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(54) Title: MUTANT CELLS FOR PROTEIN SECRETION AND LIGNOCELLULOSE DEGRADATION

(57) Abstract: The present disclosure provides mutant cells for the secretion of proteins and for the degradation of lignocellulosic biomass. Methods for the use of these cells are also provided. Specifically, the utility of combined genetic deletions of β-glucosidases and the catabolite repressor gene creA/*cre-1* for protein secretion in fungal and yeast cells is disclosed.

MUTANT CELLS FOR PROTEIN SECRETION AND LIGNOCELLULOSE DEGRADATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/453,086, filed March 15, 2011, which is hereby incorporated 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: 677792001640SEQLIST.txt, date recorded: March 14, 2012, size: 22 KB).

FIELD

[0003] The present disclosure relates to mutant cells for the production of proteins, such as cellulases, and for the degradation of lignocellulosic biomass. In particular, mutant cells and methods for the production of proteins, such as cellulases, are provided.

BACKGROUND

[0004] Lignocellulosic biomass is an abundant and renewable raw material for biofuel production. However, the initial conversion of insoluble lignocellulosic biomass into cell-permeable and readily fermentable sugars presents a significant technical challenge and major bottleneck in the biofuel production process. Improved means to overcome this bottleneck are therefore needed to unlock the full potential of lignocellulosic biomass as a versatile energy source.

[0005] The natural degradation of biomass is achieved by fungal microorganisms through their secretion of lignocellolytic enzymes. For example, the filamentous fungus and laboratory model organism *Neurospora crassa* (*N. crassa*) is often found in the wild growing upon recently burnt plant matter, where it secretes cellulases and thereby initiates the depolymerization of plant

cell walls. Based on their natural role in lignocellulose degradation, filamentous fungi and their lignocellolytic enzymes have great potential as catalysts of biomass degradation in biotechnological production processes.

[0006] However, whereas cellulase secretion in filamentous fungi is effectively induced by insoluble plant cell wall components, such as cellulose, hemicellulose, or xylan, soluble inducers are much less effective. For example, cellobiose, the main soluble end product of cellulases, induces cellulases in several species of filamentous fungi, including *Hypocrea jecorina (Trichoderma reesei*; *T. reesei*) and *Aspergillus species (A. niger, A. nidulans*, *A. oryzae)* but at much lower levels than cellulose itself. However, one problem with insoluble inducers is that cellulase can adhere to insoluble inducers, resulting in reduced yields of secreted enzyme activity.

[0007] The processing of insoluble biomass matter is a heterogeneous process and access to biomass surfaces is limiting for fungal cells. In rich fungal cultures therefore, a large population of cells will be free-floating and not secreting high levels of active cellulase enzymes, due to their lack of contact with inducing plant surfaces. To optimize the production of proteins, including cellulase enzymes, in such cell suspensions and thereby facilitate biomass degradation, cellular systems are needed that secrete high levels of active proteins after induction with soluble small molecules, such as cellodextrin.

BRIEF SUMMARY

[0008] Provided herein are mutant cells for increasing secretion of proteins and for the degradation of lignocellulosic biomass. Also provided are methods for increasing secretion of proteins and for degrading lignocellulosic biomass using the mutant cells described herein. Moreover, the present disclosure is based, at least in part on the surprising discovery that mutating β-glucosidase genes and/or the catabolite repressor gene, *cre-1*, in filamentous fungi, such as *Neurospora crassa*, results in an increase in the secretion of proteins when induced by cellulosic biomass, such as cellobiose. Without wishing to be bound by theory, it is believed that the activity of β-glucosidase genes and the *cre-1* is involved in the transcriptional regulation of proteins (Fig. 1).

[0009] Accordingly, one aspect of the present disclosure provides a method for increasing secretion of a protein from a cell, by: (a) providing a mutant cell, where the mutant cell contains inactivating mutations in two or more β-glucosidase genes; and (b) contacting the mutant cell with cellulosic biomass, where the cellulosic biomass induces the mutant cell to secrete the protein. In certain embodiments, the mutant cell further contains an inactivating mutation in a cre-1 gene in the cell. Another aspect of the present disclosure provides a method for increasing secretion of a protein from a cell, by: (a) providing a mutant cell, where the mutant cell contains an inactivating mutation in a cre-1 gene in the cell; and (b) contacting the mutant cell with a cellulosic biomass, where the cellulosic biomass induces the mutant cell to secrete the protein. In certain embodiments, the mutant cell further contains inactivating mutations in two or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes cellobiose.

[0010] Accordingly, one aspect of the present disclosure provides a method for increasing secretion of a protein from a cell, by: (a) providing a mutant cell, where the mutant cell contains inactivating mutations in two or more β-glucosidase genes; and (b) contacting the mutant cell with a saccharide, where the saccharide induces the mutant cell to secrete the protein. In certain embodiments, the mutant cell further contains an inactivating mutation in a cre-1 gene in the cell. Another aspect of the present disclosure provides a method for increasing secretion of a protein from a cell, by: (a) providing a mutant cell, where the mutant cell contains an inactivating mutation in a cre-1 gene in the cell; and (b) contacting the mutant cell with a saccharide, where the saccharide induces the mutant cell to secrete the protein. In certain embodiments, the mutant cell further contains inactivating mutations in two or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is selected from a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is cellobiose.

[0011] In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulose-induced protein. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is selected from a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, and a cellulose binding domain-containing protein, and combinations thereof. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulase. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is encoded by a gene selected from NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137. In certain embodiments that may be combined with any of the preceding embodiments, the mutant cell further contains an inactivating mutation in at least one β-mannosidase gene. In certain embodiments that may be combined with any of the preceding embodiments, the mutant cell further contains an inactivating mutation in at least one phospholipase gene or phospholipase-like gene. In certain embodiments that may be combined with any of the preceding embodiments, the inactivating mutations are deletions. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a recombinant cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a fungal or yeast cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a fungal or yeast cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is selected from *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells, *Schizophyllum commune* cells, *Postia placenta* cells, *Aspergillus oryzae* cells, and *Acremonium cellulolyticus* cells. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are three or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are four or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are five or more β-glucosidase

genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are six or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are seven or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the three or more β-glucosidase genes, four or more β-glucosidase genes, five or more β-glucosidase genes, six or more β-glucosidase genes, or seven or more β-glucosidase genes include NCU00130, NCU04952, and NCU08755. In certain embodiments that may be combined with any of the preceding embodiments, at least one of the β-glucosidase genes encodes an intracellular β-glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, at least one of the β-glucosidase genes encodes an extracellular β-glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, the at least one β-mannosidase gene is NCU00890. In certain embodiments that may be combined with any of the preceding embodiments, the at least one phospholipase gene or phospholipase-like gene is NCU06650.

[0012] Another aspect of the present disclosure provides a method for increasing secretion of a protein from a cell, by: (a) providing a recombinant cell, where the recombinant cell exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell; and (b) contacting the recombinant cell with cellulosic biomass, where the cellulosic biomass induces the recombinant cell to secrete the protein. In certain embodiments, the recombinant cell further exhibits reduced expression of a cre-1 gene compared to the expression of the expression of the cre-1 gene in a corresponding non-recombinant cell. Another aspect of the present disclosure provides a method for increasing secretion of a protein from a cell, by: (a) providing a recombinant cell, where the recombinant cell exhibits reduced expression of a cre-1 gene compared to the expression of the expression of the cre-1 gene in a corresponding non-recombinant cell; and (b) contacting the recombinant cell with cellulosic biomass, where the cellulosic biomass induces the recombinant cell to secrete the protein. In certain embodiments, the recombinant cell further exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes one or more

of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes cellobiose.

[0013] Another aspect of the present disclosure provides a method for increasing secretion of a protein from a cell, by: (a) providing a recombinant cell, where the recombinant cell exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell; and (b) contacting the recombinant cell with a saccharide, where the saccharide induces the recombinant cell to secrete the protein. In certain embodiments, the recombinant cell further exhibits reduced expression of a cre-1 gene compared to the expression of the expression of the cre-1 gene in a corresponding non-recombinant cell. Another aspect of the present disclosure provides a method for increasing secretion of a protein from a cell, by: (a) providing a recombinant cell, where the recombinant cell exhibits reduced expression of a cre-1 gene compared to the expression of the expression of the cre-1 gene in a corresponding non-recombinant cell; and (b) contacting the recombinant cell with a saccharide, where the saccharide induces the recombinant cell to secrete the protein. In certain embodiments, the recombinant cell further exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is selected from a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is cellobiose.

[0014] In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulose-induced protein. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is selected from a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, and a cellulose binding domain-containing protein, and combinations thereof. In certain embodiments that may be combined with any of the preceding embodiments,

the secreted protein is a cellulase. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is encoded by a gene selected from NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137. In certain embodiments that may be combined with any of the preceding embodiments, the function of creA/cre-1 is reduced by overexpression of a dominant negative mutant or a protein inhibitor. In certain embodiments that may be combined with any of the preceding embodiments, the recombinant cell further exhibits reduced expression of at least one β-mannosidase gene compared to the expression of the at least one β-mannosidase genes in a corresponding non-recombinant cell. In certain embodiments that may be combined with any of the preceding embodiments, the recombinant cell further exhibits reduced expression of at least one phospholipase gene or phospholipase-like gene compared to the expression of the at least one phospholipase gene or phospholipase-like gene in a corresponding non-recombinant cell. In certain embodiments that may be combined with any of the preceding embodiments, gene expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are three or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are four or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are five or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are six or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are seven or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the three or more β-glucosidase genes, four or more β-glucosidase genes, five or more β-glucosidase genes, six or more β-glucosidase genes, or seven or more β-glucosidase genes include NCU00130, NCU04952, and NCU08755. In certain embodiments that may be combined with any of the preceding embodiments, at least one of the β-glucosidase genes encodes an intracellular β-glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, at least one of the β-glucosidase genes

encodes an extracellular β-glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, the at least one β-mannosidase gene is NCU00890. In certain embodiments that may be combined with any of the preceding embodiments, the at least one phospholipase gene or phospholipase-like gene is NCU06650. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a stable cell line or a transiently transfected cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a fungal or yeast cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a filamentous fungus of the ascomycete or basidiomycete species. In certain embodiments that may be combined with any of the preceding embodiments, the cell is selected from *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells, *Schizophyllum commune* cells, *Postia placenta* cells, *Aspergillus oryzae* cells, and *Acremonium cellulolyticus* cells.

[0015] Another aspect of the present disclosure provides a mutant cell containing inactivating mutations in two or more β-glucosidase genes, where cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the two or more β-glucosidase genes. In certain embodiments, the mutant cell further contains an inactivating mutation in a cre-1 gene in the cell, where cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell lacking the mutation in the cre-1 gene. In certain embodiments that may be combined with any of the preceding embodiments, the mutant cell further contains an inactivating mutation in at least one β-mannosidase gene, where cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell lacking the mutation in the at least one β-mannosidase gene. In certain embodiments that may be combined with any of the preceding embodiments, the mutant cell further contains an inactivating mutation in at least one phospholipase gene or phospholipase-like gene, where cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell lacking the mutation in the at least one phospholipase gene or phospholipase-like gene. In certain embodiments that may be combined with any of the preceding

embodiments, the cellulosic biomass includes one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes cellobiose.

[0016] Another aspect of the present disclosure provides a mutant cell containing inactivating mutations in two or more β-glucosidase genes, where a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the two or more β-glucosidase genes. In certain embodiments, the mutant cell further contains an inactivating mutation in a cre-1 gene in the cell, where a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell lacking the mutation in the cre-1 gene. In certain embodiments that may be combined with any of the preceding embodiments, the mutant cell further contains an inactivating mutation in at least one β-mannosidase gene, where a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell lacking the mutation in the at least one β-mannosidase gene. In certain embodiments that may be combined with any of the preceding embodiments, the mutant cell further contains an inactivating mutation in at least one phospholipase gene or phospholipase-like gene, where a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell lacking the mutation in the at least one phospholipase gene or phospholipase-like gene. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is selected from a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is cellobiose.

[0017] In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulose-induced protein. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is selected from a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, and a cellulose binding domain-containing protein, and combinations thereof. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulase. In certain embodiments that may be combined with any of the

preceding embodiments, the secreted protein is encoded by a gene selected from NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137. In certain embodiments that may be combined with any of the preceding embodiments, the inactivating mutations are deletions. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a recombinant cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a fungal or yeast cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a filamentous fungus of the ascomycete or basidiomycete species. In certain embodiments that may be combined with any of the preceding embodiments, the cell is selected from *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells, *Schizophyllum commune* cells, *Postia placenta* cells, *Aspergillus oryzae* cells, and *Acremonium cellulolyticus* cells. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are three or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are four or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are five or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are six or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are seven or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the three or more β-glucosidase genes, four or more β-glucosidase genes, five or more β-glucosidase genes, six or more β-glucosidase genes, or seven or more β-glucosidase genes include NCU00130, NCU04952, and NCU08755. In certain embodiments that may be combined with any of the preceding embodiments, at least one of the β-glucosidase genes encodes an intracellular β-glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, at least one of the β-glucosidase genes

encodes an extracellular β-glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, the at least one β-mannosidase gene is NCU00890. In certain embodiments that may be combined with any of the preceding embodiments, the at least one phospholipase gene or phospholipase-like gene is NCU06650.

[0018] Another aspect of the present disclosure provides a recombinant cell exhibiting reduced expression of at least two β-glucosidase genes compared to the expression of the at least twoβ-glucosidase genes in a corresponding non-recombinant cell, where the expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and where cellulosic biomass induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the at least twoβ-glucosidase genes is not reduced In certain embodiments, the cell further exhibits reduced expression of a cre-1 gene compared to the expression of the cre-1 gene in a corresponding non-recombinant cell, where the expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and where cellulosic biomass induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the cre-1 gene is not reduced. In certain embodiments, the function of creA/cre-1 is reduced by overexpression of a dominant negative mutant or a protein inhibitor, where cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell in which the dominant negative mutant is not overexpressed. In certain embodiments that may be combined with any of the preceding embodiments, the cell further exhibits reduced expression of at least one β-mannosidase gene compared to the expression of the at least one β-mannosidase gene in a corresponding non-recombinant cell, where expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and where cellulosic biomass induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the least one β-mannosidase gene is not reduced. In certain embodiments that may be combined with any of the preceding embodiments, the cell further exhibits reduced expression of at least one phospholipase gene or phospholipase-like gene compared to the expression of the at least one phospholipase gene or phospholipase-like gene in a corresponding non-recombinant cell, where expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and where cellulosic biomass induces the cell to secrete higher levels of a protein than the non-recombinant cell in which the expression of the least one

phospholipase gene or phospholipase-like gene is not reduced. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes cellobiose.

[0019] Another aspect of the present disclosure provides a recombinant cell exhibiting reduced expression of at least two β-glucosidase genes compared to the expression of the at least twoβ-glucosidase genes in a corresponding non-recombinant cell, where the expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and where a saccharide induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the at least twoβ-glucosidase genes is not reduced In certain embodiments, the cell further exhibits reduced expression of a cre-1 gene compared to the expression of the cre-1 gene in a corresponding non-recombinant cell, where the expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and where a saccharide induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the cre-1 gene is not reduced. In certain embodiments, the function of creA/cre-1 is reduced by overexpression of a dominant negative mutant or a protein inhibitor, where a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell in which the dominant negative mutant is not overexpressed. In certain embodiments that may be combined with any of the preceding embodiments, the cell further exhibits reduced expression of at least one β-mannosidase gene compared to the expression of the at least one β-mannosidase gene in a corresponding non-recombinant cell, where expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and where a saccharide induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the least one β-mannosidase gene is not reduced. In certain embodiments that may be combined with any of the preceding embodiments, the cell further exhibits reduced expression of at least one phospholipase gene or phospholipase-like gene compared to the expression of the at least one phospholipase gene or phospholipase-like gene in a corresponding non-recombinant cell, where expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and

where a saccharide induces the cell to secrete higher levels of a protein than the non-recombinant cell in which the expression of the least one phospholipase gene or phospholipase-like gene is not reduced. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is selected from a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is cellobiose.

[0020] In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulose-induced protein. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is selected from a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, and a cellulose binding domain-containing protein, and combinations thereof. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulase. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is encoded by a gene selected from NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are three or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are four or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are five or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are six or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the two or more β-glucosidase genes are seven or more β-glucosidase genes. In certain embodiments that may be combined with any of the preceding embodiments, the three or more β-glucosidase genes, four or more β-glucosidase genes, five or more β-glucosidase genes, six or more β-glucosidase genes, or seven or more β-glucosidase genes include NCU00130, NCU04952, and NCU08755. In certain embodiments that may be combined

with any of the preceding embodiments, at least one of the β-glucosidase genes encodes an intracellular β-glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, at least one of the β-glucosidase genes encodes an extracellular β-glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, the at least one β-mannosidase gene is NCU00890. In certain embodiments that may be combined with any of the preceding embodiments, the at least one phospholipase gene or phospholipase-like gene is NCU06650. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a stable cell line or a transiently transfected cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a fungal or yeast cell. In certain embodiments that may be combined with any of the preceding embodiments, the cell is a filamentous fungus of the ascomycete or basidiomycete species. In certain embodiments that may be combined with any of the preceding embodiments, the cell is selected from *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells, *Schizophyllum commune* cells, *Postia placenta* cells, *Aspergillus oryzae* cells, and *Acremonium cellulolyticus* cells.

[0021] Another aspect of the present disclosure relates to a method for the degradation of biomass, by: (a) providing lignocellulosic biomass; (b) providing the cell of any of the preceding embodiments, or a cell containing an inactivating mutation in the cre-1 gene; (c) inducing the cell to secrete a protein by contacting the cell with a cellulosic biomass; and (d) contacting the induced cell with the lignocellulosic biomass, where the secreted protein degrades the lignocellulosic biomass. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the cellulosic biomass includes cellobiose. Another aspect of the present disclosure relates to a method for the degradation of biomass, by: (a) providing lignocellulosic biomass; (b) providing the cell of any of the preceding embodiments, or a cell containing an inactivating mutation in the cre-1 gene; (c) inducing said cell to secrete a protein

by contacting the cell with a saccharide; and (d) contacting the induced cell with the lignocellulosic biomass, where the secreted protein degrades the lignocellulosic biomass. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is selected from a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain embodiments that may be combined with any of the preceding embodiments, the saccharide is cellobiose. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulose-induced protein. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is selected from a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, and a cellulose binding domain-containing protein, and combinations thereof. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is a cellulase. In certain embodiments that may be combined with any of the preceding embodiments, the secreted protein is encoded by a gene selected from NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] **Figure 1** shows a model for transcriptional regulation of cellulases in β-glucosidase deletion strains of *N. crassa*. Both transcriptional de-repression and specific induction are required to achieve maximal transcriptional activation of cellulase gene expression. Arrows indicate possible pathways for cellulose metabolites. Blue lines indicate pathways believed to be minimized in the Δ3βG and Δ3βGΔ*cre* deletion strains; and red lines indicate pathways believed to be most active in the Δ3βG and Δ3βGΔ*cre* deletion strains.

[0023] **Figure 2** shows gene expression time courses for cellulase enzymes in *N. crassa*. **Figure 2A** shows the cellobiohydrolase I (*cbh-1*, NCU07340) time course. **Figure 2B** shows the endoglucanase 2 (*gh5-1*, NCU00762) time course. Expression levels for all genes were normalized to 1 when induced with 2% sucrose. Strains were grown in minimal media with 2% sucrose for 16 hours followed by 4 hours growth in minimal media with 2% Avicel®. Actin

(NCU04173) gene expression levels were used as an endogenous control in all samples. Each reaction was done in triplicate and error bars indicate a 95% confidence interval.

[0024] **Figure 3** shows gene expression of select cellulases after 4 hours induction with 0.2% cellobiose or 1% Avicel® in WT, Δ3βG and Δ3βGΔcre. Gene expression levels of *cbh-1*, *gh6-2*, and *gh5-1* were normalized to 1 when induced with 1% sucrose. Actin was used as an endogenous control in all samples. Each strain was grown in triplicate and error bars indicate 1 standard deviation.

[0025] **Figure 4** shows gene expression levels of cellulases cellobiohydrolase I (*cbh-1*, NCU07340) and endoglucanase 2 (*gh5-1*, NCU00762) in *Δcre-1* at 4 hrs post transfer to minimal media with 2% sucrose. Expression levels for both genes were normalized to 1 for wild type induction with 2% sucrose. Actin (NCU04173) gene expression levels were used as an endogenous control. Each reaction was done in triplicate and error bars indicate a 95% confidence interval.

[0026] **Figure 5** shows gene expression levels of cellulases cellobiohydrolase I (*cbh-1*, NCU07340) and endoglucanase 2 (*gh5-1*, NCU00762) under starvation conditions in wild type (WT), *Δcre-1, Δ4952Δ8755Δ130*, and *Δ4952Δ8755Δ130Δcre-1*. Expression levels for all genes were normalized to 1 when induced with 2% sucrose. Strains were grown in minimal media with 2% sucrose for 16 hrs followed by 4 hrs growth in minimal media with no carbon source added. Actin (NCU04173) gene expression levels were used as an endogenous control in all samples. Each reaction was done in triplicates and error bars indicate a 95% confidence interval.

[0027] **Figure 6** shows gene expression levels of cellulases cellobiohydrolase I (*cbh-1*, NCU07340) and endoglucanase 2 (*gh5-1*, NCU00762) after 4 hour induction with either 10mM or 1mM cellobiose. **Figure 6A** shows results for the wild type. **Figure 6B** shows results for the *Δ4952Δ8755Δ130* deletion mutant. **Figure 6C** shows results for *Δ4952Δ8755Δ130Δcre-1* deletion mutant. Expression levels for all genes were normalized to 1 when induced with 2% sucrose. Strains were grown in minimal media with 2% sucrose for 16 hrs followed by 4 hrs growth in minimal media with 1mM cellobiose, 10mM cellobiose, or 2% sucrose. Actin

(NCU04173) gene expression levels were used as an endogenous control in all samples. Each reaction was done in triplicates and error bars indicate a 95% confidence interval.

[0028] **Figure 7** summarizes protein production and enzyme activity in WT, Δ3βG, and Δ3βGΔcre strains after induction with cellobiose or Avicel®. **Figure 7A** shows the production of cellulases in a bioreactor using Δ3βG induced with cellobiose. **Figure 7B** shows the production of cellulases in a bioreactor using Δ3βGΔcre induced with cellobiose. **Figure 7C** shows the production of cellulases in a bioreactor using WT induced with cellobiose. **Figure 7D** shows the production of cellulases in a bioreactor using WT grown 5 days on Avicel®. Cellobiose-induced strains were pre-grown in minimal media with 1% sucrose for 24 hours before induction with 0.2% cellobiose for 36 hours. The concentration of sucrose, glucose, fructose (in glucose equivalents; triangle) cellobiose (circle), protein production (square), and biomass accumulation (diamond) were measured. **Figure 7E** shows 24-hour induced supernatant activity from 7A, 7B, and 7D towards Avicel®. Cellulase activity of culture supernatant from Δ3βG (squares) and Δ3βGΔcre (triangle) induced with cellobiose for 24 hours compared to culture supernatants from WT grown on Avicel® for 5 days (diamond). Error bars are 1 standard deviation. **Figure 7F** shows Azo-CMC (endoglucanase) activity time course from bioreactor culture supernatants in 7A and 7B. Azo-CMC activity is expressed as a percentage of activity from WT culture supernatant grown on 2% Avicel® for 5 days.

[0029] **Figure 8** compares MuLac activities (cellobiohydrolase I) in culture filtrates from wild type (WT), *Δcre-1*, *Δ4952Δ8755Δ130*, and *Δ4952Δ8755Δ130Δcre-1*. **Figure 8A** shows MuLac activity expressed as a percentage of the wild type activity on Avicel® after 4 days on Avicel®. **Figure 8B** shows MuLac activity expressed as μg purified recombinant Cbh-1 equivalents. Strains were grown in 2% sucrose for 16 hrs followed by 4 days in 2% sucrose, 2% cellobiose, or 2% Avicel® with time points taken at both 2 and 4 days. Exoglucanase activity in the culture supernatant was measured using a 4-Methylumbelliferyl-β-D-cellobioside (MuLac) assay.

[0030] **Figure 9** compares Azo-CM-cellulose (endo-1,4-β-glucanase) activities in culture filtrates from wild type (WT), *Δcre-1*, *Δ4952Δ8755Δ130*, and *Δ4952Δ8755Δ130Δcre-1*. Strains were grown in 1% sucrose for 24 hrs followed by 4 days 2% sucrose, 1% Avicel®, or 2%

cellobiose. The endo-1,4-β-glucanase activity is presented as a percentage of the wild type activity on Avicel® after 4 days and of activity. Note: No data is shown for sucrose cultures because Azo-CM-cellulose activity was not detectable for any of the 4 strains.

[0031] **Figure 10** compares the phenotypes of *N. crassa* wild type (WT) and *Δcre-1* strains. **Figure 10A** shows an SDS-PAGE analysis of secreted proteins in culture filtrates from WT and *Δcre-1* strains grown on Avicel® for 7 days. Protein bands representing β-glucosidase (NCU04952), cellobiohydrolase 1 (*cbh-1*, NCU07340) and 2 (*cbh-2*, NCU09680), and endoglucanase 2 (*gh5-1*, NCU00762) are marked. **Figure 10B** compares the endoglucanase activity on Azo-CMC, protein concentrations, and glucose and cellobiose concentrations as determined by Avicelase assays of 7-day culture supernatants from WT and *Δcre-1* strains.

[0032] **Figure 11** shows a SDS-PAGE analysis of secreted proteins in culture filtrates from wild type (WT), *Δcre-1*, *Δ4952Δ8755Δ130*, and *Δ4952Δ8755Δ130Δcre-1* deletion mutants. Strains were grown in 1% sucrose for 24 hours followed by 4 days in 2% sucrose, 2% cellobiose, 1% sucrose and 1% cellobiose, or 1% sucrose and 1% Avicel® with samples taken at 24 hour time points. 15µl filtered culture supernatant was run on a Criterion 10% Tris-HCl polyacrylamide gel and stained with Thermo Scientific GelCode Blue Stain Reagent. Note: The protein running at 72kDa in *Δcre-1* and *Δ4952Δ8755Δ130Δcre-1* on sucrose has been identified using Mass Spectrometry as NCU01517 (Glucoamylase 1).

[0033] **Figure 12** shows a SDS-PAGE analysis of secreted proteins in culture filtrates from wild type *N. crassa* and *Δ4952Δ8755Δ130 (β-G tKO)*. Strains were grown in 1% sucrose for 24 hrs followed by 5 days in 2% sucrose, 2% cellobiose, or 2% Avicel®. Protein bands representing cellobiohydrolase 1 (*cbh-1*, NCU07340), cellobiohydrolase 2 (*cbh-2*, NCU09680), and endoglucanase 2 (*gh5-2*, NCU00762) are marked. In addition, β-glucosidase (NCU04952) is marked in the wild type and its absence is outlined in the triple deletion *Δ4952Δ8755Δ130*.

[0034] **Figure 13** shows ClustalW alignments for NCU00130 (SEQ ID NO: 1), NCU04952 (SEQ ID NO: 2), and NCU08755 (SEQ ID NO: 3) orthologues in closely related fungi. The entire sequence is provided for the *N. crassa* gene with orthologues displaying only divergent amino acids. A "." indicates an identical residue and "-" indicates an insertion or deletion.

[0035] **Figure 14** shows the evolutionary relationships of β-glucosidase NCU00130 orthologues. The evolutionary history was inferred using the Neighbor-Joining method (*Saitou N. and Nei M., 1987*). The optimal tree with the sum of branch length = 1.56132503 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (*Zuckerkandl E. and Pauling L., 1965*) and are in the units of the number of amino acid substitutions per site. The analysis involved 11 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 447 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (*Tamura K., Dudley J., Nei M., and Kumar S., 2007).*

[0036] **Figure 15** shows the evolutionary relationships of β-glucosidase NCU04952 orthologues. The optimal tree with the sum of branch length = 2.84018960 is shown. The analysis involved 11 amino acid sequences. There were a total of 690 positions in the final dataset.

[0037] **Figure 16** shows the evolutionary relationships of β-glucosidase NCU08755 orthologues. The optimal tree with the sum of branch length = 2.37353896 is shown. The analysis involved 11 amino acid sequences. There were a total of 709 positions in the final dataset.

[0038] **Figure 17** shows cellulase induction in WT and Δ3βG after induction with cellodextrins. **Figure 17A** shows *cbh-1*, *gh5-1*, and *gh6-2* expression in WT after a 4-hour induction with Avicel®, cellobiose, cellotriose, or cellotetraose. **Figure 17B** shows *cbh-1*, *gh5-1*, and *gh6-2* expression in Δ3βG after a 4-hour induction with Avicel®, cellobiose, cellotriose, or cellotetraose. Gene expression levels of *cbh-1*, *gh5-1* and *gh6-2* were normalized to 1 when induced with 1% sucrose. Actin (NCU04173) gene expression levels were used as an endogenous control in all samples. Error bars indicate 1 standard deviation.

[0039] **Figure 18** shows cellulase expression levels in WT and β-glucosidase deletion strains after induction with cellobiose or Avicel®. **Figure 18A** shows an SDS-PAGE analysis of secreted proteins in culture filtrates from WT, Δ3βG, and Δ3βGΔcre strains. Protein bands

representing CBH-1, GH6-2, and GH5-1 are marked. In addition, the absence of the extracellular β−glucosidase (NCU04952) is marked in the triple knockout. The presence of glucoamylase I (NCU01517) correlates with the deletion of the *cre-1* gene. Cultures were grown in 1% sucrose for 24 hours followed by the addition of 2% sucrose or 0.2% cellobiose. Supernatant was harvested after 24 hours (WT, Δ3βG and Δ3βGΔcre) or 72 hours (Δ3βG). The WT Avicel® culture was grown for 5 days on 2% Avicel®, Δ3βG was grown in 1% sucrose for 24 hours followed by 48 hours in 1% Avicel® and Δ3βGΔcre was grown in 1% sucrose for 24 hours followed by 24 hours in 1% Avicel®. **Figure 18B** shows activity of supernatant from 18A towards Avicel®. Glucose (dark grey) and cellobiose (light grey) were measured after 24 hours of incubation with 1% Avicel® at 50°C. Error bars are 1 standard deviation.

[0040] **Figure 19** shows cellulase induction in WT and Δ3βG after induction with sophorose, lactose or D-(+)-galactose. **Figure 19A** shows cbh-1 expression in WT and Δ3βG after a 4 hour induction with 1mM sophorose, 1mM lactose or 1mM D-(+)-galactose. **Figure 19B** shows gh6-2 expression in WT and Δ3βG after a 4 hour induction with 1mM sophorose, 1mM lactose or 1mM D-(+)-galactose. Gene expression levels of cbh-1 and gh6-2 were normalized to 1 when induced with 1% sucrose. Actin (NCU04173) gene expression levels were used as an endogenous control in all samples. Error bars indicate 1 standard deviation.

[0041] **Figure 20** shows RNA sequencing of the WT and Δ3βG strains. **Figure 20A** shows hierarchical clustering analysis of 318 genes differentially induced in WT *N. crassa* by Avicel®, compared to induction by cellobiose. Light color indicates higher relative expression and dark color indicates lower relative expression. **Figure 20B** shows cellulase expression in FPKMs (fragments per kilobase of exon per million fragments mapped) for the WT induced with cellobiose or Avicel® compared to Δ3βG induced with cellobiose. All strains were grown for 16 hours on 2% sucrose, followed by a transfer to no carbon source (Vogels salt solution only), 0.2% cellobiose or 1% Avicel® for 4 hours.

