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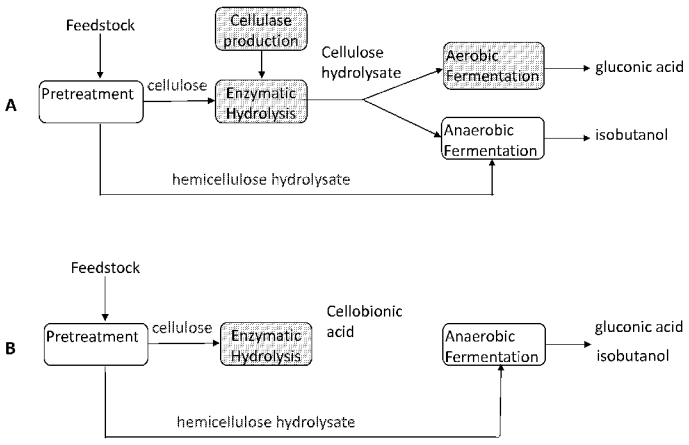
(54) Title: BIOLOGICAL PLATFORM FOR PRODUCTION OF COMMODITY CHEMICALS

FIG. 1



(57) Abstract: The present disclosure generally relates to biological platforms for the conversion of cellulosic biomass into fuels and chemicals. More specifically, the present disclosure relates to the conversion of cellulosic materials into sugar acids or their salts, which may then be used to produce commodity chemicals. In one aspect, the present disclosure relates to a recombinant host cell including: reduced activity of one or more polypeptides having P-glucosidase activity as compared to a corresponding wild type cell, where each of said one or more polypeptides are encoded by a gene that has at least 80% sequence identity to a gene.

BIOLOGICAL PLATFORM FOR PRODUCTION OF COMMODITY CHEMICALS

CROSS-REFERENCE(S) TO RELATED APPLICATION(S)

[0001] This application claims the benefit of U.S. Provisional Application No. - 61/985,379 filed on April 28, 2014, which is incorporated herein by reference in its entirety.

SUBMISSION OF SEQUENCE LISTING ON ASCII TEXT FILE

[0002] The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 514112007840SEQLIST.TXT, date recorded: April 23, 2015, size: 97 KB).

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH

[0003] This invention was made with government support under Grant No. 2011-67009-20060 awarded by United States Department of Agriculture-National Institute of Food and Agriculture (USDA-NIFA). The government has certain rights in the invention. This invention was also made with State of California support under California Energy Commission Grant number 55779A/08-03. The Energy Commission has certain rights to this invention.

FIELD

[0004] The present disclosure generally relates to biological platforms for the conversion of cellulosic biomass into fuels and chemicals. More specifically, the present disclosure relates to the conversion of cellulosic materials into sugar acids or their salts, which may then be used to produce commodity chemicals.

BACKGROUND

[0005] Cellulosic biomass, which is available at low cost and in large abundance, is one of the only foreseeable sustainable sources for organic fuels, chemicals and materials (Lynd *et al.*, 2005; Lynd *et al.*, 2002, Lynd *et al.*, 1999). In particular, ethanol production from cellulosic biomass has near-zero greenhouse emissions and offers many other environmental benefits (Lynd *et al.*, 2005; Lynd *et al.*, 1999; Lynd *et al.*, 1991). The primary obstacle

impeding production of ethanol and other chemicals from cellulosic biomass is the lack of technology for low-cost production (Lynd *et al.*, 1999).

[0006] Traditional biochemical platforms or methods for fuel- and chemical-production generate sugars from cellulosic feedstock as reactive intermediates. These sugars can then be fermented to produce fuels and chemicals. There are five key steps involved in the current biochemical platform: (1) pre-treatment, (2) cellulase production, (3) enzymatic hydrolysis, (4) fermentation, and (5) product recovery. The first three steps: pre-treatment, cellulase production, and enzymatic hydrolysis are the three most costly steps in the production process, constituting approximately 65% of the overall processing cost.

[0007] The first step, pre-treatment, is a process to remove hemicellulose and lignin to increase the susceptibility of cellulose to subsequent enzymatic hydrolysis, thus allowing the exposed cellulose to be hydrolyzed into sugars by cellulases. The pre-treatment process tends to be thermo-chemical. Techniques used in the process include treatment with acid or base, or through steam or ammonia explosions. Most of the techniques are energy-intensive, expensive, and often polluting. In addition, capital cost for pre-treatment reactors are extremely high due to specific material requirements for acid or alkali resistance at elevated temperatures.

[0008] After the pre-treatment step, cellulases are added in a second step to hydrolyze cellulose, resulting in the production of sugars. While cellulase production costs have dropped significantly due to industrial production of enzymes, costs of this step still remain high. Lowering the processing costs of the two aforementioned steps is crucial for the realization of cost-effective production of ethanol and chemicals from biomass. Thus, there exists a need for improved compositions and methods for conversion of cellulosic biomass into commodity chemicals.

BRIEF SUMMARY

[0009] In one aspect, the present disclosure relates to a recombinant host cell including: a) reduced activity of one or more polypeptides having β-glucosidase activity as compared to a corresponding wild type cell, where each of said one or more polypeptides are encoded by a gene that has at least 80% sequence identity to a gene selected from the group of NCU00130, NCU04952, NCU05577, NCU07487, NCU08755, and NCU03641 genes; b) reduced activity of a polypeptide having cellobionate phosphorylase activity as compared to a corresponding

wild type cell, where said polypeptide is encoded by a gene that has at least 80% sequence identity to NCU09425 (NdvB); c) reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08807 (CRE-1) as compared to a corresponding wild type cell; and d) reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU09333 (ACE-1) as compared to a corresponding wild type host cell. In some embodiments, the host cell is a fungal cell. In some embodiments, the fungal cell is *Neurospora crassa*. In some embodiments that may be combined with any of the preceding embodiments, reduced activity of one or more of the polypeptides having β-glucosidase activity is due to a genetic mutation. In some embodiments, a genetic mutation is present in two or more, three or more, four or more, five or more, or six or more genes encoding the polypeptides having β-glucosidase activity. In some embodiments, at least one of the genetic mutations is a knockout mutation. In some embodiments that may be combined with any of the preceding embodiments, reduced activity a polypeptide having cellobionate phosphorylase activity is due to a genetic mutation. In some embodiments, the genetic mutation is a knockout mutation. In some embodiments that may be combined with any of the preceding embodiments, reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08807 (CRE-1) is due to a genetic mutation. In some embodiments, the genetic mutation is a knockout mutation. In some embodiments that may be combined with any of the preceding embodiments, reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU09333 (ACE-1) is due to a genetic mutation. In some embodiments, the genetic mutation is a knockout mutation. In some embodiments that may be combined with any of the preceding embodiments, the host cell further comprises reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08290 (MUS51) as compared to a corresponding wild type cell. In some embodiments, reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08290 (MUS51) is due to a genetic mutation. In some embodiments, the genetic mutation is a knockout mutation. In some embodiments that may be combined with any of the preceding embodiments, the host cell further comprises a polypeptide having increased laccase expression or activity as compared to a corresponding wild type cell. In some embodiments, the polypeptide is encoded by a gene that has at least 80% sequence identity to NCU04528. In some embodiments, expression of the polypeptide is under the control of a constitutive promoter. In some embodiments that may be combined with any of the preceding embodiments, the host cell further comprises a polypeptide having increased cellobiose dehydrogenase expression or activity as compared to a corresponding

wild type cell. In some embodiments, expression of the polypeptide having increased cellobiose dehydrogenase expression or activity is under the control of a constitutive promoter. In some embodiments that may be combined with any of the preceding embodiments, the host cell produces a sugar acid from cellulose. In some embodiments that may be combined with any of the preceding embodiments, the host cell produces cellobiose. In some embodiments that may be combined with any of the preceding embodiments, the sugar acid is cellobionate. In some embodiments, consumption of cellobionate by the host cell is reduced by at least 80% as compared to a corresponding wild type cell.

[0010] In another aspect, the present disclosure relates to a recombinant *N. crassa* cell including: a) a mutation in each of NCU00130, NCU04952, NCU05577, NCU07487, NCU08755, and NCU03641 genes, where said mutation reduces β-glucosidase activity of polypeptides encoded by said genes; b) a mutation in the NCU09425 (NdvB) gene, where said mutation reduces cellobionate phosphorylase activity of the polypeptides encoded by said gene; c) a mutation in each of NCU08807 (CRE-1) and NCU09333 (ACE-1) genes, where said mutations reduce activity of polypeptides encoded by said genes; d) a laccase having increased expression or activity as compared to a wild type *N. crassa* cell, and where the recombinant *N. crassa* cell produces cellobionate from cellulose.

[0011] In another aspect, the present disclosure relates to a method of producing sugar acids, the method including: a) providing a host cell having i) reduced activity of one or more polypeptides having β-glucosidase activity as compared to a corresponding wild type cell, where each of said one or more polypeptides are encoded by a gene that has at least 80% sequence identity to a gene selected from the group of NCU00130, NCU04952, NCU05577, NCU07487, NCU08755, and NCU03641 genes; ii) reduced activity of a polypeptide having cellobionate phosphorylase activity as compared to a corresponding wild type cell, where said polypeptide is encoded by a gene that has at least 80% sequence identity to NCU09425 (NdvB); iii) reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08807 (CRE-1) as compared to a corresponding wild type cell; and iv) reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU09333 (ACE-1) as compared to a corresponding wild type host cell; b) culturing the host cell in a medium including cellulose, where the host cell produces a sugar acid from the cellulose.

[0012] In another aspect, the present disclosure relates to a method of producing sugar acids, the method including a) providing a host cell of any of the preceding embodiments, and b) culturing the host cell in a medium including cellulose, where the host cell produces a sugar acid from the cellulose. In some embodiments, the method further includes a step of substantially purifying the sugar acid from the medium. In some embodiments that may be combined with any of the preceding embodiments, the host cell produces cellobiose. In some embodiments that may be combined with any of the preceding embodiments, the sugar acid is cellobionate. In some embodiments, consumption of cellobionate by the host cell is reduced by at least 80% as compared to a corresponding wild type cell. In some embodiments that may be combined with any of the preceding embodiments, the host cell is cultured in the presence of an exogenous source of laccase. In some embodiments that may be combined with any of the preceding embodiments, the host cell is cultured in the presence of an exogenous source of cellobiose dehydrogenase. In some embodiments that may be combined with any of the preceding embodiments, the medium further includes a redox mediator.

DESCRIPTION OF THE FIGURES

[0013] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the office upon request and payment of the necessary fee.

[0014] **FIG. 1A** illustrates a flow chart of the conventional steps used in methods of producing commodity chemicals from cellulosic biomass. **FIG. 1B** illustrates a flow chart for methods of direct enzymatic hydrolysis of cellulose.

[0015] **FIG. 2** illustrates the chemical conversion of cellobiose to cellobionic acid.

[0016] **FIG. 3** illustrates the cellulose degradation mechanism for some cellulolytic microorganisms, such as *Neurospora crassa*.

[0017] **FIG. 4A** illustrates cellobiose production by the F5 strain with or without exogenous cellobiose dehydrogenase addition. **FIG. 4B** illustrates cellobionate production by the F5 strain with or without exogenous cellobiose dehydrogenase addition.

[0018] **FIG. 5A** illustrates conversion of gluconate to ethanol and acetate by the *Escherichia coli* strain KO 11. **FIG. 5B** illustrates the conversion of a glucose and gluconate mixture to ethanol and acetate by the *Escherichia coli* strain KO 11.

[0019] **FIG. 6** illustrates a summary of cellulose conversion and mycelial mass production.

[0020] **FIG. 7** illustrates the impact of laccase and ABTS on cellobiose and cellobionate production in various strains.

[0021] **FIG. 8A** illustrates cellobiose and cellobionate levels in various strains. **FIG. 8B** illustrates theoretical and retained yields of cellobionate.

[0022] **FIG. 9A** illustrates cellobiose and cellobionate levels in various strains with cells and cellulose filtered prior to the conversion reaction. **FIG. 9B** illustrates theoretical and retained yields of cellobionate.

[0023] **FIG. 10A** illustrates a table depicting theoretical cellobiose and cellobionate concentrations. **FIG. 10B** illustrates theoretical conversions of cellobionate based on either measured or predicted cellobiose levels.

[0024] **FIG. 11A** illustrates cellobionate levels in various strains. **FIG. 11B** illustrates cellobiose levels in various strains. **FIG. 11C** illustrates cellobiose dehydrogenase (CDH) activity in various strains.

[0025] **FIG. 12** illustrates cellobionate utilization by the F5Δcre-1Δace strain and the F5Δcre-1ΔaceΔndvB strains.

[0026] **FIG. 13** illustrates cellobionate production by the F5Δcre-1Δace strain and the F5Δcre-1ΔaceΔndvB +lac strain.

[0027] **FIG. 14A** illustrates cellobiose production in various strains. **FIG. 14B** illustrates cellobionate production in various strains. All strains were grown on 20g/L Avicel.

[0028] **FIG. 15** illustrates the conversion of cellobionic acid to α-D-glucose-1-phosphate and D-gluconic acid.

[0029] **FIG. 16** illustrates the enzymatic oxidation of substrate (cellobiose/lactose) by CDH. The reduced CDH is oxidized by a redox mediator (ABTS), which is in turn oxidized by laccase. The reduced laccase is oxidized by oxygen, with water as the only byproduct.

[0030] **FIG. 17A** and **FIG. 17B** illustrate the effect of pH on the conversion of cellobiose to CBA via CDH-ABTS-laccase mediated conversion. Acidic conditions (pH 4 and pH 6) used 50 mM sodium citrate buffer; pH 7.2 and pH 8 used 50 mM sodium phosphate buffer. The Vogels pH 6 condition used 1x Vogel's salts medium. The results shown are the means of biological duplicates with the error bars representing the standard deviation.

[0031] **FIG. 18A** and **FIG. 18B** illustrate the effect of potassium phosphate buffer concentration and the conversion of cellobiose to CBA via CDH-ABTS-laccase mediated conversion with the *F5Δace-1Δcre-1ΔndvB* strain in 1x Vogel's medium and 20 g/L Avicel. The values shown are the means of biological duplicates with the error bars representing the standard deviations.

[0032] **FIG. 19A** and **FIG. 19B** illustrate optimization of laccase (0.05 U/mL) and ABTS (0.01 mM) addition time with the *F5Δace-1Δcre-1ΔndvB* strain grown in 1x Vogel's medium and 20 g/L Avicel. The values shown are the means of biological triplicates with the error bars representing the standard deviations.

[0033] **FIG. 20** illustrates laccase production by *N. crassa* with induction by 3 μM cycloheximide induction at 48 hours into the fermentation. Results shown are the means of biological triplicates with the error bars representing the standard deviations.

[0034] **FIG. 21A** and **FIG. 21B** illustrate comparisons of the *N. crassa* laccase to the *P. ostreatus* laccase in the conversion of cellobiose to CBA using the CDH-ABTS-laccase conversion system. The data shown are the means of biological duplicates with the error bars representing the standard deviations.

[0035] **FIG. 22** illustrates laccase production by recombinant *N. crassa* strains.

[0036] **FIG. 23** illustrates cellobionate production in the indicated strains with ABTS addition (with ABTS addition at 0 hr).

[0037] **FIG. 24** illustrates cellobionate production in the indicated strains with or without the addition of ABTS.

DETAILED DESCRIPTION

[0038] The following description is presented to enable a person of ordinary skill in the art to make and use the various embodiments. Descriptions of specific devices, techniques,

and applications are provided only as examples. Various modifications to the examples described herein will be readily apparent to those of ordinary skill in the art, and the general principles defined herein may be applied to other examples and applications without departing from the spirit and scope of the various embodiments. Thus, the various embodiments are not intended to be limited to the examples described herein and shown, but are to be accorded the scope consistent with the claims.

Overview

[0039] The present disclosure generally relates to biological platforms for the conversion of cellulosic biomass into fuels and chemicals. More specifically, the present disclosure relates to the conversion of cellulosic materials into sugar acids or their salts, which may then be used to produce commodity chemicals.

[0040] **FIG. 1A** illustrates the conventional steps used in the production of gluconic acid and isobutanol from cellulosic biomass, which involves the production of cellulase and a step of aerobic fermentation. Applicants propose an alternative route, outlined in **FIG. 1B**, which circumvents the need for cellulase production, but is instead based on the direct enzymatic hydrolysis of cellulose to produce sugar acids, such as cellobionic acid (See also **FIG. 2**), and subsequent downstream products.

[0041] Cellulolytic microorganisms are capable of hydrolyzing cellulose, and thus such organisms may be used as work horses for the production of commodity chemicals. **FIG. 3** shows the cellulose degradation mechanism for some cellulolytic microorganisms, including *Neurospora crassa*. Cellulose is hydrolyzed by endoglucanases and exoglucanases to cello-oligosaccharides, with cellobiose as the main component. The resultant cellobiose as the main component is oxidized by cellobiose dehydrogenase to produce cellobiono-1,5-lactone, which reacts spontaneously with water to form cellobionic acid. In addition, cellobionic acid is released from cellulose by the combined action of lytic polysaccharide monooxygenase and cellobiohydrolase. The cellobionic acid is transported into the cytoplasm, followed by phosphorolysis by cellobionic acid phosphorylase (NCU09425) to produce αGlc1P and D-gluconic acid. The released αGlc1P is converted into D-glucose 6-phosphate by a-phosphoglucomutase (EC 2.7.5.1) to enter the glycolysis pathway. The released D-gluconic acid is converted into ribulose 5-phosphate via 6- phosphogluconate by the sequential reaction of gluconokinase (EC 2.7.1.12) and 6-phosphogluconate dehydrogenase (EC

1.1.1.44) to enter the pentose phosphate pathway (Nihira *et al.*, 2013). One of the notable features of the metabolic pathway for cellulose described herein is that the fungus uses ATP-derived energy efficiently, because it is possible to phosphorylate a D-glucose residue of cellobionic acid directly without consuming ATP by ATP-dependent carbohydrate kinase. Cellobionic acid appears to be a less ideal substrate for β-glucosidase because the hydrolysate of this reaction, D-gluconic acid, is a non-competitive inhibitor of β-glucosidase (Nihira *et al.*, 2013). In addition, it should be noted that a variety of cellulolytic fungi of the phylum Ascomycota also possess a cellobionic acid phosphorylase homologous protein.

[0042] After the generation of cello-oligosaccharides , β-glucosidase enzymes can then hydrolyze cello-oligosaccharides into glucose, which can be metabolized by fungi. If β-glucosidase enzyme activity is reduced and cellobiose dehydrogenase (CDH) activity is increased, carbon flow can be directed toward aldonic acid production. These organic acids can be continuously removed from the broth, such as by calcium hydroxide precipitation, to avoid any possible inhibition. In practice, some β-glucosidase activity must remain intact in the cell to support cell growth and enzyme production. Gluconic acid utilization can be prevented by reducing the activity of gluconokinases, thus leaving this compound available for subsequent ethanol fermentation. Gluconic acid was found to be a non-competitive product inhibitor to the β-glucosidase for *A. niger*, and its presence could inhibit any noticeable hydrolysis of cellobionate by the residual β-glucosidase activity. Further, hydrolysis experiments revealed that cellobionic acid was hydrolyzed by β-glucosidase at a rate almost 10-fold lower than for cellobiose and the formed gluconic acid was an inhibitor of the β-glucosidase (Cannella, 2012).

[0043] Applicants disclose herein compositions and methods for conversion of cellulose into downstream products. The present disclosure is based, at least in part, on Applicant's discovery that a *Neurospora crassa* engineered to contain mutations in six β-glucosidase genes, a mutation in the CRE-1 gene, a mutation in the ACE-1 gene, a mutation in the NdvB cellobionate phosphorylase gene, and either engineered to overexpress a laccase gene or cultured in the presence of exogenous laccase enzyme, produced surprisingly high amounts of cellobionic acid (cellobionate). This engineered strain concomitantly failed to metabolize the cellobionate that was produced, allowing cellobionate to accumulate. As described above, cellobionate may be used for the production of downstream products, such as gluconic acid and isobutanol.

Polypeptides Having Modified Activity

[0044] The present disclosure relates to recombinant host cells having modified activity of various polypeptides as compared to corresponding control cells, such as wild type cells. As used herein, a "polypeptide" is an amino acid sequence including a plurality of consecutive polymerized amino acid residues (e.g., at least about 15 consecutive polymerized amino acid residues). As used herein, "polypeptide" refers to an amino acid sequence, oligopeptide, peptide, protein, or portions thereof, and the terms "polypeptide" and "protein" are used interchangeably.

β-Glucosidase Polypeptides

[0045] Host cells of the present disclosure have reduced activity of one or more β-glucosidase polypeptides as compared to a corresponding wild type cell.

[0046] β-glucosidase (*bgl*) genes of the present disclosure encode β-glucosidase enzymes. As used herein, "β-glucosidase" refers to a β-D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing β-D-glucose residues with the release of β-D-glucose. A β-glucosidase is any enzyme that catalyzes the hydrolysis of terminal non-reducing residues in β-D-glucosides, such as cellodextrins, with release of glucose.

[0047] β-glucosidases of the present disclosure may be either intracellular β-glucosidases or extracellular β-glucosidases. As used herein "intracellular β-glucosidases" are expressed within lignocellulolytic cells and hydrolyze cellodextrins transported into the cell. As used herein "extracellular β-glucosidases" are expressed and secreted from lignocellulolytic cells or expressed on the surface of lignocellulolytic cells.

[0048] In certain embodiments, the β-glucosidase is a glycosyl hydrolase family 1 member. Members of this group can be identified by the motif, [LIVMFSTC] - [LIVFYS] - [LIV] - [LIVMST] - E - N - G - [LIVMFAR] - [CSAGN] (SEQ ID NO: 23). Here, E is the catalytic glutamate. In some embodiments, the β-glucosidase is from *N. crassa*. Other β-glucosidases may include those from the glycosyl hydrolase family 3. These β-glucosidases can be identified by the following motif according to PROSITE: [LIVM](2) - [KR] - x - [EQKRD] - x(4) - G - [LIVMFTC] - [LIVT] - [LIVMF] - [ST] - D - x(2) - [SGADNIT] (SEQ ID NO: 24). Here D is the catalytic aspartate. Typically, any β-glucosidase may be used that

contains the conserved domain of β-glucosidase/6-phospho-β-glucosidase/β-galactosidase found in NCBI sequence COG2723.

[0049] In certain embodiments, β-glucosidases of the present disclosure include, for example, *N. crassa* β-glucosidases encoded by NCU00130, NCU04952, NCU05577, NCU07487, NCU08054, NCU08755, and NCU03641. Suitable β-glucosidases of the present disclosure also include homologs, orthologs, and paralogs of NCU00130, NCU04952, NCU05577, NCU07487, NCU08054, NCU08755, and NCU03641.

[0050] Intracellular β-glucosidases of the present disclosure include, for example, those encoded by NCU00130, NCU05577, NCU07487, NCU08054, homologs thereof, orthologs thereof, and paralogs thereof. Extracellular β-glucosidases of the present disclosure include, for example, those encoded by NCU04952, NCU08755, NCU03641, homologs thereof, orthologs thereof, and paralogs thereof.

[0051] In some embodiments, the polypeptide having reduced β-glucosidase activity is a polypeptide encoded by NCU00130, NCU04952, NCU05577, NCU07487, NCU08755, and/or NCU03641. The amino acid sequence of the protein encoded by NCU00130 is set forth herein as SEQ ID NO: 1. The amino acid sequence of the protein encoded by NCU04952 is set forth herein as SEQ ID NO: 3. The amino acid sequence of the protein encoded by NCU05577 is set forth herein as SEQ ID NO: 5. The amino acid sequence of the protein encoded by NCU07487 is set forth herein as SEQ ID NO: 7. The amino acid sequence of the protein encoded by NCU08755 is set forth herein as SEQ ID NO: 9. The amino acid sequence of the protein encoded by NCU03641 is set forth herein as SEQ ID NO: 11. In some embodiments, the polypeptide having reduced β-glucosidase activity is a polypeptide encoded by a gene that has at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to NCU00130, NCU04952, NCU05577, NCU07487, NCU08755, or NCU03641 genes. The nucleotide sequence of NCU00130 is set forth herein as SEQ ID NO: 2. The nucleotide sequence of NCU04952 is set forth herein as SEQ ID NO: 4. The nucleotide sequence of NCU05577 is set forth herein as SEQ ID NO: 6. The nucleotide sequence of NCU07487 is set forth herein as SEQ ID NO: 8. The amino acid sequence of NCU08755 is set forth herein as SEQ ID NO: 10. The nucleotide sequence of NCU03641 is set forth herein as SEQ ID NO: 12.

[0052] Various other β-glucosidases are well-known in the art and may be used in the methods and compositions of the present disclosure.

[0053] One or more β-glucosidases may have reduced activity in host cells of the present disclosure. Host cells of the present disclosure may have reduced activity of two or more, three or more, four or more, five or more, or six or more polypeptides having β-glucosidase activity.

Cellobionate Phosphorylase Polypeptides

[0054] Host cells of the present disclosure have reduced activity of one or more cellobionate phosphorylase proteins as compared to a corresponding wild type cell.

[0055] Cellobionate phosphorylases are well-known in the art and are described herein. Cellobionate phosphorylase catalyzes the reaction outlined in **FIG. 15** and has activity associated with EC 2.4.1.321. In this reaction, cellobionate phosphorylase catalyzes the phosphorolysis of cellobionic acid (4-O-β-D-glucopyranosyl-D-gluconate) to produce α-D-glucose-1-phosphate and D-gluconic acid. In some embodiments, cellobionate phosphorylases of the present disclosure contain one or more glycosyltransferase family 36 protein domains.

[0056] In some embodiments, the polypeptide having reduced cellobionate phosphorylase activity is a polypeptide encoded by NCU09425, which encodes the NdvB protein from *Neurospora crassa*. The amino acid sequence of the protein encoded by NCU09425 is set forth herein as SEQ ID NO: 13. In some embodiments, the polypeptide having reduced cellobionate phosphorylase activity is a polypeptide encoded by a gene that has at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to NCU09425. The nucleotide sequence of NCU09425 is set forth herein as SEQ ID NO: 14. Various other cellobionate phosphorylases are well-known in the art and may be used in the methods and compositions of the present disclosure. Other exemplary cellobionate phosphorylases include, for example, a glycoside hydrolase family 94 cellobionate phosphorylase from the bacterium *Xanthomonas campestris*.

CRE-1 Polypeptides

[0057] Host cells of the present disclosure have reduced activity of a CRE-1 protein as compared to a corresponding wild type cell. CRE-1 proteins are well-known in the art and are described herein. CRE-1 is a transcription factor protein. Transcription factor polypeptides, generally speaking, are polypeptides involved in the regulation of gene expression in a cell. Transcription factor polypeptides may have activity relating to the control of expression of a nucleic acid, such as DNA. Transcription factor activity is well-known in the art. For example, transcription factor polypeptide activity may include DNA-binding activity or activity relating to the regulation of gene expression.

[0058] It is thought that CRE-1 is involved in catabolite repression. Deletion of the cre has been shown to increase cellulase expression. A cre- strain has decreased growth rate on preferred carbon sources; however, on Avicel it produces 30-50% more cellulase and consumes avicel faster (Sun et al., 2011), and cre expression correlates with cellulase expression.

