Production of glycoproteins having increased N-glycosylation site occupancy

FIELD OF THE INVENTION

The present disclosure relates to compositions and methods useful for the production of heterologous proteins, e.g recombinant antibodies, in filamentous fungal cells.

BACKGROUND

Posttranslational modification of eukaryotic proteins, particularly therapeutic proteins such as immunoglobulins, is often necessary for proper protein folding and function. Because standard prokaryotic expression systems lack the proper machinery necessary for such modifications, alternative expression systems have to be used in production of these therapeutic proteins. Even where eukaryotic proteins do not have posttranslational modifications, prokaryotic expression systems often lack necessary chaperone proteins required for proper folding. Yeast and fungi are attractive options for expressing proteins as they can be easily grown at a large scale in simple media, which allows low production costs, and yeast and fungi have posttranslational machinery and chaperones that perform similar functions as found in mammalian cells. Moreover, tools are available to manipulate the relatively simple genetic makeup of yeast and fungal cells as well as more complex eukaryotic cells such as mammalian or insect cells (De Pourcq et al., Appl Microbiol Biotechnol, 87(5):1617-31).

However, posttranslational modifications occurring in yeast and fungi may still be a concern for the production of recombinant therapeutic protein. In particular, insufficient N-glycosylation is one of the biggest hurdles to overcome in the production of biopharmaceuticals for human applications in fungi.

N-glycosylation, which refers to the attachment of sugar molecule to a nitrogen atom of an asparagine side chain, has been shown to modulate the pharmacokinetics and pharmacodynamics of therapeutic proteins.

When recombinant proteins are expressed in filamentous fungal cells such as *Trichoderma* fungus cells, the proportion of N-glycosylation sites that are indeed glycosylated is generally lower than for the same protein expressed in a mammalian system, such as CHO cells.

WO2011/106389, entitled “Methods for increasing N-glycosylation site occupancy on therapeutic glycoproteins produced in *Pichia pastoris*”, describes *Pichia pastoris* cells that overexpress heterologous single-subunit oligotransferase, and are able to produce glycoproteins with improved N-glycosylation.

Similarly, Choi et al. describe improved N-glycosylation of recombinant proteins by heterologous expression of heterologous single-subunit oligotransferase (Choi et al., Appl Microbiol Biotechnol, 95(3): 671-82).

The same authors have also described, in WO2013062939, methods for increasing N-glycan occupancy and reducing production of hybrid N-glycans in Pichia pastoris strains lacking alpha-1,3 mannosyltransferase activity (Alg3p disruption).

Reports of fungal cell expression systems expressing human-like fucosylated N-glycans are lacking. Indeed, due to the industry's focus on mammalian cell culture technology for such a long time, the fungal cell expression systems such as *Trichoderma* are not as well established for therapeutic protein production as mammalian cell culture and therefore suffer from drawbacks when expressing mammalian proteins. In particular, a need remains in the art for improved filamentous fungal cells, such as *Trichoderma* fungus cells, that can stably produce heterologous proteins with increased N-glycosylation site occupancy, preferably at high levels of expression.

SUMMARY

The present invention relates to improved methods for producing glycoproteins with increased N-glycosylation site occupancy in filamentous fungal expression systems, and more specifically, glycoproteins, such as antibodies or related immunoglobulins or fusion proteins.

The present invention is based in part on the surprising discovery that filamentous fungal cells, such as *Trichoderma* cells, can be genetically modified to express oligosaccharyl transferase activity, without adversely affecting yield of produced glycoproteins.

Accordingly, in a first aspect, the invention relates to a filamentous fungal cell comprising

i. one or more mutation that reduces or eliminates one or more endogenous protease activity compared to a parental filamentous fungal cell which does not have said mutation(s),
ii. a polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase, and
iii. a polynucleotide encoding a heterologous glycoprotein,

wherein said catalytic subunit of oligosaccharyl transferase is selected from *Leishmania* oligosaccharyl transferase catalytic subunits.

In one embodiment, said filamentous fungal cell has at least a two-fold reduction, preferably at least a three-fold reduction, even more preferably at least a four-fold reduction, at least a five-fold reduction, in total protease activity compared to a parental filamentous fungal cell which does not have the protease-deficient mutations(s).

In one embodiment of the invention, said filamentous fungal cell is a *Trichoderma*, *Neurospora*, *Myceliophtora*, *Chrysosporium*, *Aspergillus*, or *Fusarium* cell.

In one embodiment of the invention, the polynucleotide encoding the heterologous catalytic subunit of oliogaccharyl transferase comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 9, SEQ ID NO: 88 and SEQ ID NO: 90 or a polynucleotide encoding a functional variant polypeptide having at least 50%, at least 60%, at least 70% identity, at least 80% identity, at least 90% identity, or at least 95% identity with SEQ ID NO: 1, SEQ ID NO: 8, SEQ ID NO: 89 or SEQ ID NO: 91, said functional variant polypeptide having oligosaccharyltransferase activity.

In another embodiment, said polynucleotide encoding the heterologous catalytic subunit of oligosaccharyl transferase is under the control of a promoter for constitutive expression of said oligosaccharyl transferase in said cell.

In one embodiment of the invention, the N-glycosylation site occupancy of the heterologous glycoprotein expressed in filamentous fungal cell is at least 80%, at least 90%, at least 95%, at least 99%, or 100%.

In a specific embodiment, the N-glycosylation site occupancy of the heterologous glycoprotein is at least 95% and Man3, Man5, G0, G1 and/or G2 glycoforms represent at least 50% of total neutral N-glycans of the heterologous glycoprotein.

In one embodiment of the invention, the filamentous fungal cell is a *Trichoderma* cell, for example, *Trichoderma reesei*, and said cell comprises mutations that reduce or eliminate the activity of

- the three endogenous proteases pep1, tsp1, and slp1;
- the three endogenous proteases gap1, slp1, and pep1;
- the three endogenous proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep8, pep9, pep11, pep12, tsp1, slp1, slp2, slp3, slp7, gap1 and gap2;
- three to six proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, tsp1, slp1, slp2, slp3, gap1 and gap2;

- seven to ten proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep7, pep8, pep9, tsp1, slp1, slp2, slp3, slp5, slp6, slp7, slp8, tpp1, gap1 and gap2.

In one embodiment, the fungal cell further comprises a mutation in the gene encoding ALG3 that reduces or eliminates the corresponding ALG3 expression compared to the level of expression of ALG3 gene in a parental cell which does not have such mutation.

In one embodiment, the fungal cell further comprises a polynucleotide encoding an N-acetylglucosaminyltransferase I catalytic domain and an N-acetylglucosaminyltransferase II catalytic domain.

In one embodiment, the fungal cell further comprises one or more polynucleotides encoding a polypeptide selected from the group consisting of:

i. α1, 2 mannosidase;
ii. N-acetylglucosaminyltransferase I catalytic domain;
iii. α-mannosidase II;
iv. N-acetylglucosaminyltransferase II catalytic domain;
v. β1,4 galactosyltransferase; and,
vi. fucosyltransferase.

In one embodiment of the invention, the heterologous glycoprotein is a mammalian glycoprotein.

In a specific embodiment, said mammalian glycoprotein is selected from the group consisting of an antibody, an immunoglobulin or a protein fusion comprising Fc fragment of an immunoglobulin.

In another specific embodiment, said mammalian glycoprotein is a therapeutic antibody.

In another aspect, the invention also relates to a method of increasing N-glycosylation site occupancy of heterologous glycoprotein produced in a filamentous fungal host cell, comprising:

a) providing a filamentous fungal host cell, for example a *Trichoderma* cell, having a *Leishmania* STT3D gene encoding a catalytic subunit of oligosaccharyl transferase, or a functional variant thereof, and a polynucleotide encoding a heterologous glycoprotein,

b) culturing the host cell under appropriate conditions for expression of the STT3D gene or its functional variant, or said functional variant, and the production of the heterologous glycoprotein;

wherein the expressed heterologous glycoproteins exhibit increased N-glycosylation site occupancy compared to the heterologous glycoproteins expressed in a corresponding parental filamentous fungal cell which does not express said oligosaccharyl transferase catalytic subunit.

The invention also relates to a method of producing a heterologous glycoprotein composition, with increased N-glycosylation site occupancy, comprising:

a) providing a filamentous fungal cell, for example a *Trichoderma* cell, having a *Leishmania* STT3D gene encoding a catalytic subunit of oligosaccharyl transferase, or a functional variant thereof, and a polynucleotide encoding a heterologous glycoprotein,

b) culturing the cell under appropriate conditions for expression of the STT3D gene or its functional variant, and the production of the heterologous glycoprotein composition; and,

c) recovering and, optionally, purifying the heterologous glycoprotein composition.

In certain embodiments of the method of the invention, said *Leishmania* STT3D gene encoding a catalytic subunit of oligosaccharyl transferase comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 9, SEQ ID NO: 88 and SEQ ID NO: 90, or a polynucleotide encoding a functional variant polypeptide having at least 50%, at least 60%, at least 70% identity, at least 80% identity, at least 90% identity, or at least 95% identity with SEQ ID NO: 1, SEQ ID NO: 8, SEQ ID NO: 89 or SEQ ID NO: 91, said functional variant polypeptide having oligosaccharyltransferase activity.

In one embodiment, said polynucleotide encoding said heterologous glycoprotein further comprises a polynucleotide encoding CBH1 catalytic domain and linker as a carrier protein and/or cbh1 promoter.

In one embodiment of the invention, the culturing is in a medium comprises a protease inhibitor.

In a specific embodiment, the culturing is in a medium comprising one or two protease inhibitors selected from SBTI and chymostatin.

In one embodiment of the method of the invention, the N-glycosylation site occupancy of the produced glycoprotein composition is at least 80%, at least 90%, at least 95%, at least 99%, or 100%.

In one aspect, the invention also relates to a glycoprotein composition obtainable by the method described above.

In one aspect, the invention relates to an antibody composition obtainable by the method described above.

In one embodiment the invention relates to the antibody composition described above, wherein N-glycosylation site occupancy is at least 80%, at least 90%, at least 95%, at least 99%, or 100%.

In one embodiment the invention relates to the antibody composition described above, wherein said antibody composition further comprises, as a major glycoform, either:

i. Manα3[Manα6(Manα3)Manα6]Manβ4GlcNAβ4GlcNAc (Man5 glycoform);
ii. GlcNAcβ2Manα3[Manα6(Manα3)Manα6]Manβ4GlcNAβ4GlcNAc (GlcNAcMan5 glycoform);
iii. Manα6(Manα3)Manβ4GlcNAβ4GlcNAc (Man3 glycoform);
iv. Manα6(GlcNAcβ2Manα3)Manβ4GlcNAβ4GlcNAc (GlcNAcMan3 glycoform); or,
v. complex type N-glycans selected from the G0, G1, or G2 glycoform.

DESCRIPTION OF THE FIGURES

Figure 1. Schematic expression cassette design for *Leishmania major* STT3 targeted to the xylanase 1 locus.

Figure 2. Example spectra of parental strain M317 (pyr4- of M304) and L. major STT3 clone 26B-a (M421). K means lysine.

Figure 3. Schematic map of the STT3 expression cassettes.

Figure 4. Glycan structures produced in Δalg3 strains.

Figure 5. Normalized protease activity data from culture supernatants from the protease deletion supernatants and the parent strain. Protease activity was measured at pH 5.5 in first 5 strains and at pH 4.5 in the last three deletion strains. Protease activity is against green fluorescent casein. The six protease deletion strain has only 6% of the wild type parent strain and the 7 protease deletion strain protease activity was about 40% less than the 6 protease deletion strain activity.

DETAILED DESCRIPTION

Definitions

As used herein, an “expression system” or a “host cell” refers to the cell that is genetically modified to enable the transcription, translation and proper folding of a polypeptide or a protein of interest, typically of mammalian protein.

The term “polynucleotide” or “oligonucleotide” or “nucleic acid” as used herein typically refers to a polymer of at least two nucleotides joined together by a phosphodiester bond and may consist of either ribonucleotides or deoxynucleotides or their derivatives that can be introduced into a host cell for genetic modification of such host cell. For example, a polynucleotide may encode a coding sequence of a protein, and/or comprise control or regulatory sequences of a coding sequence of a protein, such as enhancer or promoter sequences or terminator. A polynucleotide may for example comprise native coding sequence of a gene or their fragments, or variant sequences that have been optimized for optimal gene expression in a specific host cell (for example to take into account codon bias).

As used herein, the term, "optimized" with reference to a polynucleotide means that a polynucleotide has been altered to encode an amino acid sequence using codons that are preferred in the production cell or organism, for example, a filamentous fungal cell such as a *Trichoderma* cell. Heterologous nucleotide sequences that are transfected in a host cell are typically optimized to retain completely or as much as possible the amino acid sequence originally encoded by the original (not optimized) nucleotide sequence. The optimized sequences herein have been engineered to have codons that are preferred in the corresponding production cell or organism, for example the filamentous fungal cell. The amino acid sequences encoded by optimized nucleotide sequences may also be referred to as optimized.

As used herein, a “peptide” or a “polypeptide” is an amino acid sequence including a plurality of consecutive polymerized amino acid residues. The peptide or polypeptide may include modified amino acid residues, naturally occurring amino acid residues not encoded by a codon, and non-naturally occurring amino acid residues. As used herein, a “protein” may refer to a peptide or a polypeptide or a combination of more than one peptide or polypeptide assembled together by covalent or non-covalent bonds. Unless specified, the term “protein” may encompass one or more amino acid sequences with their post-translation modifications, and in particular with either O-mannosylation or N-glycan modifications.

As used herein, the term “glycoprotein” refers to a protein which comprises at least one N-linked glycan attached to at least one asparagine residue of a protein, or at least one mannose attached to at least one serine or threonine resulting in O-mannosylation. Since glycoproteins as produced in a host cell expression system are usually produced as a mixture of different glycosylation patterns, the terms “glycoprotein” or “glycoprotein composition” encompass the mixtures of glycoproteins as produced by a host cell, with different glycosylation patterns, unless specifically defined.

The terms “N-glycosylation” or “oligosaccharyl transferase activity” are used herein to refer to the covalent linkage of at least an oligosaccharide chain to the side-chain amide nitrogen of asparagine residue (Asn) of a polypeptide.

