGACAAGACGTACATTGCCACGGGTGTCGGCCAGCACCA
GATGTGGGTTGCCCAGCACTTCCGCTGGAGGCACCCTCGATCCATGATTACCTCTG
GTGGTCTGGGCACCATGGGCTACGGTCTCCCCGCGGCCATTGGCGCCAAGGTGGC
CCAGCCCGACGCTCTCGTAATTGACGTTGATGGCGATGCCTCGTTTAACATGACGC
TGACGGAGCTGTCGACTGCTGCACAGTTCAACATTGGCGTCAAGGTGGTTGTGCT
CAACAACGAGGAGCAGGGCATGGTGACGCAGTGGCAGAACCTCTTTTACGAGGA
CCGATATGCCCACACGCACCAGAAGAACCCCGACTTCATGAAGCTGGCCGACGCC
ATGGGCGTTCAGCACCAGCGCGTGACGGAGCCGGAGAAGCTGGTCGATGCCCTG
ACGTGGCTGATCAACACCGATGGCCCGGCCCTGTTGGAGGTTGTCACGGACAAGA
AGGTGCCTGTCCTGCCCATGGTGCCCGCCGGATCGGCCCTGCACGAGTTCCTCGT
CTTTGAACCTGGTGAGTCTACTTCAGACATATTGCTTGCGCATTGCAGATACTAAC
ACTCTCACAGAAAAGGATAAGCAGCGCCGTGAGCTGATGAAGGAGAGAACAAAG
GGTGTGCACTCCTAAAGCGATGATGTCTGCGAGGGGTTCTTCGTTGAACCCTAGTT
CAGGCACCATCTTACCCTCTTATTTTTTCCCGTGGGCTTTCATTTTGTGTCATCCGA
GCATGACGTTGTAGGGTTGGAGTTTCTTCCTTTTTATCTTGTCATTTACTGGTACCC
ATAGGCGCGAGACTAGGCTTCCATGTTTTGTTTTGCGACTTTCAAAAAGTACTTTT
AGTGGTTTGGGGCACGACGAGGGGGGGCAACCTCTTCTGTCGAAAAAGGTGGCT
GGATGGATGAGATGAGATGAGATGAGGGTGAAGATAGATACCTGCAGTGTTTTTG

ACGCGACGGGATGGCGATCGCAGCACCCCCGACAGAACTCGTCGAGACTGTGCA
GCCTCATATCGATGCACTGATTCACGCTGCAGACGTGAAGAAAGGTACTGATTCCA
TTACATATGCTTCTCTGCACACTGATGTTTGATTTGTGCTAACGCCCCCCTTAGTGC
CGCCCAAGGCCAAGGGCAAGCGCCAAAGAGAAACAGTTAAACCCATCTCGGGAC
TGGATGTGGATGCCCTTCTGGGAGAAGAGCAGAAAGGTTCCATTAGTCCGGAGAA
TGCCATTCCGGACTTCAAACGAGCCCTCAACTCGTCCGAAGAAGTCGAGCAGATT
GCCGACGCCACAAAACAAATGGGGGCCATTGTGCGGTCTCTCATTACGGACAGCT
TCGGGGATAGCAAATATGCCCAGGCAATGGAAGGCATTGGTGCGATGCGTGAGGA
GCTGATCAACCTGGAAGAGCCTGGCCTGTACAACGACTTTGTGCGCGACTTGAAG
AAAAGTTTGCTATCTGGAGCCTTGGGTGGTGACAGGCGAGATTTCTGGTTCAAGA
TGAGGTGGGCGAAGCTGGGCCTGATTGACAAGAAACAGTCGGAGGTGTCTTCGG
TCACTCTTGAGGAGGCGGACGAGGTGAGTGGTGCAGCATGCTGTCGGATTATACG
GACGTTGTTTGCTAACTTGTGGGATAGTTTTACAAGTCGAGGTGAGGTATCTACGT
TGACCAAGAATGGGACCATGTATATGAGCGGTGTAACAACAGAATCCTGTGCTTTG
AGCATTGTATGATATGATTATTGATGAACCGGACAAAAGGGGGTAGGGGATTGATG
CCATCACGACCGATTGACCAGACCTGGATTCTCGCACAGCATGGCTGCTGATTTTG
TTGACCTTGCGACGTAACATCCCTGAAGAACAACCTACTATTAACCTATCATTTAGC
AGAAGCTCTGTAACCTTCTTGATTCTTGTATTCAGCTTCTGAGTCTGTCAAATGTAA
TCATTTCGAGGTTGTGTAATTCCGGCCAAGCAGGCGGCCGTCTGCCAGCGCCTGC
CTAGGCTGCACCGCAATCTGCCCAATCAGCTGCCCTTCAGTTTCGTTTGACCTTGC
AGCTGCCCTTCATCCTTTATCTGCACACAATTCTTTTTCCTCTGCTCTGCGCATTCTT
CTCTCTCTCGTCTCCCTTCTCAAGCTCAACTTCACCTCATCCGCTCCACTACAAGC
CCTCCCGTCGTCGTCTCGCATCCTCATCTCGACTGCGGCCAGCAAAACAAGCAAA
GCCGTGATCGATCCTCAGCATGGCTACCTTCAACCTCACCGTCCGCCTGGAGATGC
TCAAAGAAATTGGAATCACCGTCCAATACGGCGAGCATGTAGCGAAAGAAGCAGC
CAGCAACGAAGCAGCGATGGCATTCGAAGAAGAAGAGTTCCCCGCCGTTGT
GCCGCCCAAGGCAGAACAGCACGCCTCTGAACACGACGCTGGCCACGATGCTTG
GGACGCGGCTGCCCACATCTCGACTTCGGCGCAAGAACAGCAGAAGCCCCAGGA
GATGGACGACTCGTCTATCGTGATGCCGCTGGACTACTCCAAGTTTGTCGTTGGAG
AGCCTGCGGACGAATCCATCAGCTTTTGCTCGTGGAAGGTCGTCGAGGCTTATCCT
GACCAGTTTATCGGCAAGGCAAACAGGCCTCGTGTATGTAGCGATTGCTTTCTCTG
CATTATGGGAATCTCAAGAGAGTATGGTAGAAGATAACTGACAACTTGCAGGCCA
AGCCGTACTTTGACAAGATTTTGGAAGACAGAGTCTGGGATTTGTGAGGATCTTG
ATTGATGTGCATATGGCGACATGCCTGCTAATATCATTGTAGCTTCTATCTCTACAAC

CCCGAGAAGCCTTCAGAGAAGCCTCGCGTGCTGGTGCCCACTGTTCAGCTCGAA
GGCTTTCTCAAAAGCATCAACAGAGCGCTCGGTACTTCTCTCACCATTCCAGGAG
GGGCAAACCAGGACCGTTTTTATCTGAGGTTCGGCCAGGGAGACACCCCAAGGC
CTCGATATCTACAGAGGTCGAGAGACCAGAAATCCCTAAAGATTGAAACGTTCCC
CGATTTTCAACAGGCGGACTACGACAGCTTTAGGAACGCGCATGGCGCCATCCAG
GAGGACTGGTTGAAGAACTGGCAGATGCTGGTACCTCGGCCGAGTTTCGACAAG
AAGAAAAATGCAGACAAAAGAGCAGCCAAGAGAAGGCTCGAGCGAGAGCGAAT
GCTTCACAATACGCAGGAATTTCTTCATTTGGCAGGTAAGGGCAAAGGGGCTGAC
GTGG.

[121] Creation of the Archy2 strain from the *T. reesei Δku80* quad deleted derivative strain. The *pyr2* gene was deleted from the *ku80* knockout strain. The *pyr2* deletion cassette contains the *T. reesei* cbh1 promoter, a hygromycin resistance gene and a partial *amdS* selectable marker flanked by 5′ and 3′ pyr2 sequences, schematically shown in Figure 3. Use of this vector permits screening for resistance to hygromycin and fluoroorotic acid of *pyr2* knockout transformants. The partial *amdS* gene contains the 3' portion of the gene, but lacks a promoter and the amino-terminal portion of the coding region, and is consequently non-functional. The nucleotide sequence of the pyr2 knockout cassette is 9259 base pairs in length: bases 1-1994 correspond to the *pyr2* 3' homologous region; bases 2002-3497 correspond to the *T. reesei cbh1* promoter; bases 3563-5449 correspond to the hygromycin resistance selectable marker; bases 5450-7440 correspond to the *A. nidulans amdS* 3' partial marker; and bases 7441-9259 correspond to the *pyr2* 5' homologous region. The nucleotide sequence of the *pyr2* knockout cassette is provided as SEQ ID NO: 39:

ATCACGCCCTCGCATAAAAGACCCTCAAGAGTCCATGTGCCCTATCTGCCTGATCT
TCCTAACCCTTATTTAACATTGGCCCTATCACAACCTAGTTCTTCTTCAGCCTGCTT
TGTCAACACTTGTCACGGTTCAACTCAACGTAATCAGCAGGTAGCAGGACGAGGA
TAGGGAGAGAAACGAAGAGAAGAAGAGGAGAGAGGAAGAAAAAAAAAAAGAAA
AGAAAGAAAAAGGGAAAAGGAAAGAAGGAGGAAAAGAGAAGAAAGTCAGATG
AAGAAGCAAGAAGACGCCATGGTAGCCACCGCTTGTCAGGGCTCCTTAGCAACG
AACAACTCTAGCTTGGGGACTTGTCGATGTGTCGTTTCCTTCCTACCCATCAGCAC
CAACGATGAGTTCGATATAGACGAGGACCTCATGGAAGTAGAGACCATTGGGTTC
GACAGGATCTCTCAGTTTCACTTCTATGAGGTCTGTCGCTCGGATGACTTTTTGAG
GAGCTTCCCCTTCTGCTTCAACCCCAAACTCTCTTTCCTGAAACCGCAGCACGTTG
GCACGGCCGTGTTGCTGGAGCAGTTTGCTTTCGAGCACTCTCAGCGTGGTTTCAG
CAGCCCACTGGTGAGTGGCCTCCTTTGACGTCCACACCTTGCTCCTGTCGCATGC
GTATCTGGTGGGAACGACTGCTCCAAGGAGGATTGCTAACGAGGTTGTAGGCCGA
ATATCGCATCAGATTCTCCGGTAACCTTAGCTACGGCCTCTTCAACATCTGTGACAT
GACGGAGCGCAAGTACTGGTGGTTGGCGACCAAGATGCGCGGCTGGAACATCGA
CGGCTGCCCCGAAGACGTCAGGAGACTCATGTTTGTTCACATCATCGCCACCCTG
GGATGCAGCCCCGTCGTGACGGATGAAGACATGGACTACCCCAAGAACTGGGCG

GCAATTCTCCACGGTAGAGACAGATATCCGAGTGAACCTGTGGCCACCGGCCTC
ATGGGCGCACCATCTGCCTCCACTCGGTGGCCGTCTGCCCTCGTCTCCAGGGCTTG
GGTCTCGGTACTGCGACTCTGAAGTCGTATGTGCAGCGCATGAACAGCCTCGGCG
CCGCGGACCGTGTTGCTCTCGTTTGCCGCAAGCCCGAGACGAGATTTTTGAAAG
ATGCGGCTTCAGGAACAGCGGCCGGAGTAGTATCAAGACTCTGGTCGGCGAATAC
TACAACATGGTGTGTGCTTCCACATCGACTTGGCCAGACTCTATACGATTTTCAAA
CCTCGCTATACGTCATATTGACTTGTTTCTTTAGGTCTTCGATTTGCCCGGGCCCAA
AGACTTTATCGACTGGAATAGCATTGCCGACGCTGCCAAGAAGATGTGAACCATTT
GACTGATACGATGTGTGCTACGCATGTCGACCTTCTTTGTTTGTTTCTTTGGCGGCT
CTTTGTATACCTTGGGACACGGCAGACGCATGTCTATGTGAAGAAAACGTTCACG
GCGCTGTTTGCATCAGGAATATGATCATTAAACATGGAGCGTAATGGTATTAATGAT
CAACTAGAAAAATGGTATGGAAGGGCGAGAGGGCGATCAACAAAGCAGCCCGGG
GCATAGTCTGGAAGCAGCAGGAATTGGAAGGGAAAAGGAAGCTGCACAATGAAG
GGATATCGTGAGCGGAGTGGCTCACGAGAGTATCAACAGACTGGCGAAAGCAAG
CAATTGCCAACGCCGGCTATTAGGCCATAAGATGGCCTGTTGTGAGTCCCAGTTGC
ACGTATCCCCATATGACTGCTCTGTCGCTGACTTGAAAAAAAATAGGGAGGATAAA
GGAGAAAGAAAGTGAGACAACCCGTGAGGGACTTGGGGTAGTAGGAGAACACAT
GGGCAACCGGGCAATACACGCGATGTGAGACGAGTTCAACGGCGAATGGAAAAT
CTTGAAAAACAAAATAAAATAACTGCCCTCCATACGGGTATCAAATTCAAGCAGTT
GTACGGAGGCTAGCTAGAGTTGTGAAGTCGGTAATCCCGCTGTATAGTAATACGAG
TCGCATCTAAATACTCCGAAGCTGCTGCGAACCCGGAGAATCGAGATGTGCTGGA
AAGCTTCTAGCGAGCGGCTAAATTAGCATGAAAGGCTATGAGAAATTCTGGAGAC
GGCTTGTTGAATCATGGCGTTCCATTCTTCGACAAGCAAAGCGTTCCGTCGCAGTA
GCAGGCACTCATTCCCGAAAAAACTCGGAGATTCCTAAGTAGCGATGGAACCGGA
ATAATATAATAGGCAATACATTGAGTTGCCTCGACGGTTGCAATGCAGGGGTACTG
AGCTTGGACATAACTGTTCCGTACCCCACCTCTTCTCAACCTTTGGCGTTTCCCTG
ATTCAGCGTACCCGTACAAGTCGTAATCACTATTAACCCAGACTGACCGGACGTGT
TTTGCCCTTCATTTGGAGAAATAATGTCATTGCGATGTGTAATTTGCCTGCTTGACC
GACTGGGGCTGTTCGAAGCCCGAATGTAGGATTGTTATCCGAACTCTGCTCGTAGA
GGCATGTTGTGAATCTGTGTCGGGCAGGACACGCCTCGAAGGTTCACGGCAAGGG
AAACCACCGATAGCAGTGTCTAGTAGCAACCTGTAAAGCCGCAATGCAGCATCAC
TGGAAAATACAAACCAATGGCTAAAAGTACATAAGTTAATGCCTAAAGAAGTCATA
TACCAGCGGCTAATAATTGTACAATCAAGTGGCTAAACGTACCGTAATTTGCCAAC
GGCTTGTGGGGTTGCAGAAGCAACGGCAAAGCCCCACTTCCCCACGTTTGTTTCT
TCACTCAGTCCAATCTCAGCTGGTGATCCCCCAATTGGGTCGCTTGTTTGTTCCGG
TGAAGTGAAAGAAGACAGAGGTAAGAATGTCTGACTCGGAGCGTTTTGCATACA
ACCAAGGGCAGTGATGGAAGACAGTGAAATGTTGACATTCAAGGAGTATTTAGCC
AGGGATGCTTGAGTGTATCGTGTAAGGAGGTTTGTCTGCCGATACGACGAATACTG
TATAGTCACTTCTGATGAAGTGGTCCATATTGAAATGTAAAGTCGGCACTGAACAG
GCAAAAGATTGAGTTGAAACTGCCTAAGATCTCGGGCCCTCGGGCCTTCGGCCTT
TGGGTGTACATGTTTGTGCTCCGGGCAAATGCAAAGTGTGGTAGGATCGAACACA
CTGCTGCCTTTACCAAGCAGCTGAGGGTATGTGATAGGCAAATGTTCAGGGGCCA
CTGCATGGTTTCGAATAGAAAGAGAAGCTTAGCCAAGAACAATAGCCGATAAAGA
TAGCCTCATTAAACGGAATGAGCTAGTAGGCAAAGTCAGCGAATGTGTATATATAA
AGGTTCGAGGTCCGTGCCTCCCTCATGCTCTCCCCATCTACTCATCAACTCAGATC
CTCCAGGAGACTTGTACACCATCTTTTGAGGCACAGAAACCCAATAGTCAACCGC
GGACTGCGCATCATGTATCGGAAGTTGGCCGTCATCTCGGCCTTCTTGGCCACACC
TCGTGCTAGACTAGGCGCGCCAGGAAGCCCGGAAGGTAAGTGGATTCTTCGCCGT
GGCTGGAGCAACCGGTGGATTCCAGCGTCTCCGACTTGGACTGAGCAATTCAGCG
TCACGGATTCACGATAGACAGCTCAGACCGCTCCACGGCTGGCGGCATTATTGGTT