[0042] **Figure 21** shows enzyme activity in WT, Δ3βG, and Δ3βGΔcre strains after induction with cellobiose or Avicel®. **Figure 21A** shows the 24-hour induced supernatant activity towards Avicel®. Cellulase activity of culture supernatant from Δ3βG (square) and Δ3βGΔcre (diamond) strains when induced with cellobiose for 24 hours compared to culture

supernatants from WT grown on Avicel® for 5 days (triangle). **Figure 21B** shows breakdown of cellobiose (light grey) and glucose (dark grey) produced in the Avicel® hydrolysis assay (from A) after 36 hours. Error bars are 1 standard deviation.

[0043] **Figure 22** summarizes the proteins identified by Mass Spectrometry in wild type (Avicel®), Δ3βG (cellobiose), and Δ3βGΔcre (cellobiose) *Neurospora crassa* strains.

[0044] **Figure 23** compares MuLac activity (cellulase activity) in culture filtrates from *Neurospora crassa* strains Δ3βG, Δ3βGΔcre, Δ3βGΔ890, Δ3βGΔ6650, Δ3βGΔ6650Δ890, Δ3βGΔcreΔ6650, Δ3βGΔcreΔ890, and Δ3βGΔcreΔ6650Δ890. The strains with Δ890 have a deletion in the β-mannosidase gene NCU00890. The strains with Δ6650 have a deletion in the phospholipase gene or phospholipase-like gene NCU06650. Strains were grown in 2% sucrose for 36 hrs followed by 24 hrs in 0.2% cellobiose. Exoglucanase activity in the culture supernatant was measured using a 4-Methylumbelliferyl-β-D-cellobioside (MuLac) assay. Results are shown as relative fluorescence between strains.

DETAILED DESCRIPTION

Overview

[0045] The present disclosure relates to mutant cells and recombinant cells that exhibit increased secretion of a protein, such as a cellulase, in response to induction by cellulosic biomass or a saccharide; and to methods of using such cells to increase secretion of a protein. The secreted proteins may find use in degrading lignocellulosic biomass. As disclosed herein, mutant cells of the present disclosure contain inactivating mutations in at least one gene, such as a β-glucosidase gene, a cre-1 gene, a β-mannosidase gene, or a phospholipase or phospholipase-like gene. As disclosed herein, recombinant cells of the present disclosure exhibit reduced expression of at least one gene, such as a β-glucosidase gene, a cre-1 gene, a β-mannosidase gene, or a phospholipase or phospholipase-like gene, compared to the expression of the at least one gene in a corresponding non-recombinant cell.

Inducers of Protein Secretion

[0046] Cellulosic biomass is mass obtained from living matter, such as plants, algae, fungi, bacteria, and bacterial biofilms that contains polysaccharides and polysaccharide components. Cellulose is the predominant polysaccharide in cellulosic biomass. Cellulose is a homopolymer of anhydrocellobiose (a linear beta-(1-4)-D-glucan), and includes glucose units linked together in β-1,4-glycosidic linkages. Although generally polymorphous, cellulose is found in plant tissue primarily as an insoluble crystalline matrix of parallel glucan chains. Cellulosic biomass may be raw biomass, pre-treated biomass, or processed biomass. Cellulosic biomass may also include one or more saccharides.

[0047] Suitable cellulosic biomass of the present disclosure may include, without limitation, saccharides, polysaccharides, oligosaccharides, purified cellulose, and cellulose derivatives. Purified celluloses include holocelluloses, such as Solka Flok, and microcrystalline celluloses, such as Avicel® and Sigmacell®. Cellulose derivatives include, without limitation, cellodextrins, β-methylumbelliferyl-oligosaccharides, p-nitrophenol-oligosaccharides, long chain cellulose derivatives, carboxymethyl cellulose (CMC), and hydroxyethyl cellulose (HEC).

[0048] As used herein, "cellodextrin(s)" refers to a β(1→4) glucose polymers of varying length and includes, without limitation, cellobiose (2 glucose monomers), cellotriose (3 glucose monomers), cellotetraose (4 glucose monomers), cellopentose (5 glucose monomers), and cellohexose (6 glucose monomers). Advantageously, short-chain cellodextrins, such as cellobiose are soluble. Moreover, secreted proteins of the present disclosure do not adhere to short-chain cellodextrins, such as cellobiose.

[0049] In certain aspects, cellulosic biomass of the present disclosure may be raw biomass material that is degraded by the cells of the present disclosure. The degraded biomass may include, without limitation, polysaccharides, such as cellulose and microcrystalline cellulose; or oligosaccharides, such as cellodextrin and cellobiose. In other aspects, cellulosic biomass of the present disclosure may include purified polysaccharides, such as cellulose and microcrystalline cellulose; or oligosaccharides, such as cellodextrin and cellobiose. In still other aspects, biomass of the present disclosure may include a mixture of polysaccharides, such as cellulose and microcrystalline cellulose; and oligosaccharides, such as cellodextrin and cellobiose.

[0050] In certain aspects, cellulosic biomass of the present disclosure is directly added to mutant cells or recombinant cells of the present disclosure to induce secretion of a protein.

[0051] In other aspects, secretion of a protein is induced from mutant cells or recombinant cells of the present disclosure by one or more cellulose derivatives, such as cellodextrin or cellobiose, that are generated *in situ* by the cells via degradation of the cellulosic biomass. In certain aspects, a sufficient amount of the cellulosic biomass to generate cellulose derivatives that induce secretion from the cell, but that does not adhere to or otherwise sequester the one or more types of proteins secreted from the cell.

[0052] Additionally, saccharides may be used to induce secretion of a protein from a mutant cell or recombinant cell of the present disclosure. Suitable saccharides include, without limitation, polysaccharides, oligosaccharides, sophorose, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

Secreted Proteins

[0053] In certain aspects, mutant cells and recombinant cells of the present disclosure exhibit increased secretion of at least one, at least two, at least three, at least four, at least five, or more types of proteins in response to induction by cellulosic biomass or a saccharide.

[0054] As used herein, increased secretion refers to increased levels of secretion of a protein of the present disclosure. Secretion involves the movement of the protein from inside the cell to outside of the cell. The increased levels of secretion may be the result of increasing expression or production of the protein of interest. Alternatively, the increased levels of secretion may be the result of increasing transport of the protein of interest from the cell. The methods of the present disclosure may also increase level of secretion of a protein by altering a pathway involved in the production and secretion of a protein that results in overall increased levels of secretion of the protein.

[0055] Types of proteins of the present disclosure that may be secreted include, without limitation, endogenous proteins and heterologous proteins. Endogenous proteins of the present disclosure are proteins endogenous to a cell of the present disclosure, or naturally produced by a

cell of the present disclosure. Heterologous proteins of the present disclosure are proteins that are not normally expressed in a cell of the present disclosure. Heterologous proteins may be recombinantly expressed in the cell by any method known in the art. Generally, the recombinant nucleic acid encoding a heterologous protein is operably linked to a regulatory sequence, such as a promoter. Any suitable regulatory sequence known in the art may be used. Suitable promoters include, without limitation, constitutive promoters or inducible promoters. Additionally, the heterologous protein may contain a secretion peptide that directs its secretion from the cell. Any secretion peptide known in the art suitable for use in the methods of the present disclosure may be used.

[0056] In certain aspects, the secreted protein is a cellulose-induced protein. As used herein, "cellulose-induced protein" refers to a protein whose expression and secretion in a wild-type cell (e.g., non-mutant or non-recombinant cell) is induced by cellulose. For example, cellulose-induced proteins are described in C.M. Phillips et al., 2011 (Phillips, CM et al., *Proteome Res.* 2011 Sep 2;10(9):4177-85. Epub 2011 Aug 1).

[0057] Secreted cellulose-induced proteins of the present disclosure include, without limitation, cellulases, GH61 enzymes, cellobiose dehydrogenases, lactonases, carbohydrate esterases, polysaccharide lyases, and cellulose binding domain-containing proteins.

[0058] As used herein, a "cellulase" or "cellulase polypeptide" refers to a polypeptide having enzymatic activity that catalyzes the hydrolysis of cellulose, lichenin, and cereal β-D-glucans. For example, cellulases may have hydrolyze 1,4-β-D-glucosidic linkages in cellulose. Ccellulases of the present disclosure include, without limitation, endocellulases, endoglucanases, endo-1,4-β-glucanases, endo-1,4-β-D-glucanases, carboxymethyl cellulases (CMCases), β-1,4-glucanases, β-1,4-endoglucan hydrolases, and celludextrinases; exocellulases, such as exoglucanases; cellobiases; cellobiohydrolases; oxidative cellulases, such as cellobiose dehydrogenases; and cellulose phosphorylases.

[0059] As used herein, "GH61 enzyme(s)" refers to Glycoside Hydrolase Family 61 enzymes. GH61 enzymes of the present disclosure are capable of enhancing cellulase activity. Examples of GH61 enzymes include, without limitation, polysaccharide monooxygenases. In

certain aspects, a GH61 hydrolase of the present disclosure is encoded by a *GH61-1* gene, a *GH61-2* gene, a *GH61-5* gene, the NCU07898 gene, the NCU08760 gene, homologues thereof, and orthologues thereof.

[0060] Cellobiose dehydrogenases are enzymes with oxidoreductase activity, and include enzymes having EC 1.1.99.18 activity. In certain aspects, a cellobiose dehydrogenase of the present disclosure is encoded by NCU00206, the *cdh-1* gene, homologues thereof, and orthologues thereof.

[0061] Lactonases are enzymes that can hydrolyze the ester bond of the homoserine lactone ring of acylated homoserine lactones. In certain aspects, a lactonase of the present disclosure is encoded by NCU07143, the *lac-2* gene, homologues thereof, and orthologues thereof.

[0062] Carbohydrate esterases are enzymes that have EC 3.1.1.- and EC 3.1.2- activity. Examples of carbohydrate esterases include, without limitation, acetyl xylan esterases, cinnamoyl esterases, feruloyl esterases, carboxylesterases, and S-formylglutathione hydrolases. In certain aspects, a carbohydrate esterase of the present disclosure is encoded by NCU09491, NCU09664, homologues thereof, and orthologues thereof.

[0063] Polysaccharide lyases are enzymes that have EC 4.2.2- activity. In certain aspects, a polysaccharide lyase of the present disclosure is encoded by NCU05598, homologues thereof, and orthologues thereof.

[0064] As used herein, "cellulose binding domain-containing protein(s)" refers to a protein that contains a cellulose binding domain. A cellulose binding domain is a protein domain found in cellulose-active enzymes, such as glycoside hydrolases. Generally, cellulose binding domains have carbohydrate-binding activity. In certain aspects, a cellulose binding domain-containing protein of the present disclosure is encoded by NCU09764, homologues thereof, and orthologues thereof.

[0065] In certain aspects, a secreted cellulose-induced protein of the present disclosure is a protein encoded by NCU05137, homologues thereof, and orthologues thereof.

Mutant Cells

[0066] One aspect of the present disclosure relates to mutant cells exhibiting increased secretion of a protein in response to cellulosic biomass or a saccharide; and to methods of using such cells to increase secretion of a protein from the cell, and to degrade lignocellulosic biomass. As disclosed herein, mutant cells of the present disclosure contain inactivating mutations in at least one gene. Examples of suitable inactivating mutations include, without limitation, deletions, point mutations, loss-of-function mutations, truncations, duplications, amplifications, translocations, and/or inversions that result inhibit the function of the protein encoded by the gene. Methods of generating one or more inactivating mutations in a gene of interest are well known in the art and include, without limitation, PCR mutagenesis, insertional mutagenesis, chemical mutagenesis, and irradiation.

[0067] In one aspect of the present disclosure the mutant cells are fungal or yeast cells. In another aspect of the present disclosure the mutant cells may be ascomycete basidiomycete fungal cells, *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells, *Schizophyllum commune* cells, *Postia placenta* cells, *Aspergillus oryzae* cells, or *Acremonium cellulolyticus* cells. Preferably, the mutant cells are mutant *N. crassa* cells. In another aspect of the present disclosure the mutant cells are recombinant cells. Preferably, the mutant, recombinant cells are *N. crassa* mutant, recombinant cells.

β-Glucosidase Mutant Cells

[0068] β-Glucosidase genes encode β-glucosidase enzymes. As used herein, "β-glucosidase(s)" refers to a β-D-glucoside glucohydrolase that catalyzes the hydrolysis of terminal non-reducing β-D-glucose residues with the release of glucose. β-Glucosidases are highly conserved enzymes.

[0069] In one aspect a mutant cell of the present disclosure contains inactivating mutations in at least two *β*-glucosidase genes, which cause a loss of the *β*-glucosidase function encoded by

the at least two genes. Inactivating mutations of the at least two β-glucosidase genes include, without limitation, deletion mutations, point mutations, nonsense mutations, truncations, and insertions. Inactivating mutations may completely abolish β-glucosidase activity or inhibit β-glucosidase activity by at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more. Inactivating mutations may affect the expression levels of mutated genes or affect the functional activity of proteins or RNAs encoded by mutated genes. Inactivating mutations may also be *cis*- or *trans*-acting. Inactivating mutations may be introduced by random mutagenesis, including irradiation or exposure to mutagenic chemicals, or they may be introduced in a targeted manner, including homologous recombination and crossing of strains that include inactivating mutations.

[0070] β-Glucosidases of the present disclosure that contain inactivating mutations may be intracellular β-glucosidases or extracellular (i.e., secreted) β-glucosidases. Examples of suitable β-glucosidases containing inactivating mutations include, without limitation, those encoded by the *N. crassa* genes NCU00130, NCU04952, NCU08755, homologues thereof, and orthologues thereof. Examples of NCU00130 orthologues, NCU04952 orthologues, and NCU08755 orthologues include, without limitation, those listed in Figures 13-16.

[0071] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell to transcribe 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000 or 100,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating β-glucosidase mutations.

[0072] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell to secrete 1.2, 1.4, 1.6, 1.8, 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, or 10,000-fold higher levels of at least one type of protein after a two day induction compared to that of a cell lacking the inactivating β-glucosidase mutations.

[0073] In another specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell to secrete 1.2, 1.4, 1.6, 1.8, 2, 3, 4, 5, 6, 7, 8, 9, or 10-fold higher

levels of total protein after a two day induction compared to that of a cell lacking the inactivating β-glucosidase mutations.

[0074] In another specific aspect of the present disclosure, the mutant cells may transcribe 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000 or 100,000–fold higher levels of at least one type of protein after induction with at least 1 nM, at least 5 nM, at least 10 nM, 15 nM, at least 20 nM, at least 25 nM, 30 nM, at least 35 nM, at least 40 nM, 45 nM, at least 50 nM, at least 55 nM, 60 nM, at least 65 nM, at least 70 nM, at least 75 nM, 80 nM, at least 85 nM, 90 nM, at least 95 nM, at least 100 nM, at least 125 nM, 150 nM, at least 175 nM, 200 nM, at least 225 nM, at least 250 nM, at least 275 nM, 300 nM, at least 325 nM, 350 nM, at least 375 nM, at least 400 nM, at least 425 nM, at least 450 nM, at least 475 nM, 500 nM, at least 525 nM, at least 550 nM, at least 575 nM, 600 nM, at least 625 nM, 650 nM, at least 675 nM, at least 700 nM, at least 725 nM, at least 750 nM, at least 775 nM, 800 nM, at least 825 nM, at least 850 nM, at least 875 nM, 900 nM, at least 925 nM, 950 nM, at least 975 nM, at least 1 μM, at least 2 μM, at least 3 μM, at least 4 μM, at least 5 μM, at least 6 μM, at least 7 μM, at least 8 μM, at least 9 μM, at least 10 μM, at least 15μM, at least 20 μM, at least 25 μM, at least 30 μM, at least 35 μM, at least 40 μM, at least 45 μM, at least 50 μM, at least 55 μM, at least 60 μM, at least 65 μM, at least 70 μM, at least 75 μM, at least 80 μM, at least 85 μM, at least 90 μM, at least 95 μM, at least 100 μM, at least 125 μM, at least 150 μM, at least 175 μM, at least 200 μM, at least 225 μM, at least 250 μM, at least 275 μM, at least 300 μM, at least 325 μM, at least 350 μM, at least 375 μM, at least 400 μM, at least 425 μM, at least 450 μM, at least 475 μM, at least 500 μM, at least 525 μM, at least 550 μM, at least 575 μM, at least 600 μM, at least 625 μM, at least 650 μM, at least 675 μM, at least 700 μM, at least 725 μM, at least 750 μM, at least 775 μM, at least 800 μM, at least 825 μM, at least 850 μM, at least 875 μM, at least 900 μM, at least 925 μM, at least 950 μM, at least 975 μM, at least 1 mM, at least 2 mM, at least 3 mM, at least 4 mM, at least 5 mM, at least 6 mM, at least 7 mM, at least 8 mM, at least 9 mM, at least 10 mM, at least 11 mM, at least 12 mM, at least 13 mM, at least 14 mM, at least 15 mM, at least 16 mM, at least 17 mM, at least 18 mM, at least 19 mM, at least 20 mM, or more cellulosic biomass or saccharide compared to a cell lacking the inactivating β-glucosidase mutations.

[0075] In another specific aspect of the present disclosure, the mutant cells may secrete 2, 4, 8, 16, 32, 64, 128, or 256 –fold higher levels of at least one type of protein after induction with at

least 1 nM, at least 5 nM, at least 10 nM, 15 nM, at least 20 nM, at least 25 nM, 30 nM, at least 35 nM, at least 40 nM, 45 nM, at least 50 nM, at least 55 nM, 60 nM, at least 65 nM, at least 70 nM, at least 75 nM, 80 nM, at least 85 nM, 90 nM, at least 95 nM, at least 100 nM, at least 125 nM, 150 nM, at least 175 nM, 200 nM, at least 225 nM, at least 250 nM, at least 275 nM, 300 nM, at least 325 nM, 350 nM, at least 375 nM, at least 400 nM, at least 425 nM, at least 450 nM, at least 475 nM, 500 nM, at least 525 nM, at least 550 nM, at least 575 nM, 600 nM, at least 625 nM, 650 nM, at least 675 nM, at least 700 nM, at least 725 nM, at least 750 nM, at least 775 nM, 800 nM, at least 825 nM, at least 850 nM, at least 875 nM, 900 nM, at least 925 nM, 950 nM, at least 975 nM, at least 1 μM, at least 2 μM, at least 3 μM, at least 4 μM, at least 5 μM, at least 6 μM, at least 7 μM, at least 8 μM, at least 9 μM, at least 10 μM, at least 15μM, at least 20 μM, at least 25 μM, at least 30 μM, at least 35 μM, at least 40 μM, at least 45 μM, at least 50 μM, at least 55 μM, at least 60 μM, at least 65 μM, at least 70 μM, at least 75 μM, at least 80 μM, at least 85 μM, at least 90 μM, at least 95 μM, at least 100 μM, at least 125 μM, at least 150 μM, at least 175 μM, at least 200 μM, at least 225 μM, at least 250 μM, at least 275 μM, at least 300 μM, at least 325 μM, at least 350 μM, at least 375 μM, at least 400 μM, at least 425 μM, at least 450 μM, at least 475 μM, at least 500 μM, at least 525 μM, at least 550 μM, at least 575 μM, at least 600 μM, at least 625 μM, at least 650 μM, at least 675 μM, at least 700 μM, at least 725 μM, at least 750 μM, at least 775 μM, at least 800 μM, at least 825 μM, at least 850 μM, at least 875 μM, at least 900 μM, at least 925 μM, at least 950 μM, at least 975 μM, at least 1 mM, at least 2 mM, at least 3 mM, at least 4 mM, at least 5 mM, at least 6 mM, at least 7 mM, at least 8 mM, at least 9 mM, at least 10 mM, at least 11 mM, at least 12 mM, at least 13 mM, at least 14 mM, at least 15 mM, at least 16 mM, at least 17 mM, at least 18 mM, at least 19 mM, at least 20 mM, or more cellulosic biomass or saccharide compared to a cell lacking the inactivating β-glucosidase mutations.

[0076] In another specific aspect of the present disclosure the at least two β-glucosidase genes are at least three β-glucosidase genes, at least four β-glucosidase genes, at least five β-glucosidase genes, at least six β-glucosidase genes, at least seven β-glucosidase genes or more β-glucosidase genes.

[0077] In one preferred embodiment of the present disclosure the β-glucosidase genes NCU00130, NCU04952, and NCU08755 are deleted in a *N. crassa* cell.

[0078] In another aspect of the present disclosure, the mutant cell including inactivating mutations that reduce the activities of at least two β-glucosidases further includes an inactivating mutation in the *cre-1* gene, where cellulosic biomass or a saccharide induces the cell to secrete higher levels of at least one protein than a cell lacking a mutation in the *cre-1* gene. Inactivating mutations may affect the expression levels of mutated genes or affect the functional activity of proteins or RNAs encoded by mutated genes. Inactivating mutations may be *cis*- or *trans*-acting. Inactivating mutations may be introduced by random mutagenesis, including irradiation or exposure to mutagenic chemicals, or they may be introduced in a targeted manner, including homologous recombination and crossing of strains that include single or multiple inactivating mutations.

[0079] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell to transcribe 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000, or 100,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating *creA/cre-1* mutation.

[0080] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell to secrete 1.2, 1.4, 1.6, 1.8, 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, or 10,000-fold higher levels of at least one type of protein compared to that of a cell lacking the β-glucosidase mutations or the *cre-1* mutation.

[0081] In another specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell to secrete 1.2, 1.4, 1.6, 1.8, 2, 3, 4, 5, 6, 7, 8, 9, or 10-fold higher levels of total protein after a two-day induction than a cell lacking the β-glucosidase mutations or the *cre-1* mutation.

[0082] In another specific aspect of the present disclosure, the mutant cell may transcribe 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000 or 100,000 –fold higher levels of at least one type of protein after induction with 1 nM, at least 5 nM, at least 10 nM, 15 nM, at least 20 nM, at least

25 nM, 30 nM, at least 35 nM, at least 40 nM, 45 nM, at least 50 nM, at least 55 nM, 60 nM, at least 65 nM, at least 70 nM, at least 75 nM, 80 nM, at least 85 nM, 90 nM, at least 95 nM, at least 100 nM, at least 125 nM, 150 nM, at least 175 nM, 200 nM, at least 225 nM, at least 250 nM, at least 275 nM, 300 nM, at least 325 nM, 350 nM, at least 375 nM, at least 400 nM, at least 425 nM, at least 450 nM, at least 475 nM, 500 nM, at least 525 nM, at least 550 nM, at least 575 nM, 600 nM, at least 625 nM, 650 nM, at least 675 nM, at least 700 nM, at least 725 nM, at least 750 nM, at least 775 nM, 800 nM, at least 825 nM, at least 850 nM, at least 875 nM, 900 nM, at least 925 nM, 950 nM, at least 975 nM, at least 1 μM, at least 2 μM, at least 3 μM, at least 4 μM, at least 5 μM, at least 6 μM, at least 7 μM, at least 8 μM, at least 9 μM, at least 10 μM, at least 15μM, at least 20 μM, at least 25 μM, at least 30 μM, at least 35 μM, at least 40 μM, at least 45 μM, at least 50 μM, at least 55 μM, at least 60 μM, at least 65 μM, at least 70 μM, at least 75 μM, at least 80 μM, at least 85 μM, at least 90 μM, at least 95 μM, at least 100 μM, at least 125 μM, at least 150 μM, at least 175 μM, at least 200 μM, at least 225 μM, at least 250 μM, at least 275 μM, at least 300 μM, at least 325 μM, at least 350 μM, at least 375 μM, at least 400 μM, at least 425 μM, at least 450 μM, at least 475 μM, at least 500 μM, at least 525 μM, at least 550 μM, at least 575 μM, at least 600 μM, at least 625 μM, at least 650 μM, at least 675 μM, at least 700 μM, at least 725 μM, at least 750 μM, at least 775 μM, at least 800 μM, at least 825 μM, at least 850 μM, at least 875 μM, at least 900 μM, at least 925 μM, at least 950 μM, at least 975 μM, at least 1 mM, at least 2 mM, at least 3 mM, at least 4 mM, at least 5 mM, at least 6 mM, at least 7 mM, at least 8 mM, at least 9 mM, at least 10 mM, at least 11 mM, at least 12 mM, at least 13 mM, at least 14 mM, at least 15 mM, at least 16 mM, at least 17 mM, at least 18 mM, at least 19 mM, at least 20 mM, or more cellulosic biomass or saccharide compared to that of a cell lacking the *β*-glucosidase mutations or the *cre-1* mutation.

[0083] In another specific aspect of the present disclosure, the mutant cell may secrete 1.2, 1.4, 1.6, 1.8, 2, 4, 8, 16, 32, 64, 128, or 256 –fold higher levels of at least one type of protein after induction with 1 nM, at least 5 nM, at least 10 nM, 15 nM, at least 20 nM, at least 25 nM, 30 nM, at least 35 nM, at least 40 nM, 45 nM, at least 50 nM, at least 55 nM, 60 nM, at least 65 nM, at least 70 nM, at least 75 nM, 80 nM, at least 85 nM, 90 nM, at least 95 nM, at least 100 nM, at least 125 nM, 150 nM, at least 175 nM, 200 nM, at least 225 nM, at least 250 nM, at least 275 nM, 300 nM, at least 325 nM, 350 nM, at least 375 nM, at least 400 nM, at least 425 nM, at

least 450 nM, at least 475 nM, 500 nM, at least 525 nM, at least 550 nM, at least 575 nM, 600 nM, at least 625 nM, 650 nM, at least 675 nM, at least 700 nM, at least 725 nM, at least 750 nM, at least 775 nM, 800 nM, at least 825 nM, at least 850 nM, at least 875 nM, 900 nM, at least 925 nM, 950 nM, at least 975 nM, at least 1 μM, at least 2 μM, at least 3 μM, at least 4 μM, at least 5 μM, at least 6 μM, at least 7 μM, at least 8 μM, at least 9 μM, at least 10 μM, at least 15μM, at least 20 μM, at least 25 μM, at least 30 μM, at least 35 μM, at least 40 μM, at least 45 μM, at least 50 μM, at least 55 μM, at least 60 μM, at least 65 μM, at least 70 μM, at least 75 μM, at least 80 μM, at least 85 μM, at least 90 μM, at least 95 μM, at least 100 μM, at least 125 μM, at least 150 μM, at least 175 μM, at least 200 μM, at least 225 μM, at least 250 μM, at least 275 μM, at least 300 μM, at least 325 μM, at least 350 μM, at least 375 μM, at least 400 μM, at least 425 μM, at least 450 μM, at least 475 μM, at least 500 μM, at least 525 μM, at least 550 μM, at least 575 μM, at least 600 μM, at least 625 μM, at least 650 μM, at least 675 μM, at least 700 μM, at least 725 μM, at least 750 μM, at least 775 μM, at least 800 μM, at least 825 μM, at least 850 μM, at least 875 μM, at least 900 μM, at least 925 μM, at least 950 μM, at least 975 μM, at least 1 mM, at least 2 mM, at least 3 mM, at least 4 mM, at least 5 mM, at least 6 mM, at least 7 mM, at least 8 mM, at least 9 mM, at least 10 mM, at least 11 mM, at least 12 mM, at least 13 mM, at least 14 mM, at least 15 mM, at least 16 mM, at least 17 mM, at least 18 mM, at least 19 mM, at least 20 mM, or more cellulosic biomass or saccharide compared to that of a cell lacking the *β*-glucosidase mutations or the *cre-1* mutation.

[0084] In another specific aspect of the present disclosure the at least two *β*-glucosidases are at least three *β*-glucosidases.

[0085] In another preferred embodiment of the present disclosure the *β*-glucosidase genes NCU00130, NCU04952, and NCU08755 and the *cre-1* gene are deleted in a *N. crassa* cell.

CreA/cre-1 Mutant Cells

[0086] In one aspect, a mutant cell of the present disclosure contains an inactivating mutation in the *creA/cre-1* gene, which causes a loss of the CreA/CRE-1 function encoded by the gene. Inactivating mutations of the *creA/cre-1* gene include, without limitation, deletion mutations, point mutations, nonsense mutations, truncations, and insertions. Inactivating mutations may

completely abolish CreA/CRE-1 activity or inhibit CreA/CRE-1 activity by at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more. Inactivating mutations may affect the expression levels of mutated genes or affect the functional activity of proteins or RNAs encoded by mutated genes. Inactivating mutations may also be *cis*- or *trans*-acting. Inactivating mutations may be introduced by random mutagenesis, including irradiation or exposure to mutagenic chemicals, or they may be introduced in a targeted manner, including homologous recombination and crossing of strains that include inactivating mutations. As used herein, "*cre-1* gene" and "*creA/cre-1* gene" are used interchangeably.

[0087] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell to transcribe 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000, or 100,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating *cre-1* mutation.

[0088] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell including the inactivating mutation of cre-1 to secrete 1.2, 1.4, 1.6, 1.8, 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, or 10,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating *cre-1* mutation.

[0089] In another specific aspect of the present disclosure, the mutant cells exhibit elevated basal levels of expression of genes involved in C-compound/carbohydrate metabolism, extracellular metabolism, proteins with binding function or cofactor requirement, C-compound/carbohydrate transport, transport facilities, and protein synthesis relative to a cell lacking the *cre-1* mutation.

[0090] In one preferred example of the present disclosure the *cre-1* gene is deleted in a *N. crassa* cell.

[0091] In another aspect of the present disclosure, the mutant cell including an inactivating mutation in the *cre-1* gene further includes inactivating mutations, which abolish the β-glucosidase activity encoded by the at least two β-glucosidase genes. Inactivating mutations of

the at least two β-glucosidase genes include deletions, point mutations, nonsense mutations, truncations, and insertions. Inactivating mutations may completely abolish β-glucosidase activity or inhibit the activity by at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more. Inactivating mutations may affect the expression levels of mutated genes or affect the functional activity of proteins or RNAs encoded by mutated genes. Inactivating mutations may be *cis*- or *trans*-acting. Inactivating mutations may be introduced by random mutagenesis, including irradiation or exposure to mutagenic chemicals, or they may be introduced in a targeted manner, including homologous recombination and crossing of strains that include inactivating mutations. The β-glucosidases may be intracellular or extracellular (i.e., secreted) β-glucosidases.

[0092] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell to transcribe 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000, or 100,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating β-glucosidase mutations.

[0093] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce induces the mutant cell to secrete 1.2, 1.4, 1.6, 1.8, 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, or 10,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating mutations in the at least two β-glucosidase mutations.

[0094] In another specific aspect of the present disclosure, the at least two β-glucosidases are three β-glucosidases.

[0095] In one preferred embodiment of the present disclosure the mutant cell is a *N. crassa* cell including deletions of the β-glucosidases genes NCU00130, NCU04952, and NCU08755 and a deletion of *cre-1*.

β-Mannosidase Mutant Cells

[0096] In another aspect of the present disclosure, mutant cells containing inactivating mutations that reduce the activities of at least two *β*-glucosidases of the present disclosure; mutant cells containing an inactivating mutation in the *cre-1* gene of the present disclosure; and/or mutant cells containing inactivating mutations that reduce the activities of at least two *β*-glucosidases and an inactivating mutation in the*cre-1* gene further include a mutation that reduces the activity of at least one β-mannosidase gene.

