Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 2961280 C 2022/08/30

(11)(21) **2 961 280**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 2015/09/22
(87) Date publication PCT/PCT Publication Date: 2016/03/31
(45) Date de délivrance/Issue Date: 2022/08/30
(85) Entrée phase nationale/National Entry: 2017/03/14
(86) N° demande PCT/PCT Application No.: CN 2015/090204
(87) N° publication PCT/PCT Publication No.: 2016/045569
(30) Priorités/Priorities: 2014/09/23 (CN PCT/CN2014/087191); 2014/12/11 (CN PCT/CN2014/093589); 2015/04/27 (CN PCT/CN2015/077496)

(51) Cl.Int./Int.Cl. *C12N 1/19* (2006.01), *C12N 1/22* (2006.01), *C12P 1/00* (2006.01), *C12P 7/06* (2006.01)

(72) Inventeurs/Inventors:
DIAO, LIUYANG, CN;
SUTTON, KATE BRANDON, US;
JIANG, YU, CN;
YANG, SHENG, CN;
QIAN, FENGHUI, CN;
LIU, YINGMIAO, CN

(73) Propriétaire/Owner:
NOVOZYMES A/S, DK

(74) Agent: NORTON ROSE FULBRIGHT CANADA LLP/S.E.N.C.R.L., S.R.L.

(54) Titre : PROCEDES DE PRODUCTION D'ETHANOL ET ORGANISMES DE FERMENTATION
(54) Title: PROCESSES FOR PRODUCING ETHANOL AND FERMENTING ORGANISMS

(57) Abrégé/Abstract:
Processes for producing ethanol comprise saccharifying cellulosic material with a cellulolytic enzyme composition and fermenting the saccharified cellulosic material with a fermenting microorganism to produce ethanol. The fermenting organism is Saccharomyces cerevisiae CIBTS1260 (deposited under Accession No. NRRL Y-50973 at the Agricultural Research Service Culture Collection (NRRL), Illinois 61604 U.S.A.) or a fermenting organism that has properties that the same or about the same as that of Saccharomyces cerevisiae CIBTS1260).

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 March 2016 (31.03.2016)

(10) International Publication Number
WO 2016/045569 A1

(51) **International Patent Classification**:
C12N 1/19 (2006.01) *C12P 1/00* (2006.01)
C12N 1/22 (2006.01) *C12P 7/06* (2006.01)

(21) **International Application Number**: PCT/CN2015/090204

(22) **International Filing Date**: 22 September 2015 (22.09.2015)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
PCT/CN2014/087191 23 September 2014 (23.09.2014) CN
PCT/CN2014/093589 11 December 2014 (11.12.2014) CN
PCT/CN2015/077496 27 April 2015 (27.04.2015) CN

(71) **Applicant**: **NOVOZYMES A/S** [DK/DK]; Krogshoejvej 36, DK-2880 Bagsvaerd, DK- (DK).

(72) **Inventor; and**
(71) **Applicant** *(for BW only)*: **DIAO, Liuyang** [CN/CN]; Room 501, No. 143, 55 Yifeng Road, Pudong New District, Shanghai 201210 (CN).

(72) **Inventors**: **SUTTON, Kate Brandon**; 7208 Bolero Circle, Raleigh, 27615 (US). **JIANG, Yu**; 16-1402, 199 Baiyang Road, Shanghai 201201 (CN). **YANG, Sheng**; 7-501, 333 Liyuan Road, Shanghai 200011 (CN). **QIAN, Fenghui**; Room 401, No.2, Fourth village in Nanyuan, Jiading District, Shanghai 201821 (CN). **LIU, Yingmiao**; Room 603, Building 9, Chuanhuan South Road 1049, Lane 45, Pudong, Shanghai 201200 (CN).

(74) **Agent**: **LIU, SHEN & ASSOCIATES**; 10th Floor, Building 1, 10 Caihefang Road, Haidian District, Beijing 100080 (CN).

(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, BN, 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, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, 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, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, 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, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2016/045569 A1

(54) **Title**: PROCESSES FOR PRODUCING ETHANOL AND FERMENTING ORGANISMS

(57) **Abstract**: Processes for producing ethanol comprise saccharifying cellulosic material with a cellulolytic enzyme composition and fermenting the saccharified cellulosic material with a fermenting microorganism to produce ethanol. The fermenting organism is Saccharomyces cerevisiae CIBTS1260 (deposited under Accession No. NRRL Y-50973 at the Agricultural Research Service Culture Collection (NRRL), Illinois 61604 U.S.A.) or a fermenting organism that has properties that the same or about the same as that of Saccharomyces cerevisiae CIBTS1260).

PROCESSES FOR PRODUCING ETHANOL AND FERMENTING ORGANISMS

FIELD OF THE INVENTION

The present invention relates to improved processes for producing ethanol from cellulosic material and improved fermenting organisms.

BACKGROUND OF THE INVENTION

Ethanol is a transportation fuel commonly blending into gasoline. Cellulosic material is used as a feedstock in ethanol production processes. There are several processes in the art for making cellulose and hemicelluloses hydrolysates containing glucose, mannose, xylose and arabinose. Glucose and mannose are efficiently converted to ethanol during natural anaerobic metabolism. By far the most efficient ethanol producing microorganism is the yeast *Saccharomyces cerevisiae*. However, *Saccharomyces cerevisiae* lacks the necessary enzymes to convert the dominant sugar xylose into xylulose and is therefore unable to utilize xylose as a carbon source. To do so requires genetic engineering of *Saccharomyces cerevisiae* to express enzymes that can convert xylose into xylulose. One of the enzymes needed is xylose isomerase (E.C. 5.3.1.5) which converts xylose into xylulose, which can then be converted into ethanol during fermentation by *Saccharomyces cerevisiae*.

WO 2003/062430 discloses that the introduction of a functional *Piromyces* xylose isomerase (XI) into *Saccharomyces cerevisiae* allows slow metabolism of xylose via the endogenous xylulokinase (EC 2.7.1.17) encoded by *XKS1* and the enzymes of the non-oxidative part of the pentose phosphate pathway and confers to the yeast transformants the ability to grow on xylose.

US patent no. 8,586,336-B2 disclosed a *Saccharomyces cerevisiae* yeast strain expressing a xylose isomerase obtained by bovine rumen fluid. The yeast strain can be used to produce ethanol by culturing under anaerobic fermentation conditions.

Despite significant improvement of ethanol production processes from cellulosic material over the past decade there is still a desire and need for providing improved processes. To produce ethanol economically a fermentation organism that is biologically efficient is required.

SUMMARY OF THE INVENTION

The present invention also relates to processes of producing ethanol, comprising:

(a) saccharifying a cellulosic material with a cellulolytic enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting microorganism to produce ethanol; wherein the fermenting organism is *Saccharomyces cerevisiae* CIBTS1260 (deposited under Accession No. NRRL Y-50973 at the Agricultural Research Service Culture Collection (NRRL), Illinois 61604 U.S.A.) or a fermenting organism strain having properties that
5 are about the same as that of *Saccharomyces cerevisiae* CIBTS1260.

In a preferred embodiment the process comprises recovering the ethanol from the fermentation.

In an embodiment the yeast cell pitch is between 0.1 and 20 g DWC *Saccharomyces cerevisiae* CIBTS1260 /L fermentation medium, such as 0.2-10 g/L, preferably 0.3-5 g/L, such
10 as 0.4 g/L, such as around 1g DWC/L or around 2 g DWC/L.

In another aspect the invention relates to recombinant fermenting organisms having properties that are the same as that of *Saccharomyces cerevisiae* CIBTS1260 (deposited under Accession No. NRRL Y-50973 at the Agricultural Research Service Culture Collection (NRRL), Illinois 61604 U.S.A.) or a fermenting organism having properties that are about the same as
15 that of *Saccharomyces cerevisiae* CIBTS1260.

In a preferred embodiment the fermenting organism of the invention having properties that are about the same as that of *Saccharomyces cerevisiae* CIBTS1260 has one or more, such as all, of the following properties:

- higher xylose consumption compared to BSGX001 after 48 hours fermentation at 1 g
20 DWC/L, 35ºC, pH 5.5, in particular as described in Example 3;
- higher glucose consumption compared to BSGX001 after 48 hours fermentation at 1 g DWC/L, 35ºC, pH 5.5, in particular as described in Example 3;
- higher ethanol production compared to BSGX001 after 48 hours fermentation at 1 g DWC/L, 35ºC, pH 5.5, in particular as described in Example 3.

25 In an embodiment the fermenting organism of the invention comprises a gene encoding the amino acid sequence having xylose isomerase activity shown in SEQ ID NO: 2 in US 8586336B2 or SEQ ID NO: 13 herein, or an amino acid sequence being at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to SEQ ID NO: 2 in
30 US 8586336B2 or SEQ ID NO: 13 herein. The gene in the fermenting organism encoding the xylose isomerase may be the one shown in SEQ ID NO: 1 in US patent no. 8,586,336-B2 or SEQ ID NO: 20 herein or a sequence having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%,

Date reçue / Date received 2021-12-16

such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical thereto.

In an embodiment the fermenting organism of the invention has one or more, such as all, of the following genetic modifications:

5 - xylose isomerases gene (Ru-XI) obtained from bovine rumen fluid, in particular the one shown in SEQ ID NO: 20 herein, encoding the xylose isomerase shown in SEQ ID NO: 13 herein;

- optionally a pentose transporter gene (GXF1) from *Candida intermedia,* in particular the one shown in SEQ ID NO: 18;

10 - xylulokinase gene (XKS), in particular from a type strain of *Saccharomyces cerevisiae;*

- ribulose 5 phosphate 3-epimerase gene (RPE1), in particular from a type strain of *Saccharomyces cerevisiae*;

- ribulose 5 phosphate isomerase gene (RKI1), in particular from a type strain of *Saccharomyces cerevisiae*;

15 - transketolase gene (TKL1) and transaldolase gene (TAL1), in particular from a type strain of *Saccharomyces cerevisiae*.

In a specific embodiment the fermenting organism is *Saccharomyces cerevisiae* CIBTS1260 (deposited under Accession No. NRRL Y-50973 at the Agricultural Research Service Culture Collection (NRRL), Illinois 61604 U.S.A.).

20

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows a plasmid map of the plasmid pYIE2-mgXI-GXF1-delta harboring the mgXI and GXF expression cassettes.

Fig. 2 shows a plasmid map of the plasmid used pSH47-hyg.

25 Fig. 3 shows a map of the resulting plasmid pYIE2-XKS1-PPP-δ.

Fig. 4 shows a fermentation comparison of CIBTS1260 versus BSGX001 in NREL Acid Pretreated Corn Stover Hydrolysate at 1 g DCW/L yeast pitch, 35ºC, pH 5.5, in 72 hours.

Fig. 5 shows a comparison of *Saccharomyces cerevisiae* vs. BSGX001 in model media: 2/L yeast pitch, 32°C, pH 5.5, 72 hours.

30 Fig. 6 shows a fermentation comparison of Cellulolytic Enzyme Composition CA and Cellulolytic Enzyme Composition CB generated bagasse hydrolysate with CIBTS1260 at 1 g/L yeast pitch in 72 hours.

Fig. 7 shows percentage reduction of DP2 concentration during fermentation of hydrolysates generated with Cellulase CA or CB at 1g/L yeast pitch, 35˚C, pH 5.5, 72 hours.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides improved processes for producing ethanol from lignocellulosic material using a fermenting organism.

5

Definitions

Auxiliary Activity 9: The term "Auxiliary Activity 9" or "AA9" means a polypeptide classified as a lytic polysaccharide monooxygenase (Quinlan *et al.*, 2011, *Proc. Natl. Acad. Sci. USA* 208: 15079-15084; Phillips *et al.*, 2011, *ACS Chem. Biol.* 6: 1399-1406; Lin *et al.*, 2012,
10 *Structure* 20: 1051-1061). AA9 polypeptides were formerly classified into the glycoside hydrolase Family 61 (GH61) according to Henrissat, 1991, *Biochem. J.* 280: 309-316, and Henrissat and Bairoch, 1996, *Biochem. J.* 316: 695-696.

AA9 polypeptides enhance the hydrolysis of a cellulosic material by an enzyme having cellulolytic activity. Cellulolytic enhancing activity can be determined by measuring the increase
15 in reducing sugars or the increase of the total of cellobiose and glucose from the hydrolysis of a cellulosic material by cellulolytic enzyme under the following conditions: 1-50 mg of total protein/g of cellulose in pretreated corn stover (PCS), wherein total protein is comprised of 50-99.5% w/w cellulolytic enzyme protein and 0.5-50% w/w protein of an AA9 polypeptide for 1-7 days at a suitable temperature, such as 40C-80°C, *e.g.*, 50°C, 55°C, 60°C, 65°C, or 70°C, and a
20 suitable pH, such as 4-9, *e.g.*, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, or 8.5, compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS).

AA9 polypeptide enhancing activity can be determined using a mixture of CELLUCLAST™ 1.5L (Novozymes A/S, Bagsværd, Denmark) and beta-glucosidase as the
25 source of the cellulolytic activity, wherein the beta-glucosidase is present at a weight of at least 2-5% protein of the cellulase protein loading. In one aspect, the beta-glucosidase is an *Aspergillus oryzae* beta-glucosidase (*e.g.*, recombinantly produced in *Aspergillus oryzae* according to WO 02/095014). In another aspect, the beta-glucosidase is an *Aspergillus fumigatus* beta-glucosidase (*e.g.*, recombinantly produced in *Aspergillus oryzae* as described in
30 WO 02/095014).

AA9 polypeptide enhancing activity can also be determined by incubating an AA9 polypeptide with 0.5% phosphoric acid swollen cellulose (PASC), 100 mM sodium acetate pH 5, 1 mM $MnSO_4$, 0.1% gallic acid, 0.025 mg/ml of *Aspergillus fumigatus* beta-glucosidase, and

0.01% TRITON® X-100 (4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol) for 24-96 hours at 40°C followed by determination of the glucose released from the PASC.

AA9 polypeptide enhancing activity can also be determined according to WO 2013/028928 for high temperature compositions.

5 AA9 polypeptides enhance the hydrolysis of a cellulosic material catalyzed by enzyme having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at least 1.01-fold, *e.g.*, at least 1.05-fold, at least 1.10-fold, at least 1.25-fold, at least 1.5-fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 10-fold, or at least 20-fold.

10 **Beta-glucosidase:** The term “beta-glucosidase” means a beta-D-glucoside glucohydrolase (E.C. 3.2.1.21) that catalyzes the hydrolysis of terminal non-reducing beta-D-glucose residues with the release of beta-D-glucose. Beta-glucosidase activity can be determined using *p*-nitrophenyl-beta-D-glucopyranoside as substrate according to the procedure of Venturi *et al.*, 2002, *J. Basic Microbiol.* 42: 55-66. One unit of beta-glucosidase is 15 defined as 1.0 µmole of *p*-nitrophenolate anion produced per minute at 25°C, pH 4.8 from 1 mM *p*-nitrophenyl-beta-D-glucopyranoside as substrate in 50 mM sodium citrate containing 0.01% TWEEN® 20.

Beta-xylosidase: The term “beta-xylosidase” means a beta-D-xyloside xylohydrolase (E.C. 3.2.1.37) that catalyzes the exo-hydrolysis of short beta (1→4)-xylooligosaccharides to 20 remove successive D-xylose residues from non-reducing termini. Beta-xylosidase activity can be determined using 1 mM *p*-nitrophenyl-beta-D-xyloside as substrate in 100 mM sodium citrate containing 0.01% TWEEN® 20 at pH 5, 40°C. One unit of beta-xylosidase is defined as 1.0 µmole of *p*-nitrophenolate anion produced per minute at 40°C, pH 5 from 1 mM *p*-nitrophenyl-beta-D-xyloside in 100 mM sodium citrate containing 0.01% TWEEN® 20.

25 **Catalase:** The term “catalase” means a hydrogen-peroxide:hydrogen-peroxide oxidoreductase (EC 1.11.1.6) that catalyzes the conversion of 2 H_2O_2 to O_2 + 2 H_2O. For purposes of the present invention, catalase activity is determined according to U.S. Patent No. 5,646,025. One unit of catalase activity equals the amount of enzyme that catalyzes the oxidation of 1 µmole of hydrogen peroxide under the assay conditions.

30 **Cellobiohydrolase:** The term “cellobiohydrolase” means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91 and E.C. 3.2.1.176) that catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing end (cellobiohydrolase I) or non-reducing end (cellobiohydrolase II) of the chain (Teeri, 1997, *Trends in Biotechnology* 15: 160-167; Teeri *et*

al., 1998, *Biochem. Soc. Trans.* 26: 173-178). Cellobiohydrolase activity can be determined according to the procedures described by Lever *et al.*, 1972, *Anal. Biochem.* 47: 273-279; van Tilbeurgh *et al.*, 1982, *FEBS Letters* 149: 152-156; van Tilbeurgh and Claeyssens, 1985, *FEBS Letters* 187: 283-288; and Tomme *et al.*, 1988, *Eur. J. Biochem.* 170: 575-581.