[0059] In some embodiments, the polypeptide having reduced CRE-1 activity is a polypeptide encoded by NCU08807, which encodes a CRE-1 protein from *Neurospora crassa*. The amino acid sequence of the protein encoded by NCU08807 is set forth herein as SEQ ID NO: 15. In some embodiments, the polypeptide having reduced CRE-1 activity is a polypeptide encoded by a gene that has at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to NCU08807. The nucleotide sequence of NCU08807 is set forth herein as SEQ ID NO: 16. Various other CRE-1 proteins are well-known in the art and may be used in the methods and compositions of the present disclosure.

ACE-1 Polypeptides

[0060] Host cells of the present disclosure have reduced activity of an ACE-1 protein as compared to a corresponding wild type cell. ACE-1 proteins are well-known in the art and are described herein. ACE-1 is a zinc finger transcription factor protein. Mutation of the ace-1 gene (ace-) resulted in higher cellulase expression in *T. reesei* (Aro *et al.*, 2003).

[0061] In some embodiments, the polypeptide having reduced ACE-1 activity is a polypeptide encoded by NCU09333, which encodes an ACE-1 protein from *Neurospora crassa*. The amino acid sequence of the protein encoded by NCU09333 is set forth herein as SEQ ID NO: 17. In some embodiments, the polypeptide having reduced ACE-1 activity is a polypeptide encoded by a gene that has at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to NCU09333. The nucleotide sequence of NCU09333 is set forth herein as SEQ ID NO: 18. Various other ACE-1 proteins are well-known in the art and may be used in the methods and compositions of the present disclosure.

MUS51 Polypeptides

[0062] Host cells of the present disclosure may have reduced activity of a MUS51 protein as compared to a corresponding wild type cell. MUS51 proteins are well-known in the art and are described herein. MUS51 is an ATP-dependent DNA helicase II subunit 1 protein (E.C. 3.6.4.12). MUS51 proteins are involved in non-homologous end joining (NHEJ) DNA double strand break repair. Without wishing to be bound by theory, it is thought that reducing MUS51 protein activity in a host cell will increase the chances for and/or frequency of homologous recombination in the host cell, which may make genetic modification of the host cell easier.

[0063] In some embodiments, the polypeptide having reduced MUS51 activity is a polypeptide encoded by NCU08290, which encodes a MUS51 protein from *Neurospora crassa*. In some embodiments, the polypeptide having reduced MUS51 activity is a polypeptide encoded by a gene that has at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to NCU08290. Various other MUS51 proteins are well-known in the art and may be used in the methods and compositions of the present disclosure.

Laccase Polypeptides

[0064] Host cells of the present disclosure may have increased expression or activity of a laccase protein as compared to a corresponding wild type cell. Laccase proteins may also be

added exogenously to culture media in methods of the present disclosure. Laccase proteins are well-known in the art and are described herein. Laccases are "blue" copper containing oxidases (E.C. 1.10.3.2). Laccases catalyze the following reaction: 4 benzenediol + O_2 = 4 benzosemiquinone + 2 H_2O. These multi copper enzymes have low specificity acting on both o- and p-quinols, and often acting also on aminophenols and phenylenediamine. Laccases may also act on phenols and similar molecules, performing one-electron oxidations. Because laccase proteins belong to the oxidase enzyme family, these enzymes require oxygen as a second substrate for enzymatic action.

[0065] In some embodiments, the polypeptide having increased laccase expression or activity is a polypeptide encoded by NCU04528, which encodes a laccase protein from *Neurospora crassa*. The amino acid sequence of the protein encoded by NCU04528 is set forth herein as SEQ ID NO: 19. In some embodiments, the polypeptide having increased laccase expression or activity is a polypeptide encoded by a gene that has at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to NCU04528. The nucleotide sequence of NCU04528 is set forth herein as SEQ ID NO: 20. Various other laccase proteins are well-known in the art and may be used in the methods and compositions of the present disclosure.

Cellobiose Dehydrogenase Polypeptides

[0066] Host cells of the present disclosure may have increased expression or activity of a cellobiose dehydrogenase protein as compared to a corresponding wild type cell. Cellobiose dehydrogenase proteins may also be added exogenously to culture media in methods of the present disclosure. Cellobiose dehydrogenase (CDH) proteins are well-known in the art and are described herein. CDH enzymes catalyze the following reaction: cellobiose + acceptor = cellobiono-1,5-lactone + reduced acceptor (E.C. 1.1.99.18). CDH proteins contain an N-terminal heme domain and a C-terminal dehydrogenase domain. Some CDH proteins also contain a cellulose binding module (CBM) at the C-terminus of the protein. Orthologs of the CDH heme domain are found only in fungal proteins, whereas orthologs of the dehydrogenase domain are found in proteins throughout all domains of life; the dehydrogenase domain is part of the larger GMC oxidoreductase superfamily.

[0067] In some embodiments, the polypeptide having increased cellobiose dehydrogenase expression or activity is a polypeptide encoded by NCU00206, which encodes a cellobiose dehydrogenase protein from *Neurospora crassa*. The amino acid sequence of the protein encoded by NCU00206 is set forth herein as SEQ ID NO: 21. In some embodiments, the polypeptide having increased cellobiose dehydrogenase expression or activity is a polypeptide encoded by a gene that has at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identity to NCU00206. The nucleotide sequence of NCU00206 is set forth herein as SEQ ID NO: 22. Various other cellobiose dehydrogenase proteins are well-known in the art and may be used in the methods and compositions of the present disclosure. Other cellobiose dehydrogenases include, for example, the polypeptides of Accession Numbers: XM_411367, BAD32781, BAC20641, XM_389621, AF257654, AB187223, XM_360402, U46081, AF081574, AY187232, AF074951, and AF029668.

Polynucleotides Encoding Polypeptides

[0068] The present disclosure further relates to polynucleotides that encode polypeptides having modified activity of the present disclosure. Polynucleotides that encode a polypeptide are also referred to herein as "genes." For example, polynucleotides encoding any known or putative β-glucosidase, cellobionate phosphorylase, CRE-1 protein, ACE-1 protein, MUS51 protein, laccase, or cellobiose dehydrogenase polypeptide as described herein are provided. Methods for determining the relationship between a polypeptide and a polynucleotide that encodes the polypeptide are well-known to one of skill in the art. Similarly, methods of determining the polypeptide sequence encoded by a polynucleotide sequence are well-known to one of skill in the art.

[0069] As used herein, the terms "polynucleotide," nucleic acid sequence," "nucleic acid," and variations thereof shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), to any other type of polynucleotide that is an N-glycoside of a purine or pyrimidine base, and to other polymers containing non-nucleotidic backbones, provided that the polymers contain nucleobases in a configuration that allows for base pairing and base stacking, as found in DNA and RNA. Thus, these terms include known types of nucleic acid sequence modifications, for example, substitution of one or more of the naturally occurring nucleotides with an analog, and inter-

nucleotide modifications. As used herein, the symbols for nucleotides and polynucleotides are those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature.

[0070] Sequences of the polynucleotides of the present disclosure may be prepared by various suitable methods known in the art, including, for example, direct chemical synthesis or cloning. For direct chemical synthesis, formation of a polymer of nucleic acids typically involves sequential addition of 3 '-blocked and 5 '-blocked nucleotide monomers to the terminal 5'-hydroxyl group of a growing nucleotide chain, wherein each addition is effected by nucleophilic attack of the terminal 5'-hydroxyl group of the growing chain on the 3 '-position of the added monomer, which is typically a phosphorus derivative, such as a phosphotriester, phosphoramidite, or the like. Such methodology is known to those of ordinary skill in the art and is described in the pertinent texts and literature (*e.g.*, in Matteucci *et al.*, (1980) Tetrahedron Lett 21:719-722; U.S. Pat. Nos. 4,500,707; 5,436,327; and 5,700,637). In addition, the desired sequences may be isolated from natural sources by splitting DNA using appropriate restriction enzymes, separating the fragments using gel electrophoresis, and thereafter, recovering the desired polynucleotide sequence from the gel via techniques known to those of ordinary skill in the art, such as utilization of polymerase chain reactions (PCR; *e.g.*, U.S. Pat. No. 4,683,195).

Methods of Identifying Sequence Similarity

[0071] Various methods are known to those of skill in the art for identifying similar (*e.g.* homologs, orthologs, paralogs, etc.) polypeptide and/or polynucleotide sequences, including phylogenetic methods, sequence similarity analysis, and hybridization methods.

[0072] Phylogenetic trees may be created for a gene family by using a program such as CLUSTAL (Thompson et al. Nucleic Acids Res. 22: 4673-4680 (1994); Higgins et al. *Methods Enzymol* 266: 383-402 (1996)) or MEGA (Tamura et al. *Mol. Biol. & Evo.* 24:1596-1599 (2007)). Once an initial tree for genes from one species is created, potential orthologous sequences can be placed in the phylogenetic tree and their relationships to genes from the species of interest can be determined. Evolutionary relationships may also be inferred using the Neighbor-Joining method (Saitou and Nei, *Mol. Biol.* & Evo. 4:406-425 (1987)). Homologous sequences may also be identified by a reciprocal BLAST strategy. Evolutionary distances may be computed using the Poisson correction method (Zuckerkandl

and Pauling, pp. 97-166 in *Evolving Genes and Proteins*, edited by V. Bryson and H.J. Vogel. Academic Press, New York (1965)).

[0073] In addition, evolutionary information may be used to predict gene function. Functional predictions of genes can be greatly improved by focusing on how genes became similar in sequence (i.e. by evolutionary processes) rather than on the sequence similarity itself (Eisen, *Genome Res.* 8: 163-167 (1998)). Many specific examples exist in which gene function has been shown to correlate well with gene phylogeny (Eisen, *Genome Res.* 8: 163-167 (1998)). By using a phylogenetic analysis, one skilled in the art would recognize that the ability to deduce similar functions conferred by closely-related polypeptides is predictable.

[0074] When a group of related sequences are analyzed using a phylogenetic program such as CLUSTAL, closely related sequences typically cluster together or in the same clade (a group of similar genes). Groups of similar genes can also be identified with pair-wise BLAST analysis (Feng and Doolittle, *J. Mol. Evol.* 25: 351-360 (1987)). Analysis of groups of similar genes with similar function that fall within one clade can yield sub-sequences that are particular to the clade. These sub-sequences, known as consensus sequences, can not only be used to define the sequences within each clade, but define the functions of these genes; genes within a clade may contain paralogous sequences, or orthologous sequences that share the same function (see also, for example, Mount, Bioinformatics: Sequence and Genome Analysis Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., page 543 (2001)).

[0075] To find sequences that are homologous to a reference sequence, BLAST nucleotide searches can be performed with the BLASTN program, score=100, wordlength=12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the disclosure. BLAST protein searches can be performed with the BLASTX program, score=50, wordlength=3, to obtain amino acid sequences homologous to a protein or polypeptide of the disclosure. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al. (1997) supra. When utilizing BLAST, Gapped BLAST, or PSI-BLAST, the default parameters of the respective programs (*e.g.*, BLASTN for nucleotide sequences, BLASTX for proteins) can be used.

[0076] Methods for the alignment of sequences and for the analysis of similarity and identity of polypeptide and polynucleotide sequences are well-known in the art.

[0077] As used herein "sequence identity" refers to the percentage of residues that are identical in the same positions in the sequences being analyzed. As used herein "sequence similarity" refers to the percentage of residues that have similar biophysical / biochemical characteristics in the same positions (e.g. charge, size, hydrophobicity) in the sequences being analyzed.

[0078] Methods of alignment of sequences for comparison are well-known in the art, including manual alignment and computer assisted sequence alignment and analysis. This latter approach is a preferred approach in the present disclosure, due to the increased throughput afforded by computer assisted methods. As noted below, a variety of computer programs for performing sequence alignment are available, or can be produced by one of skill.

[0079] The determination of percent sequence identity and/or similarity between any two sequences can be accomplished using a mathematical algorithm. Examples of such mathematical algorithms are the algorithm of Myers and Miller, CABIOS 4:11-17 (1988); the local homology algorithm of Smith et al., Adv. Appl. Math. 2:482 (1981); the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970); the search-for-similarity-method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85:2444-2448 (1988); the algorithm of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 87:2264-2268 (1990), modified as in Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-5877 (1993).

[0080] Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity and/or similarity. Such implementations include, for example: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the AlignX program, version10.3.0 (Invitrogen, Carlsbad, CA) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins et al. *Gene* 73:237-244 (1988); Higgins et al. *CABIOS* 5:151-153 (1989); Corpet et al., *Nucleic Acids Res.* 16:10881-90 (1988); Huang et al. *CABIOS* 8:155-65 (1992); and Pearson et al.,

Meth. Mol. Biol. 24:307-331 (1994). The BLAST programs of Altschul et al. *J. Mol. Biol.* 215:403-410 (1990) are based on the algorithm of Karlin and Altschul (1990) supra.

[0081] Polynucleotides homologous to a reference sequence can be identified by hybridization to each other under stringent or under highly stringent conditions. Single stranded polynucleotides hybridize when they associate based on a variety of well characterized physical-chemical forces, such as hydrogen bonding, solvent exclusion, base stacking and the like. The stringency of a hybridization reflects the degree of sequence identity of the nucleic acids involved, such that the higher the stringency, the more similar are the two polynucleotide strands. Stringency is influenced by a variety of factors, including temperature, salt concentration and composition, organic and non-organic additives, solvents, etc. present in both the hybridization and wash solutions and incubations (and number thereof), as described in more detail in references cited below (e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. ("Sambrook") (1989); Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology, vol. 152 Academic Press, Inc., San Diego, Calif. ("Berger and Kimmel") (1987); and Anderson and Young, "Quantitative Filter Hybridisation." In: Hames and Higgins, ed., Nucleic Acid Hybridisation, A Practical Approach. Oxford, TRL Press, 73-111 (1985)).

[0082] Encompassed by the disclosure are polynucleotide sequences that are capable of hybridizing to the disclosed polynucleotide sequences and fragments thereof under various conditions of stringency (see, for example, Wahl and Berger, *Methods Enzymol.* 152: 399-407 (1987); and Kimmel, *Methods Enzymo.* 152: 507-511, (1987)). Full length cDNA, homologs, orthologs, and paralogs of polynucleotides of the present disclosure may be identified and isolated using well-known polynucleotide hybridization methods.

Vectors for Expressing Polynucleotides

[0083] Each polynucleotide of the present disclosure may be incorporated into an expression vector. "Expression vector" or "vector" refers to a compound and/or composition that transduces, transforms, or infects a host cell, thereby causing the cell to express polynucleotides and/or proteins other than those native to the cell, or in a manner not native to the cell. An "expression vector" contains a sequence of polynucleotides (ordinarily RNA or DNA) to be expressed by the host cell. Optionally, the expression vector also includes

materials to aid in achieving entry of the polynucleotide into the host cell, such as a virus, liposome, protein coating, or the like. The expression vectors contemplated for use in the present disclosure include those into which a polynucleotide sequence can be inserted, along with any preferred or required operational elements. Further, the expression vector must be one that can be transferred into a host cell and replicated therein. Preferred expression vectors are plasmids, particularly those with restriction sites that have been well-documented and that contain the operational elements preferred or required for transcription of the polynucleotide sequence. Such plasmids, as well as other expression vectors, are well-known in the art.

[0084] Incorporation of the individual polynucleotides may be accomplished through known methods that include, for example, the use of restriction enzymes (such as *Bam*HI, *Eco*RI, *Hha*I, *Xho*I, *Xma*I, and so forth) to cleave specific sites in the expression vector, *e.g.*, plasmid. The restriction enzyme produces single stranded ends that may be annealed to a polynucleotide having, or synthesized to have, a terminus with a sequence complementary to the ends of the cleaved expression vector. Annealing is performed using an appropriate enzyme, *e.g.*, DNA ligase. As will be appreciated by those of ordinary skill in the art, both the expression vector and the desired polynucleotide are often cleaved with the same restriction enzyme, thereby assuring that the ends of the expression vector and the ends of the polynucleotide are complementary to each other. In addition, DNA linkers may be used to facilitate linking of polynucleotide sequences into an expression vector.

[0085] A series of individual polynucleotides can also be combined by utilizing methods that are known in the art (*e.g.*, U.S. Pat. No. 4,683,195). For example, each of the desired polynucleotides can be initially generated in a separate PCR. Thereafter, specific primers are designed such that the ends of the PCR products contain complementary sequences. When the PCR products are mixed, denatured, and reannealed, the strands having the matching sequences at their 3' ends overlap and can act as primers for each other. Extension of this overlap by DNA polymerase produces a molecule in which the original sequences are "spliced" together. In this way, a series of individual polynucleotides may be "spliced" together and subsequently transduced into a host cell simultaneously. Thus, expression of each of the plurality of polynucleotides is affected.

[0086] Individual polynucleotides, or "spliced" polynucleotides, are then incorporated into an expression vector. The present disclosure is not limited with respect to the process by

which the polynucleotide is incorporated into the expression vector. Those of ordinary skill in the art are familiar with the necessary steps for incorporating a polynucleotide into an expression vector. A typical expression vector contains the desired polynucleotide preceded by one or more regulatory regions, along with a ribosome binding site, *e.g.*, a nucleotide sequence that is 3-9 nucleotides in length and located 3-11 nucleotides upstream of the initiation codon in *E. coli*. See Shine and Dalgarno (1975) Nature 254(5495):34-38 and Steitz (1979) Biological Regulation and Development (ed. Goldberger, R. F.), 1:349–399 (Plenum, New York).

[0087] The term "operably linked" as used herein refers to a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of the DNA sequence or polynucleotide such that the control sequence directs the expression of a polypeptide.

[0088] Regulatory regions include, for example, those regions that contain a promoter and an operator. A promoter is operably linked to the desired polynucleotide, thereby initiating transcription of the polynucleotide via an RNA polymerase enzyme. An operator is a sequence of polynucleotides adjacent to the promoter, which contains a protein-binding domain where a repressor protein can bind. In the absence of a repressor protein, transcription initiates through the promoter. When present, the repressor protein specific to the protein-binding domain of the operator binds to the operator, thereby inhibiting transcription. In this way, control of transcription is accomplished, based upon the particular regulatory regions used and the presence or absence of the corresponding repressor protein. Examples include lactose promoters (Lad repressor protein changes conformation when contacted with lactose, thereby preventing the Lad repressor protein from binding to the operator) and tryptophan promoters (when complexed with tryptophan, TrpR repressor protein has a conformation that binds the operator; in the absence of tryptophan, the TrpR repressor protein has a conformation that does not bind to the operator). Another example is the tac promoter (see de Boer *et al.*, (1983) Proc Natl Acad Sci USA 80(1):21-25).

[0089] Methods of producing host cells of the disclosure may include the introduction or transfer of the expression vectors containing recombinant nucleic acids of the disclosure into the host cell. Such methods for transferring expression vectors into host cells are well-known to those of ordinary skill in the art. For example, one method for transforming cells with an expression vector involves a calcium chloride treatment where the expression vector is

introduced via a calcium precipitate. Other salts, e.g., calcium phosphate, may also be used following a similar procedure. In addition, electroporation (i.e., the application of current to increase the permeability of cells to nucleic acid sequences) may be used to transfect the host cell. Cells also may be transformed through the use of spheroplasts (Schweizer, M, Proc. Natl. Acad. Sci., 78: 5086-5090 (1981). Also, microinjection of the nucleic acid sequences provides the ability to transfect host cells. Other means, such as lipid complexes, liposomes, and dendrimers, may also be employed. Those of ordinary skill in the art can transfect a host cell with a desired sequence using these or other methods.

[0090] In some cases, cells are prepared as protoplasts or spheroplasts prior to transformation. Protoplasts or spheroplasts may be prepared, for example, by treating a cell having a cell wall with enzymes to degrade the cell wall. Fungal cells may be treated, for example, with chitinase.

[0091] The vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host, or a transposon may be used.

[0092] The vectors preferably contain one or more selectable markers which permit easy selection of transformed host cells. A selectable marker is a gene the product of which provides, for example, biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Selection of bacterial cells may be based upon antimicrobial resistance that has been conferred by genes such as the amp, gpt, neo, and hyg genes.

[0093] Selectable markers for use in fungal host cells may include, for example, amdS (acetamidase), argB (ornithine carbamoyltransferase), bar (phosphinothricin acetyltransferase), hph (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase), sC (sulfate adenyltransferase), and trpC (anthranilate synthase), as well as equivalents thereof.

[0094] The vectors may contain an element(s) that permits integration of the vector into the host's genome or autonomous replication of the vector in the cell independent of the genome.

[0095] For integration into the host genome, the vector may rely on the gene's sequence or any other element of the vector for integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the host. The additional nucleotide sequences enable the vector to be integrated into the host genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, or 800 to 10,000 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host by non-homologous recombination.

[0096] For autonomous replication, the vector may further contain an origin of replication enabling the vector to replicate autonomously in the host in question. The origin of replication may be any plasmid replicator mediating autonomous replication which functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a sequence that enables a plasmid or vector to replicate *in vivo*.

[0097] Various promoters for regulation of expression of a recombinant nucleic acid of the disclosure in a vector are well-known in the art and include, for example, constitutive promoters and inducible promoters. Promoters are described, for example, in Sambrook, et al. Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, (2001). Promoter can be viral, bacterial, fungal, mammalian, or plant promoters. Additionally, promoters can be constitutive promoters, inducible promoters, environmentally regulated promoters, or developmentally regulated promoters. Examples of suitable promoters for regulating recombinant nucleic acid of the disclosure may include, for example, the *N. crassa* ccg-1 constitutive promoter, which is responsive to the *N. crassa* circadian rhythm and nutrient conditions; the *N. crassa* gpd-1 (glyceraldehyde 3-phosphate

dehydrogenase-1) strong constitutive promoter; the *N. crassa* vvd (light) inducible promoter; the *N. crassa* qa-2 (quinic acid) inducible promoter; the *Aspergillus nidulans* gpdA promoter; the *Aspergillus nidulans* trpC constitutive promoter; the *N. crassa* tef-1 (transcription elongation factor) highly constitutive promoter; and the *N. crassa* xlr-1 (XlnR homolog) promoter, which is used frequently in Aspergillus species. In some embodiments, expression of a recombinant polypeptide of the disclosure is under the control of a heterologous promoter. In some preferred embodiments, the promoter is the TEF1 promoter to achieve overexpression of a polypeptide.

[0098] More than one copy of a gene may be inserted into the host to increase production of the gene product. An increase in the copy number of the gene can be obtained by integrating at least one additional copy of the gene into the host genome or by including an amplifiable selectable marker gene with the nucleotide sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the gene, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

[0099] The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present disclosure are well-known to one skilled in the art (see, e.g., Sambrook et al., 1989, supra). When only a single expression vector is used (without the addition of an intermediate), the vector will contain all of the nucleic acid sequences necessary.

Host Cells Having Modified Polypeptide Activity

[0100] Recombinant host cells of the present disclosure having modified polypeptide activity are capable of producing sugar acids, such as cellobionate, and commodity chemicals from cellulose or cellulosic biomass. The ability to metabolize cellulose is a trait exhibited by lignocellulolytic cells, and thus host cells of the present disclosure are preferably lignocellulolytic cells. As disclosed herein, the lignocellulolytic cells may be either aerobic cells or anaerobic cells. In certain preferred embodiments, the lignocellulolytic cells are aerobic cells.

[0101] Lignocellulolytic cells of the present disclosure produce enzymes that degrade lignocellulose or components thereof. The lignocellulolytic cells may degrade the lignocellulose or components thereof under aerobic (i.e., oxygen rich), or anaerobic (i.e., oxygen deficient) conditions. In certain embodiments, the lignocellulolytic cells of the

present disclosure are capable of pretreating lignocellulosic biomass. Such lignocellulolytic cells simultaneously degrade lignin, solubilize lignin, or change lignin to a revised form, such as de-methylized lignin. Lignin is an energy-rich compound that can be utilized for energy production (e.g. electricity). In other embodiments, lignocellulolytic cells of the present disclosure produce one or more cellulases, hemicellulases, lignin-solubilizing enzymes, or combinations thereof. In certain embodiments, the one or more hemicellulases and/or lignin-solubilizing enzymes are recombinantly expressed in the lignocellulolytic cells. Accordingly, lignocellulolytic cells of the present disclosure can produce monosaccharides (e.g., glucose) and cellodextrins (e.g., cellobiose, cellotriose, cellotetrose, cellopentose, etc.) from lignocellulosic biomass. Additionally, lignocellulolytic cells of the present disclosure can also produce hemicellulose oligosaccharides, such as xylobiose, from lignocellulosic biomass.

[0102] Lignocellulolytic cells of the present disclosure may include, for example, fungi and bacteria. Suitable lignocellulolytic fungi of the present disclosure may include, for example, White Rot Fungi, Brown Rot Fungi, Soft Rot Fungi, and ascomycetes fungi. Suitable lignocellulolytic bacteria of the present disclosure may include, for example, *Clostridium sp.* and *Thermanaerobacterium sp.* Additional examples of suitable host cells may include, for example, *Trichoderma reesei*, *Clostridium thermocellum, Clostridium papyrosolvens C7, Podospera anserine, Chaetomium globosum, Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Phanerochaete chrysosporium*, *Sporotrichum thermophile (Myceliophthora thermophila)*, *Gibberella zeae*, *Sclerotinia sclerotiorum*, *Botryotinia fuckelian*, *Aspergillus niger*, *Thielavia terrestris*, *Fusarium spp.*, *Rhizopus spp.*, *Neocallimastix frontalis*, *Orpinomyces sp.*, *Piromyces sp.*, *Penicillium chrysogenum* cells, *Schizophyllum commune*, *Postia placenta*, *Acremonium cellulolyticus, Yarrowia lipolytica, Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, *Chrysosporium lucknowense*, *Aspergillus sp.*, *Trichoderma sp.*, *Caldocellulosiruptor sp.*, *Butyrivibrio sp.*, *Butyrivibrio sp.*, *Eubacterium sp.*, *Clostridium sp.*, *Bacteroides sp.*, *,Acetivibrio sp.*, *Thermoactinomyces sp.*, *Caldibacillus sp.*, *Bacillus sp.*, *Acidothermus sp.*, *Cellulomonas sp.*, *Micromonospora sp.*, *Actinoplanes sp.*, *Streptomyces sp.*, *Thermobifida sp.*, *Thermomonospora sp.*, *Microbispora sp.*, *Microbispora sp.*, *Fibrobacter sp.*, *Sporocytophaga sp.*, *Cytophaga sp.*, *Flavobacterium sp.*, *Achromobacter sp.*, *Xanthomonas sp.*, *Cellvibrio sp.*, *Pseudomonas sp.*, *Myxobacter sp.*, *Clostridium phytofermentans*, *Clostridium japonicas*, *and Thermoanaerobacterium saccharolyticum* cells.

[0103] In certain embodiments, host cells of the present disclosure are filamentous fungal cells including, for example, *Neurospora*, *Trichoderma*, and *Aspergillus* cells. In certain preferred embodiments, the filamentous fungal cells are *Neurospora crassa* cells.