[0097] β-Mannosidase genes of the present disclosure encode β-mannosidase enzymes. As used herein, "β-mannosidase," "mannan endo-1,4-β-mannosidase," "endo-1,4-β-mannanase," "endo-β-1,4-mannase," "β-mannanase B," "β-1, 4-mannan 4-mannanohydrolase," "endo-β-mannanase," "β-D-mannanase," and "1,4-β-D-mannan mannanohydrolase" are used interchangeably and refer to an enzymes capable of the random hydrolysis of 1,4-β-D-mannosidic linkages in mannans, galactomannans and glucomannans (EC 3.2.1.78). In certain aspects, the at least one β-mannosidase gene is NCU00890, *T. reesei* protein ID 62166, *T. reesei* protein ID 57857, homologues thereof, and orthologues thereof.

[0098] In one aspect a mutant cell of the present disclosure contains inactivating mutations in at least one β-mannosidase gene, which causes a loss of the β-mannosidase function encoded by the gene. Inactivating mutations of the at least one β-mannosidase gene include, without limitation, deletion mutations, point mutations, nonsense mutations, truncations, and insertions. Inactivating mutations may completely abolish β-mannosidase activity or inhibit β-mannosidase activity by at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more. Inactivating mutations may affect the expression levels of mutated genes or affect the functional activity of proteins or RNAs encoded by mutated genes. Inactivating mutations may also be *cis*- or *trans*-acting. Inactivating mutations may be introduced by random mutagenesis, including irradiation or exposure to mutagenic chemicals, or they may be introduced in a targeted manner, including homologous recombination and crossing of strains that include inactivating mutations.

[0099] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell further containing the inactivating mutation of at least one β-mannosidase gene to transcribe 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000, or 100,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating mutation of the at least one β-mannosidase gene.

[00100] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell further containing the inactivating mutation of at least one β-mannosidase gene to secrete 1.2, 1.4, 1.6, 1.8, 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, or 10,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating mutation of the at least one β-mannosidase gene.

[00101] In one preferred example of the present disclosure the at least one β-mannosidase gene is deleted in a *N. crassa* cell.

Phospholipase Mutant Cells

[00102] In another aspect of the present disclosure, mutant cells containing inactivating mutations that reduce the activities of at least two *β*-glucosidases of the present disclosure; mutant cells containing an inactivating mutation in the *creA/cre-1* gene of the present disclosure; mutant cells containing inactivating mutations that reduce the activities of at least two *β*-glucosidases and an inactivating mutation in the *creA/cre-1* gene; and/or mutant cells containing inactivating mutations that reduce the activities of at least two *β*-glucosidases, an inactivating mutation in the *creA/cre-1* gene, and an inactivating mutation that reduces the activity of at least one β-mannosidase gene of the present disclosure further contain an inactivating mutation that reduces the activity of at least one phospholipase gene or phospholipase-like gene.

[00103] As used here, a "phospholipase-like gene" is a gene having sequence homology to a phospholipase gene, or a gene encoding a protein having amino acid sequence homology to a phospholipase. For example, a phospholipase-like gene of the present disclosure may be NCU06650. While NCU06650 has not been shown to encode a protein having phospholipase activity, the closest related homologues of the encoded amino acid sequence are phospholipases.

[00104] Phospholipase genes of the present disclosure encode phospholipase enzymes. As used herein, phospholipase enzymes include, without limitation, any enzyme that hydrolyzes phospholipids into, for example, fatty acids and other lipophilic molecules. Phospholipase-encoding genes may include, without limitation, genes that encode a phospholipase A1, a phospholipase A2, a phospholipase B, a phospholipase C, a phospholipase D, or a phosphodiesterase.

[00105] Accordingly, in certain aspects, the at least one phospholipase gene or phospholipase-like gene is NCU06650, *T. reesei* protein ID 67579, homologues thereof, and orthologues thereof.

[00106] In one aspect a mutant cell of the present disclosure contains inactivating mutations in at least one phospholipase gene or phospholipase-like gene, which causes a loss of the protein function encoded by the gene. Inactivating mutations of the at least one phospholipase gene or phospholipase-like gene include, without limitation, deletion mutations, point mutations, nonsense mutations, truncations, and insertions. Inactivating mutations may completely abolish phospholipase activity or inhibit phospholipase activity by at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more. Inactivating mutations may affect the expression levels of mutated genes or affect the functional activity of proteins or RNAs encoded by mutated genes. Inactivating mutations may also be *cis*- or *trans*-acting. Inactivating mutations may be introduced by random mutagenesis, including irradiation or exposure to mutagenic chemicals, or they may be introduced in a targeted manner, including homologous recombination and crossing of strains that include inactivating mutations.

[00107] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell further containing the inactivating mutation of at least one phospholipase gene or phospholipase-like gene to transcribe 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000, or 100,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating mutation of the at least one phospholipase gene or phospholipase-like gene.

[00108] In one specific aspect of the present disclosure, cellulosic biomass or a saccharide may induce the mutant cell further containing the inactivating mutation of at least one phospholipase gene or phospholipase-like gene to secrete 1.2, 1.4, 1.6, 1.8, 2, 4, 6, 8, 10, 50, 100, 500, 1,000, 5,000, or 10,000-fold higher levels of at least one type of protein compared to that of a cell lacking the inactivating mutation of the at least one phospholipase gene or phospholipase-like gene.

[00109] In one preferred example of the present disclosure the at least one phospholipase gene or phospholipase-like gene is deleted in a *N. crassa* cell.

Recombinant Cells

[00110] Another aspect of the present disclosure relates to recombinant cells exhibiting reduced expression of at least two β-glucosidase genes or a cre-1 gene in the cell, that also exhibit increased secretion at least one, at least two, at least three, at least four, at least five, or more types of proteins in response to cellulosic biomass or a saccharide; and to methods of using such cells to increase secretion of a protein from the cell, and to degrade lignocellulosic biomass. Recombinant cells of the present disclosure may be stable cell lines or transiently transfected cells.

[00111] Recombinant cells of the present disclosure exhibiting reduced expression of a gene of interest (e.g., a β-glucosidase gene, a cre-1 gene, a β-mannosidase gene, or a phospholipase gene or phospholipase-like gene) may contain a mutation that reduces expression of the gene of interest. Methods for generating and characterizing mutations are well known in the art, such as mutational screening. Alternatively, recombinant cells of the present disclosure may be transgenic cells that contain a recombinant construct, such as an inhibitory oligonucleotide, that targets and reduces expression of the gene of interest. Non-limiting examples of inhibitory oligonucleotides include siRNA, miRNA, antisense DNA. Additionally, the expression of a gene of interest may be reduced by gene silencing techniques, such quelling and meiotic silencing. Gene silencing techniques can target the gene of interest, RNA of the gene of interest, a regulator protein of the gene of interest.

[00112] Types of proteins that may be secreted by recombinant cells of the present disclosure include, without limitation, cellulose-induced proteins. Non-limiting examples of cellulose-induced proteins include, without limitation, cellulases, GH61 enzymes, cellobiose dehydrogenases, lactonases, carbohydrate esterases, polysaccharide lyases, and cellulose binding domain-containing proteins. In certain aspects, a secreted protein of the present disclosure is encoded by NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, or NCU05137. In certain aspects, recombinant cells of the present disclosure have increased secretion of at least one, at least two, at least three, at least four, at least five, or more types of proteins.

[00113] In certain aspects, a recombinant cell of the present disclosure exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least twoβ-glucosidase genes in a corresponding non-recombinant cell. In other embodiments, a recombinant cell of the present disclosure exhibits reduced expression of a *cre-1* gene compared to the expression of the *cre-1* gene in a corresponding non-recombinant cell.

[00114] As used herein, a "corresponding non-recombinant cell" refers to a cell that is of the same species as the recombinant cell and has been cultured under the same conditions as the recombinant cell, but lacks the modification of the recombinant cell that results in reduced gene expression in the recombinant cell. "Reduced expression" of a gene of the present disclosure refers to decreased levels of expression of a gene in a modified cell as compared to the levels of expression of the gene in a corresponding non-modified cell.

[00115] In certain aspects, the expression of the at least two β-glucosidase genes may be reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In other aspects, the expression of the cre-1 gene may be reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[00116] In a specific aspect of the present disclosure, the recombinant cell exhibiting reduced expression of at least two β-glucosidase genes further exhibits reduced expression of the gene creA/*cre-1*. Means for the reduction of *creA/cre-1* expression may be gene silencing techniques, including siRNA, miRNA, antisense DNA, quelling or meiotic silencing. Gene silencing techniques may target creA/*cre-1* or a creA/*cre-1* regulator protein or RNA. C*reA/cre-1* expression may be reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[00117] In another specific aspect of the present disclosure, the recombinant cell exhibiting reduced expression of at least two β-glucosidase genes may also be a cell in which the functional activity of the C*reA/CRE-1* transcription factor has been reduced by overexpression of a dominant negative mutant or protein inhibitor. *CreA/CRE-1* function may be reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[00118] In another specific aspect of the present disclosure, the recombinant cell exhibiting reduced expression of at least two β-glucosidase genes, and/or the recombinant cell exhibiting reduced expression levels of at least two β-glucosidase genes and reduced expression levels of the gene creA/*cre-1*, may further exhibit reduced expression of at least one β-mannosidase gene of the present disclosure. Means for the reduction of the β-mannosidase expression include, without limitation, gene silencing techniques, including siRNA, miRNA, antisense DNA, quelling or meiotic silencing. Gene silencing techniques may target the at least one β-mannosidase gene or a β-mannosidase gene regulator protein or RNA. β-Mannosidase gene expression may be reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In certain aspects, expression of the β-mannosidase gene NCU00890 is reduced in a recombinant *N. crassa* cell.

[00119] In another specific aspect of the present disclosure, the recombinant cell exhibiting reduced expression of at least two β-glucosidase genes; the recombinant cell exhibiting reduced expression of at least two β-glucosidase genes and reduced expression of the gene creA/*cre-1*; and/or the recombinant cell exhibiting reduced expression of at least two β-glucosidase genes, reduced expression of the gene creA/*cre-1*, and reduced expression of at least one β-mannosidase gene may further exhibit reduced expression of at least one phospholipase gene or phospholipase-like gene of the present disclosure. Means for the reduction of the phospholipase expression include, without limitation, gene silencing techniques, including siRNA, miRNA, antisense DNA, quelling or meiotic silencing. Gene silencing techniques may target the at least one phospholipase gene or phospholipase-like gene or a gene regulator protein or RNA. Phospholipase gene or phospholipase-like gene expression may be reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In certain aspects, expression of the gene NCU06650 is reduced in a recombinant *N. crassa* cell.

[00120] In a preferred embodiment of the present disclosure, expression of the β-glucosidase genes NCU00130, NCU04952, and NCU08755 is reduced in a recombinant *N. crassa* cell. In another preferred embodiment of the present disclosure, expression of the β-glucosidases genes NCU00130, NCU04952, and NCU08755, and expression of the *cre-1* gene is reduced in a recombinant *N. crassa* cell. In still another preferred embodiment of the present disclosure, expression of the β-glucosidases genes NCU00130, NCU04952, and NCU08755, expression of the *cre-1* gene, and expression of the β-mannosidase gene NCU00890 is reduced in a recombinant *N. crassa* cell. In a further preferred embodiment of the present disclosure, expression of the β-glucosidases genes NCU00130, NCU04952, and NCU08755, expression of the *cre-1* gene, expression of the β-mannosidase gene NCU00890, and expression of the gene NCU06650 is reduced in a recombinant *N. crassa* cell.

[00121] In one aspect of the present disclosure, the recombinant cell exhibits reduced expression of the *cre-1* gene. Means for the reduction of *cre-1* expression include, without limitation, gene silencing techniques, including siRNA, miRNA, antisense DNA, quelling or

meiotic silencing. Gene silencing techniques may target *cre-1* or a *cre-1* regulator protein or RNA. C*re-1* expression may be reduced in recombinant cells by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[00122] In another aspect of the present disclosure, the functional activity of the C*reA/CRE-1* transcription factor has been reduced in a recombinant cell by overexpression of a dominant negative mutant or a protein inhibitor. *CreA/cre-1* function may be reduced by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

Variants, Sequence Identity, and Sequence Similarity

[00123] Methods of alignment of sequences for comparison are well-known in the art. For example, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) CABIOS 4:11 17; the local homology algorithm of Smith et al. (1981) Adv. Appl. Math. 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443 453; the search-for-similarity-method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444 2448; the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 872264, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873 5877.

[00124] Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0) 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. (1988) Gene 73:237 244 (1988); Higgins et al. (1989) CABIOS 5:151 153; Corpet et al. (1988) Nucleic Acids Res. 16:10881 90; Huang et al. (1992) CABIOS 8:155 65; and Pearson et al. (1994) Meth. Mol. Biol. 24:307 331. The ALIGN program is based on the algorithm of Myers and Miller (1988) supra. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul et al. (1990) J. Mol. Biol. 215:403 are based on the algorithm of Karlin and Altschul (1990) supra. 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 present 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 present 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. See http://www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

[00125] As used herein, sequence identity or identity in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins, it is recognized that residue positions which are not identical and often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (*e.g.*, charge or hydrophobicity), do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have sequence similarity or similarity. Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby

increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, *e.g.*, as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

[00126] The nucleic acids may be synthesized, isolated, or manipulated using standard molecular biology techniques such as those described in Sambrook, J. et al. 2000. Molecular Cloning: A Laboratory Manual (Third Edition). Techniques may include cloning, expression of cDNA libraries, and amplification of mRNA or genomic DNA.

[00127] The nucleic acids of the present disclosure, or subsequences thereof, may be incorporated into a cloning vehicle including an expression cassette or vector. The cloning vehicle can be a viral vector, a plasmid, a phage, a phagemid, a cosmid, a fosmid, a bacteriophage, or an artificial chromosome. The viral vector can include an adenovirus vector, a retroviral vector, or an adeno-associated viral vector. The cloning vehicle can include a bacterial artificial chromosome (BAC), a plasmid, a bacteriophage P1-derived vector (PAC), a yeast artificial chromosome (YAC), or a mammalian artificial chromosome (MAC).

[00128] The nucleic acids may be operably linked to a promoter. The promoter can be a viral, bacterial, mammalian or plant promoter. The promoter can be a constitutive promoter, an inducible promoter, a tissue-specific promoter, or an environmentally regulated or a developmentally regulated promoter.

Methods for Increasing Secretion of a Protein

[00129] Other aspects of the present disclosure relate to methods for increasing secretion of a protein from a cell by providing any of the cells of the present disclosure capable of secreting at least one, at least two, at least three, at least four, at least five, or more types of proteins in response to cellulosic biomass or a saccharide; and inducing the cell to secrete the at least two, at least three, at least four, at least five, or more types of proteins by contacting the cell with cellulosic biomass or a saccharide.

[00130] Cellulosic biomass that may be used with the methods of the present disclosure may include, without limitation, one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain preferred embodiments, the cellulosic biomass includes cellobiose.

[00131] Saccharides that may be used with the methods of the present disclosure include, without limitation, a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, cellohexose, and sophorose. In certain preferred embodiments, the saccharide is cellobiose

[00132] Types of proteins that may be secreted by recombinant cells of the present disclosure include, without limitation, cellulose-induced proteins. Non-limiting examples of cellulose-induced proteins include, without limitation, cellulases, GH61 enzymes, cellobiose dehydrogenases, lactonases, carbohydrate esterases, polysaccharide lyases, and cellulose binding domain-containing proteins. In certain aspects, a secreted protein of the present disclosure is encoded by NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, or NCU05137.

[00133] Accordingly, certain aspects of the present disclosure provide methods for increasing secretion of a protein from a cell by: providing a mutant cell, where the mutant cell contains inactivating mutations in two or more β-glucosidase genes, or contains an inactivating mutation in a cre-1 gene in the cell; and contacting the mutant cell with cellulosic biomass or a saccharide, where the cellulosic biomass or saccharide induces the mutant cell to secrete the protein. In certain aspects, the cellulosic biomass or saccharide induces the cell to secrete at least two, at least three, at least four, at least five, or more types of proteins.

[00134] In some aspects the method for increasing secretion of a protein from a cell includes the step of inducing the secretion of the protein in the presence of *β*-glucosidase inhibitors. Preferably, the *β*-glucosidase inhibitor is nojirimycin.

[00135] Other aspects of the present disclosure provide methods for increasing secretion of a protein from a cell, by: providing a recombinant cell, where the recombinant cell exhibits reduced expression of two or more β-glucosidase genes compared to the expression of the at least twoβ-glucosidase genes in a corresponding non-recombinant cell, or exhibits reduced expression of a cre-1 gene compared to the expression of the cre-1 gene in a corresponding non-recombinant cell; and contacting the recombinant cell with cellulosic biomass or a saccharide, where the cellulosic biomass or saccharide induces the recombinant cell to secrete the protein. In certain aspects, the cellulosic biomass or saccharide induces the cell to secrete at least two, at least three, at least four, at least five, or more types of proteins.

Methods for Degrading Lignocellulosic Biomass

[00136] Further aspects of the present disclosure relate to methods for the degradation of biomass by providing lignocellulosic biomass; providing any of the mutant or recombinant cells of the present disclosure; inducing the cell to secrete at least one, at least two, at least three, at least four, at least five, or more types of proteins by contacting the cell with cellulosic biomass or a saccharide; and contacting the induced cell with the lignocellulosic biomass, where the secreted at least one, at least two, at least three, at least four, at least five, or more types of proteins degrade the lignocellulosic biomass.

[00137] Lignocellulosic biomass generally refers to plant biomass containing cellulose and other carbohydrate polymers that are tightly bound to lignin. Examples of suitable lignocellulosic biomass include, without limitation, plant material, municipal solid waste, municipal paper waste, wood residues, sawmill and paper mill discards, and agricultural residues. Examples of suitable plant material includes, without limitation, Miscanthus, energy grass, elephant grass, switchgrass, cord grass, rye grass, reed canary grass, common reed, wheat straw, barley straw, canola straw, oat straw, corn stover, soybean stover, oat hulls, oat spelt, sorghum, rice hulls, sugarcane bagasse, corn fiber, barley, oats, flax, wheat, linseed, citrus pulp, cottonseed, groundnut, rapeseed, sunflower, peas, lupines, palm kernel, coconut, konjac, locust bean gum, gum guar, soy beans, Distillers Dried Grains with Solubles (DDGS), Blue Stem, corncobs, pine, conifer softwood, eucalyptus, birchwood, willow, aspen, poplar wood, hybrid

poplar, energy cane, short-rotation woody crop, crop residue, yard waste, and combinations thereof.

[00138] Cellulosic biomass that may be used with the methods of the present disclosure may include, without limitation, one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. In certain preferred embodiments, the cellulosic biomass includes cellobiose.

[00139] Saccharides that may be used with the methods of the present disclosure include, without limitation, a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, cellohexose, and sophorose. In certain preferred embodiments, the saccharide is cellobiose

[00140] Types of proteins that may be secreted by recombinant cells of the present disclosure include, without limitation, cellulose-induced proteins. Non-limiting examples of cellulose-induced proteins include, without limitation, cellulases, GH61 enzymes, cellobiose dehydrogenases, lactonases, carbohydrate esterases, polysaccharide lyases, and cellulose binding domain-containing proteins. In certain aspects, a secreted protein of the present disclosure is encoded by NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, or NCU05137.

[00141] In one aspect of the present disclosure, the method for degrading lignocellulosic biomass includes the step of contacting lignocellulosic biomass with mutant cells of the present disclosure as described above in the presence of β-glucosidase inhibitors. Preferably, the β-glucosidase inhibitor is nojirimycin.

Applications

[00142] The methods described herein can be practiced in combination with other methods useful for degrading lignocellulosic biomass.

[00143] For example, lignocellulosic biomass may be subjected to pretreatment including ammonia fiber expansion (AFEX), steam explosion, treatment with alkaline aqueous solutions, acidic solutions, organic solvents, ionic liquids (IL), electrolyzed water, phosphoric acid, and combinations thereof. Pretreatments that remove lignin from the plant material may increase the overall amount of sugar released from the hemicellulose.

EXAMPLES

[00144] The following Examples are merely illustrative and are not meant to limit any aspects of the present disclosure in any way.

Example 1

[00145] The following example relates to the characterization of the cellulase transcription and cellulolytic enzyme production induced in the *N. crassa* triple *β*-glucosidase gene deletion strain and the triple *β*-glucosidase and *cre-1* gene deletion strain.

Materials and Methods

Strains

[00146] Strains were obtained from the Fungal Genetics Stock Center (FGSC) including the *Neurospora crassa* wild-type (WT) (FGSC 2489), the *cre-1* gene deletion (Δ*cre-1*) (FGSC 10372), and deletion strains for the intracellular β-glucosidase NCU00130 (FGSC 11822 and FGSC 11823), and extracellular β-glucosidases: NCU08755 (FGSC 18387 and FGSC 18388) and NCU04952 (FGSC 13731 and FGSC 13732).

[00147] The quadruple deletion produced by performing sequential crosses of the single deletions using the method described by the FGSC (http://www.fgsc.net/neurosporaprotocols/How%20to%20make%20a%20cross-2.pdf). The genotype of all deletion strains was confirmed by using a gene-specific primer and a common primer for the hygromycin (hph) cassette. The forward primer for hph was:

hph Middle FWD: 5'-CGA CAG ACG TCG CGG TGA GTT CAG-3' [SEQ ID NO: 4]

Reverse primers were:

NCU00130:	5'- TAG TGT ACA AAC CCC AAG C -3'	[SEQ ID NO: 5]
NCU004953:	5'-AAC ACA CAC ACA CAC ACT GG-3'	[SEQ ID NO: 6]
NCU08755:	5'-ACA GTG GAG GTG AGA AAG G-3'	[SEQ ID NO: 7]
NCU08807:	5'-GTA CTT ACG CAG TAG CGT GG-3'	[SEQ ID NO: 8]

Transcriptional Studies Growth

[00148] Strains were inoculated at an OD595 equal to .05 in 50ml Vogel's salts with 2% (wt/vol) sucrose in a 250ml Erlenmeyer flask and grown under constant light at 200 rpm for 16 hours. The biomass was then spun at 4000rpm for 10 minutes and washed in Vogel's twice to remove any excess sucrose. The biomass was then added to a new 50ml culture with 2% (wt/vol) sucrose, cellobiose (Sigma) or Avicel® PH 101 (Avicel®). Cultures were induced for 4 hours under constant light at 200 rpm. The culture biomass was then harvested by filtration over a Whatman glass microfiber filter (GF/F) on a Buchner funnel and washed with 50ml Vogel's, biomass was flash frozen in liquid Nitrogen and stored at -80°C.

RNA Isolation

[00149] Total RNA from frozen samples was isolated using Zirconia/Silica beads (.2g, 0.5mm diameter; Biospec) and a Mini-Beadbeater-96 (Biospec) with 1mL TRIzol reagent (Invitrogen) according to manufacturer's instructions. Total RNA was further purified by digestion with TURBO DNA-free (Ambion) and an RNeasy kit (Qiagen). RNA integrity was checked by Nanodrop and agarose gel electrophoresis.

Quantitative Real-time RT-PCR

[00150] Quantitative RT-PCR was performed using the EXPRESS One-Step SYBR GreenER Kit (Invitrogen) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Reactions were performed in triplicate with a total reaction volume of 10ul including 300nM each forward and reverse primers and 75ng template RNA. Data Analysis was performed by the StepOne

Software (Applied Biosystems) using Relative Quantitation/Comparative CT (ΔΔCT). Data was normalized to the endogenous control actin with expression on sucrose as the reference sample. Error bars indicate a 95% confidence interval. The RT-PCR primers were used as described in (Tian et al., 2009).

Actin:	5'-TGA TCT TAC CGA CTA CCT-3'	[SEQ ID NO: 9]
	5'-CAG AGC TTC TCC TTG ATG-3'	[SEQ ID NO: 10]
CBHI (NCU07340)	5'-ATC TGG GAA GCG AAC AAA G-3'	[SEQ ID NO: 11]
	5'-TAG CGG TCG TCG GAA TAG-3'	[SEQ ID NO: 12]
CBHII (NCU09680)	5'-CCC ATC ACC ACT ACT ACC-3'	[SEQ ID NO: 13]
	5'-CCA GCC CTG AAC ACC AAG-3'	[SEQ ID NO: 14]
Endoglucanase 2	5'- GAG TTC ACA TTC CCT GAC A-3'	[SEQ ID NO: 15]
(NCU00762)	5'-CGA AGC CAA CAC GGA AGA-3'	[SEQ ID NO: 16]
GH61 (NCU07898)	5'-TCA AGC CCG GTT ACT ATC-3'	[SEQ ID NO: 17]
	5'-AAC CTG TCA CCT GCA ACT-3'	[SEQ ID NO: 18]
CRE- 1	5'-CTACTGCCATGTCCTCTC-3'	[SEQ ID NO: 19]
	5'-TATCAGGACCACTTTGGCTTC-3'	[SEQ ID NO: 20]
β-Glucosidase	5'-GTTCGGCGTTACCTATGT-3'	[SEQ ID NO: 21]
(NCU00130)	5'-AGAGTCAAAGAGCGGCTTC-3'	[SEQ ID NO: 22]

Protein Secretion/Enzyme Activity Studies

[00151] Strains were inoculated at an OD595 equal to 0.05 in 100ml Vogel's salts with 1% (wt/vol) sucrose in a 250ml Erlenmeyer flask and grown under constant light at 200 rpm for 24 hours. Cultures were then induced with 2% sucrose, 2% cellobiose, 1% sucrose/1% cellobiose, or 1% sucrose/1% Avicel®. Cultures continued to grow under constant light at 200 rpm for 5

days with supernatant collected at 1, 2, 3, 4 and 5 day. The collected supernatant was filtered through a .2μm PES filter to remove biomass before being stored at -20°C until all samples were collected. To visualize the secreted proteins, 15μl of unconcentrated supernatant was run on a Criterion 10% Tris-HCL polyacrylamide gel and stained with Thermo Scientific GelCode Blue Stain Reagent.

[00152] Endo-1,4-β-Glucanase activity was measured using Azo-CM-Cellulose (Megazyme) according to the manufacturers suggested method. Briefly, 100μl Azo-CM-Cellulose substrate solution pre-heated to 37°C was mixed with 96.5μl culture supernatant and 3.5μl 3M sodium acetate pH5.0 in a deep-well 96-well plate. Following mixing, the plate was incubated for 10 minutes at 37°C. The reaction was stopped by the addition of .5ml Precipitant Solution and centrifuged for 10 minutes at 1000g. 50μl sample was transferred to a flat-bottom 96-well assay plate in triplicate and the absorbance was read in a Beckman Coulter Paradigm plate reader at an optical density of 590nm. The data is presented as a percentage of the wild type activity on Avicel® after 4 days.

[00153] Exoglucanase (Cellobiohydrolase I) activity was measured using a 4-Methylumbelliferyl β-D-cellobioside (MuLac) assay. This assay mainly measures the activity of CBH-1 and activity is expressed as the change in fluorescence over time resulting in the slope of a best-fit line as an indication of enzyme activity. Prior to performing this assay, any excess sugars in the culture supernatant were removed by passing the supernatant over a 5,000 Dalton concentrator (sartorius stedim Vivaspin 500). Retained proteins were washed twice with 50mM sodium acetate pH 5 and diluted to 2μg/μl to assure that the assay remained in the linear range. The assay was performed in a total volume of 100μl containing 10μg total protein and had a final concentration of 1.0mM MuLac and 50mM sodium acetate pH 5. The assay was performed in a Beckman Coulter Paradigm plate reader set at 40°C with excitation/emission wavelengths of 360/465nm with readings every 30 seconds for 10 minutes. The slope of the best-fit line represents the MuLac activity for an individual culture supernatant. The MuLac activity is normalized to the initial dilution required to obtain a 2μg/μl concentration in order to represent the undiluted activity. The activity of recombinant cellobiohydrolase-1 was used as a standard and data is presented as a percentage of the wild type activity on Avicel® after 4 days.

[00154] Avicelase activity was determined according to Tian et al. (Tian et al, 2009) as a measure for glucose and cellobiose concentrations in 7-day culture supernatants from WT, Δ*cre-1* and other deletion strains. In brief, one volume of 7-day culture supernatants from WT and Δ*cre-1* strains were mixed with one volume of substrate solution containing 5mg/ml Avicel® and 50mM NaAc buffer, pH 5.0 at 37°C. After 5 hours of shaking glucose and cellobiose concentrations were measured by coupled enzyme assays.

Results

Induction of cellulase transcription in triple β-glucosidase gene deletion

[00155] In order to study early time points of cellulase induction in *N. crassa*, cultures were first grown for 16-hours on sucrose to produce a significant amount of biomass and subsequently transferred to a fresh culture containing an alternate carbon source. An initial time course on the WT strain demonstrated that a four-hour induction period provides a maximal difference in gene expression on sucrose versus Avicel® for the cellobiohydrolase I gene *cbh-1* and the endoglucanase 2 gene *gh5-1* (Fig. 2).

[00156] Three β-glucosidases (NCU08755, NCU04952, and NCU00130) have been shown to be significantly increased at the transcriptional level during growth of WT *N. crassa* on Avicel® or *Miscanthus* (Tian et al, 2009). Additionally, NCU04952 was identified as a secreted protein by mass spectrometry (Tian et al, 2009). To determine whether these three β-glucosidases play a role in the induction of cellulases on cellobiose, three strains containing deletions of single β-glucosidases were screened by qRT-PCR for cellobiose-mediated induction of cellulases. However, no single β-glucosidase deletion mutant showed a significant effect. To overcome the problem of redundancy of β-glucosidase activities and the possibility of strong catabolite repression at minimal glucose concentrations, a triple β-glucosidase deletion strain was constructed.

[00157] On Avicel®, the triple β-glucosidase deletion strain showed a similar induction phenotype as the WT strain for the three cellulases examined (Fig. 3). However, on 2% cellobiose, while the WT strain only shows a 20-fold induction for *cbh-1*, and no change for *cbh-*

2 or *eg-2* compared to their relative expression on sucrose, the triple β-glucosidase mutant shows a very different picture: *cbh-1* has a 6,500-fold increase in relative expression over expression on sucrose; *cbh-2* has a 2,100-fold increase in relative expression and *eg-2* has a 2,200-fold increase in relative expression (Fig. 3).

Induction of cellulase transcription in triple β-glucosidase and cre-1 gene deletion

[00158] By crossing the triple β-glucosidase deletion with the *Δcre-1* strain a mutant was generated that transcriptionally responds to cellobiose in the same way as the WT strain responds to Avicel®. Induction of this mutant with either cellobiose or Avicel® shows similar transcriptional induction of *cbh-1*, *cbh-2* and *eg-2* as the triple β-glucosidase deletion on cellobiose or Avicel® and as the WT strain on Avicel® (Fig. 3). These results demonstrate that *cre-1* acts as a general cellulolytic regulon and that *cre*-1 deletion causes the permanent de-repression of *N. crassa* cellulases.

[00159] C*re-1* deletion is known to moderately increase the transcription and secretion of cellulases on Avicel®. Similarly, Cre-1 is known to allow for approximately 7-fold increases in the basal level of transcription for *cbh-1* and *eg-2* on sucrose compared to WT expression on sucrose (Fig. 4). When induced with cellobiose, *Δcre-1* shows a 600-fold increase in transcription of *cbh-1* and an 80-fold increase in *eg-2* above their expression on sucrose in the same strain (Fig. 3). While this increase in expression is significant relative to expression on sucrose, it is dwarfed by the 11,000-fold increase in *cbh-1* and the 8000-fold increase in *eg-2* seen when *Δcre-1* is induced with Avicel® (Fig. 3).

[00160] In order to show that the transcriptional response to cellobiose is specific and not due to a general starvation effect, a no carbon control experiment was performed. After an initial 16-hour pre-growth phase on sucrose a gentle wash was conducted with minimal media to remove any remaining sucrose and finally the biomass was transferred to a culture containing minimal media without any added carbon source. Because these cultures were processed in the same way as the cultures with added sucrose, cellobiose or Avicel®, the transcriptional data obtained through RT-PCR will show the general affect of starvation on cellulase transcription.