As used herein, “glycan” refers to an oligosaccharide chain that can be linked to a carrier such as an amino acid, peptide, polypeptide, lipid or a reducing end conjugate. In certain embodiments, the invention relates to N-linked glycans (“N-glycan”) conjugated to a polypeptide N-glycosylation site such as -Asn-Xaa-Ser/Thr- by N-linkage to side-chain amide nitrogen of asparagine residue (Asn), where Xaa is any amino acid residue except Pro. The invention may further relate to glycans as part of dolichol-phospho-oligosaccharide (Dol-P-P-OS) precursor lipid structures, which are precursors of N-linked glycans in the endoplasmic reticulum of eukaryotic cells. The precursor oligosaccharides are linked from their reducing end to two phosphate residues on the dolichol lipid. For example, α3-mannosyltransferase Alg3 modifies the Dol-P-P-oligosaccharide precursor of N-glycans. Generally, the glycan structures described herein are terminal glycan structures, where the non-reducing residues are not modified by other monosaccharide residue or residues.

As used throughout the present disclosure, glycolipid and carbohydrate nomenclature is essentially according to recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (e.g. Carbohydrate Res. 1998, 312, 167; Carbohydrate Res. 1997, 297, 1; Eur. J. Biochem. 1998, 257, 29). It is assumed that Gal (galactose), Glc (glucose), GlcNAc (N-acetylglucosamine), GalNAc (N-acetylgalactosamine), Man (mannose), and Neu5Ac are of the D-configuration, Fuc of the L-configuration, and all the monosaccharide units in the pyranose form (D-Galp, D-Glcp, D-GlcpNAc, D-GalpNAc, D-Manp, L-Fucp, D-Neup5Ac). The amine group is as defined for natural galactose and glucosamines on the 2-position of GalNAc or GlcNAc. Glycosidic linkages are shown partly in shorter and partly in longer nomenclature, the linkages of the sialic acid SA/Neu5X-residues α3 and α6 mean the same as α2-3 and α2-6, respectively, and for hexose monosaccharide residues α1-3, α1-6, β1-2, β1-3, β1-4, and β1-6 can be shortened as α3, α6, β2, β3, β4, and β6, respectively. Lactosamine refers to type II N-acetyllactosamine, Galβ4GlcNAc, and/or type I N-acetyllactosamine. Galβ3GlcNAc and sialic acid (SA) refer to N-acetylneuraminic acid (Neu5Ac),

N-glycolylneuraminic acid (Neu5Gc), or any other natural sialic acid including derivatives of Neu5X. Sialic acid is referred to as NeuNX or Neu5X, where preferably X is Ac or Gc. Occasionally Neu5Ac/Gc/X may be referred to as NeuNAc/NeuNGc/NeuNX.

The sugars typically constituting N-glycans found in mammalian glycoprotein, include, without limitation, N-acetylglucosamine (abbreviated hereafter as "GlcNAc"), mannose (abbreviated hereafter as "Man"), glucose (abbreviated hereafter as "Glc"), galactose (abbreviated hereafter as "Gal"), and sialic acid (abbreviated hereafter as "Neu5Ac"). N-glycans share a common pentasaccharide referred to as the "core" structure $Man_3GlcNAc_2$ (Manα6(Manα3)Manβ4GlcNAβ4GlcNAc, referred to as Man3).

In some embodiments Man3 glycan or its derivative Manα6(GlcNAcβ2Manα3)Manβ4GlcNAβ4GlcNAc is the major glycoform. When a fucose is attached to the core structure, preferably α6-linked to reducing end GlcNAc, the N-glycan or the core of N-glycan, may be represented as $Man_3GlcNAc_2$(Fuc). In an embodiment the major N-glycan is Manα3[Manα6(Manα3)Manα6]Manβ4GlcNAβ4GlcNAc (Man5).

Preferred hybrid type N-glycans comprise GlcNAcβ2Manα3[Manα6(Manα3)Manα6]Manβ4GlcNAβ4GlcNAc ("GlcNAcMan5"), or b4-galactosylated derivatives thereof Galβ4GlcNAcMan3, G1, G2, or GalGlcNAcMan5 glycoform.

A "complex N-glycan" refers to a N-glycan which has at least one GlcNAc residue, optionally by GlcNAcβ2-residue, on terminal 1,3 mannose arm of the core structure and at least one GlcNAc residue, optionally by GlcNAcβ2-residue, on terminal 1,6 mannose arm of the core structure.

Such complex N-glycans include, without limitation, $GlcNAc_2Man_3GlcNAc_2$ (also referred as G0 glycoform), $Gal_1GlcNAc_2Man_3GlcNAc_2$ (also referred as G1 glycoform), and $Gal_2GlcNAc_2Man_3GlcNAc_2$ (also referred as G2 glycoform), and their core fucosylated glycoforms FG0, FG1 and FG2, respectively $GlcNAc_2Man_3GlcNAc_2$(Fuc), $Gal_1GlcNAc_2Man_3GlcNAc_2$(Fuc), and $Gal_2GlcNAc_2Man_3GlcNAc_2$(Fuc).

As used herein, the expression "neutral N-glycan" has its general meaning in the art. It refers to non-sialylated N-glycans. In contrast, sialylated N-glycans are acidic.

"Increased" or "Reduced activity of an endogenous enzyme": The filamentous fungal cell may have increased or reduced levels of activity of various endogenous enzymes. A reduced level of activity may be provided by inhibiting the activity of the endogenous enzyme with an inhibitor, an antibody, or the like. In certain embodiments, the filamentous fungal cell is genetically modified in

ways to increase or reduce activity of various endogenous enzymes. “Genetically modified” refers to any recombinant DNA or RNA method used to create a prokaryotic or eukaryotic host cell that expresses a polypeptide at elevated levels, at lowered levels, or in a mutated form. In other words, the host cell has been transfected, transformed, or transduced with a recombinant polynucleotide molecule, and thereby been altered so as to cause the cell to alter expression of a desired protein.

“Genetic modifications” which result in a decrease or deficiency in gene expression, in the function of the gene, or in the function of the gene product (i.e., the protein encoded by the gene) can be referred to as inactivation (complete or partial), knock-out, deletion, disruption, interruption, blockage, silencing, or down-regulation, or attenuation of expression of a gene. For example, a genetic modification in a gene which results in a decrease in the function of the protein encoded by such gene, can be the result of a complete deletion of the gene (i.e., the gene does not exist, and therefore the protein does not exist), a mutation in the gene which results in incomplete (disruption) or no translation of the protein (e.g., the protein is not expressed), or a mutation in the gene which decreases or abolishes the natural function of the protein (e.g., a protein is expressed which has decreased or no enzymatic activity or action). More specifically, reference to decreasing the action of proteins discussed herein generally refers to any genetic modification in the host cell in question, which results in decreased expression and/or functionality (biological activity) of the proteins and includes decreased activity of the proteins (e.g., decreased catalysis), increased inhibition or degradation of the proteins as well as a reduction or elimination of expression of the proteins. For example, the action or activity of a protein can be decreased by blocking or reducing the production of the protein, reducing protein action, or inhibiting the action of the protein. Combinations of some of these modifications are also possible. Blocking or reducing the production of a protein can include placing the gene encoding the protein under the control of a promoter that requires the presence of an inducing compound in the growth medium. By establishing conditions such that the inducer becomes depleted from the medium, the expression of the gene encoding the protein (and therefore, of protein synthesis) could be turned off. Blocking or reducing the action of a protein could also include using an excision technology approach similar to that described in U.S. Pat. No. 4,743,546. To use this approach, the gene encoding the protein of interest is cloned between specific genetic sequences that allow specific, controlled excision of the gene from the genome. Excision could be prompted by, for example, a shift in the cultivation temperature of the culture, as in U.S. Pat. No. 4,743,546, or by some other physical or nutritional signal.

In general, according to the present invention, an increase or a decrease in a given characteristic of a mutant or modified protein (e.g., enzyme activity) is made with reference to the same characteristic of a parent (i.e., normal, not modified) protein that is derived from the same organism

(from the same source or parent sequence), which is measured or established under the same or equivalent conditions. Similarly, an increase or decrease in a characteristic of a genetically modified host cell (e.g., expression and/or biological activity of a protein, or production of a product) is made with reference to the same characteristic of a wild-type host cell of the same species, and preferably the same strain, under the same or equivalent conditions. Such conditions include the assay or culture conditions (e.g., medium components, temperature, pH, etc.) under which the activity of the protein (e.g., expression or biological activity) or other characteristic of the host cell is measured, as well as the type of assay used, the host cell that is evaluated, etc. As discussed above, equivalent conditions are conditions (e.g., culture conditions) which are similar, but not necessarily identical (e.g., some conservative changes in conditions can be tolerated), and which do not substantially change the effect on cell growth or enzyme expression or biological activity as compared to a comparison made under the same conditions.

Preferably, a genetically modified host cell that has a genetic modification that increases or decreases (reduces) the activity of a given protein (e.g., a protease) has an increase or decrease, respectively, in the activity or action (e.g., expression, production and/or biological activity) of the protein, as compared to the activity of the protein in a parent host cell (which does not have such genetic modification), of at least about 5%, and more preferably at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55 60%, 65%, 70%, 75 80%, 85 90%, 95%, or any percentage, in whole integers between 5% and 100% (e.g., 6%, 7%, 8%, etc.).

In another aspect of the invention, a genetically modified host cell that has a genetic modification that increases or decreases (reduces) the activity of a given protein (e.g., a protease) has an increase or decrease, respectively, in the activity or action (e.g., expression, production and/or biological activity) of the protein, as compared to the activity of the wild-type protein in a parent host cell, of at least about 2-fold, and more preferably at least about 5-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 75-fold, 100-fold, 125-fold, 150-fold, or any whole integer increment starting from at least about 2-fold (e.g., 3-fold, 4-fold, 5-fold, 6-fold, etc.).

As used herein, the terms “identical” or “per cent identity,” in the context of two or more nucleic acid or amino acid sequences, refers to two or more sequences or subsequences that are the same. Two sequences are “substantially identical” if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (i.e., 29% identity, optionally 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or 100% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10

amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200, or more amino acids) in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. When comparing two sequences for identity, it is not necessary that the sequences be contiguous, but any gap would carry with it a penalty that would reduce the overall percent identity. For blastn, the default parameters are Gap opening penalty=5 and Gap extension penalty=2. For blastp, the default parameters are Gap opening penalty=11 and Gap extension penalty=1.

A “comparison window,” as used herein, includes reference to a segment of any one of the number of contiguous positions including, but not limited to from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman (1981), by the homology alignment algorithm of Needleman and Wunsch (1970) J Mol Biol 48(3):443-453, by the search for similarity method of Pearson and Lipman (1988) Proc Natl Acad Sci USA 85(8):2444-2448, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection [see, e.g., Brent et al., (2003) Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou Ed)].

Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1997) Nucleic Acids Res 25(17):3389-3402 and Altschul et al. (1990) J. Mol Biol 215(3)-403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) or 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix [see Henikoff and Henikoff,

(1992) Proc Natl Acad Sci USA 89(22):10915-10919] alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, (1993) Proc Natl Acad Sci USA 90(12):5873-5877). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

"Functional variant" or "functional homologous gene" as used herein refers to a coding sequence or a protein having sequence similarity with a reference sequence, typically, at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% identity with the reference coding sequence or protein, and retaining substantially the same function as said reference coding sequence or protein. A functional variant may retain the same function but with reduced or increased activity. Functional variants include natural variants, for example, homologs from different species or artificial variants, resulting from the introduction of a mutation in the coding sequence. Functional variant may be a variant with only conservatively modified mutations.

"Conservatively modified mutations" as used herein include individual substitutions, deletions or additions to an encoded amino acid sequence which result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the disclosure. The following eight groups contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (see, e.g., Creighton, Proteins (1984)).

Filamentous fungal cells

As used herein, "filamentous fungal cells" include cells from all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth et al., In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK). Filamentous fungal cells are generally characterized by a mycelial wall composed of chitin, cellulose, glucan,

chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

Preferably, the filamentous fungal cell is not adversely affected by the transduction of the necessary nucleic acid sequences, the subsequent expression of the proteins (e.g., mammalian proteins), or the resulting intermediates. General methods to disrupt genes of and cultivate filamentous fungal cells are disclosed, for example, for *Penicillium,* in Kopke *et al.* (2010) Appl Environ Microbiol. 76(14):4664-74. doi: 10.1128/AEM.00670-10, for *Aspergillus,* in Maruyama and Kitamoto (2011), Methods in Molecular Biology, vol. 765, DOI10.1007/978-1-61779-197-0_27; for Neurospora, in Collopy et al. (2010) Methods Mol Biol. 2010;638:33-40. doi: 10.1007/978-1-60761-611-5_3; and for *Myceliophthora* or *Chrysosporium* PCT/NL2010/000045 and PCT/EP98/06496.

Examples of suitable filamentous fungal cells include, without limitation, cells from an *Acremonium, Aspergillus, Fusarium, Humicola, Mucor, Myceliophthora, Neurospora, Penicillium, Scytalidium, Thielavia, Tolypocladium*, or *Trichoderma/Hypocrea* strain.

In certain embodiments, the filamentous fungal cell is from a *Trichoderma* sp., *Acremonium, Aspergillus, Aureobasidium, Cryptococcus, Chrysosporium, Chrysosporium lucknowense, Filibasidium, Fusarium, Gibberella, Magnaporthe, Mucor, Myceliophthora, Myrothecium, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Piromyces, Schizophyllum, Talaromyces, Thermoascus, Thielavia,* or *Tolypocladium* strain.

In some embodiments, the filamentous fungal cell is a *Myceliophthora or Chrysosporium, Neurospora, Aspergillus, Fusarium* or *Trichoderma* strain.

Aspergillus fungal cells of the present disclosure may include, without limitation, *Aspergillus aculeatus, Aspergillus awamori, Aspergillus clavatus, Aspergillus flavus, Aspergillus foetidus, Aspergillus fumigatus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae*, or *Aspergillus terreus*.

Neurospora fungal cells of the present disclosure may include, without limitation, *Neurospora crassa*.

