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(71) Applicant(s)
DANISCO US INC.

(72) Inventor(s)
Larenas, Edmund A.;Lantz, Suzanne E.;Power, Scott D.;Caldwell, Robert M.

(74) Agent / Attorney
Houlihan2, Level 1 70 Doncaster Road, BALWYN NORTH, VIC, 3104

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

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **POWER, Scott D.** [US/US]; DANISCO US INC., GENENCOR DIVISION, 925 Page Mill Road, Palo Alto, CA 94304 (US). **CALDWELL, Robert M.** [US/US]; DANISCO US INC., GENENCOR DIVISION, 925 Page Mill Road, Palo Alto, CA 94304 (US). **LANTZ, Suzanne E.** [US/US]; DANISCO US INC., GENENCOR DIVISION, 925 Page Mill Road, Palo Alto, CA 94304 (US). **LARENAS, Edmund, A.** [US/US]; DANISCO US INC., GENENCOR DIVISION, 925 Page Mill Road, Palo Alto, CA 94304 (US).

(74) Agent: **TODD, Stephen**; DANISCO US INC., GENENCOR DIVISION, 925 Page Mill Road, Palo Alto, CA 94304 (US).

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(54) **Title**: HEMICELLULASE ENRICHED COMPOSITIONS FOR ENHANCING HYDROLYSIS OF BIOMASS

(57) **Abstract**: Described are compositions and methods relating to cellulase/hemicellulase enzyme blends for improving the enzymatic hydrolysis of cellulosic and hemicellulosic materials, as commonly found in biomass

HEMICELLULASE ENRICHED COMPOSITIONS FOR ENHANCING HYDROLYSIS OF BIOMASS

PRIORITY

[001] The present application claim priority to U.S. Provision Application Serial No. 61/038,520, filed on March 21, 2008, which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[002] The present compositions and methods relate to cellulase/hemicellulase enzyme blends for improving the enzymatic hydrolysis of cellulosic materials.

BACKGROUND

[003] The principal components of biomass are cellulose and hemicellulose. Cellulose consists of polymers of β-1,4-linked glucose residues that are organized into higher order fibrillar structures. Hemicelluloses are heteropolysaccharides that include monosaccharides other than glucose, such as D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, and 4-O-methyl-D-glucuronic acid linked together not only by glycosidic linkages but also by ester linkages. The composition and structure of hemicellulose are more complicated than that of cellulose and can vary quantitatively and qualitatively in various woody plant species, grasses, and cereals.

[004] Cellulose can be converted into sugars, such as glucose, and used as an energy source by numerous microorganisms including bacteria, yeast and fungi for industrial purposes. Cellulosic materials can also be converted into sugars by commercially available enzymes, and the resulting sugars can be used as a feedstock for industrial microorganisms to produce products such as plastics and ethanol. However, current cellulase products generally lack the ability to hydrolyze hemicellulosic materials, which remain unconsumed in the biomass compositions and may interfere with the handling and disposal of the biomass.

[005] Accordingly, there remains a need to develop efficient enzyme systems for hydrolyzing both cellulose and hemicellulose, including the coproduction or blending of

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an optimized set of enzymes for converting hemicellulosic oligomers and polymers into free pentose for fermentation. Such optimized enzyme systems are desired to improve the efficiency and economics of biomass.

SUMMARY

[006] The present teachings provides optimized bioconverting enzyme blends, methods for producing the same, as well as methods of using the optimized bioconverting enzyme blend for converting biomass to sugar. The bioconverting enzyme blend comprises a mixture of a whole cellulase and one or more hemicellulases, the selection of which is dictated by the intended biomass substrate and processing conditions.

[007] In one aspect, an enzyme blend composition is provided, comprising:

(a) a first enzyme composition comprising a cellulase,

(b) a second enzyme composition comprising at least one xylanase selected from a GH10 or GH11 xylanase, and

(c) a third enzyme composition comprising at least one additional hemicellulase that is not a GH10 or GH11 xylanase or not the same GH10 or GH11 xylanase as in (b),

wherein the enzyme blend composition provides (i) enhanced glucan conversion and (ii) enhanced xylan conversion from a mixture of cellulosic and hemicellulosic materials as compared to the glucan and xylan conversion levels achieved by an equivalent enzyme blend composition lacking the at least one additional hemicellulase.

[008] In some embodiments, the first enzyme composition is a whole cellulase blend from a filamentous fungus. In some embodiments, the first enzyme composition is a whole cellulase blend from a filamentous fungus supplemented with an additional amount of β-glucosidase.

[009] In some embodiments, the second enzyme composition comprises xylanase XYN2 from *Trichoderma reesei*. In some embodiments, the second enzyme composition comprises xylanase XYN3 from *Trichoderma reesei*.

[0010] In some embodiments, the at least one xylanase has an amino acid sequence having at least 80% identity to an amino acid sequence selected from SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the at least one xylanase has an amino acid

sequence having 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% amino acid sequence identity to identity to an amino acid sequence selected from SEQ ID NO: 1 or SEQ ID NO: 2. In particular embodiments, the at least one xylanase has an amino acid sequence selected from SEQ ID NO: 1 or SEQ ID NO: 2.

[0011] In some embodiments, the at least one additional hemicellulase is selected from the group consisting of a GH54 hemicellulase, a GH62 hemicellulase, a GH27 hemicellulase, a GH36 hemicellulase, a GH5 hemicellulase, a GH74 hemicellulase, a GH67 hemicellulase, a GH28 hemicellulase, a GH11 hemicellulase, a GH10 hemicellulase, a GH3 hemicellulase, and a CE5 hemicellulase.

[0012] In some embodiments, the at least one additional hemicellulase is a β-xylosidase or an arabinofuranosidase. In particular embodiments, the β-xylosidase is BXL1 from *Trichoderma reesei* and the arabinofuranosidase is ABF1, ABF2, or ABF3 from *Trichoderma reesei*. In some embodiments, the at least one additional hemicellulase is a combination of a β-xylosidase and an arabinofuranosidase.

[0013] In some embodiments, the first enzyme composition is a whole cellulase blend from a filamentous fungus supplemented with an addition amount of β-glucosidase, the second enzyme composition comprises a xylanase, and the at least one additional hemicellulase is a combination of a β-xylosidase and arabinofuranosidase.

[0014] In some embodiments, the at least one additional hemicellulase is a *Trichoderma reesei* hemicellulase selected from the group consisting of α-arabinofuranosidase I (ABF1), α-arabinofuranosidase II (ABF2), α-arabinofuranosidase III (ABF3), α-galactosidase I (AGL1), α-galactosidase II (AGL2), α-galactosidase III (AGL3), acetyl xylan esterase I (AXE1), acetyl xylan esterase III (AXE3), endoglucanase VI (EG6), endoglucanase VIII (EG8), α-glucuronidase I (GLR1), β-mannanase (MAN1), polygalacturonase (PEC2), xylanase I (XYN1), xylanase II (XYN2), xylanase III (XYN3), and β-xylosidase (BXL1).

[0015] In some embodiments, the at least one additional hemicellulase has an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of 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, and SEQ ID NO: 17. In some embodiments, the at least one additional hemicellulase has an amino acid sequence having 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% amino acid sequence identity to one of the aforementioned amino acid sequences. In particular embodiments, the at least one additional hemicellulase has an amino acid sequence corresponding to one of aforementioned amino acid sequences.

[0011] In another aspect, a method for hydrolyzing a mixture of cellulosic and hemicellulosic materials is provided, comprising contacting the mixture of cellulosic and hemicellulosic materials with:

(a) a first enzyme composition comprising a cellulase,

(b) a second enzyme composition comprising at least one xylanase selected from a GH10 or GH11 xylanase, and

(c) a third enzyme composition comprising at least one additional hemicellulase that is not a GH10 or GH11 xylanase or not the same GH10 or GH11 xylanase as in (b),

thereby hydrolyzing the mixture of cellulosic and hemicellulosic materials,

wherein the contacting results in (i) enhanced glucan conversion and (ii) enhanced xylan conversion compared to equivalent contacting in the absence of the at least one additional hemicellulase.

[0017] In some embodiments, the first enzyme composition is a whole cellulase blend from a filamentous fungus. In some embodiments, the first enzyme composition is a whole cellulase blend from a filamentous fungus supplemented with an additional amount of β-glucosidase.

[0018] In some embodiments, the second enzyme composition comprises xylanase XYN2 from *Trichoderma reesei*. In some embodiments, the second enzyme composition comprises xylanase XYN3 from *Trichoderma reesei*.