[00161] Figure 5 shows that while the WT strain, Δ*cre-1*, and the triple β-glucosidase deletion show a slight induction due to starvation (three to thirty-fold increases), the triple β-glucosidase/*cre-1* deletion has a larger increase in transcription of *cbh-1* and *eg-2* under these conditions. Relative to triple β-glucosidase/*cre-1* deletion growth on sucrose, its response to starvation is a 340 and 200-fold induction of *cbh-1* and *eg-2* respectively. These effects are minor compared to the 10,000 to 20,000-fold induction of *cbh-1* and *eg-2* observed on Avicel® or cellobiose. These results therefore demonstrate that the transcriptional response of these *N. crassa* cellulases on Avicel® and cellobiose is specific and not a general response to starvation.

[00162] During cellulose hydrolysis in nature cellobiose and glucose do not accumulate to levels high enough to cause significant glucose repression. This phenomenon can be reproduced *in vitro* by varying the experimental concentrations of cellobiose. Figure 6 shows that while induction of cellulases in the wild type strain is significantly reduced relative to the deletion strains, the WT cellulase expression is concentration dependent, with the lower 1mM cellobiose concentration acting as a better inducer than the higher 10 mM concentration. By removing the activity of the major β-glucosidases, a 25-fold increase over wild type induction is achieved at either cellobiose concentration. Moreover, after deleting the catabolite repressor CRE-1, increasing cellobiose concentrations no longer limit enzyme induction (Fig. 6).

Deletion of β-glucosidase genes shows increased cellulolytic enzyme production when induced with cellobiose

[00163] The triple *β*-glucosidase deletion shows a very similar result to the wild type culture when induced with sucrose or Avicel®. On sucrose, there is very little secreted protein (180μg/ml) after 2 days (Fig. 7), and those that are secreted have no activity towards MuLac (Fig. 8). When induced with Avicel®, by day 4 we can see a significant concentration of proteins in the supernatant (Fig. 7). This culture supernatant has an Azo-CM-Cellulose and MuLac activity similar to that for the wild type cultures at the same time point (5.5μg CBHI equivalent) (Figs. 8 and 9). While this deletion is similar to wild type for the sucrose and Avicel® cultures, it responds very differently to cellobiose. At two days on cellobiose, we can see MuLac activity equivalent to 1.4μg recombinant CBHI and by 4 days, this value has significantly increased to 4.84μg recombinant CBHI equivalent (Fig. 8B), and the Azo-CM-Cellulose activity is similar to

the wild type when grown on Avicel® (Fig. 9). This specific enzyme activity indicates that in addition to inducing transcription, cellobiose can directly stimulate the secretion of active cellulases when we minimize the affect of carbon catabolite repression.

Deletion of cre-1 increased cellulolytic enzyme production

[00164] To investigate the role of CRE-1 for *N. crassa* growth on cellulolytic media the relative growth rates of Δ*cre-1* and wild type (WT) strains were compared on different carbon sources. When grown on 2% Avicel® medium as a sole carbon source, the Δ*cre-1* strain consumed Avicel® faster than WT (e.g. 3-4 days vs. 5-6 days), secreted 30% more extracellular protein and showed 50% higher endoglucanase activity (Figs. 10A and 10B). An aggregate Avicelase assay (which measures combined β-glucosidase, endo- and exo-cellulase activity) showed 20% higher glucose concentrations in the Δ*cre-1* strain as compared to WT (Fig. 10B). However, less cellobiose was detected, suggesting increased secretion of β-glucosidase (which converts cellobiose into glucose; Fig. 10B) in the Δ*cre-1* strain.

[00165] Compared to the WT strain, Δ*cre-1* generally appears to produce more secreted proteins. This is evident not only in the cellobiose and Avicel® induced cultures, but also at 48 hours on sucrose (Figs. 7 and 11) where the secreted protein concentration is approximately twice that seen for WT (481μg/ml vs. 270 μg/ml). While the Δ*cre-1* secretes more protein on sucrose, these proteins do not exhibit any activity towards MuLac (Fig. 8). While the most obvious band at 70kDa, runs at the same molecular weight as CBH-1/2, the lack of activity implies that this is either an inactive form of CBH-1/2 or a different non-cellulolytic protein. Similar to what was seen in the transcriptional studies, Δ*cre-1* shows a slight increase in cellulase secretion on cellobiose (653μg/ml) (Fig. 7), which results in a modest increase in activity towards MuLac (.8ug) (Fig. 8B). In addition, when induced with Avicel®, the MuLac activity at 4 days is less than the activity of wild type (2.8ug CBHI equivalent) (Fig. 8B). However, this effect might be due to starvation as the overall protein-banding pattern is generally lighter at 4 days as compared to 3 days (Fig 12).

Deletion of β-glucosidase genes and cre-1 shows increased cellulolytic enzyme production when induced with cellobiose

[00166] The triple *β*-glucosidase/*Δcre-1* deletion strain is similar to the *cre-1* deletion strain in that it seems to constitutively secrete more enzymes than the wildtype and the pattern visible on a protein gel under sucrose or Avicel® induction look very similar for these two strains (Fig. 11). The major difference between the *Δcre-1* and triple *β*-glucosidase/*Δcre-1* deletion strains is the affect of cellobiose on the activity of the secreted proteins (Fig. 8). By four days on cellobiose, this mutant is capable of producing more than 11μg CBHI equivalent, which is even more than is produced on Avicel® at this same timepoint (Fig. 8B). In addition, the Azo-CMC activity assay indicates that this strain produces a similar amount of endo-1,4-β-glucanase activity in either an Avicel® or cellobiose inducing culture (Fig. 9).

Example 2

[00167] The following example relates to the identification of orthologues of the *N. crassa β*-glucosidase genes NCU00130, NCU04952, and NCU08755.

Materials and Methods

[00168] BLASTp searches were conducted using the National Center for Biotechnology Information (NCBI) non-redundant amino acid database using the NCU00130, NCU04952, and NCU08755 amino acid sequences as queries. Sequence hits from the BLASTp searches were aligned in MEGA5 using ClustalW2.

[00169] Phylogenetic trees were generated using the Neighbor-Joining method (Saitou N. and Nei M., 1987). The evolutionary distances were computed using the Poisson correction method (*Zuckerkandl E. and Pauling L., 1965*) and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA5 (*Tamura K., Dudley J., Nei M., and Kumar S., 2007*).

Results

[00170] The results of the ClustalW amino acid sequence alignments for NCU00130, NCU04952, and NCU08755 orthologues in closely related fungi are shown in Figure 13.

[00171] The phylogenetic tree of the β-glucosidase NCU00130 is depicted in Figure 14.

[00172] The phylogenetic tree of the β-glucosidase NCU04952 is depicted in Figure 15.

[00173] The phylogenetic tree of the β-glucosidase NCU08755 is depicted in Figure 16.

Example 3

[00174] The following example relates to the identification and characterization of the proteins secreted at higher levels from the triple β-glucosidase gene deletion *N. crassa* strain, and the triple β-glucosidase and *cre-1 N. crassa* deletion strain.

Materials and Methods

Strains

[00175] Strains obtained from the Fungal Genetics Stock Center (FGSC) include the wild type (FGSC 2489), and deletion strains for the intracellular β-glucosidase NCU00130 (FGSC 11822 and FGSC 11823), and extracellular β-glucosidases: NCU08755 (FGSC 18387 and FGSC 18388) and NCU04952 (FGSC 13731 and FGSC 13732). The homokaryon *cre-1* deletion strain (NCU08807) is described in (44). Multiple deletion strains were made by performing sequential crosses. The genotype of each multiple deletion strain was confirmed using a gene-specific primer and a common primer for the hygromycin (hph) cassette. The hph forward primer used was SEQ ID NO: 4 from Example 1. The reverser primers used for NCU00130, NCU004953, NCU08755, and NCU08807 were the same as those used for Example 1. In particular, the reverse primer for NCU00130 was SEQ ID NO: 5, the reverse primer for NCU004953 was SEQ ID NO: 6, the reverse primer for NCU08755 was SEQ ID NO: 7, and the reverse primer for NCU08807 was SEQ ID NO: 8.

Transcriptional Studies

[00176] Conidia from strains were inoculated at an OD595 equal to .05 in 50 ml Vogel's salts (45) with 2% w/v sucrose in a 250ml Erlenmeyer flask and grown under constant light at 200 rpm for 16 hours. Biomass was then spun at 4200 rpm for 10 minutes and washed in Vogel's salts twice to remove any excess sucrose. Biomass was then added to a new flask containing 50 ml Vogel's salts supplemented with 1% w/v sucrose, 0.2% w/v cellobiose (Sigma) or 1% w/v Avicel® PH 101 (Sigma). Cultures were induced for 4 hrs under constant light at 200 rpm. The culture biomass was then harvested by filtration over a Whatman glass microfiber filter (GF/F) on a Buchner funnel and washed with 50ml Vogel's salts. The biomass was flash frozen in liquid nitrogen and stored at -80°C. Three independent biological duplicates (flasks) were evaluated for each time point.

RNA Isolation

[00177] Total RNA from frozen samples was isolated using Zirconia/Silica beads (0.5mm diameter; Biospec) and a Mini-Beadbeater-96 (Biospec) with 1mL TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA was further purified by digestion with TURBO DNA-free (Ambion) and an RNeasy kit (Qiagen). RNA concentration and integrity was checked by Nanodrop and agarose gel electrophoresis.

RT-PCR

[00178] Quantitative RT-PCR was performed using the EXPRESS One-Step SYBR GreenER Kit (Invitrogen) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Reactions were performed in triplicate with a total reaction volume of 10 µl including 300nM each forward and reverse primers and 75ng template RNA. Data Analysis was performed by the StepOne Software (Applied Biosystems) using the Relative Quantitation/Comparative CT (ΔΔCT) setting. Data was normalized to the endogenous control actin with expression on sucrose as the reference sample.

[00179] The RT-PCR primers used for actin (NCU4173) were SEQ ID NO: 9 and SEQ ID NO: 10 from Example 1; the RT-PCR primers used for *cbh-1* (NCU07340) were SEQ ID NO: 11

and SEQ ID NO: 12 from Example 1; the RT-PCR primers used for *gh6-2* (NCU09680) were SEQ ID NO: 13 and SEQ ID NO: 14 from Example 1; and the RT-PCR primers used for *gh5-1* (NCU00762) were SEQ ID NO: 15 and SEQ ID NO: 16 from Example 1 (46, 47).

mRNA Sequencing

[00180] mRNA sequencing was performed using an Illumina kit (RS-100-0801) with isolated RNA. The final cDNA library was quantified by an Agilent bioanalyzer 2000 and sequenced using an Illumina Genome Analyzer-II using standard Illumina operating procedures.

Phylogenetic Analysis

[00181] GenBank accession numbers (PID), Joint Genome Institute protein ID (JGI), or Broad Institute Fusarium Comparative Database Genes (FGSG) numbers for B-G's used in phylogenetic analysis are as follows; NCU08755: *Myceliophthora thermophila*, JGI 80304; *Aspergillus niger*, PID 254674400; *Phanerochaete chrysosporium*, PID 19352194; *Trichoderma reesei*, JGI 121735; *Fusarium graminearum*, FGSG_06605; *Sclerotinia sclerotiorum*, PID 156051478; *Botryotinia fuckeliana*, PID 154301968; *Penicillium chrysogenum*, PID 255942539; *Schizophyllum commune*, JGI 256304; *Postia placenta*, JGI 107557. NCU00130: *Myceliophthora thermophila*, JGI 115968; *Aspergillus niger*, PID 213437; *Phanerochaete chrysosporium*, PID 127920; *Trichoderma reesei*, JGI 120749; *Fusarium graminearum*, FGSG_07274; *Sclerotinia sclerotiorum*, PID 156037816; *Botryotinia fuckeliana*, PID 156037816; *Penicillium chrysogenum*, PID 255941826; *Schizophyllum commune*, JGI 57050; *Postia placenta*, JGI 45922. NCU04952: *Myceliophthora thermophila*, JGI 66804; *Aspergillus terreus*, PID 115401928; *Phanerochaete chrysosporium*, PID 3320413; *Trichoderma reesei*, JGI 76672; *Sclerotinia sclerotiorum*, PID 156050519; *Botryotinia fuckeliana*, PID 154293970; *Penicillium chrysogenum,* PID 255945487; *Schizophyllum commune*, PID 302694815.

[00182] All proteins used in the alignments were identified using BLASTp. Homologous proteins sequences were aligned in MEGA5 using ClustalW. Maximum Likelihood phylogeny was determined using the Poisson model to estimate distances and the Nearest-Neighborhood-Interchange (NNI) tree searching strategy with 500 bootstrap replications (48, 49).

Analysis of Differential Expression

[00183] To establish biological variation, triplicate cultures were sampled and analyzed for the WT strain on cellulose and sucrose at 4 hours after the media shift. For all other strains and conditions, a single RNAseq library was analyzed.

[00184] Sequenced libraries were mapped against predicted transcripts from the *N. crassa* OR74A genome (version 10) with Tophat (version 1.1.4) (50). Transcript abundance was estimated with Cufflinks (version 0.9.2) in FPKMs (fragments per kilobase of exon per million fragments mapped) (51) using upper quartile normalization and mapping against reference isoforms from the Broad Institute.

Hierarchical Clustering Analysis

[00185] Genes exhibiting statistically significant expression changes between strains or growth conditions were identified with Cuffdiff, using upper quartile normalization and a minimum of mapped reads per locus. These genes were then filtered to select only those exhibiting a two-fold change in estimated abundance between all biological replicates of each strain/condition tested and only those genes with an FPKM consistently above 10 in at least one strain/condition.

[00186] The hierarchical clustering analysis was performed using Cluster 3.0 (52) according to the FPKMs in the WT strain on cellulose, WT on cellobiose, mutant strains on cellobiose and mutant strains on cellulose. Prior to clustering, FPKMs were log transformed, normalized across strains/conditions on a per-gene basis and centered on the mean value across strains/conditions. The Pearson correlation coefficient (uncentered) was used as the similarity metric and average linkage as the clustering method.

Shake Flask Studies

[00187] Cultures were grown in 1% sucrose for 24 hours followed by the addition of 2% sucrose or 0.2% cellobiose. Supernatant was harvested after 24 (WT, Δ3βG and Δ3βGΔ*cre*) or 72 hours (Δ3βG). The WT Avicel® culture was grown for 5 days on 2% Avicel®, Δ3βG was

grown in 1% sucrose for 24 hours followed by 48 hours in 1% Avicel® and Δ3βGΔ*cre* was grown in 1% sucrose for 24 hours followed by 24 hours in 1% Avicel®.

Bioreactor Studies

[00188] Cellulase production was carried out in a 3.7 L bioreactor (BioEngineering AG) at an operating volume of 1 L. The bioreactor was equipped with one 48 mm Rushton impeller and four equally spaced baffles to provide adequate mixing. Impeller speed was controlled at 200 rpm for 8 hours to allow spore germination followed by 500 rpm for the remainder of the experiment. The temperature was maintained at 25°C, and medium pH was controlled at 5.5 using 40% phosphoric acid and 1:5 diluted ammonium hydroxide. The dissolved oxygen was maintained at a level greater than 20% of the saturation value of the medium by varying the aeration rate between 0.5 and 3 VVM in response to the dissolved oxygen tension. Minimal growth medium with 1% w/v sucrose as the sole carbon source (unless otherwise noted) was inoculated with 10^9 conidia. After 24 hours initial growth, cellulase production was induced with either cellobiose or Avicel® added to a final concentration of 0.2% w/v. Supernatant samples were collected at timepoint 0, 12 hours before induction, at induction, as well as 4, 8, 12, 24 and 36 hours post induction. Samples were spun at 4000rpm for 5 minutes to pellet biomass and the supernatant was filtered through a .2 μm PES filter before being stored at -20°C until all samples were collected.

Enzyme Activity Assays

[00189] Total secreted proteins were measured using the Bio-Rad Protein Assay kit (Bio-Rad) and visualized by running 15 μl of unconcentrated supernatant on a Criterion 4-14% Tris-HCL polyacrylamide gel and stained with Thermo Scientific GelCode Blue Stain Reagent.

[00190] Total Avicelase activity was conducted in 250 mL media bottles incubated at 50°C on a orbital shaker at 200rpm. Each bottle contained 1% cellulose (Avicel®) and 50 mM (pH 5.0) sodium acetate in a working volume of 50 mL. Tetracycline (10μg/mL) was added to prevent microbial contamination. Bioreactor culture broth samples were buffer exchanged using a 10 kDa MWCO centrifugal filter to remove any soluble sugars prior to initiating hydrolysis experiments. After pre-incubating the hydrolysis mixture to 50°C, enzyme was added (1 mL

filtered culture broth). Samples were taken every 4 hours for the first 12 hours and then every 12 hours thereafter for a total of 48 hours. Hydrolysis experiments were performed in triplicate.

Sugar Analysis

[00191] Sucrose, fructose, glucose and cellobiose were measured on a DIONEX ICS-3000 HPLC (Dionex Corp., Sunnyvale, CA) using a CarboPac PA20 Analytical Column (3x150mm) and a CarboPac PA20 guard column(3x30mm) at 30°C. Following injection of 25µl of diluted samples, elution was performed with 100mM KOH (isocratic) at .4ml/min. Sugars were detected using PAD, Four-Potential Carbohydrate Waveform and Peaks were analyzed using the Chromeleon software package.

Mass Spectrometry

[00192] Acetonitrile (Fisher Optima grade, 99.9%) and formic acid (Pierce, 1 mL ampules, 99+%) purchased from Fisher Scientific (Pittsburgh, PA), and water purified to a resistivity of 18.2 MΩ·cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA), were used to prepare mobile phase solvents for liquid chromatography-mass spectrometry.

[00193] Trypsin-digested proteins were analyzed using an orthogonal acceleration quadrupole time-of-flight (Q-tof) mass spectrometer that was connected in-line with an ultraperformance liquid chromatograph (UPLC). Peptides were separated using a nanoAcquity UPLC (Waters, Milford, MA) equipped with C_{18} trapping (180 µm × 20 mm) and analytical (100 µm × 100 mm) columns and a 10 µL sample loop. Solvent A was 99.9% water/0.1% formic acid and solvent B was 99.9% acetonitrile/0.1% formic acid (v/v). Sample solutions contained in 0.3 mL polypropylene snap-top vials sealed with septa caps (Wheaton Science, Millville, NJ) were loaded into the nanoAcquity autosampler compartment prior to analysis. Following sample injection (10 µL), trapping was performed for 3 min with 100% A at a flow rate of 15 µL/min. The injection needle was washed with 500 µL each of solvents A and B after injection to avoid cross-contamination between samples. The elution program consisted of a linear gradient from 8% to 35% B over 30 min, a linear gradient to 95% B over 0.33 min, isocratic conditions at 95% B for 3.67 min, a linear gradient to 1% B over 0.33 min, and isocratic conditions at 1% B for

11.67 min, at a flow rate of 500 nL/min. The analytical column and sample compartment were maintained at 35°C and 8°C, respectively.

[00194] The UPLC column exit was connected to a Universal NanoFlow Sprayer nanoelectrospray ionization (nanoESI) emitter that was mounted in the nanoflow ion source of the mass spectrometer (Q-tof Premier, Waters, Milford, MA). The nanoESI emitter tip was positioned approximately 3 mm from the sampling cone aperture. The nanoESI source parameters were as follows: nanoESI voltage 2.4 kV, nebulizing gas (nitrogen) pressure 0.15 mbar, sample cone voltage 35 V, extraction cone and ion guide voltages 4 V, and source block temperature 80°C. No cone gas was used. The collision cell contained argon gas at a pressure of 8×10^{-3} mbar. The Tof analyzer was operated in "V" mode. Under these conditions, a mass resolving power (53) of 1.0×10^4 (measured at $m/z = 771$) was routinely achieved, which was sufficient to resolve the isotopic distributions of the singly and multiply charged precursor and fragment ions measured in this study. Thus, an ion's mass and charge were determined independently, i.e., the ion charge was determined from the reciprocal of the spacing between adjacent isotope peaks in the *m/z* spectrum. External mass calibration was performed immediately prior to analysis using a solution of sodium formate. Survey scans were acquired in the positive ion mode over the range $m/z = 400\text{-}1500$ using a 0.45 s scan integration and a 0.05 s interscan delay. In the data-dependent mode, up to five precursor ions exceeding an intensity threshold of 20 counts/second (cps) were selected from each survey scan for tandem mass spectrometry (MS/MS) analysis. Real-time deisotoping and charge state recognition were used to select 2+, 3+, and 4+ charge state precursor ions for MS/MS. Collision energies for collisionally activated dissociation (CAD) were automatically selected based on the mass and charge state of a given precursor ion. MS/MS spectra were acquired over the range $m/z = 100\text{-}2000$ using a 0.20 s scan integration and a 0.05 s interscan delay. Ions were fragmented to achieve a minimum total ion current (TIC) of 30,000 cps in the cumulative MS/MS spectrum for a maximum of 2 s. To avoid the occurrence of redundant MS/MS measurements, real-time dynamic exclusion was used to preclude re-selection of previously analyzed precursor ions over an exclusion width of ±0.2 *m/z* unit for a period of 300 s.

[00195] Data resulting from LC-MS/MS analysis of trypsin-digested proteins were processed using ProteinLynx Global Server software (version 2.3, Waters), which performed background

subtraction (threshold 35% and fifth order polynomial), smoothing (Savitzky-Golay, 10 times, over three channels), and centroiding (top 80% of each peak and minimum peak width at half height four channels) of mass spectra and MS/MS spectra. Processed data were searched against the *Neurospora crassa* protein database (Broad Institute, Cambridge, MA). The following criteria were used for the database search: precursor ion mass tolerance 100 ppm, fragment ion mass tolerance 0.15 Da, digest reagent trypsin, allowing for up to three missed cleavages, and methionine oxidation as a variable modification. The identification of at least three consecutive fragment ions from the same series, i.e., b or y-type fragment ions (54), was required for assignment of a peptide to an MS/MS spectrum. MS/MS spectra were inspected to verify the presence of fragment ions that identify the peptides. A protein was determined to be present if at least 1 peptide was detected in 2 out of 3 biological replicates (whole supernatant, PASC bound or PASC unbound)

Results

Induction of cellulase transcripts in cellodextrin-induced cultures of N. crassa lacking three β-glucosidase genes

[00196] Lignocellulolytic genes were not induced, nor was cellulolytic enzyme activity detected when wild-type *N. crassa* (WT) was grown on sucrose, cellobiose, cellotriose, or cellotetraose as the sole carbon source (Fig. 17A). It was believed that when *N. crassa* is grown on cellodextrins, glucose produced by action of β-glucosidase enzymes may mask its inducing capacity (Fig. 1). While the genome of *N. crassa* has at least 7 genes encoding predicted β-glucosidase enzymes, only three (NCU00130, NCU04952 and NCU08755) show a significant increase in transcription during growth on Avicel® or *Miscanthus* (20). All three of these β-glucosidases showed significant homology to both predicted and experimentally verified β-glucosidase enzymes in other filamentous fungi. Based on expression data, we believed that GH1-1 (NCU00130), GH3-3 (NCU08755), and GH3-4 (NCU04952) would be the most relevant enzymes in converting cellobiose to glucose when *N. crassa* is grown on either Avicel® or cellodextrins as sole carbon sources.

[00197] To determine whether cellobiose induces cellulase gene expression in *N. crassa*, we tested whether the expression of three major cellulase genes (*cbh-1*, NCU07340; *gh6-2,* NCU09680 and *gh5-1,* NCU00762) were induced in strains carrying deletions in the β-glucosidase genes *gh1-1*, *gh3-3* or *gh3-4* via a transfer experiment. To eliminate the possibility of redundancy between the β-glucosidase enzymes, double and triple mutant strains carrying different combinations of β-glucosidase gene deletion sets were also constructed and tested. Following a 4 hr induction with 0.2% cellobiose, the individual β-glucosidase deletion strains (Δ*gh1-1,* Δ*gh3-3* or Δ*gh3-4*) did not show a significant induction of *cbh-1*, *gh6-2*, or *gh5-1* expression; whereas a Δ*gh1-1*Δ*gh3-3* double mutant showed some cellulase gene induction. However, a strain carrying deletions for all three β-glucosidase genes (Δ*gh1-1,* Δ*gh3-3* and Δ*gh3-4*; Δ3βG) showed similar relative expression levels of *cbh-1, gh5-1* and *gh6-2* when shifted to 0.2% cellobiose as did a WT culture shifted to Avicel® (Fig. 18A). In addition, the Δ3βG strain showed similar relative expression levels of *cbh-1, gh5-1* and *gh6-2* when shifted to cellobiose, cellotriose, or cellotetraose (Fig. 17B). The transcriptional response in the Δ3βG mutant was specific for cellobiose and was not due to starvation as the expression of *cbh-1* and *gh5-1* in WT and the Δ3βG strain when transferred to media lacking any carbon source showed only a small increase in transcription levels (less than 50-fold induction). These values are negligible when compared to the ~20,000-fold (minimum) induction of *cbh-1* and *gh5-1* by Avicel® in WT *N. crassa* and in the Δ3βG strain shifted to cellobiose.

[00198] The most widely used soluble inducers of cellulases in the industrial species *T. reesei* are sophorose and lactose (25). We therefore examined whether exposure to sophorose or lactose induced cellulase gene expression in *N. crassa* using both the WT and the Δ3βG deletion strain. As observed for other filamentous fungal species (15), transfer of either WT or the Δ3βG mutant to media containing sophorose, lactose or D-(+)-galactose (a degradation product of lactose), did not significantly induce cellulase gene expression (Fig. 19).

[00199] Carbon catabolite repression (CCR) acts in filamentous fungi to repress cellulase and hemicellulase gene expression in the presence of preferred carbon sources, such as glucose or sucrose, even when lignocellulose is present (4). The C2H2 zinc finger transcription factor CreA/CRE1/CRE-1 (26) plays a key role in CCR as strains lacking CreA/CRE1/CRE-1 in *Aspergillus sp., T. reesei* and *N. crassa,* respectively, produce increased amounts of both

cellulases and hemicellulases when grown on cellulose or hemicellulose (21, 27, 28). Quantitative RT-PCR analysis of RNA isolated from an *N. crassa cre-1* deletion strain (ΔNCU08807) showed that the basal expression of *cbh-1* and *gh5-1* increased about ten-fold relative to a WT strain. When shifted from sucrose to 0.2% cellobiose for 4 hrs, the Δ*cre-1* strain showed increased induction of *cbh-1, gh5-1* and *gh6-2* (3,000, 500, and 85-fold, respectively). However, the level of induction in the Δ*cre-1* mutant was significantly lower than induction levels obtained for WT exposed to Avicel® or the Δ3βG mutant exposed to cellobiose. Notably, a Δ3βG strain that also carried the Δ*cre-1* deletion (Δ3βGΔ*cre*) exhibited stronger induction of *cbh-1, gh5-1* and *gh6-2* than either the WT strain shifted to Avicel® or the Δ3βG strain shifted to cellobiose (Fig. 18A). These data indicate that the induction of cellulase gene expression in the Δ3βG mutant when exposed to cellobiose is comparable to induction by cellulose and is not a consequence of relief from CCR.

Recapitulation of wild-type N. crassa cellulolytic response in the triple β-glucosidase mutant on cellobiose

[00200] High throughput sequencing (RNA-Seq) was used to assess whether the full genomic response in the Δ3βG strains to cellobiose was similar to or different from a WT strain exposed to Avicel®. The full genomic pattern of gene expression changes showed that the response of the Δ3βG mutant to cellobiose closely matched that of WT induced by Avicel®, but was significantly different from the response of WT to cellobiose or when subjected to starvation. To identify which genes were significantly and specifically induced in WT *N. crassa* in response to Avicel®, a pairwise analysis was performed between expression profiles of WT transferred to Avicel® versus WT transferred to no added carbon source. These analyses identified 321 genes (including the three deleted β-glucosidase genes) that were significantly and specifically induced in WT cultures in response to Avicel® (cellulose regulon). This gene set included 16 predicted cellulase and 12 predicted hemicellulase genes. Additional genes in the cellulose regulon included 41 genes encoding proteins predicted to be active on carbohydrates by CAZy (29) and 111 genes encoding secreted proteins (signalP) (30). Of the 321 genes in the cellulose regulon, 156 encode proteins that are characterized as unclassified proteins (MIPS FunCat database) (31). Of specific interest, the orthologue for *xlnR/xyr1* (NCU06971), which plays a major role in the regulation of cellulases in *Aspergilli* (32) and *T. reesei (33)*, falls into the cellulose regulon.

However, although NCU06971 was previously identified as a *xlnR/xyr1* homolog in *N. crassa* (34), its role in plant cell wall degradation is unknown.

[00201] Hierarchical clustering of genes within the cellulose regulon from expression data of WT transferred to media containing no carbon source, cellobiose or Avicel® and the Δ3βG strain transferred to media containing cellobiose or Avicel® resulted in the identification of four distinct expression clusters (Fig. 20A). The largest cluster (cluster 2) contained 210 genes that showed high expression in the WT strain on Avicel®, as well as in the Δ3βG strain on either cellobiose or under Avicel®-induced conditions. This group of 210 genes contained all 16 predicted cellulases (NCU00762, *gh5-1*; NCU00836, *gh61-7*; NCU01050, *gh61-4*; NCU02240, *gh61-1*; NCU02344, *gh61-12*; NCU02916, *gh61-3*; NCU03328, *gh61-6*; NCU04854, *gh7-2*; NCU05057, *gh7-1*; NCU05121, *gh45-1*; NCU07190, *gh6-3*; NCU07340, *cbh-1*; NCU07760, *gh61-2*; NCU07898, *gh61-13*; NCU08760, *gh61-5*; NCU09680, *gh6-2*) as well as 3 genes identified to be accessory proteins for cellulose degradation (NCU00206, *cdh-1*; NCU07143, *lac-2*; NCU09764, CBM1 containing protein) (20, 35). This cluster also contained 9 hemicellulase genes (NCU02343, *gh51-1*; NCU02855, *gh11-1*; NCU04997, *gh10-3*; NCU05924, *gh10-1*; NCU05955, *gh74-1*; NCU07225, *gh11-2*; NCU07326, *gh43-6*; NCU08189, *gh10-2*; NCU09775, *gh54-1*). Of the 182 proteins remaining in this cluster, 29 are predicted to be active on carbohydrates by CAZy (29) and 76 are predicted to be secreted by signalP, with 25 genes falling into both categories. The remaining 102 genes were grouped into their predicted functional category (31) resulting in 10 genes expected to be involved in C-compound and carbohydrate metabolism; 8 genes involved in protein folding, modification, or transport; and 62 genes encoding unclassified proteins.

[00202] A small cluster of 36 genes (cluster 1) showed high expression levels in either the WT or Δ3βG deletion strain when exposed to Avicel® (Fig. 20A), but had lower expression levels in the Δ3βG deletion strain on cellobiose. This group contained a predicted β-xylosidase gene (NCU09652, *gh43-5*) and several other genes encoding proteins active on hemicellulose (NCU00710, acetyl xylan esterase; NCU01900, xylosidase/arabinosidase; NCU00891, xylitol dehydrogenase; and NCU08384, xylose reductase). These results suggest that these genes were induced by the 0.5-1.0% hemicellulose found in Avicel® (20) and are not part of the regulon induced by cellobiose.