[0104] Host cells of the present disclosure are living biological cells that are manipulated to alter, for example, the activity of one or more polypeptides in the cell. For example, host cells may be transformed via insertion of recombinant DNA or RNA. Such recombinant DNA or RNA can be in an expression vector. Further, host cells may be subject to mutagenesis to induce mutations in polypeptide-encoding polynucleotides. Host cells that have been genetically modified are recombinant host cells.

[0105] The host cells of the present disclosure may be genetically modified. For example, recombinant nucleic acids may have been introduced into the host cells or the host cells may have mutations introduced into endogenous and/or exogenous polynucleotides, and as such the genetically modified host cells do not occur in nature. A suitable host cell may be, for example, one that is capable of expressing one or more nucleic acid constructs for different functions such as, for example, recombinant protein expression and/or targeted gene silencing.

[0106] "Recombinant nucleic acid" or "heterologous nucleic acid" or "recombinant polynucleotide", "recombinant nucleotide" or "recombinant DNA" as used herein refers to a polymer of nucleic acids where at least one of the following is true: (a) the sequence of nucleic acids is foreign to (*i.e.*, not naturally found in) a given host cell; (b) the sequence may be naturally found in a given host cell, but is expressed in an unnatural (*e.g.*, greater than expected) amount; or (c) the sequence of nucleic acids contains two or more subsequences that are not found in the same relationship to each other in nature. For example, regarding instance (c), a recombinant nucleic acid sequence will have two or more sequences from unrelated genes arranged to make a new functional nucleic acid. For example, the present disclosure describes the introduction of an expression vector into a host cell, where the expression vector contains a nucleic acid sequence coding for a protein that is not normally found in a host cell or contains a nucleic acid coding for a protein that is normally found in a cell but is under the control of different regulatory sequences. With reference to the host cell's genome, then, the nucleic acid sequence that codes for the protein is recombinant. As used herein, the term "recombinant polypeptide" refers to a polypeptide generated from a

"recombinant nucleic acid" or "heterologous nucleic acid" or "recombinant polynucleotide", "recombinant nucleotide" or "recombinant DNA" as described above.

[0107] In some embodiments, the host cell naturally produces any of the polypeptides of the present disclosure. In some embodiments, the genes encoding the desired polypeptides may be heterologous to the host cell or these genes may be endogenous to the host cell but are operatively linked to heterologous promoters and/or control regions that result in, for example, the higher expression of the gene(s) in the host cell or the decreased expression of the gene(s) in the host cell.

[0108] In some embodiments, host cells of the present disclosure may be modified to facilitate the metabolism of cellulose. For example, host cells of the present disclosure may be modified to contain one or more cellodextrin transporters. Cellodextrins are glucose polymers of varying length and include, for example, cellobiose (2 glucose monomers), cellotriose (3 glucose monomers), cellotetraose (4 glucose monomers), cellopentaose (5 glucose monomers), and cellohexaose (6 glucose monomers). A cellodextrin transporter is any transmembrane protein that transports a cellodextrin molecule from outside of the cell to the inside of the cell and/or from inside of the cell to outside of the cell. Examples of suitable cellodextrin transporters may include, for example, NCU00801, NCU00809, NCU8114, XP_001268541.1, and LAC2.

Methods of Modifying Polypeptide Activity

[0109] Host cells of the present disclosure have modified polypeptide activity as compared to a corresponding control cell, such as a corresponding wild type cell, to facilitate and/or increase the production of sugar acids. Polypeptide activity may be modified such that one or more polypeptides of the present disclosure have increased activity or decreased activity. In some embodiments, a host cell may have one or more polypeptides with increased activity as well as one or more polypeptides with decreased activity. Methods of modifying (*e.g.* increasing and/or decreasing) the activity of one or more polypeptides of the present disclosure are well-known in the art and are described herein.

Decreased Polypeptide Activity

[0110] Host cells of the present disclosure may contain one or more polypeptides with decreased activity as compared to a corresponding control cell, such as a wild type cell. In

some embodiments, one or more β-glucosidases, cellobionate phosphorylases, CRE-1 proteins, ACE-1 proteins, and/or MUS51 proteins have decreased activity in a host cell as compared to a corresponding control cell. Methods of decreasing the expression, abundance, and/or activity of a polypeptide are well-known in the art and are described herein.

[0111] In some embodiments, decreasing activity of a polypeptide involves overexpressing a polypeptide that is an inhibitor of the polypeptide. Host cells may overexpress an inhibitor that inhibits the expression and/or activity of one or more β-glucosidases, cellobionate phosphorylases, CRE-1 proteins, ACE-1 proteins, and/or MUS51 proteins of the present disclosure. In some embodiments, a recombinant β-glucosidase, cellobionate phosphorylase, CRE-1 protein, ACE-1 protein, and/or MUS51 protein may be expressed in host cells such that the recombinant polypeptide interferes with and decreases the activity of the endogenous polypeptide.

[0112] In some embodiments, decreasing the activity of a polypeptide such as, for example, one or more β-glucosidases, cellobionate phosphorylases, CRE-1 proteins, ACE-1 proteins, and/or MUS51 proteins involves decreasing the expression of a nucleic acid encoding the polypeptide.

[0113] Decreasing the expression of a nucleic acid may be accomplished by introducing a genetic mutation into a target nucleic acid. Mutagenesis approaches may be used to disrupt or "knockout" the expression of a target gene by generating mutations. In some embodiments, the mutagenesis results in a partial deletion of the target gene. In other embodiments, the mutagenesis results in a complete deletion of the target gene. Methods of mutagenizing microorganisms are well known in the art and include, for example, random mutagenesis and site-directed mutagenesis to induce mutations. Examples of methods of random mutagenesis include, for example, chemical mutagenesis (e.g., using ethane methyl sulfonate), insertional mutagenesis, and irradiation.

[0114] One method for reducing or inhibiting the expression of a target gene is by genetically modifying the target gene and introducing it into the genome of a host cell to replace the wild-type version of the gene by homologous recombination (for example, as described in U.S. Pat. No. 6,924,146).

[0115] Another method for reducing or inhibiting the expression of a target gene is by insertion mutagenesis using the T-DNA of *Agrobacterium tumefaciens*, or transposons (see

Winkler et al., Methods Mol. Biol. 82:129-136, 1989, and Martienssen Proc. Natl. Acad. Sci. 95:2021-2026, 1998). After generating the insertion mutants, the mutants can be screened to identify those containing the insertion in a target gene. Methods to disrupt a target gene by insertional mutagenesis are described in for example, U.S. Pat. No. 5,792,633. Methods to disrupt a target gene by transposon mutagenesis are described in for example, U.S. Pat. No. 6,207,384.

[0116] A further method to disrupt a target gene is by use of the cre-lox system (for example, as described in U.S. Pat. No. 4,959,317).

[0117] Another method to disrupt a target gene is by use of PCR mutagenesis (for example, as described in U.S. Pat. No. 7,501,275).

[0118] Endogenous gene expression may also be reduced or inhibited by means of RNA interference (RNAi), which uses a double-stranded RNA having a sequence identical or similar to the sequence of the target gene. RNAi may include the use of micro RNA, such as artificial miRNA, to suppress expression of a gene.

[0119] RNAi is the phenomenon in which when a double-stranded RNA having a sequence identical or similar to that of the target gene is introduced into a cell, the expressions of both the inserted exogenous gene and target endogenous gene are suppressed. The double-stranded RNA may be formed from two separate complementary RNAs or may be a single RNA with internally complementary sequences that form a double-stranded RNA.

[0120] Thus, in some embodiments, reduction or inhibition of gene expression is achieved using RNAi techniques. For example, to achieve reduction or inhibition of the expression of a DNA encoding a protein using RNAi, a double-stranded RNA having the sequence of a DNA encoding the protein, or a substantially similar sequence thereof (including those engineered not to translate the protein) or fragment thereof, is introduced into a host cell of interest. As used herein, RNAi and dsRNA both refer to gene-specific silencing that is induced by the introduction of a double-stranded RNA molecule, see e.g., U.S. Pat. Nos. 6,506,559 and 6,573,099, and includes reference to a molecule that has a region that is double-stranded, e.g., a short hairpin RNA molecule. The resulting cells may then be screened for a phenotype associated with the reduced expression of the target gene, e.g., reduced cellulase expression, and/or by monitoring steady-state RNA levels for transcripts of the target gene. Although the sequences used for RNAi need not be completely

identical to the target gene, they may be at least 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more identical to the target gene sequence. See, e.g., U.S. Patent Application Publication No. 2004/0029283. The constructs encoding an RNA molecule with a stem-loop structure that is unrelated to the target gene and that is positioned distally to a sequence specific for the gene of interest may also be used to inhibit target gene expression. See, e.g., U.S. Patent Application Publication No. 2003/0221211.

[0121] The RNAi nucleic acids may encompass the full-length target RNA or may correspond to a fragment of the target RNA. In some cases, the fragment will have fewer than 100, 200, 300, 400, or 500 nucleotides corresponding to the target sequence. In addition, in some aspects, these fragments are at least, e.g., 50, 100, 150, 200, or more nucleotides in length. Interfering RNAs may be designed based on short duplexes (i.e., short regions of double-stranded sequences). Typically, the short duplex is at least about 15, 20, or 25-50 nucleotides in length (e.g., each complementary sequence of the double stranded RNA is 15-50 nucleotides in length), often about 20-30 nucleotides, e.g., 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In some cases, fragments for use in RNAi will correspond to regions of a target protein that do not occur in other proteins in the organism or that have little similarity to other transcripts in the organism, e.g., selected by comparison to sequences in analyzing publicly-available sequence databases. Similarly, RNAi fragments may be selected for similarity or identity with a conserved sequence of a gene family of interest, such as those described herein, so that the RNAi targets multiple different gene transcripts containing the conserved sequence.

[0122] RNAi may be introduced into a host cell as part of a larger DNA construct. Often, such constructs allow stable expression of the RNAi in cells after introduction, e.g., by integration of the construct into the host genome. Thus, expression vectors that continually express RNAi in cells transfected with the vectors may be employed for this disclosure. For example, vectors that express small hairpin or stem-loop structure RNAs, or precursors to microRNA, which get processed in vivo into small RNAi molecules capable of carrying out gene-specific silencing (Brummelkamp et al, Science 296:550-553, (2002); and Paddison, et al., Genes & Dev. 16:948-958, (2002)) can be used. Post-transcriptional gene silencing by double-stranded RNA is discussed in further detail by Hammond et al., Nature Rev Gen 2: 110-119, (2001); Fire et al., Nature 391: 806-811, (1998); and Timmons and Fire, Nature 395: 854, (1998).

[0123] Methods for selection and design of sequences that generate RNAi are well-known in the art (e.g. U.S. Pat. Nos. 6,506,559; 6,511,824; and 6,489,127).

[0124] A reduction or inhibition of gene expression in a host cell of a target gene may also be obtained by introducing into host cells antisense constructs based on a target gene nucleic acid sequence. For antisense suppression, a target sequence is arranged in reverse orientation relative to the promoter sequence in the expression vector. The introduced sequence need not be a full length cDNA or gene, and need not be identical to the target cDNA or a gene found in the cell to be transformed. Generally, however, where the introduced sequence is of shorter length, a higher degree of homology to the native target sequence is used to achieve effective antisense suppression. In some aspects, the introduced antisense sequence in the vector will be at least 30 nucleotides in length, and improved antisense suppression will typically be observed as the length of the antisense sequence increases. In some aspects, the length of the antisense sequence in the vector will be greater than 100 nucleotides. Transcription of an antisense construct as described results in the production of RNA molecules that are the reverse complement of mRNA molecules transcribed from an endogenous target gene. Suppression of a target gene expression can also be achieved using a ribozyme. The production and use of ribozymes are disclosed in U.S. Pat. Nos. 4,987,071 and 5,543,508.

[0125] Expression cassettes containing nucleic acids that encode target gene expression inhibitors, e.g., an antisense or siRNA, can be constructed using methods well known in the art. Constructs include regulatory elements, including promoters and other sequences for expression and selection of cells that express the construct. Typically, fungal and/or bacterial transformation vectors include one or more cloned coding sequences (genomic or cDNA) under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such transformation vectors typically also contain a promoter (e.g., a regulatory region controlling inducible or constitutive, environmentally-or developmentally-regulated expression), a transcription initiation start site, an RNA processing signal (such as intron splice sites), a transcription termination site, and/or a polyadenylation signal.

[0126] In certain embodiments, a portion of the target nucleic acid may be modified, such as the region encoding the catalytic domain, the coding region, or a control sequence required for expression of the coding region. Such a control sequence of the gene may be a promoter sequence or a functional part thereof, *i.e.*, a part that is sufficient for affecting expression of

the gene. For example, a promoter sequence may be inactivated resulting in no expression or a weaker promoter may be substituted for the native promoter sequence to reduce expression of the coding sequence. Other control sequences for possible modification may include, for example, a leader sequence, a propeptide sequence, a signal sequence, a transcription terminator, and a transcriptional activator.

Increased Polypeptide Activity

[0127] Host cells of the present disclosure may contain one or more polypeptides with increased expression and/or activity as compared to a corresponding control cell, such as a corresponding wild type cell. In some embodiments, host cells of the disclosure contain a recombinant nucleic acid encoding a recombinant polypeptide of the disclosure such as, for example, a recombinant laccase and/or a recombinant cellobiose dehydrogenase. In certain embodiments, the recombinant nucleic acid is mis-expressed in the host cell (e.g., constitutively expressed, inducibly expressed, etc.) such that mis-expression results in increased polypeptide activity as compared to a corresponding control cell. In some embodiments, a host cell that contains a recombinant nucleic acid encoding a recombinant polypeptide contains a greater amount of the polypeptide than a corresponding control cell that does not contain the corresponding recombinant nucleic acid. When a protein or nucleic acid is produced or maintained in a host cell at an amount greater than normal, the protein or nucleic acid is "overexpressed." In some embodiments, host cells of the disclosure overexpress a polypeptide such as, for example, a laccase and/or cellobiose dehydrogenase. Host cells may overexpress one or more of a laccase and/or cellobiose dehydrogenase polypeptide such that the activity of one or more of these proteins is increased in the host cell as compared to a corresponding control cell. The corresponding control cell may be, for example, a cell that does not overexpress one or more of the polypeptides overexpressed in the host cell, such as a wild type cell. Various control cells will be readily apparent to one of skill in the art.

[0128] Various methods of increasing the expression of a polypeptide are known in the art. For example, other genetic regions involved in controlling expression of the nucleic acid encoding the polypeptide, such as an enhancer sequence, may be modified such that expression of the nucleic acid is increased. The level of expression of a nucleic acid may be assessed by measuring the level of mRNA encoded by the gene, and/or by measuring the level or activity of the polypeptide encoded by the nucleic acid.

[0129] In some embodiments, host cells overexpress a polypeptide that is an activator of one or more of a laccase and/or cellobiose dehydrogenase polypeptide. Overexpression of an activator polypeptide may lead to increased abundance and activity of the polypeptide activated by the activator. The activator may increase expression of one or more of a laccase and/or cellobiose dehydrogenase polypeptide. The activator may increase activity of one or more of a laccase and/or cellobiose dehydrogenase polypeptide.

[0130] Increasing the abundance of a polypeptide of the disclosure such as, for example, a laccase and/or cellobiose dehydrogenase polypeptide, to increase polypeptide activity may be achieved by overexpressing the polypeptide. Other methods of increasing abundance of a polypeptide are known in the art. For example, decreasing degradation of the polypeptide by cellular degradation machinery, such as the proteasome, may increase the stability and the abundance of the polypeptide. The polypeptides may be genetically modified such that they have increased resistance to cellular proteolysis, but exhibit no change in molecular activity. Polypeptides that are inhibitors of cellular factors involved in the degradation of one or more of a laccase and/or cellobiose dehydrogenase polypeptides may be introduced into host cells to increase abundance of the one or more polypeptides. Further, host cells may be treated with chemical inhibitors of the proteasome, such as cycloheximide, to increase the abundance of one or more polypeptides of the disclosure.

Methods of Producing Sugar Acids

[0131] Host cells of the present disclosure having modified polypeptide activity are capable of producing sugar acids, such as cellooligosaccharide aldonates, from cellulose or cellulosic materials. Host cells of the present disclosure are capable of metabolizing cellulose (lignocellulolytic cells) and may be able to do so based on the presence of endogenous genetic machinery that facilitates cellulose metabolism, or may be genetically engineered to metabolize cellulose. Thus, host cells of the present disclosure shall express one or more of the following enzymes: cellulases, xylanases, ligninases, oxidases, dehydrogenases, and laccases. Once products from the breakdown of cellulose, such as cellobiose, are generated, these breakdown products may be used for the production of sugar acids by host cells of the present disclosure.

[0132] Methods of the present disclosure involve the use of host cells having modified polypeptide activity to produce sugar acids, such as cellobionate, and commodity chemicals

from cellulose or cellulosic biomass. Sugar acids, also known as saccharide aldonic acids (SAAs), refer to molecules in which the CHO aldehyde functional group of a saccharide has been replaced with a carboxylic acid functional group (COOH). Sugar acids can be divided into four general categories: (1) oligosaccharide aldonic acid (OAA), (2) di-saccharide aldonic acid (DAA), (3) monosaccharide aldonic acid (MAA), and (4) heteropolysaccharide aldonic acid (HSAA). Examples of oligosaccharide aldonic acid (OAA) include: cellotrionic acid, cellotetraonic acid; celloheptonic acid, xylotrionic acid, and xylopentaonic acid. Examples of di-saccharide aldonic acid (DAA) include: cellobionic acid (CBA), xylobionic acid, galactonic acid, 4-O-β-D-galactopyranosylgluconic acid, and 6-O- β -D-galactopyranosylgluconic acid. Examples of monosaccharide aldonic acids (MAA) include gluconic acid, xylonic acid, galactonic acid, arabinic acid, and mannonic acid. An example of HSAA is 4-O-methyl-α-D-glucuronopyranosyl acid.

[0133] In the case of DAA, OAA and HSAA, the connection between sugar units and between the sugar and the end of the aldonic acid could be straight–chain or branched-chain. For example, gluconic acids could be connected glycosidically on the oxygen atoms in the 1-, 3-, 4- or 6-position of a sugar unit. There could be any combination of sugar and terminal aldonic acid.

[0134] In aqueous solution, sugar acids generally exist in salt form. Examples of inorganic and organic salts are ammonium, lithium, sodium, magnesium, calcium and aluminum salts, as well as the salts with ethanolamine, triethanolamine, morpholine, pyridine, and piperidine.

[0135] The methods of the present disclosure involve culturing a host cell of the present disclosure in a cellulose-containing medium, where the host cell produces a sugar acids a sugar acid from the cellulose. Host cells of the present disclosure such as, for example, host cells having decreased activity of one or more β-glucosidases, cellobionate phosphorylases, CRE-1 proteins, ACE-1 proteins, and/or MUS51 proteins, and/or increased expression or activity of one or more laccases and/or cellobiose dehydrogenases, are cultured in a growth medium under conditions suitable for the production of sugar acids from cellulose by the host cell.

[0136] Host cells of the present disclosure are capable of utilizing cellulose as a carbon source in a growth/culture medium. According to some aspects of the disclosure, the culture

media contains cellulose or a source of cellulose as a carbon source for the host cell. "Carbon source" generally refers to a substrate or compound suitable to be used as a source of carbon for cell growth, such as, for example, cellulose or a source of cellulose. In some embodiments, the culture media contains only cellulose or a source of cellulose as the sole carbon source. In other embodiments, the culture media may contain additional carbon sources other than cellulose. Carbon sources may be in various forms such as, for example, polymers, carbohydrates, acids, alcohols, aldehydes, ketones, amino acids, peptides, etc. These include, for example, various monosaccharides, oligosaccharides, polysaccharides, a biomass polymer such as cellulose or hemicellulose, xylose, arabinose, disaccharides, such as sucrose, saturated or unsaturated fatty acids, succinate, lactate, acetate, ethanol, etc., or mixtures thereof. In addition to an appropriate carbon source, culture media may contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the pathways involved in the production of sugar acids. Standard culture environments for microorganisms, such as those used in methods of the present disclosure, are well-known in the art and are described herein.

[0137] Sources of cellulose are well-known in the art and are described herein. For example, cellulose may be purchased commercially and added to culture media as a carbon source. The source of cellulose may be a material that contains cellulose or whose breakdown releases cellulose. As used herein, a "cellulose-containing material" is any material that contains or is capable of generating cellulose, including biomass, such as biomass containing plant material. Biomass suitable for use with the currently disclosed methods include various cellulose-containing materials such as, for example, Miscanthus, switchgrass, cord grass, rye grass, reed canary grass, elephant grass, common reed, wheat straw, barley straw, canola straw, oat straw, corn stover, soybean stover, oat hulls, sorghum, rice hulls, rye hulls, wheat hulls, sugarcane bagasse, copra meal, copra pellets, palm kernel meal, corn fiber, Distillers Dried Grains with Solubles (DDGS), Blue Stem, corncobs, pine wood, birch wood, willow wood, aspen wood, poplar wood, energy cane, waste paper, sawdust, forestry wastes, municipal solid waste, waste paper, crop residues, other grasses, and other woods. The source of cellulose may require a pre-treatment to generate and/or liberate cellulose or cellulose breakdown products such as, for example, treatment with high temperature or pressure. Such treatments are well-known to those skilled in the art.

[0138] In some embodiments, host cells of the present disclosure are cultured in the presence of exogenously added laccase and/or cellobiose dehydrogenase enzymes. These enzymes may be purchased from a commercial vendor, or they may be recombinantly expressed and purified as will be readily understood by one of skill in the art. In some embodiments, such as those embodiments where exogenous laccase is added to the culture media, the culture media may further contain a compounds which is a redox mediator such as, for example, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)).

[0139] In some embodiments, after a host cell of the present disclosure has produced a sugar acid, the sugar acid may be precipitated, removed, or substantially purified from the culture media. Methods of substantially purifying sugar acids from culture media are known in the art.

[0140] In some embodiments, the methods of the present disclosure involve host cells having an increased sugar acid production rate as compared to a corresponding control cell. Host cells having an increased sugar acid production rate may be those having decreased activity of one or more β-glucosidases, cellobionate phosphorylases, CRE-1 proteins, ACE-1 proteins, and/or MUS51 proteins, and/or increased expression or activity of one or more laccases and/or cellobiose dehydrogenases. The rate of sugar acid production in host cells of the present disclosure may be at least 0.1-fold, at least 0.2-fold, at least 0.3-fold, at least 0.4-fold, at least 0.5-fold, at least 0.6-fold, at least 0.7-fold, at least 0.8-fold, at least 0.9-fold, at least 1-fold, at least 1.25 fold, at least 1.5-fold, at least 1.75-fold, at least 2-fold, at least 2.25-fold, at least 2.5-fold, at least 2.75-fold, at least 3-fold, at least 3.25-fold, at least 3.5-fold, at least 3.75-fold, at least 4-fold, at least 4.25-fold, at least 4.5-fold, at least 4.75-fold, at least 5-fold, at least 5.25-fold, at least 5.5-fold, at least 5.75-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, at least 20-fold, or at least 30-fold or more higher than the sugar acid production rate of a corresponding control cell such as, for example, a wild type cell or a cell not having modified polypeptide activity. In some embodiments, the corresponding control cell does not accumulate any sugar acids. In some embodiments, the sugar acid produced by the host cell is cellobionate.

[0141] In some embodiments, the methods of the present disclosure involve host cells having a decreased rate of sugar acid consumption as compared to a corresponding control cell. Host cells having a decreased rate of sugar acid consumption may be those having decreased activity of one or more β-glucosidases, cellobionate phosphorylases, CRE-1

proteins, ACE-1 proteins, and/or MUS51 proteins, and/or increased expression or activity of one or more laccases and/or cellobiose dehydrogenases. The consumption of a sugar acid by a host cell of the present disclosure may be reduced by about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, or by about 100% as compared to a corresponding control cell such as, for example, a wild type cell or a cell not having modified polypeptide activity. In some embodiments, the consumption of cellobionate by a host cell of the present disclosure is reduced by about 100% as compared to a corresponding control cell.

[0142] Methods of measuring the accumulation, production, and/or consumption/degradation rate of a sugar acid by a host cell or a population of cells are well-known in the art and are described herein. For example, determining the sugar acid consumption/degradation rate of a cell or cell population in culture may involve allowing cells to grow in medium containing a sugar acid and measuring the depletion of the sugar acid from the growth medium over time at specified time points and determining the slope of the resulting plotted line to determine sugar consumption/degradation rate (e.g. cellobionate/L/h). Similarly, determining sugar acid accumulation or production may involve culturing a cell or population of cells in a medium containing cellulose and measuring the production of a sugar acid from the cellulose over time at specified time points.

[0143] Sugar acids produced using the methods of the present disclosure may be used as starting materials for the production of commodity chemicals, such as fuels or fuel additives. Exemplary commodity chemicals include, for example, alcohol, ethanol, propanol, isopropanol, acetone, butanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, phenylethanol, a fatty alcohol, isopentenol, an aldehyde, acetylaldehyde, propionaldehyde, butryaldehyde, isobutyraldehyde, 2-methyl-1-butanal, 3-methyl-1-butanal, phenylacetaldehyde, a fatty aldehyde, a hydrocarbon, an alkane, an alkene, an isoprenoids, a fatty acid, a wax ester, an ethyl ester, hydrogen, and combinations thereof.

EXAMPLES

[0144] The following Examples are offered to illustrate provided embodiments and are not intended to limit the scope of the present disclosure.

Example 1: Metabolic pathway for direct sugar acid and sugar oligomer production from cellulose

[0145] This Example demonstrates a pathway for production of sugar acids directly from cellulose. A fungal strain was constructed by deleting various copies of β-glucosidase genes, over-expressing laccase gene, and deleting a cellobionate phosphorylase gene. The engineered fungus was able to directly produce sugar acids (such as cellobionate) directly from cellulose without any exogenous cellulase addition. Sugar acids produced can be used as the starting feedstock for the production of various fuels and chemicals.

Materials and Methods

Materials

[0146] Wild type *Neurospora crassa* (2489) was obtained from the Fungal Genetics Stock Center (FGSC). The F5 strain used in this study is strain 2489 with six out of seven of its beta-glucosidase (bgl) genes knocked out. Laccase from *Pleurotus ostreatus*, an efficient laccase producer, was obtained from Sigma Aldrich and used in any studies requiring the exogenous addition of laccase.

[0147] 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) was obtained from Sigma Aldrich. For experiments requiring exogenous CDH, a recombinant *Pichia pastoris* strain developed by Applicants was used to generate CDH (Zhang *et al.*, 2011).

Strain Construction

[0148] The strain F5Δcre-1 and F5Δacre1 was constructed using the method of targeted gene deletion with marker recycling as previously described (Szewczyk *et al.*, 2013). The F5Δcre-1 and F5 Δacre1 was constructed by genetic crossing (Fan *et al.*, 2012).

[0149] Cellobionate phosphorylase (NdvB) knockout (F5 Δcre-1Δace1ΔndvB) was constructed using the method described using the method of targeted gene deletion with marker recycling as previously described (Szewczyk *et al.*, 2013).