Myceliophthora fungal cells of the present disclosure may include, without limitation, *Myceliophthora thermophila.*

In a preferred embodiment, the filamentous fungal cell is a *Trichoderma* fungal cell. *Trichoderma* fungal cells of the present disclosure may be derived from a wild-type *Trichoderma* strain or a mutant thereof. Examples of suitable *Trichoderma* fungal cells include, without limitation, *Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei, Trichoderma atroviride, Trichoderma virens, Trichoderma viride*; and alternative sexual form thereof (i.e., *Hypocrea*).

In a more preferred embodiment, the filamentous fungal cell is a *Trichoderma reesei*, and for example, strains derived from ATCC 13631 (QM 6a), ATCC 24449 (radiation mutant 207 of QM 6a), ATCC 26921 (QM 9414; mutant of ATCC 24449), VTT-D-00775 (Selinheimo et al., FEBS J., 2006, 273: 4322-4335), Rut-C30 (ATCC 56765), RL-P37 (NRRL 15709) or T. harzianum isolate T3 (Wolffhechel, H., 1989).

The invention described herein relates to a filamentous fungal cell, for example selected from *Trichoderma*, *Neurospora*, *Myceliophthora* or a *Chrysosporium* cells, such as *Trichoderma reesei* fungal cell, comprising:

i. one or more mutation that reduces or eliminates one or more endogenous protease activity compared to a parental filamentous fungal cell which does not have said mutation(s),

ii. a polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase, and

iii. a polynucleotide encoding a heterologous glycoprotein,

wherein said catalytic subunit of oligosaccharyl transferase is selected from *Leishmania* oligosaccharyl transferase catalytic subunits.

Proteases with reduced activity

It has been found that reducing protease activity enables to increase substantially the production of heterologous mammalian protein. Indeed, such proteases found in filamentous fungal cells that express a heterologous protein normally catalyse significant degradation of the expressed recombinant protein. Thus, by reducing the activity of proteases in filamentous fungal cells that express a heterologous protein, the stability of the expressed protein is increased, resulting in an increased level of production of the protein, and in some circumstances, improved quality of the produced protein (e.g., full-length instead of degraded).

Proteases include, without limitation, aspartic proteases, trypsin-like serine proteases, subtilisin proteases, glutamic proteases, and sedolisin proteases. Such proteases may be identified and isolated from filamentous fungal cells and tested to determine whether reduction in their activity

affects the production of a recombinant polypeptide from the filamentous fungal cell. Methods for identifying and isolating proteases are well known in the art, and include, without limitation, affinity chromatography, zymogram assays, and gel electrophoresis. An identified protease may then be tested by deleting the gene encoding the identified protease from a filamentous fungal cell that expresses a recombinant polypeptide, such a heterologous or mammalian polypeptide, and determining whether the deletion results in a decrease in total protease activity of the cell, and an increase in the level of production of the expressed recombinant polypeptide. Methods for deleting genes, measuring total protease activity, and measuring levels of produced protein are well known in the art and include the methods described herein.

Aspartic Proteases

Aspartic proteases are enzymes that use an aspartate residue for hydrolysis of the peptide bonds in polypeptides and proteins. Typically, aspartic proteases contain two highly-conserved aspartate residues in their active site which are optimally active at acidic pH. Aspartic proteases from eukaryotic organisms such as *Trichoderma* fungi include pepsins, cathepsins, and renins. Such aspartic proteases have a two-domain structure, which is thought to arise from ancestral gene duplication. Consistent with such a duplication event, the overall fold of each domain is similar, though the sequences of the two domains have begun to diverge. Each domain contributes one of the catalytic aspartate residues. The active site is in a cleft formed by the two domains of the aspartic proteases. Eukaryotic aspartic proteases further include conserved disulfide bridges, which can assist in identification of the polypeptides as being aspartic acid proteases.

Ten aspartic proteases have been identified in *Trichoderma* fungal cells: *pep1* (tre74156); *pep2* (tre53961); *pep3* (tre121133); *pep4* (tre77579), *pep5* (tre81004), and *pep7* (tre58669), *pep8* (tre122076), *pep9* (tre79807), *pep11* (121306), and *pep12* (tre119876).

Examples of suitable aspartic proteases include, without limitation, *Trichoderma reesei* pep1 (SEQ ID NO: 22), *Trichoderma reesei* pep2 (SEQ ID NO: 18), *Trichoderma reesei* pep3 (SEQ ID NO: 19); *Trichoderma reesei* pep4 (SEQ ID NO: 20), *Trichoderma reesei* pep5 (SEQ ID NO: 21) and *Trichoderma reesei* pep7 (SEQ ID NO:23), *Trichoderma reesei* EGR48424 pep8 (SEQ ID NO:85), *Trichoderma reesei* *pep9* (SEQ ID NO:87), *Trichoderma reesei* EGR49498 *pep11* (SEQ ID NO:86), *Trichoderma reesei* EGR52517 *pep12* (SEQ ID NO:35), and homologs thereof. Examples of homologs of pep1, pep2, pep3, pep4, pep5, pep7, pep8, pep11 and pep12 proteases identified in other organisms are also described in PCT/EP/2013/050186, the content of which being incorporated by reference.

Trypsin-Like Serine Proteases

Trypsin-like serine proteases are enzymes with substrate specificity similar to that of trypsin. Trypsin-like serine proteases use a serine residue for hydrolysis of the peptide bonds in polypeptides and proteins. Typically, trypsin-like serine proteases cleave peptide bonds following a positively-charged amino acid residue. Trypsin-like serine proteases from eukaryotic organisms such as *Trichoderma* fungi include trypsin 1, trypsin 2, and mesotrypsin. Such trypsin-like serine proteases generally contain a catalytic triad of three amino acid residues (such as histidine, aspartate, and serine) that form a charge relay that serves to make the active site serine nucleophilic. Eukaryotic trypsin-like serine proteases further include an "oxyanion hole" formed by the backbone amide hydrogen atoms of glycine and serine, which can assist in identification of the polypeptides as being trypsin-like serine proteases.

One trypsin-like serine protease has been identified in *Trichoderma* fungal cells: tsp1 (tre73897). As discussed in PCT/EP/2013/050186, tsp1 has been demonstrated to have a significant impact on expression of recombinant glycoproteins, such as immunoglobulins.

Examples of suitable tsp1 proteases include, without limitation, *Trichoderma reesei* tsp1 (SEQ ID NO: 24) and homologs thereof. Examples of homologs of tsp1 proteases identified in other organisms are described in PCT/EP/2013/050186.

Subtilisin Proteases

Subtilisin proteases are enzymes with substrate specificity similar to that of subtilisin. Subtilisin proteases use a serine residue for hydrolysis of the peptide bonds in polypeptides and proteins. Generally, subtilisin proteases are serine proteases that contain a catalytic triad of the three amino acids aspartate, histidine, and serine. The arrangement of these catalytic residues is shared with the prototypical subtilisin from Bacillus licheniformis. Subtilisin proteases from eukaryotic organisms such as *Trichoderma* fungi include furin, MBTPS1, and TPP2. Eukaryotic trypsin-like serine proteases further include an aspartic acid residue in the oxyanion hole.

Seven subtilisin proteases have been identified in *Trichoderma* fungal cells: slp1 (tre51365); slp2 (tre123244); slp3 (tre123234); slp5 (tre64719), slp6 (tre121495), slp7 (tre123865), and slp8 (tre58698). Subtilisin protease *slp7* resembles also sedolisin protease *tpp1*.

Examples of suitable slp proteases include, without limitation, *Trichoderma reesei* slp1 (SEQ ID NO: 25), slp2 (SEQ ID NO: 26); slp3 (SEQ ID NO: 27); slp5 (SEQ ID NO: 28), slp6 (SEQ ID NO: 29), slp7 (SEQ ID NO: 30), and slp8 (SEQ ID NO: 31), and homologs thereof. Examples of homologs of slp proteases identified in other organisms are described in in PCT/EP/2013/050186.

Glutamic Proteases

Glutamic proteases are enzymes that hydrolyse the peptide bonds in polypeptides and proteins. Glutamic proteases are insensitive to pepstatin A, and so are sometimes referred to as pepstatin insensitive acid proteases. While glutamic proteases were previously grouped with the aspartic proteases and often jointly referred to as acid proteases, it has been recently found that glutamic proteases have very different active site residues than aspartic proteases.

Two glutamic proteases have been identified in *Trichoderma* fungal cells: gap1 (tre69555) and gap2 (tre106661).

Examples of suitable gap proteases include, without limitation, *Trichoderma reesei* gap1 (SEQ ID NO: 32), *Trichoderma reesei* gap2 (SEQ ID NO: 33), and homologs thereof. Examples of homologs of gap proteases identified in other organisms are described in PCT/EP/2013/050186.

Sedolisin Proteases and homologs of proteases

Sedolisin proteases are enzymes that use a serine residue for hydrolysis of the peptide bonds in polypeptides and proteins. Sedolisin proteases generally contain a unique catalytic triad of serine, glutamate, and aspartate. Sedolisin proteases also contain an aspartate residue in the oxyanion hole. Sedolisin proteases from eukaryotic organisms such as *Trichoderma* fungi include tripeptidyl peptidase.

Examples of suitable tpp1 proteases include, without limitation, *Trichoderma reesei* tpp1 tre82623 (SEQ ID NO: 34) and homologs thereof. Examples of homologs of tpp1 proteases identified in other organisms are described in PCT/EP/2013/050186.

As used in reference to protease, the term “homolog” refers to a protein which has protease activity and exhibit sequence similarity with a known (reference) protease sequence. Homologs may be identified by any method known in the art, preferably, by using the BLAST tool to compare a reference sequence to a single second sequence or fragment of a sequence or to a database of sequences. As described in the “Definitions” section, BLAST will compare sequences based upon percent identity and similarity.

Preferably, a homologous protease has at least 30% identity with (optionally 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or 100% identity over a specified region, or, when not specified, over the entire sequence), when compared to one of the protease sequences listed above, including *T. reesei pep1, pep2, pep3, pep4, pep5, pep7, pep8, pep9,*

pep11, pep12, tsp1, slp1, slp2, slp3, slp5, slp6, slp7, slp8, tpp1, gap1 and *gap2*. Corresponding homologous proteases from *N. crassa* and *M. thermophila* are shown in SEQ ID NO: 136-169.

Reducing the Activity of Proteases in the filamentous fungal cell of the invention

The filamentous fungal cells according to the invention have reduced activity of at least one endogenous protease, typically 2, 3, 4, 5 or more, in order to improve the stability and production of the protein with increased N-glycosylation site occupancy in said filamentous fungal cell, preferably in a *Trichoderma* cell.

Total protease activity can be measured according to standard methods in the art and, for example, as described herein using protease assay kit (QuantiCleave protease assay kit, Pierce #23263) with succinylated casein as substrate.

The activity of proteases found in filamentous fungal cells can be reduced by any method known to those of skill in the art. In some embodiments reduced activity of proteases is achieved by reducing the expression of the protease, for example, by promoter modification or RNAi.

In further embodiments, the reduced or eliminated expression of the proteases is the result of anti-sense polynucleotides or RNAi constructs that are specific for each of the genes encoding each of the proteases. In one embodiment, an RNAi construct is specific for a gene encoding an aspartic protease such as a pep-type protease, a trypsin-like serine proteases such as a tsp1, a glutamic protease such as a gap-type protease, a subtilisin protease such as a slp-type protease, or a sedolisin protease such as a tpp1 or a slp7 protease. In one embodiment, an RNAi construct is specific for the gene encoding a slp-type protease. In one embodiment, an RNAi construct is specific for the gene encoding slp2, slp3, slp5 or slp6. In one embodiment, an RNAi construct is specific for two or more proteases. In one embodiment, two or more proteases are any one of the pep-type proteases, any one of the trypsin-like serine proteasess, any one of the slp-type proteases, any one of the gap-type proteases and/or any one of the sedolisin proteases. In one embodiment, two or more proteases are slp2, slp3, slp5 and/or slp6. In one embodiment, RNAi construct comprises any one of the following nucleic acid sequences (see also PCT/EP/2013/050186).

RNAi Target sequence
GCACACTTTCAAGATTGGC (SEQ ID NO: 15)
GTACGGTGTTGCCAAGAAG (SEQ ID NO: 16)
GTTGAGTACATCGAGCGCGACAGCATTGTGCACACCATGCTTCCCCTCGAGTC CAAGGACAGCATCATCGTTGAGGACTCGTGCAACGGCGAGACGGAGAAGCAG GCTCCCTGGGGTCTTGCCCGTATCTCTCACCGAGAGACGCTCAACTTTGGCTC CTTCAACAAGTACCTCTACACCGCTGATGGTGGTGAGGGTGTTGATGCCTATGT CATTGACACCGGCACCAACATCGAGCACGTCGACTTTGAGGGTCGTGCCAAGT

GGGGCAAGACCATCCCTGCCGGCGATGAGGACGAGGACGGCAACGGCCACG
GCACTCACTGCTCTGGTACCGTTGCTGGTAAGAAGTACGGTGTTGCCAAGAAG
GCCCACGTCTACGCCGTCAAGGTGCTCCGATCCAACGGATCCGGCACCATGTC
TGACGTCGTCAAGGGCGTCGAGTACG (SEQ ID NO:17)

In other embodiments, reduced activity of proteases is achieved by modifying the gene encoding the protease. Examples of such modifications include, without limitation, a mutation, such as a deletion or disruption of the gene encoding said endogenous protease activity.

Accordingly, the invention relates to a filamentous fungal cell, such as a *Trichoderma* cell, which has a mutation that reduces or eliminates at least one endogenous protease activity compared to a parental filamentous fungal cell which does not have such protease deficient mutation, said filamentous fungal cell further comprising a polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase from *Leishmania*.

Deletion or disruption mutation includes without limitation knock-out mutation, a truncation mutation, a point mutation, a missense mutation, a substitution mutation, a frameshift mutation, an insertion mutation, a duplication mutation, an amplification mutation, a translocation mutation, or an inversion mutation, and that results in a reduction in the corresponding protease activity. Methods of generating at least one mutation in a protease encoding gene of interest are well known in the art and include, without limitation, random mutagenesis and screening, site-directed mutagenesis, PCR mutagenesis, insertional mutagenesis, chemical mutagenesis, and irradiation.