[00203] When comparing the induction of the Δ3βG strain on cellobiose versus WT on Avicel®, a striking pattern appears (Fig. 20B). Genes induced in the WT by Avicel® are very close to the value seen in the Δ3βG mutant. For example, the FPKM for *cbh-1* in the WT on Avicel® is 126,816 ± 53,016, while the FPKM in Δ3βG on cellobiose is 130,865. This pattern extends even to the lesser-induced cellulases like NCU07760 (*gh61-2*), which has a FPKM of 239±62 for WT on Avicel® and 538 for Δ3βG mutant on cellobiose. In contrast, some hemicellulase genes in the Δ3βG mutant were induced in response to cellobiose, but had lower expression levels than in Avicel®-induced WT or Δ3βG cultures. For example, while NCU05924 (endoxylanase, *gh10-1*) has 20,023 ± 9,888 FPKMs in WT induced with Avicel®, an expression level of 10,000 FPKMs was observed in the Δ3βG mutant induced with cellobiose. These results indicate that while all of the cellulase genes are in the same regulon, the hemicellulase genes are divided into those that are coordinately regulated with cellulases and those that require additional signals for full induction.

Transcription of plant cell wall degrading enzymes in the Δ3βG mutant correlates with cellulase secretion and activity

[00204] To determine whether the transcriptional response of the Δ3βG and Δ3βGΔ*cre* strains in response to cellobiose corresponded to an increase in functional protein, we assessed secreted proteins and cellulase activity of the Δ3βG and Δ3βGΔ*cre* strains in response to induction with either cellobiose or Avicel® (SI Materials and Methods), as compared to WT cultures. As expected, supernatants from all sucrose-grown cultures (Δ3βG, Δ3βGΔ*cre* and WT) were unable to produce glucose or cellobiose from crystalline cellulose in an Avicel® hydrolysis assay (Materials and Methods), while supernatants from all three Avicel®–induced cultures (Δ3βG, Δ3βGΔ*cre* and WT) were able to degrade crystalline cellulose to cellobiose and glucose (Fig. 18C). When grown on cellobiose, the Δ3βG and Δ3βGΔ*cre* strains displayed a secreted protein pattern similar to WT Avicel®-grown cultures (Fig. 18B) (20). Importantly, supernatants from both the Δ3βG and Δ3βGΔ*cre* deletion strains induced by cellobiose hydrolyzed crystalline cellulose, while supernatants from WT cellobiose grown cultures did not. The Δ3βG and Δ3βGΔ*cre* strains, which lack three β-glucosidases, produced mostly cellobiose. These data are consistent with the role of the three β-glucosidase enzymes in providing the bulk of the glucose-generating activity in WT cultures (37).

[00205] Industrial filamentous fungi are grown in submerged cultures for high-level production of a variety of products (38). We therefore examined the induction of cellulases in the Δ3βG and Δ3βGΔ*cre* deletion strains in a controlled bioreactor process (Figs.7A-D). After 24 hours growth on sucrose, WT, Δ3βG and Δ3βGΔ*cre* produce a similar amount of biomass (~3.5g/L) (Figs. 7A-C). After induction with 0.2% cellobiose, WT did not secrete a significant amount of protein (.05mg/mL; Fig.7C). In contrast, the Δ3βG and Δ3βGΔ*cre* cultures produced 0.12 mg/mL and 0.24 mg/mL protein, respectively, in the supernatant (Figs.7A and 7B). In addition, the cellobiose-induced Δ3βG and Δ3βGΔ*cre* cultures showed a significant increase in endoglucanase activity over this same period of induction (Fig. 7F). Examining the aggregate Avicelase activity from the 24-hour time point indicated that the Δ3βGΔ*cre* strain produced 60% more glucose equivalents (0.424 mg/mL) as compared to the Δ3βG strain (0.296 mg/mL) (Fig. 7E). However, when the total concentration of protein was normalized, the Δ3βGΔ*cre* strain had less specific activity than either the WT or Δ3βG culture supernatants (Fig. 21).

Proteomic analysis of secreted proteins

[00206] In order to compare the identity of proteins secreted by WT *N. crassa* grown on Avicel® versus the Δ3βG strains when induced with cellobiose, we analyzed the secretome using a shotgun proteomics approach (Table 1). There were 39 proteins identified in the WT Avicel®-grown culture supernatant. In cellobiose-grown cultures, 38 proteins were identified in the Δ3βG broth and 24 were identified in the Δ3βGΔ*cre* broth (Fig. 22). Using quantitative mass spectrometry it was concluded that 76% of the WT *N. crassa* secretome on Avicel® is composed of 6 individual proteins (35). All of these proteins were identified in the WT, Δ3βG, and Δ3βGΔ*cre* culture broths (except for the deleted β-glucosidase, *gh3-4*) (Table 1). In addition to the cellulases, we identified a number of lower abundance accessory proteins which make up a total of 6.5% of the secretome (35): a cellobiose dehydrogenase (CDH-1), a type 2 lactonase (LAC-2), and two hypothetical proteins: NCU09764, a CBM1-containing protein of unknown function and NCU05137, a gene which when deleted leads to an increase in cellulase activity (20). These data indicate that, similar to the transcriptional response of the Δ3βG mutant to cellobiose, the identity of proteins secreted and the amount of protein secreted in the Δ3βG strain on cellobiose mimicked the WT *N. crassa* response to Avicel®.

Table 1

Gene	Annotation	Wild Type	Δ3βG	Δ3βGΔcre	Secretome Percentage
Cellulases					
NCU07340	CBH-1	+	+	+	39.5%
NCU09680	GH6-2	+	+	+	13.4%
NCU07898	GH61-2	+		+	6.6%
NCU00762	GH5-1	+	+	+	5.9%
NCU08760	GH61-5	+	+	+	4.6%
NCU05057	GH7-1	+	+	+	4.0%
NCU02240	GH61-1	+		+	3.4%
NCU07190	GH6-3	+	+	+	3.2%
Accessory Proteins					
NCU04952	GH3-4	+	N/A	N/A	3.8%
NCU00206	CDH-1	+	+	+	2.4%
NCU09764	N/A	+	+	+	1.6%
NCU05137	NCW-1	+	+	+	1.5%
NCU07143	LAC-2	+	+	+	1.0%

[00207] In Table 1, GH refers to glycoside hydrolase, and N/A refers to gene knockout. For secretome percentage, Avicel®-induced secretome was identified by AQUA Mass Spectrometry (35). Thirteen proteins represent 91% of the total secretome with all other proteins representing less than 1% of the secretome.

Example 4

[00208] The following example relates to the characterization of cellulase activity in *N. crassa* strains containing deletions of the *N. crassa* gene NCU00890 and the *N. crassa* gene NCU06650.

Materials and Methods

[00209] The *N. crassa* triple β-glucosidases gene deletion strain, and the *N. crassa* triple β-glucosidases gene deletion and *cre-1* gene deletion strain were generated as described in Example 1.

[00210] Deletion strains for NCU06650 (FGSC 11246 and 11247) and NCU00890 (FGSC 16749) were obtained from the Fungal Genetics Stock Center (FGSC). Multiple deletion strains were generated by performing sequential crosses. The genotype of each multiple deletion strain

was confirmed using a gene-specific primer and a common primer for the hygromycin (hph) cassette. The forward primer for hph was:

hph Middle FWD: 5'-CGA CAG ACG TCG CGG TGA GTT CAG-3' [SEQ ID NO: 4]

Reverse primers were:

NCU06650: 5'- CAT CTC ATA CTC CCT CAT CC -3' [SEQ ID NO: 23]

NCU00890: 5'- GGT TGT CTC GGT CGA CAT TG -3' [SEQ ID NO: 24]

[00211] Exoglucanase (Cellobiohydrolase I) activity was measured using a 4-Methylumbelliferyl β-D-cellobioside (MuLac) assay. This assay mainly measures the activity of CBH-1 and activity is expressed as the change in fluorescence over time resulting in the slope of a best-fit line as an indication of enzyme activity. The assay was performed in a total volume of 100µl containing 20µl total culture supernatant and had a final concentration of 1.0mM MuLac and 50mM sodium acetate pH 5. The assay was performed in a Beckman Coulter Paradigm plate reader set at 40°C with excitation/emission wavelengths of 360/465nm with readings every 30 seconds for 10 minutes. The slope of the best-fit line represents the MuLac activity for an individual culture supernatant.

Results

[00212] Given that both NCU00890 and NCU06650 deletions have both been characterized as hypersecretors, we wanted to examine if combining these deletions with the triple β-glucosidase and cre-1 deletion strain would increase cellulase secretion. The NCU00890 geneencodes a β-mannosidase. The NCU06650 gene encodes an characterized polypeptide having closest homology to a phospholipase.

[00213] As shown in Figure 23, when either the NCU00890 or the NCU06650 deletion is combined with the triple β-glucosidase deletion strain, we see a modest increase in cellobiohydrolase I activity after 24 hours induction by cellobiose. Moreover, by also including the cre-1 deletion the sextuple mutant (containing the triple β-glucosidase deletion and both the

NCU00890 and the NCU06650 deletion) had even higher cellobiohydrolase I activity after 24 hours induction by cellobiose.

[00214] Homologues of the β-mannosidase gene NCU00890 were identified in *Trichoderma reesei*. The *T. reesei* homologues are found on Scaffold 10, 258215-260779, protein ID 62166; and on Scaffold 4, 877954-880802, protein ID 57857.

[00215] Additionally, a homologue of the gene NCU06650 was identified in T. *reesei*. The homologue was found on Scaffold 22, 490155-490769, protein ID 67579.

[00216] The *T. reesei* homologues were identified by performing a BLASTp search of either NCU00890 or NCU06650 using the DOE Joint Genome Institute *T. reesei* database.

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CLAIMS

What is claimed:

1. A method for increasing secretion of a protein from a cell, the method comprising:

   (a) providing a mutant cell, wherein the mutant cell comprises inactivating mutations in two or more β-glucosidase genes; and

   (b) contacting said mutant cell with cellulosic biomass, wherein said cellulosic biomass induces the mutant cell to secrete said protein.

2. The method of claim 1, wherein the mutant cell further comprises an inactivating mutation in a cre-1 gene in said cell.

3. A method for increasing secretion of a protein from a cell, the method comprising:

   (a) providing a mutant cell, wherein the mutant cell comprises an inactivating mutation in a cre-1 gene in said cell; and

   (b) contacting said mutant cell with cellulosic biomass, wherein said cellulosic biomass induces the mutant cell to secrete said protein.

4. The method of claim 3, wherein the mutant cell further comprises inactivating mutations in two or more β-glucosidase genes.

5. The method of any one of claims 1-4, wherein the cellulosic biomass comprises one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

6. The method of any one of claims 1-4, wherein the cellulosic biomass comprises cellobiose.

7. A method for increasing secretion of a protein from a cell, the method comprising:

   (a) providing a mutant cell, wherein the mutant cell comprises inactivating mutations in two or more β-glucosidase genes; and

(b) contacting said mutant cell with a saccharide, wherein said saccharide induces the mutant cell to secrete said protein.

8. The method of claim 7, wherein the mutant cell further comprises an inactivating mutation in a cre-1 gene in said cell.

9. A method for increasing secretion of a protein from a cell, the method comprising:

(a) providing a mutant cell, wherein the mutant cell comprises an inactivating mutation in a cre-1 gene in said cell; and

(b) contacting said mutant cell with a saccharide, wherein said saccharide induces the mutant cell to secrete said protein.

10. The method of claim 9, wherein the mutant cell further comprises inactivating mutations in two or more β-glucosidase genes.

11. The method of any one of claims 7-10, wherein the saccharide is selected from the group consisting of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

12. The method of any one of claims 7-10, wherein the saccharide is cellobiose.

13. The method of any one of claims 1-12, wherein the secreted protein is a cellulose-induced protein.

14. The method of any one of claims 1-13, wherein the secreted protein is selected from the group consisting of a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, a cellulose binding domain-containing protein, and combinations thereof.

15. The method of any one of claims 1-13, wherein the secreted protein is a cellulase.

16. The method of any one of claims 1-13, wherein the secreted protein is encoded by a gene selected from the group consisting of NCU07340, NCU09680, NCU07898, NCU00762,

NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137.

17. The method of any one of claims 1-16, wherein the mutant cell further comprises an inactivating mutation in at least one β-mannosidase gene in said cell.

18. The method of any one of claims 1-17, wherein the mutant cell further comprises an inactivating mutation in at least one phospholipase gene or phospholipase-like gene in said cell.

19. The method of any one of claims 1-18, wherein the inactivating mutations are deletions.

20. The method of any one of claims 1-19, wherein the cell is a recombinant cell.

21. The method of any one of claims 1-20, wherein the cell is a fungal or yeast cell.

22. The method of any one of claims 1-21, wherein the cell is a filamentous fungus of the ascomycete or basidiomycete species.

23. The method of any one of claims 1-22, wherein the cell is selected from the group consisting of *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells, *Schizophyllum commune* cells, *Postia placenta* cells, *Aspergillus oryzae* cells, and *Acremonium cellulolyticus* cells.

24. The method of any one of claims 1-23, wherein the two or more β-glucosidase genes are three or more β-glucosidase genes.

25. The method of any one of claims 1-23, wherein the two or more β-glucosidase genes are four or more β-glucosidase genes.

26. The method of any one of claims 1-23, wherein the two or more β-glucosidase genes are five or more β-glucosidase genes.

27. The method of any one of claims 1-23, wherein the two or more β-glucosidase genes are six or more β-glucosidase genes.

28. The method of any one of claims 1-23, wherein the two or more β-glucosidase genes are seven or more β-glucosidase genes.

29. The method of any one of claims 24-28, wherein the three or more β-glucosidase genes, four or more β-glucosidase genes, five or more β-glucosidase genes, six or more β-glucosidase genes, or seven or more β-glucosidase genes comprise NCU00130, NCU04952, and NCU08755.

30. The method of any one of claims 1-29, wherein at least one of the β-glucosidase genes encodes an intracellular β-glucosidase.

31. The method of any one of claims 1-29, wherein at least one of the β-glucosidase genes encodes an extracellular β-glucosidase.

32. The method of any one of claims 1-31, wherein the at least one β-mannosidase gene is NCU00890.

33. The method of any one of claims 1-32, wherein the at least one phospholipase gene or phospholipase-like gene is NCU06650.

34. A method for increasing secretion of a protein from a cell, the method comprising:

(a) providing a recombinant cell, wherein the recombinant cell exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell; and

(b) contacting said recombinant cell with cellulosic biomass, wherein said cellulosic biomass induces the recombinant cell to secrete said protein.

35. The method of claim 34, wherein the recombinant cell further exhibits reduced expression of a cre-1 gene compared to the expression of the expression of the cre-1 gene in a corresponding non-recombinant cell.

36. A method for increasing secretion of a protein from a cell, the method comprising:

(a) providing a recombinant cell, wherein the recombinant cell exhibits reduced expression of a cre-1 gene compared to the expression of the expression of the cre-1 gene in a corresponding non-recombinant cell; and

(b) contacting said recombinant cell with cellulosic biomass, wherein said cellulosic biomass induces the recombinant cell to secrete said protein.

37. The method of claim 36, wherein the recombinant cell further exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell.

38. The method of any one of claims 34-37, wherein the cellulosic biomass comprises one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

39. The method of any one of claims 34-37, wherein the cellulosic biomass comprises cellobiose.

40. A method for increasing secretion of a protein from a cell, the method comprising:

(a) providing a recombinant cell, wherein the recombinant cell exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell; and

(b) contacting said mutant cell with a saccharide, wherein said saccharide induces the mutant cell to secrete said protein.

41. The method of claim 40, wherein the recombinant cell further exhibits reduced expression of a cre-1 gene compared to the expression of the expression of the cre-1 gene in a corresponding non-recombinant cell.

42. A method for increasing secretion of a protein from a cell, the method comprising:

(a) providing a recombinant cell, wherein the recombinant cell exhibits reduced expression of a cre-1 gene compared to the expression of the expression of the cre-1 gene in a corresponding non-recombinant cell; and

(b) contacting said mutant cell with a saccharide, wherein said saccharide induces the mutant cell to secrete said protein.

43. The method of claim 42, wherein the recombinant cell further exhibits reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell.

44. The method of any one of claims 40-43, wherein the saccharide is selected from the group consisting of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

45. The method of any one of claims 40-43, wherein the saccharide is cellobiose.

46. The method of any one of claims 34-45, wherein the secreted protein is a cellulose-induced protein.

47. The method of any one of claims 34-46, wherein the secreted protein is selected from the group consisting of a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, a cellulose binding domain-containing protein, and combinations thereof.

48. The method of any one of claims 34-46, wherein the secreted protein is a cellulase.

49. The method of any one of claims 34-46, wherein the secreted protein is encoded by a gene selected from the group consisting of NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137.

50. The method of any one of claims 34-49, wherein the function of creA/cre-1 is reduced by overexpression of a dominant negative mutant or a protein inhibitor.

51. The method of any one of claims 34-50, wherein the recombinant cell further exhibits reduced expression of at least one β-mannosidase gene compared to the expression of the at least one β-mannosidase genes in a corresponding non-recombinant cell.

52. The method of any one of claims 34-51, wherein the recombinant cell further exhibits reduced expression of at least one phospholipase gene or phospholipase-like gene compared to the expression of the at least one phospholipase gene or phospholipase-like gene in a corresponding non-recombinant cell.

53. The method of any one of claims 34-52, wherein gene expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing.

54. The method of any one of claims 34-53, wherein the two or more β-glucosidase genes are three or more β-glucosidase genes.

55. The method of any one of claims 34-53, wherein the two or more β-glucosidase genes are four or more β-glucosidase genes.

56. The method of any one of claims 34-53, wherein the two or more β-glucosidase genes are five or more β-glucosidase genes.

57. The method of any one of claims 34-53, wherein the two or more β-glucosidase genes are six or more β-glucosidase genes.

58. The method of any one of claims 34-53, wherein the two or more β-glucosidase genes are seven or more β-glucosidase genes.

59. The method of any one of claims 54-58, wherein the three or more β-glucosidase genes, four or more β-glucosidase genes, five or more β-glucosidase genes, six or more β-glucosidase genes, or seven or more β-glucosidase genes comprise NCU00130, NCU04952, and NCU08755.

60. The method of any one of claims 34-59, wherein at least one of the β-glucosidase genes encodes an intracellular β-glucosidase.

61. The method of any one of claims 34-59, wherein at least one of the β-glucosidase genes encodes an extracellular β-glucosidase.

62. The method of any one of claims 34-61, wherein the at least one β-mannosidase gene is NCU00890.

63. The method of any one of claims 34-62, wherein the at least one phospholipase gene or phospholipase-like gene is NCU06650.

64. The method of any one of claims 34-63, wherein the cell is a stable cell line or a transiently transfected cell.

65. The method of any one of claims 34-64, wherein the cell is a fungal or yeast cell.

66. The method of any one of claims 34-65, wherein the cell is a filamentous fungus of the ascomycete or basidiomycete species.

67. The method of any one of claims 34-66, wherein the cell is selected from the group consisting of *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells,

Schizophyllum commune cells, *Postia placenta* cells, *Aspergillus oryzae* cells, and *Acremonium cellulolyticus* cells.

68. A mutant cell comprising inactivating mutations in two or more β-glucosidase genes, wherein cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the two or more β-glucosidase genes.

69. The mutant cell of claim 68, further comprising an inactivating mutation in a cre-1 gene in said cell, wherein cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the cre-1 gene.

70. The mutant cell of claim 68 or claim 69, further comprising an inactivating mutation in at least one β-mannosidase gene in said cell, wherein cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the at least one β-mannosidase gene.

71. The mutant cell of any one of claims 68-70, further comprising an inactivating mutation in at least one phospholipase gene or phospholipase-like gene in said cell, wherein cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the at least one phospholipase gene or phospholipase-like gene.

72. The mutant cell of any one of claims 68-71, wherein the cellulosic biomass comprises one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

73. The mutant cell of any one of claims 68-71, wherein the cellulosic biomass comprises cellobiose.

74. A mutant cell comprising inactivating mutations in two or more β-glucosidase genes, wherein a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the two or more β-glucosidase genes.

75. The mutant cell of claim 74, further comprising an inactivating mutation in a cre-1 gene in said cell, wherein a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the cre-1 gene.

76. The mutant cell of claim 74 or claim 75, further comprising an inactivating mutation in at least one β-mannosidase gene in said cell, wherein a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the at least one β-mannosidase gene.

77. The mutant cell of any one of claims 74-76, further comprising an inactivating mutation in at least one phospholipase gene or phospholipase-like gene in said cell, wherein a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell lacking said mutation in the at least one phospholipase gene or phospholipase-like gene.

78. The mutant cell of any one of claims 74-77, wherein the saccharide is selected from the group consisting of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

79. The mutant cell of any one of claims 74-77, wherein the saccharide is cellobiose.

80. The mutant cell of any one of claims 68-79, wherein the secreted protein is a cellulose-induced protein.

81. The mutant cell of any one of claims 68-80, wherein the secreted protein is selected from the group consisting of a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, a cellulose binding domain-containing protein, and combinations thereof.

82. The mutant cell of any one of claims 68-80, wherein the secreted protein is a cellulase.

83. The mutant cell of any one of claims 68-80, wherein the secreted protein is encoded by a gene selected from the group consisting of NCU07340, NCU09680, NCU07898,

NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137.

84. The mutant cell of any one of claims 68-83, wherein the inactivating mutations are deletions.

85. The mutant cell of any one of claims 68-84, wherein the cell is a recombinant cell.

86. The mutant cell of any one of claims 68-85, wherein the cell is a fungal or yeast cell.

87. The mutant cell of any one of claims 68-86, wherein the cell is a filamentous fungus of the ascomycete or basidiomycete species.

88. The mutant cell of any one of claims 68-87, wherein the cell is selected from the group consisting of *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells, *Schizophyllum commune* cells, *Postia placenta* cells, *Aspergillus oryzae* cells, and *Acremonium cellulolyticus* cells.

89. The mutant cell of any one of claims 68-88, wherein the two or more β-glucosidase genes are three or more β-glucosidase genes.

90. The mutant cell of any one of claims 68-88, wherein the two or more β-glucosidase genes are four or more β-glucosidase genes.

91. The mutant cell of any one of claims 68-88, wherein the two or more β-glucosidase genes are five or more β-glucosidase genes.

92. The mutant cell of any one of claims 68-88, wherein the two or more β-glucosidase genes are six or more β-glucosidase genes.

93. The mutant cell of any one of claims 68-88, wherein the two or more β-glucosidase genes are seven or more β-glucosidase genes.

94. The mutant cell of any one of claims 89-93, wherein the three or more β-glucosidase genes, four or more β-glucosidase genes, five or more β-glucosidase genes, six or more β-glucosidase genes, or seven or more β-glucosidase genes comprise NCU00130, NCU04952, and NCU08755.

95. The mutant cell of any one of claims 68-94, wherein at least one of the β-glucosidase genes encodes an intracellular β-glucosidase.

96. The mutant cell of any one of claims 68-94, wherein at least one of the β-glucosidase genes encodes an extracellular β-glucosidase.

97. The mutant cell of any one of claims 68-96, wherein the at least one β-mannosidase gene is NCU00890.

98. The mutant cell of any one of claims 68-97, wherein the at least one phospholipase gene or phospholipase-like gene is NCU06650.

99. A recombinant cell exhibiting reduced expression of at least two β-glucosidase genes compared to the expression of the at least two β-glucosidase genes in a corresponding non-recombinant cell, wherein said expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and wherein cellulosic biomass induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the at least two β-glucosidase genes is not reduced.

100. The recombinant cell of claim 99, wherein the cell further exhibits reduced expression of a cre-1 gene compared to the expression of the cre-1 gene in a corresponding non-recombinant cell, wherein said expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and wherein cellulosic biomass induces

the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the cre-1 gene is not reduced.

101. The recombinant cell of claim 100, wherein the function of creA/cre-1 is reduced by overexpression of a dominant negative mutant or a protein inhibitor, wherein cellulosic biomass induces the cell to secrete higher levels of a protein than a corresponding cell in which the dominant negative mutant is not overexpressed.

102. The recombinant cell of any one of claims 99-101, wherein the cell further exhibits reduced expression of at least one β-mannosidase gene compared to the expression of the at least one β-mannosidase gene in a corresponding non-recombinant cell, wherein said expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and wherein cellulosic biomass induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the least one β-mannosidase gene is not reduced.

103. The recombinant cell of any one of claims 99-102, wherein the cell further exhibits reduced expression of at least one phospholipase gene or phospholipase-like gene compared to the expression of the at least one phospholipase gene or phospholipase-like gene in a corresponding non-recombinant cell, wherein said expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and wherein cellulosic biomass induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the least one phospholipase gene or phospholipase-like gene is not reduced.

104. The recombinant cell of any one of claims 99-103, wherein the cellulosic biomass comprises one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

105. The recombinant cell of any one of claims 99-103, wherein the cellulosic biomass comprises cellobiose.

106. A recombinant cell exhibiting reduced expression of at least two β-glucosidase genes compared to the expression of the at least twoβ-glucosidase genes in a corresponding non-recombinant cell, wherein said expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and wherein a saccharide induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the at least twoβ-glucosidase genes is not reduced.

107. The recombinant cell of claim 106, wherein the cell further exhibits reduced expression of a cre-1 gene compared to the expression of the cre-1 gene in a corresponding non-recombinant cell, wherein said expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and wherein a saccharide induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the cre-1 gene is not reduced.

108. The recombinant cell of claim 107, wherein the function of creA/cre-1 is reduced by overexpression of a dominant negative mutant or a protein inhibitor, wherein a saccharide induces the cell to secrete higher levels of a protein than a corresponding cell in which the dominant negative mutant is not overexpressed.

109. The recombinant cell of any one of claims 106-108, wherein the cell further exhibits reduced expression of at least one β-mannosidase gene compared to the expression of the at least one β-mannosidase gene in a corresponding non-recombinant cell, wherein said expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and wherein a saccharide induces the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the least one β-mannosidase gene is not reduced.

110. The recombinant cell of any one of claims 106-109, wherein the cell further exhibits reduced expression of at least one phospholipase gene or phospholipase-like gene compared to the expression of the at least one phospholipase gene or phospholipase-like gene in a corresponding non-recombinant cell, wherein said expression is reduced by siRNA, antisense DNA, quelling, or meiotic silencing, and wherein a saccharide induces

the cell to secrete higher levels of a protein than the corresponding non-recombinant cell in which the expression of the least one phospholipase gene or phospholipase-like gene is not reduced.

111. The recombinant cell of any one of claims 106-110, wherein the saccharide is selected from the group consisting of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

112. The recombinant cell of any one of claims106-110, wherein the saccharide is cellobiose.

113. The recombinant cell of any one of claims 99-112, wherein the secreted protein is a cellulose-induced protein.

114. The recombinant cell of any one of claims 99-113, wherein the secreted protein is selected from the group consisting of a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, a cellulose binding domain-containing protein, and combinations thereof.

115. The recombinant cell of any one of claims 99-113, wherein the secreted protein is a cellulase.

116. The recombinant cell of any one of claims 99-113, wherein the secreted protein is encoded by a gene selected from the group consisting of NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137.

117. The recombinant cell of any one of claims 99-116, wherein the two or more β-glucosidase genes are three or more β-glucosidase genes.

118. The recombinant cell of any one of claims 99-116, wherein the two or more β-glucosidase genes are four or more β-glucosidase genes.

119. The recombinant cell of any one of claims 99-116, wherein the two or more β-glucosidase genes are five or more β-glucosidase genes.

120. The recombinant cell of any one of claims 99-116, wherein the two or more β-glucosidase genes are six or more β-glucosidase genes.

121. The recombinant cell of any one of claims 99-116, wherein the two or more β-glucosidase genes are seven or more β-glucosidase genes.

122. The mutant cell of any one of claims 117-121, wherein the three or more β-glucosidase genes, four or more β-glucosidase genes, five or more β-glucosidase genes, six or more β-glucosidase genes, or seven or more β-glucosidase genes comprise NCU00130, NCU04952, and NCU08755.

123. The mutant cell of any one of claims 99-122, wherein at least one of the β-glucosidase genes encodes an intracellular β-glucosidase.

124. The mutant cell of any one of claims 99-122, wherein at least one of the β-glucosidase genes encodes an extracellular β-glucosidase.

125. The recombinant cell of any one of claims 99-124, wherein the at least one β-mannosidase gene is NCU00890.

126. The recombinant cell of any one of claims 99-125, wherein the at least one phospholipase gene or phospholipase-like gene is NCU06650.

127. The recombinant cell of any one of claims 99-126, wherein the cell is a stable cell line or a transiently transfected cell.

128. The recombinant cell of any one of claims 99-127, wherein the cell is a fungal or yeast cell.

129. The recombinant cell of any one of claims 99-128, wherein the cell is a filamentous fungus of the ascomycete or basidiomycete species.

130. The recombinant cell of any one of claims 99-129, wherein the cell is selected from the group consisting of *Neurospora crassa* (*N. crassa*) cells, *Aspergillus nidulans* cells, *Trichoderma reesei* cells, *Phanerochaete chrysosporium* cells, *Sporotrichum thermophile (Myceliophthora thermophila)* cells, *Gibberella zeae* cells, *Sclerotinia sclerotiorum* cells, *Botryotinia fuckeliana* cells, *Aspergillus niger* cells, *Penicillium chrysogenum* cells, *Schizophyllum commune* cells, *Postia placenta* cells, *Aspergillus oryzae* cells, and *Acremonium cellulolyticus* cells.

131. A method for the degradation of biomass, the method comprising:

(a) providing lignocellulosic biomass;

(b) providing the cell of any one of claims 68-130, or a cell comprising an inactivating mutation in the cre-1 gene;

(c) inducing said cell to secrete a protein by contacting the cell with cellulosic biomass; and

(d) contacting the induced cell with the lignocellulosic biomass, wherein the secreted protein degrades the lignocellulosic biomass.

132. The method of claim 131, wherein the cellulosic biomass comprises one or more of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

133. The method of claim 131, wherein the cellulosic biomass comprises cellobiose.

134. A method for the degradation of biomass, the method comprising:

(a) providing lignocellulosic biomass;

(b) providing the cell of any one of claims 68-130, or a cell comprising an inactivating mutation in the cre-1 gene;

(c) inducing said cell to secrete a protein by contacting the cell with a saccharide; and

(d) contacting the induced cell with the lignocellulosic biomass, wherein the secreted protein degrades the lignocellulosic biomass.

135. The method of claim 134, wherein the saccharide is selected from the group consisting of a polysaccharide, an oligosaccharide, cellulose, microcrystalline cellulose, cellodextrin, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose.

136. The method of claim 134, wherein the saccharide is cellobiose.

137. The method of any one of claims 131-136, wherein the secreted protein is a cellulose-induced protein.

138. The method of any one of claims 131-137, wherein the secreted protein is selected from the group consisting of a cellulase, a GH61 enzyme, a cellobiose dehydrogenase, a lactonase, a carbohydrate esterase, a polysaccharide lyase, a cellulose binding domain-containing protein, and combinations thereof.

139. The method of any one of claims 131-137, wherein the secreted protein is a cellulase.

140. The method of any one of claims 131-137, wherein the secreted protein is encoded by a gene selected from the group consisting of NCU07340, NCU09680, NCU07898, NCU00762, NCU08760, NCU05057, NCU02240, NCU07190, NCU07898, NCU08760, NCU00206, NCU07143, NCU09491, NCU09664, NCU05598, NCU09764, and NCU05137.