AACCCGGAAACTCAGTCTCCTTGGCCCCGTCCCGAAGGGACCCGACTTACCAGGC
TGGGAAAGCCAGGGATAGAATACACTGTACGGGCTTCGTACGGGAGGTTCGGCGT
AGGGTTGTTCCCAAGTTTTACACACCCCCCAAGACAGCTAGCGCACGAAAGACGC
GGAGGGTTTGGTGAAAAAAGGGCGAAAATTAAGCGGGAGACGTATTTAGGTGCT
AGGGCCGGTTTCCTCCCCATTTTTCTTCGGTTCCCTTTCTCTCCTGGAAGACTTTCT
CTCTCTCTCTTCTTCTCTTCTTCCATCCTCAGTCCATCTTCCTTTCCCATCATCCATC
TCCTCACCTCCATCTCAACTCCATCACATCACAATCGATATGAAAAAGCCTGAACT
CACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGAC
CTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAG
GGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGT
TATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATT
GGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCA
CGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGA
GGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCA
TTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGAT
TGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGT
CCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGT
CCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGC
CGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACG
AGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGCAGCAGAC
GCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCG
TATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTC
GATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCG
GGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATG
GCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAG
GGCAAAGGAATAGAGTAGATGCCGACCGGGATCCACTTAACGTTACTGAAATCAT
CAAACAGCTTGACGAATCTGGATATAAGATCGTTGGTGTCGATGTCAGCTCCGGAG
TTGAGACAAATGGTGTTCAGGATCTCGATAAGATACGTTCATTTGTCCAAGCAGCA
AAGAGTGCCTTCTAGTGATTTAATAGCTCCATGTCAACAAGAATAAAACGCGTTTC
GGGTTTACCTCTTCCAGATACAGCTCATCTGCAATGCATTAATGCATTGGACCTCGC
AACCCTAGTACGCCCTTCAGGCTCCGGCGAAGCAGAAGAATAGCTTAGCAGAGTC
TATTTTCATTTTCGGGAGACTAGCATTCTGTAAACGGGCAGCAATCGCCCAGCAGT
TAGTAGGGTCCCCTCTACCTCTCAGGGAGATGTAACAACGCCACCTTATGGGACTA
TCAAGCTGACGCTGGCTTCTGTGCAGACAAACTGCGCCCACGAGTTCTTCCCTGA
CGCCGCTCTCGCGCAGGCAAGGGAACTCGATGAATACTACGCAAAGCACAAGAG
ACCCGTTGGTCCACTCCATGGCCTCCCCATCTCTCTCAAAGACCAGCTTCGAGTCA
AGGTACACCGTTGCCCCTAAGTCGTTAGATGTCCCTTTTTGTCAGCTAACATATGCC
ACCAGGGCTACGAAACATCAATGGGCTACATCTCATGGCTAAACAAGTACGACGA
AGGGGACTCGGTTCTGACAACCATGCTCCGCAAAGCCGGTGCCGTCTTCTACGTC
AAGACCTCTGTCCCGCAGACCCTGATGGTCTGCGAGACAGTCAACAACATCATCG
GGCGCACCGTCAACCCACGCAACAAGAACTGGTCGTGCGGCGGCAGTTCTGGTG
GTGAGGGTGCGATCGTTGGGATTCGTGGTGGCGTCATCGGTGTAGGAACGGATAT
CGGTGGCTCGATTCGAGTGCCGGCCGCGTTCAACTTCCTGTACGGTCTAAGGCCG
AGTCATGGGCGGCTGCCGTATGCAAAGATGGCGAACAGCATGGAGGGTCAGGAG
ACGGTGCACAGCGTTGTCGGGCCGATTACGCACTCTGTTGAGGGTGAGTCCTTCG
CCTCTTCCTTCTTTTCCTGCTCTATACCAGGCCTCCACTGTCCTCCTTTCTTGCTTTT
TATACTATATACGAGACCGGCAGTCACTGATGAAGTATGTTAGACCTCCGCCTCTTC
ACCAAATCCGTCCTCGGTCAGGAGCCATGGAAATACGACTCCAAGGTCATCCCCA
TGCCCTGGCGCCAGTCCGAGTCGGACATTATTGCCTCCAAGATCAAGAACGGCGG
GCTCAATATCGGCTACTACAACTTCGACGGCAATGTCCTTCCACACCCTCCTATCCT

GCGCGGCGTGGAAACCACCGTCGCCGCACTCGCCAAAGCCGGTCACACCGTGAC
CCCGTGGACGCCATACAAGCACGATTTCGGCCACGATCTCATCTCCCATATCTACG
CGGCTGACGGCAGCGCCGACGTAATGCGCGATATCAGTGCATCCGGCGAGCCGGC
GATTCCAAATATCAAAGACCTACTGAACCCGAACATCAAAGCTGTTAACATGAAC
GAGCTCTGGGACACGCATCTCCAGAAGTGGAATTACCAGATGGAGTACCTTGAGA
AATGGCGGGAGGCTGAAGAAAAGGCCGGGAAGGAACTGGACGCCATCATCGCGC
CGATTACGCCTACCGCTGCGGTACGGCATGACCAGTTCCGGTACTATGGGTATGCC
TCTGTGATCAACCTGCTGGATTTCACGAGCGTGGTTGTTCCGGTTACCTTTGCGGA
TAAGAACATCGATAAGAAGAATGAGAGTTTCAAGGCGGTTAGTGAGCTTGATGCC
CTCGTGCAGGAAGAGTATGATCCGGAGGCGTACCATGGGGCACCGGTTGCAGTGC
AGGTTATCGGACGGAGACTCAGTGAAGAGAGGACGTTGGCGATTGCAGAGGAAG
TGGGGAAGTTGCTGGGAAATGTGGTGACTCCATAGCTAATAAGTGTCAGATAGCA
ATTTGCACAAGAAATCAATACCAGCAACTGTAAATAAGCGCTGAAGTGACCATGC
CATGCTACGAAAGAGCAGAAAAAAACCTGCCGTAGAACCGAAGAGATATGACAC
GCTTCCATCTCTCAAAGGAAGAATCCCTTCAGGGTTGCGTTTCCAGTAGTGATTTT
ACCGCTGATGAAATGACTGGACTCCCTCCTCCTGCTCTTATACGAAAAATTGCCTG
ACTCTGCAAAGGTTGTTTGTCTTGGAAGATGATGTGCCCCCCCATCGCTCTTATCT
CATACCCCGCCATCTTTCTAGATTCTCATCTTCAACAAGAGGGGCAATCCATGATCT
GCGATCCAGATGTGCTTCTGGCCTCATACTCTGCCTTCAGGTTGATGTTCACTTAAT
TGGTGACGAATTCAGCTGATTTGCTGCAGTATGCTTTGTGTTGGTTCTTTCCAGGC
TTGTGCCAGCCATGAGCGCTTTGAGAGCATGTTGTCACTTATAAACTCGAGTAACG
GCCACATATTGTTCACTACTTGAATCACATACCTAATTTTGATAGAATTGACATGTTT
AAAGAGCTGAGGTAGCTTTAATGCCTCTGAAGTATTGTGACACAGCTTCTCACAG
AGTGAGAATGAAAAGTTGGACTCCCCCTAATGAAGTAAAAGTTTCGTCTCTGAAC
GGTGAAGAGCATAGATCCGGCATCAACTACCTGGCTAGACTACGACGTCAATTCTG
CGGCCTTTTGACCTTTATATATGTCCATTAATGCAATAGATTCTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTGCCCAATTTCGCAGATCAAAGTGGACGTTATAG
CATCATAACTAAGCTCAGTTGCTGAGGGAAGCCGTCTACTACCTTAGCCCATCCAT
CCAGCTCCATACCTTGATACTTTAGACGTGAAGCAATTCACACTGTACGTCTCGCA
GCTCTCCTTCCCGCTCTTGCTTCCCCACTGGGGTCCATGGTGCGTGTATCGTCCCCT
CCACAATTCTATGCCATGGTACCTCCAGCTTATCAATGCCCCGCTAACAAGTCGCCT
CTTTGCCTTGATAGCTTATCGATAAAACTTTTTTTCCGCCAGAAAGGCTCCGCCCA
CAGACAAGAAAAAAATTCACCGCCTAGCCTTTGGCCCCGGCATTTGGCTAAACC
TCGAGCCTCTCTCCCGTCTTGGGGTATCAGGAAGAAAAGAAAAAAATCCATCGCC
AAGGGCTGTTTTGGCATCACCACCCGAAAACAGCACTTCCTCGATCAAAAGTTGC
CCGCCATGAAGACCACGTGGAAGGACATCCCTCCGGTGCCTACGCACCAGGAGTT
TCTGGACATTGTGCTGAGCAGGACCCAGCGCAAACTGCCCACTCAGATCCGTGCC
GGCTTCAAGATTAGCAGAATTCGAGGTACGTCGCATTGCCCATCGCAGGATGTCTC
ATTATCGGGGTCCTTGGAGAACGATCATGATTGCATGGCGATGCTAACACATAGAC
AGCCTTCTACACTCGAAAGGTCAAGTTCACCCAGGAGACGTTTTCCGAAAAGTTC
GCCTCCATCCTCGACAGCTTCCCTCGCCTCCAGGACATCCACCCCTTCCACAAGG
ACCTTCTCAACACCCTCTACGATGCCGACCACTTCAAGATTGCCCTTGGCCAGATG
TCCACTGCCAAGCACCTGGTCGAGACCATCTCGCGCGACTACGTCCGTCTCTTGA
AATACGCCCAGTCGCTCTACCAGTGCAAGCAGCTCAAGCGGGCCGCTCTCGGTCG
CATGGCCACGCTGGTCAAGCGCCTCAAGGACCCCCTGCTGTACCTGGACCAGGTC
CGCCAGCATCTCGGCCGTCTTCCCTCCATCGACCCCAACACCAGGACCCTGCTCAT
CTGCGGTTACCCCAATGTTGGCAAGTCCAGCTTCCTGCGAAGTATCACCCGCGCC
GATGTGGACGTCCAGCCCTATGCTTTCACCACCAAGAGTCTGTTTGTCGGCCACTT
TGACTACAAGTACCTGCGATTCCAGGCCATTGATACCCCCGGTATTCTGGACCACC
CTCTTGAGGAGATGAACACTATCGAAATGCAGAGGTATGTGGCGCGGCTA.

[122] Creation of the Archy3 strain from the Archy2 *T. reesei* strain. The Archy 2 strain was transformed with a vector to integrate at the same pyr2 locus and replace the hygromycin resistance gene with the coding region of the pyr2 gene. The hygromycin deletion cassette is shown in Figure 4. This re-introduction of the *pyr2* gene back into the pyr2 locus placed it between the *T. reesei* cbh1 promoter and the partial amdS selectable marker. This strain could be selected for uridine prototrophy and sensitivity to hygromycin. The nucleotide sequence of the *hygR* knockout cassette is 9088 base pairs in length: bases 1-1994 correspond to the *pyr2* 3' homologous region; bases 1995-3497 correspond to the *T. reesei* *cbh1* promoter; bases 3564-5137 correspond to the *pyr2* selectable marker; bases 5280-7270 correspond to the *A. nidulans amdS* 3' partial marker; bases 7271-9088 correspond to the *pyr2* 5' homologous region. The nucleotide sequence of the *hygR* knockout cassette is provided as SEQ ID NO: 40:

ATCACGCCCTCGCATAAAAGACCCTCAAGAGTCCATGTGCCCTATCTGCCTGATCT
TCCTAACCCTTATTTAACATTGGCCCTATCACAACCTAGTTCTTCTTCAGCCTGCTT
TGTCAACACTTGTCACGGTTCAACTCAACGTAATCAGCAGGTAGCAGGACGAGGA
TAGGGAGAGAAACGAAGAGAAGAAGAGGAGAGAGGAAGAAAAAAAAAAGAAA
AGAAAGAAAAAGGGAAAAGGAAAGAAGGAGGAAAAGAGAAGAAAGTCAGATG
AAGAAGCAAGAAGACGCCATGGTAGCCACCGCTTGTCAGGGCTCCTTAGCAACG
AACAACTCTAGCTTGGGGACTTGTCGATGTGTCGTTTCCTTCCTACCCATCAGCAC
CAACGATGAGTTCGATATAGACGAGGACCTCATGGAAGTAGAGACCATTGGGTTC
GACAGGATCTCTCAGTTTCACTTCTATGAGGTCTGTCGCTCGGATGACTTTTTGAG
GAGCTTCCCCTTCTGCTTCAACCCCAAACTCTCTTTCCTGAAACCGCAGCACGTTG
GCACGGCCGTGTTGCTGGAGCAGTTTGCTTTCGAGCACTCTCAGCGTGGTTTCAG
CAGCCCACTGGTGAGTGGCCTCCTTTGACGTCCACACCTTGCTCCTGTCGCATGC
GTATCTGGTGGGAACGACTGCTCCAAGGAGGATTGCTAACGAGGTTGTAGGCCGA
ATATCGCATCAGATTCTCCGGTAACCTTAGCTACGGCCTCTTCAACATCTGTGACAT
GACGGAGCGCAAGTACTGGTGGTTGGCGACCAAGATGCGCGGCTGGAACATCGA
CGGCTGCCCCGAAGACGTCAGGAGACTCATGTTTGTTCACATCATCGCCACCCTG
GGATGCAGCCCCGTCGTGACGGATGAAGACATGGACTACCCCAAGAACTGGGCG
GCAATTCTCCACGGTAGAGACAGATATCCGAGTGAACCTGTGGGCCACCGGCCTC
ATGGGCGCACCATCTGCCTCCACTCGGTGGCCGTCTGCCCTCGTCTCCAGGGCTTG
GGTCTCGGTACTGCGACTCTGAAGTCGTATGTGCAGCGCATGAACAGCCTCGGCG
CCGCGGACCGTGTTGCTCTCGTTTGCCGCAAGCCCGAGACGAGATTTTTGAAAG
ATGCGGCTTCAGGAACAGCGGCCGGAGTAGTATCAAGACTCTGGTCGGCGAATAC
TACAACATGGTGTGTGCTTCCACATCGACTTGGCCAGACTCTATACGATTTTCAAA
CCTCGCTATACGTCATATTGACTTGTTTCTTTAGGTCTTCGATTTGCCCGGGCCCAA
AGACTTTATCGACTGGAATAGCATTGCCGACGCTGCCAAGAAGATGTGAACCATTT
GACTGATACGATGTGTGCTACGCATGTCGACCTTCTTTGTTTGTTTCTTTGGCGGCT
CTTTGTATACCTTGGGACACGGCAGACGCATGTCTATGTGAAGAAAACGTTCACG
GCGCTGTTTGCATCAGGAATATGATCATTAAACATGGAGCGTAATGGTATTAATGAT
CAACTAGAAAAATGGTATGGAAGGGCGAGAGGGCGATCAACAAAGCAGCCCGGG
GCATAGTCTGGAAGCAGCAGGAATTGGAAGGGAAAAGGAAGCTGCACAATGAAG
GGATATCGTGAGCGGAGTGGCTCACGAGAGTATCAACAGACTGGCGAAAGCAAG

CAATTGCCAACGCCGGCTATTAGGCCATAAGATGGCCTGTTGTGAGTCCCAGTTGC
ACGTATCCCCATATGACTGCTCTGTCGCTGACTTGAAAAAAAATAGGGAGGATAAA
GGAGAAAGAAAGTGAGACAACCCGTGAGGGACTTGGGGTAGTAGGAGAACACAT
GGGCAACCGGGCAATACACGCGATGTGAGACGAGTTCAACGGCGAATGGAAAAT
CTTGAAAAACAAAATAAAATAACTGCCCTCCATACGGGTATCAAATTCAAGCAGTT
GTACGGAGGCTAGATAGAGTTGTGAAGTCGGTAATCCCGCTGTATAGTAATACGAG
TCGCATCTAAATACTCCGAAGCTGCTGCGAACCCGGAGAATCGAGATGTGCTGGA
AAGCTTCTAGCGAGCGGCTAAATTAGCATGAAAGGCTATGAGAAATTCTGGAGAC
GGCTTGTTGAATCATGGCGTTCCATTCTTCGACAAGCAAAGCGTTCCGTCGCAGTA
GCAGGCACTCATTCCCGAAAAAACTCGGAGATTCCTAAGTAGCGATGGAACCGGA
ATAATATAATAGGCAATACATTGAGTTGCCTCGACGGTTGCAATGCAGGGGTACTG
AGCTTGGACATAACTGTTCCGTACCCCACCTCTTCTCAACCTTTGGCGTTTCCCTG
ATTCAGCGTACCCGTACAAGTCGTAATCACTATTAACCCAGACTGACCGGACGTGT
TTTGCCCTTCATTTGGAGAAATAATGTCATTGCGATGTGTAATTTGCCTGCTTGACC
GACTGGGGCTGTTCGAAGCCCGAATGTAGGATTGTTATCCGAACTCTGCTCGTAGA
GGCATGTTGTGAATCTGTGTCGGGCAGGACACGCCTCGAAGGTTCACGGCAAGGG
AAACCACCGATAGCAGTGTCTAGTAGCAACCTGTAAAGCCGCAATGCAGCATCAC
TGGAAAATACAAACCAATGGCTAAAAGTACATAAGTTAATGCCTAAAGAAGTCATA
TACCAGCGGCTAATAATTGTACAATCAAGTGGCTAAACGTACCGTAATTTGCCAAC
GGCTTGTGGGGTTGCAGAAGCAACGGCAAAGCCCCACTTCCCCACGTTTGTTTCT
TCACTCAGTCCAATCTCAGCTGGTGATCCCCCAATTGGGTCGCTTGTTTGTTCCGG
TGAAGTGAAAGAAGACAGAGGTAAGAATGTCTGACTCGGAGCGTTTTGCATACA
ACCAAGGGCAGTGATGGAAGACAGTGAAATGTTGACATTCAAGGAGTATTTAGCC
AGGGATGCTTGAGTGTATCGTGTAAGGAGGTTTGTCTGCCGATACGACGAATACTG
TATAGTCACTTCTGATGAAGTGGTCCATATTGAAATGTAAAGTCGGCACTGAACAG
GCAAAAGATTGAGTTGAAACTGCCTAAGATCTCGGGCCCTCGGGCCTTCGGCCTT
TGGGTGTACATGTTTGTGCTCCGGGCAAATGCAAAGTGTGGTAGGATCGAACACA
CTGCTGCCTTTACCAAGCAGCTGAGGGTATGTGATAGGCAAATGTTCAGGGGCCA
CTGCATGGTTTCGAATAGAAAGAGAAGCTTAGCCAAGAACAATAGCCGATAAAGA
TAGCCTCATTAAACGGAATGAGCTAGTAGGCAAAGTCAGCGAATGTGTATATATAA
AGGTTCGAGGTCCGTGCCTCCCTCATGCTCTCCCCATCTACTCATCAACTCAGATC
CTCCAGGAGACTTGTACACCATCTTTTGAGGCACAGAAACCCAATAGTCAACCGC
GGACTGCGCATCATGTATCGGAAGTTGGCCGTCATCTCGGCCTTCTTGGCCACACC
TCGTGCTAGACTAGGCGCGTCAATATGTGGCCGTTACTCGAGTTTATAAGTGACAA
CATGCTCTCAAAGCGCTCATGGCTGGCACAAGCCTGGAAAGAACCAACACAAAG
CATACTGCAGCAAATCAGCTGAATTCGTCACCAATTAAGTGAACATCAACCTGAA
GGCAGAGTATGAGGCCAGAAGCACATCTGGATCGCAGATCATGGATTGCCCCTCTT
GTTGAAGATGAGAATCTAGAAAGATGGCGGGGTATGAGATAAGAGCGATGGGGG
GCACATCATCTTCCAAGACAAACAACCTTTGCAGAGTCAGGCAATTTTTCGTATAA
GAGCAGGAGGAGGGAGTCCAGTCATTTCATCAGCGGTAAAATCACTCTAGACAAT
CTTCAAGATGAGTTCTGCCTTGGGTGACTTATAGCCATCATCATACCTAGACAGAA
GCTTGTGGGATACTAAGACCAACGTACAAGCTCGCACTGTACGCTTTGACTTCCAT
GTGAAAACTCGATACGGCGCGCCTCTAAATTTTATAGCTCAACCACTCCAATCCAA
CCTCTGCATCCCTCTCACTCGTCCTGATCTACTGTTCAAATCAGAGAATAAGGACA
CTATCCAAATCCAACAGAATGGCTACCACCTCCCAGCTGCCTGCCTACAAGCAGG
ACTTCCTCAAATCCGCCATCGACGGCGGCGTCCTCAAGTTTGGCAGCTTCGAGCT
CAAGTCCAAGCGGATATCCCCCTACTTCTTCAACGCGGGCGAATTCCACACGGCG
CGCCTCGCCGGCGCCATCGCCTCCGCCTTTGCAAAGACCATCATCGAGGCCCAGG
AGAAGGCCGGCCTAGAGTTCGACATCGTCTTCGGCCCGGCCTACAAGGGCATCCC
GCTGTGCTCCGCCATCACCATCAAGCTCGGCGAGCTGGCGCCCCAGAACCTGGAC