5 **Cellulolytic enzyme composition or cellulase:** The term "cellulolytic enzyme composition" or "cellulase" means one or more (*e.g.*, several) enzymes that hydrolyze a cellulosic material. Such enzymes include endoglucanase(s), cellobiohydrolase(s), beta-glucosidase(s), or combinations thereof. The two basic approaches for measuring cellulolytic enzyme activity include: (1) measuring the total cellulolytic enzyme activity, and (2) measuring
10 the individual cellulolytic enzyme activities (endoglucanases, cellobiohydrolases, and beta-glucosidases) as reviewed in Zhang *et al.*, 2006, *Biotechnology Advances* 24: 452-481. Total cellulolytic enzyme activity can be measured using insoluble substrates, including Whatman №1 filter paper, microcrystalline cellulose, bacterial cellulose, algal cellulose, cotton, pretreated lignocellulose, etc. The most common total cellulolytic activity assay is the filter paper assay
15 using Whatman №1 filter paper as the substrate. The assay was established by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987, *Pure Appl. Chem.* 59: 257-68).

Cellulolytic enzyme activity can be determined by measuring the increase in production/release of sugars during hydrolysis of a cellulosic material by cellulolytic enzyme(s)
20 under the following conditions: 1-50 mg of cellulolytic enzyme protein/g of cellulose in pretreated corn stover (PCS) (or other pretreated cellulosic material) for 3-7 days at a suitable temperature such as 40°C-80°C, *e.g.*, 50°C, 55°C, 60°C, 65°C, or 70°C, and a suitable pH such as 4-9, *e.g.*, 5.0, 5.5, 6.0, 6.5, or 7.0, compared to a control hydrolysis without addition of cellulolytic enzyme protein. Typical conditions are 1 ml reactions, washed or unwashed PCS, 5% insoluble solids
25 (dry weight), 50 mM sodium acetate pH 5, 1 mM $MnSO_4$, 50°C, 55°C, or 60°C, 72 hours, sugar analysis by AMINEX® HPX-87H column chromatography (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cellulosic material: The term "cellulosic material" means any material containing cellulose. The predominant polysaccharide in the primary cell wall of biomass is cellulose, the
30 second most abundant is hemicellulose, and the third is pectin. The secondary cell wall, produced after the cell has stopped growing, also contains polysaccharides and is strengthened by polymeric lignin covalently cross-linked to hemicellulose. Cellulose is a homopolymer of anhydrocellobiose and thus a linear beta-(1-4)-D-glucan, while hemicelluloses include a variety of compounds, such as xylans, xyloglucans, arabinoxylans, and mannans in complex branched

structures with a spectrum of substituents. Although generally polymorphous, cellulose is found in plant tissue primarily as an insoluble crystalline matrix of parallel glucan chains. Hemicelluloses usually hydrogen bond to cellulose, as well as to other hemicelluloses, which help stabilize the cell wall matrix.

5 Cellulose is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. The cellulosic material can be, but is not limited to, agricultural residue, herbaceous material (including energy crops), municipal solid waste, pulp and paper mill residue, waste paper, and wood (including forestry residue) (see, for example, Wiselogel *et al.*, 1995, in Handbook on Bioethanol (Charles E. Wyman, editor), pp. 10 105-118, Taylor & Francis, Washington D.C.; Wyman, 1994, *Bioresource Technology* 50: 3-16; Lynd, 1990, *Applied Biochemistry and Biotechnology* 24/25: 695-719; Mosier *et al.*, 1999, Recent Progress in Bioconversion of Lignocellulosics, in *Advances in Biochemical Engineering/Biotechnology*, T. Scheper, managing editor, Volume 65, pp. 23-40, Springer-Verlag, New York). It is understood herein that the cellulose may be in the form of 15 lignocellulose, a plant cell wall material containing lignin, cellulose, and hemicellulose in a mixed matrix. In one aspect, the cellulosic material is any biomass material. In another aspect, the cellulosic material is lignocellulose, which comprises cellulose, hemicelluloses, and lignin.

In an embodiment, the cellulosic material is agricultural residue, herbaceous material (including energy crops), municipal solid waste, pulp and paper mill residue, waste paper, or 20 wood (including forestry residue).

In another embodiment, the cellulosic material is arundo, bagasse, bamboo, corn cob, corn fiber, corn stover, miscanthus, rice straw, switchgrass, or wheat straw.

In another embodiment, the cellulosic material is aspen, eucalyptus, fir, pine, poplar, spruce, or willow.

25 In another embodiment, the cellulosic material is algal cellulose, bacterial cellulose, cotton linter, filter paper, microcrystalline cellulose (*e.g.*, AVICEL®), or phosphoric-acid treated cellulose.

In another embodiment, the cellulosic material is an aquatic biomass. As used herein the term "aquatic biomass" means biomass produced in an aquatic environment by a 30 photosynthesis process. The aquatic biomass can be algae, emergent plants, floating-leaf plants, or submerged plants.

The cellulosic material may be used as is or may be subjected to pretreatment, using conventional methods known in the art, as described herein. In a preferred aspect, the cellulosic material is pretreated.

Endoglucanase: The term "endoglucanase" means a 4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4) that catalyzes endohydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3-1,4 glucans such as cereal beta-D-glucans or
5 xyloglucans, and other plant material containing cellulosic components. Endoglucanase activity can be determined by measuring reduction in substrate viscosity or increase in reducing ends determined by a reducing sugar assay (Zhang *et al.*, 2006, *Biotechnology Advances* 24: 452-481). Endoglucanase activity can also be determined using carboxymethyl cellulose (CMC) as substrate according to the procedure of Ghose, 1987, *Pure and Appl. Chem.* 59: 257-268, at pH
10 5, 40°C.

Hemicellulolytic enzyme or hemicellulase: The term "hemicellulolytic enzyme" or "hemicellulase" means one or more (*e.g.*, several) enzymes that hydrolyze a hemicellulosic material. See, for example, Shallom and Shoham, 2003, *Current Opinion In Microbiology* 6(3): 219-228). Hemicellulases are key components in the degradation of plant biomass. Examples of
15 hemicellulases include, but are not limited to, an acetylmannan esterase, an acetylxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. The substrates for these enzymes, hemicelluloses, are a heterogeneous group of branched and linear polysaccharides that are bound via hydrogen
20 bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose a highly complex structure. The variable structure and organization of hemicelluloses require the concerted action of many enzymes for its complete degradation. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze glycosidic bonds, or
25 carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. These catalytic modules, based on homology of their primary sequence, can be assigned into GH and CE families. Some families, with an overall similar fold, can be further grouped into clans, marked alphabetically (*e.g.*, GH-A). A most informative and updated classification of these and other carbohydrate active enzymes is available in the Carbohydrate-
30 Active Enzymes (CAZy) database. Hemicellulolytic enzyme activities can be measured according to Ghose and Bisaria, 1987, *Pure & Appl. Chem.* 59: 1739-1752, at a suitable temperature such as 40°C-80°C, *e.g.*, 50°C, 55°C, 60°C, 65°C, or 70°C, and a suitable pH such as 4-9, *e.g.*, 5.0, 5.5, 6.0, 6.5, or 7.0.

Pretreated corn stover: The term "Pretreated Corn Stover" or "PCS" means a cellulosic material derived from corn stover by treatment with heat and dilute sulfuric acid, alkaline pretreatment, neutral pretreatment, or any pretreatment known in the art.

Xylan-containing material: The term "xylan-containing material" means any material comprising a plant cell wall polysaccharide containing a backbone of beta-(1-4)-linked xylose residues. Xylans of terrestrial plants are heteropolymers possessing a beta-(1-4)-D-xylopyranose backbone, which is branched by short carbohydrate chains. They comprise D-glucuronic acid or its 4-*O*-methyl ether, L-arabinose, and/or various oligosaccharides, composed of D-xylose, L-arabinose, D- or L-galactose, and D-glucose. Xylan-type polysaccharides can be divided into homoxylans and heteroxylans, which include glucuronoxylans, (arabino)glucuronoxylans, (glucurono)arabinoxylans, arabinoxylans, and complex heteroxylans. See, for example, Ebringerova *et al.*, 2005, *Adv. Polym. Sci.* 186: 1-67.

In the processes of the present invention, any material containing xylan may be used. In a preferred aspect, the xylan-containing material is cellulosic material.

Xylan degrading activity or xylanolytic activity: The term "xylan degrading activity" or "xylanolytic activity" means a biological activity that hydrolyzes xylan-containing material. The two basic approaches for measuring xylanolytic activity include: (1) measuring the total xylanolytic activity, and (2) measuring the individual xylanolytic activities (*e.g.*, endoxylanases, beta-xylosidases, arabinofuranosidases, alpha-glucuronidases, acetylxylan esterases, feruloyl esterases, and alpha-glucuronyl esterases). Recent progress in assays of xylanolytic enzymes was summarized in several publications including Biely and Puchard, 2006, *Journal of the Science of Food and Agriculture* 86(11): 1636-1647; Spanikova and Biely, 2006, *FEBS Letters* 580(19): 4597-4601; Herrimann *et al.*, 1997, *Biochemical Journal* 321: 375-381.

Total xylan degrading activity can be measured by determining the reducing sugars formed from various types of xylan, including, for example, oat spelt, beechwood, and larchwood xylans, or by photometric determination of dyed xylan fragments released from various covalently dyed xylans. A common total xylanolytic activity assay is based on production of reducing sugars from polymeric 4-O-methyl glucuronoxylan as described in Bailey *et al.*, 1992, Interlaboratory testing of methods for assay of xylanase activity, *Journal of Biotechnology* 23(3): 257-270. Xylanase activity can also be determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% TRITON® X-100 and 200 mM sodium phosphate pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 µmole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6.

Xylan degrading activity can be determined by measuring the increase in hydrolysis of birchwood xylan (Sigma Chemical Co., Inc., St. Louis, MO, USA) by xylan-degrading enzyme(s) under the following typical conditions: 1 ml reactions, 5 mg/ml substrate (total solids), 5 mg of xylanolytic protein/g of substrate, 50 mM sodium acetate pH 5, 50°C, 24 hours, sugar analysis
5 using *p*-hydroxybenzoic acid hydrazide (PHBAH) assay as described by Lever, 1972, *Anal. Biochem.* 47: 273-279.

Xylanase: The term "xylanase" means a 1,4-beta-D-xylan-xylohydrolase (E.C. 3.2.1.8) that catalyzes the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans. Xylanase activity can be determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% TRITON® X-100 and
10 200 mM sodium phosphate pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 µmole of azurine produced per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6.

Xylose Isomerase: The term "Xylose Isomerase" or "XI" means an enzyme which can catalyze D-xylose into D-xylulose in vivo, and convert D-glucose into D-fructose in vitro. Xylose
15 isomerase is also known as "glucose isomerase" and is classified as E.C. 5.3.1.5. As the structure of the enzyme is very stable, the xylose isomerase is one of the good models for studying the relationships between protein structure and functions (Karimaki et al., Protein Eng Des Sel, 12004, 17 (12):861-869).Moreover, the extremely important industrial application value makes the xylose isomerase is seen as important industrial enzyme as protease and amylase
20 (Tian Shen et al. , Microbiology Bulletin, 2007, 34 (2): 355-358; Bhosale et al. , Microbiol Rev, 1996, 60 (2): 280-300). The scientists keep high concern and carried out extensive research on xylose isomerase. Since 1970s, the applications of the xylose isomerase have focused on the production of high fructose syrup and fuel ethanol. In recent years, scientists have found that under certain conditions, the xylose isomerase can be used for producing many important rare
25 sugars, which are the production materials in the pharmaceutical industry, such as ribose, mannose, arabinose and lyxose (Karlmaki et al., Protein Eng Des Se, 12004, 17 (12): 861-869).These findings bring new vitality in the research on the xylose isomerase.

Processes of The Invention

30 The present invention also relates to processes of producing ethanol, comprising:

(a) saccharifying a cellulosic material with a cellulolytic enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting microorganism to produce ethanol; wherein the fermenting organism is *Saccharomyces cerevisiae* CIBTS1260 (deposited under Accession No. NRRL Y-50973 at the Agricultural Research Service Culture

Collection (NRRL), Illinois 61604 USA.) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* CIBTS1260.

In a preferred embodiment the process comprises recovering the ethanol from the fermentation medium.

5 The processing of the cellulosic material according to the present invention can be accomplished using methods conventional in the art. Moreover, the processes of the present invention can be implemented using any conventional biomass processing apparatus configured to operate in accordance with the invention.

Saccharification (i.e., hydrolysis) and fermentation, separate or simultaneous, include, 10 but are not limited to, separate hydrolysis and fermentation (SHF); simultaneous saccharification and fermentation (SSF); simultaneous saccharification and co-fermentation (SSCF); hybrid hydrolysis and fermentation (HHF); separate hydrolysis and co-fermentation (SHCF); hybrid hydrolysis and co-fermentation (HHCF).

SHF uses separate process steps to first enzymatically hydrolyze the cellulosic material 15 to fermentable sugars, *e.g.*, glucose, cellobiose, and pentose monomers, and then ferment the fermentable sugars to ethanol. In SSF, the enzymatic hydrolysis of the cellulosic material and the fermentation of sugars to ethanol are combined in one step (Philippidis, G. P., 1996, Cellulose bioconversion technology, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212). SSCF involves the co-20 fermentation of multiple sugars (Sheehan and Himmel, 1999, *Biotechnol. Prog.* 15: 817-827). HHF involves a separate hydrolysis step, and in addition a simultaneous saccharification and hydrolysis step, which can be carried out in the same reactor. The steps in an HHF process can be carried out at different temperatures, *i.e.*, high temperature enzymatic saccharification followed by SSF at a lower temperature that the fermentation organismcan tolerate. It is 25 understood herein that any method known in the art comprising pretreatment, enzymatic hydrolysis (saccharification), fermentation, or a combination thereof, can be used in the practicing the processes of the present invention.

A conventional apparatus can include a fed-batch stirred reactor, a batch stirred reactor, a continuous flow stirred reactor with ultrafiltration, and/or a continuous plug-flow column reactor 30 (de Castilhos Corazza *et al.*, 2003, *Acta Scientiarum. Technology* 25: 33-38; Gusakov and Sinitsyn, 1985, *Enz. Microb. Technol.* 7: 346-352), an attrition reactor (Ryu and Lee, 1983, *Biotechnol. Bioeng.* 25: 53-65). Additional reactor types include fluidized bed, upflow blanket, immobilized, and extruder type reactors for hydrolysis and/or fermentation.

Pretreatment

In an embodiment the cellulosic material is pretreated before saccharification in step (a).

In practicing the processes of the present invention, any pretreatment process known in the art can be used to disrupt plant cell wall components of the cellulosic material (Chandra *et al.*, 2007, *Adv. Biochem. Engin./Biotechnol.* 108: 67-93; Galbe and Zacchi, 2007, *Adv. Biochem. Engin./Biotechnol.* 108: 41-65; Hendriks and Zeeman, 2009, *Bioresource Technology* 100: 10-18; Mosier *et al.*, 2005, *Bioresource Technology* 96: 673-686; Taherzadeh and Karimi, 2008, *Int. J. Mol. Sci.* 9: 1621-1651; Yang and Wyman, 2008, *Biofuels Bioproducts and Biorefining-Biofpr.* 2: 26-40).

The cellulosic material can also be subjected to particle size reduction, sieving, pre-soaking, wetting, washing, and/or conditioning prior to pretreatment using methods known in the art.

Conventional pretreatments include, but are not limited to, steam pretreatment (with or without explosion), dilute acid pretreatment, hot water pretreatment, alkaline pretreatment, lime pretreatment, wet oxidation, wet explosion, ammonia fiber explosion, organosolv pretreatment, and biological pretreatment. Additional pretreatments include ammonia percolation, ultrasound, electroporation, microwave, supercritical CO_2, supercritical H_2O, ozone, ionic liquid, and gamma irradiation pretreatments.

In a preferred embodiment the cellulosic material is pretreated before saccharification (i.e., hydrolysis) and/or fermentation. Pretreatment is preferably performed prior to the hydrolysis. Alternatively, the pretreatment can be carried out simultaneously with enzyme hydrolysis to release fermentable sugars, such as glucose, xylose, and/or cellobiose. In most cases the pretreatment step itself results in some conversion of biomass to fermentable sugars (even in absence of enzymes).

Steam Pretreatment. In steam pretreatment, the cellulosic material is heated to disrupt the plant cell wall components, including lignin, hemicellulose, and cellulose to make the cellulose and other fractions, *e.g.*, hemicellulose, accessible to enzymes. The cellulosic material is passed to or through a reaction vessel where steam is injected to increase the temperature to the required temperature and pressure and is retained therein for the desired reaction time. Steam pretreatment is preferably performed at 140-250°C, *e.g.*, 160-200°C or 170-190°C, where the optimal temperature range depends on optional addition of a chemical catalyst. Residence time for the steam pretreatment is preferably 1-60 minutes, *e.g.*, 1-30 minutes, 1-20 minutes, 3-12 minutes, or 4-10 minutes, where the optimal residence time depends on the temperature and optional addition of a chemical catalyst. Steam pretreatment allows for

relatively high solids loadings, so that the cellulosic material is generally only moist during the pretreatment. The steam pretreatment is often combined with an explosive discharge of the material after the pretreatment, which is known as steam explosion, that is, rapid flashing to atmospheric pressure and turbulent flow of the material to increase the accessible surface area by fragmentation (Duff and Murray, 1996, *Bioresource Technology* 855: 1-33; Galbe and Zacchi, 2002, *Appl. Microbiol. Biotechnol.* 59: 618-628; U.S. Patent Application No. 2002/0164730). During steam pretreatment, hemicellulose acetyl groups are cleaved and the resulting acid autocatalyzes partial hydrolysis of the hemicellulose to monosaccharides and oligosaccharides. Lignin is removed to only a limited extent.