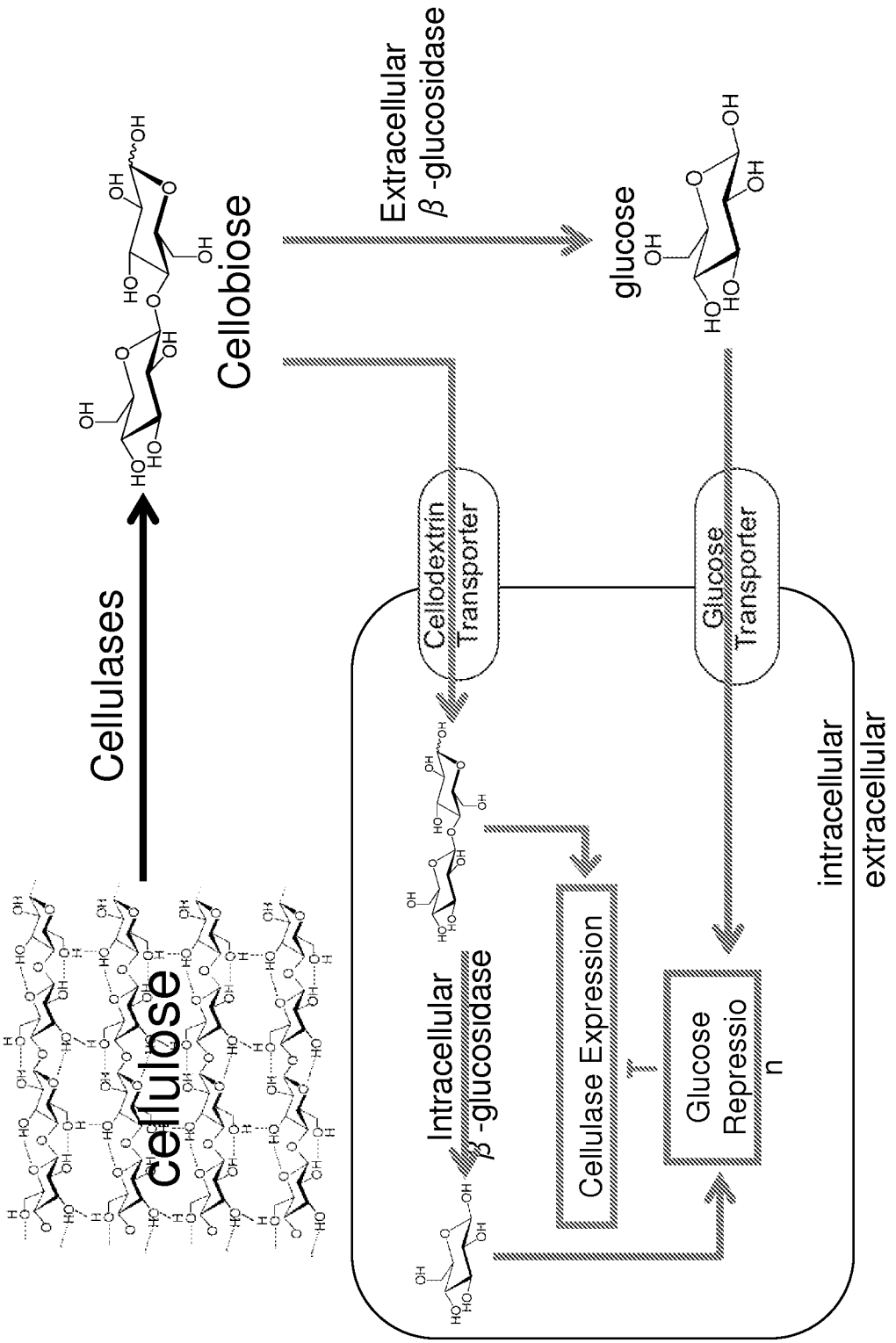

Cellulose
Cellulases
Cellobiose
Extracellular
β -glucosidase
glucose
Cellodextrin
Transporter
Glucose
Transporter
Intracellular
β -glucosidase
Cellulase Expression
Glucose
Repressio
n
intracellular
extracellular

Figure 1

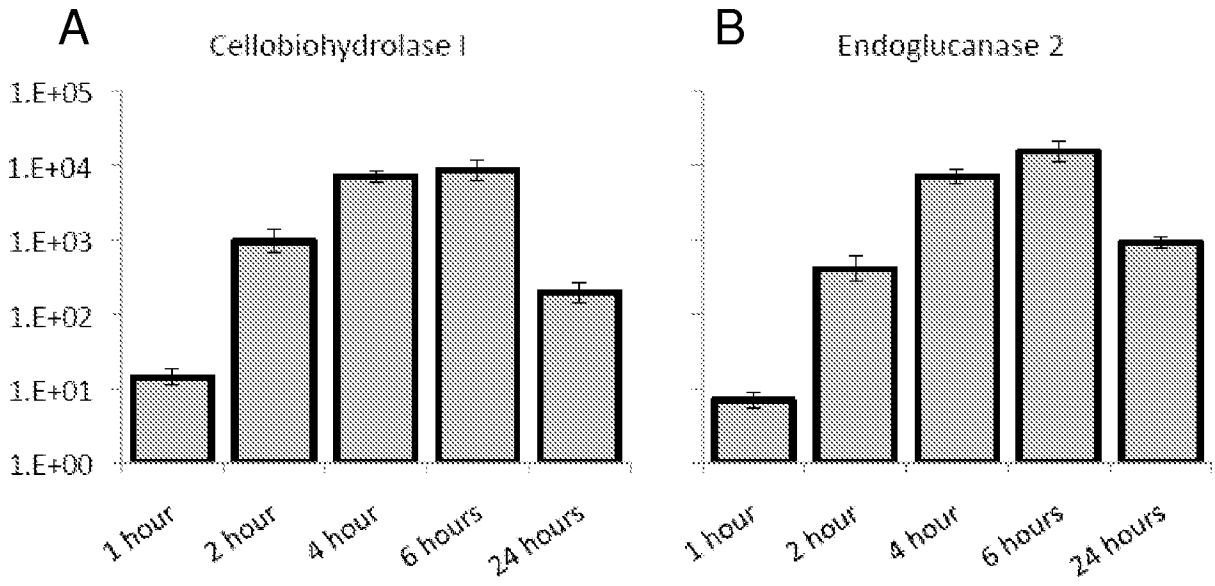

A
Cellobiohydrolase I
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
1 hour
2 hour
4 hour
6 hours
24 hours
B
Endoglucanase 2
1 hour
2 hour
4 hour
6 hours
24 hours

Figure 2

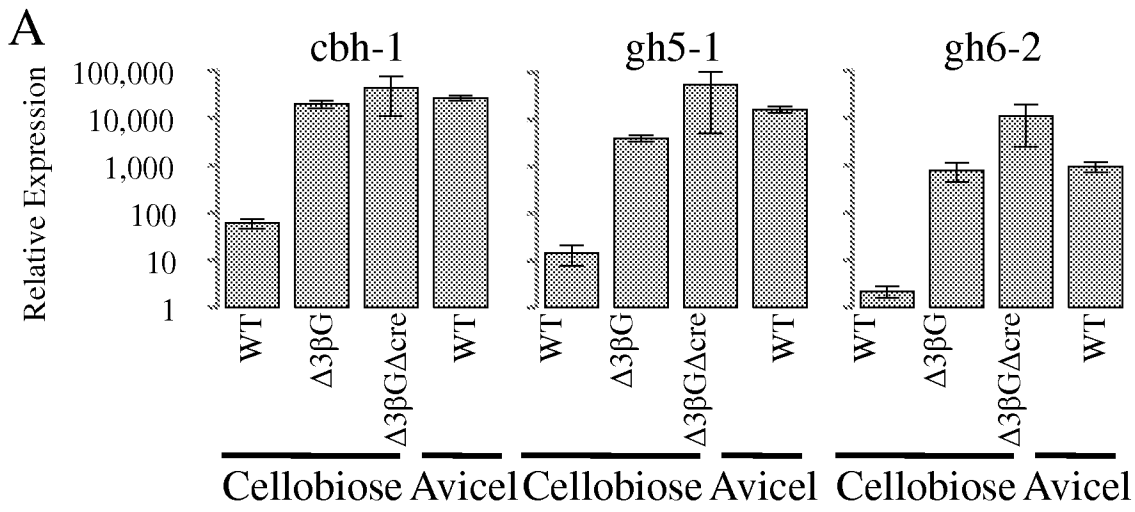

A
Relative Expression
100,000
10,000
1,000
100
10
1
cbh-1
gh5-1
gh6-2
WT
Δ3βG
Δ3βGΔcre
WT
Cellobiose
Avicel

Figure 3

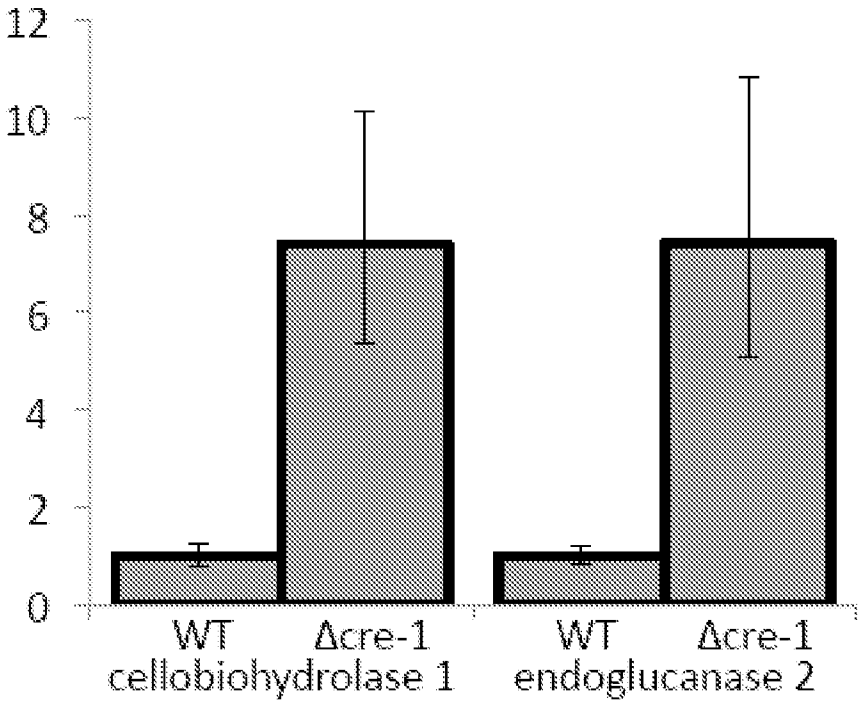

12
10
8
6
4
2
0
WT
Δcre-1
cellobiohydrolase 1
WT
Δcre-1
endoglucanase 2

Figure 4

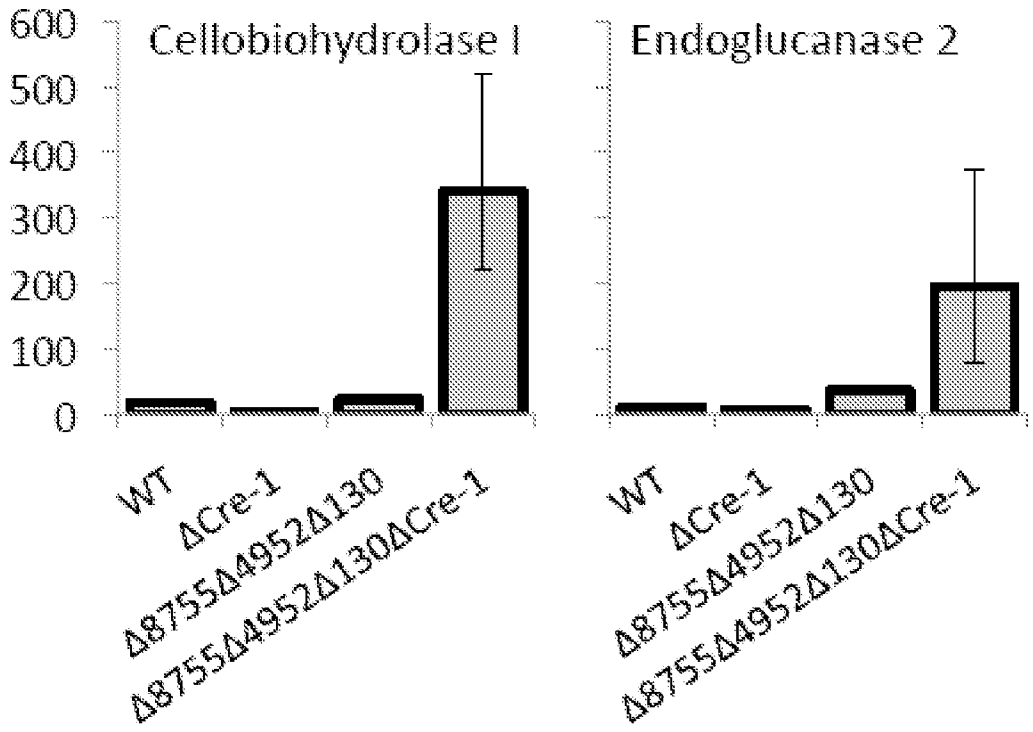

600
500
400
300
200
100
0
Cellobiohydrolase I
Endoglucanase 2
WT
ΔCre-1
Δ8755Δ4952Δ130
Δ8755Δ4952Δ130ΔCre-1
WT
ΔCre-1
Δ8755Δ4952Δ130
Δ8755Δ4952Δ130ΔCre-1

Figure 5

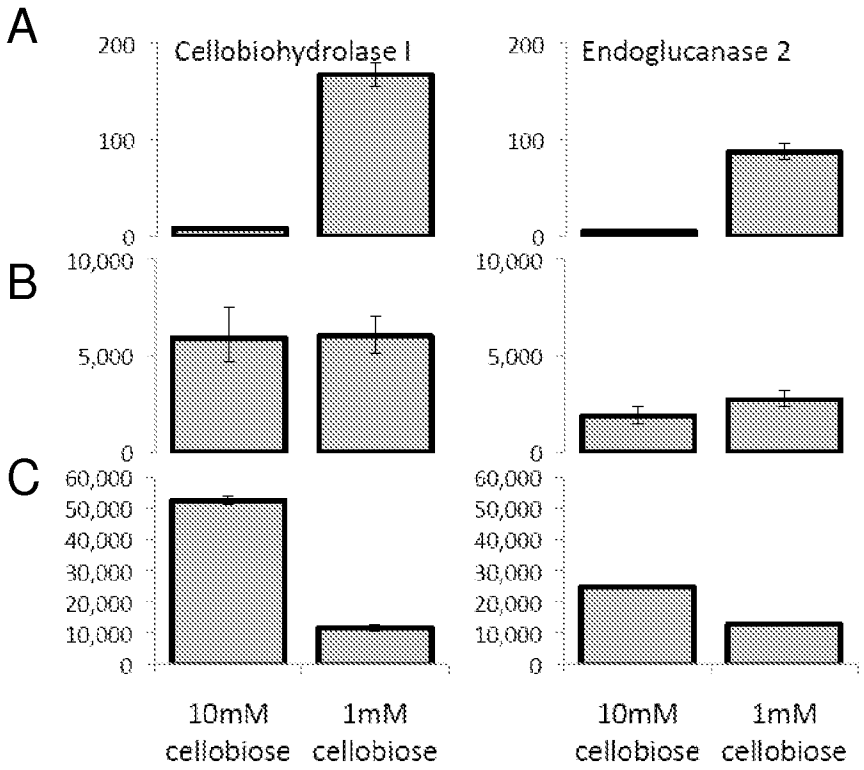

A
B
C
Cellobiohydrolase I
Endoglucanase 2
200
100
0
10,000
5,000
0
60,000
50,000
40,000
30,000
20,000
10,000
0
10mM cellobiose
1mM cellobiose
10mM cellobiose
1mM cellobiose

Figure 6

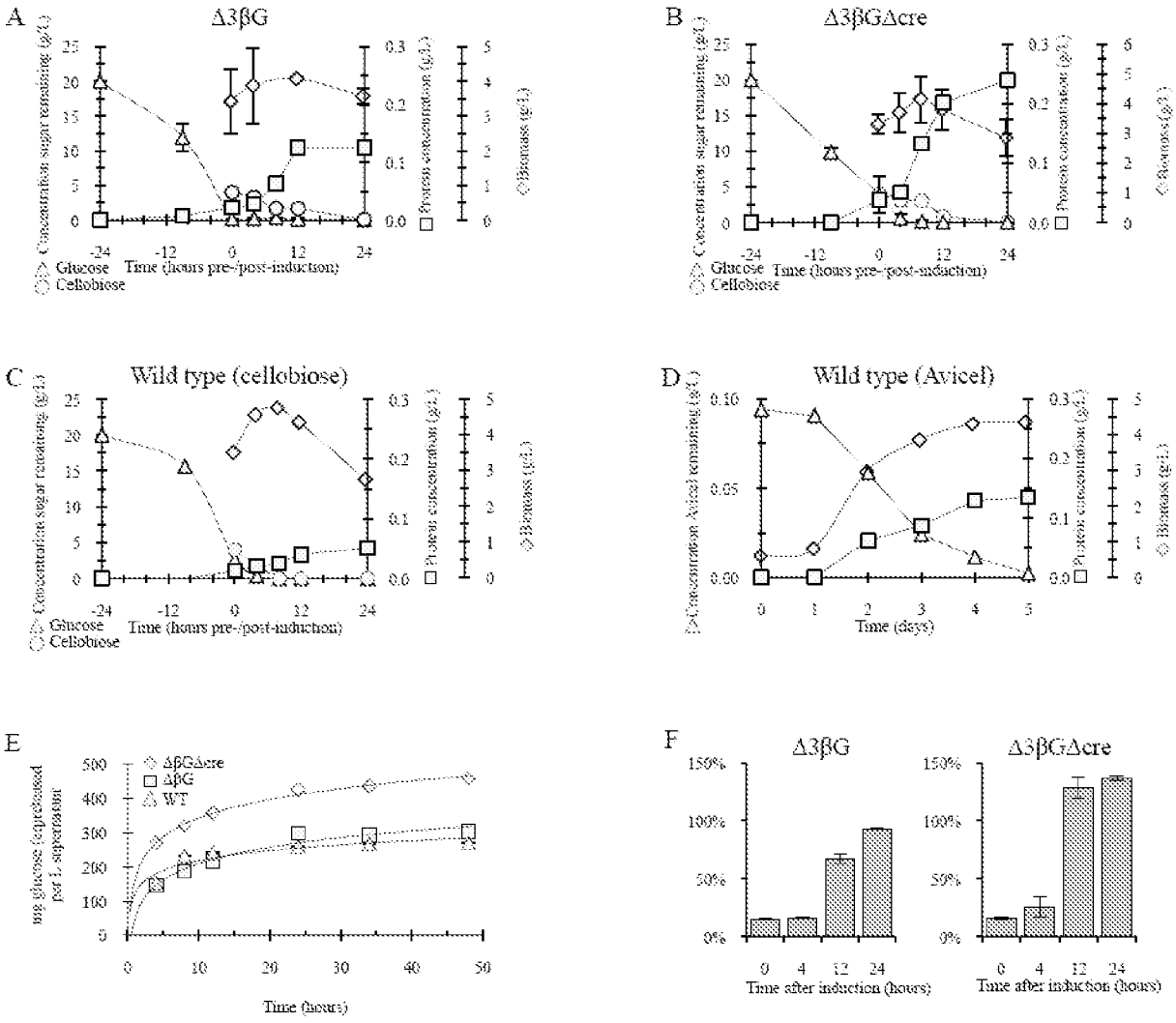
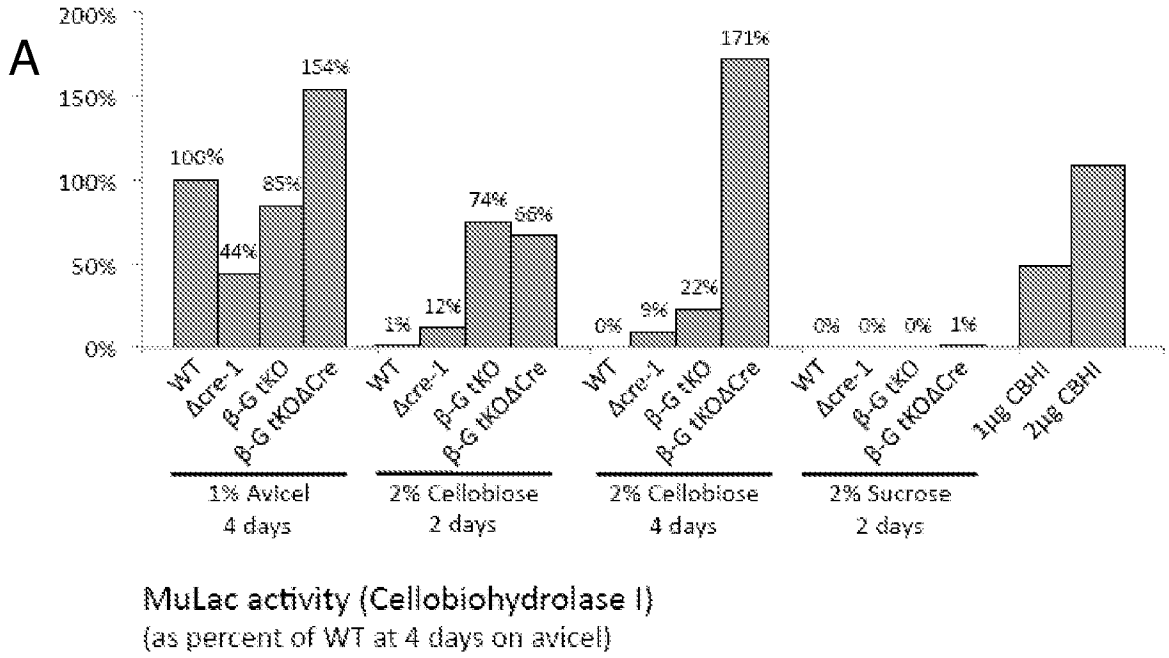

A
Δ3βG
Concentration sugar remaining (g/L)
Protein concentration (g/L)
Biomass (g/L)
Glucose
Cellobiose
Time (hours pre-/post-induction)
B
Δ3βGΔcre
C
Wild type (cellobiose)
D
Wild type (Avicel)
Concentration Avicel remaining (g/L)
Time (days)
E
ΔβGΔcre
ΔβG
WT
mg glucose (glycolysis) produced per 1 mg biomass
Time (hours)
F
Δ3βG
Δ3βGΔcre
Time after induction (hours)

Figure 7


A
200%
150%
100%
50%
0%
100%
44%
85%
154%
1%
12%
74%
68%
0%
9%
22%
171%
0%
0%
0%
1%
WT
Δcre-1
β-G tKO
β-G tKOΔCre
1% Avicel
4 days
2% Cellobiose
2 days
2% Cellobiose
4 days
2% Sucrose
2 days
1μg CBHI
2μg CBHI
MuLac activity (Cellobiohydrolase I)
(as percent of WT at 4 days on avicel)

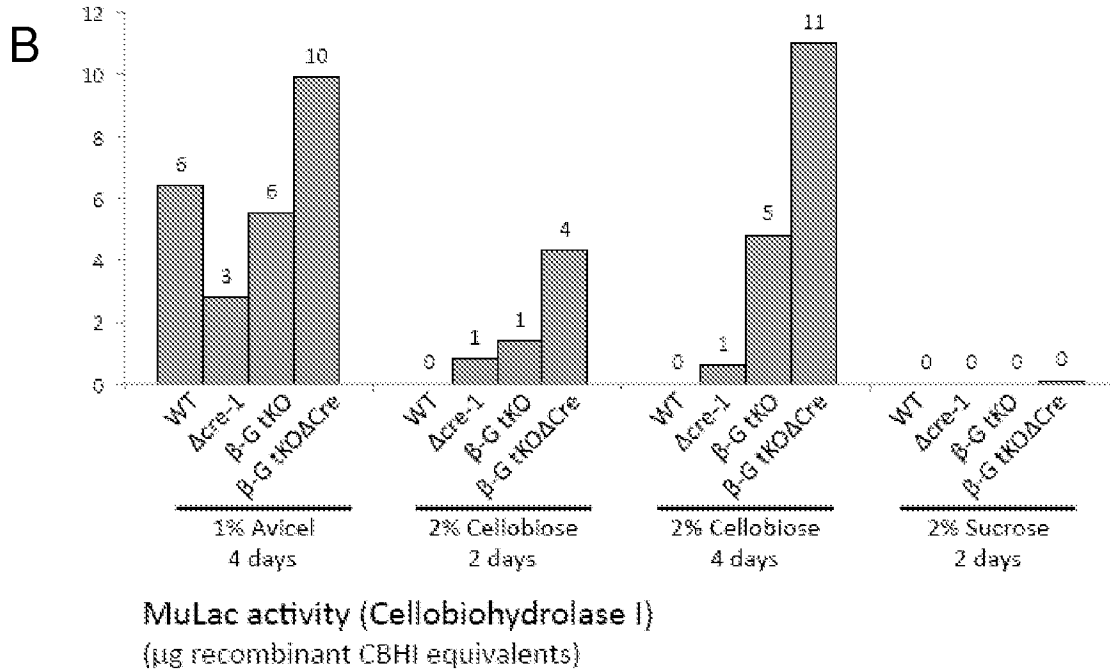

B
12
10
8
6
4
2
0
5
3
6
10
0
1
1
4
0
1
5
11
0
0
0
0
WT
Δcre-1
β-G tKO
β-G tKOΔCre
1% Avicel
4 days
2% Cellobiose
2 days
2% Cellobiose
4 days
2% Sucrose
2 days
MuLac activity (Cellobiohydrolase I)
(μg recombinant CBHI equivalents)

Figure 8

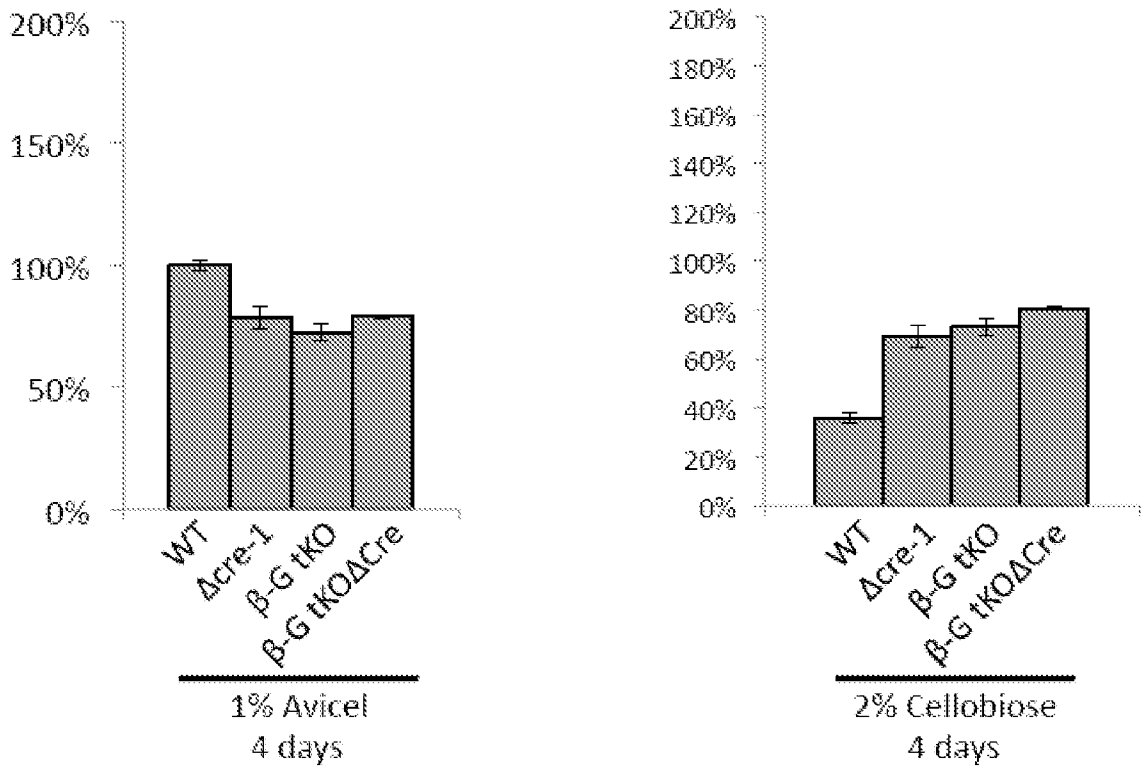

200%
150%
100%
50%
0%
WT
Δcre-1
β-G tKO
β-G tKOΔCre
1% Avicel
4 days
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
WT
Δcre-1
β-G tKO
β-G tKOΔCre
2% Cellobiose
4 days

Figure 9

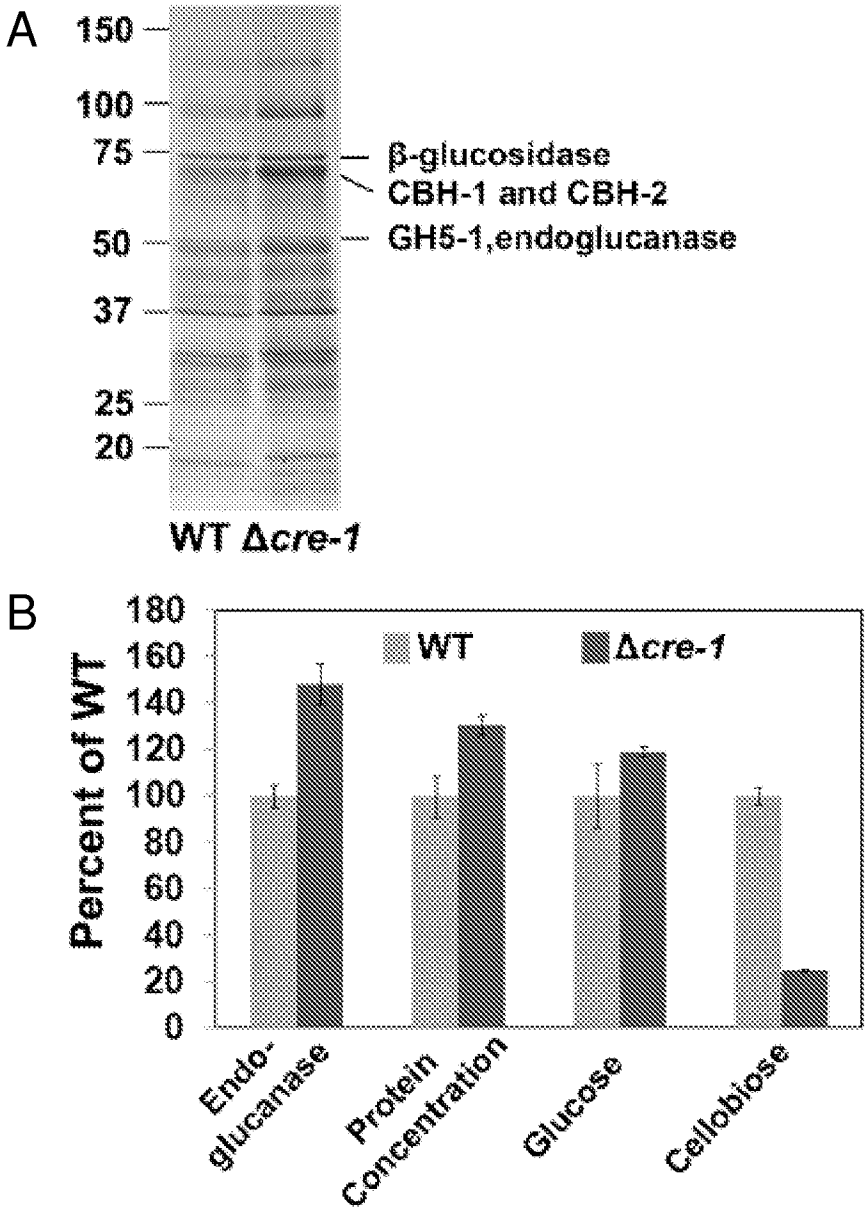

A
150
100
75
50
37
25
20
β-glucosidase
CBH-1 and CBH-2
GH5-1,endoglucanase
WT Δcre-1
B
Percent of WT
180
160
140
120
100
80
60
40
20
0
WT
Δcre-1
Endo-glucanase
Protein Concentration
Glucose
Cellobiose