[0019] In some embodiments, the at least one xylanase has an amino acid sequence having at least 80% identity to an amino acid sequence selected from SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the at least one xylanase has an amino acid sequence having 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%

amino acid sequence identity to identity to an amino acid sequence selected from SEQ ID NO: 1 or SEQ ID NO: 2. In particular embodiments, the at least one xylanase has an amino acid sequence selected from SEQ ID NO: 1 or SEQ ID NO: 2.

[0020] In some embodiments, the at least one additional hemicellulase is selected from the group consisting of a GH54 hemicellulase, a GH62 hemicellulase, a GH27 hemicellulase, a GH36 hemicellulase, a GH5 hemicellulase, a GH74 hemicellulase, a GH67 hemicellulase, a GH28 hemicellulase, a GH11 hemicellulase, a GH10 hemicellulase, a GH3 hemicellulase, and a CE5 hemicellulase.

[0021] In some embodiments, the at least one additional hemicellulase is a β-xylosidase or an arabinofuranosidase. In particular embodiments, the β-xylosidase is BXL1 from *Trichoderma reesei* and the arabinofuranosidase is ABF1, ABF2, or ABF3 from *Trichoderma reesei*. In some embodiments, the at least one additional hemicellulase is a combination of a β-xylosidase and an arabinofuranosidase.

[0022] In some embodiments, the first enzyme composition is a whole cellulase blend from a filamentous fungus supplemented with an addition amount of β-glucosidase, the second enzyme composition comprises xylanase, and the at least one additional hemicellulase is a combination of a β-xylosidase and arabinofuranosidase.

[0023] In some embodiments, the at least one additional hemicellulase is a *Trichoderma reesei* hemicellulase selected from the group consisting of α-arabinofuranosidase I (ABF1), α-arabinofuranosidase II (ABF2), α-arabinofuranosidase III (ABF3), α-galactosidase I (AGL1), α-galactosidase II (AGL2), α-galactosidase III (AGL3), acetyl xylan esterase I (AXE1), acetyl xylan esterase III (AXE3), endoglucanase VI (EG6), endoglucanase VIII (EG8), α-glucuronidase I (GLR1), β-mannanase (MAN1), polygalacturonase (PEC2), xylanase I (XYN1), xylanase II (XYN2), xylanase III (XYN3), and β-xylosidase (BXL1).

[0024] In some embodiments, the at least one additional hemicellulase has an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of 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, and SEQ ID NO: 17. In some embodiments, the at least one additional hemicellulase has an amino

acid sequence having 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% amino acid sequence identity to one of the aforementioned amino acid sequences. In particular embodiments, the at least one additional hemicellulase has an amino acid sequence corresponding to one of aforementioned amino acid sequences.

[0025] In some embodiments, contacting the mixture of cellulosic and hemicellulosic materials with the first enzyme composition, the second enzyme composition, and the third enzyme composition are performed simultaneously.

[0026] In some embodiments, the first enzyme composition, the second enzyme composition, and the third enzyme composition are provided in a single composition enzyme blend.

[0027] These and other aspect and embodiments of the present compositions and methods will be apparent from the following description.

DETAILED DESCRIPTION

I. Definitions

[0028] 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. The headings provided herein are not limitations of the various aspects or embodiments of the invention described under one heading may apply to the compositions and methods as a whole. Both the foregoing general description and the following detailed description are exemplary and explanatory and are not restrictive of the compositions and methods described herein. The use of the singular includes the plural unless specifically stated otherwise, and the use of “or” means “and/or” unless state otherwise. The terms “comprise,” “comprising,” “comprises,” “include,” “including,” and “includes” are not intended to be limiting. All patents and publications, including all amino acid and nucleotide sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference. The following terms are defined for clarity:

[0029] As used herein, the term "cellulose: refers a polysaccharide consisting of β(1→4) linked D-glucose units having the general formula $(C_6H_{10}O_5)_n$. Cellulose is the structural

component of the primary cell wall of green plants, many forms of algae and the oomycetes.

[0030] As used herein, the term "cellulase" refers to an enzyme capable of hydrolyzing cellulose polymers to shorter oligomers and/or glucose.

[0031] As used herein, the term "whole cellulase composition/preparation/mixture" or the like refers to both naturally occurring and non-naturally occurring compositions that include a plurality of cellulases produced by an organism, for example a filamentous fungus. One example of a whole cellulase composition is medium in which filamentous fungi are cultured, which includes secreted cellulases, such as one or more cellobiohydrolases, one or more endoglucanases, and one or more β-glucosidases at a predetermined ratio.

[0032] As used herein, “hemicellulose” is a polymer component of plant materials that contains sugar monomers other than glucose, in contrast to cellulose, which contains only glucose. In addition to glucose, hemicellulose may include xylose, mannose, galactose, rhamnose, and arabinose, with xylose being the most common sugar monomer. Hemicelluloses contain most of the D-pentose sugars, and occasionally small amounts of L-sugars. The sugars in hemicellulose may be linked by ester linkages as well as glycosidic linkages. Exemplary forms of hemicellulose include but are not limited to are galactan, mannan, xylan, arabanan, arabinoxylan, glucomannan, galactomanan, and the like.

[0033] As used herein, the term "hemicellulase" refers to a class of enzymes capable of breaking hemicellulose into its component sugars or shorter polymers, and includes endo-acting hydrolases, exo-acting hydrolases, and various esterases.

[0034] As used herein, the term “xylanase" refers to a protein or polypeptide domain of a protein or polypeptide derived from a microorganism, *e.g.*, a fungus, bacterium, or from a plant or animal, and that has the ability to catalyze cleavage of xylan at one or more of various positions of xylan's carbohydrate backbone, including branched xylans and xylooligosaccharides. Note that a xylanase is a type of hemicellulase.

[0035] As used herein, a “biomass substrate” is a material containing both cellulose and hemicellulose.

[0036] As used herein, a "naturally occurring" composition is one produced in nature or by an organism that occurs in nature.

[0037] As used herein, a “variant” protein differ from the “parent” protein from which it is derived by the substitution, deletion, or addition of a small number of amino acid residues, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more amino acid residues. In some cases, the parent protein is a “wild-type,” “native,” or “naturally-occurring” polypeptides. Variant proteins may be described as having a certain percentage sequence identity with a parent protein, *e.g.*, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, 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%, at even at least 99%, which can be determined using any suitable software program known in the art, for example those described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel *et al.* (eds) 1987, Supplement 30, section 7.7.18).

[0038] Preferred programs include the Vector NTI Advance™ 9.0 (Invitrogen Corp. Carlsbad, CA), GCG Pileup program, FASTA (Pearson *et al.* (1988) *Proc. Natl, Acad. Sci USA* 85:2444–2448), and BLAST (BLAST Manual, Altschul *et al.*, Natl Cent. Biotechnol. Inf., Natl Lib. Med. (NCIB NLM NIH), Bethesda, Md., and Altschul *et al.* (1997) *NAR* 25:3389–3402). Another preferred alignment program is ALIGN Plus (Scientific and Educational Software, PA), preferably using default parameters. Another sequence software program that finds use is the TFASTA Data Searching Program available in the Sequence Software Package Version 6.0 (Genetics Computer Group, University of Wisconsin, Madison, WI).

II. Bioconverting enzyme blend compositions and methods of use, thereof

[0039] Cellulose is a homopolymer of anhydrocellobiose and thus a linear β-(1-4)-D-glucan. In contrast, hemicelluloses include a variety of compounds, such as xylans, xyloglucans, arabinoxylans, and mannans, in complex branched structures, and with a spectrum of substituents. As a consequence of the complex branching and heterogenous composition of hemicelluloses, particularly arabinoxylans, the enzymatic degradation of plant material requires the action of a battery of both debranching and

depolymerizing activities. Additionally, the degradation of plant materials requires enzymes that act on hemicelluloses containing both five-carbon sugars (pentoses), such as xylose and arabinose, and six-carbon sugars (hexoses), such as mannose and glucose.

[0040] Enzyme hydrolysis of hemicellulose to its monomers requires the participation of several hemicellulase enzymes with different functions. Hemicellulases can be placed into three general categories: endo-acting enzymes that attack internal bonds within the polysaccharide, exo-acting enzymes that act processively from either the reducing or nonreducing end of a polysaccharide chain, and the accessory enzymes, acetylesterases, and esterases that hydrolyze lignin glycoside bonds, such as coumaric acid esterase and ferulic acid esterase.