CGCGTCTCCTACTCGTTTGACCGCAAGGAGGCCAAGGACCACGGCGAGGCGGC
AACATCGTCGGCGCTTCGCTCAAGGGCAAGAGGGTCCTGATTGTCGACGACGTCA
TCACCGCCGGCACCGCCAAGAGGGACGCCATTGAGAAGATCACCAAGGAGGGCG
GCATCGTCGCCGGCATCGTCGTGGCCCTGGACCGCATGGAGAAGCTCCCCGCTGC
GGATGGCGACGACTCCAAGCCTGGACCGAGTGCCATTGGCGAGCTGAGGAAGGA
GTACGGCATCCCCATCTTTGCCATCCTCACTCTGGATGACATTATCGATGGCATGAA
GGGCTTTGCTACCCCTGAGGATATCAAGAACACGGAGGATTACCGTGCCAAGTAC
AAGGCGACTGACTGATTGAGGCGTTCAATGTCAGAAGGGAGAGAAAGACTGAAA
AGGTGGAAAGAAGAGGCAAATTGTTGTTATTATTATTATTCTATCTCGAATCTTCTA
GATCTTGTCGTAAATAAACAAGCGTAACTAGCTAGCCTCCGTACAACTGCTTGAAT
TTGATACCCGTATGGAGGGCAGTTATTTTATTTTGTTTTTCAAGATTTTCCATTCGCC
GTTGAACTCGTCTCACATCGCGTGTATTGCCCGGTTGCCCATGTGTACGCGTTTCG
GGTTTACCTCTTCCAGATACAGCTCATCTGCAATGCATTAATGCATTGGACCTCGCA
ACCCTAGTACGCCCTTCAGGCTCCGGCGAAGCAGAAGAATAGCTTAGCAGAGTCT
ATTTTCATTTTCGGGAGACTAGCATTCTGTAAACGGGCAGCAATCGCCCAGCAGTT
AGTAGGGTCCCCTCTACCTCTCAGGGAGATGTAACAACGCCACCTTATGGACTAT
CAAGCTGACGCTGGCTTCTGTGCAGACAAACTGCGCCCACGAGTTCTTCCCTGAC
GCCGCTCTCGCGCAGGCAAGGGAACTCGATGAATACTACGCAAAGCACAAGAGA
CCCGTTGGTCCACTCCATGGCCTCCCCATCTCTCTCAAAGACCAGCTTCGAGTCAA
GGTACACCGTTGCCCCTAAGTCGTTAGATGTCCCTTTTTGTCAGCTAACATATGCCA
CCAGGGCTACGAAACATCAATGGGCTACATCTCATGGCTAAACAAGTACGACGAA
GGGGACTCGGTTCTGACAACCATGCTCCGCAAAGCCGGTGCCGTCTTCTACGTCA
AGACCTCTGTCCCGCAGACCCTGATGGTCTGCGAGACAGTCAACAACATCATCGG
GCGCACCGTCAACCCACGCAACAAGAACTGGTCGTGCGGCGGCAGTTCTGGTGG
TGAGGGTGCGATCGTTGGGATTCGTGGTGGCGTCATCGGTGTAGGAACGGATATC
GGTGGCTCGATTCGAGTGCCGGCCGCGTTCAACTTCCTGTACGGTCTAAGGCCGA
GTCATGGGCGGCTGCCGTATGCAAAGATGGCGAACAGCATGGAGGGTCAGGAGA
CGGTGCACAGCGTTGTCGGGCCGATTACGCACTCTGTTGAGGGTGAGTCCTTCGC
CTCTTCCTTCTTTTCCTGCTCTATACCAGGCCTCCACTGTCCTCCTTTCTTGCTTTTT
ATACTATATACGAGACCGGCAGTCACTGATGAAGTATGTTAGACCTCCGCCTCTTC
ACCAAATCCGTCCTCGGTCAGGAGCCATGGAAATACGACTCCAAGGTCATCCCCA
TGCCCTGGCGCCAGTCCGAGTCGGACATTATTGCCTCCAAGATCAAGAACGGCGG
GCTCAATATCGGCTACTACAACTTCGACGGCAATGTCCTTCCACACCCTCCTATCCT
GCGCGGCGTGGAAACCACCGTCGCCGCACTCGCCAAAGCCGGTCACACCGTGAC
CCCGTGGACGCCATACAAGCACGATTTCGGCCACGATCTCATCTCCCATATCTACG
CGGCTGACGGCAGCGCCGACGTAATGCGCGATATCAGTGCATCCGGCGAGCCGGC
GATTCCAAATATCAAAGACCTACTGAACCCGAACATCAAAGCTGTTAACATGAAC
GAGCTCTGGGACACGCATCTCCAGAAGTGGAATTACCAGATGGAGTACCTTGAGA
AATGGCGGGAGGCTGAAGAAAAGGCCGGGAAGGAACTGGACGCCATCATCGCGC
CGATTACGCCTACCGCTGCGGTACGGCATGACCAGTTCCGGTACTATGGGTATGCC
TCTGTGATCAACCTGCTGGATTTCACGAGCGTGGTTGTTCCGGTTACCTTTGCGGA
TAAGAACATCGATAAGAAGAATGAGAGTTTCAAGGCGGTTAGTGAGCTTGATGCC
CTCGTGCAGGAAGAGTATGATCCGGAGGCGTACCATGGGGCACCGGTTGCAGTGC
AGGTTATCGGACGGAGACTCAGTGAAGAGAGGACGTTGGCGATTGCAGAGGAAG
TGGGGAAGTTGCTGGGAAATGTGGTGACTCCATAGCTAATAAGTGTCAGATAGCA
ATTTGCACAAGAAATCAATACCAGCAACTGTAAATAAGCGCTGAAGTGACCATGC
CATGCTACGAAAGAGCAGAAAAAAACCTGCCGTAGAACCGAAGAGATATGACAC
GCTTCCATCTCTCAAAGGAAGAATCCCTTCAGGGTTGCGTTTCCAGTAGTGATTTT
ACCGCTGATGAAATGACTGGACTCCCTCCTCCTGCTCTTATACGAAAAATTGCCTG
ACTCTGCAAAGGTTGTTTGTCTTGGAAGATGATGTGCCCCCCCATCGCTCTTATCT

CATACCCCGCCATCTTTCTAGATTCTCATCTTCAACAAGAGGGGCAATCCATGATCT
GCGATCCAGATGTGCTTCTGGCCTCATACTCTGCCTTCAGGTTGATGTTCACTTAAT
TGGTGACGAATTCAGCTGATTTGCTGCAGTATGCTTTGTGTTGGTTCTTTCCAGGC
TTGTGCCAGCCATGAGCGCTTTGAGAGCATGTTGTCACTTATAAACTCGAGTAACG
GCCACATATTGTTCACTACTTGAATCACATACCTAATTTTGATAGAATTGACATGTTT
AAAGAGCTGAGGTAGCTTTAATGCCTCTGAAGTATTGTGACACAGCTTCTCACAG
AGTGAGAATGAAAAGTTGGACTCCCCCTAATGAAGTAAAAGTTTCGTCTCTGAAC
GGTGAAGAGCATAGATCCGGCATCAACTACCTGGCTAGACTACGACGTCAATTCTG
CGGCCTTTTGACCTTTATATATGTCCATTAATGCAATAGATTCTTTTTTTTTTTTTTTT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTGCCCAATTTCGCAGATCAAAGTGGACGTTATAG
CATCATAACTAAGCTCAGTTGCTGAGGGAAGCCGTCTACTACCTTAGCCCATCCAT
CCAGCTCCATACCTTGATACTTTAGACGTGAAGCAATTCACACTGTACGTCTCGCA
GCTCTCCTTCCCGCTCTTGCTTCCCCACTGGGGTCCATGGTGCGTGTATCGTCCCCT
CCACAATTCTATGCCATGGTACCTCCAGCTTATCAATGCCCCGCTAACAAGTCGCCT
CTTTGCCTTGATAGCTTATCGATAAAACTTTTTTTCCGCCAGAAAGGCTCCGCCCA
CAGACAAGAAAAAAAATTCACCGCCTAGCCTTTGGCCCCGGCATTTGGCTAAACC
TCGAGCCTCTCTCCCGTCTTGGGGTATCAGGAAGAAAAGAAAAAAATCCATCGCC
AAGGGCTGTTTTGGCATCACCACCCGAAAACAGCACTTCCTCGATCAAAAGTTGC
CCGCCATGAAGACCACGTGGAAGGACATCCCTCCGGTGCCTACGCACCAGGAGTT
TCTGGACATTGTGCTGAGCAGGACCCAGCGCAAACTGCCCACTCAGATCCGTGCC
GGCTTCAAGATTAGCAGAATTCGAGGTACGTCGCATTGCCCATCGCAGGATGTCTC
ATTATCGGGGTCCTTGGAGAACGATCATGATTGCATGGCGATGCTAACACATAGAC
AGCCTTCTACACTCGAAAGGTCAAGTTCACCCAGGAGACGTTTTCCGAAAAGTTC
GCCTCCATCCTCGACAGCTTCCCTCGCCTCCAGGACATCCACCCCTTCCACAAGG
ACCTTCTCAACACCCTCTACGATGCCGACCACTTCAAGATTGCCCTTGGCCAGATG
TCCACTGCCAAGCACCTGGTCGAGACCATCTCGCGCGACTACGTCCGTCTCTTGA
AATACGCCCAGTCGCTCTACCAGTGCAAGCAGCTCAAGCGGGCCGCTCTCGGTCG
CATGGCCACGCTGGTCAAGCGCCTCAAGGACCCCCTGCTGTACCTGGACCAGGTC
CGCCAGCATCTCGGCCGTCTTCCCTCCATCGACCCCAACACCAGGACCCTGCTCAT
CTGCGGTTACCCCAATGTTGGCAAGTCCAGCTTCCTGCGAAGTATCACCCGCGCC
GATGTGGACGTCCAGCCCTATGCTTTCACCACCAAGAGTCTGTTTGTCGGCCACTT
TGACTACAAGTACCTGCGATTCCAGGCCATTGATACCCCCGGTATTCTGGACCACC
CTCTTGAGGAGATGAACACTATCGAAATGCAGAGGTATGTGGCGCGGCT.

Example 2: Laccase variants with added glycosylation sites

[123] Seven glycosylation sites were engineered on the surface of the *Cerrena* laccase D polypeptide. Briefly, seven pairs of oligonucleotides were prepared for use in use in standard techniques to introduce the following amino acid residue changes at the indicated positions, referring to SEQ ID NO: 11: NKD to NAT at residues 12 to 14 (variant mut1), GGT to NGT at residues 28 to 30 (variant mut2), NVI to NVT at residues 47 to 49 (variant mut3), QTV to NTT at residues 157 to 159 (variant mut4), NAV to NAT at residues 317 to 319 (variant mut5), NAQ to NAS at residues 362 to 364 (variant mut6), and SAS to NAS at residues 492 to 494 (variant mut7). The PCR-mediated mutagenesis reaction contained 2 µl of template plasmid DNA (*i.e.*, pKB409, a pENTR plasmid that includes the nucleotide sequence encoding the signal sequence of the *Trichoderma* CBH1 gene and the mature *Cerrena* laccase

D1 protein, without an amdS marker; 5 ng/μl), 5 μl of standard 10x buffer, 1.5 μl of 100 mM dNTPs, 1.25 μl of 100 ng/μl forward primer, 1.25 μl of 100 ng/μl reverse primer, and 1 μl of *Pfu* Ultra II polymerase (Stratagene, La Jolla, CA, USA) in a 50 μl reaction volume. The PCR products were digested with the *Dpn*I restriction enzyme (Roche) and 5 μl of each mixture containing nicked plasmid DNA was transformed into *E. coli* cells. DNA was prepared from each of the transformants and the engineered nucleotide changes were confirmed by DNA sequencing.

[124] The mutated coding sequences were then cloned into expression plasmid pTrex3g using the gateway cloning method in a reaction containing 0.5 μl of plasmid DNA, 0.5 μl of pTrex3g, 3 μl of TE buffer, and 1 μl of LRII mixture (Invitrogen), which was reacted at room temperature for one hour and then transformed to *E. coli* cells. pTrex3g, described in U.S. Patent Pub. No. 20100304468, is based on the *E. coli* vector pSL1180 (Pharmacia Inc., Piscataway, N.J.), which is a pUC118 phagemid based vector (Brosius, J. (1989) *DNA* 8:759) with an extended multiple cloning site containing 64 hexamer restriction enzyme recognition sequences. The vector is designed as a Gateway destination vector (Hartley *et al.* (2000) *Genome Research* 10:1788-95) to allow insertion using Gateway technology (Invitrogen) of any desired open reading frame between the promoter and terminator regions of the *T. reesei* cbh1 gene. The vector also contains the *Aspergillus nidulans* amdS gene for use as a selective marker. DNA prepared from each of the transformants was subjected to nucleotide sequence analysis to confirm the engineered nucleotide changes.

[125] Three to six 100μl PCR replicates were performed using each of the seven variant coding sequences as templates. The resulting PCR fragments were used to transform the Archy3 strain of *T. reesei*. Briefly, frozen Archy3 strain protoplasts were thawed on ice and 100-μl portions were transferred to 15 ml tubes. 5-15 μl of each PCR fragment were separately added to the protoplasts, and the DNA and protoplast mixtures were left on ice for 20 minutes. 2 ml of FF4 [25% PEG 6000, 50 mM CaCl, and 10 mM Tris (pH 7.5)] buffer was then added to each tube of protoplasts, followed by incubation at room temperature for 5 minutes. 4 ml of FF3 [1.2 M sorbitol, 10 mM CaCl, and 10 mM Tris (pH 7.5)] were added and the entire volume was transferred to a new tube for equal distribution onto two petri dishes. 25 ml overlay [amdS-sorbitol-agarose (2%) + uridine (0.5mg/ml)] were overlayed on each of the plates, which were then incubated for 5-6 days at 28°C. Detailed methods for using amdS marker system in the transformation of industrially important filamentous fungi are established in the art (*e.g.*, in *Aspergillus niger* (see, *e.g.*, Kelly and Hynes (1985) *EMBO J.* 4:475-79; Wang *et al.* (2008) *Fungal Genet Biol.* 45:17-27); in *Penicillium chrysogenum* (see, *e.g.*, Beri and Turner (1987) *Curr. Genet.* 11:639-41); in *Trichoderma reesei* (see, *e.g.*, Pentilla *et al.* (1987) *Gene* 61:155-64); in *Aspergillus oryzae* (see, *e.g.*, Christensen *et al.* (1988) *Bio/technology* 6:1419-22); in *Trichoderma harzianum* (see, *e.g.*, Pe'er *et al.* (1991) *Soil Biol. Biochem.* 23:1043-46); and in U.S. Pat. No. 6,548,285; each of which references is hereby incorporated by reference.

[126] For each transformation, five colonies were selected and transferred to a conventional potato dextrose agar (PDA) plate contain 1.2 mg/ml 5-FOA and 0.5 mg/ml uridine. The Archy3 strain with integrated plasmid expressing wild type laccase was used as control. The plates were grown at 28°C for 2 days and left at room temperature for one day to encourage sporulation. All five clones were transferred to a 96-well microtiter filter plate (MTP, Corning 3505) filled with 200 µl defined medium with glucose/sophorose (33.0 g/L PIPPS buffer; 9.0 g/L casamino acids; 5.0 g/L $(NH_4)_2SO_4$; 4.5 g/L KH_2PO_4; 1.0 g/L $MgSO_4 \cdot 7H_2O$; 1.0 g/L $CaCl_2$; 26 ml/L 60% glucose/sophorose mixture; 2.5 ml/L 400X *T. reesei* trace elements: 175 g/L citric acid anhydrous; 200 g/L $FeSO_4 \cdot 7H_2O$; 16 g/L $ZnSO_4 \cdot 7H_2O$; 3.2 g/L $CuSO_4 \cdot 5H_2O$; 1.4 g/L $MnSO_4 \cdot H_2O$; 0.8 g/L H_3BO_3; and 0.5 mg/ml uridine; pH 5.5). The MTP filter plate was grown at 28°C with a constant oxygen supply and without shaking for 5 days. 10 µl of 5-days old liquid cultures were transferred to a new plate and 150 µl 100 mM NaOAc, pH 5, and 20 µl 4.5 mM ABTS were added.

[127] The OD_{420} was measured using a Spectra Max spectrophotomoeter for 5 minutes at 20-second intervals. The laccase activity present in the liquid cultures containing filamentous fungi transformed with each of the seven glycosylation mutants is shown in the graph of Figure 5. Error bars in this and other graphs indicate standard deviation. Mut6, which has the NAQ to NAS change, demonstrated a 7% average increase in laccase activity compared to wild-type based on the average laccase activity, although the error bars suggest that the difference may not be significant.