Chemical Pretreatment. The term “chemical treatment” refers to any chemical pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin. Such a pretreatment can convert crystalline cellulose to amorphous cellulose. Examples of suitable chemical pretreatment processes include, for example, dilute acid pretreatment, lime pretreatment, wet oxidation, ammonia fiber/freeze expansion (AFEX), ammonia percolation (APR), ionic liquid, and organosolv pretreatments.

A chemical catalyst such as H_2SO_4 or SO_2 (typically 0.3 to 5% w/w) is sometimes added prior to steam pretreatment, which decreases the time and temperature, increases the recovery, and improves enzymatic hydrolysis (Ballesteros *et al.*, 2006, *Appl. Biochem. Biotechnol.* 129-132: 496-508; Varga *et al.*, 2004, *Appl. Biochem. Biotechnol.* 113-116: 509-523; Sassner *et al.*, 2006, *Enzyme Microb. Technol.* 39: 756-762). In dilute acid pretreatment, the cellulosic material is mixed with dilute acid, typically H_2SO_4, and water to form a slurry, heated by steam to the desired temperature, and after a residence time flashed to atmospheric pressure. The dilute acid pretreatment can be performed with a number of reactor designs, *e.g.*, plug-flow reactors, counter-current reactors, or continuous counter-current shrinking bed reactors (Duff and Murray, 1996, *Bioresource Technology* 855: 1-33; Schell *et al.*, 2004, *Bioresource Technology* 91: 179-188; Lee *et al.*, 1999, *Adv. Biochem. Eng. Biotechnol.* 65: 93-115). In a specific embodiment the dilute acid pretreatment of cellulosic material is carried out using 4% w/w sulfuric acid at 180ºC for 5 minutes.

Several methods of pretreatment under alkaline conditions can also be used. These alkaline pretreatments include, but are not limited to, sodium hydroxide, lime, wet oxidation, ammonia percolation (APR), and ammonia fiber/freeze expansion (AFEX) pretreatment.

Lime pretreatment is performed with calcium oxide or calcium hydroxide at temperatures of 85-150°C and residence times from 1 hour to several days (Wyman *et al.*, 2005, *Bioresource Technology* 96: 1959-1966; Mosier *et al.*, 2005, *Bioresource Technology* 96: 673-686). WO

2006/110891, WO 2006/110899, WO 2006/110900, and WO 2006/110901 disclose pretreatment methods using ammonia.

Wet oxidation is a thermal pretreatment performed typically at 180-200°C for 5-15 minutes with addition of an oxidative agent such as hydrogen peroxide or over-pressure of oxygen (Schmidt and Thomsen, 1998, *Bioresource Technology* 64: 139-151; Palonen *et al.*, 2004, *Appl. Biochem. Biotechnol.* 117: 1-17; Varga *et al.*, 2004, *Biotechnol. Bioeng.* 88: 567-574; Martin *et al.*, 2006, *J. Chem. Technol. Biotechnol.* 81: 1669-1677). The pretreatment is performed preferably at 1-40% dry matter, *e.g.*, 2-30% dry matter or 5-20% dry matter, and often the initial pH is increased by the addition of alkali such as sodium carbonate.

A modification of the wet oxidation pretreatment method, known as wet explosion (combination of wet oxidation and steam explosion) can handle dry matter up to 30%. In wet explosion, the oxidizing agent is introduced during pretreatment after a certain residence time. The pretreatment is then ended by flashing to atmospheric pressure (WO 2006/032282).

Ammonia fiber expansion (AFEX) involves treating the cellulosic material with liquid or gaseous ammonia at moderate temperatures such as 90-150°C and high pressure such as 17-20 bar for 5-10 minutes, where the dry matter content can be as high as 60% (Gollapalli *et al.*, 2002, *Appl. Biochem. Biotechnol.* 98: 23-35; Chundawat *et al.*, 2007, *Biotechnol. Bioeng.* 96: 219-231; Alizadeh *et al.*, 2005, *Appl. Biochem. Biotechnol.* 121: 1133-1141; Teymouri *et al.*, 2005, *Bioresource Technology* 96: 2014-2018). During AFEX pretreatment cellulose and hemicelluloses remain relatively intact. Lignin-carbohydrate complexes are cleaved.

Organosolv pretreatment delignifies the cellulosic material by extraction using aqueous ethanol (40-60% ethanol) at 160-200°C for 30-60 minutes (Pan *et al.*, 2005, *Biotechnol. Bioeng.* 90: 473-481; Pan *et al.*, 2006, *Biotechnol. Bioeng.* 94: 851-861; Kurabi *et al.*, 2005, *Appl. Biochem. Biotechnol.* 121: 219-230). Sulphuric acid is usually added as a catalyst. In organosolv pretreatment, the majority of hemicellulose and lignin is removed.

Other examples of suitable pretreatment methods are described by Schell *et al.*, 2003, *Appl. Biochem. Biotechnol.* 105-108: 69-85, and Mosier *et al.*, 2005, *Bioresource Technology* 96: 673-686, and U.S. Published Application 2002/0164730.

In one aspect, the chemical pretreatment is preferably carried out as a dilute acid treatment, and more preferably as a continuous dilute acid treatment. The acid is typically sulfuric acid, but other acids can also be used, such as acetic acid, citric acid, nitric acid, phosphoric acid, tartaric acid, succinic acid, hydrogen chloride, or mixtures thereof. Mild acid treatment is conducted in the pH range of preferably 1-5, *e.g.*, 1-4 or 1-2.5. In one aspect, the acid concentration is in the range from preferably 0.01 to 10 wt. % acid, *e.g.*, 0.05 to 5 wt. % acid or 0.1 to 2 wt. % acid. The

acid is contacted with the cellulosic material and held at a temperature in the range of preferably 140-200°C, *e.g.*, 165-190°C, for periods ranging from 1 to 60 minutes.

In another aspect, pretreatment takes place in an aqueous slurry. In preferred aspects, the cellulosic material is present during pretreatment in amounts preferably between 10-80 wt. %, *e.g.*, 20-70 wt. % or 30-60 wt. %, such as around 40 wt. %. The pretreated cellulosic material can be unwashed or washed using any method known in the art, *e.g.*, washed with water.

<u>Mechanical Pretreatment or Physical Pretreatment</u>: The term “mechanical pretreatment” or “physical pretreatment” refers to any pretreatment that promotes size reduction of particles. For example, such pretreatment can involve various types of grinding or milling (*e.g.*, dry milling, wet milling, or vibratory ball milling).

The cellulosic material can be pretreated both physically (mechanically) and chemically. Mechanical or physical pretreatment can be coupled with steaming/steam explosion, hydrothermolysis, dilute or mild acid treatment, high temperature, high pressure treatment, irradiation (*e.g.*, microwave irradiation), or combinations thereof. In one aspect, high pressure means pressure in the range of preferably about 100 to about 400 psi, *e.g.*, about 150 to about 250 psi. In another aspect, high temperature means temperature in the range of about 100 to about 300°C, *e.g.*, about 140 to about 200°C. In a preferred aspect, mechanical or physical pretreatment is performed in a batch-process using a steam gun hydrolyzer system that uses high pressure and high temperature as defined above, *e.g.*, a Sunds Hydrolyzer available from Sunds Defibrator AB, Sweden. The physical and chemical pretreatments can be carried out sequentially or simultaneously, as desired.

Accordingly, in a preferred aspect, the cellulosic material is subjected to physical (mechanical) or chemical pretreatment, or any combination thereof, to promote the separation and/or release of cellulose, hemicellulose, and/or lignin.

<u>Biological Pretreatment.</u> The term “biological pretreatment” refers to any biological pretreatment that promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from the cellulosic material. Biological pretreatment techniques can involve applying lignin-solubilizing microorganisms and/or enzymes (see, for example, Hsu, T.-A., 1996, Pretreatment of biomass, in *Handbook on Bioethanol: Production and Utilization*, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh and Singh, 1993, *Adv. Appl. Microbiol.* 39: 295-333; McMillan, J. D., 1994, Pretreating lignocellulosic biomass: a review, in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, chapter 15;

Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in *Advances in Biochemical Engineering/Biotechnology*, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson and Hahn-Hagerdal, 1996, *Enz. Microb. Tech.* 18: 312-331; and Vallander and Eriksson, 1990, *Adv. Biochem. Eng./Biotechnol.* 42: 63-95).

Saccharification

In the saccharification step (i.e., hydrolysis step), the cellulosic material, *e.g.*, pretreated, is hydrolyzed to break down cellulose and/or hemicellulose to fermentable sugars, such as glucose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides. The hydrolysis is performed enzymatically by a cellulolytic enzyme composition. The enzymes of the compositions can be added simultaneously or sequentially.

Enzymatic hydrolysis is preferably carried out in a suitable aqueous environment under conditions that can be readily determined by one skilled in the art. In one aspect, hydrolysis is performed under conditions suitable for the activity of the enzymes(s), *i.e.*, optimal for the enzyme(s). The hydrolysis can be carried out as a fed batch or continuous process where the cellulosic material is fed gradually to, for example, an enzyme containing hydrolysis solution.

The saccharification is generally performed in stirred-tank reactors or fermentors under controlled pH, temperature, and mixing conditions. Suitable process time, temperature and pH conditions can readily be determined by one skilled in the art. For example, the saccharification can last up to 200 hours, but is typically performed for preferably about 12 to about 120 hours, *e.g.*, about 16 to about 72 hours or about 24 to about 48 hours. The temperature is in the range of preferably about 25°C to about 70°C, *e.g.*, about 30°C to about 65°C, about 40°C to about 60°C, or about 50°C to about 55°C. The pH is in the range of preferably about 3 to about 8, *e.g.*, about 3.5 to about 7, about 4 to about 6, or about 4.5 to about 5.5. The dry solids content is in the range of preferably about 5 to about 50 wt. %, *e.g.*, about 10 to about 40 wt. % or about 20 to about 30 wt. %.

Saccharification in step (a) is carried out using a cellulolytic enzyme composition. Such enzyme compositions are described below in the "Cellulolytic Enzyme Composition'-section below. The cellulolytic enzyme compositions can comprise any protein useful in degrading the cellulosic material. In one aspect, the cellulolytic enzyme composition comprises or further comprises one or more (*e.g.*, several) proteins selected from the group consisting of a cellulase, an AA9 (GH61) polypeptide, a hemicellulase, an esterase, an expansin, a ligninolytic enzyme, an oxidoreductase, a pectinase, a protease, and a swollenin.

In another aspect, the cellulase is preferably one or more (*e.g.*, several) enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

In another aspect, the hemicellulase is preferably one or more (*e.g.*, several) enzymes selected from the group consisting of an acetylmannan esterase, an acetylxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. In another aspect, the oxidoreductase is preferably one or more (*e.g.*, several) enzymes selected from the group consisting of a catalase, a laccase, and a peroxidase.

The enzymes or enzyme compositions used in a processes of the present invention may be in any form suitable for use, such as, for example, a fermentation broth formulation or a cell composition, a cell lysate with or without cellular debris, a semi-purified or purified enzyme preparation, or a host cell as a source of the enzymes. The enzyme composition may be a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a stabilized protected enzyme. Liquid enzyme preparations may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, and/or lactic acid or another organic acid according to established processes.

In one aspect, an effective amount of cellulolytic or hemicellulolytic enzyme composition to the cellulosic material is about 0.5 to about 50 mg, *e.g.*, about 0.5 to about 40 mg, about 0.5 to about 25 mg, about 0.75 to about 20 mg, about 0.75 to about 15 mg, about 0.5 to about 10 mg, or about 2.5 to about 10 mg per g of the cellulosic material.

In one aspect, such a compound is added at a molar ratio of the compound to glucosyl units of cellulose of about 10^{-6} to about 10, *e.g.*, about 10^{-6} to about 7.5, about 10^{-6} to about 5, about 10^{-6} to about 2.5, about 10^{-6} to about 1, about 10^{-5} to about 1, about 10^{-5} to about 10^{-1}, about 10^{-4} to about 10^{-1}, about 10^{-3} to about 10^{-1}, or about 10^{-3} to about 10^{-2}. In another aspect, an effective amount of such a compound is about 0.1 μM to about 1 M, *e.g.*, about 0.5 μM to about 0.75 M, about 0.75 μM to about 0.5 M, about 1 μM to about 0.25 M, about 1 μM to about 0.1 M, about 5 μM to about 50 mM, about 10 μM to about 25 mM, about 50 μM to about 25 mM, about 10 μM to about 10 mM, about 5 μM to about 5 mM, or about 0.1 mM to about 1 mM.

The term "liquor" means the solution phase, either aqueous, organic, or a combination thereof, arising from treatment of a lignocellulose and/or hemicellulose material in a slurry, or monosaccharides thereof, *e.g.*, xylose, arabinose, mannose, etc., under conditions as described in WO 2012/021401, and the soluble contents thereof. A liquor for cellulolytic enhancement of

an AA9 polypeptide (GH61 polypeptide) can be produced by treating a lignocellulose or hemicellulose material (or feedstock) by applying heat and/or pressure, optionally in the presence of a catalyst, *e.g.*, acid, optionally in the presence of an organic solvent, and optionally in combination with physical disruption of the material, and then separating the solution from the residual solids. Such conditions determine the degree of cellulolytic enhancement obtainable through the combination of liquor and an AA9 polypeptide during hydrolysis of a cellulosic substrate by a cellulolytic enzyme preparation. The liquor can be separated from the treated material using a method standard in the art, such as filtration, sedimentation, or centrifugation.

In one aspect, an effective amount of the liquor to cellulose is about 10^{-6} to about 10 g per g of cellulose, *e.g.*, about 10^{-6} to about 7.5 g, about 10^{-6} to about 5 g, about 10^{-6} to about 2.5 g, about 10^{-6} to about 1 g, about 10^{-5} to about 1 g, about 10^{-5} to about 10^{-1} g, about 10^{-4} to about 10^{-1} g, about 10^{-3} to about 10^{-1} g, or about 10^{-3} to about 10^{-2} g per g of cellulose.

Fermentation

The fermentable sugars obtained from the hydrolyzed cellulosic material can be fermented by one or more (*e.g.*, several) fermenting microorganisms capable of fermenting the sugars directly or indirectly into a desired fermentation product. “Fermentation” or “fermentation process” refers to any fermentation process or any process comprising a fermentation step. Fermentation processes also include fermentation processes used in the consumable alcohol industry (*e.g.*, beer and wine), dairy industry (*e.g.*, fermented dairy products), leather industry, and tobacco industry. The fermentation conditions depend on the desired fermentation product and fermenting organism and can easily be determined by one skilled in the art.

In the fermentation step, sugars, released from the cellulosic material as a result of the pretreatment and enzymatic hydrolysis steps, are fermented to a product, *e.g.*, ethanol, by a fermenting organism, such as yeast. Hydrolysis (saccharification) and fermentation can be separate or simultaneous.

Any suitable hydrolyzed cellulosic material can be used in the fermentation step in practicing the present invention. The material is generally selected based on economics, *i.e.*, costs per equivalent sugar potential, and recalcitrance to enzymatic conversion.

The term “fermentation medium” is understood herein to refer to a medium before the fermenting microorganism(s) is(are) added, such as, a medium resulting from a saccharification process, as well as a medium used in a simultaneous saccharification and fermentation process (SSF).

Fermenting Organism of The Invention

In this aspect the invention relates to recombinant fermenting organisms capable of converting hexoses and pentoses into ethanol.

In an embodiment the invention related to recombinant fermenting organisms having properties that are the same as that of *Saccharomyces cerevisiae* CIBTS1260 (deposited under Accession No. NRRL Y-50973 at the Agricultural Research Service Culture Collection (NRRL), Illinois 61604 U.S.A.) or a fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* CIBTS1260.

In an embodiment the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* CIBTS1260 has one or more, such as all, of the following properties:

- higher xylose consumption compared to BSGX001 after 48 hours fermentation at 1 g DWC/L, 35°C, pH 5.5, in particular as described in Example 3;
- higher glucose consumption compared to BSGX001 after 48 hours fermentation at 1 g DWC/L, 35°C, pH 5.5, in particular as described in Example 3;
- higher ethanol production compared to BSGX001 after 48 hours fermentation at 1 g DWC/L, 35°C, pH 5.5, in particular as described in Example 3.

In an embodiment the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* CIBTS1260 provides full xylose consumption by 48 hours fermentation under the process conditions in Example 3, i.e., 1g DCW/L, 35°C, pH 5.5.

In an embodiment the fermenting organism having properties that are about the same as that of *Saccharomyces cerevisiae* CIBTS1260 provides full glucose consumption by 24 hours fermentation under the process conditions in Example 3, i.e., 1g DCW/L, 35°C, pH 5.5.

In an embodiment the fermenting organism organism having properties that are about the same as that of *Saccharomyces cerevisiae* CIBTS1260 provides more than 30 g/L ethanol, such as more than 40 g/L ethanol, such as more than 45 g/L ethanol, such as approximately 47 g/L ethanol after 48 hours fermentation under the process conditions in Example 3, i.e., 1g DCW/L, 35°C, pH 5.5.