[0041] While certain fungi produce complete cellulase systems which include exo-cellobiohydrolases (or CBH-type cellulases), endoglucanases (or EG-type cellulases), and β-glucosidases (or BG-type cellulases), known cellulase enzymes and mixtures, thereof, typically have limited activity against hemicellulose, and limited value in hydrolyzing plant materials. The present bioconverting enzyme blend compositions and methods are based, in part, on the observation that certain combinations of cellulases and hemicellulases significantly increase the efficiency of plant material hydrolysis, primarily as determined by monitoring the conversion of glucan and xylan.

[0042] The exemplary cellulase composition used to identify cellulase/hemicellulase compositions that increase the hydrolysis of glucan and/or xylan is a whole cellulase compositions produced by a filamentous fungus (*i.e.*, *Trichoderma reesei*). The composition includes several exo-cellobiohydrolases and endoglucanases, and is supplemented with additional β-glucosidase to increase the release of glucose. This composition is commercially available as ACCELLERASE 1000™ (Danisco A/S, Genencor Division, Palo Alto, CA). ACCELLERASE 1000™ includes exo-cellobiohydrolases (i.e., about 50% (wt/wt) CBHI (CEL7A) and about 14% CBHII (CEL6A), endoglucanases (i.e., about 12% EGI (CEL7B) and about 10% EGII (CEL5A)), and β-glucosidase (i.e., about 5% BGLI (CEL3A). A small amount of XYN2 (i.e., less than about 1%) may also be present. Other components that are not identified are also in amounts of less than about 1%.

[0043] Other cellulase compositions may be used, including other whole cellulase mixtures and cellulase mixtures assembled from multiple individually isolated cellulases. Preferred cellulase compositions include at least one each of an exo-cellobiohydrolase, an endoglucanase, and a β-glucosidase. In some embodiments, a whole broth that includes multiple cellulases is prepared from an organism such as an *Acremonium, Aspergillus, Emericella, Fusarium, Humicola, Mucor, Myceliophthora, Neurospora, Scytalidium, Thielavia, Tolypocladium, Penicillium*, or *Trichoderma* spp., or species derived therefrom.

[0044] The composition further includes, at least one, and in some cases two, three, or more hemicellulases. Examples of suitable additional hemicellulases include xylanases, arabinofuranosidases, acetyl xylan esterase, glucuronidases, endo-galactanase, mannanases, endo or exo-arabinases, exo-galactanases, and mixtures thereof. Examples of suitable endo-acting hemicellulases include endo-arabinanase, endo-arabinogalactanase, endoglucanase, endo-mannanase, endo-xylanase, and feraxan endoxylanase. Examples of suitable exo-acting hemicellulases include α-L-arabinosidase, β-L-arabinosidase, α-1,2-L-fucosidase, α-D-galactosidase, β-D-galactosidase, β-D-glucosidase, β-D-glucuronidase, β-D-mannosidase, β-D-xylosidase, exo-glucosidase, exo-cellobiohydrolase, exo-mannobiohydrolase, exo-mannanase, exo-xylanase, xylan α-glucuronidase, and coniferin β-glucosidase. Examples of suitable esterases include acetyl esterases (acetyl xylan esterase, acetylgalactan esterase, acetylmannan esterase, and acetylxylan esterase) and aryl esterases (coumaric acid esterase and ferulic acid esterase).

[0045] Preferably, the present compositions and methods include at least one xylanase, which is a particular type of hemicellulase that cleaves the xylan main chains of hemicellulose. Preferably, the xylanase is endo-1,4-β-xylanase (E.C. 3.2.1.8). Numerous xylanases from fungal and bacterial microorganisms have been identified and characterized (see, *e.g.*, U.S. Pat. No. 5,437,992; Coughlin, M. P. *supra*; Biely, P. *et al.* (1993) *Proceedings of the second TRICEL symposium on Trichoderma reesei Cellulases and Other Hydrolases*, Espoo 1993; Souminen, P. and Reinikainen, T. (*eds*)., in *Foundation for Biotechnical and Industrial Fermentation Research* 8:125-135). Three specific xylanases (XYN1, XYN2, and XYN3) have been identified in *T. reesei*

(Tenkanen, *et al.* (1992) *Enzyme Microb. Technol.* 14:566; Torronen, *et al.* (1992) *Bio/Technology* 10:1461; and Xu, *et al.* (1998) *Appl. Microbiol. Biotechnol.* 49:718). A fourth xylanase (XYN4) isolated from *T. reesei* is described in U.S. Patent Nos. 6,555,335 and 6,768,001 to Saloheimo *et al.*, entitled *Xylanase from Trichoderma reesei*, method for production thereof, and methods employing this enzyme, which is incorporated herein by reference in its entirety.

[0046] Exemplary xylanases for use in the present compositions and methods are XYN2 and XYN3. Suitable variants of XYN2 and XYN3, and suitable related enzymes from other organisms, have 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% amino acid sequence identity to of XYN2 or XYN3 (i.e., SEQ ID NOs: 1 and 2, respectively).

[0047] In addition to the cellulase composition and a xylanase, the compositions and methods may include one or more additional hemicellulases, such as an endo-acting hemicellulase, an exo-acting hemicellulase, and/or an esterases.

[0048] Suitable endo-acting hemicellulases include but are not limited to mannan endo-1,4-β-mannosidase (E.C. 3.2.1.78, also known as β-mannase and β-mannanase), which catalyzes the random endohydrolysis of 1,4,-β-D-mannosiic linkages in mannans, galactomannans, glucomannans; α-amylase (E.C. 3.2.1.1), which catalyzes the endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units; xylan α-1,2-glucuronosidase (E.C. 3.2.1.131, also known as α-glucuronosidase), which catalyzes the hydrolysis of a-D-1,2-(4-O-methyl)glucuronosyl links in the main chain of hardwood xylans; and endoglucanase (E.C. 3.2.1.4), which catalyzes endohydrolysis of 1,4-β-D-glucosidc linkages in cellulase, lichenin, and cereal β-D-glucans. Multiple subtypes of endoglucanase have been identified and are suitable for use in the compositions and methods, for example, endoglucanase I, endoglucanase II, endoglucanase III, endoglucanase V, and endoglucanase VI.

[0049] Suitable exo-acting hemicellulases include but are not limited to α-arabinofuranosidase, α-galactosidase, and β-xylosidase. α-arabinofuranosidase, also known as a-*N*-arabinofuranosidase (E.C. 3.2.1.55), catalyzes the hydrolysis of terminal

non-reducing α-L-arabinofuranoside residues in α-L-arabinosides Any of the at least three known subtypes of α arabinofuranosidase (*i.e.*, abf1, abf2 and abf3) can be used. α-galactosidase (E.C. 3.2.1.22) catalyzes the hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides including galactose oligosaccharides and galactomannans. Any of the three known subtypes, *i.e.*, α-galactosidase I (agl1), α-galactosidase II (agl2) and α-galactosidase III (agl3) can be used. Glucoamylase, also known as glucan 1,4-α-glucosidase (E.C. 3.2.1.3), catalyzes hydrolysis of terminal 1,4-linked α-D-glucose residues successively from non-reducing ends of the chains with release of β-D-glucose. β-glucosidase (E.C. 3.2.1.21) catalyzes the hydrolysis of terminal, non-reducing β-D-glucose residues with release of β-D-glucose. β-xylosidase, also known as xylan 1,4-β-xylosidase (E.C. 3.2.1.37), catalyzes hydrolysis of 1,4-β-D-xylans, to remove successive D-xylose residues from the non-reducing termini. Compositions that included a whole cellulase mixture, along with a xylanase and either an α-arabinofuranosidase or a β-xylosidase were particularly effective in glucan and/or xylan conversion.

[0050] Suitable esterases include but are not limited to ferulic acid esterase and acetyl xylan esterase. Ferulic acid esterase, also known as ferulate esterase (E.C. 3.1.1.73), catalyses the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose in "natural" substrates. Known microbial ferulic acid esterases are secreted into the culture medium. Any of the three known subtypes of ferulic acid esterase (fae1, fae2, and fae3) can be used in the present compositions and methods. Acetyl xylan esterase I (E.C. 3.1.1.72) catalyzes the deacetylation of xylans and xylo-oligosaccharides, and can also be used in the compositions and methods. U.S. Patent Nos. 5,426,043 and 5,681,732 to De Graaff *et al.* describe the cloning and expression of acetyl xylan esterases from fungal origin. EP 507 369 discloses a DNA sequence encoding an acetyl xylan esterase isolated from *Aspergillus niger*. U.S. Patent No. 5,830,734 to Christgau *et al.*, entitled *Enzyme with acetyl esterase activity*, describes the isolation of a variety of esterases for use in the food industry.