Example 3: Laccase variants with additional positively or negatively charged amino acid residues

[128] Five positively or five negatively charged amino acid residues were introduced on the surface of the *Cerrena* laccase. Briefly, ten pairs of oligonucleotides (*i.e.*, forward and reverse primers) were prepared to introduce the following amino acid residue changes at the indicated positions (referring to SEQ ID NO: 11): Q21E (variant S1), N130E (variant S2), T232E (variant S3), N335E (variant S4), Q479E (variant S5), Q21R (variant S6), N130R (variant S7), T232R (variant S8), N335R (variant S9), and Q479R (variant S10). PCR-mediated mutagenesis, *E. coli* transformation, and verification of the mutations, were performed as in Example 2.

[129] The PCR fragments were used to transform the Archy3 strain of *T. reesei* as in Example 2. Transformants were selected and transferred to an amdS plate (*supra*) containing 1.2 mg/ml 5-FOA and 0.5 mg/ml of uridine and grown at 28°C for 2 days. For each variant, four colonies were selected and transferred to a PDA plate containing 1.2 mg/ml 5-FOA and 0.5 mg/ml uridine. The Archy3 strain with integrated plasmid expressing wild type laccase was used as control. The plates were grown at 28°C for 1 day and left at room temperature for 3 days to encourage sporulation. All clones were transferred to a 96-well microtiter filter plate (MTP, Corning 3505) filled with 200 µl NREL defined medium with glucose/sophomores and 0.5 mg/ml uridine. The MTP filter plate was grown at 28°C with a constant oxygen supply and without shaking for 5 days. 10 µl of 5-days old liquid cultures were transferred to a new plate and 150 µl 100 mM NaOAc, pH 5, and 20 µl 4.5 mM ABTS were added.

[130] The OD_{420} was measured as in Example 2. The results are shown in Figure 6 (variants S1 to S5, which include a neutral amino acid residue changed to a negatively charged residue) and Figure 7 (variants S7 to S10, which include a neutral amino acid residue changed to a positively charged residue). Variant S2 demonstrated 17% increased laccase activity compared to wild-type. Variant S9 demonstrated a 10% increased laccase activity compared to wild-type, although this latter difference may not be significant.

Example 4: Site Evaluation Library #1 (SEL1) variants

[131] A non-conservative, hydrophobic amino acid residue (I265) located on the surface of *Cerrena* laccase was selected for further engineering. Briefly, a pair of complementary oligonucleotide primers overlapping the I265 codon were prepared and used to introduce amino acid residue changes at this position. PCR-mediated mutagenesis reaction was performed as in Example 2. The PCR products were digested with *Dpn*I restriction enzyme for 2 hours at 37°C and purified using a Qiagen column. The SEL library variants were then cloned to expression plasmid pTrex3g using the gateway cloning method in a reaction containing of 3 µl of PCR product, 0.5 µl of pTrex3g, 0.5 µl of TE buffer, and 1 µl of LRII mixture (Invitrogen), which were incubated at room temperature for one hour. The mixture was transformed into *E. coli* cells.

[132] DNA was prepared from 28 clones and subjected to DNA sequence analysis. A total of 13 variants were obtained. The codons and corresponding amino acid residues at position

265 are listed in Figure 8. 5 µl DNA of each of the 13 variants was pooled and used as DNA template for PCR fragment amplification. Ten tubes of 100-µl PCR mixes were prepared and used to transform the Archy3 strain of *T. reesei* as in Example 2, except that 10 mls of overlays containing 1.2 mg/ml 5-FOA and 0.5 mg/ml uridine were added after 24 hours incubation at 28°C. Transformants were selected and transferred to two 48-well MTPs filled with 1 ml of PDA containing 1.2 mg/ml 5-FOA and 0.5 mg/ml uridine. The MTPs were grown at 28°C for 2 days and left at room temperature for 2 day to promote sporulation. All clones were individually transferred to a 96-well filter plate and incubated for 5 days. The ABTS assay was performed as in Example 2. Figure 9 shows the ABTS activity assay result for all 88 transformants screened. The identity of the variant laccase sequences were unknown at the time of screening, therefore the X-axis has no labels. Six clones showing higher ABTS activity were selected for further study, hence corresponding mycellia from the filter plate were grown in YEGfor genomic DNA extraction. A 600 bp fragment was amplified using two primers flanking the codon corresponding to amino acid position 265. The PCR fragments were sequenced to identify the mutations present. Figure 10 shows the variants that produced the highest laccase expression or activity, *i.e.*, I265R, I265H, and I265V.

Example 5: Site Evaluation Library #2 (SEL2) variants

[133] A non-conservative, hydrophobic amino acid residue (V287) located on the surface of *Cerrena* laccase was selected for further engineering. Briefly, pairs of complementary primers overlapping the V287 codon were prepared and used to introduce amino acid residue changes. The SEL2 library variants were generated in same way as the SEL1 variants described in Example 4, except that all *E.coli* transformants were pooled and plasmids were extracted from pooled *E. coli* cells and used as mixed DNA template for PCR fragment amplification.

[134] Ten tubes of 100 µl PCR reactions were prepared, and the PCR fragments were transformed into the Archy3 strain of *T. reesei* as in Example 4. Transformants were selected and transferred to 96-well MTPs filled with 0.2 ml of PDA containing 1.2 mg/ml 5-FOA and 0.5 mg/ml of uridine. The MTPs were incubated at 28°C for 2 days and left at room temperature for 3 days to promote sporulation. All clones were transferred to a 96-well filter plate using a metal replicator for 96 well plates (Boekel). The filter plate was incubated for 5 days and the ABTS assay was performed as in Example 2. Figure 11 shows the ABTS activity assay result for all 88 transformants screened. As in Example 4, the identity of the variant laccase sequences were unknown at the time of screening, therefore the X-axis has no labels.

[135] Genomic DNA was prepared using 5-day old mycellium from the filter plate, and subjected to DNA sequence analysis. A total of 14 variants were identified, *i.e.*, V287A, V287D, V287E, V287F, V287G, V287H, V287L, V287N, V287P, V287Q, V287R, V287S, V287T, and V287W. Figure 12 shows data obtained from the three best variants, *i.e.*, B1, which includes the V287P mutation, C2, which includes the V287H mutation as well as another mutation (F68L), presumably resulting from a PCR error, and G3, which includes the V287G mutation.

Example 6: Site Evaluation Library #3 (SEL3) variants

[136] A non-conservative, hydrophobic amino acid residue (V319) located on the surface of *Cerrena* laccase was selected for further engineering. Briefly, pairs of complementary primers overlapping the V319 codon were prepared and used to introduce amino acid residue changes. The SEL3 library variants were generated in same way as the SEL1 variants in Example 4, except that all *E.coli* colonies were subjected to DNA sequence analysis. *E. coli* cultures of 17 variants were pooled and plasmid DNA was extracted. Figure 13 lists the 17 variants identified. The DNA was then used as template for PCR, and the PCR fragments were transformed into the Archy3 strain of *T. reesei* as in Example 4.

[137] 65 transformants were selected and transferred to a 96-well MTP filled with 0.2 ml of PDA containing 1.2 mg/ml 5-FOA and 0.5 mg/ml uridine using a colony picker (CP-7200, Norgren Systems). The MTP was incubated at 28°C for 2 days and left at room temperature for 3 days to promote sporulation. All clones were transferred to a 96-well filter plate, which was incubated for 5 days. An ABTS assay was performed as Example 2. Figure 14 shows the ABTS activity for all 65 transformants. The four transformants showing higher ABTS activity were selected for further analysis, and the mutations were identified as in Example 4. Figure 15 shows the variants that produced the highest laccase expression or activity, *i.e.*, V319W and V319T.

Example 7: Site Evaluation Library #4 (SEL4) variants

[138] A non-conservative, hydrophobic amino acid residue (V293) located on the surface of *Cerrena* laccase was selected for further engineering. Briefly, pairs of complementary primers overlapping the V293 codon were prepared and used to introduce amino acid residue changes. The SEL4 library variants were generated in same way as the SEL1 variants in

Example 4. DNA from *E. coli* colonies were subjected to DNA sequence analysis. DNA from each of 16 different variants was used as template in separate PCR reactions. Three tubes of 100 µl of PCR reactions were performed for each variant, and the resulting 16 different PCR fragments were separately transformed into the Archy3 strain of *T. reesei* as in Example 4.

[139] Four transformants corresponding to each variant were selected and transferred to a 96-well MTP filled with 0.2 ml of PDA containing 1.2 mg/ml 5-FOA and 0.5 mg/ml uridine. The MTP was incubated at 28°C for 1 days and left at room temperature for 3 days to promote sporulation. All clones were transferred to a 96-well filter plate, which was incubated for 5 days. The ABTS assay was performed as in Example 2. Figure 16 showed the ABTS activity for all the transformants screened. The results indicate that two variants (V293N and V293T) demonstrated higher laccase expression or activity than the wild type control.

Example 8: Combinatorial variants

[140] Three mutations (*i.e.*, V287G, V293T, and V319T) were selected for combination with mutation I265R. Three primers (*i.e.*, the V287G reverse primer, the V293T forward primer, and the V319 forward primer) were prepared and used to introduce amino acid residue changes at all three position and to generate all possible combinations that include the I265R mutation. As in Example 4, a single PCR reaction was used. Five variants were obtained, *i.e.*, I265R/V287G, I265R/V293T, I265R/V319T, I265R/V287G/V319T, and I265R/V287G/V293T/V319T.

[141] Plasmid DNA corresponding to each of the five different variants was then used as a template for PCR. Three tubes of 100-µl PCR reactions were prepared using each template, and each of the five resulting PCR fragments was transformed the Archy3 strain of *T. reesei* as in Example 2. Six transformants from each variant were picked to a 96-well MTP filled with 0.2 ml of PDA containing 1.2 mg/ml 5-FOA and 0.5 mg/ml uridine. The MTP was incubated at 28°C for over 1 day and left at room temperature for 2 days to promote sporulation. Spores were transferred to a 96-well filter plate and incubated for 5 days at 28°C. An ABTS assay was performed as in Example 2. Figure 17 shows the ABTS activity assay for all transformants screened. The results indicate that combinations of mutations produced laccase variants having greater expression and/or specific activity that the wild-type laccase.

[142] Four additional mutations (F68L, V287P, N335R, and N130E) were also selected for combination with mutation I265R. Four pairs of primers were prepared and used to introduce the indicated amino acid residue changes in combination with variant I265R, as in Example 4. Four variants were obtained, *i.e.*, I265R/V287P, F68L/I265R, I265R/N335R, and I265R/N130E. Plasmid DNA corresponding to each of the four different variants was then used as template for PCR. Three tubes of 100-µl PCR reactions were prepared using each template, and each of the four resulting PCR fragments was transformed the Archy3 strain of *T. reesei* as in Example 2. Six transformants from each variant were picked to a 96-well MTP filled with 0.2 ml of PDA containing 1.2 mg/ml 5-FOA and 0.5 mg/ml uridine. The MTP was incubated at 28°C for over 1 day and left at room temperature for more than 2 days to promote sporulation. Spores were transferred to a 96-well filter plate and incubated for 5 days at 28°C. An ABTS assay was performed as in Example 2.

[143] Figure 18 shows the ABTS activity assay for all the transformants screened. The results indicate that the laccase variant having the combination of the F68L and I265R mutations had much greater expression and/or specific activity that the wild-type laccase, or other variants tested. Plasmid DNA encoding the F68L/I265R laccase variant and plasmid DNA encoding the wild-type laccase were separately transformed into *T. reesei* cell using biolistic transformation. A total of 12 stable F68L/I265R transformants (*i.e.*, the “67” clones) and 14 wild-type transformants (*i.e.*, the “42” clones) were obtained. 8 stable transformants of each type were grown in shake flasks and tested for laccase activity. As shown in Figure 19, the variant laccase (*i.e.*, the “67” clones) demonstrated more than a 4-fold increases in expression and/or specific activity compared to the wild type laccase (*i.e.*, the “42” clones).

CLAIMS

What is claimed is:

1. A variant laccase enzyme derived from a parental laccase enzyme, the variant laccase enzyme having:

(a) a mutation at a position corresponding to position 68 of the amino acid sequence of SEQ ID NO: 11;

(b) a mutation that alters the surface charge of the parental laccase enzyme;

(c) a mutation that alters the surface hydrophobicity of the parental laccase enzyme; or

(d) a mutation at an amino acid position corresponding to a non-conservative, hydrophobic amino acid residue located on the surface of the parental laccase enzyme;

wherein the mutation is a substitution to a different amino acid residue compared to the parental laccase.

2. The variant laccase enzyme of claim 1, having a mutation at a position corresponding to position 68 of the amino acid sequence of SEQ ID NO: 11, wherein the mutation is a substitution of an aromatic amino acid residue to a non-aromatic amino acid residue.

3. The variant laccase enzyme of claim 2, wherein the mutation is a substitution of an aromatic amino acid residue to an aliphatic amino acid residue.

4. The variant laccase enzyme of claim 3, wherein the mutation is a substitution of an aromatic amino acid residue to A, V, L, or I.

5. The variant laccase enzyme of claim 4, wherein the mutation is equivalent to F68L in SEQ ID NO: 11.

6. The variant laccase enzyme of claim 1, having a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at a position equivalent to position 130, 265, 287, 293, or 319, in SEQ ID NO: 11.

7. The variant laccase enzyme of claim 1, having a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at a position equivalent to position 130 in SEQ ID NO: 11.

8. The variant laccase enzyme of claim 1, having a mutation that alters the surface charge or alters the surface hydrophobicity of the parental laccase enzyme, wherein the mutation is at:

(a) an amino acid position equivalent to position 130 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from D, E, R, and K;

(b) an amino acid position equivalent to position 265 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from R, H, and V;

(c) an amino acid position equivalent to position 287 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from P, H, and G;

(d) an amino acid position equivalent to position 293 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from N, T, and S; or

(e) an amino acid position equivalent to position 319 in SEQ ID NO: 11, wherein the residue in the parental laccase is substituted with a different residue selected from W, T, and S.

9. The variant laccase enzyme of claim 1, having mutations equivalent to:

(a) I265R/V287G,
(b) I265R/V293T;
(c) I265R/V319T;
(d) I265R/V287G/V319T;
(e) I265R/V287G/V293T/V319T;
(f) I265R/V287P;
(g) I265R/N335R;
(h) I265R/N130E;
(i) F68L/I265R;
(j) F68L/I265R/V287G;
(k) F68L/I265R/V293T;
(l) F68L/I265R/V319T;

(m) F68L/I265R/V287G/V319T;
(n) F68L/I265R/V287G/V293T/V319T;
(o) F68L/I265R/V287P;
(p) F68L/I265R/N335R; or
(q) F68L/I265R/N130E;
in SEQ ID NO: 11.

10. The variant laccase enzyme of any of the preceding claims, wherein the parental laccase is obtainable from a *Cerrena* species.

11. The variant laccase enzyme of any of the preceding claims, wherein the parental laccase is obtainable from *Cerrena unicolor*.

12. The variant laccase enzyme of any of the preceding claims, wherein the parental laccase is laccase D from *C. unicolor*.

13. The variant laccase enzyme of any of the preceding claims, wherein the parental laccase has an amino acid sequence selected from the group consiting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, and SEQ ID NO: 28.

14. The variant laccase enzyme of any of the preceding claims, having an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO: 11.

15. The variant laccase enzyme of any of the preceding claims, having an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO: 11.

16. The variant laccase enzyme of any of the preceding claims, having an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO: 11.

17. The variant laccase enzyme of any of the preceding claims, having an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 11.

18. The variant laccase enzyme of any of the preceding claims, further comprising a mutation that introduces a glycosylation site into the amino acid sequence of the parental laccase.

19. A composition comprising the variant laccase of any of the preceding claims.

20. The composition of claim 19, further comprising a chemical mediator.

21. The composition of claim 20, wherein the chemical mediator is a phenolic compound.

22. The composition of claim 21, wherein the chemical mediator is a phenolic compound is selected from the group consisting of syringonitrile, acetosyringone, and methyl syringate.