In certain embodiments, a portion of the protease encoding gene is modified, such as the region encoding the catalytic domain, the coding region, or a control sequence required for expression of the coding region. Such a control sequence of the gene may be a promoter sequence or a functional part thereof, i.e., a part that is sufficient for affecting expression of the gene. For example, a promoter sequence may be inactivated resulting in no expression or a weaker promoter may be substituted for the native promoter sequence to reduce expression of the coding sequence. Other control sequences for possible modification include, without limitation, a leader sequence, a propeptide sequence, a signal sequence, a transcription terminator, and a transcriptional activator.

Protease encoding genes that are present in filamentous fungal cells may also be modified by utilizing gene deletion techniques to eliminate or reduce expression of the gene. Gene deletion techniques enable the partial or complete removal of the gene thereby eliminating their expression. In such methods, deletion of the gene may be accomplished by homologous recombination using a plasmid that has been constructed to contiguously contain the 5' and 3' regions flanking the gene.

The protease encoding genes that are present in filamentous fungal cells may also be modified by introducing, substituting, and/or removing one or more nucleotides in the gene, or a control sequence thereof required for the transcription or translation of the gene. For example, nucleotides may be inserted or removed for the introduction of a stop codon, the removal of the start codon, or a frame-shift of the open reading frame. Such a modification may be accomplished by methods known in the art, including without limitation, site-directed mutagenesis and peR generated mutagenesis (see, for example, Botstein and Shortie, 1985, Science 229: 4719; Lo et al., 1985, Proceedings of the National Academy of Sciences USA 81: 2285; Higuchi et al., 1988, Nucleic Acids Research 16: 7351; Shimada, 1996, Meth. Mol. Bioi. 57: 157; Ho et al., 1989, Gene 77: 61; Horton et al., 1989, Gene 77: 61; and Sarkar and Sommer, 1990, BioTechniques 8: 404).

Additionally, protease encoding genes that are present in filamentous fungal cells may be modified by gene disruption techniques by inserting into the gene a disruptive nucleic acid construct containing a nucleic acid fragment homologous to the gene that will create a duplication of the region of homology and incorporate construct nucleic acid between the duplicated regions. Such a gene disruption can eliminate gene expression if the inserted construct separates the promoter of the gene from the coding region or interrupts the coding sequence such that a nonfunctional gene product results. A disrupting construct may be simply a selectable marker gene accompanied by 5' and 3' regions homologous to the gene. The selectable marker enables identification of transformants containing the disrupted gene.

Protease encoding genes that are present in filamentous fungal cells may also be modified by the process of gene conversion (see, for example, Iglesias and Trautner, 1983, Molecular General Genetics 189:5 73-76). For example, in the gene conversion a nucleotide sequence corresponding to the gene is mutagenized in vitro to produce a defective nucleotide sequence, which is then transformed into a *Trichoderma* strain to produce a defective gene. By homologous recombination, the defective nucleotide sequence replaces the endogenous gene. It may be desirable that the defective nucleotide sequence also contains a marker for selection of transformants containing the defective gene.

Protease encoding genes of the present disclosure that are present in filamentous fungal cells that express a recombinant polypeptide may also be modified by established anti-sense techniques using a nucleotide sequence complementary to the nucleotide sequence of the gene (see, for example, Parish and Stoker, 1997, FEMS Microbiology Letters 154: 151-157). In particular, expression of the gene by filamentous fungal cells may be reduced or inactivated by introducing a nucleotide sequence complementary to the nucleotide sequence of the gene, which may be transcribed in the strain and is capable of hybridizing to the mRNA produced in the cells. Under

conditions allowing the complementary anti-sense nucleotide sequence to hybridize to the mRNA, the amount of protein translated is thus reduced or eliminated.

Protease encoding genes that are present in filamentous fungal cells may also be modified by random or specific mutagenesis using methods well known in the art, including without limitation, chemical mutagenesis (see, for example, Hopwood, The Isolation of Mutants in Methods in Microbiology (J.R. Norris and D.W. Ribbons, eds.) pp. 363-433, Academic Press, New York, 25 1970). Modification of the gene may be performed by subjecting filamentous fungal cells to mutagenesis and screening for mutant cells in which expression of the gene has been reduced or inactivated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, use of a suitable oligonucleotide, subjecting the DNA sequence to peR generated mutagenesis, or any combination thereof. Examples of physical and chemical mutagenizing agents include, without limitation, ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N'-nitrosogaunidine (NTG) O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the filamentous fungal cells, such as *Trichoderma* cells, to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and then selecting for mutants exhibiting reduced or no expression of the gene.

In certain embodiments, the at least one mutation or modification in a protease encoding gene of the present disclosure results in a modified protease that has no detectable protease activity. In other embodiments, the at least one modification in a protease encoding gene of the present disclosure results in a modified protease that has at least 25% less, at least 50% less, at least 75% less, at least 90%, at least 95%, or a higher percentage less protease activity compared to a corresponding non-modified protease.

The filamentous fungal cells or *Trichoderma* fungal cells of the present disclosure may have reduced or no detectable protease activity of at least three, or at least four proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep8, pep9, pep11, pep12, tsp1, slp1, slp2, slp3, slp5, slp6, slp7, gap1 and gap2. In preferred embodiment, a filamentous fungal cell according to the invention is a filamentous fungal cell which has a deletion or disruption in at least 3 or 4 endogenous proteases, resulting in no detectable activity for such deleted or disrupted endogenous proteases and further comprising a polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase from *Leishmania*.

In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in pep1, tsp1, and slp1. In other embodiments, the filamentous fungal

cell or *Trichoderma* cell, has reduced or no detectable protease activity in gap1, slp1, and pep1. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1 and gap1. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1 and pep4. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4 and slp1. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4, slp1, and slp3. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4, slp1, slp3, and pep3. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4, slp1, slp3, pep3 and pep2. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4, slp1, slp3, pep3, pep2 and pep5. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4, slp1, slp3, pep3, pep2, pep5 and tsp1. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4, slp1, slp3, pep3, pep2, pep5, tsp1 and slp7. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4, slp1, slp3, pep3, pep2, pep5, tsp1, slp7 and slp8. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4, slp1, slp3, pep3, pep2, pep5, tsp1, slp7, slp8 and gap2. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in at least three endogenous proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep8, pep9, pep11, pep12, tsp1, slp2, slp3, slp7, gap1 and gap2. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in at least three to six endogenous proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, tsp1, slp1, slp2, slp3, gap1 and gap2. In certain embodiments, the filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in at least seven to ten endogenous proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep7, pep8, tsp1, slp1, slp2, slp3, slp5, slp6, slp7, slp8, tpp1, gap1 and gap2.

Expression of heterologous catalytic subunits of oligosaccharyl transferase in filamentous fungal cells

As used herein, the expression “oligosaccharyl transferase” or OST refers to the enzymatic complex that transfers a 14-sugar oligosaccharide from dolichol to nascent protein. It is a type of glycosyltransferase. The sugar Glc3Man9GlcNAc2 is attached to an asparagine (Asn) residue in

the sequence Asn-X-Ser or Asn-X-Thr where X is any amino acid except proline. This sequence is called a glycosylation sequon. The reaction catalyzed by OST is the central step in the N-linked glycosylation pathway.

In most eukaryotes, OST is a hetero-oligomeric complex composed of eight different proteins, in which the STT3 component is believed to be the catalytic subunit.

According to the present invention, the heterologous catalytic subunit of oligosaccharyl transferase is selected from *Leishmania* oligosaccharyl transferase catalytic subunits. There are four STT3 paralogues in the parasitic protozoa *Leishmania,* named STT3A, STT3B, STT3C and STT3D.

In one embodiment, the heterologous catalytic subunit of oligosaccharyl transferase is STT3D from *Leishmania major* (having the amino acid sequence as set forth in SEQ ID No:1).

In another embodiment, the heterologous catalytic subunit of oligosaccharyl transferase is STT3D from *Leishmania infantum* (having the amino acid sequence as set forth in SEQ ID No:8).

In another embodiment, the heterologous catalytic subunit of oligosaccharyl transferase is STT3D from *Leishmania braziliensis* (having the amino acid sequence as set forth in SEQ ID No:89).

In another embodiment, the heterologous catalytic subunit of oligosaccharyl transferase is STT3D from *Leishmania mexicana* (having the amino acid sequence as set forth in SEQ ID No:91).

In yet another embodiment, the heterologous catalytic subunit of oligosaccharyl transferase is a functional variant polypeptide having at least 50%, preferably at least 60%, even more preferably at least 70%, 80%, 90%, 95% identity with SEQ ID NO:1, SEQ ID NO:8, SEQ ID NO: 89 or SEQ ID NO: 91.

In yet another embodiment, the heterologous catalytic subunit of oligosaccharyl transferase is a functional variant polypeptide having at least 50%, preferably at least 60%, even more preferably at least 70%, 80%, 90%, 95% identity with SEQ ID NO:1 or SEQ ID NO:8.

In one embodiment of the invention, the polynucleotide encoding heterologous catalytic subunit of oligosaccharyl transferase comprises SEQ ID NO:2.

SEQ ID NO:2 is a codon-optimized version of the STT3D gene from *L major* (gi389594572|XM_003722461.1).

In one embodiment of the invention, the polynucleotide encoding heterologous catalytic subunit of oligosaccharyl transferase comprises SEQ ID NO:9.

SEQ ID NO:9 is a codon-optimized version of the STT3D gene from *L major* (gi339899220|XM_003392747.1|).

In one embodiment of the invention, the polynucleotide encoding heterologous catalytic subunit of oligosaccharyl transferase comprises SEQ ID NO:88 or a variant or SEQ ID NO: 88 which has been codon-optimized for expression in filamentous fungal cells such as Trichoderma reesei.

In one embodiment of the invention, the polynucleotide encoding heterologous catalytic subunit of oligosaccharyl transferase comprises SEQ ID NO:90 or a variant or SEQ ID NO: 90 which has been codon-optimized for expression in filamentous fungal cells such as Trichoderma reesei.

In one embodiment of the invention, the polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase comprises a polynucleotide encoding a functional variant polypeptide of STT3D from *Leishmania major, Leishmania infantum, Leishmania braziliens or Leishmania mexicana* having at least 50%, preferably at least 60%, even more preferably at least 70%, 80%, 90%, 95% identity with SEQ ID NO:1, SEQ ID NO:8, SEQ ID NO: 89 or SEQ ID NO: 91.

In one embodiment of the invention, the polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase comprises a polynucleotide encoding a functional variant polypeptide of STT3D from *Leishmania major* or *Leishmania infantum* having at least 50%, preferably at least 60%, even more preferably at least 70%, 80%, 90%, 95% identity with SEQ ID NO:1 or SEQ ID NO:8.

In one embodiment of the invention, the polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase is under the control of a promoter for the constitutive expression of said oligosaccharyl transferase is said filamentous fungal cell.

Promoters that may be used for expression of the oligosaccharyl transferase include constitutive promoters such as gpd or cDNA1, promoters of endogenous glycosylation enzymes and glycosyltransferases such as mannosyltransferases that synthesize N-glycans in the Golgi or ER, and inducible promoters of high-yield endogenous proteins such as the cbh1 promoter.

In one embodiment of the invention, said promoter is the cDNA1 promoter from *Trichoderma reesei*.

Increasing N-glycosylation site occupancy in filamentous fungal cell of the invention

The filamentous fungal cells according to the invention have increased oligosaccharide transferase activity, in order to increase N-glycosylation site occupancy.

The N-glycosylation site occupancy can be measured by standard methods in the art (for example, Schulz and Aebi (2009) Analysis of Glycosylation Site Occupancy Reveals a Role for Ost3p and Ost6p in Site-specific N-Glycosylation Efficiency, Molecular & Cellular Proteomics, 8:357–364, or Millward *et al.* (2008), Effect of constant and variable domain glycosylation on pharmacokinetics of therapeutic antibodies in mice, Biologicals, 36:41-47, Forno *et al.* (2004) N- and O-linked carbohydrates and glycosylation site occupancy in recombinant human granulocyte-macrophage colony-stimulating factor secreted by a Chinese hamster ovary cell line, Eur. J. Biochem. 271: 907–919) or methods as described herein in the Examples.

The N-glycosylation site occupancy refers to the molar percentage (or mol%) of the heterologous glycoproteins that are N-glycosylated with respect to the total number of heterologous glycoprotein produced by the filamentous fungal cell (as described in Example 1 below).

In one embodiment of the invention, the N-glycosylation site occupancy is at least 95%, and Man3, Man5, G0, G1 and/or G2 glycoforms represent at least 50% of total neutral N-glycans of the heterologous glycoprotein.

The percentage of various glycoforms with respect to the total neutral N-glycans of the heterologous glycoprotein can be measured for example as described in WO2012069593.

In an embodiment, the heterologous protein with increased N-glycosylation site occupancy is selected from the group consisting of:

a) an immunoglobulin, such as IgG,
b) a light chain or heavy chain of an immunoglobulin,
c) a heavy chain or a light chain of an antibody,
d) a single chain antibody,
e) a camelid antibody,
f) a monomeric or multimeric single domain antibody,
g) a FAb-fragment, a FAb2-fragment, and,
h) their antigen-binding fragments.

Methods for producing glycoproteins with increased N-glycosylation site occupancy and mammalian-like N-glycans

The filamentous fungal cells according to the present invention may be useful in particular for producing heterologous glycoprotein composition, such as antibody composition, with increased N-glycosylation site occupancy and mammalian-like N-glycans, such as complex N-glycans.

Accordingly, in one aspect, the filamentous fungal cell is further genetically modified to produce a mammalian-like N-glycan, thereby enabling *in vivo* production of glycoprotein or antibody composition with increased N-glycosylation site occupancy and with mammalian-like N-glycan as major glycoforms of said glycoprotein or antibody.

In certain embodiments, this aspect includes methods of producing glycoproteins or antibodies with mammalian-like N-glycans in a *Trichoderma* cell.

In certain embodiment, the glycoprotein or antibody comprises, as a major glycoform, the mammalian-like N-glycan having the formula $[\{Gal\beta4\}_xGlcNAc\beta2]_zMan\alpha3([\{Gal\beta4\}_yGlcNAc\beta2]_wMan\alpha6)Man\beta4GlcNAc\beta[Fuc\alpha6]_aGlcNAc$, where () defines a branch in the structure, where [ ] or { } define a part of the glycan structure either present or absent in a linear sequence, and where a, x, y, z and w are 0 or 1, independently. In an embodiment w and z are 1, and x and y are 0 for a non-galactosylated G0 structure; both x and y are 1 for a G2 structure; and only either one of x or y is 1 for a G1 structure. When a is 1, the structure is core fucosylated such as a FG0, FG1 or FG2 glycan.