Overexpression of Laccase

[0150] The knock in cassette containing the native laccase gene (NCU04528; Genbank accession # J03505) flanked by the gpdA promoter and trpC terminator from *Aspergillus*

nidulans was synthesized and sequence verified by Life Technologies. The region upstream of the gpdA promoter contains a 1020 bp homologous sequence to the 3' end of *N. crassa*'s csr-1 gene, and the region downstream of the trpC terminator contains 1900 bp with homology to the 5' end of the csr-1 gene. The csr-1 gene confers sensitivity to cyclosporin A. Replacing the csr-1 gene with the knock in cassette via homologous recombination renders the strain resistant to cyclosporin A, providing a selection method for homokaryon mutants. 10-day old conidia of the strain F5 Δcre-1Δace1ΔndvB was harvested, filtered through cheese cloth, and washed and centrifuged three times with 50 mL of 1M ice cold sorbitol. The conidia was concentrated to 2.5x10^9 conidia/mL. 90μL of cells were mixed with 1000μg of DNA and chilled on ice for 5 minutes prior to electroporation (0.2cm cuvette; 1.5 kV/cm; capacitance: 25 uFD; resistance: 600 ohms). Immediately following electroporation, 750mL of 1M ice cold sorbitol was added to the cuvette, and the suspension was transferred to 2mL of recovery media containing 1x Vogel's salts and 2% yeast extract. Cells were regenerated in a rotary shaker at 30°C and 200 rpm for 6 hours. 350μL of the regenerated solution was spread on plates containing 1x Vogel's salts, 2% yeast extract, 1M sorbitol, 20 g l^{-1} sorbose, 0.5 g l^{-1} fructose, 0.5 g l^{-1} glucose, 5 μg ml^{-1} CsA, and 2% agar. After 4-7 days of growth at 30°C, colonies were transferred to slants (5 μg mL^{-1} CsA, 1x Vogel's, 2% agar) and grown for 4 days at 30°C. Positive transformants were selected by monitoring the extracellular expression of laccase for transformants grown on 1x Vogel's salts with 20g/L glucose. The parent strain does not produce any detectable laccase under these conditions. Laccase expression was measured by following the increase in absorbance of ABTS according to a previously established method (Baminger *et al.*, 2001). One unit of laccase activity was defined as the amount of enzyme oxidizing 1 mmol of ABTS per min. The strains are designated as the label F5 Δcre-1Δace1ΔndvB+lac.

Results

[0151] Applicants previously constructed seven strains of *N. crassa* which have six out of the seven β-glucosidases (bgl) genes knocked out (sextuple bgl knock out strain), as well as constructing one septuple bgl knockout strain (Fan *et al.*, 2012)(Wu *et al.*, 2013). Applicants demonstrated that cellobionate can be produced from cellulose by one of best strains, which was named F5 (Fan *et al.*, 2012). F5 contains mutations in each of the following β-glucosidase genes: NCU00130, NCU04952, NCU05577, NCU07487, NCU08755, and NCU03641. These results are reiterated in **FIG. 4**. For example, **FIG. 4A** demonstrates that

the F5 strain, which is a sextuple bgl mutant strain, was able to accumulate cellobiose. **FIG. 4B** demonstrates that the F5 strain could also accumulate cellobionate, and that the addition of exogenous cellobiose dehydrogenase (CDH) enzyme could increase cellobionate concentrations present in this strain. **FIG. 5A** and **FIG. 5B** show that sugar acid alone and sugar plus sugar acids (glucose and gluconate) can be converted to fuels and chemicals (Fan *et al.,* 2012).

[0152] Further, **FIG. 6** provides a summary of cellulose conversion and mycelial mass production. Wild type cells (which retain full β-glucosidase activity) do not accumulate cellobiose or cellobionate. In contrast, the F5 strain, with or without CDH, accumulates both cellobiose and cellobionate and produces mycelia mass at lower yields than wild-type.

Cellobiose Conversion to Cellobionate with ABTS & Laccase

[0153] To further investigate sugar acid production by cellulolytic microorganisms, Applicants generated additional genetically modified *N. crassa* strains. The F5 strain (described above) was selected for further modification, and mutations in the cre-1 gene and in the ace-1 gene were introduced into the F5 strain. The cre gene is a known catabolite repressor, and deletion of this gene has been shown to increase cellulase expression. A cre- strain has decreased growth rate on preferred carbon sources; however, on Avicel it produces 30-50% more cellulase and consumes avicel faster (Sun *et al.*, 2011), and cre expression correlates with cellulase expression. Mutation of the ace-1 gene (ace-) resulted in higher cellulase expression in *T. reesei* (Aro *et al.*, 2003).

[0154] To assay cellobionate production in the F5 and the F5Δcre-1Δace strains, these strains were grown on Vogel's medium + 20 g/L Avicel. 0.8 U/mL laccase and 0.1mM ABTS were added to the culture at 96 hours. As can be seen in **FIG. 7**, cellobiose accumulated in both the F5 and F5Δcre-1Δace strains. At 96 hours when exogenous laccase and ABTS were added, cellobionate began to accumulate while cellobiose concentrations dropped rapidly. This result suggests that all the cellobiose was converted to cellobionate upon ABTS and laccase addition.

[0155] As described above, the addition of ABTS and laccase to the growth culture of both the F5 and F5Δcre-1Δace strains induced the accumulation of cellobionate in the media, as the cellobiose in the media was converted to cellobionate. Without wishing to be bound

by theory, it is thought that laccase is involved in regenerating the activity of cellobiose dehydrogenase (CDH) enzymes present in these strains. When a flavo-enzyme, such as CDH, oxidizes a substrate such as cellobiose, it abstracts two electrons from the substrate, converting the substrate into its corresponding lactone which spontaneously hydrolyzes to an aldonic acid. In order for CDH to regain functionality, it must be oxidized. Oxygen is a thermodynamically favorable electron acceptor for CDH; however, it is extremely rate limiting. Laccase is an oxido-reductase, which, in contrast to CDH, is efficiently oxidized by oxygen, with the only byproduct being water. The FAD-containing enzyme cellobiose dehydrogenase (CDH; EC 1.1.99.18) oxidizes lactose at the C-1 position of the reducing sugar moiety to lactobionolactone, which spontaneously hydrolyzes to lactobionic acid. The cation radical of 2,20- azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is used as one possible electron acceptor (redox mediator) in this reaction, and is continuously reoxidized by laccase (LAC; EC 1.10.3.2), a multi-copper oxidase (van Hecke *et al.*, 2009).

[0156] Applicants further investigated cellobionate accumulation in the F5 and F5Δcre-1Δace strains. Additional experiments with these strains and employing laccase/ABTS in shake flask fermentation are presented in **FIG. 8A** and **FIG. 8B**. Cells were grown in Vogel's medium with 20 g/L Avicel, and 96 hours into the fermentation, laccase and ABTS were added. A similar experiment is presented in **FIG. 9A** and **FIG. 9B**, except that prior to the addition of laccase and ABTS, cells and cellulose were filtered prior to initiating conversion reaction. Further calculations are provided in **FIG. 10A** and **FIG. 10B**. The conversion calculations are based on a theoretical conversion of 1 mole of cellobiose to 1 mole of cellobionate. For example, if 15mM of cellobionate is obtained from 20mM of cellobiose, the conversion % is 75%. If 22mM of cellobionate is obtained from 20mM of cellobiose, the conversion % is 110%. As can be seen, more than 100% percent of theoretical maximal cellobiose conversion to cellobionate was observed. This may be because some other cello-oligosaccharides in the broth are converted to cellobionate via the CDH-laccase-ABTS redox-mediated system. The residual % is the amount of cellobionate detected at a given time compared to the maximum amount of cellobionate detected at a previous time point. For example, if the maximum amount of cellobionate detected is 15mM, and the amount of cellobionate detected 24 hours later is 10mM, the residual % of cellobionate is 66%. Without wishing to be bound by theory, it is thought that either cellulose is continuously converted as cellobiose is converted (relieved inhibition), or that cello-oligosaccharides are also converted to cellobionate.

NdvB Knockout Strains

[0157] Applicants further modified the F5Δcre-1Δace strain to contain mutations in the ndvB gene, which encodes a cellobionate phosphorylase. Two ndvB knockout strains (8.3.4.6 & 8.3.8.8) were chosen for evaluation in the CDH-ABTS-laccase redox system as compared to the parent strain (F5Δcre-1Δace). The two ndvB knockout strains were further modified to contain a mus51 mutation. Without wishing to be bound by theory, it is thought that a mus51 mutation increases the ease of genetic modification of this strain. Fermentation conditions for these strains were as described above. At 92.5 hours into the fermentation, laccase (0.8 U/mL and ABTS (0.1mM) were added to the fermentation broth to initiate conversion of cellobiose to cellobionate. The concentrations of cellobiose to cellobionate were monitored by HPLC. As can be seen in **FIG. 11A** and **FIG. 11B**, all the cellobiose was converted to cellobionate upon the addition of ABTS and laccase to the culture media. F5Δcre-1ΔaceΔmus51ΔndvB strains produced higher concentration of cellobionate as compared to the F5 strain. The F5Δcre-1ΔaceΔmus51ΔndvB strains also exhibited higher CDH activity (**FIG. 11C**). Without wishing to be bound by theory, it is thought that the higher CDH activity of F5Δcre-1ΔaceΔmus51ΔndvB mutant strains is due to the loss of CRE-1 and ACE-1 activity in these strains.

Analysis of Cellobionate Consumption in F5Δcre-1ΔaceΔndvB Strains

[0158] Without wishing to be bound by theory, it is thought that loss of cellobionate phosphorylase results in a block in cellobionate consumption. To explore this metabolism further, four separate F5Δcre-1ΔaceΔndvB knockout strains were constructed and compared to the F5ΔaceΔcre-1 strain for cellobionate consumption. All strains were evaluated in 1x Vogel's salts with 0.5 g/L glucose (to initiate cell growth) and 20g/L cellobionate. The concentration of glucose and cellobionate in the broth supernatant was determined by HPLC using an Aminex 87C column with 4mM CaCl2 mobile phase at 0.6mL/minute. As can be seen in **FIG. 12**, all strains analyzed consumed glucose in just over 8 hours. The F5ΔaceΔcre-1 strain consumed the 5g/L of cellobionate in just over 27 hours and only after glucose was depleted. In contrast, the F5Δcre-1ΔaceΔndvB knockout strains did not consume any cellobionate.

Laccase Overexpression in F5Δcre-1ΔaceΔndvB Strains

[0159] Applicants analyzed cellobionate accumulation in F5Δcre-1ΔaceΔndvB strains that were genetically engineered to overexpress laccase. For testing the effect of laccase enzyme overexpression, ten day old conidia of the strain F5Δcre-1ΔaceΔndvB+lac and the control strain F5Δcre-1Δace were collected and inoculated into flasks that contain 20g/L Avicel and 0.6g/L glucose. The fermentations were carried out at 27°C at 200 rpm with the light on. 0.1 mM of ABTS was added at the beginning of the culture. Samples were taken at various time intervals. Concentrations of cellobiose and cellobionate were measured by HPLC. As shown in **FIG. 13**, the F5Δcre-1Δace strain produced a small amount of cellobionate. However, the produced cellobionate was subsequently consumed by the fungus. However, the F5Δcre-1ΔaceΔndvB+lac strain produced about 7 mM of cellobionate, and no consumption of cellobionate by the strain was observed.

[0160] An additional laccase overexpression strain was constructed to confirm the results obtained in **FIG. 13**. As can be seen in **FIG. 14A** and **FIG. 14B**, mutations in cellobionate phosphorylase (ndvB) and overexpression of laccase in the F5Δcre-1Δace mutant background resulted in markedly increased cellobionate production as compared to the F5Δcre-1Δace strain.

Summary

[0161] In summary, the data presented herein demonstrates a *Neurospora crassa* that was engineered for increased production of cellobionate from cellulose. Multiple bgl gene deletions redirected carbon flow toward cellobiose/cellobionate production. Cre/ace gene deletions promote cellulase expression. NdvB gene deletion eliminates cellobionate consumption. Finally, laccase overexpression improves cellobionate production in the presence of CDH and a redox mediator.

Example 2: Production of cellobionate from cellulose using an engineered *Neurospora crassa* strain with laccase and redox mediator addition

[0162] This Example demonstrates a production process for producing cellobionic acid from cellulose using an engineered fungal strain with the exogenous addition of laccase and a redox mediator. From Example 1, an engineered strain of *Neurospora crassa* (F5Δ*ace*-

1Δcre-1ΔndvB) was shown to produce cellobionate directly from cellulose without the addition of exogenous cellulases. *N. crassa* produces cellulases (which hydrolyze cellulose to cellobiose) and cellobiose dehydrogenase (CDH)(which oxidizes cellobiose to cellobionate). However, the conversion of cellobiose to cellobionate is limited by the slow re-oxidation of CDH by molecular oxygen. By adding low concentrations of laccase and a redox mediator to the fermentation, CDH can be efficiently oxidized by the redox mediator, with in-situ re-oxidation of the redox mediator by laccase. In this Example, the conversion of cellulose to cellobionate was optimized by evaluating pH, buffer, and laccase and redox mediator addition time on the yield of cellobionate. Mass and material balances were performed, and the use of the native *N. crassa* laccase in such a conversion system was evaluated against the exogenous *Pleurotus ostreatus* laccase.

Introduction

[0163] The development of microbial fermentation platforms for the production of organic acids has gained interest in the last decade (Jang *et al.*, 2012; Sauer *et al.*, 2008) due to the reliability and cost-effectiveness of such processes (Demain *et al.*, 2007). In recent years, carboxylic acids, such as lactobionic acid (LBA), have emerged as specialty acids due to their unique physiochemical properties. LBA is a high value-added organic acid, with numerous applications that span the pharmaceutical, food, and cosmetics industries (Alonso *et al.*, 2013). In order to compete with petroleum-based processes for the production of carboxylic acids, the development of microbial processes utilizing low-cost substrates is essential (Alonso *et al.,* 2013). LBA is currently produced through chemical synthesis in an energy-intensive process requiring costly metal catalysts. Alternatively, LBA can be produced biologically by various bacterial and fungal strains using refined lactose as the substrate (Miyamoto *et al.*, 2000; Meiberg J *et al.*, 1990; Murakami H *et al.*, 2006; Murakami H *et al.*, 2003; Pedruzzi I *et al.*, 2011; Malvessi E *et al.*, 2013; Kiryu T *et al.*, 2012; Alonso S *et al.*, 2011; Alonso S *et al.*, 2012; Alonso S *et al.*, 2013). The inexpensive substrate cheese whey was also investigated as a substrate for LBA production by *Pseudomonas taetrolens* in an environmentally-friendly fermentation process (Alonso S *et al.*, 2013; Alonso S *et al.*, 2011; Alonso S *et al.*, 2012). The enzyme which catalyzes the biotransformation is lactose dehydrogenase (Miyamoto *et al.*, 2000; Meiberg J *et al.*, 1990; Murakami H *et al.*, 2006; Murakami H *et al.*, 2003; Pedruzzi I *et al.*, 2011; Malvessi E *et al.*, 2013; Kiryu T *et al.*, 2012; Alonso S *et al.*, 2011; Alonso S *et al.*, 2012; Alonso S *et al.*, 2013).

[0164] LBA could also be produced from lactose enzymatically by the action of CDH. CDH is a hemoflavoenzyme produced by several cellulolytic fungi. It contains a C-terminal flavin adenine dinucleotide (FAD) domain responsible for oxidizing lactose or cellobiose, resulting in the formation of lactobionate or cellobionate, respectively. The two electrons are subsequently transferred from the FAD domain to the N-terminal heme domain (Henriksson G *et al.*, 2000). In order for CDH to regain functionality, the reduced heme domain must be oxidized with the help of an electron acceptor. Oxygen is the electron acceptor in this system if no other electron acceptors are present. Although the overall reaction is thermodynamically favorable, the rate of re-oxidation of CDH by molecular oxygen is very slow and is the rate-limiting step in converting lactose to LBA (Roy BP *et al.*, 1996; Bao W *et al.*, 1993).

[0165] Other than oxygen, a wide variety of substrates such as metal ions, quinones, and organic dyes can be alternative electron acceptors for the heme domain of the CDH (Baminger U *et al.*, 2001). Dichlorophenolindophenol (DCPIP) and 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) are two redox mediators that can accept electrons from CDH very efficiently (Baminger U *et al.*, 2001). However, the addition of redox mediators to facilitate electron transfer in CDH to improve the conversion rate of lactose to LBA is cost prohibitive unless the redox mediator can be regenerated in-situ.

[0166] Baminger et al. reported a novel CDH-ABTS-laccase bi-enzyme system for fast oxidation of lactose to LBA (Baminger U *et al.*, 2001). Laccases are important multicopper oxidases which are also widely distributed in wood degrading fungi (Madhavi V *et al.*, 2009). They are especially prevalent in white rot and brown rot fungi, with speculative involvement in lignin degradation (Arora DS *et al.*, 2010). In contrast to CDH, laccases oxidize a large number of reduced substances and use oxygen as the final electron acceptor very efficiently. One strategy to increase the rate of lactose oxidation by CDH with oxygen as the final electron acceptor is to employ catalytic amounts of DCPIP or ABTS with in-situ regeneration of the redox mediator by laccase. As shown in **FIG. 16**, CDH is reduced. When lactose is oxidized to lactobionic acid, in turn, the reduced CDH is re-oxidized with the help of a redox mediator, which is then regenerated through oxidation by laccase. Lastly, laccase is regenerated when the electrons are passed to oxygen, the final electron acceptor. Such a bi-enzyme cascade system was found to be able to drastically boost the rate of conversion of

lactose to LBA using ABTS as a redox mediator (Baminger U *et al.*, 2001; Van Hecke W *et al.*, 2009).

[0167] Cellulosic biomass, which is available at low cost and in widespread abundance (Lynd LR *et al.*, 1991), is a potential alternative substrate for the bio-production of carboxylic acids. In Example 1, Applicants investigated a process for the production of cellobionic acid (CBA), which is a sister carboxylic acid (stereoisomer) to LBA, directly from cellulose using an engineered *Neurospora crassa* strain with exogenous laccase and redox mediator addition.

[0168] *N. crassa* was previously engineered to produce cellobiose from cellulose by deleting six out of seven β-glucosidase (BGL) genes, resulting in a strain designated F5 (Fan Z *et al.*, 2012; Wu W *et al.*, 2013). The strain also produces CDH, which can oxidize cellobiose to CBA. The strain was further engineered by Applicants to prevent CBA consumption by knocking out the cellobionate phosphorylase (*ndvB*) gene, and cellulase expression was improved by deleting carbon catabolite repression genes, *cre-1* and *ace-1*, in a strain designated F5Δ*ace-1*Δ*cre-1*Δ*ndvB* (Hildebrand A *et al.*, 2014)(*See* Example 1). From 20 g/L Avicel, the F5Δ*ace-1*Δ*cre-1*Δ*ndvB* strain produces 20 mM (7 g/L) cellobiose and 10 mM CBA (3.5 g/L). In such a system, oxygen was the final electron acceptor. The re-oxidization of CDH by oxygen is the rate limiting step, which led to incomplete conversion of most of the cellobiose to CBA.

[0169] In this Example, Applicants explored the possibility of using the engineered *N. crassa* F5Δ*ace-1*Δ*cre-1*Δ*ndvB* strain to break down cellulose to cellobiose and produce CDH. Exogenous addition of laccase and ABTS will complement CDH to form the bi-enzyme cascade system to convert cellobiose to CBA. Fermentation conditions including, pH, buffer, and laccase and ABTS concentration and addition times were optimized to maximize the yield of CBA from cellulose. In addition, a material balance on the overall fermentation is included. Lastly, the possibility of using the native *N. crassa* laccase in the CDH-ABTS-laccase system was investigated.

Materials and Methods

Strains and Reagents

[0170] Wild-type *Neurospora crassa* (2489) was obtained from the Fungal Genetics Stock Center (FGSC)(McCluskey K *et al.*, 2010). The F5 strain used in this Example is

strain 2489 with six out of seven of its *bgl* genes knocked out (Fan Z *et al.*, 2012; Wu W *et al.*, 2013). The F5 strain was engineered for increased cellulase expression and cellulose hydrolysis by knocking out the *ace-1*, *cre-1*, and *ndvB* genes as described in Hildebrand A *et al.*, 2014. The strains used in this Example and their sources are listed in **Table 1**.

Table 1: Exemplary Strains Used in this Example

Strain	**Genotype**	**Source**
FGSC 2489	Wild type	Fungal genetic source center
F5Δ*ace-1*Δ*cre-1*Δ*ndvB*	*bgl-1::hph bgl-2::hph bgl-3::hph bgl-4::hph bgl-6::hph bgl-7::hph mus-51::ace-1::six cre-1::six mat A*	(Wu W et al., 2013)

[0171] For the construction of an *N. crassa* strain overexpressing the native *N. crassa* laccase, the native laccase gene was overexpressed using a cyclosporine A-resistance based gene placement system as previously described. The gpdA promoter (from *Aspergillus niger*), laccase gene (NCU04528.7), and trpC terminator were synthesized by Life Technologies and ligated to a vector, with flanks with csr-1 homology. The plasmid was expressed in *E. coli* and linear DNA of the insertion cassette obtained by restriction digest of the plasmid. 1200µg of the linear DNA was combined with 2.5 x 10^9 conidia/mL and electroporated (0.1cm cuvette – 7.5kV/cm; capacitance: 25 uFD; resistance: 600 ohms). Following electroporation, cells were regenerated in 750mL of 1M ice cold sorbitol, which was then added to 2mL of recovery medium (1x Vogel's medium, 2% yeast extract). After 6 hours of shaking at 30°C and 200rpm, 350µL of the recovered cells were plated on agar plates (1x Vogel's, 2% yeast extract, 1M sorbitol, 20 g l^{-1} sorbose, 0.5 g l^{-1} fructose, 0.5 g l^{-1} glucose, 5 µg ml^{-1} CsA, and 2% agar). After 5 days of growth at 30°C, colonies were placed on slants (Slants (5 µg mL^{-1} CsA, 1x Vogel's, 2% agar slopes) and grown for an additional 4 days. Transformants were verified by PCR.

[0172] Laccase from *P. ostreatus* was obtained from Sigma Aldrich and used in certain experiments described involving the exogenous addition of laccase. ABTS was obtained from Sigma Aldrich. For *in vitro* experiments, the CDH used was a recombinant *N. crassa* CDH produced by an engineered *Pichia pastoris* strain. It was produced according to the method described by Zhang et al., 2011.

Conversion of cellobiose to CBA using the CDH-ABTS-laccase system

[0173] To test the suitability of the CDH-laccase-ABTS redox system on converting cellobiose to cellobionate, a concentration of approximately 30 mM cellobiose was added to 50 mL falcon tubes containing laccase, ABTS, CDH, and buffer at the indicated concentrations and pH. When investigating the effect of pH, sodium citrate buffer at a concentration of 50 mM was used for acidic conditions, sodium phosphate buffer was used at a concentration of 50 mM for basic conditions, and 1x Vogel's salts medium with no pH adjustment was used for the pH 6 condition.

Fermentation experiments

[0174] *N. crassa* strains were grown on agar with 1x Vogel's salts and 1.5% sucrose in an incubator at 30°C with light. After 3 days, flasks were removed from the incubator and grown for 7 days at room temperature. After a total of 10 days of growth, the conidia were harvested in DI water and filtered through eight layers of cheese cloth. Fermentation experiments were conducted in 250 mL unbaffled flasks with a 50 mL working volume, 1x Vogel's salts medium, 0.5 g/L of glucose to initiate growth, and 20 g/L Avicel. Conidia were inoculated at a volume to yield a final OD_{420} of 0.15. Flasks were incubated at 28°C in a rotary shaker at 200 rpm with light. Exogenous laccase and ABTS were added to the flasks as indicated in the text. Samples were taken at various time intervals to measure cellobiose or cellobionate concentration. To investigate the effect of starting pH on cellobionate yield, sodium citrate, sodium phosphate, potassium phosphate, and sodium-potassium phosphate buffers of different concentrations were added to achieve the pH indicated. To study the effect of laccase and ABTS addition time on cellobionate production, laccase and ABTS were added to the fermentation broth at final concentrations of 0.05 U/mL and 0.01 mM, respectively.

Fermentation Experiments with N. crassa Laccase Overexpression Strains

[0175] *N. crassa* was grown on agar with 1x Vogels salts and 1.5% sucrose in an incubator at 30°C with light. After 3 days, flasks were removed from the incubator and grown for 7 days at room temperature. After a total of 10 days of growth, the conidia was harvested in DI water and filtered through several layers of cheese cloth. Fermentation experiments were conducted in 250 mL unbaffled flasks with a 50 mL working volume. The growth medium contained either 1x Vogel's salts medium and 20g/L glucose to initially test laccase overexpression, or 1x Vogel's salts medium, 0.5 g/L of glucose to initiate growth, and 20 g/L Avicel. Conidia were inoculated at a volume to yield a final OD_{420} of 0.15. Flasks were incubated at 28°C in a rotary shaker at 200 rpm with light. ABTS was added at initial concentration of 0.1mM.

Laccase production by Neurospora crassa

[0176] The wild type strain was grown in Vogel's salts medium on 1.5% sucrose. Cycloheximide, an inducer for laccase production, was added at 48 hours at a final concentration of 3 μM according to literature (Huber M *et al.*, 1987; Linden RM *et al.*, 1991; Froehner SC *et al.*, 1974). Laccase expression was monitored according to the assay described below in the "enzyme concentration" section. After an additional 142 hours of fermentation, the broth was filtered to remove residual Avicel and cells in order to obtain the native laccase for evaluation in the CDH-ABTS-laccase redox system in cell-free experiments.

Sample analysis

[0177] Concentrations of cellobiose and cellobionate in the cell-free experiments and in the fermentation broth were analyzed using a Shimadzu HPLC equipped with a CARBOSep COREGEL-87C (Transgenomic, San Jose, CA, USA) column. 4 mM calcium chloride ($CaCl_2$) at a flow rate of 0.6 mL/min was used as the mobile phase.

Enzyme concentration

[0178] Laccase activity was measured by monitoring the increase in absorbance of ABTS as described previously with minor modifications (Van Hecke W *et al.*, 2009; Baminger U *et al.*, 1999). The reaction mixture contained 5 mM ABTS in 100 mM sodium acetate buffer,

pH 4.5. One unit of laccase activity is defined as the amount of enzyme oxidizing 1 μmol of ABTS per minute under the above reaction conditions.

[0179] The concentration of CDH was determined by monitoring the decrease in absorbance of DCPIP at 520 nm in a spectrophotometer according to previously established methods with slight modification (Van Hecke W *et al.*, 2009; Baminger U *et al.*, 1999). The reaction contained 0.1 mM DCPIP, 3 mM cellobiose, and 4 mM sodium fluoride in 100 mM sodium acetate buffer at pH 4.5. One unit of enzyme activity is defined as the amount of enzyme reducing 1 μmol of DCIP per minute under the above reaction conditions.