23. A method of bleaching a surface comprising contacting the surface with a composition of any of the preceding claims.

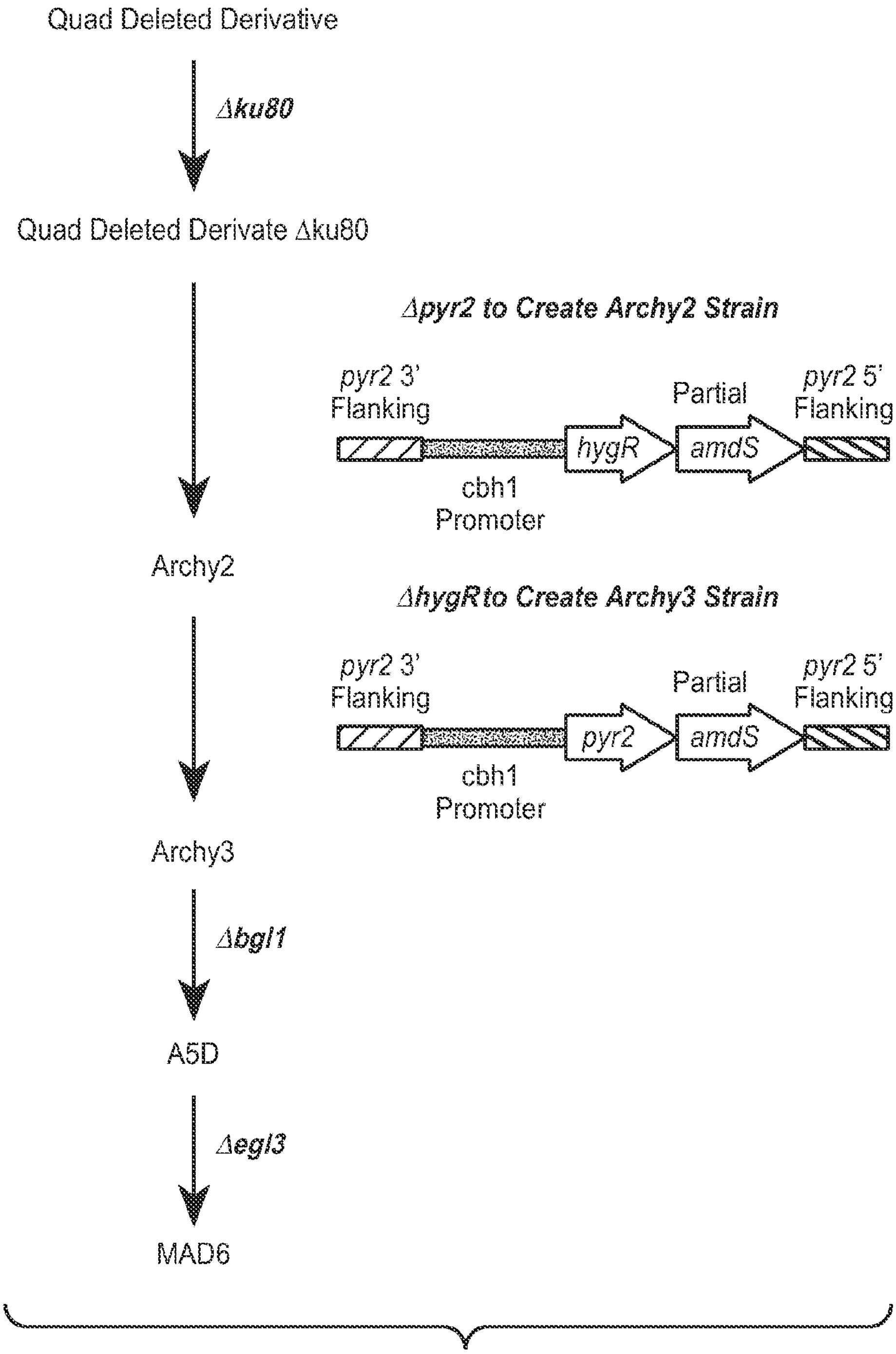

Quad Deleted Derivative
Δku80
Quad Deleted Derivate Δku80
Δpyr2 to Create Archy2 Strain
pyr2 3' Flanking
Partial
pyr2 5' Flanking
hygR
amdS
cbh1 Promoter
Archy2
ΔhygR to Create Archy3 Strain
pyr2 3' Flanking
Partial
pyr2 5' Flanking
pyr2
amdS
cbh1 Promoter
Archy3
Δbgl1
A5D
Δegl3
MAD6

FIG. 1

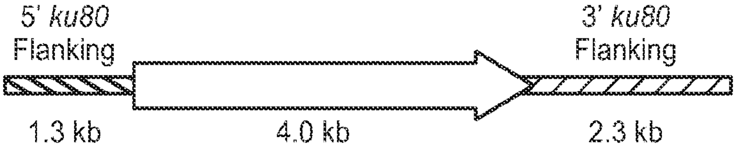


FIG. 2

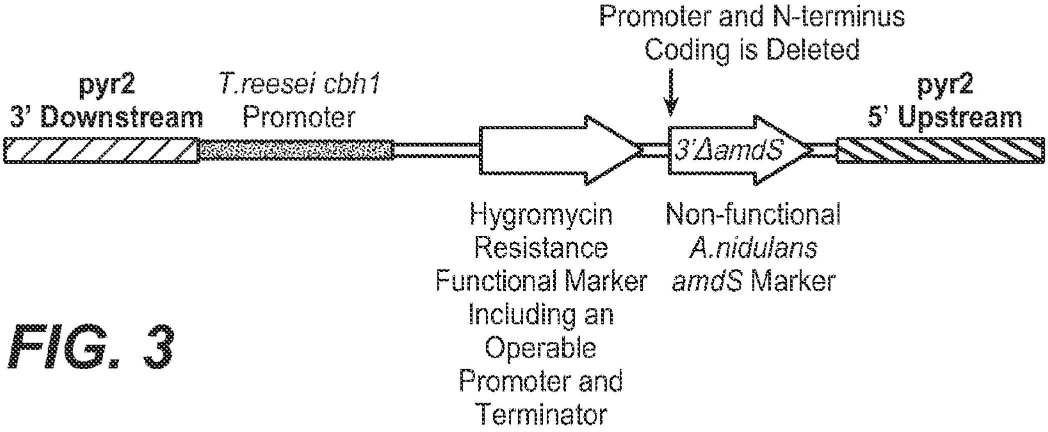


FIG. 3

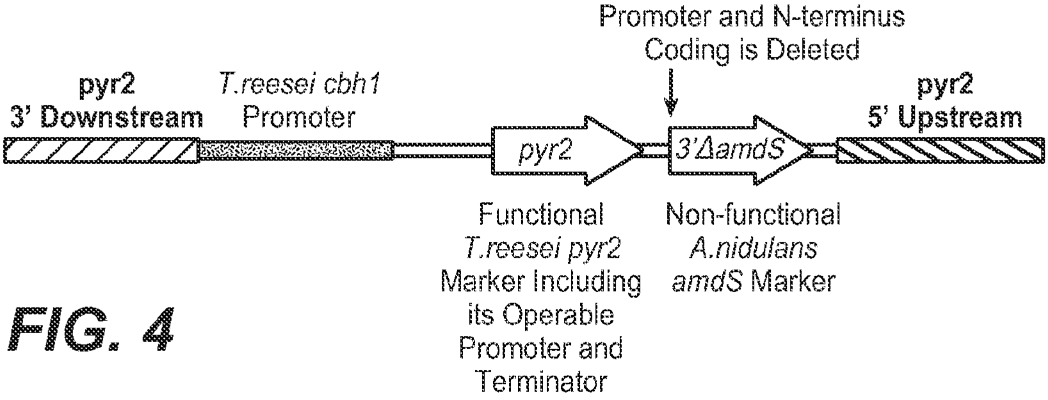


FIG. 4

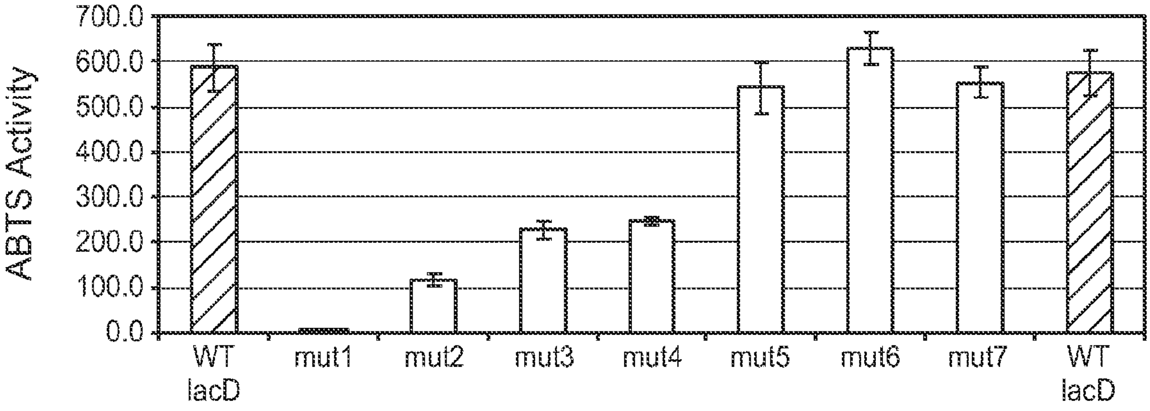


FIG. 5

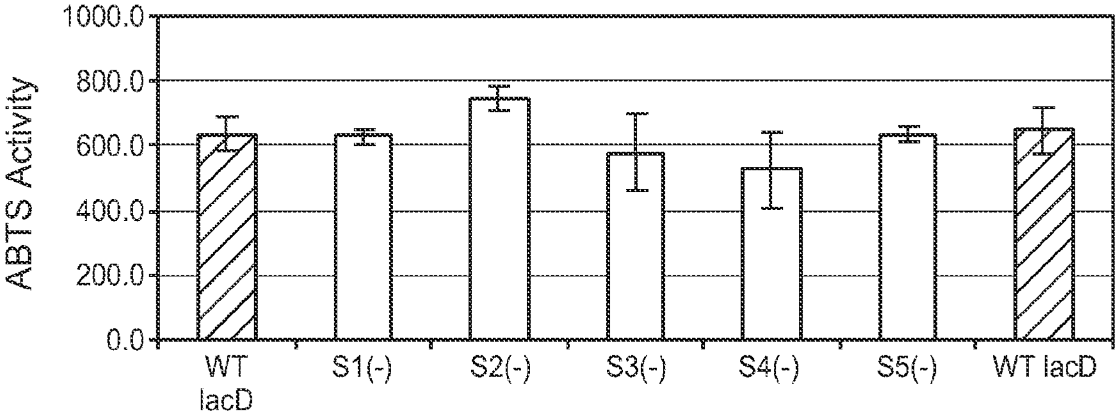


FIG. 6

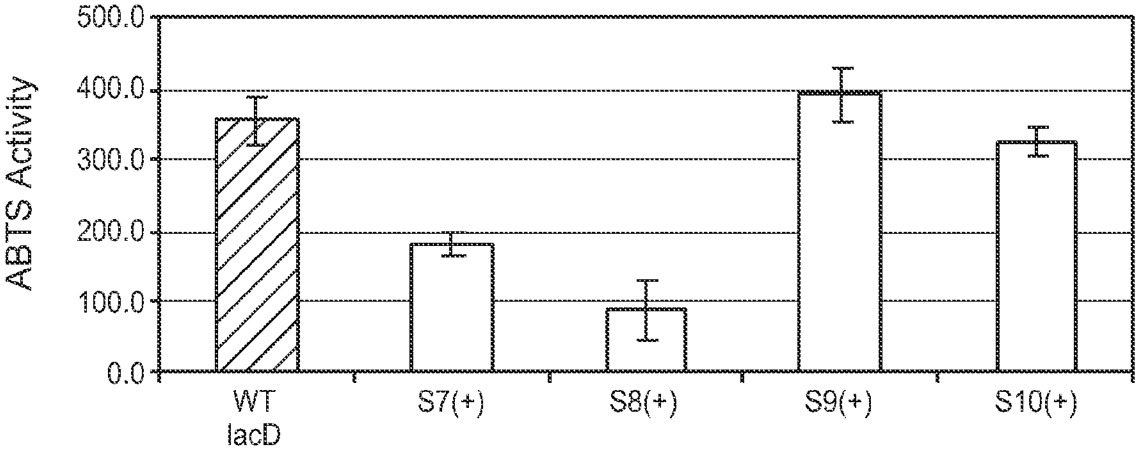


FIG. 7

I265V	GTC
I265P	CCC
I265H	CAC
I265A	CAC
I265E	GAG
I265L	CTC
I265D	GAC
I265S	TCC
I265G	GGC
I265R	AGG
I265T	ACG
I265F	TTC
I265Y	TAC

FIG. 8

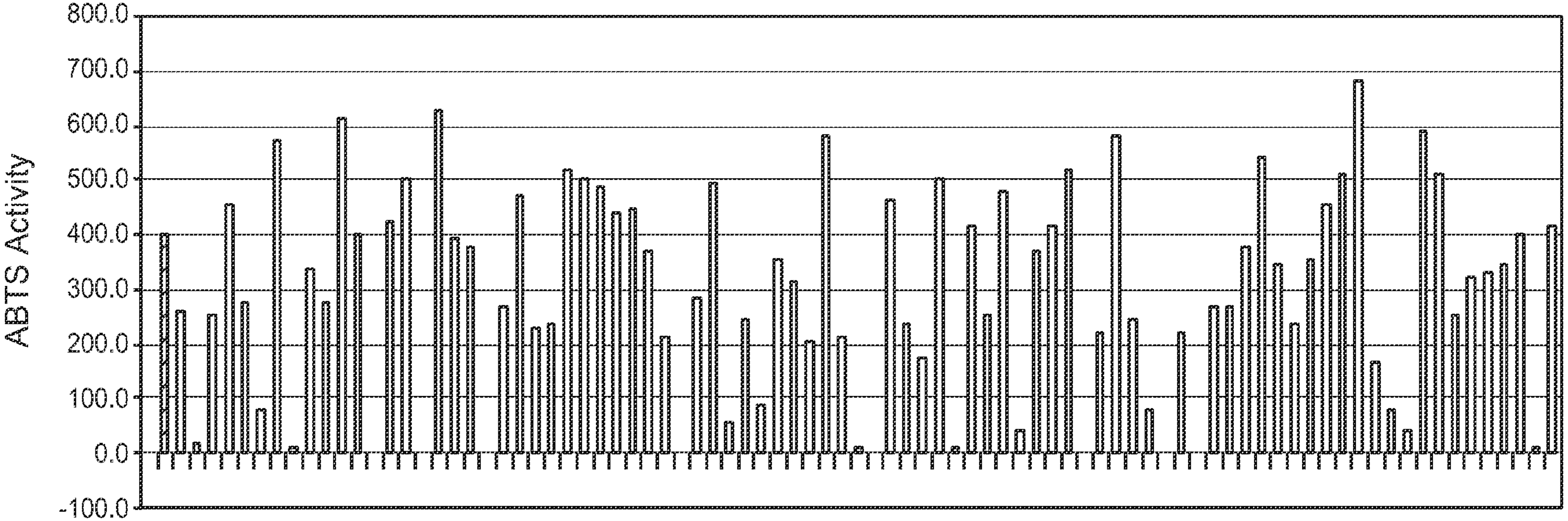


FIG. 9

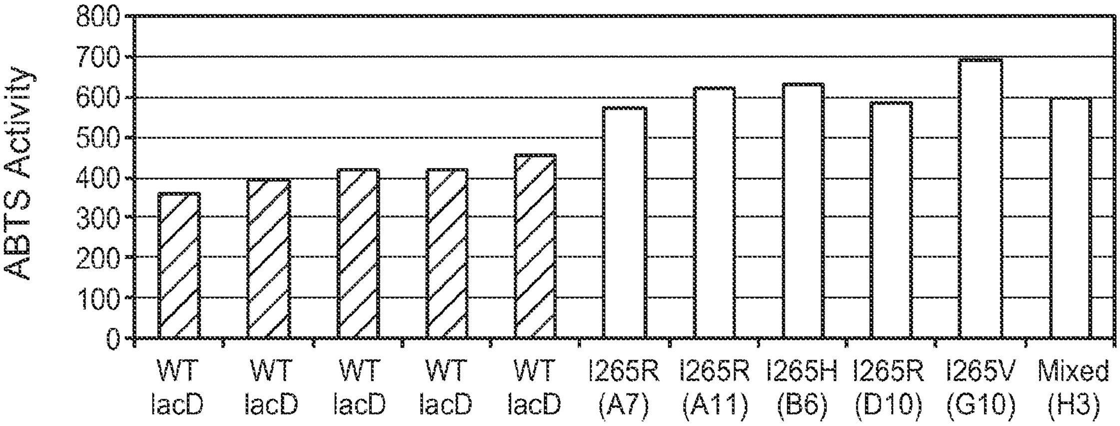


FIG. 10

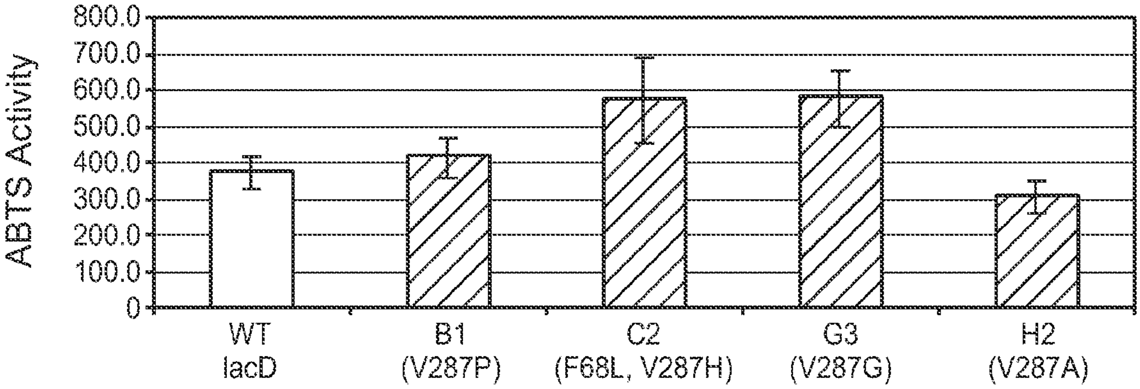


FIG. 12

A	GCC
C	TGC
D	GAC
E	GAG
F	TTC
G	GGC
H	CAC
K	AAG
L	TTG
M	ATG
N	AAC
P	CCC
Q	CAG
R	AGG
S	TCG
T	ACC
W	TGG

FIG. 13

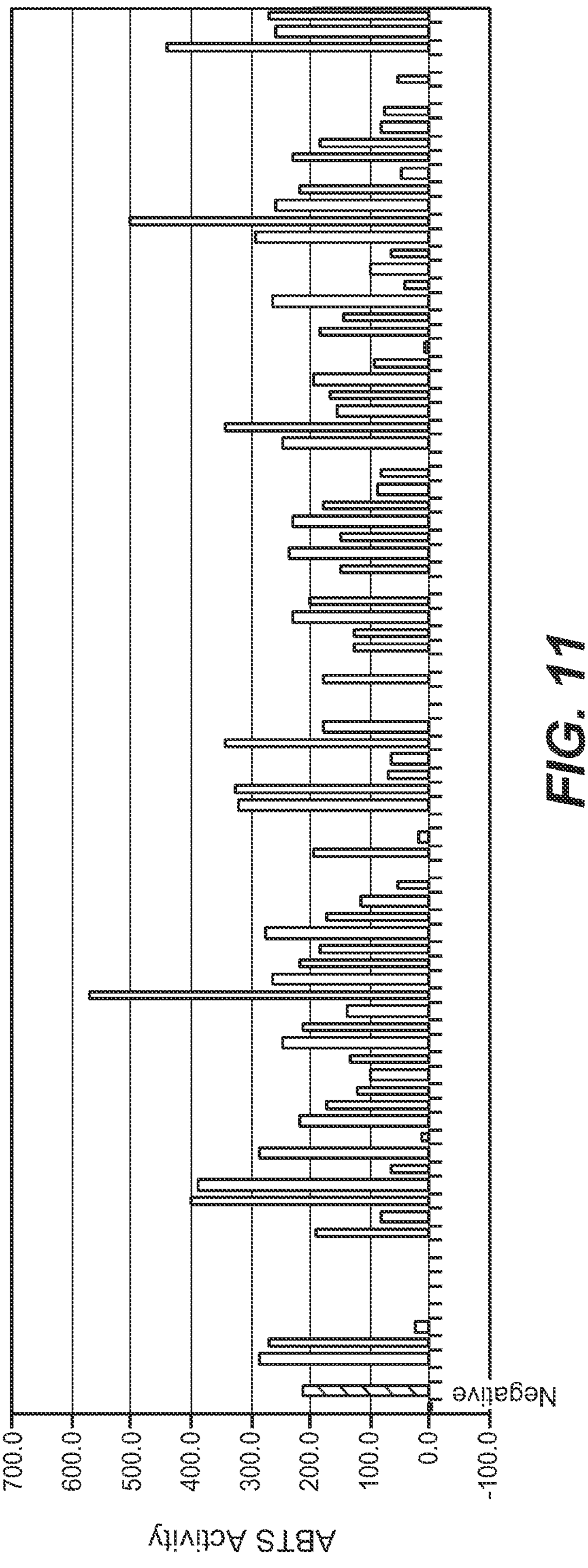

ABTS Activity
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
-100.0
Negative