In certain embodiments, the glycoprotein or antibody comprises, as a major glycoform, mammalian-like N-glycan selected from the group consisting of:

i. $Man\alpha3[Man\alpha6(Man\alpha3)Man\alpha6]Man\beta4GlcNA\beta4GlcNAc$ (Man5 glycoform);
ii. $GlcNAc\beta2Man\alpha3[Man\alpha6(Man\alpha3)Man\alpha6]Man\beta4GlcNA\beta4GlcNAc$ (GlcNAcMan5 glycoform);
iii. $Man\alpha6(Man\alpha3)Man\beta4GlcNA\beta4GlcNAc$ (Man3 glycoform);
iv. $Man\alpha6(GlcNAc\beta2Man\alpha3)Man\beta4GlcNA\beta4GlcNAc$ (GlcNAcMan3) or,
v. complex type N-glycans selected from the G0, G1, or G2 glycoform.

In an embodiment, the glycoprotein or antibody composition with mammalian-like N-glycans, preferably produced by an alg3 knock-out strain, include glycoforms that essentially lack or are devoid of glycans $Man\alpha3[Man\alpha6(Man\alpha3)Man\alpha6]Man\beta4GlcNA\beta4GlcNAc$ (Man5). In specific embodiments, the filamentous fungal cell produces heterologous glycoproteins or antibodies with, as major glycoform, the trimannosyl N-glycan structure $Man\alpha3[Man\alpha6]Man\beta4GlcNAc\beta4GlcNAc$. In other embodiments, the filamentous fungal cell produces glycoproteins or antibodies with, as major glycoform, the G0 N-glycan structure $GlcNAc\beta2Man\alpha3[GlcNAc\beta2Man\alpha6]Man\beta4GlcNAc\beta4GlcNAc$.

In certain embodiments, the filamentous fungal cell of the invention produces glycoprotein or antibody composition with a mixture of different N-glycans.

In some embodiments, Man3GlcNAc2 N-glycan (i.e. Manα3[Manα6]Manβ4GlcNAcβ4GlcNAc) represents at least 10%, at least 20%, at least at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more of total (mol%) neutral N-glycans of the heterologous glycoprotein or antibody, as expressed in a filamentous fungal cells of the invention.

In other embodiments, GlcNAc2Man3 N-glycan (for example G0 GlcNAcβ2Manα3[GlcNAcβ2Manα6]Manβ4GlcNAcβ4GlcNAc) represents at least 10%, at least 20%, at least at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more of total (mol%) neutral N-glycans of the heterologous glycoprotein or antibody, as expressed in a filamentous fungal cells of the invention.

In other embodiments, GalGlcNAc2Man3GlcNAc2 N-glycan (for example G1 N-glycan) represents at least 10%, at least 20%, at least at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more of total (mol%) neutral N-glycans of the heterologous glycoprotein or antibody, as expressed in a filamentous fungal cells of the invention.

In other embodiments, Gal2GlcNAc2Man3GlcNAc2 N-glycan (for example G2 N-glycan) represents at least 10%, at least 20%, at least at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more of total (mol%) neutral N-glycans of the heterologous glycoprotein or antibody, as expressed in a filamentous fungal cells of the invention.

In other embodiments, complex type N-glycan represents at least 10%, at least 20%, at least at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more of total (mol%) neutral N-glycans of a heterologous glycoprotein or antibody, as expressed in a filamentous fungal cells of the invention.

In other embodiments, hybrid type N-glycan represents at least 10%, at least 20%, at least at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more of total (mol%) neutral N-glycans of a heterologous glycoprotein or antibody, as expressed in a filamentous fungal cells of the invention.

In other embodiments, less than 0.5%, 0.1%, 0.05%, or less than 0.01% of the N-glycan of the heterologous glycoprotein composition or antibody composition produced by the host cell of the invention, comprises galactose. In certain embodiments, none of N-glycans comprise galactose.

The Neu5Gc and Galα- (non-reducing end terminal Galα3Galβ4GlcNAc) structures are known xenoantigenic (animal derived) modifications of antibodies which are produced in animal cells such as CHO cells. The structures may be antigenic and, thus, harmful even at low concentrations. The

filamentous fungi of the present invention lack biosynthetic pathways to produce the terminal Neu5Gc and Galα- structures. In an embodiment that may be combined with the preceding embodiments less than 0.1%, 0.01 %, 0.001 % or 0 % of the N-glycans and/or O-glycans of the glycoprotein or antibody composition comprises Neu5Gc and/or Galα- structure. In an embodiment that may be combined with the preceding embodiments, less than 0.1%, 0.01 %, 0.001 % or 0 % of the N-glycans and/or O-glycans of the heterologous glycoprotein or antibody composition comprises Neu5Gc and/or Galα- structure.

The filamentous fungal cells of the present invention lack genes to produce fucosylated heterologous proteins. In an embodiment that may be combined with the preceding embodiments, less than 0.1%, 0.01 %, 0.001 %, or 0 % of the N-glycan of the glycoprotein or antibody composition comprises core fucose structures.

The terminal Galβ4GlcNAc structure of N-glycan of mammalian cell produced glycans affects bioactivity of antibodies and Galβ3GlcNAc may be xenoantigen structure from plant cell produced proteins. In an embodiment that may be combined with one or more of the preceding embodiments, less than 0.1%, 0.01 %, 0.001 %, or 0 % of N-glycan of the heterologous glycoprotein or antibody composition comprises terminal galactose epitopes Galβ3/4GlcNAc.

Glycation is a common post-translational modification of proteins, resulting from the chemical reaction between reducing sugars such as glucose and the primary amino groups on protein. Glycation occurs typically in neutral or slightly alkaline pH in cell cultures conditions, for example, when producing antibodies in CHO cells and analysing them (see, for example, Zhang et al. (2008) Unveiling a glycation hot spot in a recombinant humanized monoclonal antibody. Anal Chem. 80(7):2379-2390). As filamentous fungi of the present invention are typically cultured in acidic pH, occurrence of glycation is reduced. In an embodiment that may be combined with the preceding embodiments, less than 1.0%, 0.5%, 0.1%, 0.01 %, 0.001 %, or 0 % of the heterologous glycoprotein or antibody composition comprises glycation structures.

In one embodiment, the glycoprotein composition, such as an antibody is devoid of one, two, three, four, five, or six of the structures selected from the group of Neu5Gc, terminal Galα3Galβ4GlcNAc, terminal Galβ4GlcNAc, terminal Galβ3GlcNAc, core linked fucose and glycation structures.

In certain embodiments, such glycoprotein protein with mammalian-like N-glycan, as produced in the filamentous fungal cell of the invention, is a therapeutic protein. Therapeutic proteins may include immunoglobulin, or a protein fusion comprising a Fc fragment or other therapeutic

glycoproteins, such as antibodies, erythropoietins, interferons, growth hormones, albumins or serum albumin, enzymes, or blood-clotting factors and may be useful in the treatment of humans or animals. For example, the glycoproteins with mammalian-like N-glycan as produced by the filamentous fungal cell according to the invention may be a therapeutic glycoprotein such as rituximab.

Methods for producing glycoproteins with mammalian-like N-glycans in filamentous fungal cells are also described for example in WO2012/069593.

In one aspect, the filamentous fungal cell according to the invention as described above, is further genetically modified to mimick the traditional pathway of mammalian cells, starting from Man5 N-glycans as acceptor substrate for GnTI, and followed sequentially by GnT1, mannosidase II and GnTII reaction steps (hereafter referred as the "traditional pathway" for producing G0 glycoforms). In one variant, a single recombinant enzyme comprising the catalytic domains of GnTI and GnTII, is used.

Alternatively, in a second aspect, the filamentous fungal cell according to the invention as described above is further genetically modified to have *alg3* reduced expression, allowing the production of core $Man_3GlcNAc_2$ N-glycans, as acceptor substrate for GnTI and GnTII subsequent reactions and bypassing the need for mannosidase α1,2 or mannosidase II enzymes (the reduced "*alg3*" pathway). In one variant, a single recombinant enzyme comprising the catalytic domains of GnTI and GnTII, is used.

In such embodiments for mimicking the traditional pathway for producing glycoproteins with mammalian-like N-glycans, a Man_5 expressing filamentous fungal cell, such as *T. reesei* strain, may be transformed with a GnTI or a GnTII/GnTI fusion enzyme using random integration or by targeted integration to a known site known not to affect Man5 glycosylation. Strains that synthesise GlcNAcMan5 N-glycan for production of proteins having hybrid type glycan(s) are selected. The selected strains are further transformed with a catalytic domain of a mannosidase II-type mannosidase capable of cleaving Man5 structures to generate GlcNAcMan3 for production of proteins having the corresponding GlcNAcMan3 glycoform or their derivative(s). In certain embodiments, mannosidase II-type enzymes belong to glycoside hydrolase family 38 (cazy.org/GH38_all.html). Characterized enzymes include enzymes listed in cazy.org/GH38_characterized.html. Especially useful enzymes are Golgi-type enzymes that cleaving glycoproteins, such as those of subfamily α-mannosidase II (Man2A1;ManA2). Examples of such enzymes include human enzyme AAC50302, D. melanogaster enzyme (Van den Elsen J.M. et al (2001) EMBO J. 20: 3008-3017), those with the 3D structure according to PDB-reference 1HTY, and others referenced with the catalytic domain in PDB. For cytoplasmic expression, the

catalytic domain of the mannosidase is typically fused with an N-terminal targeting peptide (for example as disclosed in the above Section) or expressed with endogenous animal or plant Golgi targeting structures of animal or plant mannosidase II enzymes. After transformation with the catalytic domain of a mannosidase II-type mannosidase, strains are selected that produce GlcNAcMan3 (if GnTI is expressed) or strains are selected that effectively produce GlcNAc2Man3 (if a fusion of GnTI and GnTII is expressed). For strains producing GlcNAcMan3, such strains are further transformed with a polynucleotide encoding a catalytic domain of GnTII and transformant strains that are capable of producing GlcNAc2Man3GlcNAc2 are selected.

In such embodiment for mimicking the traditional pathway, the filamentous fungal cell is a filamentous fungal cell as defined in previous sections, and further comprising one or more polynucleotides encoding a polypeptide selected from the group consisting of:

i) α1,2 mannosidase,
ii) N-acetylglucosaminyltransferase I catalytic domain,
iii) α mannosidase II,
iv) N-acetylglucosaminyltransferase II catalytic domain,
v) β1,4 galactosyltransferase, and,
vi) fucosyltransferase.

In embodiments using the reduced *alg3* pathway, the filamentous fungal cell, such as a *Trichoderma* cell, has a reduced level of activity of a dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase compared to the level of activity in a parent host cell. Dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase (EC 2.4.1.130) transfers an alpha-D-mannosyl residue from dolichyl-phosphate D-mannose into a membrane lipid-linked oligosaccharide. Typically, the dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase enzyme is encoded by an *alg3* gene. In certain embodiments, the filamentous fungal cell for producing glycoproteins with mammalian-like N-glycans has a reduced level of expression of an *alg3* gene compared to the level of expression in a parent strain.

More preferably, the filamentous fungal cell comprises a mutation of *alg3*. The ALG3 gene may be mutated by any means known in the art, such as point mutations or deletion of the entire alg3 gene. For example, the function of the alg3 protein is reduced or eliminated by the mutation of *alg3*. In certain embodiments, the *alg3* gene is disrupted or deleted from the filamentous fungal cell, such as *Trichoderma* cell. In certain embodiments, the filamentous fungal cell is a *T. reesei* cell. SEQ ID NOs: 36 and 37 provide, the nucleic acid and amino acid sequences of the *alg3* gene in *T. reesei*, respectively. In an embodiment the filamentous fungal cell is used for the production of

a glycoprotein, wherein the glycan(s) comprise or consist of Manα3[Manα6]Manβ4GlcNAcβ4GlcNAc, and/or a non-reducing end elongated variant thereof.

In certain embodiments, the filamentous fungal cell has a reduced level of activity of an alpha-1,6-mannosyltransferase compared to the level of activity in a parent strain. Alpha-1,6-mannosyltransferase (EC 2.4.1.232) transfers an alpha-D-mannosyl residue from GDP-mannose into a protein-linked oligosaccharide, forming an elongation initiating alpha-(1->6)-D-mannosyl-D-mannose linkage in the Golgi apparatus. Typically, the alpha-1,6-mannosyltransferase enzyme is encoded by an *och1* gene. In certain embodiments, the filamentous fungal cell has a reduced level of expression of an *och1* gene compared to the level of expression in a parent filamentous fungal cell. In certain embodiments, the *och1* gene is deleted from the filamentous fungal cell.

The filamentous fungal cells used in the methods of producing glycoprotein with mammalian-like N-glycans may further contain a polynucleotide encoding an N-acetylglucosaminyltransferase I catalytic domain (GnTI) that catalyzes the transfer of N-acetylglucosamine to a terminal Manα3 and a polynucleotide encoding an N-acetylglucosaminyltransferase II catalytic domain (GnTII), that catalyses N-acetylglucosamine to a terminal Manα6 residue of an acceptor glycan to produce a complex N-glycan. In one embodiment, said polynucleotides encoding GnTI and GnTII are linked so as to produce a single protein fusion comprising both catalytic domains of GnTI and GnTII.