In a preferred embodiment the recombinant fermenting organism is *Saccharomyces cerevisiae* CIBTS1260 (deposited under Accession No. NRRL Y-50973 at the Agricultural Research Service Culture Collection (NRRL), Illinois 61604 U.S.A.).

In an embodiment the fermenting organism of the invention comprises a gene encoding an amino acid sequence having xylose isomerase activity shown in SEQ ID NO: 2 in US 8586336B2 or SEQ ID NO: 13 herein, or an amino acid sequence being at least 80%, such as

at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% identical to SEQ ID NO: 2 in US 8586336B2 or SEQ ID NO: 13 herein.

In an optional embodiment the fermenting organism of the invention comprises a
5 pentose transporter gene, such as a GFX gene, in particular GFX1 from *Candida intermedia,* e.g., the sequence shown in SEQ ID NO: 18.

In an embodiment the pentose transporter gene comprised in the fermenting organism has 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
10 97%, at least 98%, at least 99% or 100% sequence identity SEQ ID NO: 18 herein.

In an embodiment the fermenting organism of the invention overexpresses a xylulokinase gene (XKS), in particular from a type strain of *Saccharomyces cerevisiae*.

In an embodiment the fermenting organism of the invention overexpresses a ribulose 5 phosphate 3-epimerase gene (RPE1), in particular from a type strain of *Saccharomyces*
15 *cerevisiae*.

In an embodiment the fermenting organism of the invention overexpresses a ribulose 5 phosphate isomerase gene (RKI1), in particular from a type strain of *Saccharomyces cerevisiae*.

In an embodiment the fermenting organism of the invention overexpresses a transketolase gene (TKL1) and overexpresses a transaldolase gene (TAL1), in particular from a
20 type strain of *Saccharomyces cerevisiae*.

In an embodiment the fermenting organism of the invention has one or more, such as one, two, three, four, five or all, of the following genetic modifications:

- xylose isomerases gene (Ru-XI) obtained from bovine rumen fluid, in particular the one shown in SEQ ID NO: 20 herein, encoding the xylose isomerase shown in SEQ ID NO: 13
25 herein;
- optionally a pentose transporter gene (GXF1) from *Candida intermedia,* in particular the one shown in SEQ ID NO: 18;
- xylulokinase gene (XKS), in particular from a type strain of *Saccharomyces cerevisiae;*
- ribulose 5 phosphate 3-epimerase gene (RPE1), in particular from a type strain of
30 *Saccharomyces cerevisiae*;
- ribulose 5 phosphate isomerase gene (RKI1), in particular from a type strain of *Saccharomyces cerevisiae*;
- transketolase gene (TKL1) and transaldolase gene (TAL1), in particular from a type strain of *Saccharomyces cerevisiae*.

For instance, in an embodiment the fermenting organism of the invention has the following genetic modifications:

- xylose isomerases gene (Ru-XI) obtained from bovine rumen fluid, in particular the one shown in SEQ ID NO: 20 herein, encoding the xylose isomerase shown in SEQ ID NO: 13 herein;
- xylulokinase gene (XKS), in particular from a type strain of *Saccharomyces cerevisiae;*
- ribulose 5 phosphate 3-epimerase gene (RPE1), in particular from a type strain of *Saccharomyces cerevisiae*;
- ribulose 5 phosphate isomerase gene (RKI1), in particular from a type strain of *Saccharomyces cerevisiae*;
- transketolase gene (TKL1) and transaldolase gene (TAL1), in particular from a type strain of *Saccharomyces cerevisiae*.

Fermentation Stimulators

A fermentation stimulator can be used in a process of the invention described herein to further improve the fermentation, and in particular, the performance of the fermenting organism, such as, rate enhancement and product yield (*e.g.*, ethanol yield). A "fermentation stimulator" refers to stimulators for growth of the fermenting organisms, in particular, yeast. Preferred fermentation stimulators for growth include vitamins and minerals. Examples of vitamins include multivitamins, biotin, pantothenate, nicotinic acid, meso-inositol, thiamine, pyridoxine, para-aminobenzoic acid, folic acid, riboflavin, and Vitamins A, B, C, D, and E. See, for example, Alfenore *et al.*, Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process, Springer-Verlag (2002). Examples of minerals include minerals and mineral salts that can supply nutrients comprising P, K, Mg, S, Ca, Fe, Zn, Mn, and Cu.

Fermentation products

The fermentation product of the invention is ethanol.

Recovery

The fermentation product, i.e., ethanol, can optionally be recovered from the fermentation medium using any method known in the art including, but not limited to, chromatography, electrophoretic procedures, differential solubility, distillation, or extraction. For example, alcohol is separated from the fermented cellulosic material and purified by

conventional methods of distillation. Ethanol with a purity of up to about 96 vol. % can be obtained, which can be used as, for example, fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

5 **ENZYMES**

Below sections describe polypeptides, enzymes and enzyme composition that may be used in processes of the invention.

Cellulolytic Enzyme Composition

10 According to the invention a cellulolytic enzyme composition is present or added during saccharification in step (a). A cellulolytic enzyme composition is an enzyme preparation containing one or more (*e.g.*, several) enzymes that hydrolyze cellulosic material. Such enzymes include endoglucanase, cellobiohydrolase, beta-glucosidase, and/or combinations thereof.

15 The cellulolytic enzyme composition may be of any origin. In an embodiment the cellulolytic enzyme composition is derived from a strain of *Trichoderma*, such as a strain of *Trichoderma reesei*; a strain of *Humicola*, such as a strain of *Humicola insolens*, and/or a strain of *Chrysosporium*, such as a strain of *Chrysosporium lucknowense*. In a preferred embodiment the cellulolytic enzyme preparation is derived from a strain of *Trichoderma reesei*.

20 The cellulolytic enzyme composition may further comprise one or more of the following polypeptides, such as enzymes: AA9 polypeptide (GH61 polypeptide) having cellulolytic enhancing activity, beta-glucosidase, xylanase, beta-xylosidase, CBH I, CBH II, or a mixture of two, three, four, five or six thereof.

The further polypeptide(s) (*e.g.*, AA9 polypeptide) and/or enzyme(s) (*e.g.*, beta-
25 glucosidase, xylanase, beta-xylosidase, CBH I and/or CBH II may be foreign to the cellulolytic enzyme composition producing organism (*e.g.*, *Trichoderma reesei*).

In an embodiment the cellulolytic enzyme preparation comprises an AA9 polypeptide having cellulolytic enhancing activity and a beta-glucosidase.

In another embodiment the cellulolytic enzyme preparation comprises an AA9
30 polypeptide having cellulolytic enhancing activity, a beta-glucosidase, and a CBH I.

In another embodiment the cellulolytic enzyme preparation comprises an AA9 polypeptide having cellulolytic enhancing activity, a beta-glucosidase, a CBH I and a CBH II.

Other enzymes, such as endoglucanases, may also be comprised in the cellulolytic enzyme composition.

As mentioned above the cellulolytic enzyme composition may comprise a number of difference polypeptides, including enzymes.

In an embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Thermoascus aurantiacus* AA9 (GH61A)
5 polypeptide having cellulolytic enhancing activity (e.g., WO 2005/074656), and *Aspergillus oryzae* beta-glucosidase fusion protein (e.g., one disclosed in WO 2008/057637, in particular shown as SEQ ID NOs: 59 and 60).

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Thermoascus aurantiacus* AA9 (GH61A)
10 polypeptide having cellulolytic enhancing activity (e.g., SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 4 herein), and *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 5 herein).

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Penicillium emersonii* AA9 (GH61A)
15 polypeptide having cellulolytic enhancing activity, in particular the one disclosed in WO 2011/041397 or SEQ ID NO: 7 herein, and *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 5 herein).

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Penicillium emersonii* AA9 (GH61A)
20 polypeptide having cellulolytic enhancing activity, in particular the one disclosed in WO 2011/041397, and *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 5 herein) or a variant disclosed in WO 2012/044915, in particular one comprising one or more such as all of the following substitutions: F100D, S283G, N456E, F512Y.

25 In an embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic composition, further comprising an AA9 (GH61A) polypeptide having cellulolytic enhancing activity, in particular the one derived from a strain of *Penicillium emersonii* (e.g., SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 7 herein), *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 5 herein) variant with one or more, in
30 particular all of the following substitutions: F100D, S283G, N456E, F512Y and disclosed in WO 2012/044915; *Aspergillus fumigatus* Cel7A CBH1, e.g., the one disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 10 herein and *Aspergillus fumigatus* CBH II, e.g., the one disclosed as SEQ ID NO: 18 in WO 2011/057140 or SEQ ID NO: 11 herein.

In a preferred embodiment the cellulolytic enzyme composition is a *Trichoderma reesei,* cellulolytic enzyme composition, further comprising a hemicellulase or hemicellulolytic enzyme composition, such as an *Aspergillus fumigatus* xylanase (e.g. SEQ ID NO: 8 herein) and *Aspergillus fumigatus* beta-xylosidase (e.g. SEQ ID NO: 9 herein).

5 In an embodiment the cellulolytic enzyme composition also comprises a xylanase (*e.g.*, derived from a strain of the genus *Aspergillus*, in particular *Aspergillus aculeatus* or *Aspergillus fumigatus;* or a strain of the genus *Talaromyces*, in particular *Talaromyces leycettanus*) and/or a beta-xylosidase (*e.g.*, derived from *Aspergillus*, in particular *Aspergillus fumigatus*, or a strain of *Talaromyces*, in particular *Talaromyces emersonii*).

10 In an embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Thermoascus aurantiacus* AA9 (GH61A) polypeptide having cellulolytic enhancing activity (e.g., WO 2005/074656 or SEQ ID NO: 4 herein), *Aspergillus oryzae* beta-glucosidase fusion protein (e.g., one disclosed in WO 2008/057637, in particular as SEQ ID NOs: 59 and 60), and *Aspergillus aculeatus* xylanase
15 (e.g., Xyl II in WO 94/21785 or SEQ ID NO: 6 herein).

In another embodiment the cellulolytic enzyme preparation comprises a *Trichoderma reesei* cellulolytic preparation, further comprising *Thermoascus aurantiacus* GH61A polypeptide having cellulolytic enhancing activity (e.g., SEQ ID NO: 2 in WO 2005/074656 or SEQ ID NO: 4 herein), *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or
20 SEQ ID NO: 5 herein) and *Aspergillus aculeatus* xylanase (Xyl II disclosed in WO 94/21785 or SEQ ID NO: 6 herein).

In another embodiment the cellulolytic enzyme composition comprises a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Thermoascus aurantiacus* AA9 (GH61A) polypeptide having cellulolytic enhancing activity (e.g., SEQ ID NO: 2 in WO
25 2005/074656 or SEQ ID NO: 4 herein), *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 5 herein) and *Aspergillus aculeatus* xylanase (e.g., Xyl II disclosed in WO 94/21785 or SEQ ID NO: 6 herein).

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Penicillium emersonii* AA9 (GH61A)
30 polypeptide having cellulolytic enhancing activity, in particular the one disclosed in WO 2011/041397 or SEQ ID NO: 7 herein, *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 5 herein) and *Aspergillus fumigatus* xylanase (e.g., Xyl III in WO 2006/078256 or SEQ ID NO: 8 herein).

In another embodiment the cellulolytic enzyme composition comprises a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Penicillium emersonii* AA9 (GH61A) polypeptide having cellulolytic enhancing activity, in particular the one disclosed in WO 2011/041397 or SEQ ID NO: 7 herein, *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 5 herein), *Aspergillus fumigatus* xylanase (e.g., Xyl III in WO 2006/078256 or SEQ ID NO: 8 herein), and CBH I from *Aspergillus fumigatus* , in particular Cel7A CBH1 disclosed as SEQ ID NO: 2 in WO2011/057140 or SEQ ID NO: 10 herein.

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Penicillium emersonii* AA9 (GH61A) polypeptide having cellulolytic enhancing activity, in particular the one disclosed in WO 2011/041397 or SEQ ID NO: 7 herein, *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 5 herein), *Aspergillus fumigatus* xylanase (e.g., Xyl III in WO 2006/078256 or SEQ ID NO: 8 herein), CBH I from *Aspergillus fumigatus*, in particular Cel7A CBH1 disclosed as SEQ ID NO: 2 in WO 2011/057140 or SEQ ID NO: 10 herein, and CBH II derived from *Aspergillus fumigatus* in particular the one disclosed as SEQ ID NO: 4 in WO 2013/028928 or SEQ ID NO: 11 herein.

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition, further comprising *Penicillium emersonii* AA9 (GH61A) polypeptide having cellulolytic enhancing activity, in particular the one disclosed in WO 2011/041397 or SEQ ID NO: 7 herein, *Aspergillus fumigatus* beta-glucosidase (e.g., SEQ ID NO: 2 of WO 2005/047499 or SEQ ID NO: 5 herein) or variant thereof with one or more, in particular all, of the following substitutions: F100D, S283G, N456E, F512Y; *Aspergillus fumigatus* xylanase (e.g., Xyl III in WO 2006/078256 or SEQ ID NO: 8 herein), CBH I from *Aspergillus fumigatus*, in particular Cel7A CBH I disclosed as SEQ ID NO: 2 in WO 2011/057140 or SEQ ID NO: 10 herein, and CBH II derived from *Aspergillus fumigatus*, in particular the one disclosed in WO 2013/028928 or SEQ ID NO: 11 herein.

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition comprising the CBH I of SEQ ID NO: 14 herein (GENSEQP Accession No. AZY49536 (WO2012/103293); a CBH II of SEQ ID NO:15 herein (GENSEQP Accession No. AZY49446 (WO2012/103288); a beta-glucosidase variant of SEQ ID NO: 5 herein (GENSEQP Accession No. AZU67153 (WO 2012/44915)), in particular with one or more, in particular all, of the following substitutions: F100D, S283G, N456E, F512Y; and AA9 (GH61 polypeptide) of SEQ ID NO: 7 herein (GENSEQP Accession No. BAL61510 (WO

2013/028912)).

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition comprising the CBH I of SEQ ID NO: 14 herein (GENSEQP Accession No. AZY49536 (WO2012/103293)); the CBH II of SEQ ID NO: 15 herein (GENSEQP
5 Accession No. AZY49446 (WO2012/103288); the GH10 xylanase of SEQ ID NO: 16 herein (GENSEQP Accession No. BAK46118 (WO 2013/019827)); and the beta-xylosidase of SEQ ID NO: 17 herein (GENSEQP Accession No. AZI04896 (WO 2011/057140)).

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition comprising the CBH I of SEQ ID NO: 14 herein (GENSEQP
10 Accession No. AZY49536 (WO2012/103293)); the CBH II of SEQ ID NO: 15 herein (GENSEQP Accession No. AZY49446 (WO2012/103288)); and the AA9 (GH61 polypeptide) of SEQ ID NO: 7 herein (GENSEQP Accession No. BAL61510 (WO 2013/028912)).

In another embodiment the cellulolytic enzyme composition is a *Trichoderma reesei* cellulolytic enzyme composition comprising the CBH I of SEQ ID NO: 14 herein (GENSEQP
15 Accession No. AZY49536 (WO2012/103293)); the CBH II of SEQ ID NO: 15 herein (GENSEQP Accession No. AZY49446 (WO2012/103288)), the AA9 (GH61 polypeptide) of SEQ ID NO: 7 herein (GENSEQP Accession No. BAL61510 (WO 2013/028912)), and the catalase of SEQ ID NO: 19 herein (GENSEQP Accession No. BAC11005 (WO 2012/130120)).

In an embodiment the cellulolytic enzyme composition is a *Trichoderma reesei*
20 cellulolytic enzyme composition comprising the CBH I of SEQ ID NO: 14 herein (GENSEQP Accession No. AZY49446 (WO2012/103288); the CBH II of SEQ ID NO: 15 herein (GENSEQP Accession No. AZY49446 (WO2012/103288)), the beta-glucosidase variant of SEQ ID NO: 5 herein (GENSEQP Accession No. AZU67153 (WO 2012/44915)), with one or more, in particular all, of the following substitutions: F100D, S283G, N456E, F512Y; the AA9 (GH61 polypeptide)
25 of SEQ ID NO: 7 herein (GENSEQP Accession No. BAL61510 (WO 2013/028912)), the GH10 xylanase of SEQ ID NO: 16 herein (GENSEQP Accession No. BAK46118 (WO 2013/019827)), and the beta-xylosidase of SEQ ID NO: 17 herein (GENSEQP Accession No. AZI04896 (WO 2011/057140)).

In an embodiment the cellulolytic composition is a *Trichoderma reesei* cellulolytic
30 enzyme preparation comprising EG I of SEQ ID NO: 21 herein (Swissprot Accession No. P07981), EG II of SEQ ID NO: 22 herein (EMBL Accession No. M19373), CBH I of SEQ ID NO: 14 herein; CBH II of SEQ ID NO: 15 herein; beta-glucosidase variant of SEQ ID NO: 5 herein with the following substitutions: F100D, S283G, N456E, F512Y; the AA9 (GH61 polypeptide) of SEQ ID NO: 7 herein, GH10 xylanase of SEQ ID NO: 16 herein; and beta-xylosidase of SEQ ID

NO: 17 herein.

All cellulolytic enzyme compositions disclosed in WO 2013/028928 are also contemplated.