FIG. 11

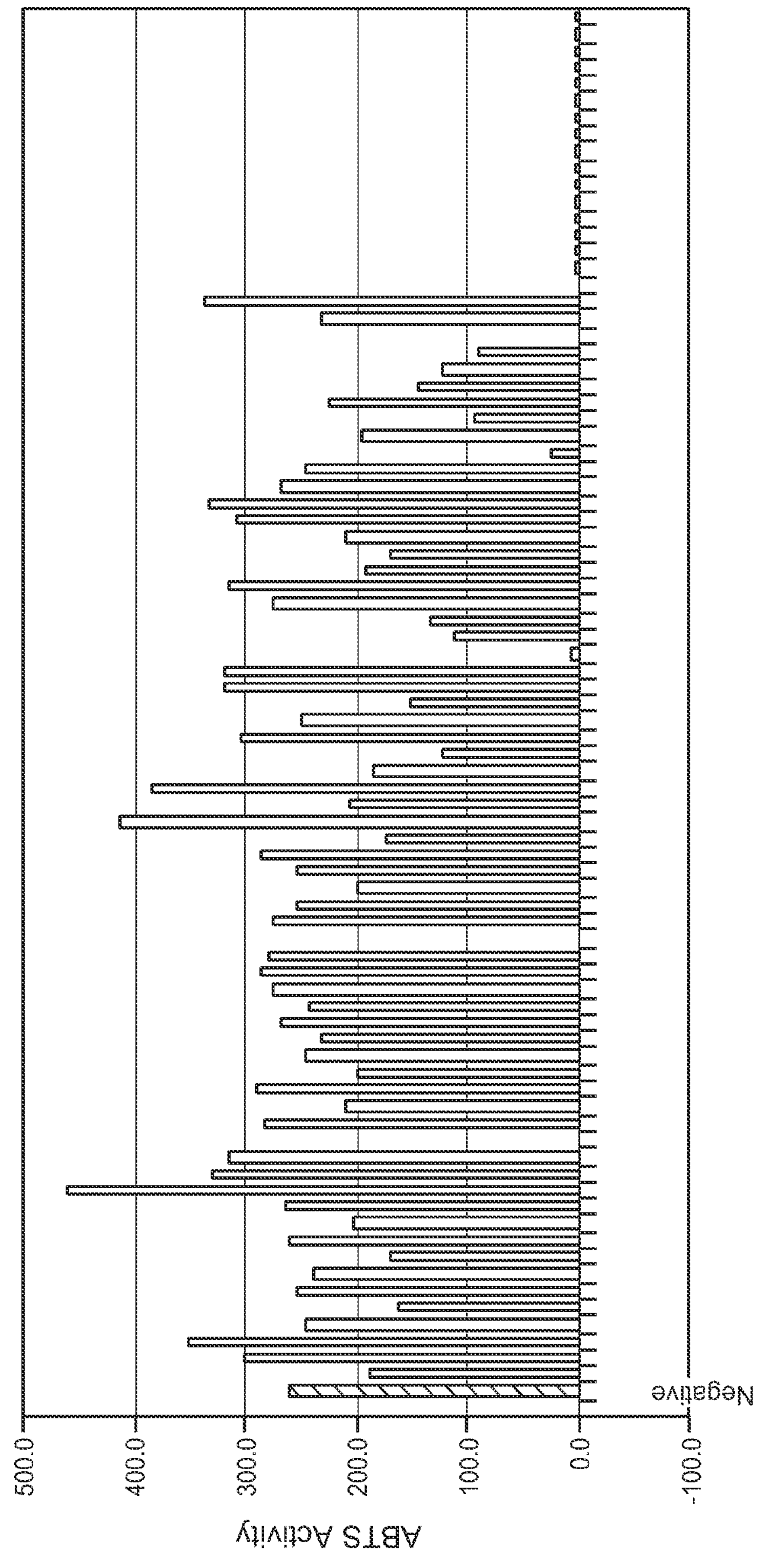


FIG. 14

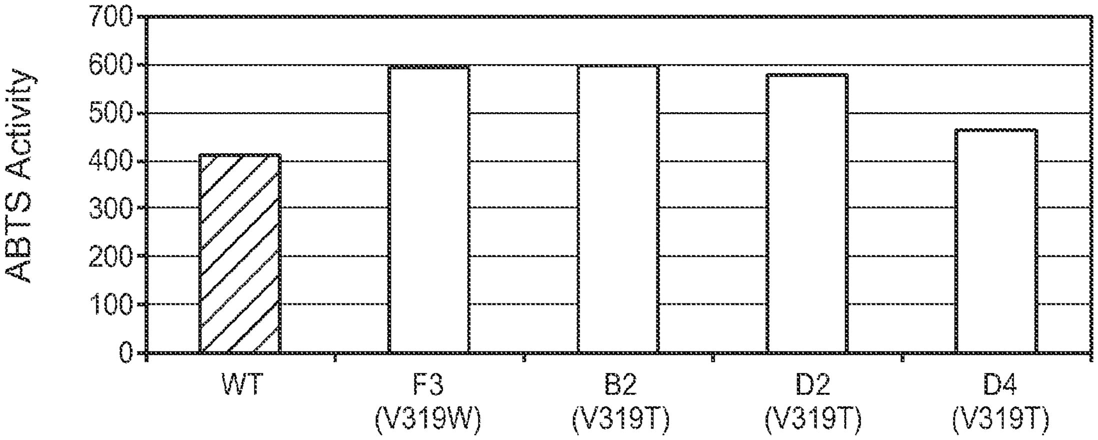


FIG. 15

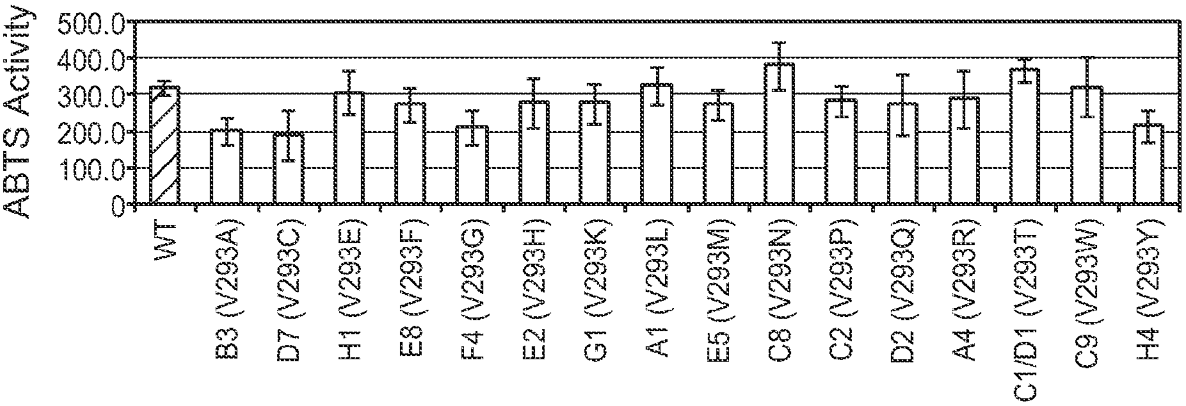


FIG. 16

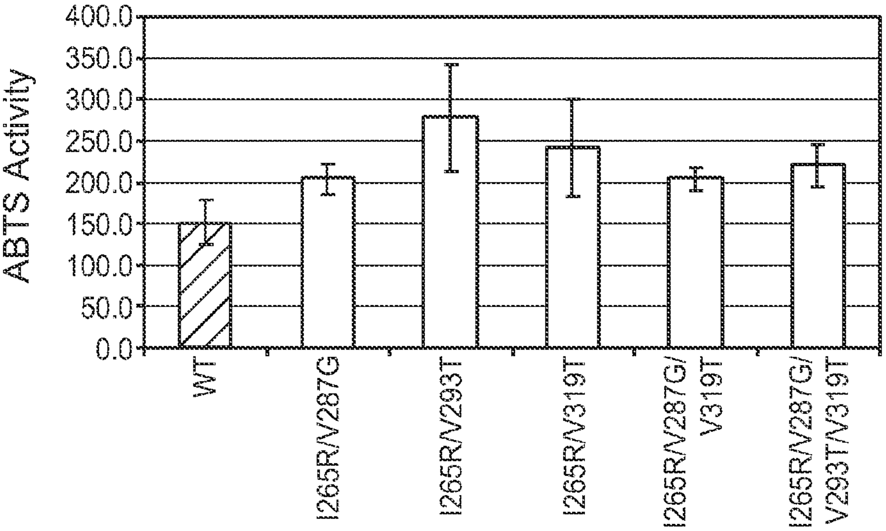


FIG. 17

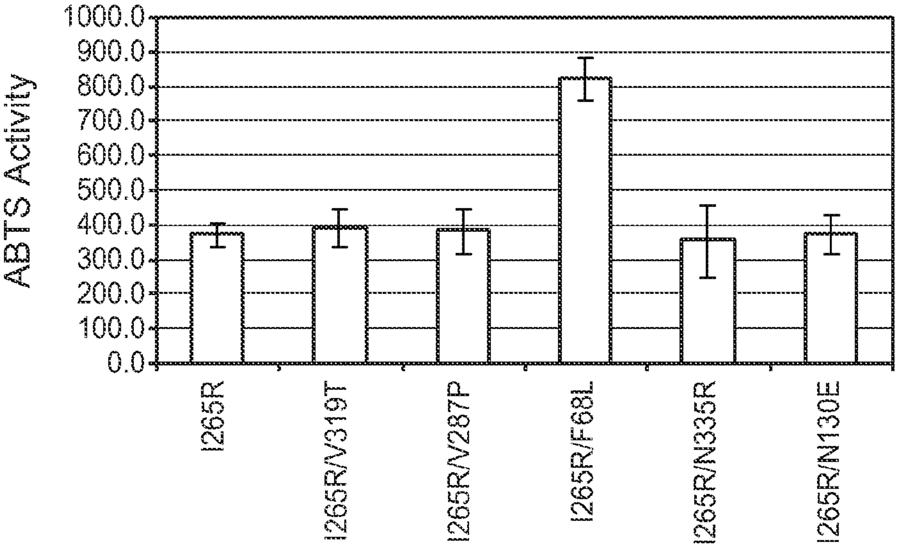

ABTS Activity
1000.0
900.0
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
I265R
I265R/V319T
I265R/V287P
I265R/F68L
I265R/N335R
I265R/N130E

FIG. 18

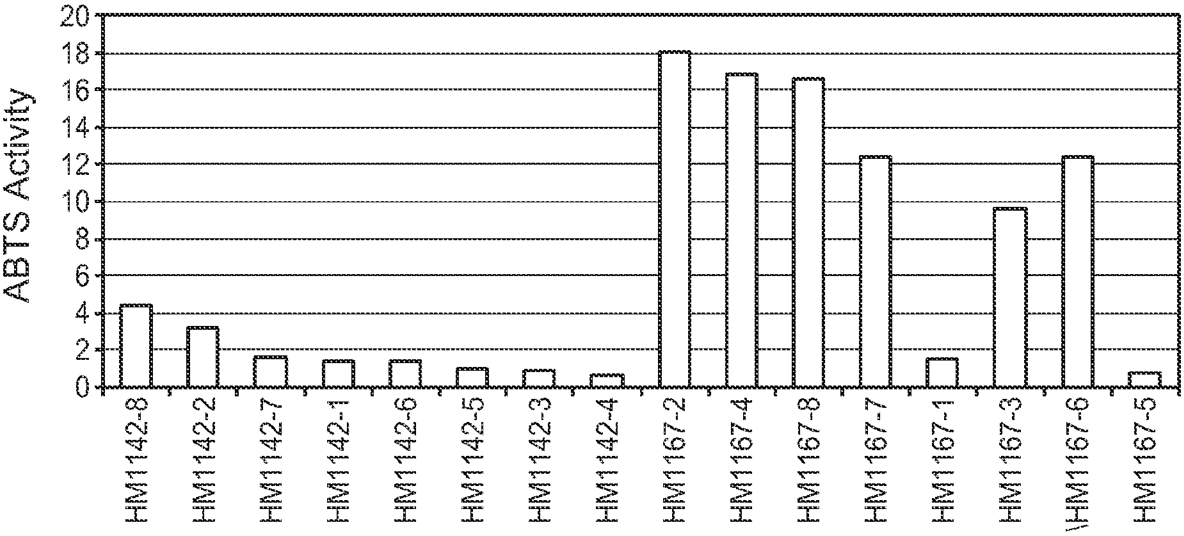

ABTS Activity
20
18
16
14
12
10
8
6
4
2
0
HM1142-8
HM1142-2
HM1142-7
HM1142-1
HM1142-6
HM1142-5
HM1142-3
HM1142-4
HM1167-2
HM1167-4
HM1167-8
HM1167-7
HM1167-1
HM1167-3
\HM1167-6
HM1167-5

FIG. 19

```
SEQ_ID_NO:7   MGLNSAITSLAILALSVGSYAAIGPVADIHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQ
SEQ_ID_NO:10  MGLNSAITSLAILALSVGSYAAIGPVADLHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQ
SEQ_ID_NO:8   MGLNSAITSLAILALSVGSYAAIGPVADIHIVNKDLAPDGVQRPTVLAGGTFPGTLITGQ
              ****************************:*******************************

SEQ_ID_NO:7   KGDNFQLNVIDDLTDDRMLTPTSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDV
SEQ_ID_NO:10  KGDNFQLNVIDDLTDDRMLTPTSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDV
SEQ_ID_NO:8   KGDNFQLNVIDDLTDDRMLTPTSIHWHGFFQKGTAWADGPAFVTQCPIIADNSFLYDFDV
              ************************************************************

SEQ_ID_NO:7   PDQAGTFWYHSHLSTQYCDGLRGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTV
SEQ_ID_NO:10  PDQAGTFWYHSHLSTQYCDGLRGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTV
SEQ_ID_NO:8   PDQAGTFWYHSHLSTQYCDGLRGAFVVYDPNDPHKDLYDVDDGGTVITLADWYHVLAQTV
              ************************************************************

SEQ_ID_NO:7   VGAATPDSTLINGLGRSQTGPADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMT
SEQ_ID_NO:10  VGAATPDSTLINGLGRSQTGPADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMT
SEQ_ID_NO:8   VGAATPDSTLINGLGRSQTGPADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMT
              ************************************************************

SEQ_ID_NO:7   VIEVDGVNTRPLTVDSIQIFAGQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAI
SEQ_ID_NO:10  VIEVDGVNTRPLTVDSIQIFAGQRYSFVLNANQPEDNYWIRAMPNIGRNTTTLDGKNAAI
SEQ_ID_NO:8   VIEVDGVNTRPLTVDSIQIFAGQRYSFVLNANQPDDNYWIRAMPNIGRNTTTLDGKNAAI
              **********************************:*************************

SEQ_ID_NO:7   LRYKNASVEEPKTVGGPAQSPLNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFS
SEQ_ID_NO:10  LRYKNASVEEPKTVGGPAQSPLNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFS
SEQ_ID_NO:8   LRYKNASVEEPKTVGGPAQSPLNEADLRPLVPAPVPGNAVPGGADINHRLNLTFSNGLFS
              ************************************************************

SEQ_ID_NO:7   INNASFTNPSVPALLQILSGAQNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLH
SEQ_ID_NO:10  INNASFTNPSVPALLQILSGAQNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLH
SEQ_ID_NO:8   INNASFTNPSVPALLQILSGAQNAQDLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLH
              ************************************************************

SEQ_ID_NO:7   GHNFWVVRSAGSDEYNFDDAILRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAG
SEQ_ID_NO:10  GHNFWVVRSAGSDEYNFDDAILRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAG
SEQ_ID_NO:8   GHNFWVVRSAGSDEYNFDDAILRDVVSIGAGTDEVTIRFVTDNPGPWFLHCHIDWHLEAG
              ************************************************************

SEQ_ID_NO:7   LAIVFAEGINQTAAANPTPQAWDELCPKYNGLSASQKVKPKKGTAI
SEQ_ID_NO:10  LAIVFAEGINQTAAANPTPQAWDELCPKYNGLSASQKVKPKKGTAI
SEQ_ID_NO:8   LAIVFAEGINQTAAANPTPQAWDELCPKYNGLSASQKVKPKKGTAI
              **********************************************
```