As disclosed herein, N-acetylglucosaminyltransferase I (GlcNAc-TI; GnTI; EC 2.4.1.101) catalyzes the reaction UDP-N-acetyl-D-glucosamine + 3-(alpha-D-mannosyl)-beta-D-mannosyl-R <=> UDP + 3-(2-(N-acetyl-beta-D-glucosaminyl)-alpha-D-mannosyl)-beta-D-mannosyl-R, where R represents the remainder of the N-linked oligosaccharide in the glycan acceptor. An N-acetylglucosaminyltransferase I catalytic domain is any portion of an N-acetylglucosaminyltransferase I enzyme that is capable of catalyzing this reaction. GnTI enzymes are listed in the CAZy database in the glycosyltransferase family 13 (cazy.org/GT13_all). Enzymatically characterized species includes *A. thaliana* AAR78757.1 (US6 653 459), *C. elegans* AAD03023.1 (Chen S. et al J. Biol.Chem 1999;274(1):288-97), *D. melanogaster* AAF57454.1 (Sarkar & Schachter Biol Chem. 2001 Feb;382(2):209-17); *C. griseus* AAC52872.1 (Puthalakath H. et al J. Biol.Chem 1996 271(44):27818-22); *H. sapiens* AAA52563.1 (Kumar R. et al Proc Natl Acad Sci U S A. 1990 Dec;87(24):9948-52); *M. auratus* AAD04130.1 (Opat As et al Biochem J. 1998 Dec 15;336 (Pt 3):593-8), (including an example of deactivating mutant), Rabbit, *O. cuniculus* AAA31493.1 (Sarkar M et al. Proc Natl Acad Sci U S A. 1991 Jan 1;88(1):234-8). Amino acid sequences for N-acetylglucosaminyltransferase I enzymes from various organisms are described for example in PCT/EP2011/070956. Additional examples of characterized active enzymes can be found at cazy.org/GT13_characterized. The 3D structure of the catalytic domain of rabbit GnTI

was defined by X-ray crystallography in Unligil UM et al. EMBO J. 2000 Oct 16;19(20):5269-80. The Protein Data Bank (PDB) structures for GnTI are 1FO8, 1FO9, 1FOA, 2AM3, 2AM4, 2AM5, and 2APC. In certain embodiments, the N-acetylglucosaminyltransferase I catalytic domain is from the human N-acetylglucosaminyltransferase I enzyme (SEQ ID NO: 38) or variants thereof. In certain embodiments, the N-acetylglucosaminyltransferase I catalytic domain contains a sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to amino acid residues 84-445 of SEQ ID NO: 38. In some embodiments, a shorter sequence can be used as a catalytic domain (e.g. amino acid residues 105-445 of the human enzyme or amino acid residues 107-447 of the rabbit enzyme; Sarkar et al. (1998) Glycoconjugate J 15:193-197). Additional sequences that can be used as the GnTI catalytic domain include amino acid residues from about amino acid 30 to 445 of the human enzyme or any C-terminal stem domain starting between amino acid residue 30 to 105 and continuing to about amino acid 445 of the human enzyme, or corresponding homologous sequence of another GnTI or a catalytically active variant or mutant thereof. The catalytic domain may include N-terminal parts of the enzyme such as all or part of the stem domain, the transmembrane domain, or the cytoplasmic domain.

As disclosed herein, N-acetylglucosaminyltransferase II (GlcNAc-TII; GnTII; EC 2.4.1.143) catalyzes the reaction UDP-N-acetyl-D-glucosamine + 6-(alpha-D-mannosyl)-beta-D-mannosyl-R <=> UDP + 6-(2-(N-acetyl-beta-D-glucosaminyl)-alpha-D-mannosyl)-beta-D-mannosyl-R, where R represents the remainder of the N-linked oligosaccharide in the glycan acceptor. An N-acetylglucosaminyltransferase II catalytic domain is any portion of an N-acetylglucosaminyltransferase II enzyme that is capable of catalyzing this reaction. Amino acid sequences for N-acetylglucosaminyltransferase II enzymes from various organisms are listed in WO2012069593. In certain embodiments, the N-acetylglucosaminyltransferase II catalytic domain is from the human N-acetylglucosaminyltransferase II enzyme (SEQ ID NO: 39) or variants thereof. Additional GnTII species are listed in the CAZy database in the glycosyltransferase family 16 (cazy.org/GT16_all). Enzymatically characterized species include GnTII of *C. elegans*, *D. melanogaster*, *Homo sapiens* (NP_002399.1), *Rattus norvegicus*, *Sus scrofa* (cazy.org/GT16_characterized). In certain embodiments, the N-acetylglucosaminyltransferase II catalytic domain contains a sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to amino acid residues from about 30 to about 447 of SEQ ID NO: 39. The catalytic domain may include N-terminal parts of the enzyme such as all or part of the stem domain, the transmembrane domain, or the cytoplasmic domain.

In embodiments where the filamentous fungal cell contains a fusion protein of the invention, the fusion protein may further contain a spacer in between the N-acetylglucosaminyltransferase I catalytic domain and the N-acetylglucosaminyltransferase II catalytic domain. In certain embodiments, the spacer is an EGIV spacer, a 2xG4S spacer, a 3xG4S spacer, or a CBHI spacer. In other embodiments, the spacer contains a sequence from a stem domain.

For ER/Golgi expression the N-acetylglucosaminyltransferase I and/or N-acetylglucosaminyltransferase II catalytic domain is typically fused with a targeting peptide or a part of an ER or early Golgi protein, or expressed with an endogenous ER targeting structures of an animal or plant N-acetylglucosaminyltransferase enzyme. In certain preferred embodiments, the N-acetylglucosaminyltransferase I and/or N-acetylglucosaminyltransferase II catalytic domain contains any of the targeting peptides of the invention as described in the section entitled "Targeting sequences". Preferably, the targeting peptide is linked to the N-terminal end of the catalytic domain. In some embodiments, the targeting peptide contains any of the stem domains of the invention as described in the section entitled "Targeting sequences". In certain preferred embodiments, the targeting peptide is a Kre2/Mnt1 targeting peptide. In other embodiments, the targeting peptide further contains a transmembrane domain linked to the N-terminal end of the stem domain or a cytoplasmic domain linked to the N-terminal end of the stem domain. In embodiments where the targeting peptide further contains a transmembrane domain, the targeting peptide may further contain a cytoplasmic domain linked to the N-terminal end of the transmembrane domain.

The filamentous fungal cells may also contain a polynucleotide encoding a UDP-GlcNAc transporter. The polynucleotide encoding the UDP-GlcNAc transporter may be endogenous (i.e., naturally present) in the host cell, or it may be heterologous to the filamentous fungal cell.

In certain embodiments, the filamentous fungal cell may further contain a polynucleotide encoding a α-1,2-mannosidase. The polynucleotide encoding the α-1,2-mannosidase may be endogenous in the host cell, or it may be heterologous to the host cell. Heterologous polynucleotides are especially useful for a host cell expressing high-mannose glycans transferred from the Golgi to the ER without effective exo-α-2-mannosidase cleavage. The α-1,2-mannosidase may be a mannosidase I type enzyme belonging to the glycoside hydrolase family 47 (cazy.org/GH47_all.html). In certain embodiments the α-1,2-mannosidase is an enzyme listed at cazy.org/GH47_characterized.html. In particular, the α-1,2-mannosidase may be an ER-type enzyme that cleaves glycoproteins such as enzymes in the subfamily of ER α-mannosidase I EC 3.2.1.113 enzymes. Examples of such enzymes include human α-2-mannosidase 1B (AAC26169), a combination of mammalian ER mannosidases, or a filamentous fungal enzyme such as α-1,2-mannosidase (MDS1) (*T. reesei* AAF34579; Maras M et al J Biotech. 77, 2000, 255, or Trire

45717). For ER expression, the catalytic domain of the mannosidase is typically fused with a targeting peptide, such as HDEL, KDEL, or part of an ER or early Golgi protein, or expressed with an endogenous ER targeting structures of an animal or plant mannosidase I enzyme.

In certain embodiments, the filamentous fungal cell may also further contain a polynucleotide encoding a galactosyltransferase. Galactosyltransferases transfer β-linked galactosyl residues to terminal N-acetylglucosaminyl residue. In certain embodiments the galactosyltransferase is a β-1,4-galactosyltransferase. Generally, β–1,4-galactosyltransferases belong to the CAZy glycosyltransferase family 7 (cazy.org/GT7_all.html) and include β-N-acetylglucosaminyl-glycopeptide β-1,4-galactosyltransferase (EC 2.4.1.38), which is also known as N-acetylactosamine synthase (EC 2.4.1.90). Useful subfamilies include β4-GalT1, β4-GalT-II, -III, -IV, -V, and -VI, such as mammalian or human β4-GalTI or β4GalT-II, -III, -IV, -V, and -VI or any combinations thereof. β4-GalT1, β4-GalTII, or β4-GalTIII are especially useful for galactosylation of terminal GlcNAcβ2-structures on N-glycans such as GlcNAcMan3, GlcNAc2Man3, or GlcNAcMan5 (Guo S. et al. Glycobiology 2001, 11:813-20). The three-dimensional structure of the catalytic region is known (e.g. (2006) J.Mol.Biol. 357: 1619-1633), and the structure has been represented in the PDB database with code 2FYD. The CAZy database includes examples of certain enzymes. Characterized enzymes are also listed in the CAZy database at cazy.org/GT7_characterized.html. Examples of useful β4GalT enzymes include β4GalT1, e.g. bovine Bos taurus enzyme AAA30534.1 (Shaper N.L. et al Proc. Natl. Acad. Sci. U.S.A. 83 (6), 1573-1577 (1986)), human enzyme (Guo S. et al. Glycobiology 2001, 11:813-20), and *Mus musculus* enzyme AAA37297 (Shaper, N.L. et al. 1998 J. Biol. Chem. 263 (21), 10420-10428); β4GalTII enzymes such as human β4GalTII BAA75819.1, Chinese hamster *Cricetulus griseus* AAM77195, *Mus musculus* enzyme BAA34385, and Japanese Medaka fish *Oryzias latipes* BAH36754; and β4GalTIII enzymes such as human β4GalTIII BAA75820.1, Chinese hamster *Cricetulus griseus* AAM77196 and *Mus musculus* enzyme AAF22221.

The galactosyltransferase may be expressed in the plasma membrane of the host cell. A heterologous targeting peptide, such as a Kre2 peptide described in Schwientek J.Biol. Chem 1996 3398, may be used. Promoters that may be used for expression of the galactosyltransferase include constitutive promoters such as gpd, promoters of endogenous glycosylation enzymes and glycosyltransferases such as mannosyltransferases that synthesize N-glycans in the Golgi or ER, and inducible promoters of high-yield endogenous proteins such as the cbh1 promoter.

In certain embodiments of the invention where the filamentous fungal cell contains a polynucleotide encoding a galactosyltransferase, the filamentous fungal cell also contains a polynucleotide encoding a UDP-Gal 4 epimerase and/or UDP-Gal transporter. In certain

embodiments of the invention where the filamentous fungal cell contains a polynucleotide encoding a galactosyltransferase, lactose may be used as the carbon source instead of glucose when culturing the host cell. The culture medium may be between pH 4.5 and 7.0 or between 5.0 and 6.5. In certain embodiments of the invention where the filamentous fungal cell contains a polynucleotide encoding a galactosyltransferase and a polynucleotide encoding a UDP-Gal 4 epimerase and/or UDP-Gal transporter, a divalent cation such as Mn2+, Ca2+ or Mg2+ may be added to the cell culture medium.

Accordingly, in certain embodiments, the filamentous fungal cell of the invention, for example, selected among *Neurospora*, *Trichoderma*, *Myceliophthora, Aspergillus, Fusarium* or *Chrysosporium* cell, and more preferably *Trichoderma reesei* cell, may comprise the following features:

a) a mutation in at least one endogenous protease that reduces or eliminates the activity of said endogenous protease, preferably the protease activity of two or three or more endogenous proteases is reduced, for example, pep1, tsp1, gap1 and/or slp1 proteases, in order to improve production or stability of a heterologous glycoprotein to be produced,

b) a polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase, preferably of SEQ ID NO:2 or NO:9,

c) a polynucleotide encoding a glycoprotein having at least one asparagine, preferably a heterologous glycoprotein, such as an immunoglobulin, an antibody, or a protein fusion comprising Fc fragment of an immunoglobulin.

d) optionally, a deletion or disruption of the alg3 gene,

e) optionally, a polynucleotide encoding N-acetylglucosaminyltransferase I catalytic domain and a polynucleotide encoding N-acetylglucosaminyltransferase II catalytic domain,

f) optionally, a polynucleotide encoding β1,4 galactosyltransferase,

g) optionally, a polynucleotide or polynucleotides encoding UDP-Gal 4 epimerase and/or transporter.

Targeting Sequences

In certain embodiments, recombinant enzymes, such as α1,2 mannosidases, GnTI, or other glycosyltransferases introduced into the filamentous fungal cells, include a targeting peptide linked to the catalytic domains. The term “linked” as used herein means that two polymers of amino acid residues in the case of a polypeptide or two polymers of nucleotides in the case of a polynucleotide are either coupled directly adjacent to each other or are within the same polypeptide or polynucleotide but are separated by intervening amino acid residues or nucleotides. A “targeting peptide”, as used herein, refers to any number of consecutive amino acid residues of the

recombinant protein that are capable of localizing the recombinant protein to the endoplasmic reticulum (ER) or Golgi apparatus (Golgi) within the host cell. The targeting peptide may be N-terminal or C-terminal to the catalytic domains. In certain embodiments, the targeting peptide is N-terminal to the catalytic domains. In certain embodiments, the targeting peptide provides binding to an ER or Golgi component, such as to a mannosidase II enzyme. In other embodiments, the targeting peptide provides direct binding to the ER or Golgi membrane.

Components of the targeting peptide may come from any enzyme that normally resides in the ER or Golgi apparatus. Such enzymes include mannosidases, mannosyltransferases, glycosyltransferases, Type 2 Golgi proteins, and MNN2, MNN4, MNN6, MNN9, MNN10, MNS1, KRE2, VAN1, and OCH1 enzymes. Such enzymes may come from a yeast or fungal species such as those of *Acremonium, Aspergillus, Aureobasidium, Cryptococcus, Chrysosporium, Chrysosporium lucknowense, Filobasidium, Fusarium, Gibberella, Humicola, Magnaporthe, Mucor, Myceliophthora, Myrothecium, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Piromyces, Schizophyllum, Talaromyces, Thermoascus, Thielavia, Tolypocladium,* and *Trichoderma*. Sequences for such enzymes can be found in the GenBank sequence database.

In certain embodiments the targeting peptide comes from the same enzyme and organism as one of the catalytic domains of the recombinant protein. For example, if the recombinant protein includes a human GnTII catalytic domain, the targeting peptide of the recombinant protein is from the human GnTII enzyme. In other embodiments, the targeting peptide may come from a different enzyme and/or organism as the catalytic domains of the recombinant protein.