[0051] In some embodiments, the at least one additional hemicellulase is selected from the group consisting of a GH54 hemicellulase, a GH62 hemicellulase, a GH27

hemicellulase, a GH36 hemicellulase, a GH5 hemicellulase, a GH74 hemicellulase, a GH67 hemicellulase, a GH28 hemicellulase, a GH11 hemicellulase, a GH10 hemicellulase, a GH3 hemicellulase, and a CE5 hemicellulase. In some embodiments, the at least one additional hemicellulase is selected from the group consisting of α-arabinofuranosidase I (ABF1), α-arabinofuranosidase II (ABF2), α-arabinofuranosidase III (ABF3), α-galactosidase I (AGL1), α-galactosidase II (AGL2), α-galactosidase III (AGL3), acetyl xylan esterase I (AXE1), acetyl xylan esterase III (AXE3), endoglucanase VI (EG6), endoglucanase VIII (EG8), α-glucuronidase I (GLR1), β-mannanase (MAN1), polygalacturonase (PEC2), xylanase I (XYN1), xylanase II (XYN2), xylanase III (XYN3), and β-xylosidase (BXL1), which may be from a filamentous fungus, such as *T. reesei*. In some embodiments, the at least one additional hemicellulase has an amino acid sequence having 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% amino acid sequence identity to an amino acid sequence selected from the group consisting of 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, and SEQ ID NO: 17.

[0052] Variants of hemicellulases (including xylanases) may include substitutions, insertions, or deletions that do not substantially affect function, or add advantageous features to the enzymes. In some embodiments, the substitutions, insertions, or deletions are not in the conserved sequence motifs but are instead limited to amino acid sequences outside the conserved motifs. Exemplary substitutions are conservative substitutions, which preserve charge, hydrophobicity, or side group size relative to the parent amino acid sequence. Examples of conservative substitutions are provided in the following Table:

Original Amino Acid Residue	Code	Acceptable Substitutions
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln

Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, b-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4- carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

[0053] It will be apparent that naturally occurring amino acids can be introduced into a polypeptide by changing the coding sequence of the nucleic acid encoding the polypeptide, while non-naturally-occurring amino acids are typically produced by chemically modifying an expressed polypeptide.

[0054] Further accessory enzymes, such as laccase (E.C. 1.10.3.2), which catalyzes oxidation of aromatic compounds, and consequent reduction of oxygen to water, can also be included in the bioconverting enzyme blends of the present compositions and methods.

[0055] In some embodiments, enzymes for use in the present bioconverting enzyme blends can be prepared from one or more strains of filamentous fungi. Suitable filamentous fungi include members of the subdivision Eumycota and Oomycota,

including but are not limited to the following genera: *Aspergillus, Acremonium, Aureobasidium, Beauveria, Cephalosporium, Ceriporiopsis, Chaetomium, Chrysosporium, Claviceps, Cochiobolus, Cryptococcus, Cyathus, Endothia, Endothia mucor, Fusarium, Gilocladium, Humicola, Magnaporthe, Myceliophthora, Myrothecium, Mucor, Neurospora, Phanerochaete, Podospora, Paecilomyces, Pyricularia, Rhizomucor, Rhizopus, Schizophylum, Stagonospora, Talaromyces, Trichoderma, Thermomyces, Thermoascus, Thielavia, Tolypocladium, Trichophyton*, and *Trametes*. In some embodiments, the filamentous fungi include, but are not limited to the following: *A. nidulans, A. niger, A. awomari, A. aculeatus, A. kawachi e.g.*, NRRL 3112, ATCC 22342 (NRRL 3112), ATCC 44733, ATCC 14331 and strain UVK 143f, *A. oryzae*, *e.g.*, ATCC 11490, *N. crassa*, *Trichoderma reesei*, *e.g.*, NRRL 15709, ATCC 13631, 56764, 56765, 56466, 56767, and *Trichoderma viride*, *e.g.*, ATCC 32098 and 32086. In a preferred implementation, the filamentous fungi is a *Trichoderma* species. A particularly preferred species and strain for use in the present invention is *T. reesei* RL-P37.

[0056] In a particular embodiment, a single engineered strain overexpresses the component enzymes at the desired ratio so that no additional purification or supplementation is necessary. In an alternative embodiment, the bioconverting enzyme blend is obtained from two or more naturally occurring or engineered strains of filamentous fungi. The desired ratio of the component enzymes can be achieved by altering the relative amount of enzyme in the final blend. Even when two or more production strains are use, the desired ratio of component enzymes may be achieved by supplementation with purified or partially purified enzyme.

[0057] In particular embodiments, a hemicellulase is prepared from *Aspergillus aculeatus, Aspergillus awamori, Aspergillus foetidus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, or Aspergillus oryzae. In another aspect, whole broth is prepared from Fusarium bactridioides, Fusarium cerealis, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminum, Fusarium heterosporum, Fusarium negundi, Fusarium oxysporum, Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium sarcochroum, Fusarium sporotrichioides, Fusarium sulphureum, Fusarium torulosum, Fusarium trichothecioides, Fusarium venenatum* or *Fusarium verticilloides.* In another aspect, the hemicellulase

complex is prepared from a *Humicola insolens, Humicola lanuginosa, Mucor miehei, Myceliophthora thermophila, Neurospora crassa, Scytalidium thermophilum,* or *Thielavia terrestris*. In other embodiments, a hemicellulase is prepared from a *Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei*, *e.g.*, RL-P37 [Sheir-Neiss *et al.* (1984) *Appl. Microbiol. Biotechnology* 20:46-53; U.S. Pat. No. 4,797,361; available as a biologically pure culture from the permanent collection of the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., U.S.A. (NRRL 15709); ATCC 13631, 56764, 56466, 56767], or *Trichoderma viride e.g.*, ATCC 32098 and 32086.

[0058] In some embodiments, a component hemicellulase enzyme is produced by expressing a gene encoding the hemicellulase enzyme. For example, xylanase can be secreted into the extracellular space of, *e.g.*, a Gram-positive organism, such as *Bacillus* or *Actinomycetes*, or a eukaryotic organism, such as *Trichoderma*, *Aspergillus*, *Saccharomyces*, or *Pichia*. It is to be understood, that in some embodiments, one or more hemicellulase enzymes can be over-expressed in a recombinant microorganism relative to the native levels. The host cell may be genetically modified to reduce expression of one or more proteins that are endogenous to the cell. In one embodiment, the cell may contain one or more native genes, particularly genes that encode secreted proteins that have been deleted or inactivated. For example, one or more protease-encoding genes (*e.g.*, an aspartyl protease-encoding gene; see Berka *et al.* (1990) *Gene* 86:153-162 and U.S. Pat. No. 6,509,171), or cellulase-encoding genes, may be deleted or inactivated. The nucleic acids encoding the hemicellulase may be present in the genome of an organism or carried in a plasmid that replicates in the organism. Where the hemicellulase is expressed from the genome, the gene and regulator sequences associate therewith, can be introduced into the genome by random or homologous integration. In certain cases, *e.g.*, when a particularly high level of expression is desired, both random and homologous integration can be used.

[0059] The biomass substrate for use as a source of cellulose and hemicellulose for hydrolysis using the present enzyme compositions and methods can be, *e.g.*, herbaceous material, agricultural residues, forestry residues, municipal solid waste, waste paper, and pulp and paper residues, and the like. Common forms of biomass

substrate include, but are not limited to trees, shrubs and grasses, wheat, wheat straw, sugar cane bagasse, corn, corn husks, corn kernel including fiber from kernels, products and by-products from milling of grains such as corn (including wet milling and dry milling) as well as municipal solid waste, waste paper and yard waste. The biomass substrate may be obtained from "virgin biomass" (such as trees, bushes, grasses, fruits, flowers, herbaceous crops, hard and soft woods.), "non-virgin biomass" (such as agricultural byproducts, commercial organic waste, construction and demolition debris, municipal solid waste and yard waste), or "blended biomass," which is a mixture of virgin and non-virgin biomass. The biomass substrate may include, *e.g.*, wood, wood pulp, papermaking sludge, paper pulp waste streams, particle board, corn stover, corn fiber, rice, paper and pulp processing waste, woody or herbaceous plants, fruit pulp, vegetable pulp, pumice, distillers grain, grasses, rice hulls, sugar cane bagasse, cotton, jute, hemp, flax, bamboo, sisal, abaca, straw, corn cobs, distillers grains, leaves, wheat straw, coconut hair, algae, switchgrass, and mixtures thereof.