Mycelial biomass measurements

[0180] The dry weight of the mycelia contained in the fermentation samples was measured by extracting ergosterol from the mycelia and measuring the amount by HPLC (Gessner MO *et al.*, 1991). The fermentation residues were collected by filtration through a 0.8 μm membrane. All the residue including the mycelia were harvested, frozen in liquid nitrogen for 1 hour, and ethanol (6 mL) was added to the frozen sample and incubated at 37 °C for 2 hours with shaking. An aliquot of KOH solution (60% w/v, 0.8 mL) was added to the mixture, which was then heated to 97 °C for 20 min. This sample was cooled and neutralized with HCl (36.5%, ~0.7 mL). The solution was extracted with hexane (3 x 5 mL), the hexane fractions were combined, and air was used to evaporate the solvent. The residue was dissolved in ethanol (1 mL), filtered through a 0.22 μm membrane filter and analyzed by HPLC with PDA detector on a reverse phase column (ZORBAX Eclipse Plus C18, 4.6 x 250 mm, 5 μm particle size, Agilent) and eluted at 1.0 mL/min with methanol-water (97:3 v/v). The amount of biomass was quantified using a standard curve prepared with known *N. crassa* dry biomass. The amount of residual Avicel was calculated by subtracting the mycelial biomass from the dry weight of the fermentation residues.

Results

Role of pH in the conversion of cellobiose to CBA using the CDH-ABTS-laccase system

[0181] Acidic, neutral, and basic conditions were tested to determine the effect of pH on the conversion of cellobiose to CBA. The time course of the conversion of cellobiose to CBA is shown in **FIG. 17A** and **FIG. 17B**. For conditions at pH 6, conversion was

completed within 24 hours with an average of 27.9 mM cellobiose converted to 28.6 mM CBA, resulting in an approximate 1:1 molar conversion, as expected. The data obtained support the efficacy of CDH-ABTS-laccase system for converting cellobiose to cellobionate. However, the presence of acidic conditions was important for the efficient conversion of cellobiose to CBA for the specific CDH and laccase used in this experiment.

The effect of starting pH on CBA production

[0182] When *F5Δace-1Δcre-1ΔndvB* is grown on 20 g/L Avicel, approximately 20 mM of cellobiose is produced along with 10 mM CBA. If laccase and ABTS are added to convert cellobiose to CBA, 30 mM of CBA is produced (Hildebrand A et al., 2014)(*See* Example 1). Because of the high level of CBA production, the pH rapidly drops from pH 6 to pH 4. The impact of buffering the fermentation medium was evaluated to determine the effect of this pH drop on the conversion of cellobiose to CBA. Three different concentrations of potassium phosphate buffer were evaluated to control the rate of pH drop and compared to the unbuffered Vogel's medium (**FIG. 18A** and **FIG. 18B**). In the case of the unbuffered Vogel's medium, the pH drops to 4 over the course of the fermentation. The addition of sodium phosphate buffer results in a slower decrease in pH and higher final pH as the buffer concentration increases. 200 mM potassium phosphate held the pH at 6 until the last day of fermentation where it dropped to 5.5. Although pH varied across the conditions tested, cellobiose and CBA production were not significantly affected. As a result, unbuffered Vogel's medium was used for the remainder of the fermentation experiments in this Example.

Production of the CBA with laccase and ABTS addition at different times

[0183] The addition of laccase and ABTS to the fermentation system employing the *F5Δace-1Δcre-1ΔndvB* strain on 20g/L Avicel was optimized. 0.05 U/mL of laccase and 0.01 mM ABTS were added at various time points. As shown in **FIG. 19A** and **FIG. 19B**, the cellobiose is completely converted to CBA within 48 hours for all addition times, with an optimal addition time at 120 hours into the fermentation. The maximum CBA concentration occurs at 168 hours with a slight decrease after that for all cases. When no laccase and ABTS are added, maximum cellobiose concentration also occurs 168 hours into the fermentation, and cellobionate production reaches a plateau at that time point as well.

[0184] In a parallel experiment with laccase and ABTS addition 120 hours into the fermentation, flasks were harvested at 168 hours to quantify biomass production and Avicel

utilization. The results, shown in **Table 2**, indicate that 67% of the Avicel is hydrolyzed, with 91% going toward CBA production and 4% going toward cell mass production. In the control case, where no laccase and ABTS are added, 62% of the Avicel is hydrolyzed, and a smaller fraction of the consumed Avicel goes toward CBA and cell mass production (29% and 3.3%, respectively). Without wishing to be bound by theory, the higher cellulose conversion in the case of with laccase and ABTS addition indicates that the conversion of cellobiose to CBA may relieve some cellulase inhibition by cellobiose, allowing for hydrolysis of the cello-oligosaccharides and subsequent conversion to CBA.

Table 2: Percentage of Avicel hydrolyzed and the percentage directed toward fermentable products for the F5Δ*ace-1*Δ*cre-1*Δ*ndvB* strain grown on 20 g/L Avicel

	Starting Avicel (g)	Residual Avicel (g)	Cellulose Conversion (%)	Mycelium produced (g)	Yield of cellobionate from consumed Avicel (mol/mol×100%)	Yield of mycelium mass from consumed Avicel (g/g×100%)
F5Δ*ace-1*Δ*cre-1*Δ*ndvB* With laccase/ABTS	1.00	0.33±0.008	67±0.8%	0.03±0.001	91±4%	4.0±0.2%
F5Δ*ace-1*Δ*cre-1*Δ*ndvB* No laccase/ABTS	1.00	0.38±0.002	62±0.2%	0.02±0.001	29±1%	3.3±0.2%

Errors are calculated based upon standard deviations and error propagation theory.

In vitro assays with native laccase from N. crassa

[0185] In the experiments where exogenous laccase was added to the fermentation, the laccase host was *Pleureotus ostreatus,* a basidiomycete that expresses high levels of laccase (Okamoto K *et al.*, 2000; More S *et al.*, 2011). While *N. crassa* does have a native laccase, it is not naturally expressed, except under stress conditions that are not suitable for efficient fermentation. However, the suitability of using the native *N. crassa* laccase in the CDH-ABTS-laccase system for conversion of cellobiose to CBA was investigated.

[0186] Cycloheximide, D-phenylalanine, and copper sulfate are possible inducers for the *N. crassa* laccase according to previous studies (Huber M *et al.*, 1987; Linden RM *et al.*, 1991; Froehner SC *et al.*, 1974; Luke AK *et al.*, 2001). To induce laccase in the *N. crassa* F5 strain, 3 μM cycloheximide was added after 48 hours of fermentation. After an additional 142 hours of fermentation, 0.15 U/mL of laccase was obtained, a suitable concentration to test in the cellobiose conversion system as shown in **FIG. 20**.

[0187] The produced laccase was tested against the *P. ostreatus* laccase in a falcon tube experiment (no cells), where cellobiose, CDH, and ABTS were added and the conversion of cellobiose to CBA was monitored as shown in **FIG. 21A** and **FIG. 21B**. The results indicate that the two laccases have comparable activities, both allowing for efficient conversion of cellobiose to CBA in the CDH-ABTS-laccase conversion system.

[0188] Cellulolytic fungi such as *N. crassa* can potentially produce all the enzymes needed to convert cellulose to CBA. *N. crassa* was able to produce cellulases and CDH. With the addition of the exogenously added laccase and ABTS, *N. crassa* was able to convert cellulose to CBA at very high yield. This Example showed that the native laccase produced by *N. crassa* works as efficiently as the *P. ostreatus* laccase. This opens up the possibility to engineer *N. crassa* to produce all the enzymes needed to convert cellulose to CBA. Homologous or heterologous expression of laccase in *N. crassa* could be achieved by engineering the native or heterologous laccase for expression under a constitutive or inducible promoter, allowing it to be produced under the tested fermentation conditions.

[0189] A report in the literature demonstrated over-expressing the native laccase in *N. crassa* at concentrations of 55 mg/L (Schilling B *et al.*, 1992). Such concentrations would be adequate to allow for the efficient conversion of cellobiose to CBA in the presence of CDH and ABTS, and thus the overexpression of native *N. crassa* laccase may be done in the strains described herein. Also, one study showed that a laccase de-repressed mutant *lah-1* produced laccase at levels even higher than when the wild type was induced with cycloheximide (Tamaru H *et al.*, 1989). The de-repression was a result of a single mutation in an unknown gene mapped between *nit-2* and *leu-3* in linkage group I. Similarly de-repressing the *F5Δace-1Δcre-1ΔndvB* strain or overexpressing laccase in this strain could create a strain which would require only the addition of a low concentration of ABTS to produce CBA from cellulose.

In vivo assays with native laccase from N. crassa

[0190] The *in vitro* experiments with the endogenous *N. crassa* laccase as described above demonstrated the potential for overexpressing this endogenous gene in lieu of adding exogenous laccase in cellobionate production assays. To explore this, an *N. crassa* strain overexpressing the native *N. crassa* laccase gene was constructed as described above in the *F5Δace-1Δcre-1ΔndvB* strain background. The native laccase was successfully expressed

when strains were grown on glucose. Three different strains produced about 0.03U/mL laccase (**FIG. 22**). The best producing transformant, 2-2, was selected for additional analysis.

[0191] Next, cellobionate production from Avicel by the recombinant strains was explored. As shown in **FIG. 23**, the strains overexpressing laccase (F5Δ*ace-1*Δ*cre-1*Δ*ndvB* (Lac+)) produced more cellobionate than the control strain (F5Δ*ace-1*Δ*cre-1*Δ*ndvB* Δ*mus51*), which does not produce laccase. Both of these strains produced more cellobionate than F5Δ*ace-1*Δ*cre-1*Δ*mus51*. 0.1mM of ABTS was added at the beginning of the fermentation.

[0192] The impact of adding ABTS on cellobionate production in the recombinant strain overexpressing the native *N. crassa* laccase was also examined. As can be seen in **FIG. 24**, the addition of ABTS increased the amount of the cellobionate produced in this strain (F5Δ*ace-1*Δ*cre-1*Δ*ndvB* (Lac+)). However, it was also observed that cellobionate could still be produced even without ABTS addition, indicating that some other reagents such as $FeSO_4$ in the medium could act as the electron mediator as well.

Discussion

[0193] ABTS was used as the redox mediator in the tested system. Although it is only needed in catalytic amount, it is very expensive for industrial application and an alternative low-cost redox mediator may be more preferable. A wide variety of inorganic metals and organic dyes may be used as redox mediators (Kunamneni, A *et al.*, 2008). Noteworthy, lignin degradation products such as vanillin, ferulic acid or p-coumaric acid, which are generated as waste in the paper and pulp industry, have been demonstrated to be very efficient naturally-occurring laccase mediators (Camarero S *et al.*, 2005; Cañas A *et al.*, 2007; Camarero S *et al.*, 2007; Gutiérrez A *et al.*, 2007). These compounds could also be used as a cheap redox mediator source (Kunamneni, A *et al.*, 2008). Further, if pretreated lignocellulosic biomass was used as the substrate instead of pure cellulose, these compounds may be naturally present in the feedstock stream, and the exogenous addition of a redox mediator could be potentially reduced or avoided.

Summary

[0194] This Example has demonstrated that CBA can be produced from cellulose by an engineered *N. crassa* strain with exogenous laccase addition or with overexpression of an

endogenous laccase gene. The native laccase produced by *N. crassa* functioned as well as the exogenously added laccase. The tested strains are useful for microbial conversion of cellulose to CBA. The conversion concept is applicable to other industrially relevant cellulolytic fungi for CBA production from cheap feedstocks (*e.g.* cellulosic biomass).

References

Lynd, L. R., Zyl, W. H., McBride, J. E., and Laser, M. (2005) Consolidated bioprocessing of cellulosic biomass: an update, Curr Opin Biotechnol.

Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S. (2002) Microbial cellulose utilization: fundamentals and biotechnology, Microbiol Mol Biol Rev 66, 506-577, table of contents.

Lynd, L. R., Wyman, C. E., and Gerngross, T. U. (1999) Biocommodity Engineering, Biotechnol Prog 15, 777-793.

Lynd, L. R., Cushman, J. H., Nichols, R. J., and Wyman, C. E. (1991) Fuel Ethanol from Cellulosic Biomass, Science 251, 1318-1323.

Fan, Z., Wu, W., Hildebrand, A., Kasuga, T., Zhang, R., and Xiong, X. (2012) A novel biochemical route for fuels and chemicals production from cellulosic biomass. PLoS One 7, e31693.

Wu, W., Hildebrand, A., Kasuga, T., Xiong, X., and Fan, Z. (2013) Direct cellobiose production from cellulose using sextuple beta-glucosidase gene deletion Neurospora crassa mutants. Enzyme and Microbial Technology 52,184-189.

Zhang, R., Fan, Z., and Kasuga, T. (2011) Expression of cellobiose dehydrogenase from Neurospora crassa in Pichia pastoris and its purification and characterization. Protein Expression and Purification 75,63-69.

Szewczyk, E., Kasuga, T., and Fan, Z. (2013) Efficient sequential repetitive gene deletions in Neurospora crassa, employing self-excising beta-recombinase/six cassette. Journal of Microbiological Methods 92,236-243.

Baminger, U., Ludwig, R., Galhaup, C., Leitner, C., Kulbe, K. D., and Haltrich, D. (2001) Continuous enzymatic regeneration of redox mediators used in biotransformation reactions employing flavoproteins. Journal of Molecular Catalysis B-Enzymatic 11,541-550.

Jang Y-S, Kim B, Shin JH, Choi YJ, Choi S, et al. (2012) Bio-based production of C2-C6 platform chemicals. Biotechnol Bioeng 109: 2437–2459.

Sauer M, Porro D, Mattanovich D, Branduardi P (2008) Microbial production of organic acids: expanding the markets. Trends Biotechnol 26: 100–108.

Demain AL (2007) The business of biotechnology. Ind Biotechnol 3: 269–283.

Alonso S, Rendueles M, Díaz M (2013) Bio-production of lactobionic acid: current status, applications and future prospects. Biotechnol Adv 31: 1275–1291.

Miyamoto Y, Ooi T, Kinoshita S (2000) Production of lactobionic acid from whey by Pseudomonas sp. LS13-1. Biotechnol Lett 22: 427–430.

Meiberg J, PM B, Sloots B (1990) A process for the fermentative oxidation of reducing disaccharides. EP 0384534 A1

Murakami H, Kiryu T, Kiso T, Nakano H (2006) Production of Aldonic Acids from Monosaccharides by Washed Cells of Burkholderia cepacia and their Calcium Binding Capability. J Appl glycosci 279: 277–279.

Murakami H, Seko A, Azumi M, Ueshima N, Yoshizumi H, et al. (2003) Fermentative Production of Lactobionic Acid by Burkholderia cepacia. J Appl glycosci 120: 117–120.

Pedruzzi I, da Silva EAB, Rodrigues AE (2011) Production of lactobionic acid and sorbitol from lactose/fructose substrate using GFOR/GL enzymes from Zymomonas mobilis cells: a kinetic study. Enzyme Microb Technol 49: 183–191.

Malvessi E, Carra S, Pasquali FC, Kern DB, da Silveira MM, et al. (2013) Production of organic acids by periplasmic enzymes present in free and immobilized cells of Zymomonas mobilis. J Ind Microbiol Biotechnol 40: 1–10.

Kiryu T, Yamauchi K, Masuyama A, Ooe K, Kimura T, et al. (2012) Optimization of Lactobionic Acid Production by Acetobacter orientalis Isolated from Caucasian Fermented Milk, "Caspian Sea Yogurt." Biosci Biotechnol Biochem 76: 361–363.

Alonso S, Rendueles M, Díaz M (2011) Efficient lactobionic acid production from whey by Pseudomonas taetrolens under pH-shift conditions. Bioresour Technol 102: 9730–9736.

Alonso S, Rendueles M, Díaz M (2012) Role of dissolved oxygen availability on lactobionic acid production from whey by Pseudomonas taetrolens. Bioresour Technol 109: 140–147.

Alonso S, Rendueles M, Díaz M (2013) Feeding strategies for enhanced lactobionic acid production from whey by Pseudomonas taetrolens. Bioresour Technol 134: 134–142.

Henriksson G, Johansson G, Pettersson G (2000) A critical review of cellobiose dehydrogenases. J Biotechnol 78: 93–113.

Roy BP, Dumonceaux T, Koukoulas AA, Archibald FS (1996) Purification and Characterization of Cellobiose Dehydrogenases from the White Rot Fungus Trametes versicolor. Appl Environ Microbiol 62: 4417–4427.

Bao W, Usha SN, Renganathan V (1993) Purification and characterization of cellobiose dehydrogenase, a novel extracellular hemoflavoenzyme from the white-rot fungus Phanerochaete chrysosporium. Arch Biochem Biophys 300: 705–713.

Baminger U, Ludwig R, Galhaup C, Leitner C, Kulbe KD, et al. (2001) Continuous enzymatic regeneration of redox mediators used in biotransformation reactions employing flavoproteins. J Mol Catal B Enzym 11: 541–550.

Madhavi V, Lele SS (2009) Laccase: properties and applications. 4: 1694–1717.

Arora DS, Sharma RK, Singh Arora D, Kumar Sharma R (2010) Ligninolytic fungal laccases and their biotechnological applications. Appl Biochem Biotechnol 160: 1760–1788.

Van Hecke W, Bhagwat A, Ludwig RR, Dewulf J, Haltrich D, et al. (2009) Kinetic Modeling of a Bi-Enzymatic System for Efficient Conversion of Lactose to Lactobionic Acid. Biotechnol Bioeng 102: 1475–1482.

Lynd LR, Cushman J, Nichols R, Wyman C (1991) Fuel ethanol from cellulosic biomass. Science 251: 1318–1323.

Fan Z, Wu W, Hildebrand A, Kasuga T, Zhang R, et al. (2012) Novel Biochemical Route for Fuels and Chemicals production from Cellulosic Biomass. PLoS One 7: 8.

Wu W, Hildebrand A, Kasuga T, Xiong X, Fan Z (2013) Direct cellobiose production from cellulose using sextuple beta -glucosidase gene deletion Neurospora crassa mutants. Enzyme Microb Technol 52: 184–189.

Hildebrand A, Szewczyk E, Lin H, Kasuga T, Fan J (2014) Engineering Neurospora crassa for improved cellobiose and cellobionate production. Appl Environ Microbiol: In press.

McCluskey K, Wiest A, Plamann M (2010) The fungal genetics stock center: A repository for 50 years of fungal genetics research. J Biosci 35: 119–126.

Zhang R, Fan Z, Kasuga T (2011) Expression of cellobiose dehydrogenase from Neurospora crassa in Pichia pastoris and its purification and characterization. Protein Expr Purif 75: 63–69.

Huber M, Lerch K (1987) The influence of copper on the induction of tyrosinase and laccase in Neurospora crassa. 219: 335–338.

Linden RM, Schilling BC, Germann U a, Lerch K (1991) Regulation of laccase synthesis in induced Neurospora crassa cultures. Curr Genet 19: 375–381.

Froehner SC, Eriksson KE (1974) Induction of Neurospora crassa laccase with protein synthesis inhibitors. J Bacteriol 120: 450–457.

Baminger U, Nidetzky B, Kulbe KD, Haltrich D (1999) A simple assay for measuring cellobiose dehydrogenase activity in the presence of laccase. 35: 253–259.

Gessner MO, Bauchrowitz MA, Escautier M, Renouvelables R, Marvig J (1991) Extraction and quantification of ergosterol as a measure of fungal biomass in leaf litter. Microb Ecol: 285–291.

Okamoto K, Yanagi SO, Sakai T (2000) Purification and characterization of extracellular laccase from Pleurotus ostreatus. Mycoscience 41: 7–13.

More S, S. RP, K. P, M. S, S.Malini, et al. (2011) Isolation, Purification, and Characterization of Fungal Laccase fromPleurotus sp. Enzyme Res 2011: 7.

Luke AK, Burton SG (2001) A novel application for Neurospora crassa: Progress from batch culture to a membrane bioreactor for the bioremediation of phenols. Enzyme Microb Technol 29: 348–356.

Schilling B, Linden RM, Kupper U, Lerch K (1992) Expression of Neurospora crassa laccase under the control of the copper-inducible metallothionein-promoter. Curr Genet 22: 197–203.

Tamaru H, Inoue H (1989) Isolation and characterization of a laccase-derepressed mutant of Neurospora crassa. J Bacteriol 171: 6288–6293.

Kunamneni, A., Camarero, S., Garcia-Burgos, C., Plou, F. J., Ballesteros, A., and Alcalde, M. (2008) Engineering and Applications of fungal laccases for organic synthesis, *Microbial cell factories 7*, 17.

Camarero S, Ibarra D, Martínez MJ, Martínez AT. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl Environ Microbiol.2005;71:1775–1784.

Cañas A, Alcalde M, Plou FJ, Martínez MJ, Martínez AT, Camarero S. Transformation of polycyclic aromatic hydrocarbons by laccase is strongly enhanced by phenolic compounds present in soil. Environ Sci Technol. 2007;41:2964–2971.

Camarero S, Ibarra D, Martínez AT, Romero J, Gutiérrez A, del Río JC. Paper pulp delignification using laccase and natural mediators. Enzyme Microb Technol. 2007;40:1264–1271.

Gutiérrez A, Rencores J, Ibarra D, Molina S, Camarero S, Romero J, del Río JC, Martínez AT. Removal of lipophilic extractives from paper pulp by laccase and lignin-derived phenols as natural mediators. Environ Sci Technol. 2007;41:4124–4129.

CLAIMS

What is claimed is:

1. A recombinant host cell comprising:
   a) reduced activity of one or more polypeptides having β-glucosidase activity as compared to a corresponding wild type cell, wherein each of said one or more polypeptides are encoded by a gene that has at least 80% sequence identity to a gene selected from the group consisting of NCU00130, NCU04952, NCU05577, NCU07487, NCU08755, and NCU03641 genes;
   b) reduced activity of a polypeptide having cellobionate phosphorylase activity as compared to a corresponding wild type cell, wherein said polypeptide is encoded by a gene that has at least 80% sequence identity to NCU09425 (NdvB);
   c) reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08807 (CRE-1) as compared to a corresponding wild type cell; and
   d) reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU09333 (ACE-1) as compared to a corresponding wild type host cell.

2. The host cell of claim 1, wherein said host cell is a fungal cell.

3. The host cell of claim 2, wherein said fungal cell is *Neurospora crassa*.

4. The host cell of any one of claims 1-3, wherein reduced activity of one or more of the polypeptides having β-glucosidase activity is due to a genetic mutation.

5. The host cell of claim 4, wherein a genetic mutation is present in two or more, three or more, four or more, five or more, or six or more genes encoding the polypeptides having β-glucosidase activity.

6. The host cell of any one of claims 4 or 5, wherein at least one of the genetic mutations is a knockout mutation.

7. The host cell of any one of claims 1-6, wherein reduced activity a polypeptide having cellobionate phosphorylase activity is due to a genetic mutation.

8. The host cell of claim 7, wherein the genetic mutation is a knockout mutation.

9. The host cell of any one of claims 1-8, wherein reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08807 (CRE-1) is due to a genetic mutation.

10. The host cell of claim 9, wherein the genetic mutation is a knockout mutation.

11. The host cell of any one of claims 1-10, wherein reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU09333 (ACE-1) is due to a genetic mutation.

12. The host cell of claim 11, wherein the genetic mutation is a knockout mutation.

13. The host cell of any one of claims 1-12, wherein the host cell further comprises reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08290 (MUS51) as compared to a corresponding wild type cell.

14. The host cell of claim 13, wherein reduced activity of a polypeptide encoded by a gene that has at least 80% sequence identity to NCU08290 (MUS51) is due to a genetic mutation.

15. The host cell of claim 14, wherein the genetic mutation is a knockout mutation.

16. The host cell of any one of claims 1-15, wherein the host cell further comprises a polypeptide having increased laccase expression or activity as compared to a corresponding wild type cell.

17. The host cell of claim 16, wherein the polypeptide is encoded by a gene that has at least 80% sequence identity to NCU04528.

18. The host cell of any one of claims 16 or 17, wherein expression of the polypeptide is under the control of a constitutive promoter.

19. The host cell of any one of claims 1-18, wherein the host cell further comprises a polypeptide having increased cellobiose dehydrogenase expression or activity as compared to a corresponding wild type cell.

20. The host cell of claim 19, wherein expression of the polypeptide is under the control of a constitutive promoter.

21. The host cell of any one of claims 1-20, wherein said host cell produces a sugar acid from cellulose.

22. The host cell of any one of claims 1-21, wherein the host cell produces cellobiose.

23. The host cell of any one of claims 21 or 22, wherein the sugar acid is cellobionate.

24. The host cell of claim 23, wherein consumption of cellobionate by the host cell is reduced by at least 80% as compared to a corresponding wild type cell.

25. A recombinant *N. crassa* cell comprising:
a) a mutation in each of NCU00130, NCU04952, NCU05577, NCU07487, NCU08755, and NCU03641 genes, wherein said mutation reduces β-glucosidase activity of polypeptides encoded by said genes;
b) a mutation in the NCU09425 (NdvB) gene, wherein said mutation reduces cellobionate phosphorylase activity of the polypeptides encoded by said gene;
c) a mutation in each of NCU08807 (CRE-1) and NCU09333 (ACE-1) genes, wherein said mutations reduce activity of polypeptides encoded by said genes;
d) a laccase having increased expression or activity as compared to a wild type *N. crassa* cell, and
wherein said recombinant *N. crassa* cell produces cellobionate from cellulose.

26. A method of producing sugar acids, the method comprising:
a) providing the host cell of any one of claims 1-20;
b) culturing the host cell in a medium comprising cellulose, whereby the host cell produces a sugar acid from the cellulose.