Examples of various targeting peptides for use in targeting proteins to the ER or Golgi that may be used for targeting the recombinant enzymes, include: Kre2/Mnt1 N-terminal peptide fused to galactosyltransferase (Schwientek, JBC 1996, 3398), HDEL for localization of mannosidase to ER of yeast cells to produce Man5 (Chiba, JBC 1998, 26298-304; Callewaert, FEBS Lett 2001, 173-178), OCH1 targeting peptide fused to GnTI catalytic domain (Yoshida et al, Glycobiology 1999, 53-8), yeast N-terminal peptide of Mns1 fused to α2-mannosidase (Martinet et al, Biotech Lett 1998, 1171), N-terminal portion of Kre2 linked to catalytic domain of GnTI or β4GalT (Vervecken, Appl. Environ Microb 2004, 2639-46), various approaches reviewed in Wildt and Gerngross (Nature Rev Biotech 2005, 119), full-length GnTI in Aspergillus nidulans (Kalsner et al, Glycocon. J 1995, 360-370), full-length GnTI in Aspergillus oryzae (Kasajima et al, Biosci Biotech Biochem 2006, 2662-8), portion of yeast Sec12 localization structure fused to C. elegans GnTI in Aspergillus (Kainz et al 2008), N-terminal portion of yeast Mnn9 fused to human GnTI in Aspergillus (Kainz et al 2008), N-terminal portion of Aspergillus Mnn10 fused to human GnTI (Kainz et al, Appl. Environ Microb 2008, 1076-86), and full-length human GnTI in *T. reesei* (Maras et al, FEBS Lett 1999, 365-70).

In certain embodiments the targeting peptide is an N-terminal portion of the Mnt1/Kre2 targeting peptide having the amino acid sequence of SEQ ID NO: 40 (for example encoded by the polynucleotide of SEQ ID NO:41). In certain embodiments, the targeting peptide is selected from human GNT2, KRE2, KRE2-like, Och1, Anp1, Van1 as shown in the Table 1 below:

Table 1: Amino acid sequence of targeting peptides

Protein	TreID	Amino acid sequence
human GNT2	-	MRFRIYKRKVLILTLVVAACGFVLWSSNGRQR KNEALAPPLLDAEPARGAGGRGGDHP (SEQ ID NO:42)
KRE2	21576	MASTNARYVRYLLIAFFTILVFYFVSNSKYEGV DLNKGTFTAPDSTKTTPK (SEQ ID NO:43)
KRE2-like	69211	MAIARPVRALGGLAAILWCFFLYQLLRPSSSY NSPGDRYINFERDPNLDPTG (SEQ ID NO:44)
Och1	65646	MLNPRRALIAAAFILTVFFLISRSHNSESASTS (SEQ ID NO:45)
Anp1	82551	MMPRHHSSGFSNGYPRADTFEISPHRFQPRA TLPPHRKRKRTAIRVGIAVVVILVLVLWFGQPR SVASLISLGILSGYDDLKLE (SEQ ID NO:46)
Van1	81211	MLLPKGGLDWRSARAQIPPTRALWNAVTRTR FILLVGITGLILLLWRGVSTSASE (SEQ ID NO:47)

Further examples of sequences that may be used for targeting peptides include the targeting sequences as described in WO2012/069593.

Uncharacterized sequences may be tested for use as targeting peptides by expressing enzymes of the glycosylation pathway in a host cell, where one of the enzymes contains the uncharacterized sequence as the sole targeting peptide, and measuring the glycans produced in view of the cytoplasmic localization of glycan biosynthesis (e.g. as in Schwientek JBC 1996 3398), or by expressing a fluorescent reporter protein fused with the targeting peptide, and analysing the localization of the protein in the Golgi by immunofluorescence or by fractionating the cytoplasmic membranes of the Golgi and measuring the location of the protein.

Methods for producing a glycoprotein having increased N-glycosylation site occupancy

The filamentous fungal cells as described above are useful in methods for producing a glycoprotein composition with increased N-glycosylation site occupancy.

Accordingly, in another aspect, the invention relates to a method for producing a glycoprotein composition with increased N-glycosylation site occupancy, comprising

a) providing a filamentous fungal cell, for example a *Trichoderma* cell, having a *Leishmania* STT3D gene encoding a catalytic subunit of oligosaccharyl transferase, or a functional variant thereof, and a polynucleotide encoding a heterologous glycoprotein,

b) culturing the cell under appropriate conditions for expression of the STT3D gene or its functional variant, and the production of the heterologous glycoprotein; and,

c) recovering said glycoprotein composition and, optionally, purifying the heterologous glycoprotein composition.

In specific embodiments of the method, the filamentous fungal cell comprises one or more mutation that reduces or eliminates one or more endogenous protease activity compared to a parental filamentous fungal cell which does not have said mutation(s), as described above.

In methods of the invention, certain growth media include, for example, common commercially-prepared media such as Luria-Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast medium (YM) broth. Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular host cell will be known by someone skilled in the art of microbiology or fermentation science. Culture medium typically has the *Trichoderma reesei* minimal medium (Penttilä et al., 1987, Gene 61, 155-164) as a basis, supplemented with substances inducing the production promoter such as lactose, cellulose, spent grain or sophorose. Temperature ranges and other conditions suitable for growth are known in the art (see, e.g., Bailey and Ollis 1986). In certain embodiments the pH of cell culture is between 3.5 and 7.5, between 4.0 and 7.0, between 4.5 and 6.5, between 5 and 5.5, or at 5.5. In certain embodiments, to produce an antibody the filamentous fungal cell or *Trichoderma* fungal cell is cultured at a pH range selected from 4.7 to 6.5; pH 4.8 to 6.0; pH 4.9 to 5.9; and pH 5.0 to 5.8.

In some embodiments of the invention, the method comprises culturing in a medium comprising one or two protease inhibitors.

In a specific embodiment of the invention, the method comprises culturing in a medium comprising one or two protease inhibitors selected from SBTI and chymostatin.

In some embodiments, the glycoprotein is a heterologous glycoprotein, preferably a mammalian glycoprotein. In other embodiments, the heterologous glycoprotein is a non-mammalian glycoprotein.

In certain embodiments, a mammalian glycoprotein is selected from an immunoglobulin, immunoglobulin or antibody heavy or light chain, a monoclonal antibody, a Fab fragment, an F(ab')2 antibody fragment, a single chain antibody, a monomeric or multimeric single domain antibody, a camelid antibody, or their antigen-binding fragments.

A fragment of a protein, as used herein, consists of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 consecutive amino acids of a reference protein.

As used herein, an "immunoglobulin" refers to a multimeric protein containing a heavy chain and a light chain covalently coupled together and capable of specifically combining with antigen. Immunoglobulin molecules are a large family of molecules that include several types of molecules such as IgM, IgD, IgG, IgA, and IgE.

As used herein, an "antibody" refers to intact immunoglobulin molecules, as well as fragments thereof which are capable of binding an antigen. These include hybrid (chimeric) antibody molecules (see, e.g., Winter et al. Nature 349:293-99225, 1991; and U.S. Pat No. 4,816,567 226); F(ab')2 molecules; non-covalent heterodimers; dimeric and trimeric antibody fragment constructs; humanized antibody molecules (see e.g., Riechmann et al. Nature 332, 323-27, 1988; Verhoeyan et al. Science 239, 1534-36, 1988; and GB 2,276,169); and any functional fragments obtained from such molecules, as well as antibodies obtained through non-conventional processes such as phage display or transgenic mice. Preferably, the antibodies are classical antibodies with Fc region. Methods of manufacturing antibodies are well known in the art.

In further embodiments, the yield of the mammalian glycoprotein, for example, the antibody, is at least 0.5, at least 1, at least 2, at least 3, at least 4, or at least 5 grams per liter.

In certain embodiments, the mammalian glycoprotein is an antibody, optionally, IgG1, IgG2, IgG3, or IgG4. In further embodiments, the yield of the antibody is at least 0.5, at least 1, at least 2, at least 3, at least 4, or at least 5 grams per liter. In further embodiments, the mammalian glycoprotein is an antibody, and the antibody contains at least 70 %, at least 80 %, at least 90 %, at least 95 %, or at least 98 % of a natural antibody C-terminus and N-terminus without additional amino acid residues. In other embodiments, the mammalian glycoprotein is an antibody, and the antibody contains at least 70 %, at least 80 %, at least 90 %, at least 95 %, or at least 98 % of a natural antibody C-terminus and N-terminus that do not lack any C-terminal or N-terminal amino acid residues.

In certain embodiments where the mammalian glycoprotein (e.g. the antibody) is purified from cell culture, the culture containing the mammalian glycoprotein contains polypeptide fragments that

make up a mass percentage that is less than 50%, less than 40%, less than 30%, less than 20%, or less than 10% of the mass of the produced polypeptides. In certain preferred embodiments, the mammalian glycoprotein is an antibody, and the polypeptide fragments are heavy chain fragments and/or light chain fragments. In other embodiments, where the mammalian glycoprotein is an antibody and the antibody purified from cell culture, the culture containing the antibody contains free heavy chains and/or free light chains that make up a mass percentage that is less than 50%, less than 40%, less than 30%, less than 20%, or less than 10% of the mass of the produced antibody. Methods of determining the mass percentage of polypeptide fragments are well known in the art and include, measuring signal intensity from an SDS-gel.

In other embodiments, the heterologous glycoprotein (e.g. the antibody) with increased N-glycosylation site occupancy, for example, the antibody, comprises the trimannosyl N-glycan structure Manα3[Manα6]Manβ4GlcNAcβ4GlcNAc. In some embodiments, the Manα3[Manα6]Manβ4GlcNAcβ4GlcNAc structure represents at least 20%, 30%; 40%, 50%; 60%, 70%, 80% (mol%) or more, of the total N-glycans of the heterologous glycoprotein (e.g. the antibody) composition obtained by the methods of the invention. In other embodiments, the heterologous glycoprotein (e.g. the antibody) comprises the G0 N-glycan structure GlcNAcβ2Manα3[GlcNAcβ2Manα6]Manβ4GlcNAcβ4GlcNAc. In other embodiments, the non-fucosylated G0 glycoform structure represents at least 20%, 30%; 40%, 50%; 60%, 70%, 80% (mol%) or more, of the total N-glycans of the heterologous glycoprotein (e.g. the antibody) composition obtained by the methods of the invention. In other embodiments, galactosylated N-glycans represents less (mol%) than 0.5%, 0.1%, 0.05%, 0.01% of total N-glycans of the culture, and/or of the heterologous glycoprotein with increased N-glycosylation site occupancy. In certain embodiments, the culture or the heterologous glycoprotein, for example an antibody, comprises no galactosylated N-glycans.

In certain embodiments of any of the disclosed methods, the method includes the further step of providing one or more, two or more, three or more, four or more, or five or more protease inhibitors. In certain embodiments, the protease inhibitors are peptides that are co-expressed with the mammalian glycoprotein. In other embodiments, the inhibitors inhibit at least two, at least three, or at least four proteases from a protease family selected from aspartic proteases, trypsin-like serine proteases, subtilisin proteases, and glutamic proteases.

In certain embodiments of any of the disclosed methods, the filamentous fungal cell or *Trichoderma* fungal cell also contains a carrier protein. As used herein, a “carrier protein” is portion of a protein that is endogenous to and highly secreted by a filamentous fungal cell or *Trichoderma* fungal cell. Suitable carrier proteins include, without limitation, those of *T. reesei* mannanase I

(Man5A, or MANI), *T. reesei* cellobiohydrolase II (Cel6A, or CBHII) (see, e.g., Paloheimo et al Appl. Environ. Microbiol. 2003 December; 69(12): 7073-7082) or *T. reesei* cellobiohydrolase I (CBHI). In some embodiments, the carrier protein is CBH1. In other embodiments, the carrier protein is a truncated *T. reesei* CBH1 protein that includes the CBH1 core region and part of the CBH1 linker region. In some embodiments, a carrier such as a cellobiohydrolase or its fragment is fused to an antibody light chain and/or an antibody heavy chain. In some embodiments, a carrier-antibody fusion polypeptide comprises a Kex2 cleavage site. In certain embodiments, Kex2, or other carrier cleaving enzyme, is endogenous to a filamentous fungal cell. In certain embodiments, carrier cleaving protease is heterologous to the filamentous fungal cell, for example, another Kex2 protein derived from yeast or a TEV protease. In certain embodiments, carrier cleaving enzyme is overexpressed. In certain embodiments, the carrier consists of about 469 to 478 amino acids of N-terminal part of the *T. reesei* CBH1 protein GenBank accession No. EGR44817.1.

In one embodiment, the polynucleotide encoding the heterologous glycoprotein (e.g. the antibody) further comprises a polynucleotide encoding CBH1 catalytic domain and linker as a carrier protein, and/or cbh1 promoter.

In certain embodiments, the filamentous fungal cell of the invention overexpress KEX2 protease. In an embodiment the heterologous glycoprotein (e.g. the antibody) is expressed as fusion construct comprising an endogenous fungal polypeptide, a protease site such as a Kex2 cleavage site, and the heterologous protein such as an antibody heavy and/or light chain. Useful 2-7 amino acids combinations preceding Kex2 cleavage site have been described, for example, in Mikosch *et al.* (1996) J. Biotechnol. 52:97-106; Goller *et al.* (1998) Appl Environ Microbiol. 64:3202-3208; Spencer *et al.* (1998) Eur. J. Biochem. 258:107-112; Jalving *et al.* (2000) Appl. Environ. Microbiol. 66:363-368; Ward *et al.* (2004) Appl. Environ. Microbiol. 70:2567-2576; Ahn *et al.* (2004) Appl. Microbiol. Biotechnol. 64:833-839; Paloheimo *et al.* (2007) Appl Environ Microbiol. 73:3215-3224; Paloheimo *et al.* (2003) Appl Environ Microbiol. 69:7073-7082; and Margolles-Clark *et al.* (1996) Eur J Biochem. 237:553-560.

The invention further relates to the glycoprotein composition, for example the antibody composition, obtainable or obtained by the method as disclosed above.