[0060] The biomass substrate can be used directly or may be subjected to pretreatment using conventional methods known in the art. Such pretreatments include chemical, physical, and biological pretreatments. For example, physical pretreatment techniques include, without limitation, various types of milling, crushing, steaming/steam explosion, irradiation and hydrothermolysis. Chemical pretreatment techniques include, without limitation, dilute acid, alkaline agents, organic solvents, ammonia, sulfur dioxide, carbon dioxide, and pH-controlled hydrothermolysis. Biological pretreatment techniques include, without limitation, applying lignin-solubilizing microorganisms.

[0061] Optimum dosage levels of bioconverting enzyme blend, and cellulases and hemicellulases, therein, vary depending on the biomass substrate and the pretreatment technologies used. Operating conditions such as pH, temperature and reaction time may also affect rates of ethanol production. Preferably, the reactive composition contains 0.1 to 200 mg bioconverting enzyme blend per gram of biomass, more preferably 1 to 100 mg bioconverting enzyme blend per gram of biomass and most preferably 10-50 mg bioconverting enzyme blend per gram of biomass. Exemplary amounts are 0.1-50, 1-40, 20-40, 1-30, 2-40, and 10-20 mg bioconverting enzyme blend per gram of biomass. Alternatively, the amount of enzyme can be determined based on

the amount of substrate in the system. In such a case, the reactive composition preferably contains 0.1 to 50 mg bioconverting enzyme blend per gram of total saccharides, more preferably, 1 to 30 mg bioconverting enzyme blend per gram of total saccharides, and more preferably 10 to 20 mg bioconverting enzyme blend per gram of total saccharides. Alternatively, the amount of enzyme can be determined based on the amount of cellulose substrate in the system. In such a case, the reactive composition preferably contains 0.2 to 100 mg bioconverting enzyme blend per gram of total glucan, more preferably, 2 to 60 mg bioconverting enzyme blend per gram of total glucan, and more preferably 20 to 40 mg bioconverting enzyme blend per gram of total glucan. Similarly, the amount of bioconverting enzyme blend utilized can be determined by the amount of hemicellulose in the substrate biomass. Accordingly, the reactive composition preferably contains 0.2 to 100 mg bioconverting enzyme blend per gram of hemicellulose, more preferably, 2 to 60 mg bioconverting enzyme blend per gram of hemicellulose, and more preferably 20 to 40 mg bioconverting enzyme blend per gram of hemicellulose.

[0062] In some embodiments, the present composition is in the form of a hemicellulose-enhanced whole cellulase composition, comprising a whole cellulase preparation and at least one hemicellulase, wherein the amount of hemicellulase is in the range of 1% to 50% of the total protein and the whole cellulase is in the range of less than 99% to 50% of total protein. For example, the hemicellulase may represent 1% of the total protein and the whole cellulase composition may represent 99% of the total protein, the hemicellulase may represent 2% of the total protein and the whole cellulase composition may represent 98% of the total protein, the hemicellulase may represent 3% of the total protein and the whole cellulase composition may represent 97% of the total protein, the hemicellulase may represent 4% of the total protein and the whole cellulase composition may represent 96% of the total protein, the hemicellulase may represent 5% of the total protein and the whole cellulase composition may represent 95% of the total protein, the hemicellulase may represent 6% of the total protein and the whole cellulase composition may represent 94% of the total protein, the hemicellulase may represent 7% of the total protein and the whole cellulase composition may represent 93% of the total protein, the hemicellulase may represent 8% of the total

protein and the whole cellulase composition may represent 92% of the total protein, the hemicellulase may represent 9% of the total protein and the whole cellulase composition may represent 91% of the total protein, the hemicellulase may represent 10% of the total protein and the whole cellulase composition may represent 90% of the total protein, the hemicellulase may represent 11% of the total protein and the whole cellulase composition may represent 89% of the total protein, the hemicellulase may represent 12% of the total protein and the whole cellulase composition may represent 88% of the total protein, the hemicellulase may represent 13% of the total protein and the whole cellulase composition may represent 87% of the total protein, the hemicellulase may represent 14% of the total protein and the whole cellulase composition may represent 86% of the total protein, the hemicellulase may represent 15% of the total protein and the whole cellulase composition may represent 85% of the total protein, the hemicellulase may represent 16% of the total protein and the whole cellulase composition may represent 84% of the total protein, the hemicellulase may represent 17% of the total protein and the whole cellulase composition may represent 83% of the total protein, the hemicellulase may represent 18% of the total protein and the whole cellulase composition may represent 82% of the total protein, the hemicellulase may represent 19% of the total protein and the whole cellulase composition may represent 81% of the total protein, the hemicellulase may represent 20% of the total protein and the whole cellulase composition may represent 80% of the total protein, the hemicellulase may represent 21% of the total protein and the whole cellulase composition may represent 79% of the total protein, the hemicellulase may represent 22% of the total protein and the whole cellulase composition may represent 78% of the total protein, the hemicellulase may represent 23% of the total protein and the whole cellulase composition may represent 77% of the total protein, the hemicellulase may represent 24% of the total protein and the whole cellulase composition may represent 76% of the total protein, the hemicellulase may represent 25% of the total protein and the whole cellulase composition may represent 75% of the total protein, the hemicellulase may represent 26% of the total protein and the whole cellulase composition may represent 74% of the total protein, the hemicellulase may represent 27% of the total protein and the whole cellulase composition may represent

73% of the total protein, the hemicellulase may represent 28% of the total protein and the whole cellulase composition may represent 72% of the total protein, the hemicellulase may represent 29% of the total protein and the whole cellulase composition may represent 71% of the total protein, the hemicellulase may represent 30% of the total protein and the whole cellulase composition may represent 70% of the total protein, the hemicellulase may represent 31% of the total protein and the whole cellulase composition may represent 69% of the total protein, the hemicellulase may represent 32% of the total protein and the whole cellulase composition may represent 68% of the total protein, the hemicellulase may represent 33% of the total protein and the whole cellulase composition may represent 67% of the total protein, the hemicellulase may represent 34% of the total protein and the whole cellulase composition may represent 66% of the total protein, the hemicellulase may represent 35% of the total protein and the whole cellulase composition may represent 65% of the total protein, the hemicellulase may represent 36% of the total protein and the whole cellulase composition may represent 64% of the total protein, the hemicellulase may represent 37% of the total protein and the whole cellulase composition may represent 63% of the total protein, the hemicellulase may represent 38% of the total protein and the whole cellulase composition may represent 62% of the total protein, the hemicellulase may represent 39% of the total protein and the whole cellulase composition may represent 61% of the total protein, the hemicellulase may represent 40% of the total protein and the whole cellulase composition may represent 60% of the total protein, the hemicellulase may represent 41% of the total protein and the whole cellulase composition may represent 59% of the total protein, the hemicellulase may represent 42% of the total protein and the whole cellulase composition may represent 58% of the total protein, the hemicellulase may represent 43% of the total protein and the whole cellulase composition may represent 57% of the total protein, the hemicellulase may represent 44% of the total protein and the whole cellulase composition may represent 56% of the total protein, the hemicellulase may represent 45% of the total protein and the whole cellulase composition may represent 55% of the total protein, the hemicellulase may represent 46% of the total protein and the whole cellulase composition may represent 54% of the total protein, the hemicellulase may

represent 47% of the total protein and the whole cellulase composition may represent 53% of the total protein, the hemicellulase may represent 48% of the total protein and the whole cellulase composition may represent 52% of the total protein, the hemicellulase may represent 49% of the total protein and the whole cellulase composition may represent 51% of the total protein, or the hemicellulase may represent 50% of the total protein and the whole cellulase composition may represent 50% of the total protein.

[0063] In use, the bioconverting enzyme blend compositions may be added to a suitable substrate material individually, *i.e.*, as separate enzyme compositions, or as a single enzyme mixtures in which all cellulases and hemicellulases are present prior to addition to the substrate. Where the cellulases and hemicellulases are separate enzyme compositions, they may be added sequentially or simultaneously to the substrate. Where the cellulases and hemicellulases are present in a single mixture, they are added simultaneously.

[0064] Other aspects and embodiments of the compositions and method may be further understood in view of the following examples, which should not be construed as limiting. It will be apparent to those skilled in the art that many modifications, both to materials and methods, may be made without departing from the present teachings.

EXAMPLES

[0065] ACCELLERASE 1000™ (Danisco A/S, Genencor Division, Palo Alto, CA), a whole broth of killed cellular material that includes a *T. reesei* whole cellulase mixture supplemented with *T. reesei* BGLU1 β-glucosidase, was used as source of cellulases. MULTIFECT® Xylanase (Danisco A/S, Genencor Division, Palo Alto, CA), a xylanase II, high pI, formulated product, was used as a source of XYN2.