Figure 10

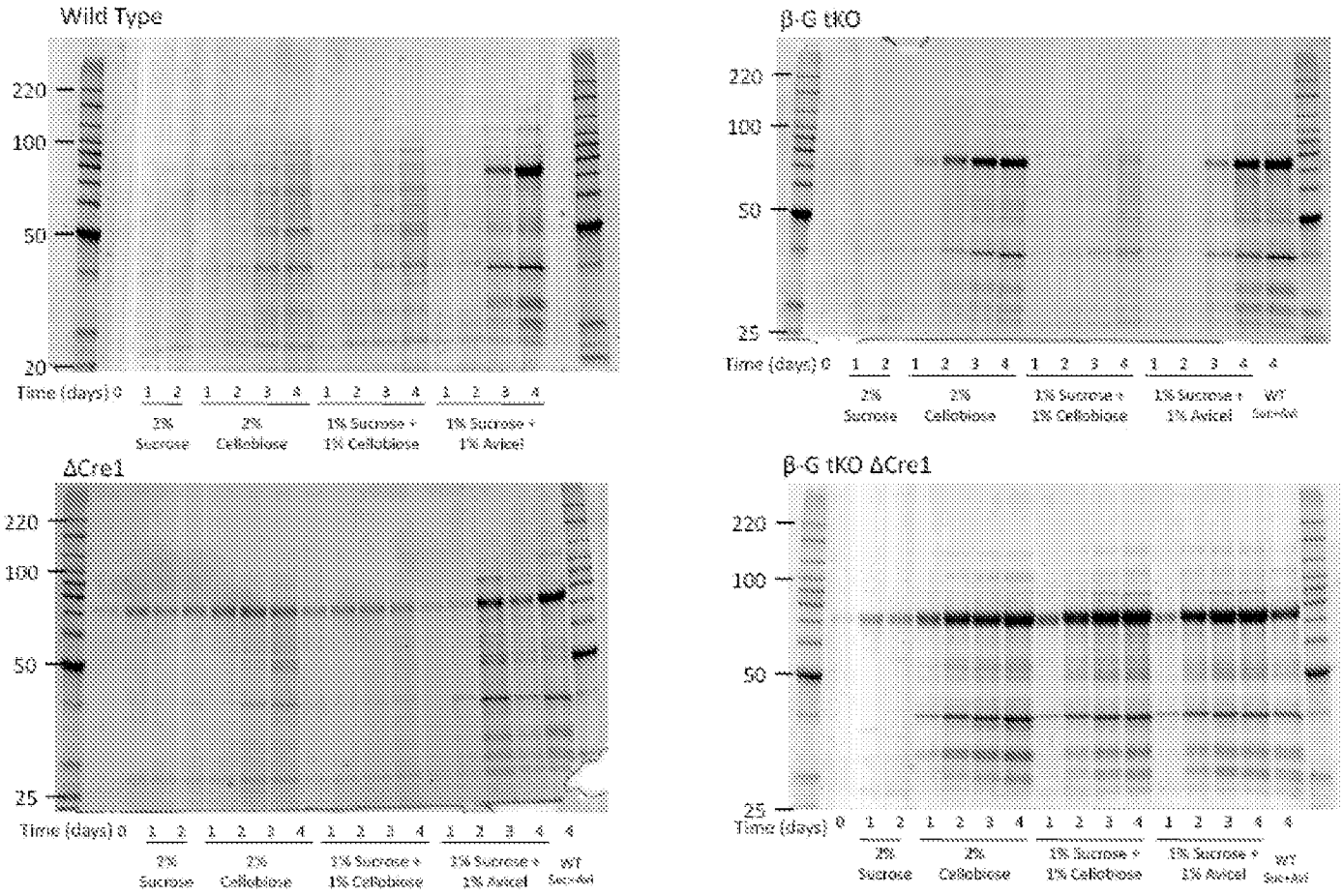

Wild Type
220
100
50
20
Time (days) 0
2% Sucrose
2% Cellobiose
1% Sucrose + 1% Cellobiose
1% Sucrose + 1% Avicel
β-G tKO
220
100
50
25
Time (days) 0
2% Sucrose
2% Cellobiose
1% Sucrose + 1% Cellobiose
1% Sucrose + 1% Avicel
WT
ΔCre1
220
100
50
25
Time (days) 0
2% Sucrose
2% Cellobiose
1% Sucrose + 1% Cellobiose
1% Sucrose + 1% Avicel
WT
β-G tKO ΔCre1
220
100
50
25
Time (days) 0
2% Sucrose
2% Cellobiose
1% Sucrose + 1% Cellobiose
1% Sucrose + 1% Avicel
WT

Figure 11

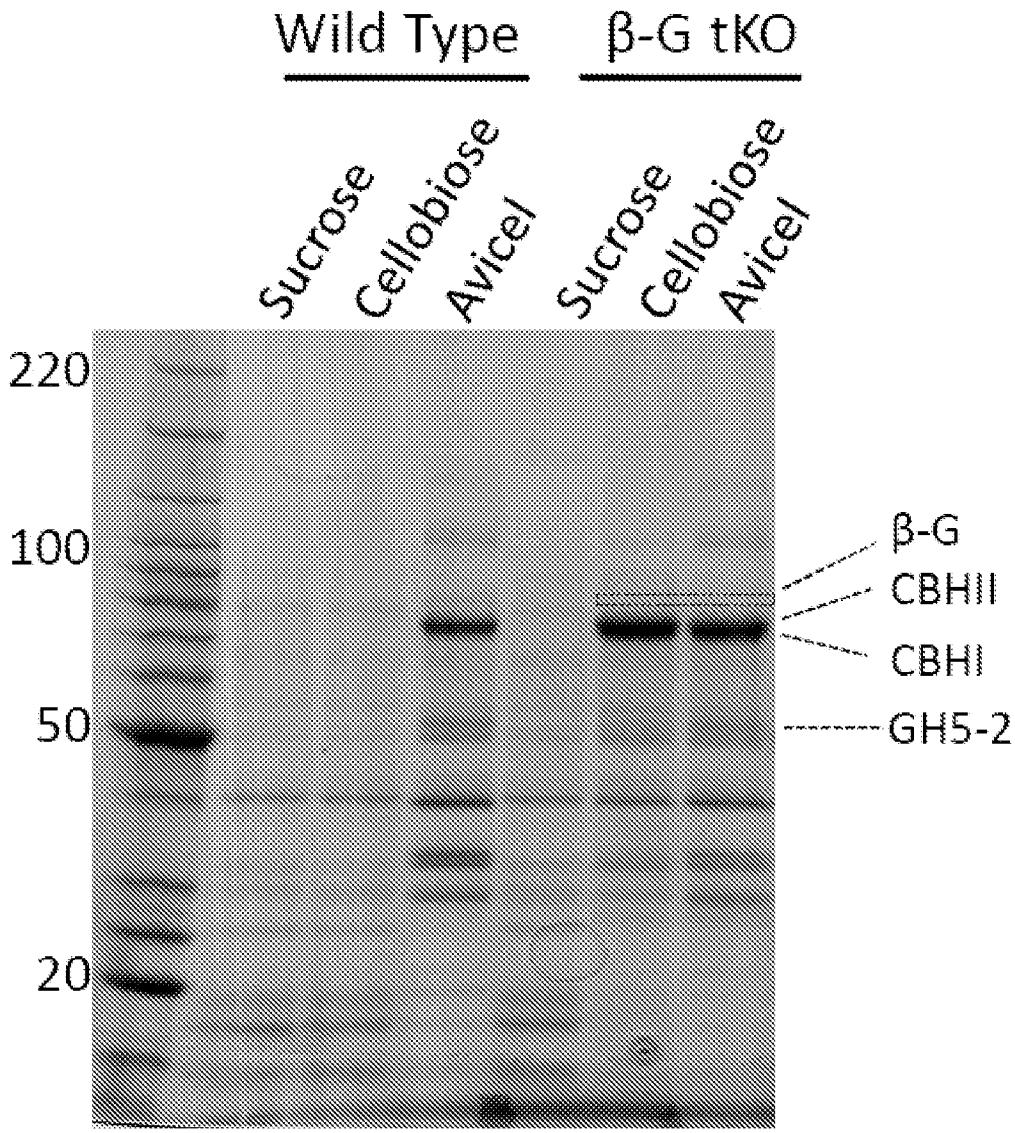

Wild Type
β-G tKO
Sucrose
Cellobiose
Avicel
Sucrose
Cellobiose
Avicel
220
100
50
20
β-G
CBHII
CBHI
GH5-2

Figure 12

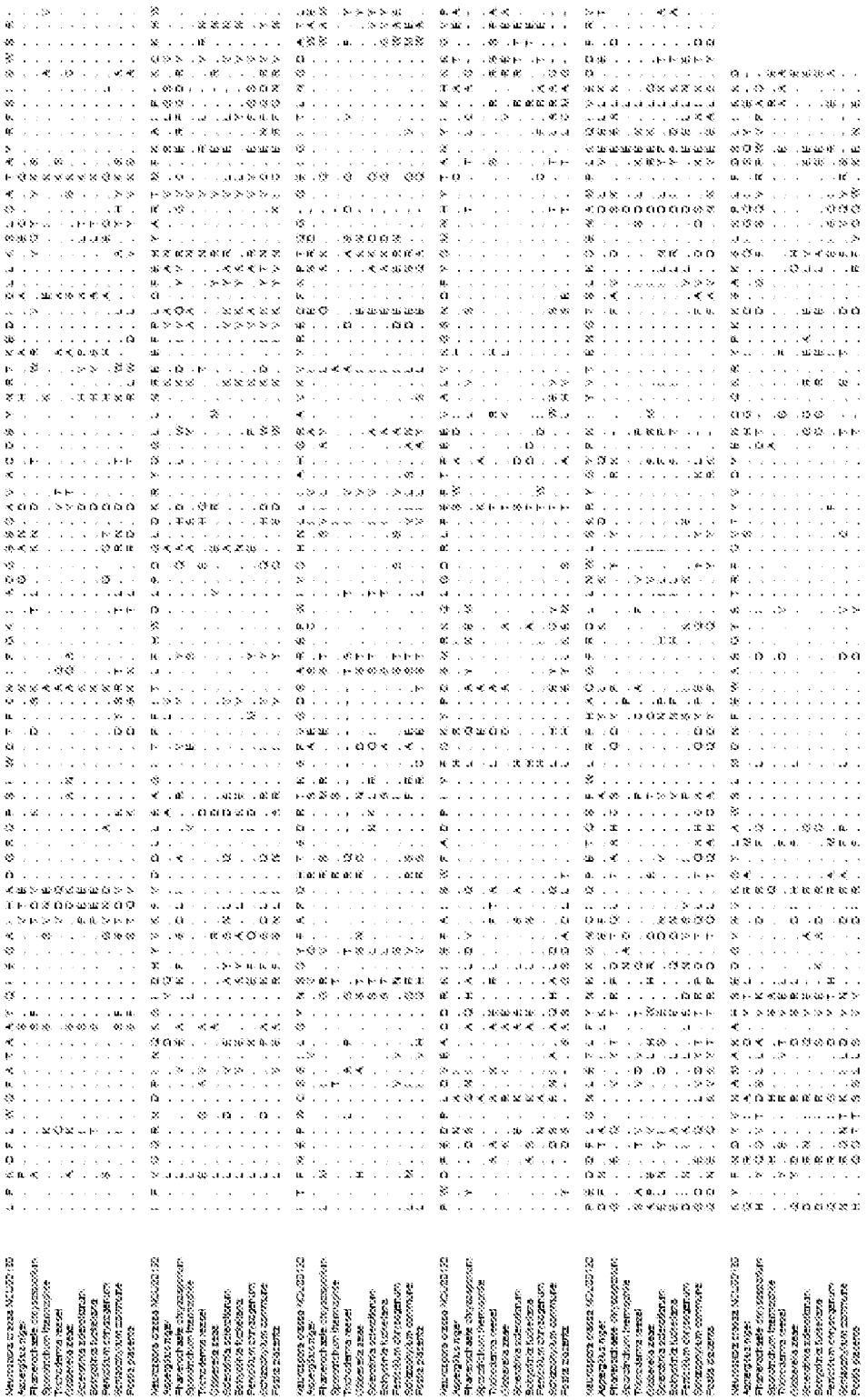


Figure 13 (1/5)

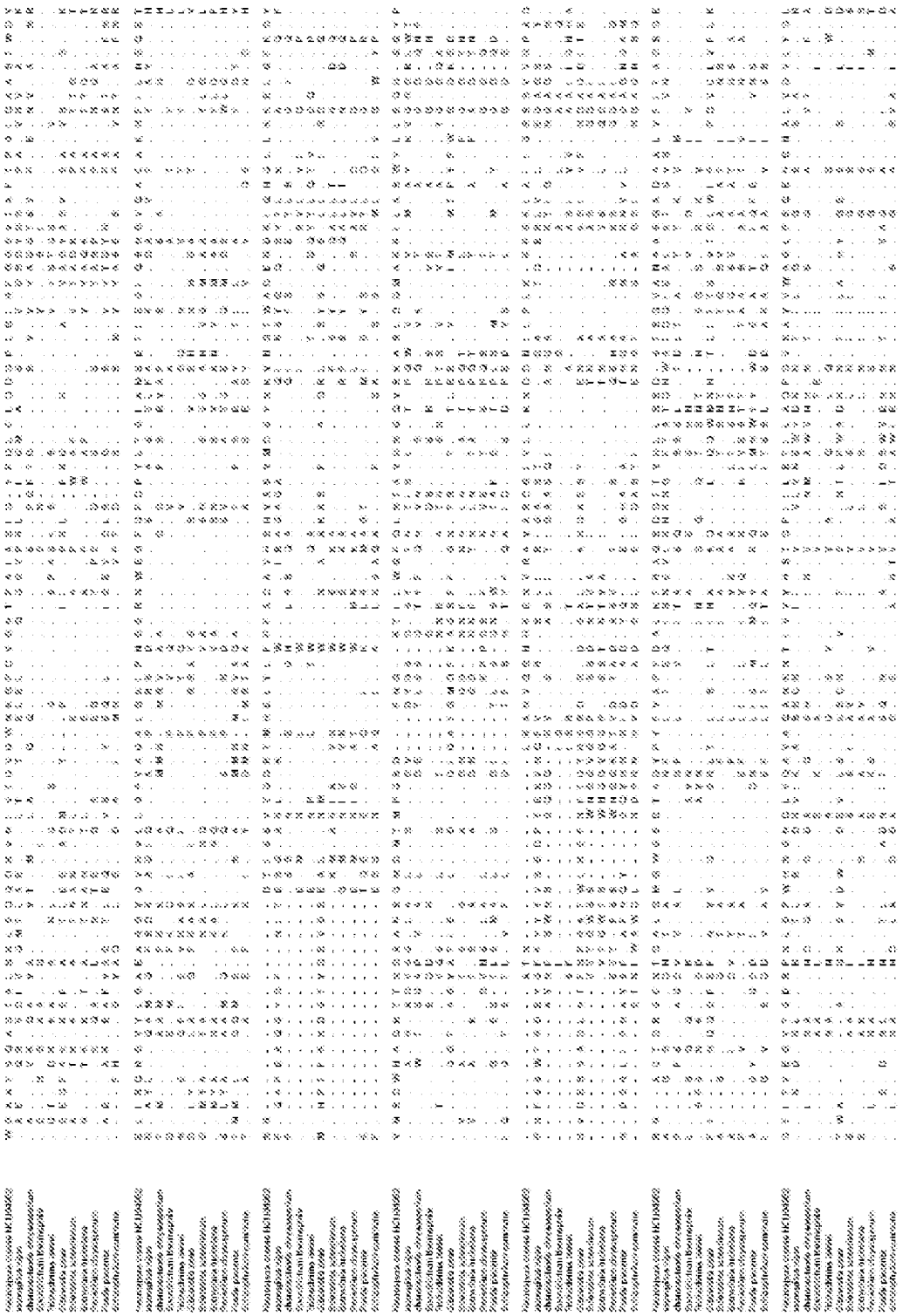


Figure 13 (2/5)

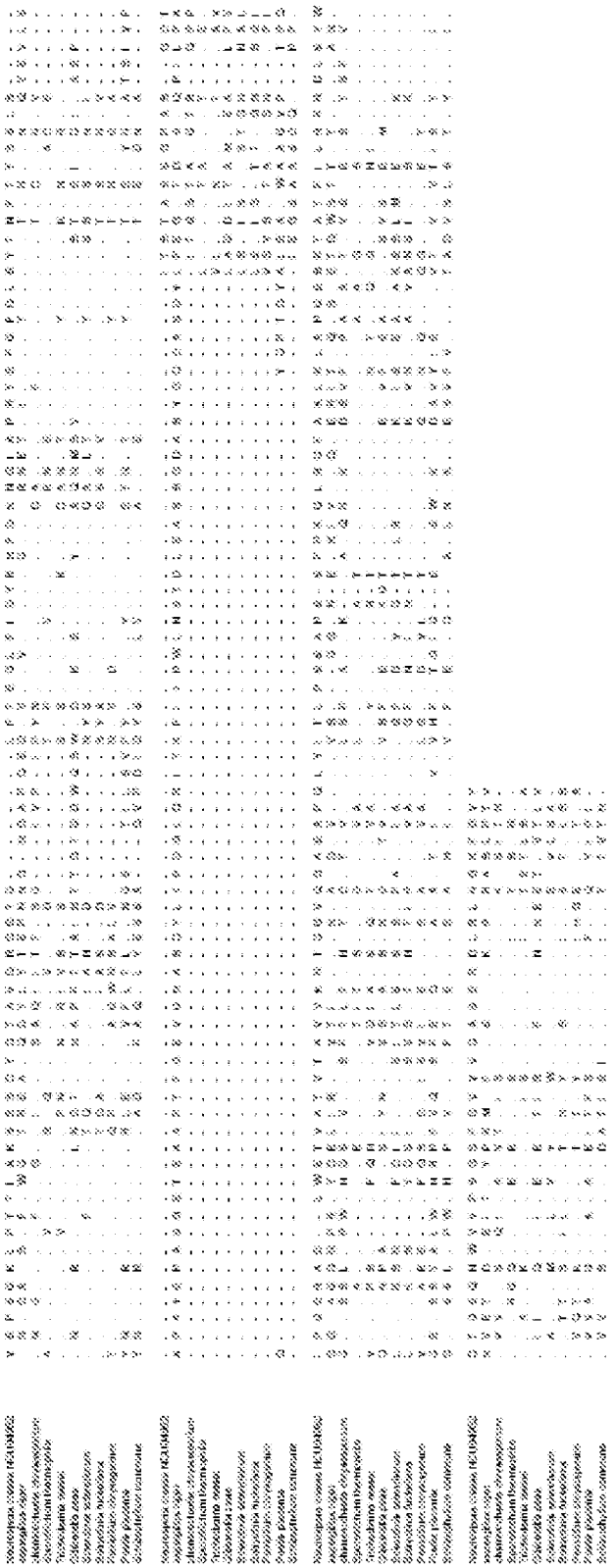


Figure 13 (3/5)

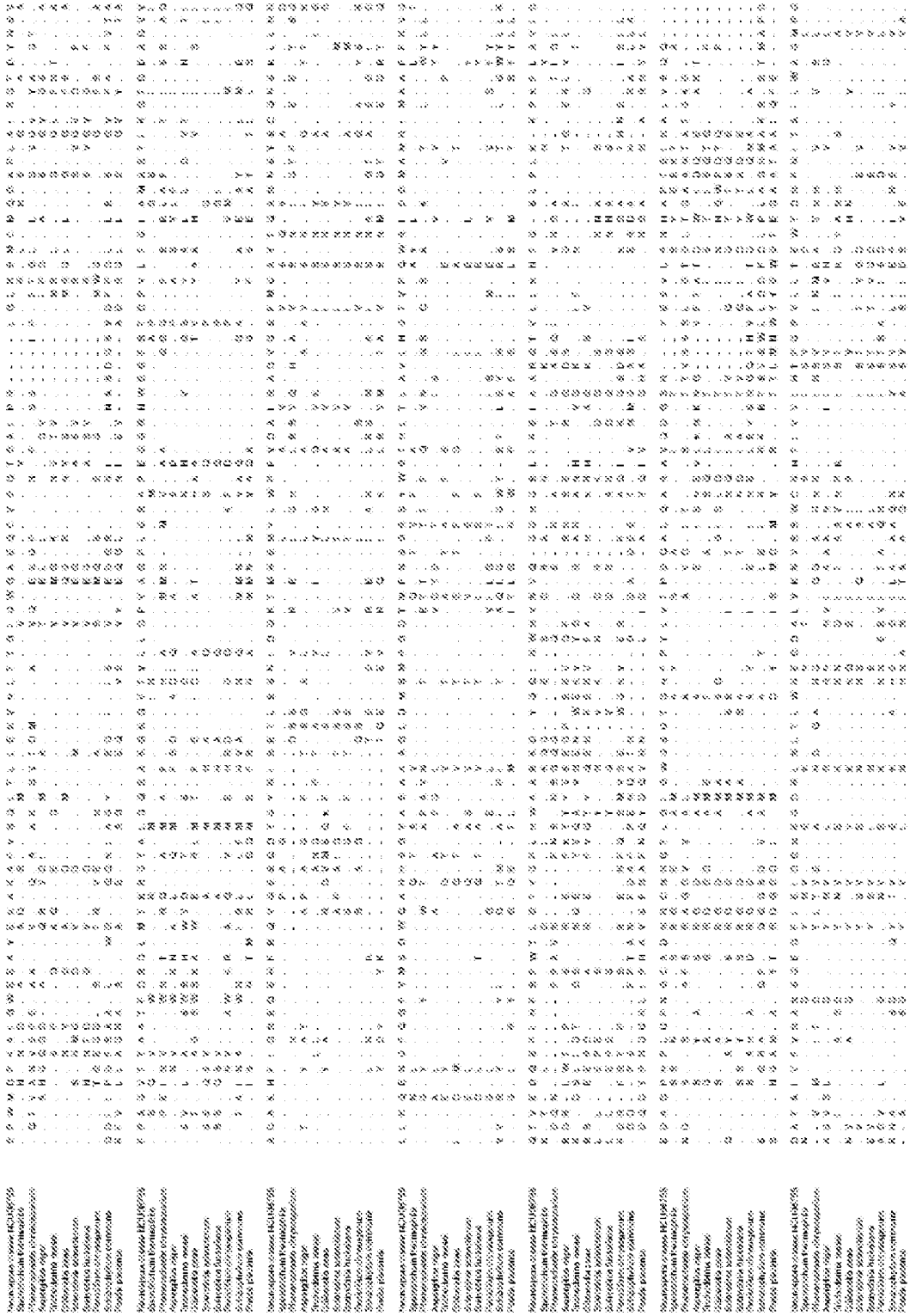


Figure 13 (4/5)

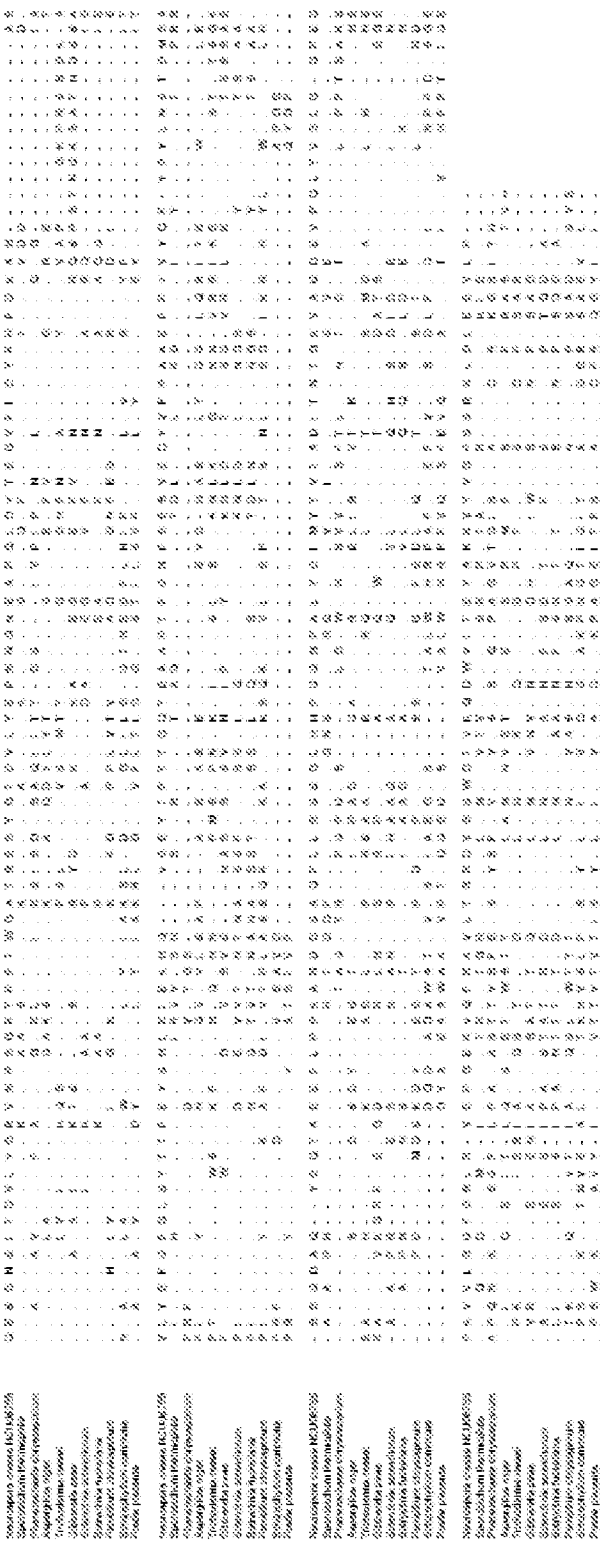


Figure 13 (5/5)

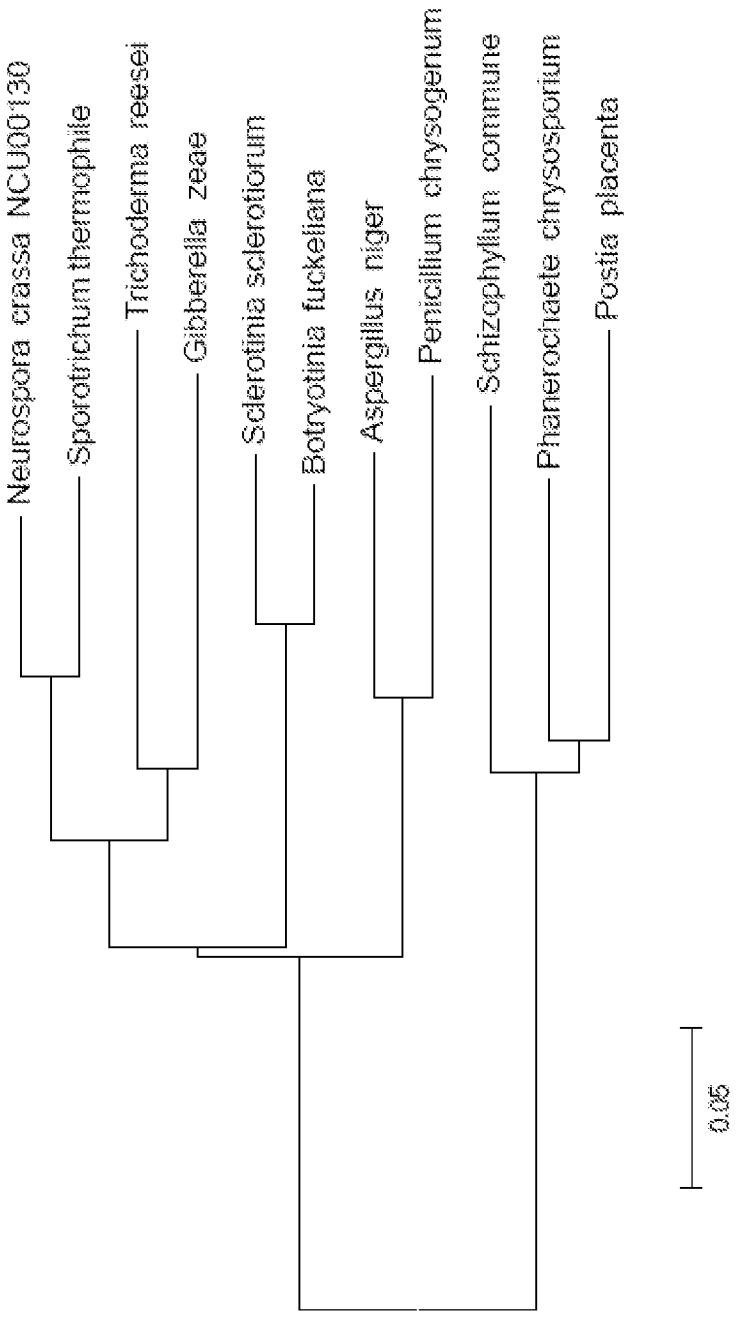

Neurospora crassa NCU00130
Sporotrichum thermophile
Trichoderma reesei
Gibberella zeae
Sclerotinia sclerotiorum
Botryotinia fuckeliana
Aspergillus niger
Penicillium chrysogenum
Schizophyllum commune
Phanerochaete chrysosporium
Postia placenta
0.05

Figure 14

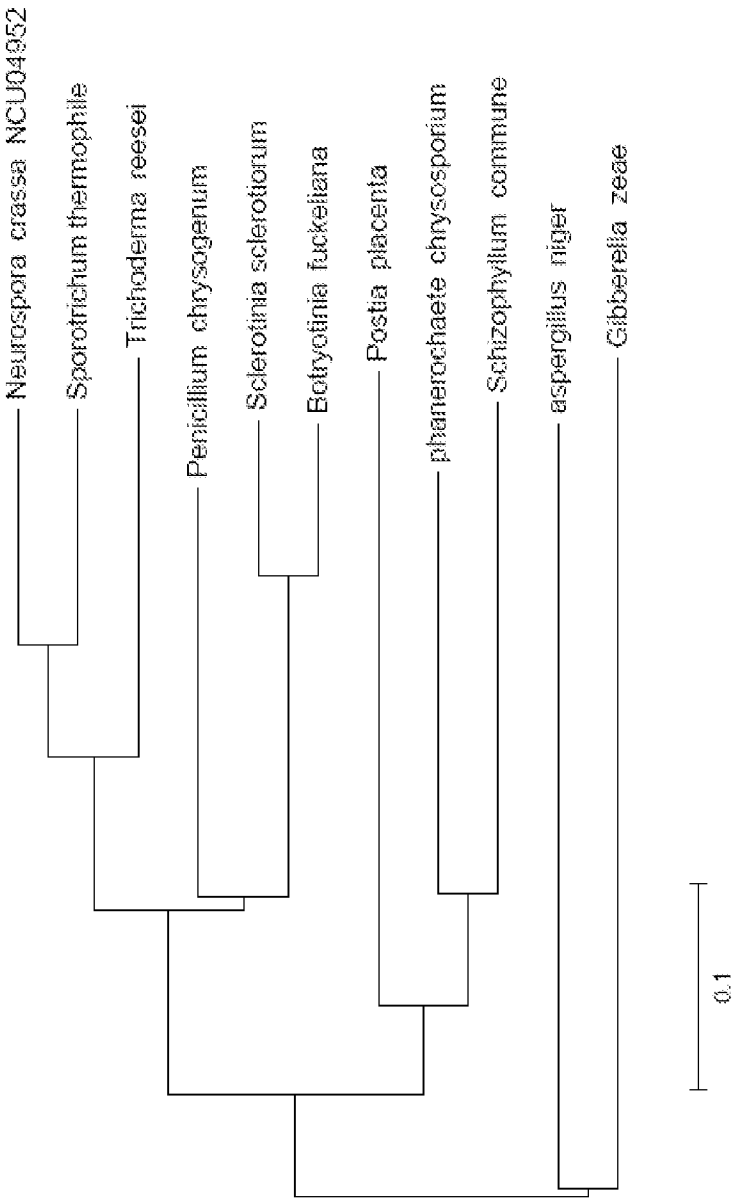

Neurospora crassa NCU04952
Sporotrichum thermophile
Trichoderma reesei
Penicillium chrysogenum
Sclerotinia sclerotiorum
Botryotinia fuckeliana
Postia placenta
phanerochaete chrysosporium
Schizophyllum commune
aspergillus niger
Gibberella zeae
0.1