FIG. 20

```
Majority                    AIGPVADLTIXNADVSPDG--FTRAAVLAGG-----VFPGPLITGNKGDRFQLNVIDNLTNHTMLKSTSIHWHGFFQKGT
                            ---------+---------+---------+---------+---------+---------+---------+---------+
                                    10        20        30        40        50        60        70        80
                            ---------+---------+---------+---------+---------+---------+---------+---------+
Cerrena laccase D           AIGPVADIHIVNKDLAPDG--VQRPTVLAGG-----TFPGTLITGQKGDNFQLNVIDDLTDDRMLTPTSIHWHGFFQKGT  73
Cerrena laccase A           GIGPVTDLRITNQDIAPDG--FTRPAVLAGG-----TFPGALITGQKGDSFQINVIDELTDASMLTQTSIHWHGFFQKGS  73
Cerrena laccase B           AIGPVTDLHIVNQNLDPDG--FNRPTVLAGG-----TFPGPLIRGNKGDNFKINVIDDLTEHSMLKATSIHWHGFFQKGT  73
Panus rudis                 AIGPVTDLHIVNDNIAPDG--FTRAAVLAGG-----TFPGPLITGNKGDNFRIDVIDDLTEESMLKSTSIHWHGFFQKGT  73
Spongipellis sp.            AVGPVADIHIVDASIAPDG--FSRPAVLAGG-----SFPGPLITGKKGDAFKLNIIDDLTNEDMLKSTSIHWHGFFQKGT  73
Curiolus versicolor CVL3    GIGPVADLTITNAAVSPDG--FSRQAVVVNG-----GTPGPLITGNMGDRFQLNVIDNLTNHTMLKSTSIHWHGFFQKGT  73
Lentinus sp.                AIGPVTDLHIVNSFIQPDG--FNRSGVLAEG-----VFPGPLITGNKGDNFQINVIDELTNGTMLLSTSIHWHGLFQKTT  73
Ceriporiopsis subvermispora AIGPVTDLEITDAFVSPDGPGLGREAVLAGG-----TFPGPLIQGNKGDNFQINVVNNLTNHTMLKTTSIHWHGLFQHGT  75
Cyathus bulleri             AIGPVLDMHIVNKVISPDG--FNRSAVLAGGTADNADFPGPLVTGNKGDHFQLNVIDSLTDTTMLRGTSIHWHGLFQHGT  78
Pycnoporus sanguineus       AIGPVADLTLTNAAVSPDG--FSREAVVVNG-----QTPGPLIAGQKGDRFQLNVIDNLTNHTMLKTTSIHWHGFFQHGT  73
Trametes villosa (1)        GIGPVADLTITNAAVSPDG--FSRQAVVVNG-----GTPGPLITGNMGDRFQLNVIDNLTNHTMVKSTSIHWHGFFQKGT  73
Trametes villosa (2)        AIGPVASLVVANAPVSPDG--FLRDAIVVNG-----VVPSPLITGKKGDRFQLNVVDTLTNHSMLKSTSIHWHGFFQAGT  73
Curiolus versicolor CVL G1  AIGPVTDLTISNADVTPDG--ITRAAVLAGG-----VFPGPLITGNKGDEFQINVIDNLTNETMLKSTTIHWHGIFQAGT  73
Trametes sp. LCC1           AIGPVASLVVANAPVSPDG--FLRDAIVVNG-----VVPSPLITGKKGDRFQLNVDDTLTNHSMLKSTSIHWHGFFQAGT  73
Ganoderma lucidum           GIGPKADLTISNANIAPDG--YTRAAVVVNG-----VFPGPLITGNKGDRFQLNVIDQLTNHTMLKTTSIHWHGFFQKGT  73
Curiolus hirsutus           AIGPTADLTISNAEVSPDG--FARQAVVVNN-----VTPGPLVAGNKGDRFQLNVIDNLTNHTMLKSTSIHWHGFFQKGT  73
Basidiomycete sp. PM1       SIGPVADLTISNGAVSPDG--FSRQAILVND-----VFPSPLITGNKGDRFQLNVIDNMTNHTMLKSTSIHWHGFFQHGT  73
Trametes sp. LCC4           AIGPVTDLTISNADVTPDG--ITRAAVLAGG-----VFPGPLITGNKGDEFQINVIDNLTNETMLKSTTIHWHGIFQAGT  73
Rigidoporus microporus      AIGPVADLHISNANISPDG--FTRAAVLAGG-----SFPGPLITGNKGDNFQINVINDLTDADQLKTTTIHWHGFFQHGT  73
Polyporus ciliatus          AIGPVADLTITNADINPDG--FTRAAVLANN-----VFPGPLITGNKGDNFQLNVVDNLSNDTMLTATTIHWHGFFQKGT  73
```

FIG. 21A

```
Majority                     NWADGPAFVNQCPIASGNSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPHASLYDVDDDSTVITLADWYH
                             ---------+---------+---------+---------+---------+---------+---------+---------+
                                    90       100       110       120       130       140       150       160
                             ---------+---------+---------+---------+---------+---------+---------+---------+
Cerrena laccase D            AWADGPAFVTQCPIIADNSFLYDFDVPDQAGTFWYHSHLSTQYCDGLRGAFVVYDPNDPHKDLYDVDDGGTVITLADWYH 153
Cerrena laccase A            AWADGPAFVTQCPIVTGNSFLYDFDVPDQPGTFWYHSHLSTQYCDGLRGPFVVYDPKDPNKRLYDIDNDHTVITLADWYH 153
Cerrena laccase B            NWADGPAFVTQCPITSGNAFLYDFNVPDQAGTFWYHSHLSTQYCDGLRGAFVVYDPNDPNKQLYDVDNGNTVITLADWYH 153
Panus rudis                  NWADGPAFVNQCPITTGHSFLYNFNVPDQAGTFWYHSHLSTQYCDGLRGAFVVYDPNDPHADLYDVDDDSTVITLADWYH 153
Spongipellis sp.             NWADGPAFVNQCPITTGNAFLYDFQVPDQAGTYWYHSHLSTQYCDGLRGAFVVYDPVDPHKALYDVDDETTVITLADWYH 153
Curiolus versicolor CVL3     NWADGPAFINQCPISSGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPAADLYDVDNDDTVITLVDWYH 153
Lentinus sp.                 NWADGPAFVNQCPIAANDSFLYNFNVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDPQDPYADLYDVDDDSTVITLADWYH 153
Ceriporiopsis subvermispora  TWADGPAFVSQCPIASGNSFLYNFNVPDQAGTFWYHSHLATQYCDGLRGPLVVYDPNDPHADLYDVDDESTVITLSDWYH 155
Cyathus bulleri              TWADGPVGVNQCPISPGNSFLYDFSVPDQAGTFWYHSHHSTQYCDGLRGPLVVYDPNDPHKSLYDVDDESTVITLADWYH 158
Pycnoporus sanguineus        NWADGPAFINQCPIASGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPQASLYDIDNDDTVITLADWYH 153
Trametes villosa (1)         NWADGPAFINQCPISSGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPAADLYDVDNDDTVITLVDWYH 153
Trametes villosa (2)         NWAEGPAFVNQCPIASGHSFLYDFHVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDNESTVITLTDWYH 153
Curiolus versicolor CVL G1   NWADGAAFVNQCPIATGNSFLYDFTVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDPDDPNASLYDVDDDTTVITLADWYH 153
Trametes sp. LCC1            NWADGPAFVNQCPIASGHSFLYDFHVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDNESTVITLTDWYH 153
Ganoderma lucidum            NWADGPAFINQCPIASGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPLKGLYDVDNDSTVITLSDWYH 153
Curiolus hirsutus            NWADGPAFVNQCPISSGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPHASLYDVDNDDTVITLADWYH 153
Basidiomycete sp.PM1         NWADGPAFVNQCPISTGHAFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPIVVYDPQDPHKSLYDVDDDSTVITLADWYH 153
Trametes sp. LCC4            NWADGAAFVNQCPIATGNSFLYDFTVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDPDDANASLYDVDDDTTVITLADWYH 153
Rigidoporus microporus       NWADGPAFINQCPIASGNSFLYNFQVPDQAGTFWYHSHLSTQYCDGLRGAFVVYDPDDPHASLYDVGDESTVIALADWYH 153
Polyporus ciliatus           NWADGPAFVNQCPISTGNSFLYNFNAPDQAGTFWYHSHLSTQYCDGLRGPMVVYDDADPHASLYDVDDESTVITLADWYH 153
```

FIG. 21B

```
Majority                    TAARLGPAFPXXADATLINGLGRSXS--GPA-ADLAVINVTQGKRYRFRLVSISCDPNYTFSIDGHNMTVIEVDGVNTQP
                            ---------+---------+---------+---------+---------+---------+---------+
                                    170       180       190       200       210       220       230       240
                            ---------+---------+---------+---------+---------+---------+---------+
Cerrena laccase D           VLAQTVVGAAT-PDSTLINGLGRSQT--GPADAELAVISVEHNKRYRFRLVSISCDPNFTFSVDGHNMTVIEVDGVNTRP 230
Cerrena laccase A           VLARTVVGVAT-PDATLINGLGRSPD--GPADAELAVINVKRGKRYRFRLVSISCDPNYIFSIDNHSMTVIEVDGVNTQS 230
Cerrena laccase B           ALAQTVTGVAV-SDATLINGLGRSAT--GPANAPLAVISVERNKRYRFRLVSISCDPNFIFSIDHHPMTVIEMDGVNTQS 230
Panus rudis                 TLARQITGVPT-PDATLINGLGRSAS--GPQDSELAVITVEQNKRYRLRLVSISCDPNYIFSIDSHNFTIIEVDGVNSKP 230
Spongipellis sp.            TLARQIVGVAV-ADTTLINGLGRNTN--GPSDAALAVINVTKGKRYRFRLVSISCDPNYVFSIDGHNMTIIEVDGVNSQP 230
Curiolus versicolor CVL3    VAANVGPAFPLGADATLINGKGRSPS--TTT-ADLSVISVTPGKRYRFRLVSLSCDPNYTFSIDGHNMTIIETDSINTAP 230
Lentinus sp.                VPAPQAGAVPT-SDATLINGLGRSVN--GPADAPFAVVNVVQGSRYRFRLVSISCDPNFLFSIDGHTFTVIEADGVNHEP 230
Ceriporiopsis subvermispora AAAST-LTFPT-FDTTLINGLGRFAGTGGSD-SNLTVITVEQGKRYRFRLVSISCDPNWVFSIDQHELTVIEVDGVNAVP 232
Cyathus bulleri             TPAPSAGLVPT-TDAVLINGKGRFPT--GPT-SPLSVINVTPGTKYRFRLVSISCDPNFVFSIDGHTFTIIEVDGVNVTP 234
Pycnoporus sanguineus       VAAKLGPRFPLGADATLINGLGRSPG--TTA-ADLAVIKVTQGKRYRFRLVSLSCDPNHTFSIDGHTMTIIETDSVNTQP 230
Trametes villosa (1)        VAAKLGPAFPLGADATLINGKGRSPS--TTT-ADLSVISVTPGKRYRFRLVSLSCDPNYTFSIDGHNMTIIETDSINTAP 230
Trametes villosa (2)        TAARLGPKFPLGADATLINGLGRSAS--TPT-AALAVINVQHGKRYRFRLVSISCDPNYTFSIDGHNLTVIEVDGINSQP 230
Curiolus versicolor CVL G1  TAAKLGPAFPAGPDSVLINGLGRFSGDGGGA-TNLTVITVTQGKRYRFRLVSISCDPNFTFSIDGHNMTIIEVDGVNHEA 232
Trametes sp. LCC1           TAARLGPRFPLGADATLINGLGRSAS--TPT-AALAVINVQHGKRYRFRLVSISCDPNYTFSIDGHNLTVIEVDGINSQP 230
Ganoderma lucidum           VAARLGPSFPLGSDSTLINGLGRSTT--NAT-AGLAVINVTQGKRYRFRLVSLSCDPNYTFSIDGHDMSVIEADGIATQP 230
Curiolus hirsutus           TAAKLGPAFPLGADATLINGLGRSPS--TTA-ADLAVINVTKGKRYRFRLVSLSCDPNHTFSIDGHDLTIIEVDSINSQP 230
Basidiomycete sp. PM1       LAAKVGPAVPT-ADATLINGLGRSIN--TLN-ADLAVITVTKGKRYRFRLVSLSCDPNHTFSIDGHSLTVIEADSVNLKP 229
Trametes sp. LCC4           TAAKLGPAFPAGPDSVLINGLGRFSGDGGGA-TNLTVITVTQGKRYRFRLVSISCDPNFTFSIDGHNMTIIEVGGVNHEA 232
rigidoporus microporus      GLARLGPKFPT-TNSTLINGLGRYDF--GPS-SDLAVISVQAGKRYRFRLVSISCDSNYVFSIDEHTFTVIEVDGVNHQP 229
Polyporus ciliatus          TAARLGPCFPLGSDSTLINGLGRFAG-GDAD-APLTVISVTSGKRYRFRLISISCDPNFTFTIQGHTMTVIEVDAVNVQP 231
```

FIG. 21C

```
Majority                    LTVDSIQIFAAQRYSFVLNANQPVD~NYWIRANPNFGT~TGFXGGINSAILRYDGAPVVEPTTTQTTS~TNPLNETNLHP
                            ~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+
                                     250       260       270       280       290       300       310       320
                            ~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+~~~~~~~~~+
Cerrena laccase D           LTVDSIQIFAGQRYSFVLNANQPDD~NYWIRAMPNIGRNTTTLDGKNAAILRYKNASVEEPKTVGGPA~QSPLNEADLRP 308
Cerrena laccase A           LTVDSIQIFAGQRYSFVLHANRPEN~NYWIRAKPNIGTDTTTDSGMNSAILRYNGAPVAEPQTVQSPS~LTPLLEQNLRP 308
Cerrena laccase B           MTVDSIQIFAGQRYSFVMQANQPVG~NYWIRAKPNVG~NTTFLGGLNSAILRYVGAPDQEPTTDQTPN~STPLVEANLRP 307
Panus rudis                 LTVDSAQIFAAQRYSVVLNANQPVG~NYRIRARPNNG~DTTFTNGRNSAILRYKGATVEEPATITPPVSQTPLIEANLKP 308
Spongipellis sp.            LNVDSIQIFAGQRYSFVLNANQNVG~NYWVRANPNLG~TTGFTGGINSAILRYQGATVAEPTTAQTTS~TNPMQEPNLHP 307
Curiolus versicolor CVL3    LVVDSIQIFAAQRYSFVLEANQAVD~NYWIRANPNFGN~VGFTGGINSAILRYDGAAAVEPTTTQTTS~TEPLNEVNLHP 307
Lentinus sp.                IVADSIQIFAAQRYSFILTANQTAD~NYWIRANPNNGH~TGFAGGINSAILRYSGAPVADPVTTQTS~~ANLLQETSLVP 306
Ceriporiopsis subvermispora LTVDAIQIFAAQRYSFVLNANQTVD~NYWIRANPNNGN~MGFANGINSAILRYVGADDVEPTSTGTTA~N~LLNEADLSP 308
Cyathus bulleri             VEVDSIQIFAGQRYSFVLNANQPVD~NYWIRAKPNIAKGVTFDGGINSAILRYAGAPDTDPTTSQTPN~SAPMVETDLHP 312
Pycnoporus sanguineus       LEVDSIQIFAAQRYSFVLDANQPVD~NYWIRANPSFGN~TGFAGGINSAILRYDGAPEVEPTTNQTTP~TKPLNEVDLHP 307
Trametes villosa (1)        LVVDSIQIFAAQRYSFVLEANQAVD~NYWIRANPNFGN~VGFTGGINSAILRYDGAAAVEPTTTQTTS~TAPLNEVNLHP 307
Trametes villosa (2)        LLVDSIQIFAAQRYSFVLNANQTVG~NYWVRANPNFGT~VGFAGGINSAILRYQGAPVAEPTTTQTPS~VIPLIETNLHP 307
Curiolus versicolor CVL G1  LDVDSIQIFAGQRYSFILNANQSID~NYWIRAIPNTGT~TDTTGGVNSAILRYDTAEDIEPTTNATTS~VIPLTETDLVP 309
Trametes LCC1               LLVDSIQIFAAQRYSFVLNANQTVG~NYWVRANPNFGT~VGFAGGINSAILRYQGAPVAEPTTTQTTS~VIPLIETNLHP 307
Ganoderma lucidum           VTANAIQIFSAQRYSFVLTANQTIG~NYWIRANPSFGN~IGFTNGINSAILRYSGADPIEPTTAQQTT~QNLLNEVDLHP 307
Curiolus hirsutus           LVVDSIQIFAAQRYSFVLNADQDVG~NYWIRANPNFGN~VGFAGGINSAILRYDGADPVEPTTTQTTP~TKPLNEVDLHP 307
Basidiomycete sp.PM1        QTVDSIQIFAAQRYSFVLNADQDVD~NYWIRALPNSGT~RNFDGGVNSAILRYDGAAPVEPTTTQTPS~TQPLVESALTT 306
Trametes sp. LCC4           LDVDSIQIFAGQRYSFILNANQSID~NYWIRAIPNTGT~TDTTGGVNSAILRYDTAEEIEPTTNATTS~VIPLTETDLVP 309
Rigidoporus microporus      VDADSLQIFAGQRYSIVVNADKSEGGNYWIRASPTPGP~QGFANGINSAILRYVGSDEVEPTTNQTTS~TNPLVEANLVP 307
Polyporus ciliatus          YEVDSIQIFAGQRYSFVLTADQAVD~NYWIQAIPSIGT~ITTDGGVNSAILRYDGADIVEPAAATVTG~SNPLVETSLVP 308
```