The cellulolytic enzyme composition comprises or may further comprise one or more (several) proteins selected from the group consisting of a cellulase, a AA9 (i.e., GH61) polypeptide having cellulolytic enhancing activity, a hemicellulase, an expansin, an esterase, a laccase, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

In one embodiment the cellulolytic enzyme composition is a commercial cellulolytic enzyme composition. Examples of commercial cellulolytic enzyme compositions suitable for use in a process of the invention include: CELLIC® CTec (Novozymes A/S), CELLIC® CTec2 (Novozymes A/S), CELLIC® CTec3 (Novozymes A/S), CELLUCLAST™ (Novozymes A/S), SPEZYME™ CP (Genencor Int.), ACCELLERASE™ 1000, ACCELLERASE 1500, ACCELLERASE™ TRIO (DuPont), FILTRASE® NL (DSM); METHAPLUS® S/L 100 (DSM), ROHAMENT™ 7069 W (Röhm GmbH), or ALTERNAFUEL® CMAX3™ (Dyadic International, Inc.). The cellulolytic enzyme composition may be added in an amount effective from about 0.001 to about 5.0 wt. % of solids, *e.g.*, about 0.025 to about 4.0 wt. % of solids or about 0.005 to about 2.0 wt. % of solids.

Endoglucanase

The cellulolytic enzyme composition used in a process of the invention may comprise an endoclucanase of any origin.

Examples of bacterial endoglucanases that can be used in the processes of the present invention, include, but are not limited to, *Acidothermus cellulolyticus* endoglucanase (WO 91/05039; WO 93/15186; U.S. Patent No. 5,275,944; WO 96/02551; U.S. Patent No. 5,536,655; WO 00/70031; WO 05/093050), *Erwinia carotovara* endoglucanase (Saarilahti *et al.*, 1990, *Gene* 90: 9-14), *Thermobifida fusca* endoglucanase III (WO 05/093050), and *Thermobifida fusca* endoglucanase V (WO 05/093050).

Examples of fungal endoglucanases that can be used in the present invention, include, but are not limited to, *Trichoderma reesei* endoglucanase I (Penttila *et al.*, 1986, *Gene* 45: 253-263, *Trichoderma reesei* Cel7B endoglucanase I (GenBank:M15665), *Trichoderma reesei* endoglucanase II (Saloheimo *et al.*, 1988, *Gene* 63:11-22), *Trichoderma reesei* Cel5A endoglucanase II (GenBank:M19373), *Trichoderma reesei* endoglucanase III (Okada *et al.*, 1988, *Appl. Environ. Microbiol.* 64: 555-563, GenBank:AB003694), *Trichoderma reesei* endoglucanase V (Saloheimo *et al.*, 1994, *Molecular Microbiology* 13: 219-228,

GenBank:Z33381), *Aspergillus aculeatus* endoglucanase (Ooi *et al.*, 1990, *Nucleic Acids Research* 18: 5884), *Aspergillus kawachii* endoglucanase (Sakamoto *et al.*, 1995, *Current Genetics* 27: 435-439), *Fusarium oxysporum* endoglucanase (GenBank:L29381), *Humicola grisea* var. *thermoidea* endoglucanase (GenBank:AB003107), *Melanocarpus albomyces* endoglucanase (GenBank:MAL515703), *Neurospora crassa* endoglucanase (GenBank:XM_324477), *Humicola insolens* endoglucanase V, *Myceliophthora thermophila* CBS 117.65 endoglucanase, *Thermoascus aurantiacus* endoglucanase I (GenBank:AF487830), *Trichoderma reesei* strain No. VTT-D-80133 endoglucanase (GenBank:M15665), *Penicillium pinophilum* endoglucanase (WO 2012/062220); and (WO 2013/019780).

In an embodiment the endoglucanase, such as one derived from *Trichoderma reesei* or homolog thereof, is selected from the group consisting of:

(i) an endoglucanase (EG) comprising the mature polypeptide of SEQ ID NO: 21 herein;

(ii) an endoglucanase (EG) an amino acid sequence having at least 60%, such as at least 70%, *e.g.*, 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 at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 21 herein.

In an embodiment the endoglucanase, such as one derived from *Trichoderma reesei* or homolog thereof, is selected from the group consisting of:

(i) an endoglucanase (EG) comprising the mature polypeptide of SEQ ID NO: 22 herein;

(ii) an endoglucanase (EG) an amino acid sequence having at least 60%, such as at least 70%, *e.g.*, 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 at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 22 herein.

AA9 (i.e., GH61) polypeptide having cellulolytic enhancing activity

The cellulolytic enzyme composition used according to the invention may in one embodiment comprise one or more AA9 (GH61) polypeptides having cellulolytic enhancing activity. The cellulolytic enzyme composition used in a process of the invention may comprise an AA9 (GH61) polypeptide of any origin.

Examples of AA9 polypeptides useful in the processes of the present invention include, but are not limited to, AA9 polypeptides from *Thielavia terrestris* (WO 2005/074647, WO 2008/148131, and WO 2011/035027), *Thermoascus aurantiacus* (WO 2005/074656 and WO 2010/065830), *Trichoderma reesei* (WO 2007/089290 and WO 2012/149344), *Myceliophthora thermophila* (WO 2009/085935, WO 2009/085859, WO 2009/085864, WO 2009/085868, and

WO 2009/033071), *Aspergillus fumigatus* (WO 2010/138754), *Penicillium pinophilum* (WO 2011/005867), *Thermoascus* sp. (WO 2011/039319), *Penicillium* sp. (*emersoni0* (WO 2011/041397 and WO 2012/000892), *Thermoascus crustaceous* (WO 2011/041504), *Aspergillus aculeatus* (WO 2012/125925), *Thermomyces lanuginosus* (WO 2012/113340, WO 2012/129699, WO 2012/130964, and WO 2012/129699), *Aurantiporus alborubescens* (WO 2012/122477), *Trichophaea saccata* (WO 2012/122477), *Penicillium thomii* (WO 2012/122477), *Talaromyces stipitatus* (WO 2012/135659), *Humicola insolens* (WO 2012/146171), *Malbranchea cinnamomea* (WO 2012/101206), *Talaromyces leycettanus* (WO 2012/101206), and *Chaetomium thermophilum* (WO 2012/101206), and *Talaromyces thermophilus* (WO 2012/129697 and WO 2012/130950).

In one aspect, the AA9 polypeptide is used in the presence of a soluble activating divalent metal cation according to WO 2008/151043, *e.g.*, manganese or copper.

In another aspect, the AA9 polypeptide is used in the presence of a dioxy compound, a bicylic compound, a heterocyclic compound, a nitrogen-containing compound, a quinone compound, a sulfur-containing compound, or a liquor obtained from a pretreated cellulosic material such as pretreated corn stover (WO 2012/021394, WO 2012/021395, WO 2012/021396, WO 2012/021399, WO 2012/021400, WO 2012/021401, WO 2012/021408, and WO 2012/021410).

In one embodiment the cellulolytic enzyme composition comprises a AA9 (GH61) polypeptide having cellulolytic enhancing activity, such as one derived from the genus *Thermoascus*, such as a strain of *Thermoascus aurantiacus*, such as the one described in WO 2005/074656 as SEQ ID NO: 2 or SEQ ID NO: 4 herein; or one derived from the genus *Thielavia*, such as a strain of *Thielavia terrestris*, such as the one described in WO 2005/074647 as SEQ ID NO: 7 and SEQ ID NO: 8 and SEQ ID NO: 2 herein; or one derived from a strain of *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the one described in WO 2010/138754 as SEQ ID NO: 2; or one derived from a strain derived from *Penicillium*, such as a strain of *Penicillium emersonii*, such as the one disclosed in WO 2011/041397 or SEQ ID NO: 7 herein.

In an embodiment the *Thermoascus aurantiacus* AA9 (GH61) polypeptide having cellulolytic enhancing activity or homolog thereof is selected from the group consisting of:

(i) a GH61 polypeptide having cellulolytic enhancing activity comprising the mature polypeptide of SEQ ID NO: 4 herein;

(ii) a GH61 polypeptide having cellulolytic enhancing activity comprising an amino acid sequence having at least 70%, *e.g.*, 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 at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 4 herein.

In another embodiment the *Penicillium* sp. AA9 (GH61) polypeptide having cellulolytic enhancing activity or homolog thereof is selected from the group consisting of:

5 (i) a GH61 polypeptide having cellulolytic enhancing activity comprising the mature polypeptide of SEQ ID NO: 7 herein;

(ii) a GH61 polypeptide having cellulolytic enhancing activity comprising an amino acid sequence having at least 70%, *e.g.*, 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
10 least 98%, or at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 7 herein.

<u>Beta-Glucosidase</u>

According to the invention a beta-glucosidase may be present and/or added in saccharification step (a). The cellulolytic enzyme composition used in a process of the invention
15 may comprise a beta-glucosidase of any origin.

Examples of beta-glucosidases useful in the present invention include, but are not limited to, beta-glucosidases from *Aspergillus aculeatus* (Kawaguchi *et al.*, 1996, *Gene* 173: 287-288), *Aspergillus fumigatus* (WO 2005/047499), *Aspergillus niger* (Dan *et al.*, 2000, *J. Biol. Chem.* 275: 4973-4980), *Aspergillus oryzae* (WO 02/095014), *Penicillium brasilianum* IBT
20 20888 (WO 2007/019442 and WO 2010/088387), *Thielavia terrestris* (WO 2011/035029), and *Trichophaea saccata* (WO 2007/019442).

The beta-glucosidase may in one embodiment be one derived from a strain of the genus *Aspergillus*, such as *Aspergillus niger* or *Aspergillus oryzae*, such as the one disclosed in WO 2002/095014 or the fusion protein having beta-glucosidase activity disclosed in WO
25 2008/057637 as SEQ ID NOs: 59 and 60, or *Aspergillus fumigatus*, such as such as one disclosed in WO 2005/047499 or SEQ ID NO: 5 herein or an *Aspergillus fumigatus* beta-glucosidase variant, such as one disclosed in WO 2012/044915, such as one with the following substitutions: F100D, S283G, N456E, F512Y (using SEQ ID NO: 5 herein for numbering).

In another embodiment the beta-glucosidase is derived from a strain of the genus
30 *Penicillium*, such as a strain of the *Penicillium brasilianum* disclosed in WO 2007/019442, or a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei*.

In an embodiment beta-glucosidase is an *Aspergillus fumigatus* beta-glucosidase or homolog thereof selected from the group consisting of:

(i) a beta-glucosidase comprising the mature polypeptide of SEQ ID NO: 5 herein;

(ii) a beta-glucosidase comprising an amino acid sequence having at least 70%, *e.g.*, 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 at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 5 herein.

5 In an embodiment the beta-glucosidase is a variant comprises a substitution at one or more (several) positions corresponding to positions 100, 283, 456, and 512 of the mature polypeptide of SEQ ID NO: 5 herein, wherein the variant has beta-glucosidase activity.

In an embodiment the beta-glucosidase is a variant of (a) a polypeptide comprising the mature polypeptide of SEQ ID NO: 5 herein; (b) a polypeptide having at least 80% sequence 10 identity to the mature polypeptide of SEQ ID NO: 5 herein or (c) a fragment of the mature polypeptide of SEQ ID NO: 5 herein, which has beta-glucosidase activity.

In an embodiment the beta-glucosidase variant has at least 80%, *e.g.*, 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 15 least 96%, at least 97%, at least 98%, at least 99%, but less than 100% sequence identity to the mature polypeptide of SEQ ID NO: 5 herein.

In an embodiment the beta-glucosidase is from a strain of *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as *Aspergillus fumigatus* beta-glucosidase (e.g., shown in SEQ ID NO: 5 herein), which comprises one or more substitutions selected from the group consisting 20 of L89M, G91L, F100D, I140V, I186V, S283G, N456E, and F512Y; such as a variant thereof with the following substitutions:

- F100D + S283G + N456E + F512Y;
- L89M + G91L + I186V + I140V;
- I186V + L89M + G91L + I140V + F100D + S283G + N456E + F512Y.

25 In an embodiment the number of substitutions is between 1 and 10, such 1 and 8, such as 1 and 6, such as 1 and 4, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 substitutions.

In an embodiment the variant comprises a substitution at a position corresponding to position 100, a substitution at a position corresponding to position 283, a substitution at a position corresponding to position 456, and/or a substitution at a position corresponding to 30 position 512.

In a preferred embodiment the beta-glucosidase variant comprises the following substitutions: Phe100Asp, Ser283Gly, Asn456Glu, Phe512Tyr in SEQ ID NO: 5 herein.

Cellobiohydrolase

The cellulolytic enzyme composition used in a process of the invention may comprise a cellobiohydrolase, such as CBH I and/or CBH II, of any origin.

Examples of cellobiohydrolases useful in the present invention include, but are not limited to, *Aspergillus aculeatus* cellobiohydrolase II (WO 2011/059740), *Aspergillus fumigatus* cellobiohydrolase I (WO 2013/028928), *Aspergillus fumigatus* cellobiohydrolase II (WO 2013/028928), *Chaetomium thermophilum* cellobiohydrolase I, *Chaetomium thermophilum* cellobiohydrolase II, *Humicola insolens* cellobiohydrolase I, *Myceliophthora thermophila* cellobiohydrolase II (WO 2009/042871), *Penicillium occitanis* cellobiohydrolase I (GenBank:AY690482), *Talaromyces emersonii* cellobiohydrolase I (GenBank:AF439936), *Thielavia hyrcanie* cellobiohydrolase II (WO 2010/141325), *Thielavia terrestris* cellobiohydrolase II (CEL6A, WO 2006/074435), *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, and *Trichophaea saccata* cellobiohydrolase II (WO 2010/057086). Cellobiohydrolase I.

Cellobiohydrolase I

The cellulolytic enzyme composition used in a process of the invention may in one embodiment comprise one or more CBH I (cellobiohydrolase I). In one embodiment the cellulolytic enzyme composition comprises a cellobiohydrolase I (CBH I), such as one derived from a strain of the genus *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the Cel7A CBH I disclosed in SEQ ID NO: 6 in WO 2011/057140 or SEQ ID NO: 10 herein; a strain of the genus *Trichoderma*, such as a strain of *Trichoderma reesei*; or a strain of the genus *Talaromyces*, such as a strain of *Talaromyces leycettanus*. preferably the one shown in SEQ ID NO: 14 herein or GENSEQP Accession No. AZY49536 (WO2012/103293).

In an embodiment the *Aspergillus fumigatus* cellobiohydrolase I or homolog thereof is selected from the group consisting of:

(i) a cellobiohydrolase I comprising the mature polypeptide of SEQ ID NO: 10 herein;

(ii) a cellobiohydrolase I comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, 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 at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 10 herein.

In another embodiment the cellobiohydrolase I, e.g., one derived from a strain of *Talaromyces leycettanus*, is selected from the group consisting of:

(i) a cellobiohydrolase I comprising the mature polypeptide of SEQ ID NO: 14 herein;

(ii) a cellobiohydrolase I comprising an amino acid sequence having at least 60%, at least

70%, *e.g.*, 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 at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 14 herein.

5 Cellobiohydrolase II

The cellulolytic enzyme composition used according to the invention may in one embodiment comprise one or more CBH II (cellobiohydrolase II). In one embodiment the cellobiohydrolase II (CBHII), such as one derived from a strain of the genus *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the one in SEQ ID NO: 11 herein or a strain of the 10 genus *Trichoderma*, such as *Trichoderma reesei*, or a strain of the genus *Thielavia*, such as a strain of *Thielavia terrestris*, such as cellobiohydrolase II CEL6A from *Thielavia terrestris*; or a strain of the genus *Talaromyces*, such as a strain of *Talaromyces leycettanus*, preferably the one shown in SEQ ID NO: 15 herein or GENSEQP Accession No. AZY49446 (WO2012/103288).

15 In an embodiment the *Aspergillus fumigatus* cellobiohydrolase II or homolog thereof is selected from the group consisting of:

(i) a cellobiohydrolase II comprising the mature polypeptide of SEQ ID NO: 11 herein;

(ii) a cellobiohydrolase II comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at 20 least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the mature polypeptide of SEQ ID NO: 11 herein.

In another embodiment the cellobiohydrolase II, e.g., one derived from a strain of *Talaromyces leycettanus*, is selected from the group consisting of:

(i) a cellobiohydrolase II comprising the mature polypeptide of SEQ ID NO: 15 herein;

25 (ii) a cellobiohydrolase II comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*, 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 at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 15 herein.

30 <u>Hemicellulases</u>

According to the invention a hemicellulase may be present and/or added during saccharification in step (a). The hemicellulase may be in the form of a hemicellulolytic enzyme composition. The hemicellulase may be of any origin, but preferably of fungal or bacterial origin.

The term “hemicellulase” or “hemicellulolytic enzyme” means one or more (several) enzymes that hydrolyze a hemicellulosic material. See, for example, Shallom and Shoham, 2003, Microbial hemicellulases. *Current Opinion In Microbiology*, 6(3): 219-228. Hemicellulases are key components in the degradation of plant biomass. Examples of hemicellulases include, but are not limited to, an acetylmannan esterase, an acetyxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. These catalytic modules, based on homology of their primary sequence, can be assigned into GH and CE families marked by numbers. Some families, with overall similar fold, can be further grouped into clans, marked alphabetically (*e.g.*, GH-A). A most informative and updated classification of these and other carbohydrate active enzymes is available on the Carbohydrate-Active Enzymes (CAZy) database. Hemicellulolytic enzyme activities can be measured according to Ghose and Bisaria, 1987, *Pure & Appl. Chem.* 59: 1739-1752.