27. The method of claim 26, further comprising a step of substantially purifying the sugar acid from the medium.

28. The method of any one of claims 26 or 27, wherein the host cell produces cellobiose.

29. The method of any one of claims 26-28, wherein the sugar acid is cellobionate.

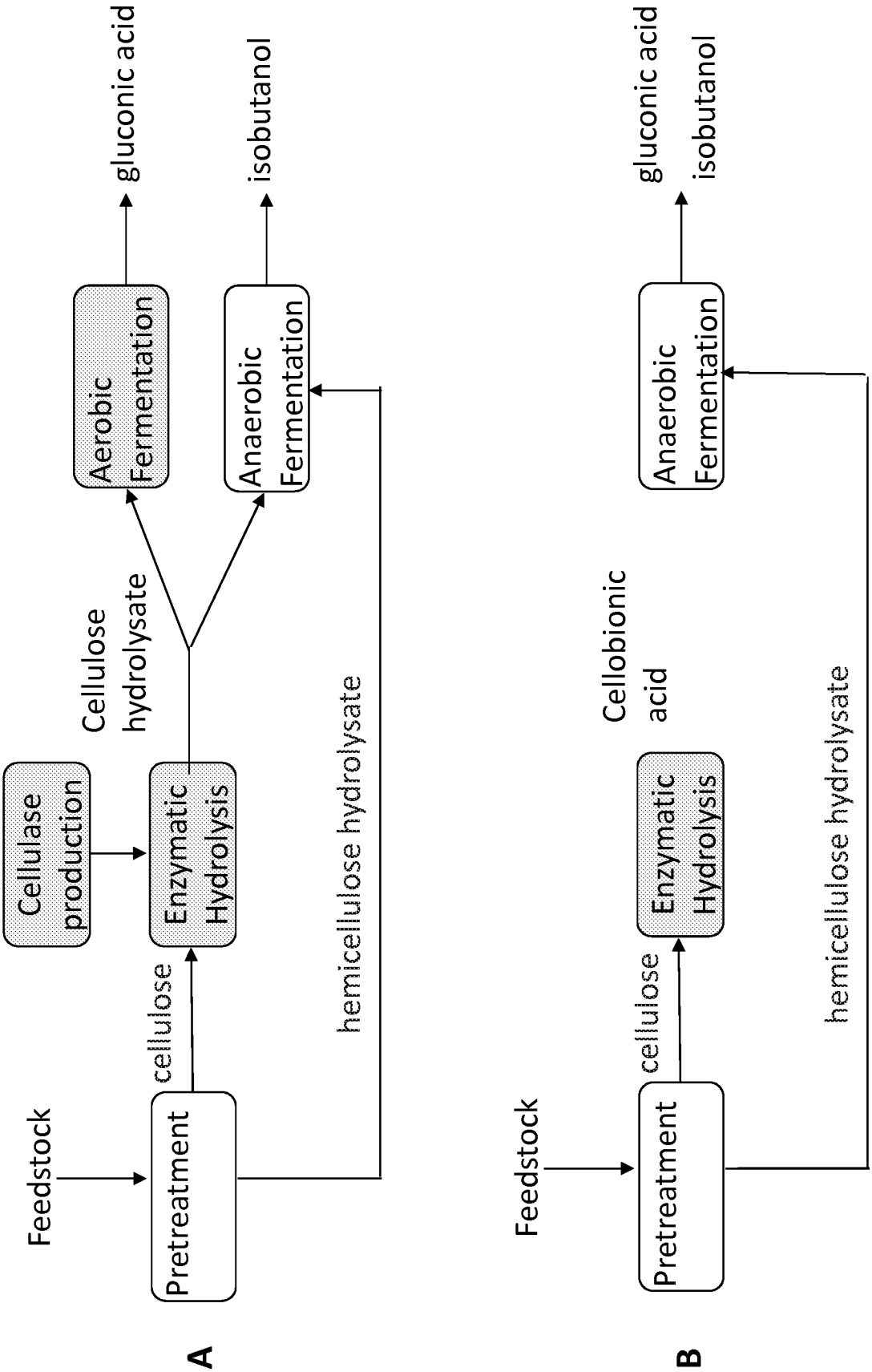
30. The method of claim 29, wherein consumption of cellobionate by the host cell is reduced by at least 80% as compared to a corresponding wild type cell.

31. The method of any one of claims 27-30, wherein the host cell is cultured in the presence of an exogenous source of laccase.

32. The method of any one of claims 27-31, wherein the host cell is cultured in the presence of an exogenous source of cellobiose dehydrogenase.

33. The method of any one of claims 27-32, wherein the medium further comprises a redox mediator.

FIG. 1


Feedstock
Pretreatment
cellulose
Cellulase production
Enzymatic Hydrolysis
Cellulose hydrolysate
Aerobic Fermentation
gluconic acid
Anaerobic Fermentation
isobutanol
hemicellulose hydrolysate
A
Feedstock
Pretreatment
cellulose
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Cellobionic acid
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gluconic acid
isobutanol
hemicellulose hydrolysate
B

FIG. 2

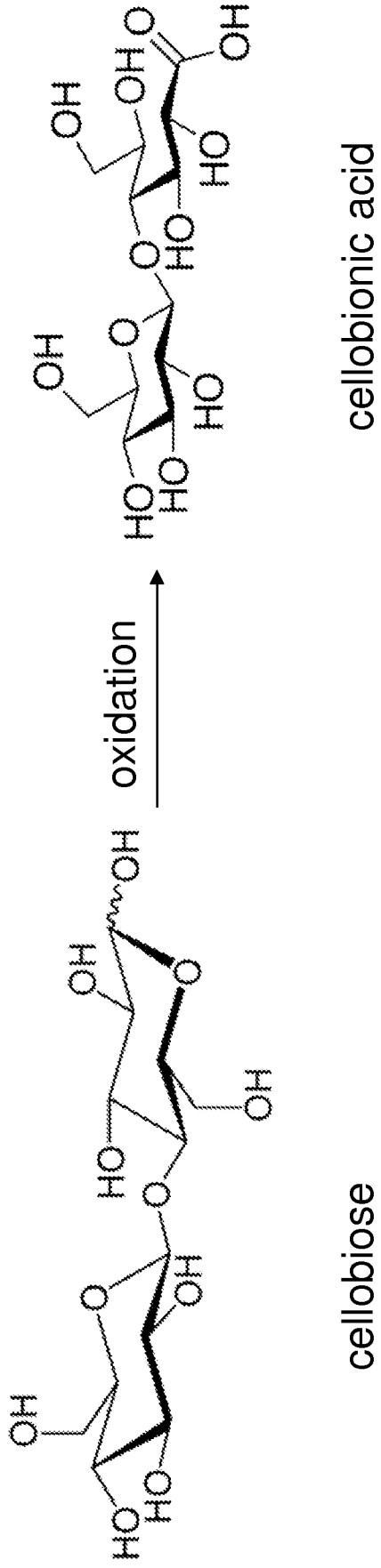


FIG. 3

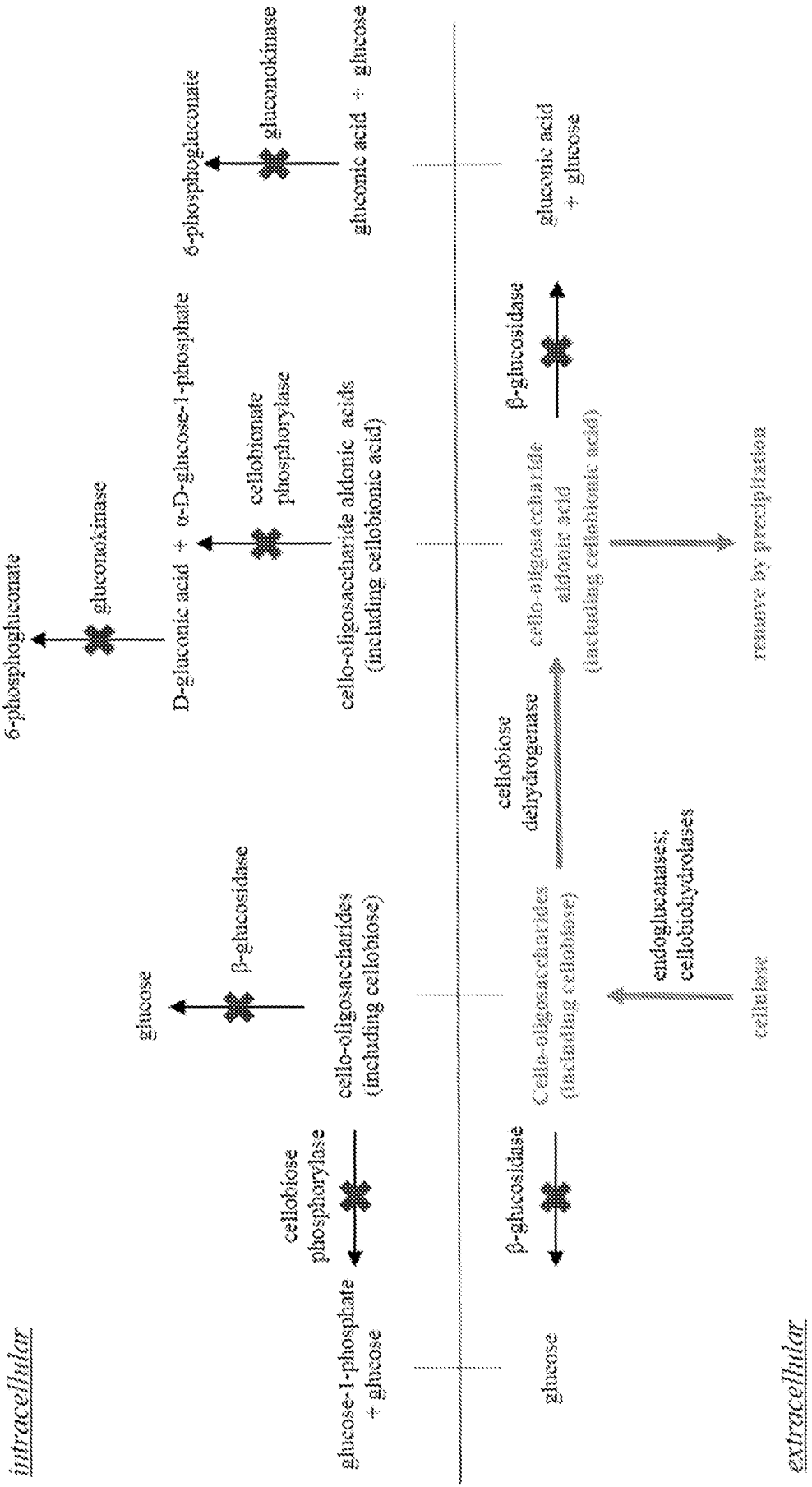
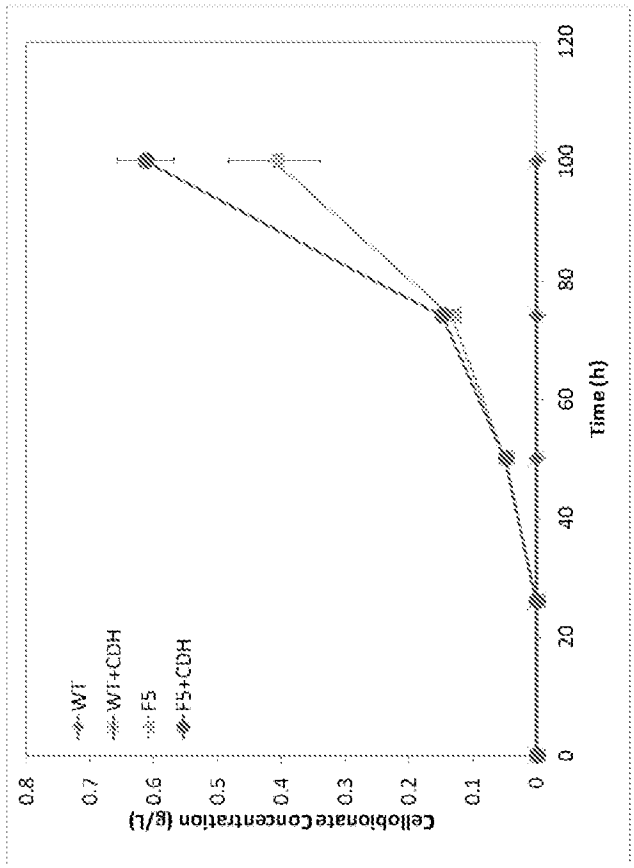

intracellular
6-phosphogluconate
gluconokinase
D-gluconic acid + α-D-glucose-1-phosphate
cellobionate phosphorylase
cello-oligosaccharide aldonic acids (including cellobionic acid)
6-phosphogluconate
gluconokinase
gluconic acid + glucose
β-glucosidase
glucose
cello-oligosaccharides (including cellobiose)
cellobiose dehydrogenase
cello-oligosaccharide aldonic acid (including cellobionic acid)
β-glucosidase
gluconic acid + glucose
cellobiose phosphorylase
glucose-1-phosphate + glucose
β-glucosidase
glucose
Cello-oligosaccharides (including cellobiose)
endoglucanases; cellobiohydrolases
cellulose
remove by precipitation
extracellular

FIG. 4


B
Cellobionate Concentration (g/L)
Time (h)
WT
WT+CDH
F5
F5+CDH
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
20
40
60
80
100
120

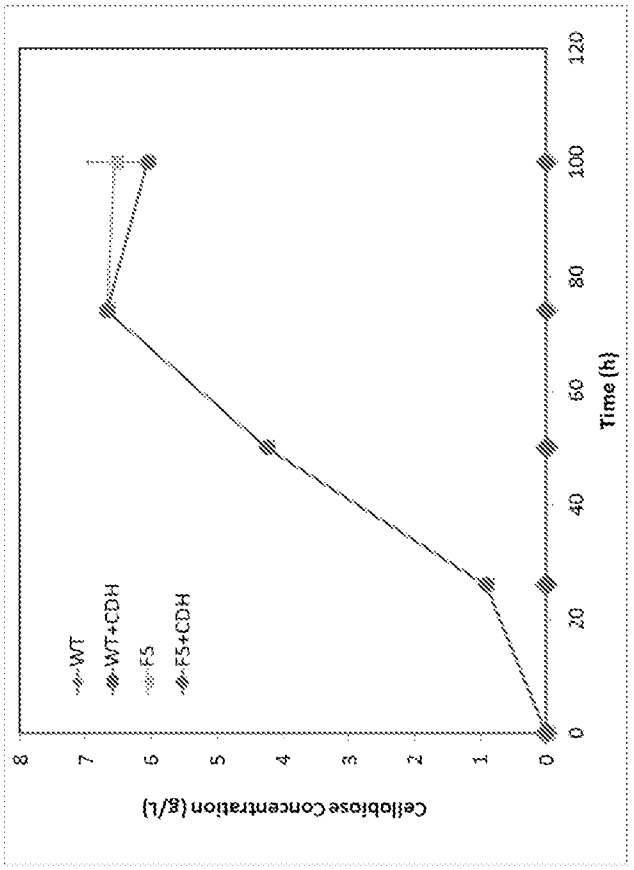
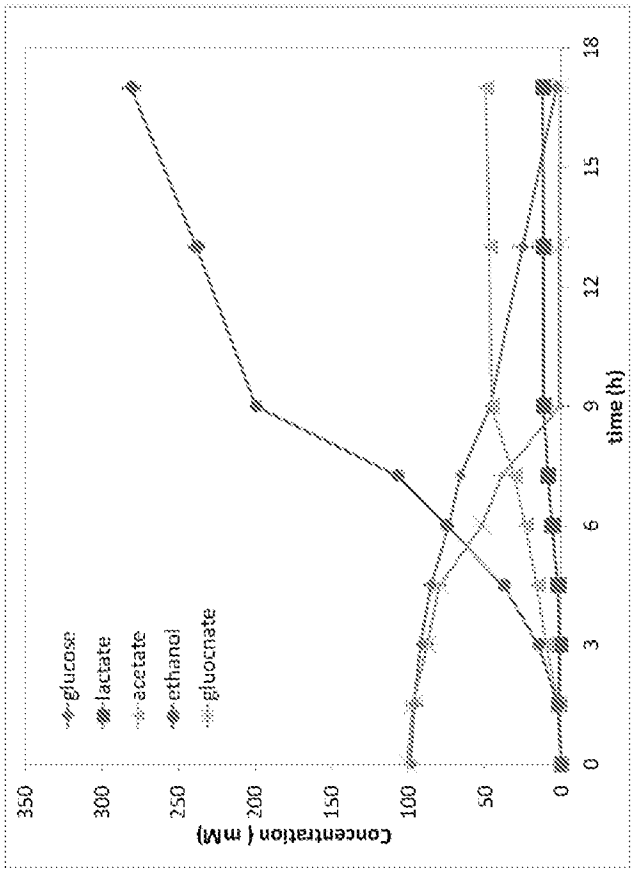

A
Cellobiose Concentration (g/L)
Time (h)
WT
WT+CDH
F5
F5+CDH
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120

FIG. 5


B
glucose
lactate
acetate
ethanol
gluconate
Concentration (mM)
time (h)
0
50
100
150
200
250
300
350
0
3
6
9
12
15
18

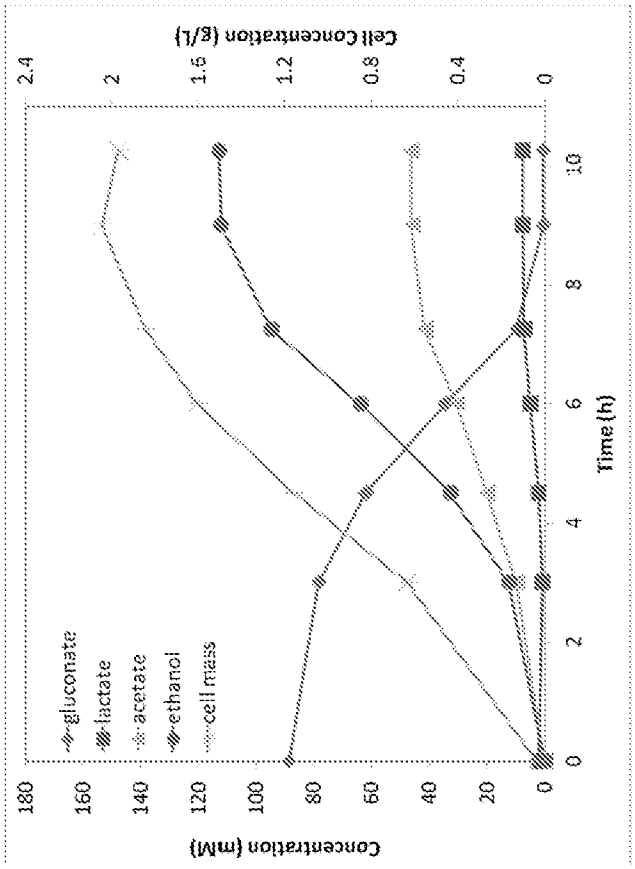

A
gluconate
lactate
acetate
ethanol
cell mass
Concentration (mM)
Cell Concentration (g/L)
Time (h)
0
20
40
60
80
100
120
140
160
180
0
0.4
0.8
1.2
1.6
2
2.4
0
2
4
6
8
10

FIG. 6

	Starting Avicel (g)	Residual Avicel (g)	Cellulose Conversion (%)	Mycelium produced (g)	Yield of cellulase and cellobiase from consumed Avicel (mol/mol×100%)	Yield of mycelium mass from consumed Avicel (g/g×100%)
Wild Type	1.0	0.47±0.05	53±3%	0.28±0.02	0	52±5%
Wild Type +CDH	1.0	0.40±0.03	60±3%	0.31±0.04	0	51±7%
F5	1.0	0.36±0.01	64±1%	0.14±0.02	52±7%	22±2%
F5+CDH	1.0	0.35±0.0002	65±0.02%	0.16±0.01	49±2%	24±1%

FIG. 7

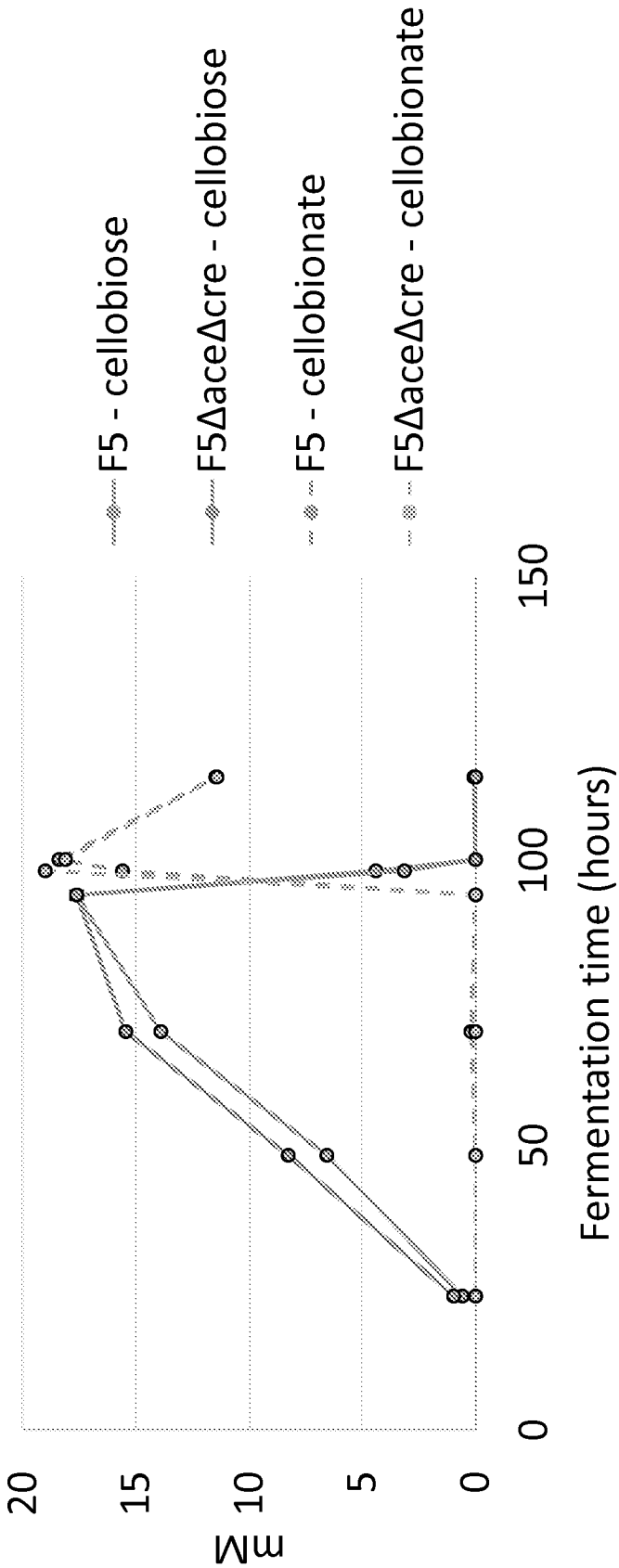

Cellobiose Conversion to Cellobionate
F5 - cellobiose
F5ΔaceΔcre - cellobiose
F5 - cellobionate
F5ΔaceΔcre - cellobionate
mM
20
15
10
5
0
0
50
100
150
Fermentation time (hours)

FIG. 8

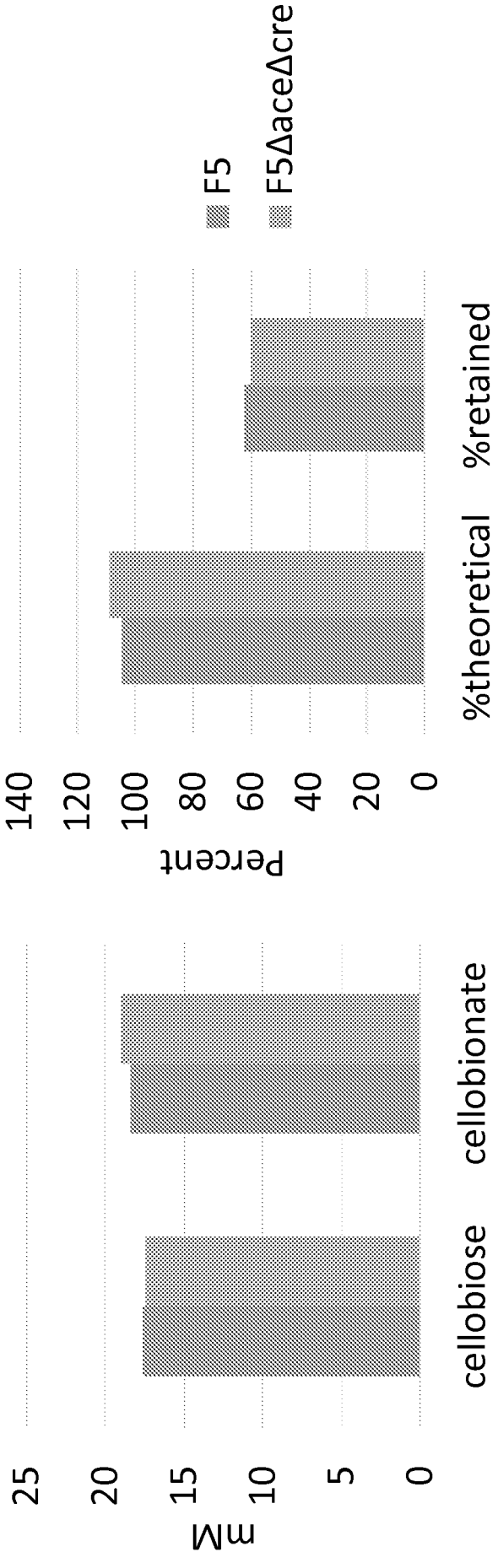

A
mM
25
20
15
10
5
0
cellobiose
cellobionate
B
Percent
140
120
100
80
60
40
20
0
%theoretical
%retained
F5
F5ΔaceΔcre

FIG. 9

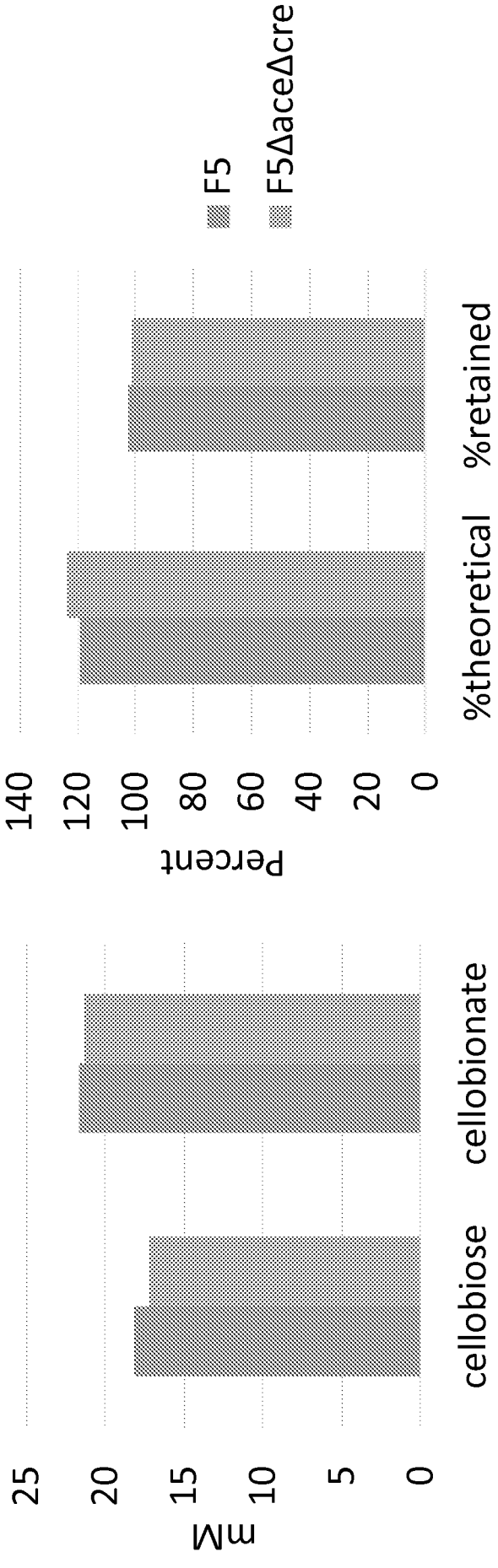

A
mM
25
20
15
10
5
0
cellobiose
cellobionate
B
Percent
140
120
100
80
60
40
20
0
%theoretical
%retained
F5
F5ΔaceΔcre

FIG. 10

A

	Cellobiose (mM)		Cellobionate (mM)
Sample	measured (HPLC)	predicted from hydrolysis	measured (HPLC)
1	5.83	7.18	7.08
2	9.26	11.33	11.41
3	8.71	10.72	10.45