[0066] *T. reesei* hemicellulases were individually over-expressed in a strain of *T. reesei* in which the genes encoding CBHI, CBHII, EG1, and EG2 were deleted, to avoid the presence of these cellulases in the resulting cellular material (*e.g.*, conditioned media or "broths"). Hemicellulases of interest ranged from <10% to 85% of total protein in these broths. In many cases, the broths were used directly; however, several hemicellulases

were further purified to demonstrate that the observed activities were not the result of other protein present in the broth.

[0067] The acronyms, polypeptide SEQ ID NOs, and Carbohydrate-Active enZymes (CAZY) family and clan designations (where known) of the particular enzymes are provided in Table 1. The aforementioned XYN2 polypeptide has the amino acid sequence of SEQ ID NO: 1 and is a family GH11 Clan C enzyme. The amino acid sequences of the immature polypeptides are also shown, below.

TABLE 1

Acronym	Enzyme	SEQ ID	Family	Clan
ABF1	α-arabinofuranosidase I	3	GH54	
ABF2	α-arabinofuranosidase II	4	GH62	F
ABF3	α-arabinofuranosidase III	5	GH54	
AGL1	α-galactosidase I	6	GH27	D
AGL2	α-galactosidase II	7	GH36	D
AGL3	α-galactosidase III	8	GH27	D
AXE1	acetyl xylan esterase I	9	CE5	
AXE3	acetyl xylan esterase III	10	CE5	
EG6	endoglucanase VI	11	GH74	
EG8	endoglucanase VIII	12	GH5	A
GLR1	α-glucuronidase I	13	GH67	
MAN1	β-mannanase	14	GH5	A
PEC2	polygalacturonase	15	GH28	N
XYN1	xylanase I	16	GH11	C
XYN3	xylanase III	2	GH10	A
BXL1	β-xylosidase	17	GH3	

XYN2 (SEQ ID NO: 1)
MVSFTSLLAASPPSRASCRPAAEVESVAVEKRQTIQPGTGYNNGYFYSYWNDGHGGVTYTNG
PGGQFSVNWSNSGNFVGGKGWQPGTKNKVINFSGSYNPNGNSYLSVYGWSRNPLIEYYIVEN
FGTYNPSTGATKLGEVTSDGSVYDIYRTQRVNQPSIIGTATFYQYWSVRRNHRSSGSVNTANH
FNAWAQQGLTLGTMDYQIVAVEGYFSSGSASITVS

XYN3 (SEQ ID NO: 2)
MKANVILCLLAPLVAALPTETIHLDPELAALRANLTERTADLWDRQASQSIDQLIKRKGKLYFGTA
TDRGLLQREKNAAIIQADLGQVTPENSMKWQSLENNQGQLNWGDADYLVNFAQQNGKSIRGH
TLIWHSQLPAWVNNINNADTLRQVIRTHVSTVVGRYKGKIRAWDVVNEIFNEDGTLRSSVFSRL
LGEEFVSIAFRAARDADPSARLYINDYNLDRANYGKVNGLKTYVSKWISQGVPIDGIGSQSHLS
GGGGSGTLGALQQLATVPVTELAITELDIQGAPTTDYTQVVQACLSVSKCVGITVWGISDKDSW
RASTNPLLFDANFNPKPAYNSIVGILQ

ABF1 (SEQ ID NO: 3)

MLSNARIIAAGCIAAGSLVAAGPCDIYSSGGTPCVAAHSTTRALFSAYTGPLYQVKRGSDGATT
AISPLSSGVANAAAQDAFCAGTTCLITIIYDQSGRGNHLTQAPPGGFSGPESNGYDNLASAIGA
PVTLNGQKAYGVFVSPGTGYRNNAASGTAKGDAAEGMYAVLDGTHYNGACCFDYGNAETNS
RDTGNGHMEAIYFGDSTVWGTGSGKGPWIMADLENGLFSGSSPGNNAGDPSISYRFVTAAIK
GQPNQWAIRGGNAASGSLSTFYSGARPQVSGYNPMSKEGAIILGIGGDNSNGAQGTFYEGVM
TSGYPSDATENSVQANIVAARYAVAPLTSGPALTVGSSISLRATTACCTTRYIAHSGSTVNTQVV
SSSSATALKQQASWTVRAGLANNACFSFESRDTSGSYIRHSNFGLVLNANDGSKLFAEDATFC
TQAGINGQGSSIRSWSYPTRYFRHYNNTLYIASNGGVHVFDATAAFNDDVSFVVSGGFA

ABF2 (SEQ ID NO: 4)
MELKALSAVVLSFVTLVAAAPATCTLPSTYRWNSTGALASPKSGWVSLKDFSHVIYNGQHLVW
GSTHDTGTIWGSMNFGLFSDWSNMATASQNKMTPGTVAPTVFYFAPKNIWVLAYQWGPTTFS
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AGL1 (SEQ ID NO: 6)
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AGL3 (SEQ ID NO: 8)
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AXE1 (SEQ ID NO: 9)
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EG6 (SEQ ID NO: 11)
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EG8 (SEQ ID NO: 12)
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GLR1 (SEQ ID NO: 13)
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MAN1 (SEQ ID NO: 14)
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PEC2 (SEQ ID NO: 15)
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XYN1 (SEQ ID NO: 16)
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BXL1 (SEQ ID NO: 17)
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FVRTSNAGPAPYPNKWLVGFDRLADIKPGHSSKLSIPIPVSALARVDSHGNRIVYPGKYELALNT
DESVKLEFELVGEEVTIENWPLEEQQIKDATPDA

[0068] Secreted protein broths expressing ABF1, ABF2, ABF3, AGL1, AGL2, AGL3, AXE1, AXE3, EG6, EG8, GLR1, MAN1, PEC2, XYN1, XYN3, and BXL1 were tested in ternary mixes. 150 µl AFEX-pretreated corn stover (31.7% glucan, 19.1% xylan, 1.83% galactan, and 3.4% of arabinan, based on dry weight, made as either a 15.6 or 12% solids slurry in pH 5 50 mM sodium acetate buffer) was added to each well of a 96-well microtiter plate (all data points are based on triplicate wells). One experiment (shown in Table 9) employed dilute ammonia-pretreated corn cob at 13.84% solids as the substrate. To selected wells was added ACCELLERASE 1000™ (CEL) alone at 20 mg/G cellulose, ACCELLERASE 1000™ at 20 mg/G + 5 mg/G MULTIFECT® Xylanase, or ACCELLERASE 1000™ at 20 mg/G + 5 mg/G MULTIFECT® Xylanase + 1 or 5 mg/G of individual hemicellulase broths all in 20 µl total enzyme volume.

[0069] Enzyme doses were adjusted for total cellulose in either substrate slurry (15.6% or 12% solids). Plates were sealed and incubated with shaking at 50° C for 72 hours. Reactions were then quenched with 100 µl 100 mM glycine, pH 10. This mix was filtered and diluted an additional 6x (20 µl + 100 µl distilled H_20) and analyzed for sugar content on an HPLC-Aminex HPX-87P column on an Agilent Chem Station HPLC instrument. HPLC peak areas were converted to sugar concentrations based on a cellobiose standard curve for cellobiose and glucose or on a xylose standard curve for xylose. Percent conversion based on starting cellulose content was calculated to include H_2O of hydrolysis for each of the three sugar polymers. Standard deviations of triplicates were also calculated.

[0070] Table 2 and 3 provide the mean conversion (± standard deviation) of glucans and of xylans for each enzyme mixture as determined in two separate executions of the protocol. These separate runs were performed with the two different AFEX substrate slurries of 15.6% (Table2) and 12 % solids (Table3) and thus include different total mgs of cellulose, though the dose as mg/G cellulose is the same.