In an embodiment the hemicellulase present and/or added in saccharification is a hemicellulolytic enzyme composition. In an embodiment the hemicellulolytic enzyme composition is cellulolytic enzyme composition from *Trichoderma reesei,* further comprising a xylanase and/or a beta-xylosidase. In a preferred embodiment the hemicellulolytic enzyme composition is a cellulolytic enzyme composition from *Trichoderma reesei,* further comprising *Aspergillus fumigatus* xylanase (XYL III shown in SEQ ID NO: 8 herein) and *Aspergillus fumigatus* beta-xylosidase (SEQ ID NO: 9 herein).

The hemicellulase or hemicellulolytic enzyme preparation may preferably be added in concentrations between 0.01 and 20 mg EP/g cellulose, such as 0.1-1 mg EP/g cellulose.

Xylanases

In a preferred embodiment the hemicellulase is a xylanase or the hemicellulolytic enzyme composition comprises a xylanase. The term “xylanase” means a 1,4-beta-D-xylan-xylohydrolase (E.C. 3.2.1.8) that catalyzes the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans. For purposes of the present invention, xylanase activity is determined with 0.2% AZCL-arabinoxylan as substrate in 0.01% TRITON® X-100 and 200 mM sodium phosphate buffer pH 6 at 37°C. One unit of xylanase activity is defined as 1.0 μmole of azurine produced

per minute at 37°C, pH 6 from 0.2% AZCL-arabinoxylan as substrate in 200 mM sodium phosphate pH 6 buffer.

Examples of xylanases useful in the processes of the present invention include, but are not limited to, xylanases from *Aspergillus aculeatus* (GeneSeqP:AAR63790; WO 94/21785), *Aspergillus fumigatus* (WO 2006/078256), *Penicillium pinophilum* (WO 2011/041405), *Penicillium* sp. (WO 2010/126772), *Thermomyces lanuginosus* (GeneSeqP:BAA22485), *Talaromyces thermophilus* (GeneSeqP:BAA22834), *Thielavia terrestris* NRRL 8126 (WO 2009/079210), and *Trichophaea saccata* (WO 2011/057083).

Examples of specifically contemplated xylanases include GH10 xylanases, such as one derived from a strain of the genus *Aspergillus*, such as a strain from *Aspergillus fumigatus*, such as the one disclosed as Xyl III in WO 2006/078256, or *Aspergillus aculeatus*, such as the one disclosed in WO 94/21785 as SEQ ID NO: 5 (Xyl II).

The xylanase may be comprised in a cellulolytic enzyme preparation which further includes a xylanase. In one embodiment hemicellulase is a cellulolytic enzyme preparation further comprising a xylanase, preferably a GH10 xylanase, such as one derived from a strain of the genus *Aspergillus*, such as a strain from *Aspergillus fumigatus*, such as the one disclosed as Xyl III in WO 2006/078256, or *Aspergillus aculeatus*, such as the one disclosed in WO 94/21785 as SEQ ID NO: 5 (Xyl II) or SEQ ID NO: 6 herein.

In an embodiment the xylanase is derived from *Aspergillus aculatues*, such as the one shown in SEQ ID NO: 6 herein. In a preferred embodiment the xylanase is derived from *Aspergillus fuminatus*, such as the one shown in SEQ ID NO: 8 herein.

Contemplated xylanases also include those comprising an amino acid sequence having at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% identity, at least 97%, at least 98%, at least 99% identity to the *Aspergillus fumigatus* xylanase in WO 2006/078256 shown as SEQ ID NO: 8 herein, or the *Aspergillus aculeatus* xylanase disclosed in WO 94/21785 as SEQ ID NO: 5 (Xyl II) or SEQ ID NO: 6 herein.

In an embodiment the xylanase, e.g., derived from a strain of *Talaromyces leycettanus,* comprised in the cellulolytic enzyme composition, has an amino acid sequence having at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% identity, at least 97%, at least 98%, at least 99% identity to SEQ ID NO: 16 herein.

Beta-Xylosidases

In a preferred embodiment the hemicellulase used in a process of the invention is a beta-xylosidase, or the hemicellulolytic enzyme composition comprises a beta-xylosidase. The

term “beta-xylosidase” means a beta-D-xyloside xylohydrolase (E.C. 3.2.1.37) that catalyzes the exo-hydrolysis of short beta (1→4)-xylooligosaccharides, to remove successive D-xylose residues from the non-reducing termini. For purposes of the present invention, one unit of beta-xylosidase is defined as 1.0 µmole of *p*-nitrophenolate anion produced per minute at 40°C, pH 5
5 from 1 mM *p*-nitrophenyl-beta-D-xyloside as substrate in 100 mM sodium citrate containing 0.01% TWEEN® 20.

Examples of beta-xylosidases useful in the processes of the present invention include, but are not limited to, beta-xylosidases from *Neurospora crassa* (SwissProt:Q7SOW4), *Trichoderma reesei* (UniProtKB/TrEMBL:Q92458), *Talaromyces emersonii* (SwissProt:Q8X212),
10 and *Talaromyces thermophilus* (GeneSeqP:BAA22816).

Examples of specifically contemplated beta-xylosidase includes the one derived from a strain of the genus *Aspergillus*, such as a strain of *Aspergillus fumigatus*, such as the one disclosed in WO 2013/028928 (Example 16 and 17) or SEQ ID NO: 9 herein, or derived from a strain of *Trichoderma*, such as a strain of *Trichoderma reesei*, such as the mature polypeptide
15 of SEQ ID NO: 58 in WO 2011/057140 or SEQ ID NO: 1 herein.

The beta-xylosidase used in a process of the invention may be comprised in a cellulolytic enzyme composition. In one embodiment the hemicellulase is a cellulolytic enzyme composition; such as *Trichoderma reesei* cellulolytic enzyme composition; further comprising a beta-xylosidase, such as one derived from a strain of the genus *Aspergillus*, such as a strain of
20 *Aspergillus fumigatus* (*e.g.*, one disclosed in WO 2011/057140 or SEQ ID NO: 9 herein), such as one disclosed in WO 2013/028928 (Examples 16 and 17), or derived from a strain of *Trichoderma*, such as a strain of *Trichoderma reesei*, such as the mature polypeptide of SEQ ID NO: 58 in WO 2011/057140.

Contemplated beta-xylosidases also include those comprising an amino acid sequence
25 having at least 60%, at least 70% at least 80%, at least 85%, at least 90%, at least 95% identity, at least 97%, at least 98%, at least 99% identity to the *Aspergillus fumigatus* beta-xylosidase disclosed as SEQ ID NO: 206 in WO 2011/057140 or SEQ ID NO: 9 herein or any of the beta-xylosidases mentioned herein.

In an embodiment the beta-xylosidase, e.g., derived from a strain of *Talaromyces*
30 *emersonii,* comprised in the cellulolytic enzyme composition, has an amino acid sequence having at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% identity, at least 97%, at least 98%, at least 99% identity to SEQ ID NO: 17 herein.

The hemicellulase used in a process of the invention may comprise a commercial hemicellulase product. Examples of commercial hemicellulase products include, for example,

SHEARZYME™ (Novozymes A/S), CELLIC™ HTec (Novozymes A/S), CELLIC™ HTec2 (Novozymes A/S), CELLIC™ HTec3 (Novozymes), VISCOZYME® (Novozymes A/S), ULTRAFLO® (Novozymes A/S), PULPZYME® HC (Novozymes A/S), MULTIFECT® Xylanase (Genencor), ECOPULP® TX-200A (AB Enzymes), HSP 6000 Xylanase (DSM), DEPOL™ 333P
5 (Biocatalysts Limit, Wales, UK), DEPOL™ 740L. (Biocatalysts Limit, Wales, UK), and DEPOL™ 762P (Biocatalysts Limit, Wales, UK).

Catalases

The cellulolytic enzyme compositions may comprise a catalase. The catalase may be
10 any catalase. The catalase may include, but is not limited to, an E.C. 1.11.1.6 or E.C. 1.11.1.21 catalase.

Examples of useful catalases include, but are not limited to, catalases from *Alcaligenes aquamarinus* (WO 98/00526), *Aspergillus lentilus*, *Aspergillus fumigatus*, *Aspergillus niger* (U.S. Patent No. 5,360,901), *Aspergillus oryzae* (JP 2002223772A; U.S. Patent No. 6,022,721),
15 *Bacillus thermoglucosidasius* (JP 1 1243961A), *Humicola insolens* (WO 2009/104622, WO 2012/130120), *Malbranchea cinnamomea*, *Microscilla furvescens* (WO 98/00526), *Neurospora crassa*, *Penicillium emersonii* (WO 2012/130120), *Penicillium pinophilum*, *Rhizomucor pusillus*, *Saccharomyces pastorianus* (WO 2007/105350), *Scytalidium thermophilum*, *Talaromyces stipitatus* (WO 2012/130120), *Thermoascus aurantiacus* (WO 2012/130120), *Thermus*
20 *brockianus* (WO 2005/044994), and *Thielavia terrestris* (WO 2010/074972).

Non-limiting examples of useful catalases are catalases from *Bacillus pseudofirmus* (UNIPROT:P30266), *Bacillus subtilis* (UNIPROT:P42234), *Humicola grisea* (GeneSeqP: AXQ55105), *Neosartorya fischeri* (UNIPROT:A1DJU9), *Penicillium emersonii* (GeneSeqP:BAC10987), *Penicillium pinophilum* (GeneSeqP:BAC10995), *Scytalidium*
25 *thermophilum* (GeneSeqP:AAW06109 or ADT89624), *Talaromyces stipitatus* (GeneSeqP:BAC10983 or BAC11039; UNIPROT:B8MT74), and *Thermoascus aurantiacus* (GeneSeqP:BAC11005).

The cellulolytic enzyme compositions may in a preferred embodiment comprise a catalase, e.g., one derived from *Thermoascus*, in particular *Thermoascus aurantiacus*, in
30 particular the one shown in WO 2012/130120 or SEQ ID NO: 19 herein.

In an embodiment the catalase, e.g., one derived from a strain of *Thermoascus auranticus*, is selected from the group consisting of:

(i) a catalase comprising the mature polypeptide of SEQ ID NO: 19 herein;

(ii) a catalase comprising an amino acid sequence having at least 60%, at least 70%, *e.g.*,

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 at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 19 herein.

The protein content of the catalase is in the range of about 0.5% to about 10%, *e.g.*, 5 about 0.5% to about 7%, about 0.5% to about 5%, about 0.5% to about 4%, about 0.5% to about 3%, about 0.5% to about 2%, and about 0.5% to about 1% of total enzyme protein in the saccharification/hydrolysis reaction.

In an embodiment, the protein ratio of catalase to cellulolytic enzyme composition is in the range of about 1:200 to about 1:10, *e.g.*, about 1:100 to about 1:15 or about 1:50 to about 10 1:25.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

15 **MATERIAL& METHODS**

Materials:

Cellulolytic Enzyme Composition CA ("CA"): Cellulolytic enzyme preparation derived from *Trichoderma reesei* further comprising GH61A polypeptide having cellulolytic enhancing activity 20 derived from a strain of *Penicillium emersonii* (SEQ ID NO: 2 in WO 2011/041397 or SEQ ID NO: 7 herein), *Aspergillus fumigatus* beta-glucosidase (SEQ ID NO: 2 in WO 2005/047499 or SEQ ID NO: 5 herein) variant F100D, S283G, N456E, F512Y) disclosed in WO 2012/044915; *Aspergillus fumigatus* Cel7A CBH1 disclosed as SEQ ID NO: 6 in WO2011/057140 and SEQ ID NO: 10 herein and *Aspergillus fumigatus* CBH II disclosed as SEQ ID NO: 18 in WO 25 2011/057140and SEQ ID NO: 11 herein. Further, Cellulolytic Enzyme Preparation CA further comprises 10% of a cellulolytic enzyme preparation from *Trichoderma reesei,* further comprising *Aspergillus fumigatus* xylanase (SEQ ID NO: 8 herein) and *Aspergillus fumigatus* beta-xylosidase (SEQ ID NO: 9 herein).

30 Cellulolytic Enzyme Composition CB ("CB"): *Trichoderma reesei* cellulolytic enzyme preparation comprising EG I of SEQ ID NO: 21 herein, EG II of SEQ ID NO: 22 herein, CBH I of SEQ ID NO: 14 herein; CBH II of SEQ ID NO: 15 herein; beta-glucosidase variant of SEQ ID NO: 5 herein with the following substitutions: F100D, S283G, N456E, F512Y; the AA9 (GH61 polypeptide) of SEQ ID NO: 7 herein, GH10 xylanase of SEQ ID NO: 16 herein; and beta-

xylosidase of SEQ ID NO: 17 herein.

CIBTS1260: *Saccharomyces cerevisiae* yeast deposited by Novozymes A/S under the terms of the Budapest Treaty with the Agricultural Research Service Culture Collection (NRRL), 1815
5 North University Street, Peoria, Illinois 61604 U.S.A.) and given the following accession number:

Deposit	Accession Number	Date of Deposit
CIBTS1260	NRRL Y-50973	September 5, 2014

10 BSGX001 is disclosed in US patent No. 8,586,336-B2 and was constructed as follows: Host *Saccharomyces cerevisiae* strain BSPX042 (phenotype: ura3-251, overexpression of XKS1; overexpression of RPE1, RKI1, TAL1, and TKL1, which are genes in PPP; knockout of aldose reductase gene GRE3; and damage of electron transport respiratory chain by deleting gene COX4 after adaptive evolution), was transformed with vector pJFE3-RuXI inserted with xylose
15 isomerase gene (SEQ ID NO: 1 in US patent No. 8,586,336-B2 or SEQ ID NO: 20 herein) encoding the RuXI shown in SEQ ID NO: 2 in US patent No. 8,586,336-B2 or SEQ ID NO: 13 herein.

Methods:

20 **Identity**

The relatedness between two amino acid sequences or between two polynucleotide sequences is described by the parameter “identity”.

For purposes of the present invention, the degree of identity between two amino acid sequences is determined by the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using the
25 LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters are Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

For purposes of the present invention, the degree of identity between two polynucleotide sequences is determined by the Wilbur-Lipman method (Wilbur and Lipman, 1983, *Proceedings*
30 *of the National Academy of Science USA* 80: 726-730) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters are Ktuple=3, gap penalty=3, and windows=20.

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

EXAMPLES

Example 1

Construction of the strain CIBTS1000.

A diploid *Saccharomyces cerevisiae* strain that is known to be an efficient ethanol producer from glucose was identified. *S. cerevisiae* strain CCTCC M94055 from the Chinese Center for Type Culture Collection (CCTCC) was used.

A xylose isomerase termed mgXI was cloned from a meta genomics project meaning that the donor organism is not known. The isolation and the characteristics of this xylose isomerase are described in CN patent application No. 102174549A or US patent Publication No. 2012/0225452.

A pentose transporter termed GXF was cloned from *Candida intermedia* using standard methods. This xylose transporter was described by D. Runquist et. al. (Runquist D, Fonseca C, Radstrom P, Spencer-Martins I, Hahn-Hägerdal B: "*Expression of the Gxf1 transporter from Candida intermedia improves fermentation performance in recombinant xylose-utilizing Saccharomyces cerevisiae*". Appl Microbiol Biotechnol 2009, 82:123-130).

The xylose isomerase gene was fused to the Triose Phosphate Isomerase (TPI) promoter from *Saccharomyces cerevisiae* and the TPI terminator using standard methods so that the expression of the xylose isomerase in *S. cerevisiae* was controlled by the TPI expression signals.

The GXF gene was fused to the TPI expression signals in the same way.

These two expression cassettes were inserted into an *Escherichia coli* cloning vector containing:

- The *E. coli* colE1 origin of replication securing that the plasmid could be propagated in *E. coli*.
- A delta (δ) sequence fragment from *Saccharomyces cerevisiae*.

- A Zeocin resistance marker from *Streptoalloteichus hindustanus* for selection of Zeocin resistant *E. coli* or *S. cerevisiae* transformants. A double promoter was fused to the 5' end of the Zeocin gene consisting of an *S. cerevisiae* Translation Elongation Factor (TEF1) promoter and an *E. coli* EM7 promoter. The *S. cerevisiae* CYC1 terminator was added to the 3' end of the Zeocin gene. The entire Zeocin expression cassette was flanked by loxP sites to enable deletion of this expression cassette by Cre-lox recombination (B. Sauer: *"Functional expression of the Cre-Lox site specific recombination system in the yeast Saccharomyces cerevisiae."* Mol. Cell. Biol. 1987, 7: 2087-2096).

The Xylose isomerase/pentose transporter expression plasmid was termed pYIE2-mgXI-GXF1-δ and is shown in Fig. 1.

The plasmid pYIE2-mgXI-GXF1-delta was first linierized by XhoI digestion and then transformed into the parental strain *Saccharomyces cerevisia* CCTCC M94055 following selection for zeocin resistant transformants. A strain termed CIBTS0912 was isolated having the plasmid integrated into a delta sequence. The zeocin resistance cassette located between the two loxP sites were then deleted by transient CRE recombinase expression resulting in the strain CIBTS0914.
The transient CRE recombinase expression was achieved similar to the yeast standard method described by Prein et. al. (Prein B, Natter K, Kohlwein SD. "*A novel strategy for constructing N-terminal chromosomal fusions to green fluorescent protein in the yeast Saccharomyces cerevisiae". FEBS Lett. 2000: 485, 29-34.*) transforming with an unstable plasmid expressing the CRE recombinase followed by curing for that plasmid again. In this work the kanamycin gene of the yeast standard vector pSH47 was replaced with a hygromycin resistance marker so that rather than selecting for kanamycin resistance, selection for hygromycin was used. A plasmid map of the plasmid used pSH47-hyg is shown in Fig. 2.