FIG. 21D

```
Majority                    LVNTAVPGSPTP--GGVDVALNLAFNFNGT--NFFINGASFTPPTVPVLLQILSGAQNAQDLLPSGSVYTLPSNXTIEIS
                            ---------+---------+---------+---------+---------+---------+---------+---------+
                                   330       340       350       360       370       380       390       400
                            ---------+---------+---------+---------+---------+---------+---------+---------+
Cerrena laccase D           LVPAPVPGNAVP--GGADINHRLNLTFSNG--LFSINNASFTNPSVPALLQILSGAQNAQDLLPTGSYIGLELGKVVELV 384
Cerrena laccase A           LVYTPVPGNPTP--GGADIVHTLDLSFDAG--RFSINGASFLDPTVPVLLQILSGTQNAQDLLPPGSVIPLELGKVVELV 384
Cerrena laccase B           LVYTPVPGQPFP--GGADIVKNLALGFNAG--RFTINGASLTPPTVPVLLQILSGTHNAQDLLPAGSVIELEQNKVVEIV 383
Panus rudis                 LASMPVPGTHTP--GVADVVKPLQFGFNPP--AFTINGASFVPPTVPVLLQILSGAKTAQEIVPSGSIIELPLNSVVELS 384
Spongipellis sp.            LVPTPVPGLPQA--GGVDVVKNLVFGFTGG--KFTINGVSFVPLTVPVLLQILSGTTNAQDLLPSGSVIELPLGKTIELT 383
Curiolus versicolor CVL3    LVATAVPGSPVA--GGVDLAINMAFNFNGT--NFFINGASFTPPTVPVLLQIISGAQNAQDLLPSGSVYSLPSNADIEIS 383
Lentinus sp.                RENPGAPGNATA--NGVDVDLNLVLSFVG--GRFEINGVSFVPPTVPVLLQILSGATTAAELLPSGSVYTLPLNSVIQLS 382
Ceriporiopsis subvermispora LVPAAAPGAPNQDFDAVDVPMNLNFTFNGT--NLFINGATFVPPSVPVLTQILSGAMTAQELLPAGSVYTLPRNATVQLS 386
Cyathus bulleri             LENPGAPG--GS--NPADVPLNLAIAFGSN-LKFTVNGATFAPPNVPVLLQILSGAQTAQDLLPTGSVYTLPANKVIEIS 387
Pycnoporus sanguineus       LTPMAVPGLPEP--GGVDKPLNMVFNFNGT--NFFINGESFVPPSVPVLLQILSGAQAAQDLVPSGSVYVLPSNSTIEIS 383
Trametes villosa (1)        LVTTAVPGSPVA--GGVDLAINMAFNFNGT--NFFINGTSFTPPTVPVLLQIISGAQNAQDLLPSGSVYSLPSNADIEIS 383
Trametes villosa (2)        LARMPVPGSPTP--GGVDKALNLAFNFNGT--NFFINNATFTPPTVPVLLQILSGAQTAQDLLPAGSVYPLPAHSTIEIT 383
Curiolus versicolor CVL G1  LDNPAAPGDPQV--GGVDLAMSLDFSFNGS--NFFINNETFVPPTVPVLLQILSGAQDAASLLPNGSVYTLPSNS-IEIS 384
Trametes sp. LCC1           LARMPVPGSPTP--GGVDKALNLAFNFNGT--NFFINNATFTPPTVPVLLQILSGAQTAQDLLPAGSVYPLPAHSTIEIT 383
Ganoderma lucidum           FVAKQTPGRATQ--GGTDVAINMVFNFNGS--NFFINNASFTPPTVPVLLQILSGAQAAQDLLPSGSVYTLPINKSIELT 383
Curiolus hirsutus           LATMAVPGSPVA--GGVDTAINMAFNFNGT--NFFINGASFVPPTVPVLLQIISGAQNAQDLLPSGSVYSLPSNADIEIS 383
Basidiomycete sp.PM1        LEGTAAPGNPTP--GGVDLALNMAFGFAGG--RFTINGASFTPPTVPVLLQILSGAQSAQDLLPSGSVYSLPANADIEIS 382
Trametes sp. LCC4           LDNPAAPGDPQV--GGVDLAMSLDFSFNGS--NFFINNETFVPPTVPVLLQILSGAQDAASLLPNGSVYTLPSNSTIEIS 385
Rigidoporus microporus      LENPGAPGDPTP--GGVDVPLNLAVAFDGTGLDFQVNGQTFIPPTVPVLLQILSGAQSATDLLPSGSVYVLPSNATVEIS 385
Polyporus ciliatus          LENLAAPGEPTI--GGVDYPLNLDFSFDGT--NFAINGATFTPPTVPVLLQIMSGAQDVADLLPSGSIYSLPSNATIELS 384
```

FIG. 21E

```
Majority                      FPATAX-----APGAPHPFHLHGHTFAVVRSAGSTTYNYDNPIFRDVVSTGTP--GDNVTIRFTTDNPGPWFLHCHIDFH
                              ---------+---------+---------+---------+---------+---------+---------+---------+
                                      410       420       430       440       450       460       470       480
                              ---------+---------+---------+---------+---------+---------+---------+---------+
Cerrena laccase D             IP-PL------AVGGPHPFHLHGHNFWVVRSAGSDEYNFDDAILRDVVSIGAG--TDEVTIRFVTDNPGPWFLHCHIDWH 455
Cerrena laccase A             IP-AG------VVGGPHPFHLHGHNFWVVRSAGTDQYNFNDAILRDVVSIGGT--GDQVTIRFVTDNPGPWFLHCHIDWH 455
Cerrena laccase B             LP-AAG-----AVGGPHPFHLHGHNFWVVRSAGQTTYNFNDAPIRDVVSIGGA--NDQVTIRFVTDNPGPWFLHCHIDWH 455
Panus rudis                   FPNPTG-----APGGPHPFHLHGHTFFVVRSAGQTTYNYDDPIARDVVSTGTA--GDNVTIRFATDNAGPWFLHCHIDWH 457
Spongipellis sp.              LA--AG-----VLGGPHPFHLHGHNFHVVRSAGQTTANYVNPIVRDVVNTGAS--PDNVTIRFTTDNPGPWFLHCHIDWH 454
Curiolus versicolor CVL3      FPATAA-----APGAPHPFHLHGHAFAVVRSAGSTVYNYDNPIFRDVVSTGTPAAGDNVTIRFRTDNPGPWFLHCHIDFH 458
Lentinus sp.                  FNTVAVA----AVGGPHPFHLHGHTFDVVRSAGSTEYNYINPPRRDVVSTGAA--TDNVTIRFTTDNAGPWFLHCHIDWH 456
Ceriporiopsis subvermispora   LPGNII------AG-PHPFHLHGHTFSVIRSAGQSDYNYVDPIQRDVVSIGGA--TDNVTIRFTTDNPGPWFFHCHIDWH 457
Cyathus bulleri               ---IPGG----TTGFPHPFHLHGHTFDVVRSAGSSVYNYDNPVRRDAVNTGGA--GDNVTIRFLTDNAGPWILHCHIDWH 458
Pycnoporus sanguineus         FPATAN-----APGAPHPFHLHGHTFAVVRSAGSSEYNYDNPIFRDVVSTGTP--GDNVTIRFETNNPGPWFLHCHIDFH 456
Trametes villosa (1)          FPATAA-----APGAPHPFHLHGHAFAVVRSAGSTVYNYDNPIFRDVVSTGTPAAGDNVTIRFRTDNPGPWFLHCHIDFH 458
Trametes villosa (2)          LPATAL-----APGAPHPFHLHGHAFAVVRSAGSTTYNYNDPIFRDVVSTGTPAAGDNVTIRFQTDNPGPWFLHCHIDFH 458
Curiolus versicolor CVL G1    FPIITTDGALNAPGAPHPFHLHGHTFSVVRSAGSSTFNYANPVRRDTVSTGNS--GDNVTIRFTTDNPGPWFLHCHIDFH 462
Trametes sp. LCC1             LPATAL-----APGAPHPFHLHGHAFAVVRSAGSTTYNYNDPIFRDVVSTGTPAAGDNVTIRFQTDNPGPWFLHCHIDFH 458
Ganoderma lucidum             FPATVN-----APGAPHPFHLHGHSFAVVRSAGSTEYNYNNPVWRDVVSTGTPAAGDNVTIRFQTDNPGPWFLHCHIDFH 458
Curiolus hirsutus             FPATAA-----APGAPHPFHLHGHAFAVVRSAGSTVYNYDNPIFRDVVSTGTPAAGDNVTIRFRTDNPGPWFLHCHIDFH 458
Basidiomycete sp.PM1          LPATSA-----APGFPHPFHLHGHTFAVVRSAGSSTYNYANPVYRDVVSTGSP--GDNVTIRFRTDNPGPWFLHCHIDFH 455
Trametes sp. LCC4             FPIITTDGALNAPGAPHPFHLHGHTFSVVRSAGSSTFNYANPVRRDTVSTGNS--GDNVTIRFTTDNPGPWFLHCHIDFH 463
Rigidoporus microporus        IPAGAV------GG-PHPIHLHGHTFDVVRSAGSSTYNYVNPPRRDVVSIGNA--GDNVTIRFRTDNPGPWFLHCHIDWH 456
Polyporus ciliatus            FPITATN----APGAPHPFHLHGHTFYVVRSAGSTEYNYVNPPQRDTVSTGTA--GDNVTIRFTTNNPGPWFLHCHIDFH 458
```

FIG. 21F

```
Majority                    LEAGFAVVFAEDIPDXAAANPVPQAWSDLCPTYDALSPSDX---------          (SEQ ID NO: 29)
                            ---------+---------+---------+---------+---------+
                                    490       500       510       520       530
                            ---------+---------+---------+---------+---------+
Cerrena laccase D           LEAGLAIVFAEGINQTAAANPTPQAWDELCPKYNGLSASQKVKPKKGTAI    505   (SEQ ID NO: 8)
Cerrena laccase A           LEAGLAIVFAEGIENTAASNLTPQAWDELCPKYNALSAQKKLNPSTT       502   (SEQ ID NO: 1)
Cerrena laccase B           LEAGFAVVFAEGINGTAAANPVPAAWNQLCPLYDALSPGDT             496   (SEQ ID NO: 3)
Panus rudis                 LDAGFAVVMAEGINQTQAANPTPDAWNQLCPTYDAL                  493   (SEQ ID NO: 12)
Spongipellis sp.            LEAGFAVVFAEGINQTNAANPTPAAWNNLCNLYNALDSGDL             495   (SEQ ID NO: 13)
Curiolus versicolor CVL3    LEAGFAVVFAEDIPDVASANPVPQAWSDL                         487   (SEQ ID NO: 14)
Lentinus sp.                LEAGFAIVFAEDAPDVAAVNPVPDAWNQLCPTYDALTPAQLGGN          500   (SEQ ID NO: 16)
Ceriporiopsis subvermispora LQAGFAIVFAEETADVASANPVPADWSALCPTYDALSDADH             498   (SEQ ID NO: 17)
Cyathus bulleri             LELGLAIVFAEDVPTIAASNP-PDAWDNLCPAYATQPTGT              497   (SEQ ID NO: 18)
Pycnoporus sanguineus       LDAGFAVVMAEDTPDTAAANPVPQSWSDLCPIYDALDPSDL             497   (SEQ ID NO: 19)
Trametes villosa (1)        LEAGFAVVFAEDIPDVASANPVPQAWSDLCPTYDALDPSDQ             499   (SEQ ID NO: 20)
Trametes villosa (2)        LDAGFAIVFAEDVADVKAANPVPKAWSDLCPIYDGLSEANQ             499   (SEQ ID NO: 21)
Curiolus versicolor CVL G1  LDAGFAIVFAEDTADTASANPVPTAWSDLCPTYDALDSSDL             503   (SEQ ID NO: 15)
Trametes Sp. LCC1           LDAGFTIVFAEDVADVKAANPVPKAWSDLCPIYDGLSEANQ             499   (SEQ ID NO: 22)
Ganoderma lucidum           LEAGFAVVFAEDTADTSLANHVPQAWSDLCPTYDALSADDH             499   (SEQ ID NO: 24)
Curiolus hirsutus           LEAGFAVVFAEDIPDVASANPVPQAWSDLCPIYDALDVNDQ             499   (SEQ ID NO: 25)
Basidiomycete sp.PM1        LEAGFAVVMAEDIPDVAATNPVPQAWSDLCPTYDALSPDDQ             496   (SEQ ID NO: 26)
Trametes sp. LCC4           LDAGFAIVFAEDTADTASANPVPTAWSDLCPTYDALDSSDL             504   (SEQ ID NO: 23)
Rigidoporus microporus      LEAGFAVVFAEDIPNVASVNSPPQAWSDLCPIYDALDPSDH             497   (SEQ ID NO: 27)
Polyporus ciliatus          LEAGFAVVFGEDIPSISDANPPSSAWEDLCPTYNSVYPNGDGN           501   (SEQ ID NO: 28)
```

FIG. 21G

AIGPVADLHIV**NKD**LAPDGVQRPTVLA**GGT**FPGTLITGQKGDNFQL**NVI**D 50

DLTDDRMLTPTSIHWHG**F**FQKGTAWADGPAFVTQCPIIADNSFLYDFDVP 100

DQAGTFWYHSHLSTQYCDGLRGAFVVYDP**N**DPHKDLYDVDDGGTVITLAD 150

WYHVLA**QTV**VGAATPDSTLINGLGRSQTGPADAELAVISVEHNKRYRFRL 200

VSISCDPNFTFSVDGHNMTVIEVDGVNTRPLTVDSIQIFAGQRYSFVLNA 250

NQPEDNYWIRAMPN**I**GRNTTTLDGKNAAILRYKNAS**V**EEPKT**V**GGPAQSP 300

LNEADLRPLVPAPVPG**NAV**PGGADINHRLNLTFS**N**GLFSINNASFTNPSV 350

PALLQILSGAQ**NAQ**DLLPTGSYIGLELGKVVELVIPPLAVGGPHPFHLHG 400

HNFWVVRSAGSDEYNFDDAILRDVVSIGAGTDEVTIRFVTDNPGPWFLHC 450

HIDWHLEAGLAIVFAEGINQTAAANPTPQAWDELCPKYNGL**SAS**QKVKPK 500

KGTAI .. (SEQ ID NO: 11) 505

FIG. 22

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/029786

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/02
ADD.

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

B. FIELDS SEARCHED

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

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

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

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2010/075402 A1 (DANISCO US INC [US]; ASHTON WAYNE [US]; KROUWER ANDREAS J [US]; MCAULI) 1 July 2010 (2010-07-01) page 19; sequences 2, 6, 20 paragraphs [0013], [0018], [0019] -----	1-5,9-23
X	DATABASE UniProt [Online] 2 March 2010 (2010-03-02), "SubName: Full=Laccase;", XP002680013, retrieved from EBI accession no. UNIPROT:D2KZ03 Database accession no. D2KZ03	1-5, 10-13
Y	the whole document -----	14-23
	-/--	

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 17 July 2012	Date of mailing of the international search report 31/08/2012
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Offermann, Stefanie

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2008/076322 A2 (DANISCO US INC GENENCOR DIV [US]; MCAULIFFE JOSEPH C [US]; WANG HUAMIN) 26 June 2008 (2008-06-26) claims 1-12	1-5,9-23
X	DATABASE UniProt [Online] 1 March 2002 (2002-03-01), "SubName: Full=Laccase; SubName: Full=Laccase 1;", XP002680014, retrieved from EBI accession no. UNIPROT:Q8WZG3 Database accession no. Q8WZG3	1-5, 10-13
Y	the whole document & YUICHI SAKAMOTO ET AL: "Heterologous expression of lcc1 from Lentinula edodes in tobacco BY-2 cells results in the production an active, secreted form of fungal laccase", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 79, no. 6, 17 May 2008 (2008-05-17), pages 971-980, XP019623660, ISSN: 1432-0614	14-23
X	DATABASE UniProt [Online] 8 March 2011 (2011-03-08), "SubName: Full=Laccase; EC=1.10.3.2;", XP002680015, retrieved from EBI accession no. UNIPROT:E7BLR0 Database accession no. E7BLR0	1-4, 10-13
Y	the whole document	14-23

International application No.
PCT/US2012/029786

INTERNATIONAL SEARCH REPORT

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-5, 9-23(all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-5, 9-23(all partially)

laccase variant derived from SEQ ID NO:1 having a mutation at a position corresponding to position 68 of SEQ ID NO:11 and subject-matter related thereto, such as compositions and methods to use said laccase

---

2-27. claims: 1-5, 9-23(all partially)

laccase variant derived from one of SEQ ID NOs:2, 3, 4,..10/11, ... 28 (SEQ ID NO:11 is the mature form of SEQ ID NO:10, thus, both sequences are considered as one invention) having a mutation at a position corresponding to position 68 of SEQ ID NO:11

---

28. claims: 1, 6-23(all partially)

laccase variant derived from SEQ ID NO:1 having a mutation that alters the surface charge of the parental laccase enzyme which corresponds to a mutation at a position equivalent to position 130 in SEQ ID NO:11.

29-32. claims: 1, 6-23(all partially)

laccase variant derived from SEQ ID NO:1 having a mutation that alters the surface charge of the parental laccase enzyme which corresponds to a mutation at a position equivalent to one of positions 265, 287, 293 or 319 in SEQ ID NO:11.

33-163. claims: 1, 6-23(all partially)

laccase variant derived from one of SEQ ID NOs:2, 3, 4,..10/11, ... 28 having a mutation that alters the surface charge of the parental laccase enzyme which corresponds to a mutation at a position equivalent to one of positions 130, 265, 287, 293 or 319 in SEQ ID NO:11

---

164. claims: 1, 6-23(all partially)

laccase variant derived from SEQ ID NO:1 having a mutation that alters the surface hydrophobicity of the parental laccase enzyme which corresponds to a mutation at a position equivalent to position 130 in SEQ ID NO:11.

165-168. claims: 1, 6, 8-23(all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

laccase variant derived from SEQ ID NO:1 having a mutation that alters the surface hydrophobicity of the parental laccase enzyme which corresponds to a mutation at a position equivalent to one of positions 265, 287, 293 or 319 in SEQ ID NO:11

\---

169-299. claims: 1, 6-23(all partially)

laccase variant derived from one of SEQ ID NOs:2, 3, 4,..10/11, ... 28 having a mutation that alters the surface hydrophobicity of the parental laccase enzyme which corresponds to a mutation at a position equivalent to one of positions 130, 265, 287, 293 or 319 in SEQ ID NO:11

\---

300-327. claims: 1, 10-23(all partially)

laccase variant derived from one of SEQ ID NOs:1, 2, 3, 4,..10/11, ... 28 having a mutation as defined in claim 1(d)

\---

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010075402 A1	01-07-2010	CA 2747813 A1	01-07-2010
		CN 102264892 A	30-11-2011
		EP 2376629 A1	19-10-2011
		JP 2012514139 A	21-06-2012
		KR 20110117064 A	26-10-2011
		US 2011302722 A1	15-12-2011
		WO 2010075402 A1	01-07-2010
WO 2008076322 A2	26-06-2008	CA 2672603 A1	26-06-2008
		CA 2672609 A1	26-06-2008
		EP 2092113 A2	26-08-2009
		EP 2094842 A2	02-09-2009
		US 2008189871 A1	14-08-2008
		US 2008196173 A1	21-08-2008
		US 2012151682 A1	21-06-2012
		WO 2008076322 A2	26-06-2008
		WO 2008076323 A2	26-06-2008