TABLE 2

Enzyme		%conversion glucan (± SD)	%conversion xylan (± SD)
20 mg/G CEL		56.31 (0.88)	39.47 (0.66)
25 mg/G CEL		61.12 (0.99)	41.82 (1.6)
30 mg/G CEL		66.48 (1.9)	46.69 (0.98)
20 mg/G CEL + 5 mg/G XYN2	---	**67.92 (1.1)**	**61.02 (1.3)**
	+ 5 mg/G ABF1	68.84 (2.1)	62.31 (0.67)
	+ 5 mg/G ABF2	74.84 (2.4)	62.36 (1.2)
	+ 5 mg/G ABF3	72.96 (1.4)	63.35 (3.7)
	+ 5 mg/G AXE1	71.93 (1.4)	64.78 (0.83)
	+ 5 mg/G BXL1	78.45 (2.8)	79.29 (4.9)
	+ 5 mg/G EG6	70.15 (2.1)	58.82 (2.7)
	+ 5 mg/G GLR1	67.81 (1.8)	65.70 (2.9)
	+ 5 mg/G MAN1	74.58 (0.80)	66.84 (0.64)
	+ 5 mg/G PEC2	72.94 (4.3)	61.99 (5.5)
	+ 5 mg/G XYN1	67.33 (1.1)	62.22 (0.44)
	+ 5 mg/G XYN3	78.82 (0.64)	73.63 (0.50)
	+ 1 mg/G XYN3 + 1 mg/G BXL1	77.37 (2.6)	74.44 (2.3)

TABLE 3

Enzyme		%conversion glucan (± SD)	%conversion xylan (±SD)
20 mg/G CEL		55.08 (1.8)	35.95 (0.94)
30 mg/G CEL		62.63 (0.96)	40.99 (0.30)
20 mg/G CEL + 5 mg/G XYN2	---	**63.96 (0.58)**	**55.06 (2.0)**
	+ 5 mg/G AGL1	67.52 (1.7)	56.00 (1.2)
	+ 5 mg/G AGL2	69.80 (2.7)	55.02 (1.8)
	+ 5 mg/G AGL3	66.51 (0.12)	55.93 (0.59)
	+ 5 mg/G AXE3	68.32 (1.4)	55.89 (0.67)
	+ 5 mg/G EG8	70.68 (3.9)	55.40 (2.7)

[0071] The addition of XYN2 was effective in increasing xylan conversion. Six enzyme mixtures with a third component (*i.e.*, XYN3, AGL2, EG8, BXL1, ABF3, or PEC2) showed further advantages in terms of glucan and/or xylan conversion compared to cellulase with XYN2. A quaternary enzyme mix was run according to the procedure described above. Table 4 provides the mean conversion (± standard deviation) of glucans and xylans for each enzyme mixture.

TABLE 4

Enzyme		%conversion glucan (±SD)	%conversion xylan (±SD)
20 mg/G CEL		55.43 (6.5)	42.29 (2.3)
20 mg/G CEL + 5 mg/G XYN2		71.27 (0.67)	63.96 (1.2)
20 mg/G CEL + 5 mg/G XYN3		85.07 (3.1)	68.69 (2.6)
20 mg/G CEL + 5 XYN2 + 5 mg/G XYN3		86.82 (1.2)	80.68 (0.33)
20 mg/G CEL + 5 mg/G XYN2 + 2.5 XYN3	---	**76.57 (0.94)**	**72.70 (0.64)**
	+ 5 mg/G ABF3	81.58 (0.76)	75.89 (0.73)
	+ 5 mg/G AGL2	78.66 (2.7)	72.49 (2.3)
	+ 5 mg/G BXL1	72.80 (6.7)	78.60 (2.1)
	+ 5 mg/G EG8	74.72 (6.0)	73.29 (2.8)
	+ 5 mg/G PEC2	78.18 (2.4)	73.90 (2.9)

[0072] In another experiment, ACCELLERASE 1000™ was mixed with purified XYN2 and/or XYN3 and assayed (Table 5). The combination of XYN2 and XYN3 produced more efficient glucan and xylan conversion.

TABLE 5

Enzyme		%conversion glucan (±SD)	%conversion xylan (±SD)
10 mg/G CEL	---	43.97 (1.4)	28.41 (1.0)
	+ 10 mg/G XYN2	59.22 (4.3)	56.83 (5.7)
	+ 10 mg/G XYN3	51.44 (8.6)	43.53 (1.6)
20 mg/G CEL	---	60.29 (1.7)	40.02 (0.33)
	+ 5 mg/G XYN2 + 5 mg/G XYN3	73.73 (0.79)	61.81 (1.2)
	+ 10 mg/G XYN2 + 10 mg/G XYN3	74.71 (1.6)	65.20 (1.4)
30 mg/G CEL		67.05 (0.74)	43.74 (0.14)

[0073] In a further example, XYN4, XYN5, FAE1 and a new lot of ABF3 with ~ 50% protein of interest (compared to previous lot at <10%) were tested as above in mixtures containing 20 mg/G ACCELLERASE 1000™ + 5 mg/G MULTIFECT® Xylanase XYN2. The results are shown in Table 6. The addition of XYN4, XYN5, or FAE1 was effective in increasing the conversion of glucan and xylan.

TABLE 6

Enzyme		%conversion glucan (± SD)	%conversion xylan (±SD)
20 mg/G CEL		57.52 (1.08)	38.37 (0.38)
30 mg/G CEL		66.21 (1.37)	44.15 (0.70)
20 mg/G CEL + 5 mg/G XYN2	---	**68.44 (0.23)**	**60.46 (0.48)**
	+ 5 mg/G ABF3	66.22 (5.99)	67.46 (3.97)
	+ 5 mg/G XYN4	72.17 (0.66)	63.47 (0.44)
	+ 5 mg/G XYN5	71.91 (3.74)	62.73 (3.37)
	+ 5 mg/G FAE1	70.98 (1.47)	67.02 (1.59)

[0074] In another experiment, ACCELLERASE 1000™ was mixed with purified BxI1 and XYN2 and/or XYN3 and assayed as above. The results are shown in Table 7. Several enzyme combinations were effective in increasing the conversion of glucan and/or xylan.

TABLE 7

Enzyme	%conversion glucan (± SD)	%conversion xylan (± SD)
35 mg/G CEL	67.95 (0.67)	40.36 (0.36)
30 mg/G CEL	66.51 (1.99)	38.63 (0.56)
20 mg/G CEL	58.03 (3.19)	32.28 (1.41)
10 mg/g CEL	45.01 (0.59)	23.85 (0.42)
10 mg/g CEL + 10 mg/G BXL1	46.89 (4.16)	48.85 (2.94)
20 CEL + 5 XYN2 + 5 BXL1	69.45 (4.88)	60.15 (1.17)
20 CEL + 5 XYN3 + 5 BXL1	65.17 (8.37)	65.36 (1.14)
20 CEL + 5 XYN2 + 5 XYN3 +5 BXL1	75.13 (1.20)	66.97 (1.07)

[0075] In another example, ABF1, ABF2 and ABF3 (ABF3 sample lot with <10% protein of interest), singly, in binary and ternary combinations were added to a background of 20 mg/G ACCELLERASE 1000™ + 5 mg/G purified XYN3 + 5 mg/G purified BXL1. The results are shown in Table 8. Several enzyme combinations were effective in increasing the conversion of glucan and/or xylan.

TABLE 8

Enzyme		%conversion glucan (± SD)	%conversion xylan (±SD)
30 mg/G CEL		67.55 (0.18)	45.05 (6.67)
45 mg/G CEL		79.39 (4.66)	56.05 (2.31)
20 mg/G CEL + 5 mg/GXYN3 + 5 mg/G BXL1	---	**73.24 (4.39)**	**79.88 (4.72)**
	+ 5 mg/G ABF1	58.21 (0.55)	86.84 (0.47)
	+ 5 mg/G ABF2	84.39 (1.01)	87.15 (1.32)
	+ 5 mg/G ABF3	65.07 (3.68)	73.46 (4.13)
	+ 5 mg/GABF1 +5 mg/G ABF2	87.65 (3.11)	87.08 (2.31)
	+ 5 mg/GABF1 +5 mg/G ABF3	67.62 (5.01)	87.77 (2.91)
	+ 5 mg/GABF2 +5 mg/G ABF3	91.21 (1.82)	89.98 (1.08)
	+ 5 mg/GABF1 +5 mg/G ABF2 + 5 mg/G ABF3	99.67 (3.45)	96.73 (4.74)

[0076] In another example 3.4 mg/G xylan of purified ABF1, ABF2 and/or ABF3 were added to a 20.7 mg/G glucan of ACCELLERASE 1000™ + 5.1 mg/G xylan each of purified XYN3 and BXL1. The results are shown in Table 9. Several enzyme combinations were effective in increasing the conversion of glucan and/or xylan.