Gene/element name	**Function**	**origin**
Cre	Recombinase that catalyse recombination between lox sites	*Saccharomyces cerevisiae.*
GAL1p	Yeast promoter induced by galactose	*Saccharomyces cerevisiae.*
ScCYC1t	Yeast terminator	*Saccharomyces cerevisiae.*

hph	Hygromycin resistance gene.	*Streptomyces hygroscopicus.*
URA3	Auxotrophic selection marker	*Saccharomyces cerevisiae.*
CEN6/ARSH4	Replication origin	*Saccharomyces cerevisiae.*
pUC Ori	E. coli replication origin	*Escherichia coli*
AmpR	Ampicillin resistance gene	*Escherichia coli*

The strain CIBTS0914 was transformed with XhoI digested pYIE2-mgXI-GXF1-δ again in order to increase the copy number of the two expression cassettes and a zeocin resistant strain, CIBTS0916 was selected.

In order to overexpress the genes of the pentose phosphate pathway, an expression
5 plasmid harboring the selected pentose phosphate pathway genes was assembled.

The genes selected for overexpression were:

1. Xylulo kinase (XKS1).
2. Trans-aldolase (TAL1).
3. Ribulose 5 phosphate epimerase (RPE1).
10 4. Trans-ketolase (TKL1).
5. Ribose 5 phosphate isomerase (RKI1)

In addition to these genes, the KanMX selection cassette surrounded by loxP sites was included as a part of the *E. coli – S. cerevisiae* shuttle vector pUG6 (Güldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH. "*A new efficient gene disruption cassette for repeated*
15 *use in budding yeast." NAR* 1996, 24:2519-24).

A map of the resulting plasmid pYIE2-XKS1-PPP-δ is shown in Fig. 3. A table listing the genetic elements used is shown below:

Base position (bp)	**Size (bp)**	**Genetic element**	**Description**	**Origion**
1- 1500	1500	ADH1p	Yeast ADH1 promoter	*Saccharomyces cerevisiae*
1501-3303	1803	XKS1	Xylulo kinase	*Saccharomyces cerevisiae*
3303-3563	260	XKS1t	Xylulo kinase terminator	*Saccharomyces cerevisiae*
3564-	586	TPI 1p	Yeast TPI promoter	*Saccharomyces*

4149				*cerevisiae*
4150-5257	1108	TAL1	Trans-aldolase	*Saccharomyces cerevisiae*
5258-5657	400	TAL1t	Trans-aldolase terminator	*Saccharomyces cerevisiae*
5658-6407	750	PGK1p	Yeast PGK promoter	*Saccharomyces cerevisiae*
6408-7124	717	RPE1	Ribulose 5 phosphate epimerase	*Saccharomyces cerevisiae*
7125-7524	400	RPE1t	Ribulose 5 phosphate epimerase terminator	*Saccharomyces cerevisiae*
7525-8344	820	FBA1p	Yeast FBA promoter	*Saccharomyces cerevisiae*
8345-10387	2043	TKL1	Trans-ketolase	*Saccharomyces cerevisiae*
10387-10667	280	TKL1t	Trans-ketolase terminator	*Saccharomyces cerevisiae*
10668-11467	800	PDC1p	Yeast PDC promoter	*Saccharomyces cerevisiae*
11468-12444	777	RKI1	Ribose 5 phosphate isomerase	*Saccharomyces cerevisiae*
12445-12644	400	RKI1t	Ribose 5 phosphate isomerase terminator	*Saccharomyces cerevisiae*
12645-12844	200	Delta up	Delta DNA upstream sequence	*Saccharomyces cerevisiae*
12845-14565	1720	pUG6	E. coli vector including ColE1 origin for E. coli replication and CEN6/ARS replication origin for yeast replication	*Escherichia coli*
14566-14865	300	Delta Dn	Delta DNA downstream sequence	*Saccharomyces cerevisiae*
14866-14907	82	Linker	Synthetic linker	Synthetic DNA

14908-14941	34	loxP	Lox recombination site	Bacteriophage P1
14942-15339	398	TEF1p	*A. gossypii* TEF promoter	*Ashbya gossypii*
15340-16149	810	KanMX	Kanamycin (G418) resistance marker	*Escherichia coli*
16150-16414	256	TEF1t	*A. gossypii* TEF terminator.	*Ashbya gossypii*
16415-16448	34	loxP	Lox recombination site	Bacteriophage P1
16449-16475	27	Linker	Synthetic linker	Synthetic DNA

The plasmid pYIE2-XKS1-PPP-δ was digested with NotI and the vector elements were removed by agarose gel electrophoresis. The linear fragment containing all of the expression cassettes were then transformed into CIBTS0916 for double homologous recombination followed by selection for kanamycin (G418) resistance. A kanamycin resistant colony was selected and termed CIBTS0931.

CIBTS0931 contains both the zeocin selection marker and the kanamycin selection marker. Both of them are flanked with loxP recombination sites.

In order to remove the zeocin and kanamycin resistance markers the strain was transformed with the episomal plasmid pSH47-hyg again, and transformants were selected on plates containing hygromycin. Subsequently, screening for transformants that had lost zeocin and kanamycin resistance was performed and after that screening for a strain that also lost the hygromycin resistance marker was done. A strain CIBTS1000 was selected and shown to have lost the plasmid pSH47-hyg.

Example 2

Adaptation of the strain CIBTS1000 to high xylose uptake and acetate resistance.

The strain CIBTS1000 was modified so that it could utilize xylose as a carbon source and ferment it to ethanol. However the xylose utilization was very inefficient. A well-known way to improve that in the field of metabolic engineering is to use adaptation. This was also done in this case. The strain CIBTS1000 was serially transferred from shakeflask to shakeflask in a medium containing xylose as sole carbon source and yeast growth inhibitors known to be

present in cellulosic biomass hydrolysates. During these serial transfers mutations are accumulated that enable the strain to grow better under the conditions provided – and thereby to utilize xylose better.

In a first round of adaptation, CIBTS1000 was serially transferred in a shake flask system using YPX medium (10 g/l Yeast extract, 20 g/l peptone and 20 g/l xylose) and YPDX (10 g/l Yeast extract, 20 g/l peptone 10 g/l glucose and 10 g/l xylose)

In a second round of adaptation serial transfer was done in YPXI (YPX supplemented with 43mM sodium formate, 50mM sodium acetate and 100mM sodium sulphate) and YPDXI (YPDX supplemented with 43mM sodium formate, 50mM sodium acetate and 100mM sodium sulphate).

In a final round of adaptation serial transfer was done using NREL dilute acid pretreated corn stover hydrolysate (see Example 3) supplemented with 10 g/l Yeast extract, 20 g/l peptone, 10 g/l glucose and 10 g/l xylose.

A strain named CIBTS1260-J132-F3 was selected as an adapted strain.

Example 3

Fermentation Comparison of CIBTS1260 and BSGX001 in NREL Dilute Acid Pretreated Corn Stover Hydrolysate

Two *Saccharomyces cerevisiae* strains, CIBTS1260 and BSGX001, were tested in NREL dilute acid pretreated corn stover hydrolysate (4% w/w sulfuric acid at 180ºC for 5 minutes). The hydrolysate was produced after 3 days of hydrolysis in a 20kg reactor at 50°C with 20 mg enzyme protein/g glucan of Cellulolytic Enzyme Composition CA. The dilute acid pretreated corn stover hydrolysate had a final composition of 63.2 g/L glucose, 44.9 g/L xylose, 0.8 g/L glycerol, and 9.5 g/L acetate. Prior to fermentation, each strain was propagated in a 30°C air shaker at 150 rpm on YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). After 24 hours of growth, these two yeast strains were tested in 50 ml of hydrolysate in 125 ml baffled Erlenmeyer flasks at a yeast pitch of 1 g dry cell weight (DCW)/L. Rubber stoppers equipped with 18 gauge blunt fill needles were used to seal each flask, and the flasks were placed in a 35°C air shaker at a speed of 150 rpm. Samples were taken at 24, 48, and 72 hours for determination of glucose, xylose, and ethanol concentrations via HPLC analysis. The results were averaged for each set of 3 replicates, and are given in Figure 1 which shows a comparison of CIBTS1260 versus BSGX001 in NREL acid pretreated corn stover hydrolysate at 1 g/L yeast pitch in 72 hours. As shown in Fig. 4, by 48 hours, the CIBTS1260 strain completed full xylose

consumption and produced approximately 47 g/L ethanol. The BSGX001 strain, however, was slow to uptake glucose for ethanol conversion and thus consumed only 3 g/L xylose. These results indicate that CIBTS1260 results in improved xylose uptake and utilization for conversion to ethanol compared to BSGX001.

5 **Example 4**

Comparison of CIBTS1260 and BSGX001 for Fermentation Performance in Model Media

The fermentation performance of CIBTS1260 and its precursor BSGX001 was compared. Prior to fermentation, each strain was propagated in a 30°C air shaker at 150 rpm on YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). After 24 hours of growth, these two
10 yeast strains were tested in YPX medium (5 g/L yeast extract, 5 g/L peptone, and 50 g/L xylose). To test fermentation performance, each strain was inoculated into 50 ml of YPX medium in 125 ml baffled Erlenmeyer flasks at a yeast pitch of 2 g DCW/L. Rubber stoppers equipped with 18 gauge blunt fill needles were used to seal each flask, and the flasks were placed in a 32°C air shaker at a speed of 150 rpm. Samples were taken at 24, 48, and 72 hours
15 for determination of glucose, xylose, and ethanol concentrations via HPLC analysis. The results were averaged for each set of 3 replicates, and are given in Fig. 5.
As shown in Fig. 5, CIBTS1260 (dotted lines) has completely utilized all available xylose in 24 hours and produced 21.3 g/L of ethanol. In the 72 hour fermentation time, BSGX001 (solid lines) consumed 1.5 g/L of xylose, and the resulting ethanol concentration was 1.3 g/L.

20

Example 5

Fermentation of Cellulolytic Enzyme Composition CA ("CA") and Cellulolytic Enzyme Composition CB ("CB") Bagasse Hydrolysate with CIBTS1260

CIBTS1260 was used in fermentation tests with NREL dilute acid pretreated bagasse
25 hydrolysates generated at Novozymes North America, USA. The hydrolysate was produced after 5 days of hydrolysis in 2L IKA reactors at 50°C with a 6 mg enzyme protein/g glucan dose of two cellulolytic enzyme compositions termed "CA" and "CB". These materials are representative benchmarks for dilute acid pretreated bagasse hydrolysates with final compositions of 40.7 and 58.7 g/L glucose, 42.5 and 44.7 g/L xylose, 0.19 and 0.08 g/L glycerol,
30 and 8.99 and 11.3 g/L acetate for "CA" and "CB", respectively. Prior to fermentation, the yeast were propagated in a 30°C air shaker at 150 rpm on 2% YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). After 24 hours of growth, CIBTS1260 was tested in 50 ml of

"CA" and "CB" hydrolysate in 125 ml baffled Erlenmeyer flasks at a yeast pitch of 1g DCW/L. Rubber stoppers equipped with 18 gauge blunt fill needles were used to seal each flask, and the flasks were placed in a 35°C air shaker at a speed of 150 rpm. Samples were taken at 24, 48, and 72 hours for determination of glucose, xylose, ethanol, acetate, and glycerol concentrations
5 via HPLC analysis. The results were averaged for each set of 3 replicates, and are given in Fig. 6. Greater than 95% of the glucose and xylose present in both systems was consumed within the 72 hour time period with ethanol yields on total sugars of 84.1% for the "CA" hydrolysate and 86.4% for the "CB" hydrolysate.

10 **Example 6**

DP2 Reduction During CIBTS1260 and BSGX001 Fermentations of Dilute Acid Pretreated Corn Stover and Sugar Cane Bagasse Hydrolysates

Dilute acid pretreated corn stover and sugar cane bagasse from National Renewable Energy
15 Laboratory (NREL), USA, were hydrolysed with a 6 mg enzyme protein/ g glucan dose of two enzyme product cocktails termed CA and CB for 5 days in 2L IKA reactors at 50°C. Prior to fermentation, the CIBTS1260 and BSGX001 yeast were propagated in a 30°C air shaker at 150 rpm on YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). After 24 hours of growth, the cells from each strain were harvested via centrifugation and added to 50 ml of CA
20 and CB hydrolysate supplemented with 2 g/L urea in 125 ml baffled Erlenmeyer flasks at a yeast pitch of 1g DCW/L (Dry Cell Weight/L), respectively. Rubber stoppers equipped with 18 gauge blunt fill needles were used to seal each flask, and the flasks were placed in a 35°C air shaker at a speed of 150 rpm. Samples were taken at 0 and 72 hours for determination DP2 concentrations via HPLC analysis. The results were averaged for each set of replicates (n=3 for
25 CIBTS1260 and n=2 for BSGX001). As shown in Figure 7, in the same hydrolysates, the DP2 concentrations were reduced more for fermentations conducted with CIBTS1260 than for fermentations with BSGX001. The DP2 peak, as measured on HPLC, contains cellobiose and short chain sugars.

30

CLAIMS

1. A process for producing ethanol, comprising:

(a) saccharifying a cellulosic material with a cellulolytic enzyme composition;

(b) fermenting the saccharified cellulosic material with a fermenting microorganism to produce the fermentation product; wherein the fermenting organism is *Saccharomyces cerevisiae* NRRL Y-50973.

2. The process of claim 1, comprising recovering the fermentation product from the fermentation.

3. The process of claim 1 or 2, wherein the cellulosic material is pretreated.

4. The process of claim 1 or 2, wherein the cellulolytic enzyme composition comprises one or more enzymes selected from the group consisting of a cellulase, a lytic polysaccharide monooxygenase, a hemicellulase, an esterase, an expansin, a ligninolytic enzyme, an oxidoreductase, a pectinase, a protease, and a swollenin.

5. The process of claim 4, wherein the cellulase is one or more enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

6. The process of claim 4 or 5, wherein the hemicellulase is one or more enzymes selected from the group consisting of a xylanase, an acetylxylan esterase, a feruloyl esterase, an arabinofuranosidase, a xylosidase, and a glucuronidase.

7. The process of any one of claims 1-6, wherein steps (a) and (b) are performed simultaneously in a simultaneous saccharification and fermentation (SSF).

8. The process of any one of claims 1-7, wherein steps (a) and (b) are performed sequentially (SHF).

9. The process of any one of claims 1-8, wherein the cellulosic material is pretreated before saccharification.

10. The process of claim 9, wherein the pretreatment is a dilute acid pretreatment.

11. A recombinant fermenting organism *Saccharomyces cerevisiae* NRRL Y-50973.

Date reçue / Date received 2021-12-16

12. The fermenting organism of claim 11, wherein the fermenting organism is capable of complete xylose consumption by 48 hours fermentation at 1g Dry Cell Weight/L, 35ºC, pH 5.5.

13. The fermenting organism of claim 11 or 12, wherein the fermenting organism is capable of complete glucose consumption by 24 hours fermentation at 1g Dry Cell Weight/L, 35ºC, pH 5.5.

14. The fermenting organism of any one of claims 11-13, wherein the fermenting organism is capable of producing more than 30 g/L ethanol after 48 hours fermentation at 1g Dry Cell Weight/L, 35ºC, pH 5.5.