Figure 15

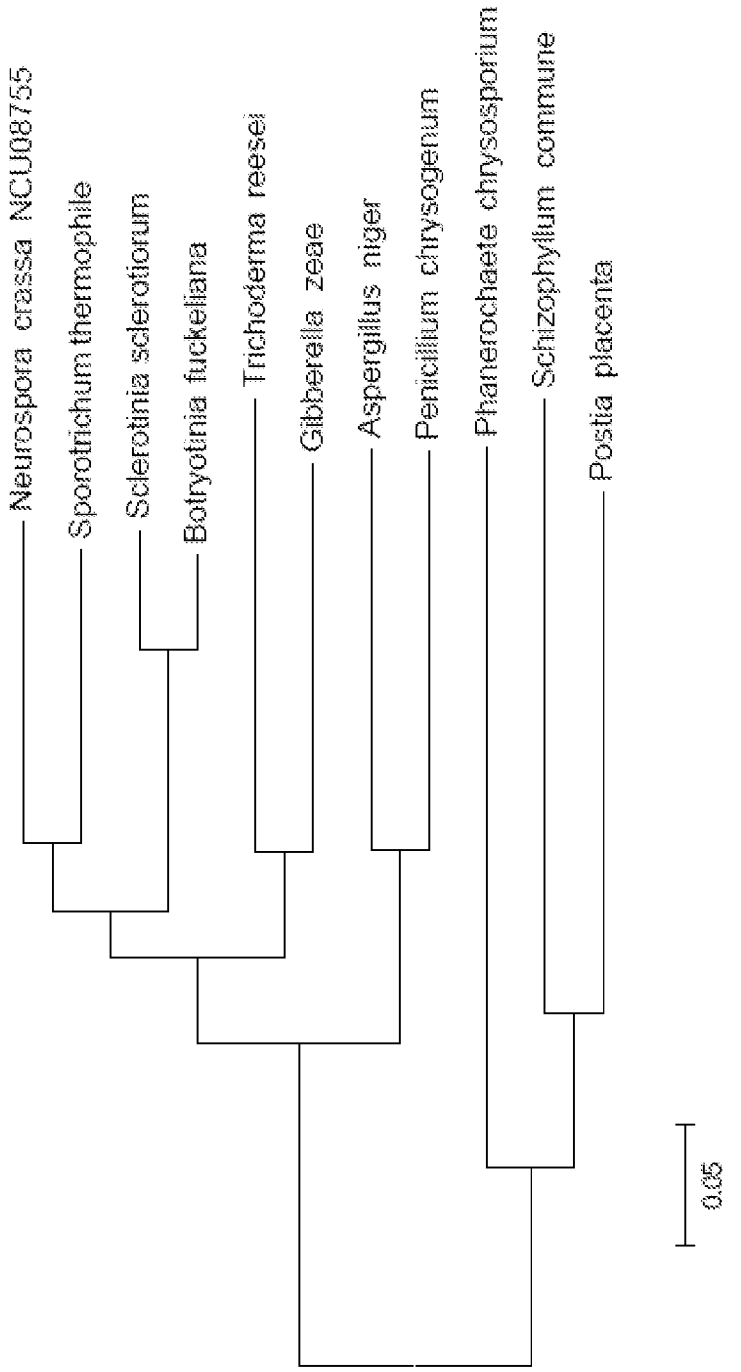

Neurospora crassa NCU08755
Sporotrichum thermophile
Sclerotinia sclerotiorum
Botryotinia fuckeliana
Trichoderma reesei
Gibberella zeae
Aspergillus niger
Penicillium chrysogenum
Phanerochaete chrysosporium
Schizophyllum commune
Postia placenta
0.05

Figure 16

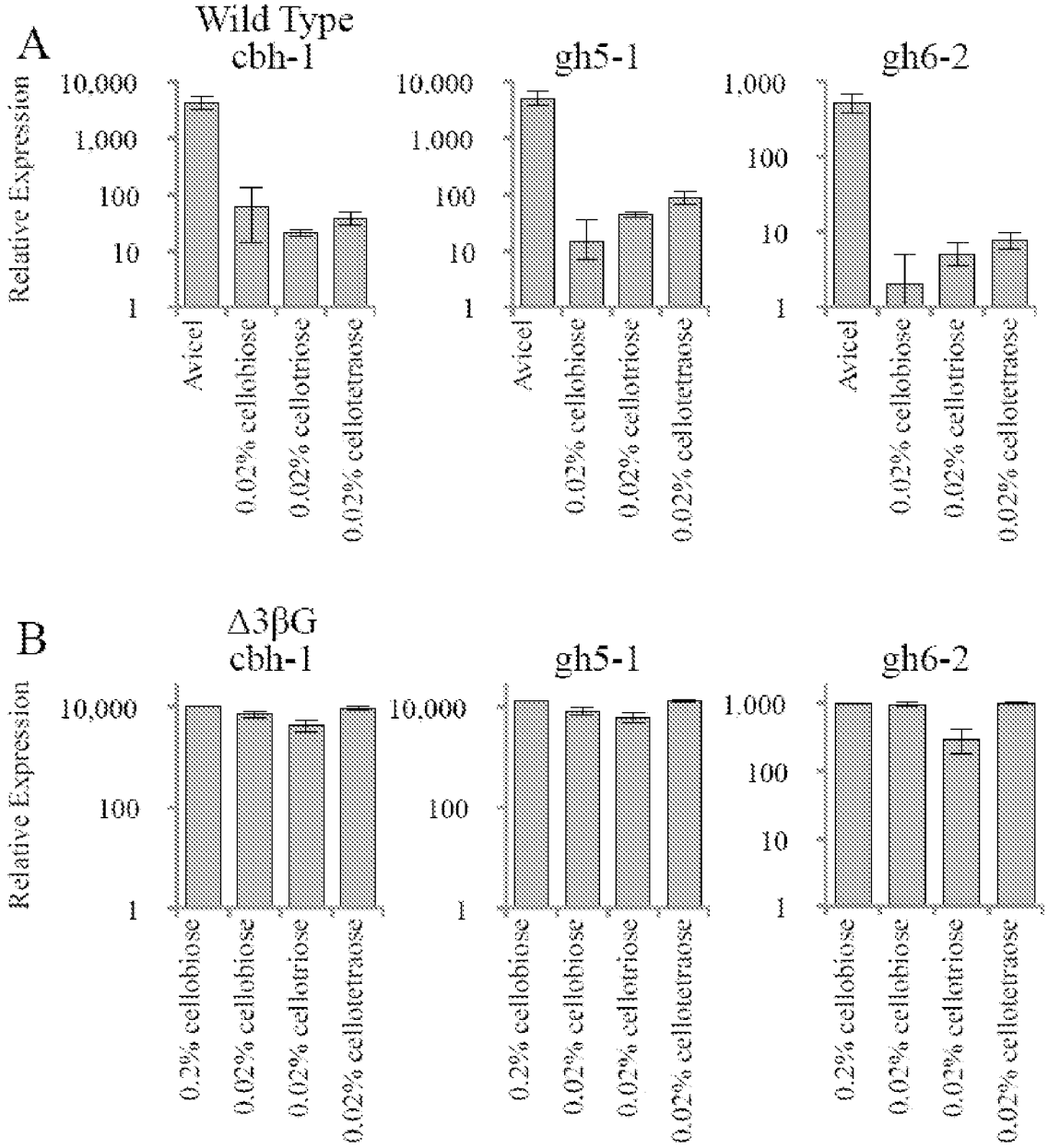

A
Wild Type
cbh-1
gh5-1
gh6-2
Relative Expression
10,000
1,000
100
10
1
Avicel
0.02% cellobiose
0.02% cellotriose
0.02% cellotetraose
B
Δ3βG
cbh-1
gh5-1
gh6-2
0.2% cellobiose
0.02% cellobiose
0.02% cellotriose
0.02% cellotetraose

Figure 17

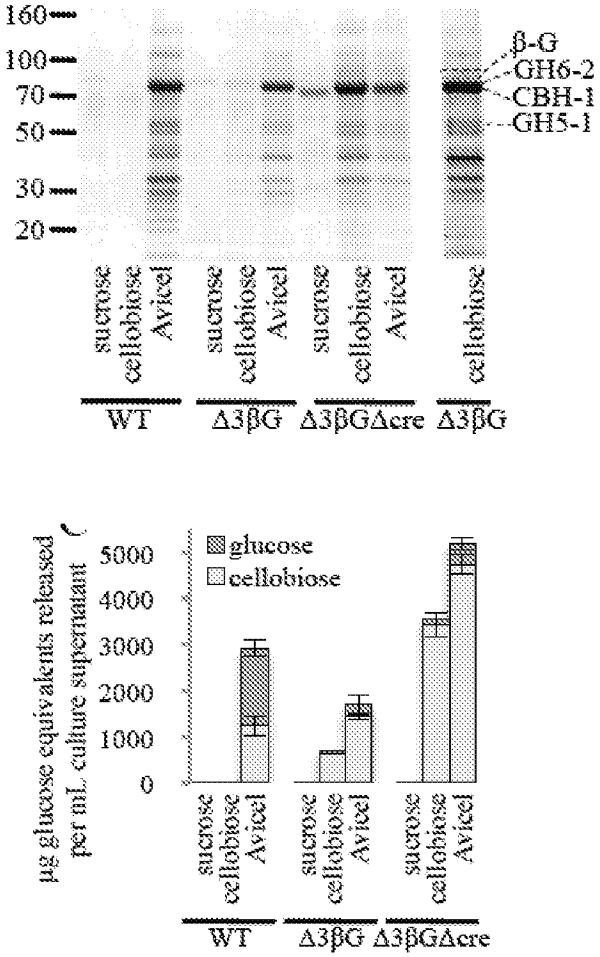

160
100
70
50
30
20
β-G
GH6-2
CBH-1
GH5-1
sucrose
cellobiose
Avicel
WT
Δ3βG
Δ3βGΔcre
μg glucose equivalents released per mL culture supernatant
5000
4000
3000
2000
1000
0
glucose
cellobiose

Figure 18

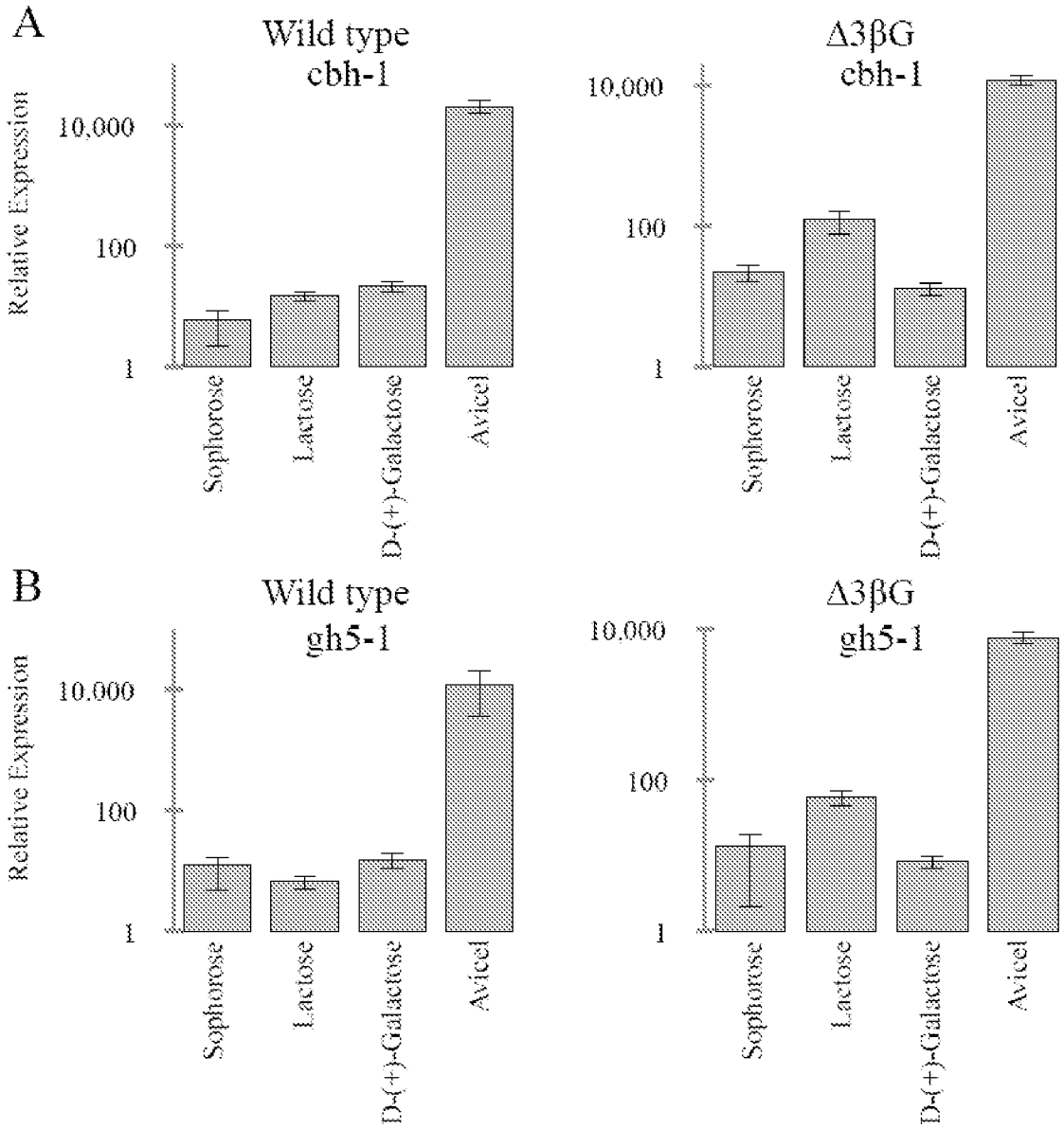
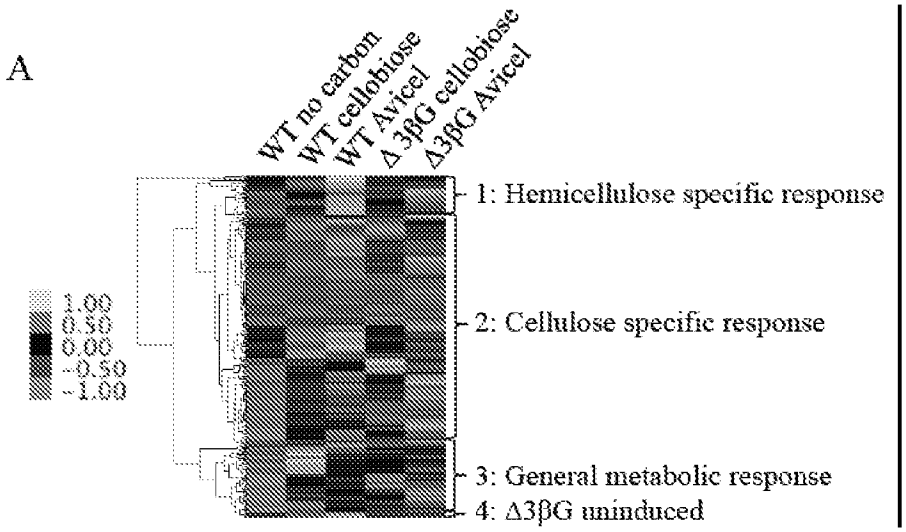

A
Wild type
cbh-1
Δ3βG
cbh-1
Relative Expression
10,000
100
1
Sophorose
Lactose
D-(+)-Galactose
Avicel
B
Wild type
gh5-1
Δ3βG
gh5-1
Relative Expression
10,000
100
1
Sophorose
Lactose
D-(+)-Galactose
Avicel

Figure 19


A
WT no carbon
WT cellobiose
WT Avicel
Δ 3βG cellobiose
Δ3βG Avicel
1.00
0.50
0.00
-0.50
-1.00
1: Hemicellulose specific response
2: Cellulose specific response
3: General metabolic response
4: Δ3βG uninduced

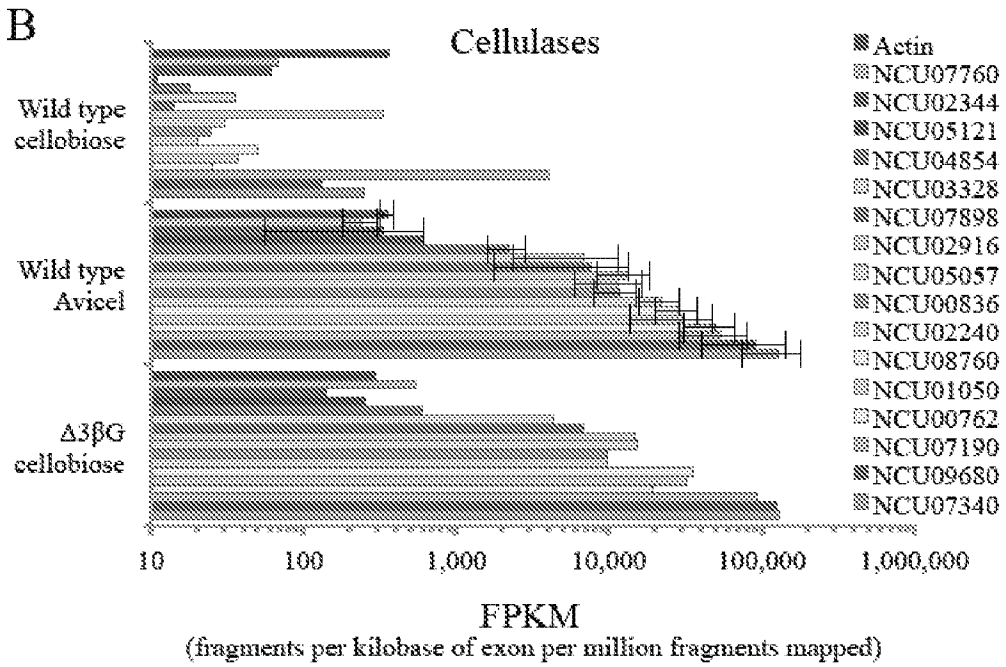
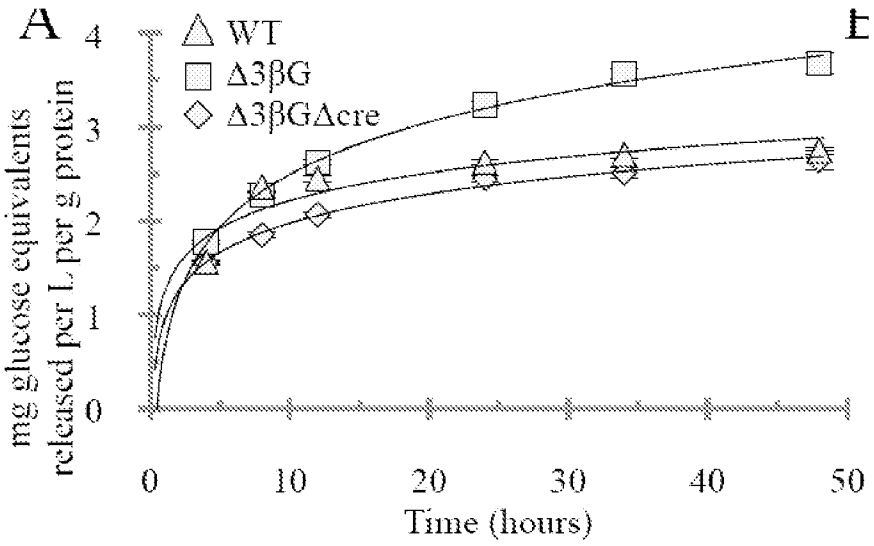

B
Cellulases
Wild type cellobiose
Wild type Avicel
Δ3βG cellobiose
Actin
NCU07760
NCU02344
NCU05121
NCU04854
NCU03328
NCU07898
NCU02916
NCU05057
NCU00836
NCU02240
NCU08760
NCU01050
NCU00762
NCU07190
NCU09680
NCU07340
10
100
1,000
10,000
100,000
1,000,000
FPKM
(fragments per kilobase of exon per million fragments mapped)

Figure 20


A
B
WT
Δ3βG
Δ3βGΔcre
mg glucose equivalents released per L per g protein
0
1
2
3
4
0
10
20
30
40
50
Time (hours)

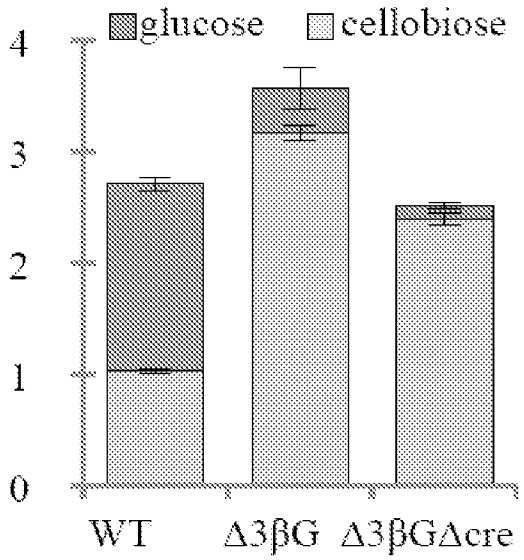

glucose
cellobiose
0
1
2
3
4
WT
Δ3βG
Δ3βGΔcre

Figure 21

		Wild type Avicel			Δ3βG cellobiose			Δ3βGΔcre cellobiose		
	GH family	Whole Supernatant*	PASC bound†	PASC unbound‡	Whole Supernatant	PASC bound	PASC unbound	Whole Supernatant	PASC bound	PASC unbound
NCU04952	3	+		+						
NCU00762	5	+	+	+	+	+		+	+	
NCU08412	5	+		+	+		+	+		+
NCU07190	6	+		+	+	+	+	+	+	+
NCU09689	6	+	+					+	+	
NCU05057	7	+		+	+		+	+		+
NCU07340	7	+	+	+	+	+	+	+		+
NCU05924	10	+		+				+		+
NCU08189	10	+		+						
NCU02855	11	+		+						
NCU07225	11	+	+	+				+	+	
NCU04431	16				+		+			
NCU05686	16				+		+			
NCU05974	16	+		+	+		+			
NCU01517	17				+		+	+		+
NCU09175	17	+			+	+	+	+		+
NCU04395	30				+		+			
NCU04265	32				+	+	+	+		+
NCU07326	43	+	+	+				+		+
NCU05121	45	+								
NCU02343	51				+		+			
NCU09775	54				+					
NCU07523	55				+	+	+			
NCU00836	61	+	+							
NCU01050	61	+	+	+				+	+	
NCU02240	61	+	+	+				+	+	
NCU07898	61	+	+	+				+	+	
NCU08760	61	+	+	+	+	+		+	+	
NCU06781	72				+	+	+			
NCU08909	72				+		+			
NCU05955	74	+	+	+		+		+	+	
NCU00206	CBM1	+		+		+		+		
NCU09764	CBM1	+	+					+	+	
NCU05159	CBM1/CE5	+	+						+	
NCU04870	CE1	+		+						
NCU08785	CE1	+		+						
NCU09491	CE1	+		+	+		+	+		+
NCU09664	CE5	+	+					+		+
NCU05852	PL20	+		+						
NCU08936	GH NC				+	+	+		+	
NCU05598	PL4	+		+	+		+			
NCU00449		+		+						
NCU00798					+		+			
NCU01595			+			+				+
NCU02133					+		+			
NCU02136					+		+			
NCU02696		+	+			+				
NCU04202					+	+				
NCU04205		+			+					
NCU04482					+					
NCU04522					+	+	+			
NCU05134					+		+			
NCU05137		+	+	+	+	+	+	+		+
NCU07143		+		+	+		+	+		+
NCU07200					+	+	+			
NCU07281					+					
NCU07787					+	+	+	+		+
NCU08171		+		+	+		+			
NCU08398		+		+						
NCU09024		+		+	+		+			
NCU09048		+		+						
NCU09267					+		+			+
NCU09992					+		+			

GH, glycoside hydrolase; CBM1, carbohydrate binding module; CE, carbohydrate esterase; PL, polysaccharide lyase; NC, Not Classified; N/A, gene knockout.

*Whole Supernatant, peptides detected from a tryptic digest of all extracellular proteins; †PASC bound, peptides detected after enrichment for proteins that bind to phosphoric acid swollen cellulose; ‡PASC unbound, proteins remaining in solution after removal of PASC bound proteins.

Figure 22

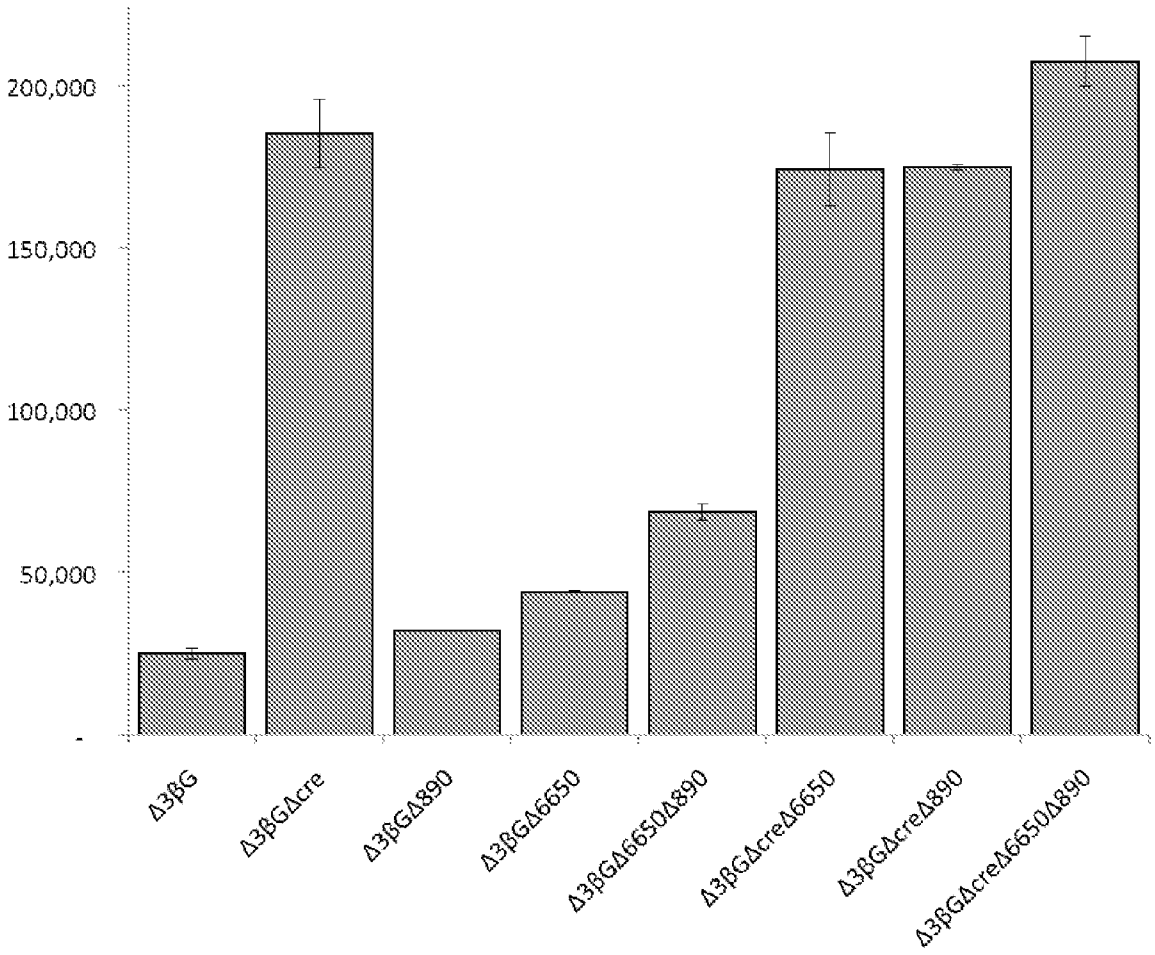

200,000
150,000
100,000
50,000
-
Δ3βG
Δ3βGΔcre
Δ3βGΔ890
Δ3βGΔ6650
Δ3βGΔ6650Δ890
Δ3βGΔcreΔ6650
Δ3βGΔcreΔ890
Δ3βGΔcreΔ6650Δ890

Figure 23

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/029293

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/24 C12P21/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P C12Y

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	JIANPING SUN ET AL: "Identification of the CRE-1 Cellulolytic Regulon in Neurospora crassa", PLOS ONE, vol. 6, no. 9, 29 September 2011 (2011-09-29), page E25654, XP055025830, DOI: 10.1371/journal.pone.0025654 the whole document -----	1,2,5-8, 11-35, 38-41, 44-140
X	WO 2004/081201 A1 (VIB VZW [BE]; UNIV GENT [BE]; CONTRERAS ROLAND [BE]; GEYSENS STEVEN [B) 23 September 2004 (2004-09-23) page 3 - page 4 examples 1-5 ----- -/--	1,2,5-8, 11-35, 38-41, 44-140

[X] Further documents are listed in the continuation of Box C.

[X] 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
9 May 2012	24/07/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Keller, Yves

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	, 1 January 2009 (2009-01-01), XP055025838, Retrieved from the Internet: URL:http://www.pnas.org/content/106/52/22157.full.pdf#page=1&view=FitH [retrieved on 2012-04-27] abstract page 22159, column 2 - page 22160, column 1 page 22161, column 1 -----	1,2,5-8, 11-35, 38-41, 44-140
X,P	, 1 January 2012 (2012-01-01), XP055025834, DOI: 10.1073/pnas.1118440109/-/DCSupplemental Retrieved from the Internet: URL:http://www.pnas.org/content/early/2012/03/30/1118440109.full.pdf [retrieved on 2012-04-27] the whole document -----	1,2,5-8, 11-35, 38-41, 44-140

3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/029293

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 and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/029293

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

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

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

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

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

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

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

see additional sheet

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

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

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

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

1, 2, 7, 8, 34, 35, 40, 41, 68-130(completely); 5, 6, 11-33, 38, 39, 44-67 131-140(partially)

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/029293

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004081201 A1	23-09-2004	CA 2518580 A1	23-09-2004
		EP 1601761 A1	07-12-2005
		US 2006110795 A1	25-05-2006
		US 2009087882 A1	02-04-2009
		WO 2004081201 A1	23-09-2004

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 1, 2, 7, 8, 34, 35, 40, 41, 68-130(completely); 5, 6, 11-33, 38, 39, 44-67, 131-140(partially)

   A mutant or recombinant cell having an inactivation of at least 2 beta glucosidase; method for increasing secretion of a protein comprising using said cells and methods for the degradation of biomass using said cells.

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2. claims: 3, 4, 9, 10, 36, 37, 42, 43(completely); 5, 6, 11-33, 38, 39, 44-67, 131-140(partially)

   A method for increasing secretion of a protein comprising providing a mutant or recombinant cell having an inactivation Cre 1.