B

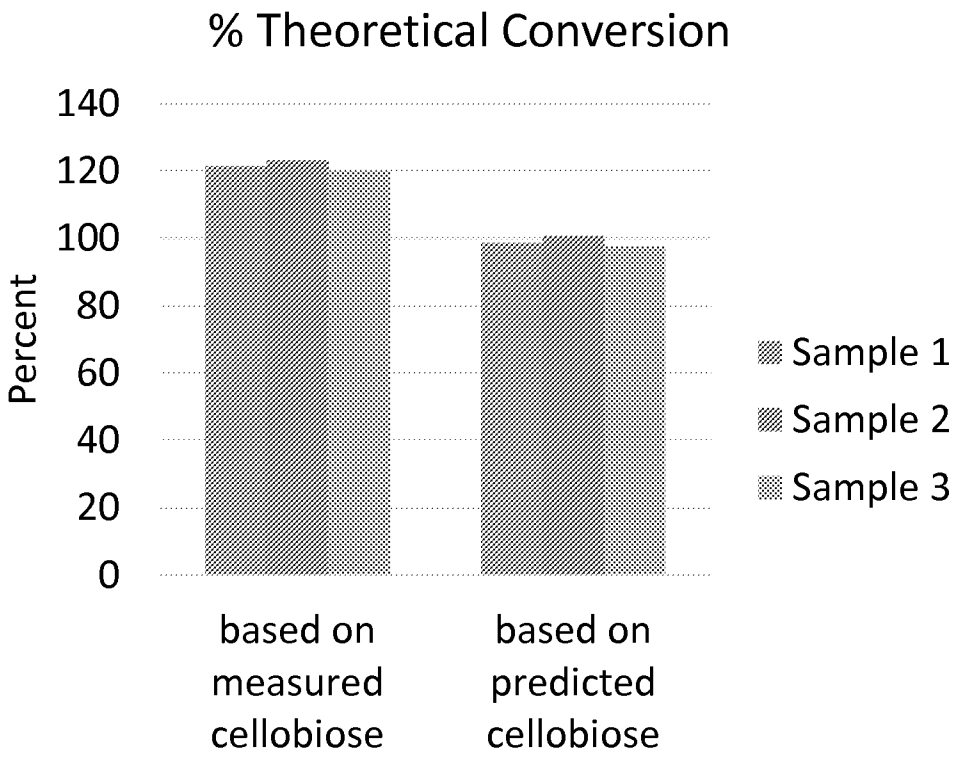
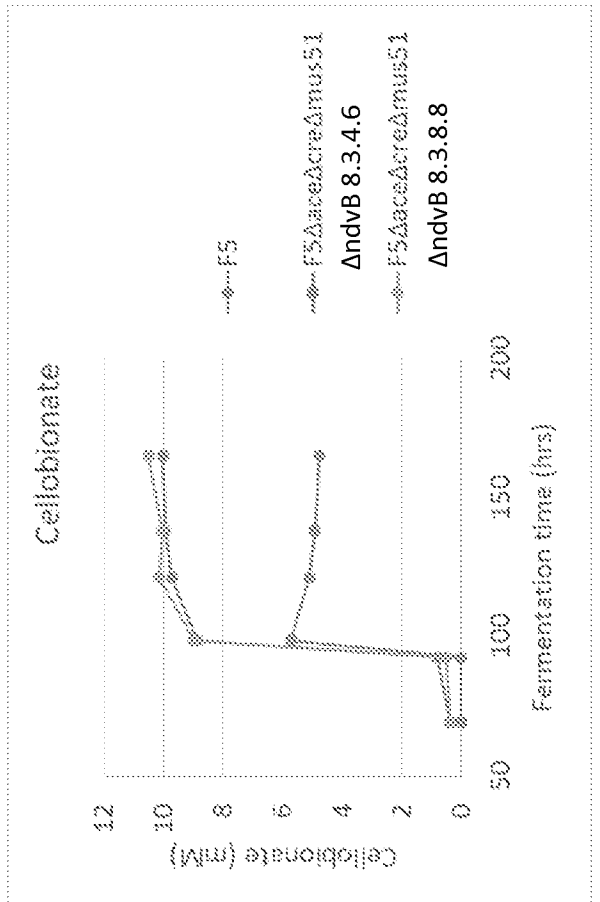
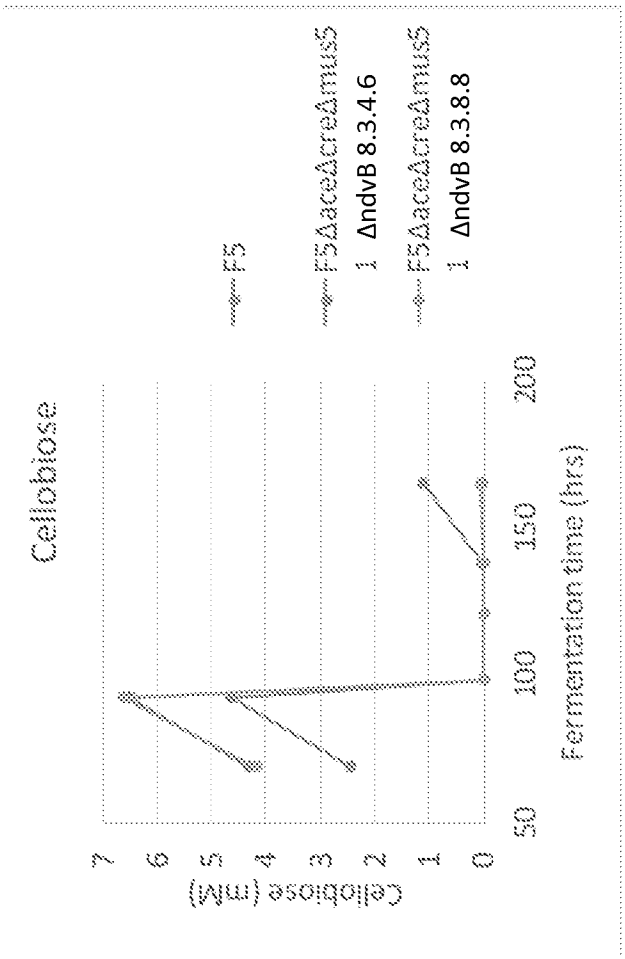
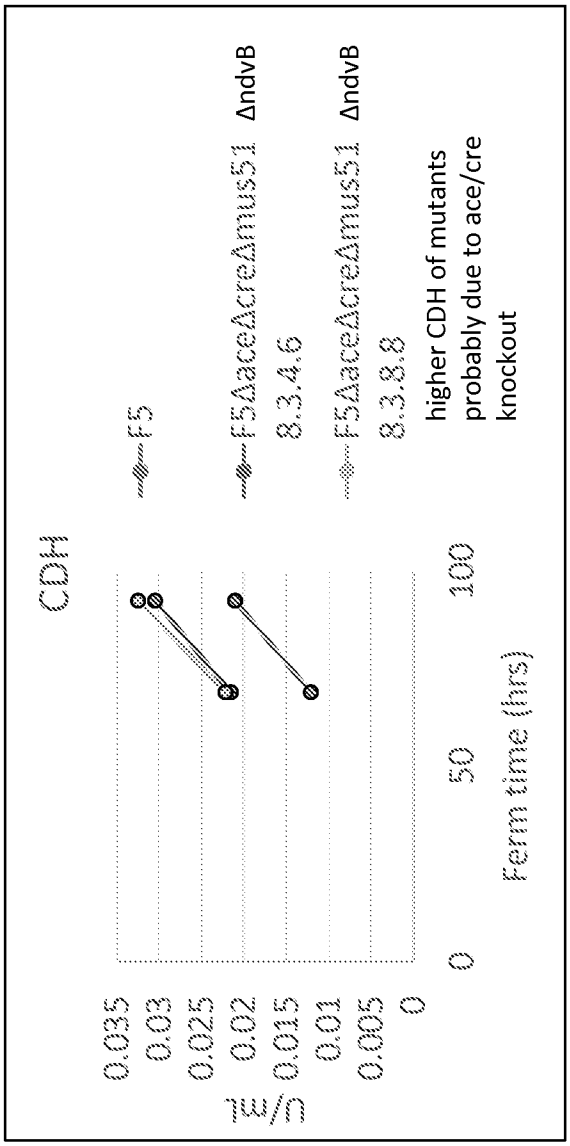


FIG. 11


A
Cellobionate
Cellobionate (mM)
Fermentation time (hrs)
F5
F5ΔaceΔcreΔmus51
ΔndvB 8.3.4.6
F5ΔaceΔcreΔmus51
ΔndvB 8.3.8.8


B
Cellobiose
Cellobiose (mM)
Fermentation time (hrs)
F5
F5ΔaceΔcreΔmus5
1 ΔndvB 8.3.4.6
F5ΔaceΔcreΔmus5
1 ΔndvB 8.3.8.8


C
CDH
U/mL
Ferm time (hrs)
F5
F5ΔaceΔcreΔmus51 ΔndvB 8.3.4.6
F5ΔaceΔcreΔmus51 ΔndvB 8.3.8.8
higher CDH of mutants probably due to ace/cre knockout

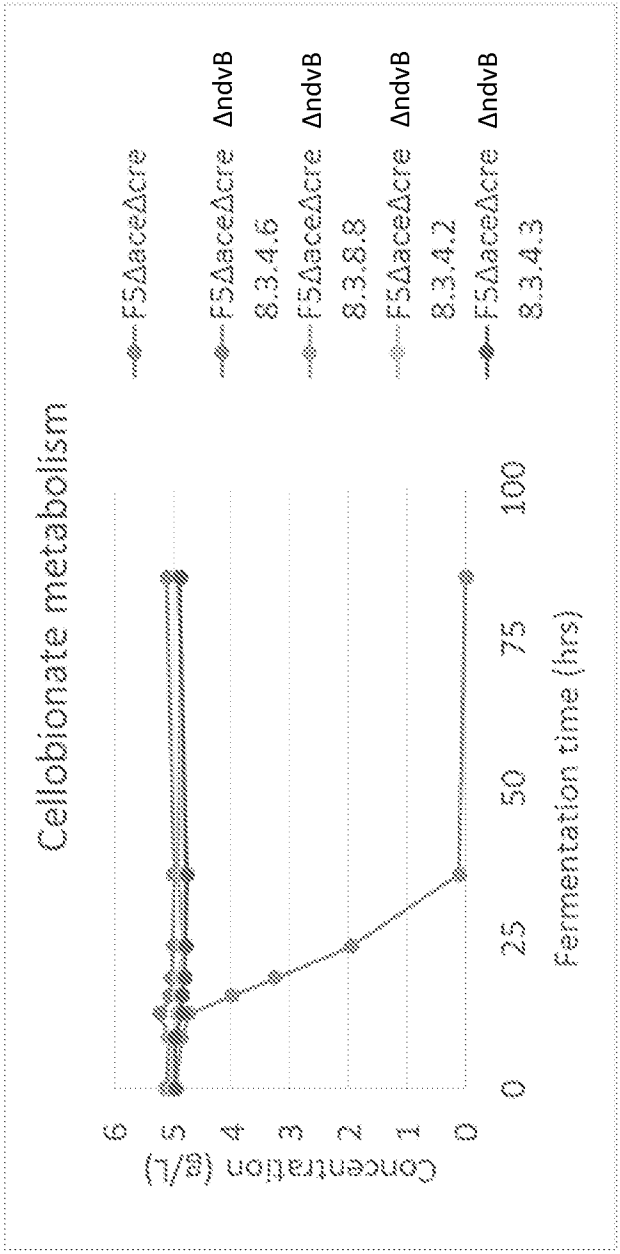

Cellobionate metabolism
F5ΔaceΔcre
F5ΔaceΔcre ΔndvB 8.3.4.6
F5ΔaceΔcre ΔndvB 8.3.8.8
F5ΔaceΔcre ΔndvB 8.3.4.2
F5ΔaceΔcre ΔndvB 8.3.4.3
Concentration (g/L)
0
1
2
3
4
5
6
0
25
50
75
100
Fermentation time (hrs)

FIG. 12

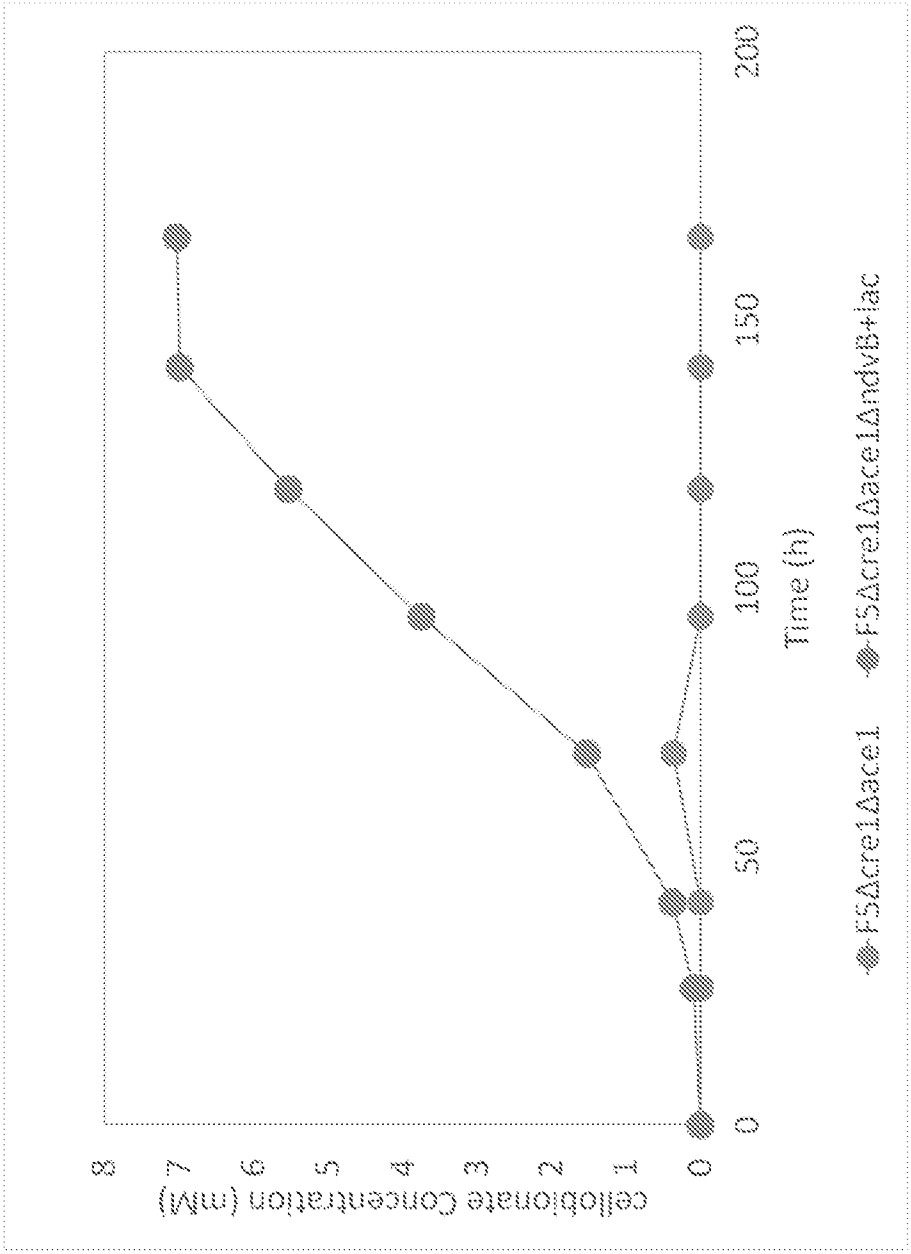

cellobionate Concentration (mM)
0
1
2
3
4
5
6
7
8
0
50
100
150
200
Time (h)
F5Δcre1Δace1
F5Δcre1Δace1ΔndvB+lac

FIG. 13

FIG. 14

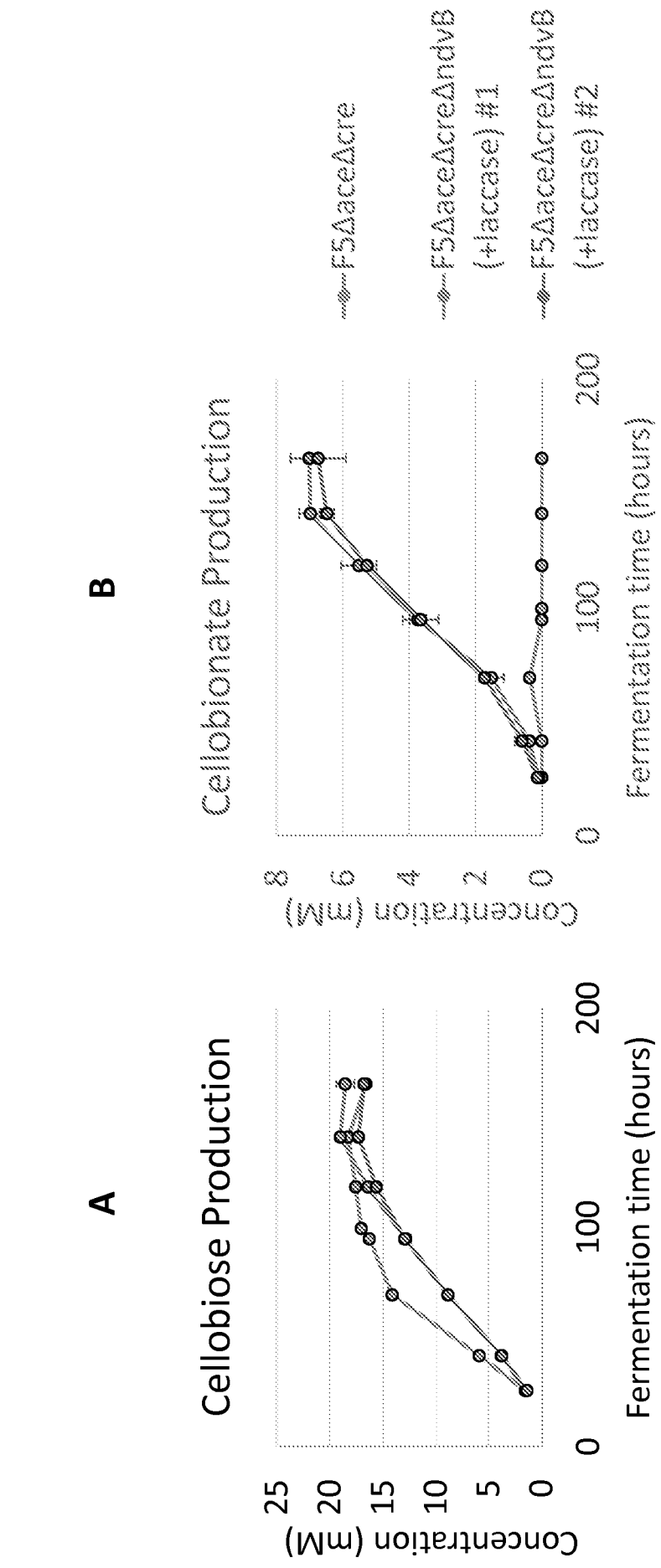

A
Cellobiose Production
Concentration (mM)
25
20
15
10
5
0
0
100
200
Fermentation time (hours)
B
Cellobionate Production
Concentration (mM)
8
6
4
2
0
0
100
200
Fermentation time (hours)
F5ΔaceΔcre
F5ΔaceΔcreΔndvB (+laccase) #1
F5ΔaceΔcreΔndvB (+laccase) #2

FIG. 15

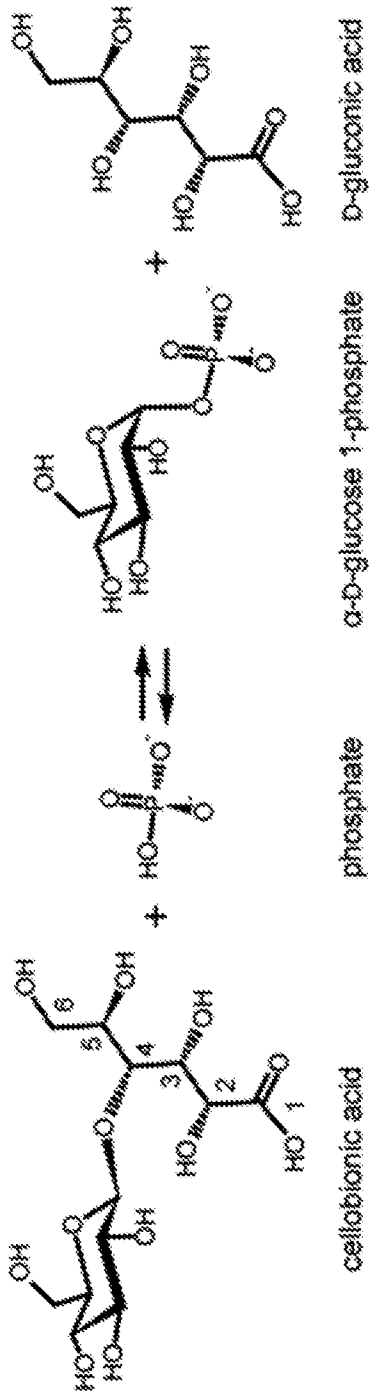

cellobionic acid
phosphate
α-D-glucose 1-phosphate
D-gluconic acid

FIG. 16

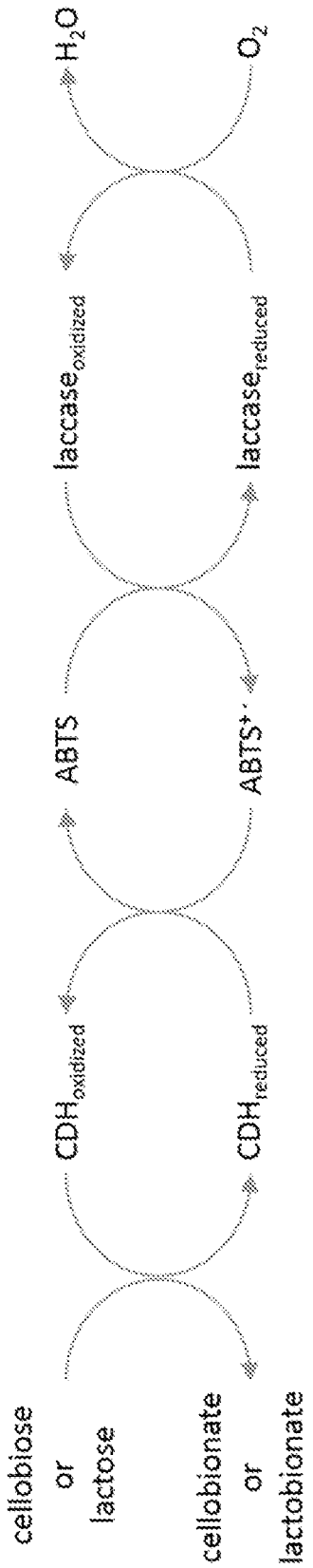
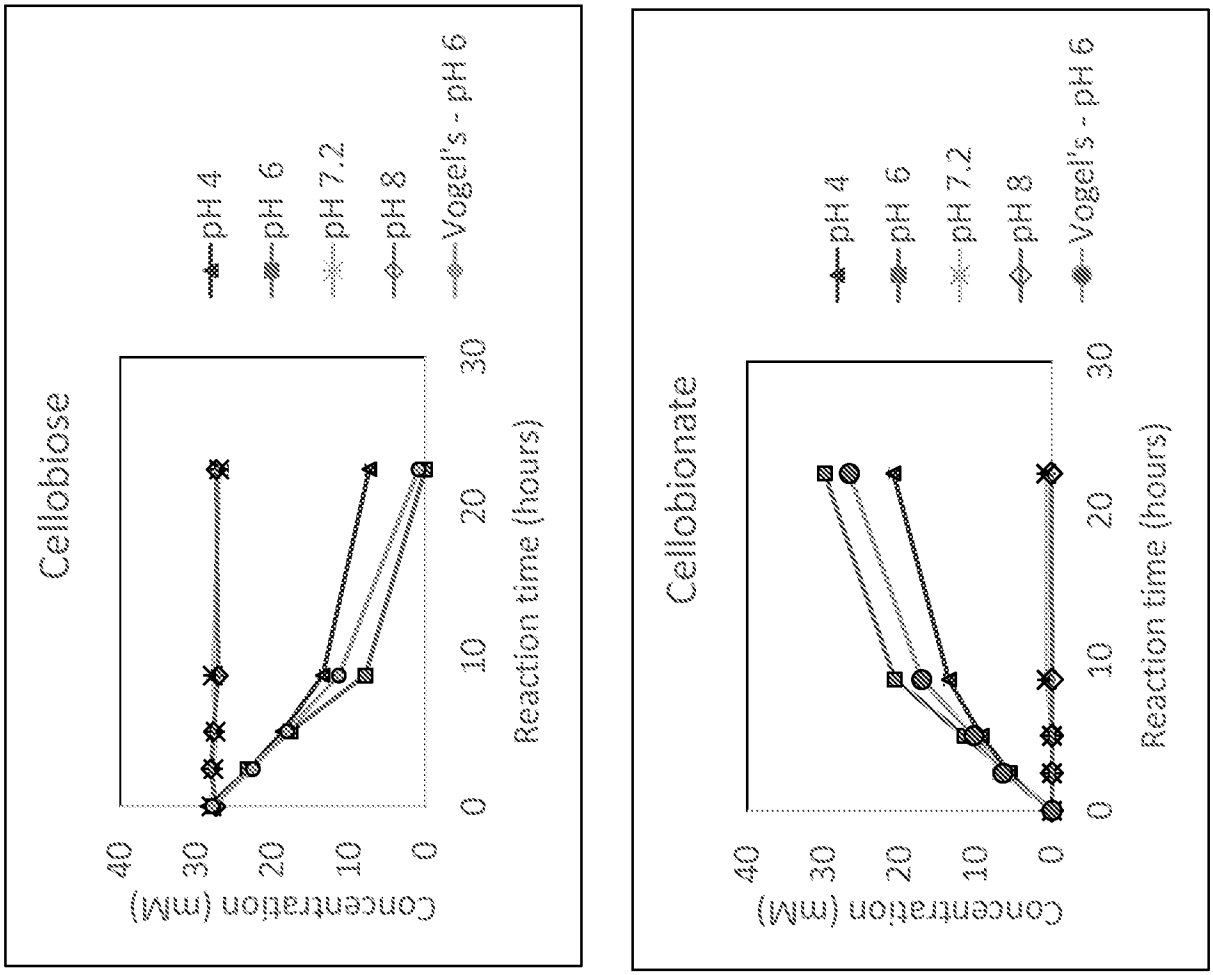

cellobiose
or
lactose
cellobionate
or
lactobionate
CDH oxidized
CDH reduced
ABTS
ABTS+·
laccase oxidized
laccase reduced
H2O
O2

FIG. 17


A
Cellobiose
Concentration (mM)
0
10
20
30
40
Reaction time (hours)
0
10
20
30
pH 4
pH 6
pH 7.2
pH 8
Vogel's - pH 6
B
Cellobionate
Concentration (mM)
0
10
20
30
40
Reaction time (hours)
0
10
20
30
pH 4
pH 6
pH 7.2
pH 8
Vogel's - pH 6