TABLE 9

Enzyme		%conversion glucan (± SD)	%conversion xylan (±SD)
30.9 mg/G CEL		66.45 (1.64)	33.84 (0.83)
41.3 mg/G CEL		67.99 (0.57)	35.95 (0.11)
20.7 mg/G CEL + 5.1 mg/GXYN3 + 5.1 mg/G BXL1	---	**76.67 (0.30)**	**63.86 (0.08)**
	+3.4 mg/G ABF1	76.37 (1.32)	64.18 (1.77)
	+3.4 mg/G ABF2	77.84(1.48)	66.59 (2.07)
	+3.4 mg/G ABF3	77.53 (1.94)	66.86 (1.84)
	+3.4 mg/G ABF1 +3.4 mg/G ABF2	78.32 (1.56)	67.65 (2.31)
	+ 3.4 mg/G ABF1 +3.4 mg/G ABF3	77.53 (1.04)	66.89 (0.51)
	+3.4mg/GABF2 +3.4 mg/G ABF3	79.92 (0.27)	68.96 (0.03)
	+ 3.4mg/G ABF1 +3.4 mg/G ABF2 + 3.4 mg/G ABF3	80.22 (1.98)	68.76 (2.22)

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[0077] Where the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification, they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component or group thereof.

[0078] Further, any prior art reference or statement provided in the specification is not to be taken as an admission that such art constitutes, or is to be understood as constituting, part of the common general knowledge in Australia.

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The Claims defining the invention are as follows:

1. An enzyme blend composition, comprising:

(a) a first enzyme composition comprising a cellulase,

(b) a second enzyme composition comprising at least one xylanase selected from a GH10 or GH11 xylanase, and

(c) a third enzyme composition comprising at least one additional hemicellulase that is not a GH10 or GH11 xylanase or not the same GH10 or GH11 xylanase as in (b),

wherein the enzyme blend composition provides (i) enhanced glucan conversion and (ii) enhanced xylan conversion from a mixture of cellulosic and hemicellulosic materials as compared to the glucan and xylan conversion levels achieved by an equivalent enzyme blend composition lacking the at least one additional hemicellulase.

2. The composition of claim 1, wherein the first enzyme composition is a whole cellulase blend produced from fermenting an engineered filamentous fungus.

3. The composition of claim 1, wherein the first enzyme composition is a whole cellulase blend produced from fermenting an engineered filamentous fungus supplemented with an additional amount of β-glucosidase.

4. The composition of any one of the preceding claims, wherein the second enzyme composition comprises xylanase XYN2 from *Trichoderma reesei* of SEQ ID NO: 1 or of a variant of XYN2 having an amino acid sequence that is at least 80% identical to SEQ ID NO: 1.

5. The composition of any one of the preceding claims, wherein the second enzyme composition comprises xylanase XYN3 from *Trichoderma reesei* of SEQ ID NO: 2 or of a variant of XYN3 having an amino acid sequence that is at least 80% identical to SEQ ID NO: 2.

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6. The composition of any one of the preceding claims, wherein the at least one additional hemicellulase is a β-xylosidase or an arabinofuranosidase.

7. The composition of claim 6, wherein the β-xylosidase is BXL1 from *Trichoderma reesei* and the arabinofuranosidase is ABF1, ABF2, or ABF3 from *Trichoderma reesei*.

8. The composition of any one of claims 1 to 5, wherein the at least one additional hemicellulase is a combination of a β-xylosidase and an arabinofuranosidase.

9. The composition of claim 8, wherein the β-xylosidase is BXL1 from *Trichoderma reesei* and the arabinofuranosidase is ABF1, ABF2, or ABF3 from *Trichoderma reesei*.

10. The composition of any one of the preceding claims, wherein the first enzyme composition is a whole cellulase blend from a filamentous fungus supplemented with an additional amount of β-glucosidase, the second enzyme composition comprises a xylanase, and the at least one additional hemicellulase is a combination of a β-xylosidase and arabinofuranosidase.

11. The composition of any one of the preceding claims, wherein the at least one additional hemicellulase is a *Trichoderma reesei* hemicellulase selected from the group consisting of α-arabinofuranosidase I (ABF1), α-arabinofuranosidase II (ABF2), α-arabinofuranosidase III (ABF3), α-galactosidase I (AGL1), α-galactosidase II (AGL2), α-galactosidase III (AGL3), acetyl xylan esterase I (AXE1), acetyl xylan esterase III (AXE3), endoglucanase VI (EG6), endoglucanase VIII (EG8), α-glucuronidase I (GLR1), β-mannanase (MAN1), polygalacturonase (PEC2), xylanase I (XYN1), xylanase II (XYN2), xylanase III (XYN3), and β-xylosidase (BXL1).

12. The composition of any one of the preceding claims, wherein the at least one additional hemicellulase has an amino acid sequence having at least 80% identity to an

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amino acid sequence selected from the group consisting of 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, and SEQ ID NO: 17.

13. A method for hydrolyzing a mixture of cellulosic and hemicellulosic materials, comprising contacting the mixture of cellulosic and hemicellulosic materials with:

(a) a first enzyme composition comprising a cellulase,

(b) a second enzyme composition comprising at least one xylanase selected from a GH10 or GH11 xylanase, and

(c) a third enzyme composition comprising at least one additional hemicellulase that is not a GH10 or GH11 xylanase or not the same GH10 or GH11 xylanase as in (b),

thereby hydrolyzing the mixture of cellulosic and hemicellulosic materials,

wherein the contacting results in (i) enhanced glucan conversion and (ii) enhanced xylan conversion compared to equivalent contacting in the absence of the at least one additional hemicellulase.

14. The method of claim 13, wherein the first enzyme composition is a whole cellulase blend produced from fermenting an engineered filamentous fungus.

15. The method of claim 13, wherein the first enzyme composition is a whole cellulase blend produced from fermenting an engineered filamentous fungus supplemented with an additional amount of β-glucosidase.

16. The method of any one of claims 13 to 15, wherein the second enzyme composition comprises xylanase XYN2 from *Trichoderma reesei*.

17. The method of any one of claims 13 to 15, wherein the second enzyme composition comprises xylanase XYN3 from *Trichoderma reesei*.

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18. The method of any one of claims 13 to 15, wherein the at least one xylanase has an amino acid sequence having at least 80% identity to an amino acid sequence selected from SEQ ID NO: 1 or SEQ ID NO: 2.

19. The method of any one of claims 13 to 15, wherein the at least one additional hemicellulase is selected from the group consisting of a GH54 hemicellulase, a GH62 hemicellulase, a GH27 hemicellulase, a GH36 hemicellulase, a GH5 hemicellulase, a GH74 hemicellulase, a GH67 hemicellulase, a GH28 hemicellulase, a GH11 hemicellulase, a GH10 hemicellulase, a GH3 hemicellulase, and a CE5 hemicellulase.

20. The method of any one of claims 13 to 15, wherein the at least one additional hemicellulase is a β-xylosidase or an arabinofuranosidase.

21. The method of claim 20, wherein the β-xylosidase is BXL1 from *Trichoderma reesei* and the arabinofuranosidase is ABF1, ABF2, or ABF3 from *Trichoderma reesei*.

22. The method of any one of claims 13 to 15, wherein the at least one additional hemicellulase is a combination of a β-xylosidase and an arabinofuranosidase.

23. The method of claim 22, wherein the β-xylosidase is BXL1 from *Trichoderma reesei* and the arabinofuranosidase is ABF1, ABF2, or ABF3 from *Trichoderma reesei*.

24. The method of any one of claims 13 to 23, wherein the first enzyme composition is a whole cellulase blend from a filamentous fungus supplemented with an additional amount of β-glucosidase, the second enzyme composition comprises xylanase, and the at least one additional hemicellulase is a combination of a β-xylosidase and arabinofuranosidase.

25. The method of any one of claims 13 to 15, wherein the at least one additional hemicellulase is a *Trichoderma reesei* hemicellulase selected from the group

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consisting of α-arabinofuranosidase I (ABF1), α-arabinofuranosidase II (ABF2), α-arabinofuranosidase III (ABF3), α-galactosidase I (AGL1), α-galactosidase II (AGL2), α-galactosidase III (AGL3), acetyl xylan esterase I (AXE1), acetyl xylan esterase III (AXE3), endoglucanase VI (EG6), endoglucanase VIII (EG8), α-glucuronidase I (GLR1), β-mannanase (MAN1), polygalacturonase (PEC2), xylanase I (XYN1), xylanase II (XYN2), xylanase III (XYN3), and β-xylosidase (BXL1).

26. The method of any one of claims 13 to 15, wherein the at least one additional hemicellulase has an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of 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, and SEQ ID NO: 17.

27. The method of any one of claims 13 to 26, wherein contacting the mixture of cellulosic and hemicellulosic materials with the first enzyme composition, the second enzyme composition, and the third enzyme composition are performed simultaneously.

28. The method of any one of claims 13 to 26, wherein the first enzyme composition, the second enzyme composition, and the third enzyme composition are provided in a single composition enzyme blend.

29. The enzyme blend composition according to claim 1, or the method according to claim 13, substantially as hereinbefore described with reference to the accompanying Examples.