Date reçue / Date received 2021-12-16

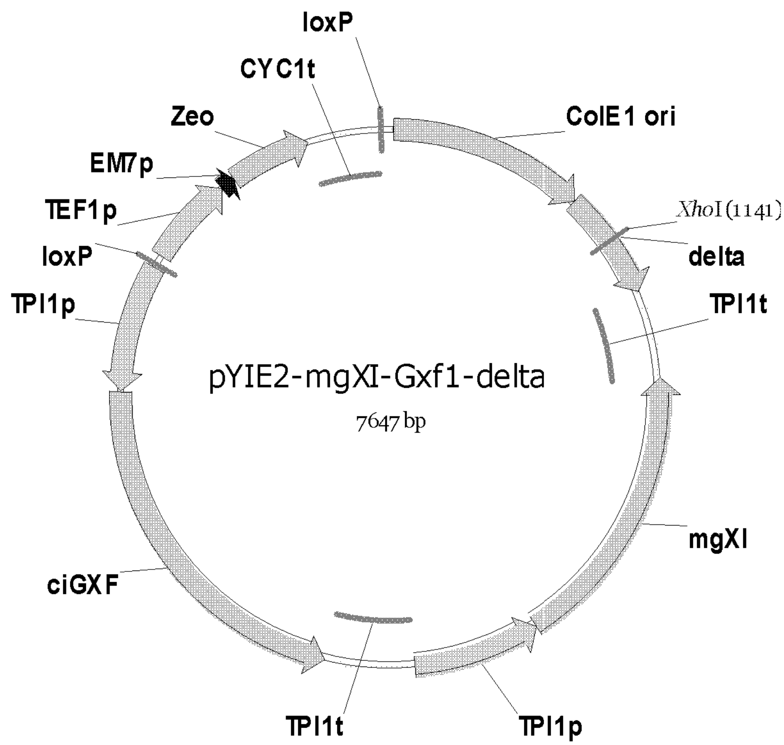

Fig. 1

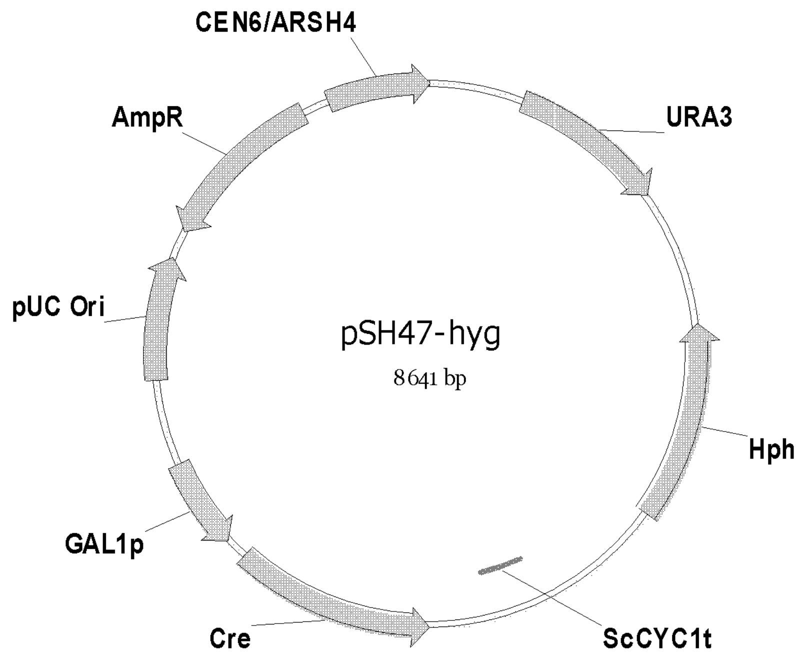

Fig. 2

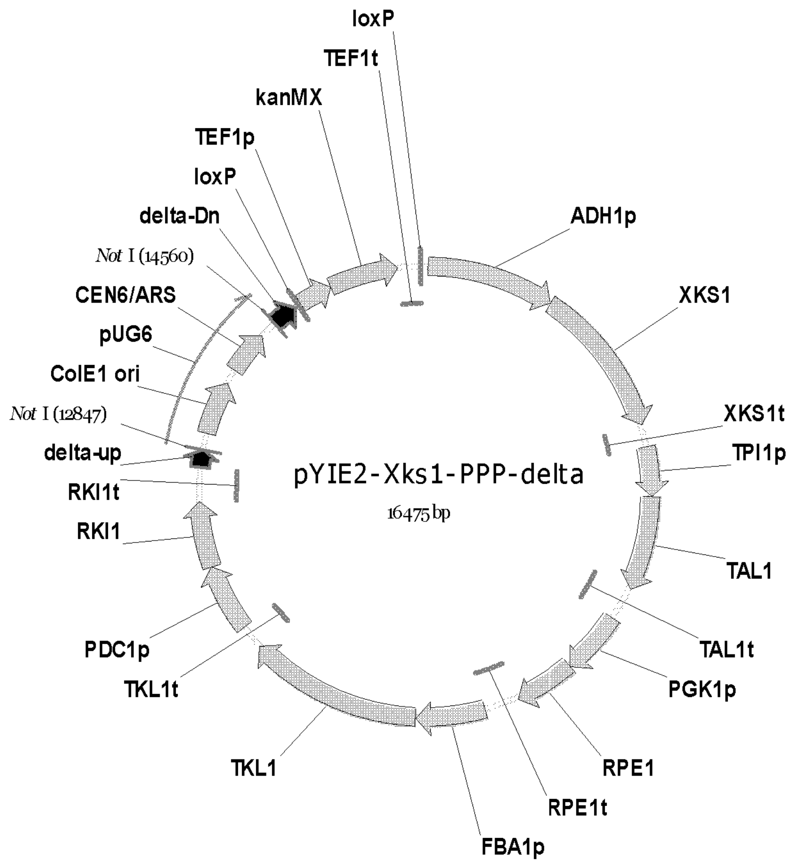

Fig. 3

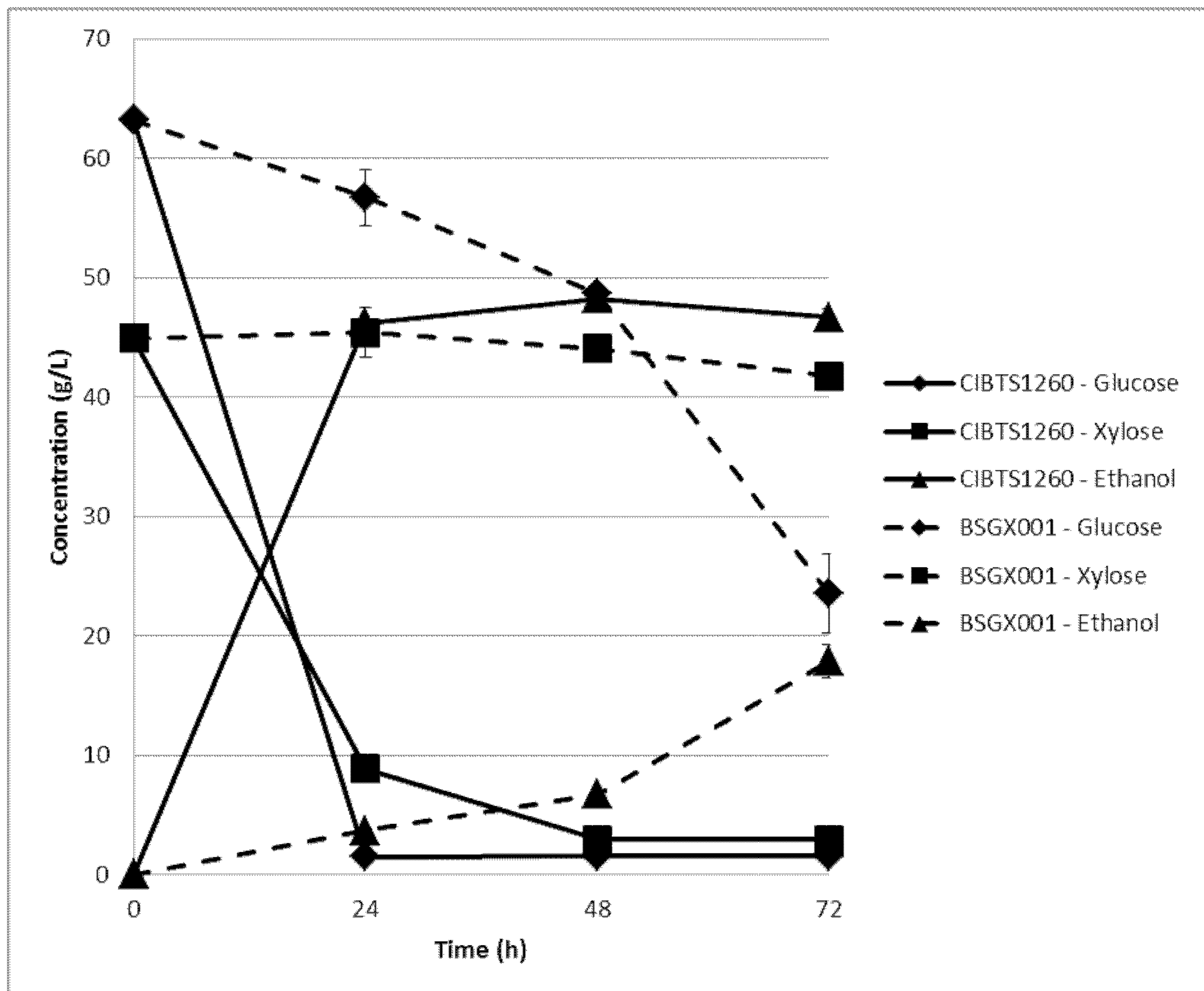

Fig. 4

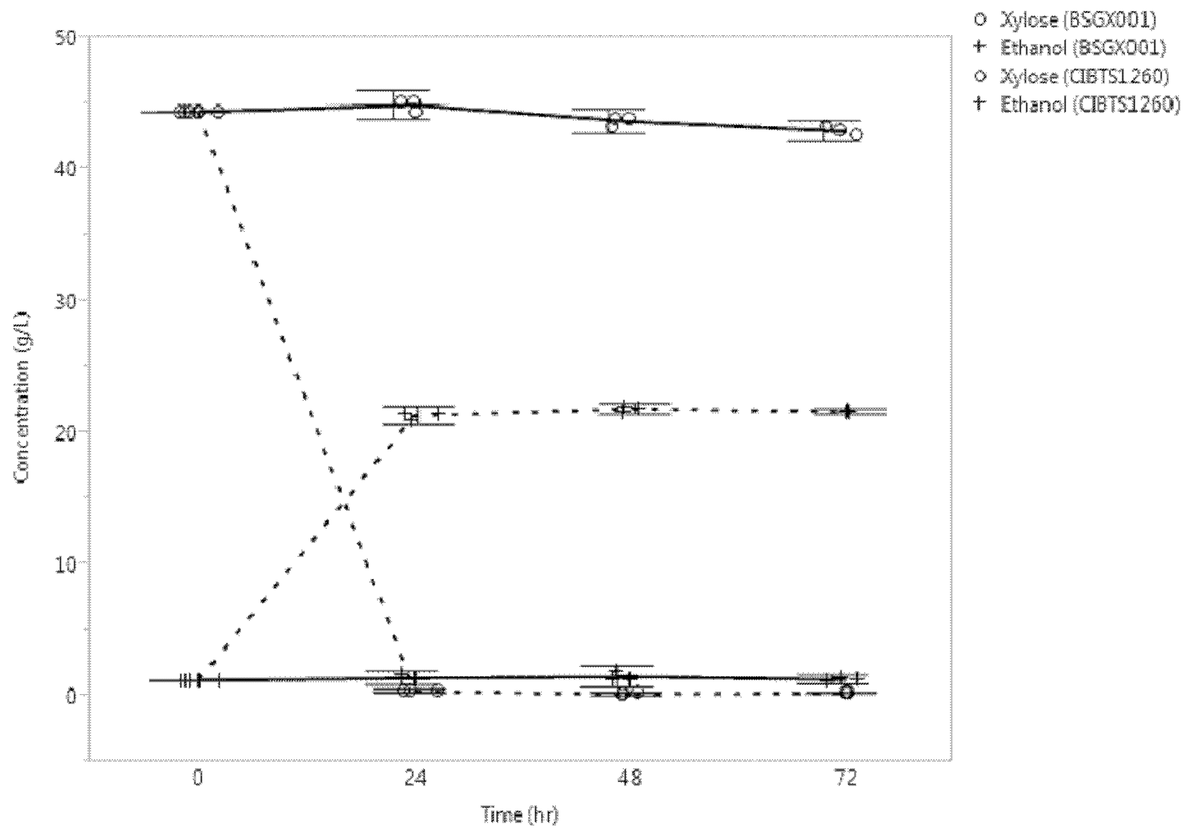

Fig. 5

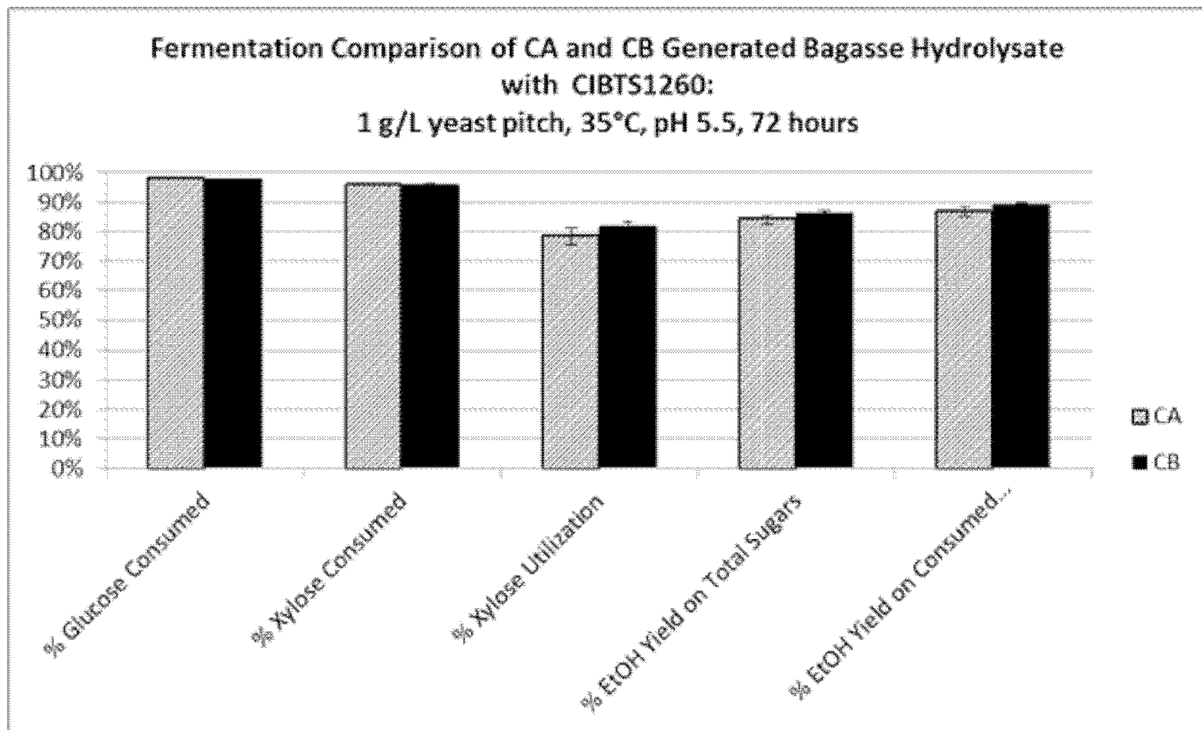

Fig. 6

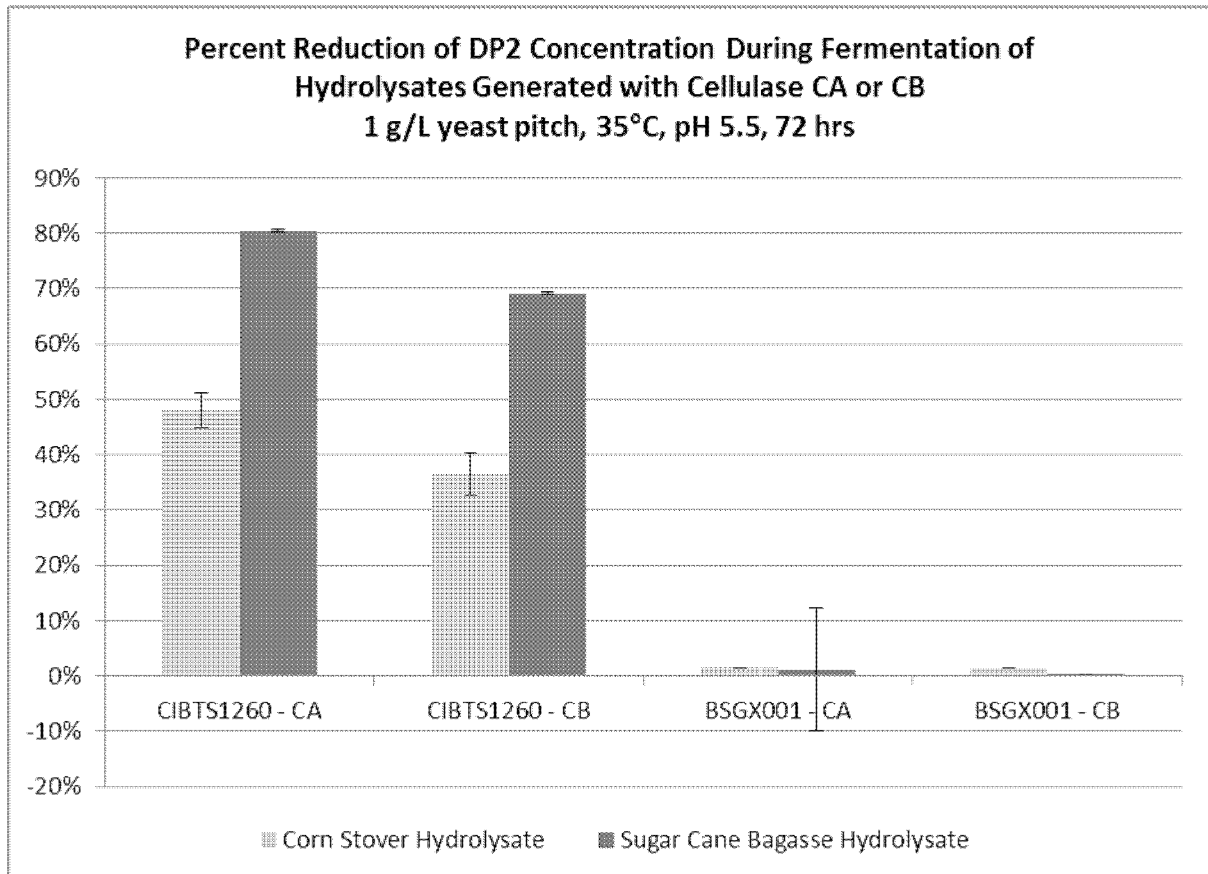

Fig. 7