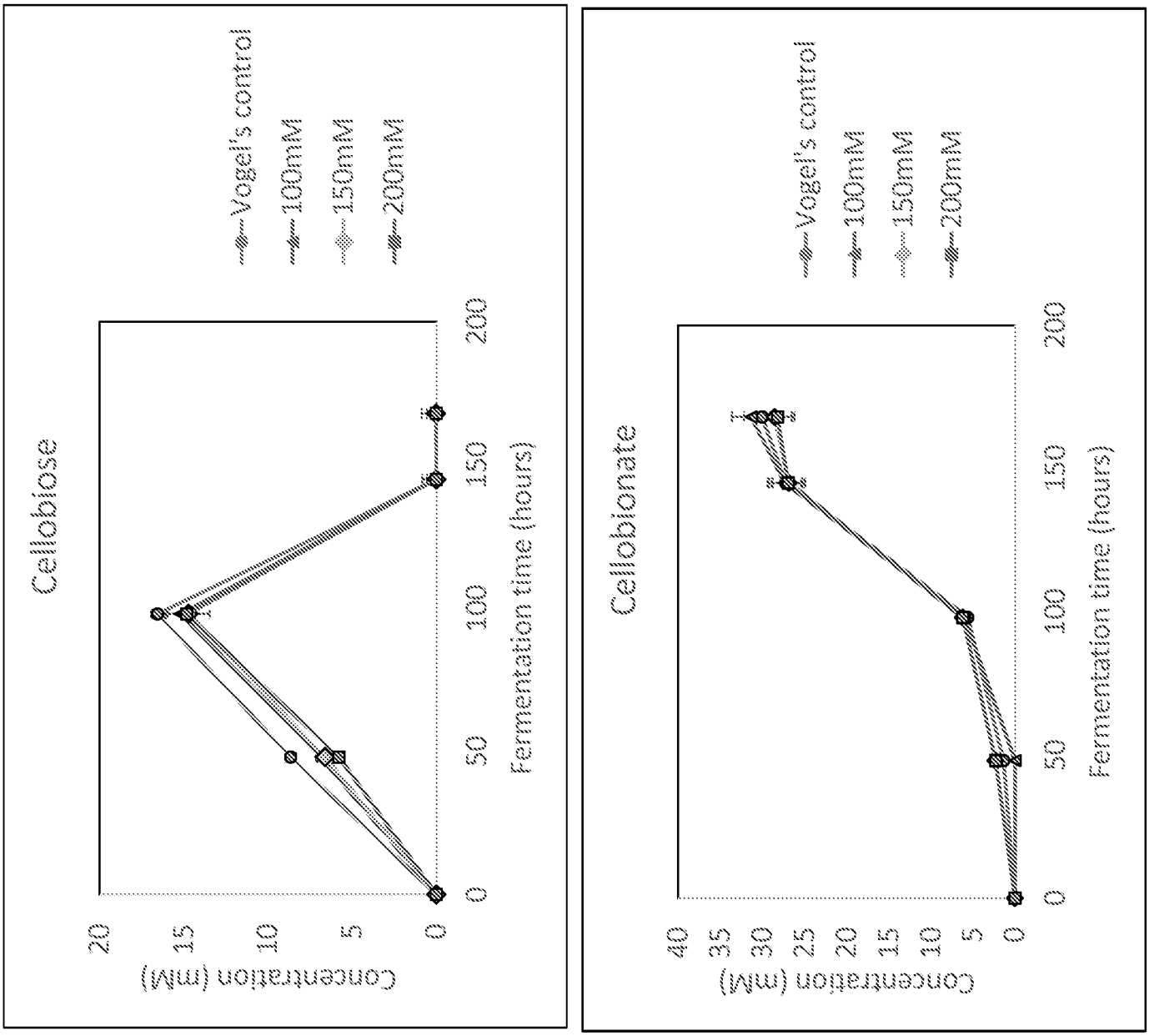
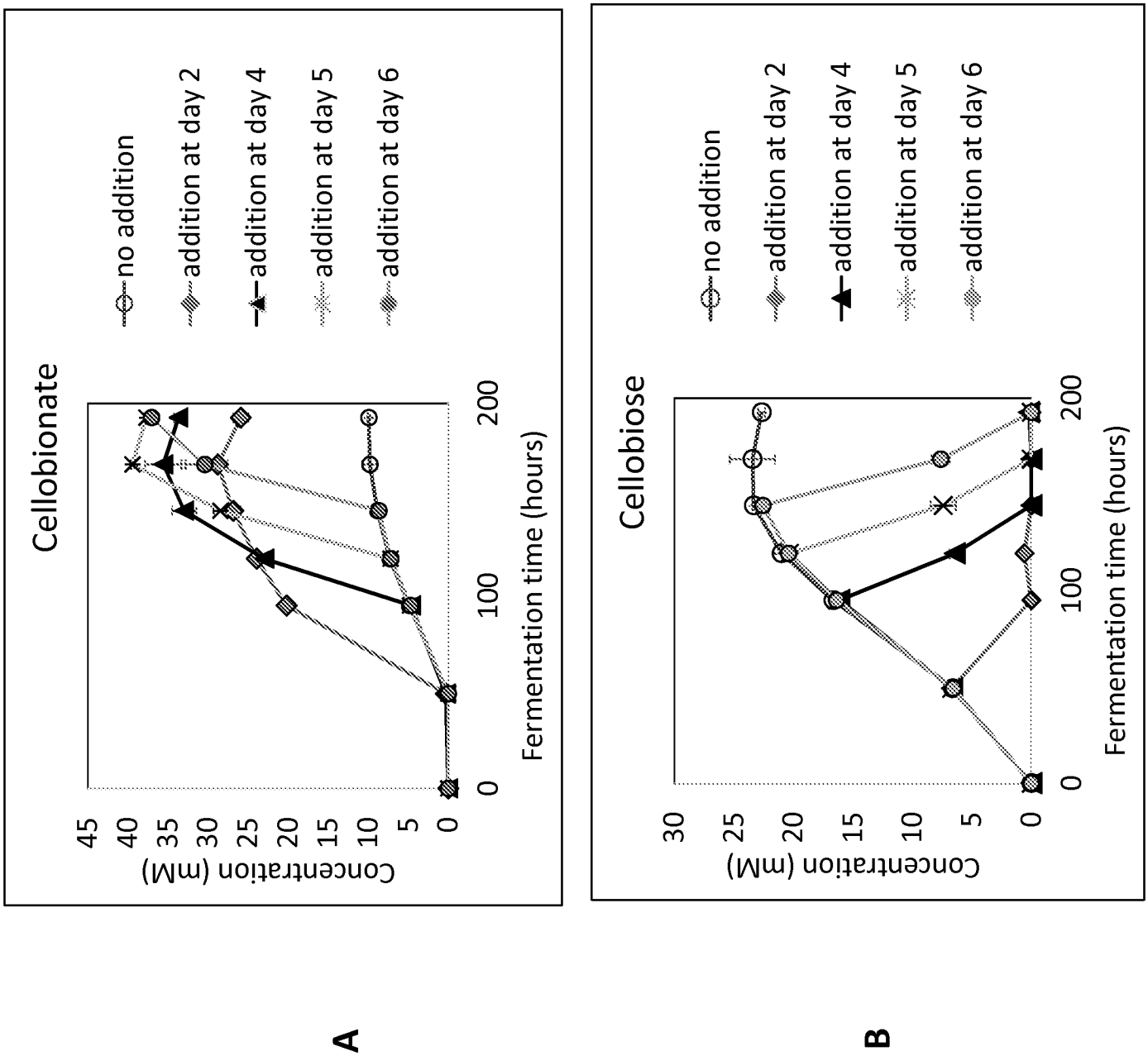

Cellobiose
Vogel's control
100mM
150mM
200mM
Concentration (mM)
Fermentation time (hours)
0
5
10
15
20
50
100
150
200
A
Cellobionate
Vogel's control
100mM
150mM
200mM
Concentration (mM)
Fermentation time (hours)
0
5
10
15
20
25
30
35
40
50
100
150
200
B

FIG. 18

FIG. 19


A
Cellobionate
Concentration (mM)
Fermentation time (hours)
no addition
addition at day 2
addition at day 4
addition at day 5
addition at day 6
B
Cellobiose
Concentration (mM)
Fermentation time (hours)
no addition
addition at day 2
addition at day 4
addition at day 5
addition at day 6

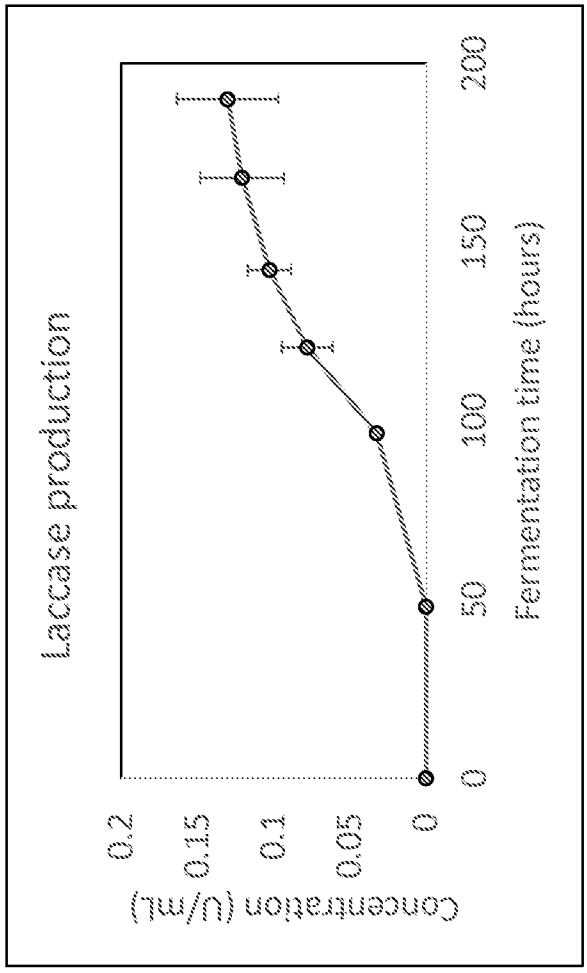

Laccase production
concentration (U/mL)
0.2
0.15
0.1
0.05
0
0
50
100
150
200
Fermentation time (hours)

FIG. 20

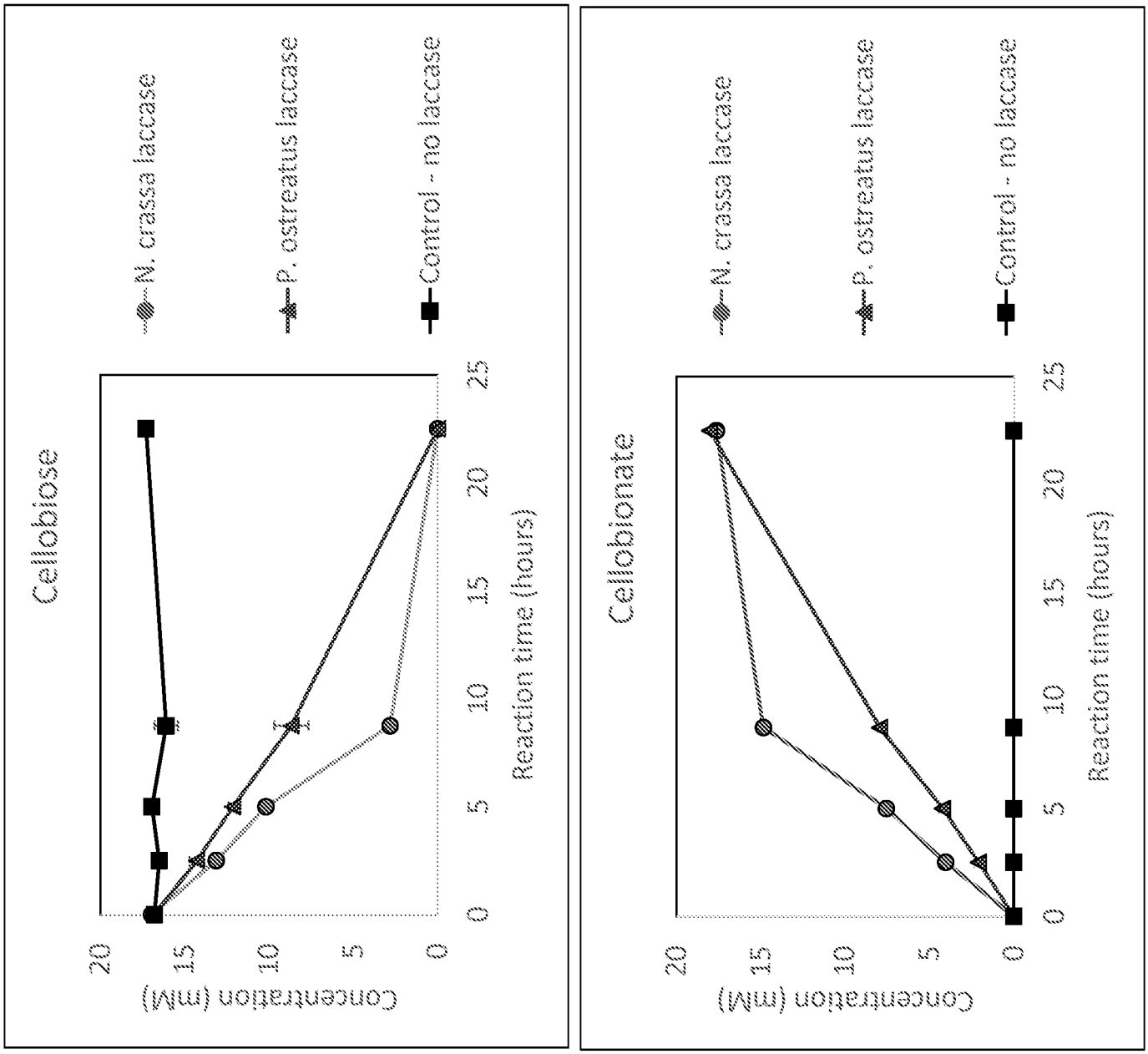

Cellobiose
N. crassa laccase
P. ostreatus laccase
Control - no laccase
Concentration (mM)
Reaction time (hours)
0
5
10
15
20
25
A
Cellobionate
N. crassa laccase
P. ostreatus laccase
Control - no laccase
Concentration (mM)
Reaction time (hours)
0
5
10
15
20
25
B

FIG. 21

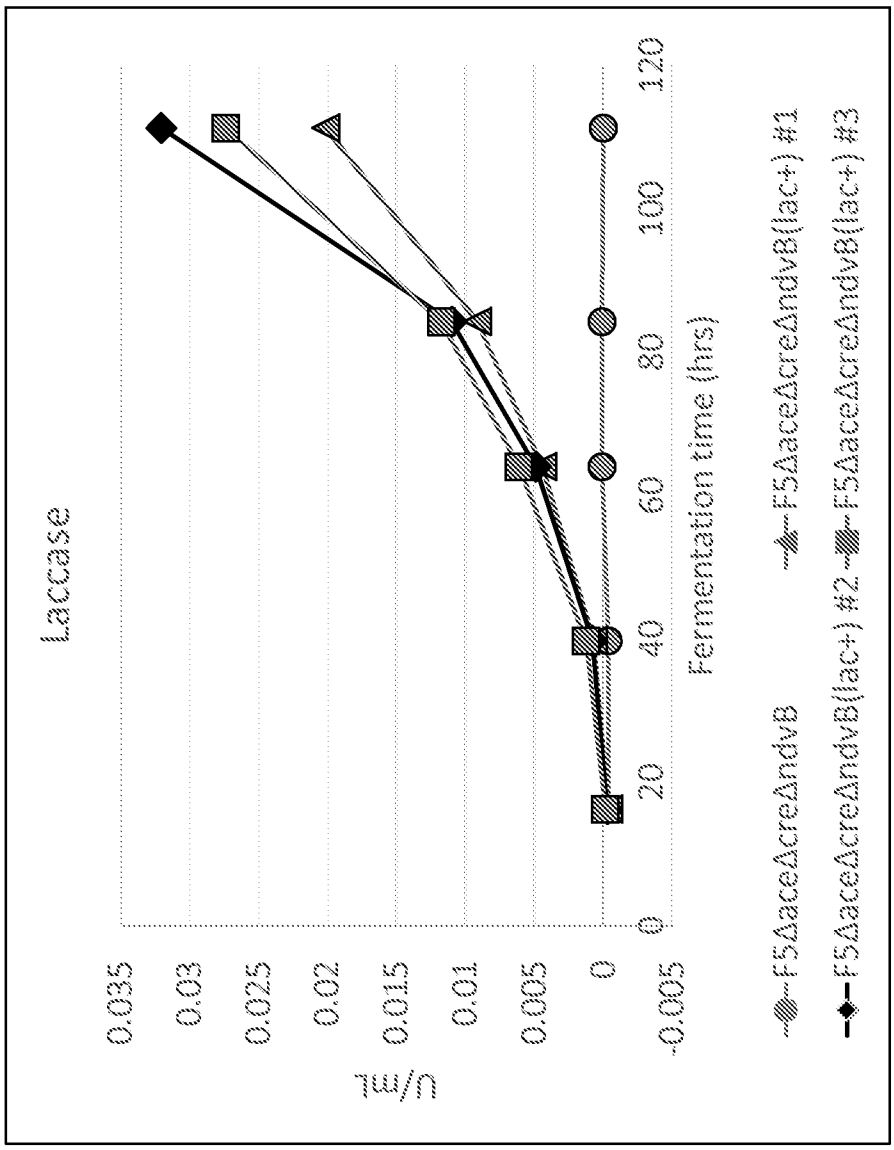
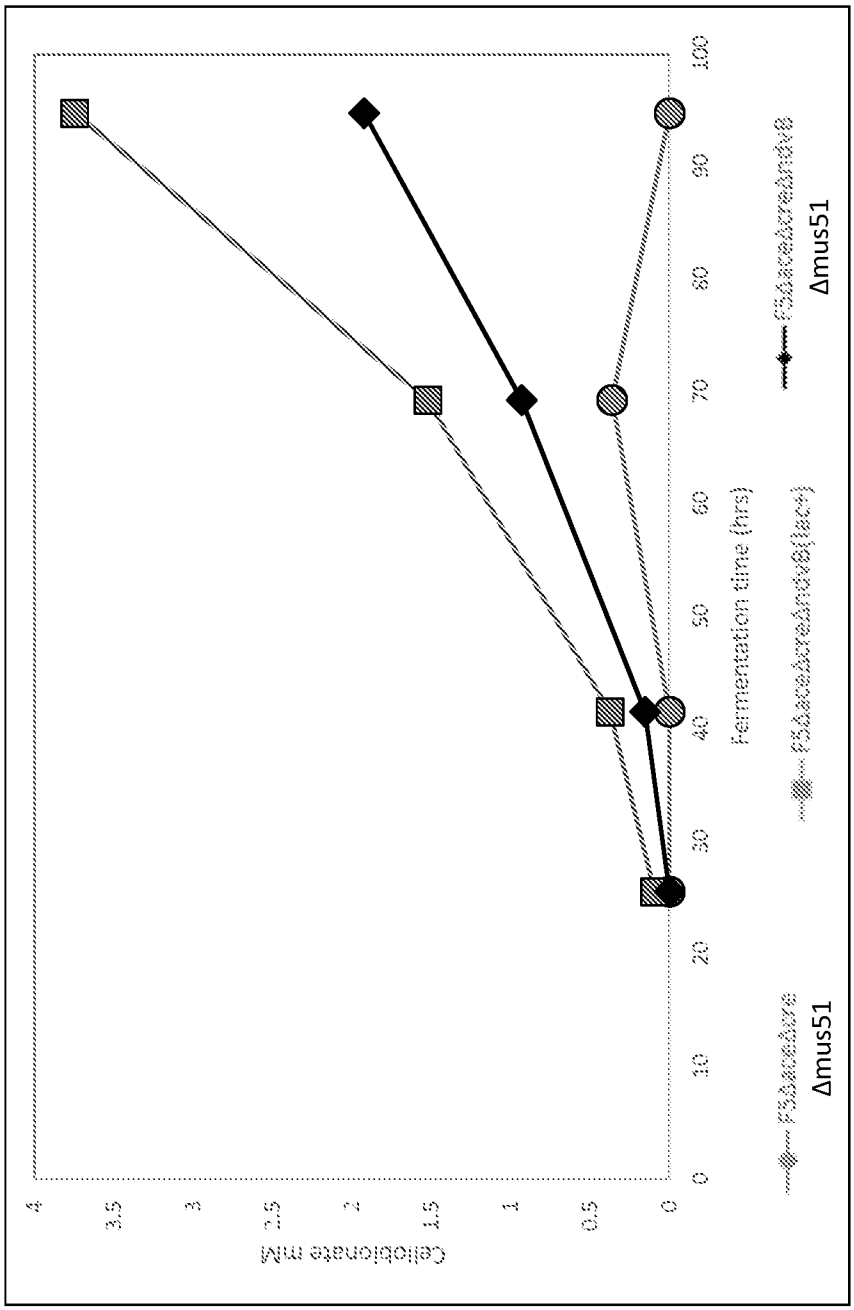

Laccase
U/mL
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
-0.005
0
20
40
60
80
100
120
Fermentation time (hrs)
F5ΔaceΔcreΔndvB
F5ΔaceΔcreΔndvB(lac+) #1
F5ΔaceΔcreΔndvB(lac+) #2
F5ΔaceΔcreΔndvB(lac+) #3

FIG. 22

FIG. 23


Cellobionate mM
4
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
90
100
Fermentation time (hrs)
FSAace4xre4nov8
Δmus51
FSAace4xre4nov8(lac+)
FSAace4xre
Δmus51

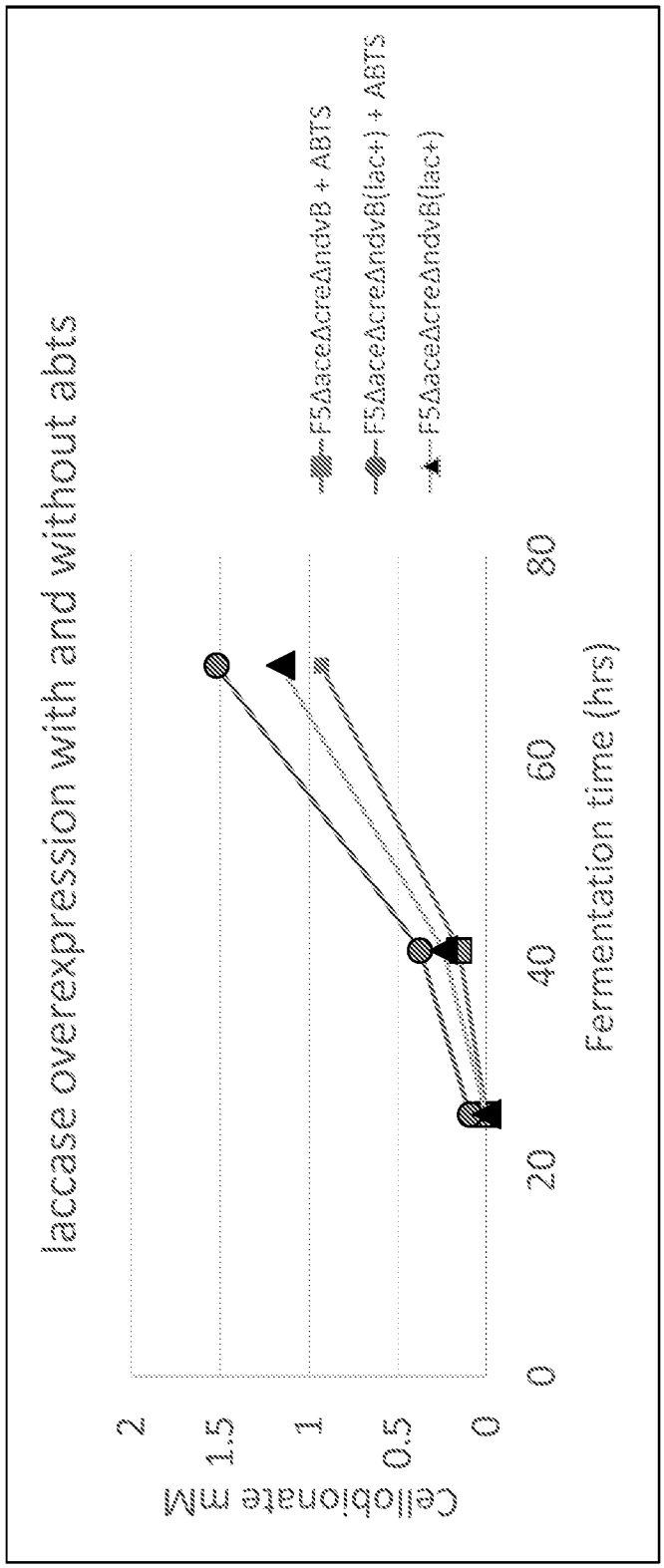

laccase overexpression with and without abts
F5ΔaceΔcreΔndvB + ABTS
F5ΔaceΔcreΔndvB(lac+) + ABTS
F5ΔaceΔcreΔndvB(lac+)
cellobionate mM
0
0.5
1
1.5
2
0
20
40
60
80
Fermentation time (hrs)

FIG. 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/028090

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/80 (2015.01)
CPC - C12N 15/80 (2015.07)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12N 15/79, 15/80; C12P 19/00, 19/02, 19/14 (2015.01)
USPC - 435/41, 72, 99, 440, 471

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12N 15/79, 15/80; C12P 19/00, 19/02, 19/14 (2015.07)
(keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, PubMed, Google
Search terms used: recombinant host cell reduced activity glucosidase cellobionate phosphorylase ndvb cre-1 ace-1 neurospora crassa mutation wild type

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0129221 A1 (FAN et al) 24 May 2012 (24.05.2012) entire document	25
Y	BEAUDOIN et al. "The Biofilm-Specific Antibiotic Resistance Gene ndvB Is Important for Expression of Ethanol Oxidation Genes in Pseudomonas aeruginosa Biofilms," Journal of Bacteriology, 13 April 2012 (13.04.2012), Vol. 194, No. 12, Pgs. 3128–3136. entire document	25
Y	WO 2013/022594 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 14 February 2013 (14.02.2013) entire document	25
Y	RAUSCHER et al. "Transcriptional Regulation of xyn1, Encoding Xylanase I, in Hypocrea jecorina," Eukaryotic Cell, 01 March 2006 (01.03.2006), Vol. 5, No. 3, Pgs. 447–456, entire document	25
Y	WO 2012/138474 A1 (DANISCO US INC. 11 October 2012 (11.10.2012) entire document	25
P, X	HILDEBRAND et al. "Production of Cellobionate from Cellulose Using an Engineered Neurospora crassa Strain with Laccase and Redox Mediator Addition," PLoS One, 07 April 2015 (07.04.2015), Vol. 10, No. 4, Pgs. 1-12. entire document	1-5, 25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2015	10 AUG 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/028090

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-20 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/028090

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

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

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

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

3. ☒ Claims Nos.: 6-24, 26-33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.