In other specific embodiments, such glycoprotein or antibody composition further comprises as 50%, 60 %, 70 % or 80 % (mole% neutral N-glycan), of the following glycoform:

(i) Manα3[Manα6(Manα3)Manα6]Manβ4GlcNAβ4GlcNAc (Man5 glycoform);

(ii) GlcNAcβ2Manα3[Manα6(Manα3)Manα6]Manβ4GlcNAβ4GlcNAc, or β4-galactosylated variant thereof;

(iii) Manα6(Manα3)Manβ4GlcNAβ4GlcNAc;

(iv) Manα6(GlcNAcβ2Manα3)Manβ4GlcNAβ4GlcNAc, or β4-galactosylated variant thereof: or,

(v) complex type N-glycans selected from the G0, G1 or G2 glycoform.

In some embodiments the N-glycan glycoform according to iii-v comprises less than 15 %, 10 %, 7 %, 5 %, 3 %, 1 % or 0.5 % or is devoid of Man5 glycan as defined in i) above.

EXAMPLES

FUNCTIONAL ASSAYS

Assay for measuring total protease activity of cells of the invention

The protein concentrations were determined from supernatant samples from day 2-7 of 1x-7x protease deficient strains (described in PCT/EP2013/050126) according to EnzChek protease assay kit (Molecular probes #E6638, green fluorescent casein substrate). Briefly, the supernatants were diluted in sodium citrate buffer to equal total protein concentration and equal amounts of the diluted supernatants were added into a black 96 well plate, using 3 replicate wells per sample. Casein FL diluted stock made in sodium citrate buffer was added to each supernatant containing well and the plates were incubated covered in plastic bag at 37°C. The fluorescence from the wells was measured after 2, 3, and 4 hours. The readings were done on the Varioskan fluorescent plate reader using 485 nm excitation and 530 nm emission. Some protease activity measurements were performed using succinylated casein (QuantiCleave protease assay kit, Pierce #23263) according to the manufacturer's protocol.

The pep1 single deletion reduced the protease activity by 1.7-fold, the pep1/tsp1 double deletion reduced the protease activity by 2-fold, the pep1/tsp1/slp1 triple deletion reduced the protease activity by 3.2-fold, the pep1/tsp1/slp1/gap1 quadruple deletion reduced the protease activity by 7.8-fold compared to the wild type M124 strain, the pep1/tsp1/slp1/gap1/gap2 5-fold deletion reduced the protease activity by 10-fold, the pep1/tsp1/slp1/gap1/gap2/pep4 6-fold deletion reduced the protease activity by 15.9-fold, and the pep1/tsp1/slp1/gap1/gap2/pep4/pep3 7-fold deletion reduced the protease activity by 18.2-fold.

Figure 5 graphically depicts normalized protease activity data from culture supernatants from each of the protease deletion supernatants (from 1-fold to 7-fold deletion mutant) and the parent strain without protease deletions. Protease activity was measured at pH 5.5 in first 5 strains and at pH 4.5 in the last three deletion strains. Protease activity is against green fluorescent casein. The six-fold protease deletion strain has only 6% of the wild type parent strain and the 7-fold protease

deletion strain protease activity was about 40% less than the 6-fold protease deletion strain activity.

Assay for measuring N-glycosylation site occupancy in a glycoprotein composition

10 – 30 µg of antibody is digested with 13.4 – 30 U of FabRICATOR (Genovis), +37°C, 60 min - overnight, producing one F(ab')2 fragment and one Fc fragment per an antibody molecule. Digested samples are purified using Poros R1 filter plate (Glyken corp.) and the Fc fragments are analysed for N-glycan site occupancy using MALDI-TOF MS. The percentage of site occupancy of an Fc is the average of two values: the one obtained from intensity values of the peaks (single and double charged) and the other from area of the peaks (single and double charged); both the values are calculated as glycosylated signal divided by the sum of non-glycosylated and glycosylated signals.

EXAMPLE 1 – Generation of *T. reesei* expressing *L. major* STT3

The *Leishmania major* oligosaccharyl transferase 4D (old GenBank No. XP_843223.1, new XP_003722509.1; SEQ ID NO: 1) coding sequence was codon optimized for *Trichoderma reesei* expression (codon optimized nucleic acid sequence SEQ ID NO: 2). The optimized coding sequence was synthesized along with cDNA1 promoter (SEQ ID NO: 3) and TrpC terminator flanking sequence (SEQ ID NO: 4). The *Leishmania major* STT3 gene was excised from the optimized cloning vector using PacI restriction enzyme digestion. The expression entry vector was also digested with PacI and dephosphorylated with calf alkaline phosphatase. The STT3 gene and the digested vector were separated with agarose gel electrophoresis and correct fragments were isolated from the gel with a gel extraction kit (Qiagen) according to manufacturer's protocol. The purified *Leishmania major* STT3 gene was ligated into the expression vector with T4 DNA ligase. The ligation reaction was transformed into chemically competent DH5α *E. coli* and grown on ampicillin (100 µg/ml) selection plates. Miniprep plasmid preparations were made from several colonies. The presence of the *Leishmania major* STT3 gene insert was checked by digesting the prepared plasmids with PacI digestion and several positive clones were sequenced to verify the gene orientation. One correctly orientated clone was chosen to be the final vector pTTv201.

The expression cassette contained the constitutive *cDNA1* promoter from *Trichoderma reesei* to drive expression of *Leishmania major STT3*. The terminator sequence included in the cassette was the TrpC terminator from *Aspergillus niger*. The expression cassette was targeted into the xylanase 1 locus (*xyn1*, tre74223) using the xylanase 1 sequence from the 5' and 3' flanks of the gene (SEQ ID NO: 5 and SEQ ID NO: 6). These sequences were included in the cassette to allow the cassette to integrate into the *xyn1* locus via homologous recombination. The cassette contained a *pyr4* loopout marker for selection. The *pyr4* gene encodes the orotidine-5'-monophosphate (OMP)

decarboxylase of *T. reesei* (Smith, J.L., et al., 1991, Current Genetics 19:27-33) and is needed for uridine synthesis. Strains deficient for OMP decarboxylase activity are unable to grow on minimal medium without uridine supplementation (i.e. are uridine auxotrophs).

To prepare the vector for transformation, the vector was cut with PmeI to release the expression cassette (Fig. 1). The digest was separated with agarose gel electrophoresis and the correct fragment was isolated from the gel with a gel extraction kit (Qiagen) according to manufacturer's protocol. The purified expression cassette DNA (5 µg) was then transformed into protoplasts of the *Trichoderma reesei* strain M317 (M317 has been described in the International Patent Application No. PCT/EP2013/050126; M317 is pyr4- of M304 and it comprises MAB01 light chain fused to *T. reesei* truncated CBH1 carrier with NVISKR Kex2 cleavage sequence, MAB01 heavy chain fused to *T. reesei* truncated CBH1 carrier with AXE1 [DGETVVKR] Kex2 cleavage sequence, Δ*pep1*Δ*tsp1*Δ*slp1*, and overexpression of *T. reesei* KEX2). Preparation of protoplasts and transformation were carried out according to methods in Penttilä et al. (1987, Gene 61:155-164) and Gruber et al (1990, Curr. Genet. 18:71-76) for pyr4 selection. The transformed protoplasts were plated onto *Trichoderma* minimal media (TrMM) plates.

Transformants were then streaked onto TrMM plates with 0.1% TritonX-100. Transformants growing fast as selective streaks were screened by PCR using the primers listed in Table 1. DNA from mycelia was purified and analyzed by PCR to look at the integration of the 5' and 3' flanks of cassette and the existence of the xylanase 1 ORF. The cassette was targeted into the xylanase 1 locus; therefore the open reading frame was not present in the positively integrated transformants. To screen for 5' integration, sequence outside of the 5' integration flank was used to create a forward primer that would amplify genomic DNA flanking *xyn1* and the reverse primer was made from sequence in the cDNA promoter of the cassette. To check for proper integration of the cassette in the 3' flank, a forward primer was made from sequence outside of the 3' integration flank that would amplify genomic DNA flanking *xyn1* and the reverse primer was made from sequence in the *pyr4* marker. Thus, one primer would amply sequence from genomic DNA outside of the cassette and the other would amply sequence from DNA in the cassette. The primer sequences are listed in Table 1. Four final strains showing proper integration and a deletion of *xyn1* orf were called M420-M423.

Shake flask cultures were conducted for four of the STT3 producing strains (M420-M423) to evaluate growth characteristics and to provide samples for glycosylation site occupancy analysis. The shake flask cultures were done in TrMM, 40 g/l lactose, 20 g/l spent grain extract, 9 g/l casamino acids, 100 mM PIPPS, pH 5.5. *L. major* STT3 expression did not affect growth negatively when compared to the parental strain M304 (Tables 2 and 3). The cell dry weight for the STT3 expressing transformants appeared to be slightly higher compared to the parent strain M304.

Table 1: List of primers used for PCR screening of STT3 transformants.

5' flank screening primers:	**1205 bp product**
T403_Xyn1_5'flank_fwd	CCGCGTTGAACGGCTTCCCA (SEQ ID NO:48)
T140_cDNA1promoter_rev	TAACTTGTACGCTCTCAGTTCGAG (SEQ ID NO:49)
3' flank screening primers:	**1697 bp product**
T404_Xyn1_3'flank_fwd	GCGACGGCGACCCATTAGCA (SEQ ID NO:50)
T028_Pyr4_flank_rev	CATCCTCAAGGCCTCAGAC (SEQ ID NO:51)
xylanase 1 orf primers:	**589 bp product**
T405_Xyn1_orf_screen_fwd	TGCGCTCTCACCAGCATCGC (SEQ ID NO:52)
T406_Xyn1_orf_screen_rev	GTCCTGGGCGAGTTCCGCAC (SEQ ID NO:53)

Table 2: Cell dry weight from large shake flask cultures.

Cell dry weight (g/L)			
	day 3	**day 5**	**day 7**
M304	2.3	3.3	4.3
M420	3.7	4.3	5.4
M421	3.7	4.6	6.3
M422	3.8	4.5	5.4
M423	3.7	4.6	5.7

Table 3: pH values from large shake flask cultures.

pH values			
	day 3	**day 5**	**day 7**
M304	5.6	6.1	6.2
M420	6.1	6.1	6.1
M421	6.0	5.9	6.0
M422	6.1	6.1	6.2
M423	6.1	6.1	6.1

Site occupancy analysis

Four transformants [pTTv201; 17A-a (M420), 26B-a (M421), 65B-a (M422) and 97A-a (M423)] and their parental strain (M317) were cultivated in shake flasks and samples at day 5 and 7 time points

were collected. MAB01 antibody was purified from culture supernatants using Protein G HP MultiTrap 96-well plate (GE Healthcare) according to manufacturer's instructions. The antibody was eluted with 0.1 M citrate buffer, pH 2.6 and neutralized with 2 M Tris, pH 9. The concentration was determined via UV absorbance in spectrophotometer against MAB01 standard curve. 10 µg of antibody was digested with 13.4 U of FabRICATOR (Genovis), +37°C, 60 min, producing one F(ab')2 fragment and one Fc fragment. Digested samples were purified using Poros R1 filter plate (Glyken corp.) and the Fc fragments were analysed for N-glycan site occupancy using MALDI-TOF MS (Fig. 2).

The overexpression of *STT3* from *Leishmania major* enhanced the site coverage compared to the parental strain. The best clone was re-cultivated in three parallel shake flasks each and the analysis results were comparable to the first analysis. Compared to parental strain the signals Fc and Fc + K are practically absent in STT3 clones.

The difference in site occupancy between parental strain and all clones of STT3 from *L. major* was significant (Fig. 2). Because the signals coming from Fc or Fc + K were practically absent, the N-glycan site occupancy of MAB01 in these shake flask cultivations was 100% (Table 4).

Table 4: Site occupancy analysis of parental strain M317 and four transformants of STT3 from *L. major*. The averages have been calculated from area and intensity from single and double charged signals from three parallel samples.

	M317	17A-a	26B-a	65B-a	97A-a
Glycosylation state	**Average %**	**Average %**	**Average %**	**Average %**	**Average %**
Non-glycosylated	13.0	0.0	0.0	0.0	0.0
Glycosylated	87.0	100.0	100.0	100.0	100.0

Fermenter cultivations

Three STT3 (L. major) clones (M420, M421 and M422) as well as parental strain M304 were cultivated in fermenter. Samples at day 3, 4, 5, 6 and 7 time points were collected and the site occupancy analysis was performed to purified antibody. STT3 overexpression strains and the respective control strain (M304) were grown in batch fermentations for 7 days, in media containing 2% yeast extract, 4% cellulose, 4% cellobiose, 2% sorbose, 5g/L KH2PO4, and 5g/L (NH4)2SO4. Culture pH was controlled at pH 5.5 (adjusted with NH3OH). The temperature was shifted from 28 °C to 22°C at 48 hours elapsed process time. Fermentations were carried out in 4 parallel 2 L glass vessel reactors with a culture volume of 1 L. Culture supernatant samples were taken during the course of the runs and stored at -20 ºC. MAB01 antibody was purified and digested with FabRICATOR as described above. The antibody titers are shown in Table 5.

Results

The site occupancy in parental strain M304 was less than 60% but in all analyzed STT3 clones the site occupancy had increased up to 98% (Table 6).

Table 5: MAB01 antibody titers of the LmSTT3 strains M420, M421 and M422 and their parental strain M304.

	Titer g/l				
Strain	**d3**	**d4**	**d5**	**d6**	**d7**
M304	0.225	0.507	0.981	1.52	1.7
M420	0.758	1.21	1.55	1.71	1.69
M421	0.76	1.24	1.54	1.67	1.6
M422	0.65	1.07	1.43	1.56	1.54

Table 6: The N-glycosylation site occupancies of MAB01 antibody of the LmSTT3 strains M420, M421 and M422 and their parental strain M304.

	Site occupancy %				
Strain	**d3**	**d4**	**d5**	**d6**	**d7**
M304	48.0	47.7	47.7	46.3	55.4
M420	97.8	97.5	96.9	94.3	94.6
M421	96.1	90.8	91.5	89.7	95.6
M422	94.4	88.5	80.9	83.6	75.2

In conclusion, overexpression of the STT3D gene from *L. major* increased the N-glycosylation site occupancy from 46%-87% in the parental strain to 98%-100% in transformants having *Leishmania* STT3 under shake flask or fermentation culture conditions.

The overexpression of the STT3D gene from *L. major* significantly increased the N-glycosylation site occupancy in strains producing an antibody as a heterologous protein. The antibody titers did not vary significantly between transformants having STT3 and parental strain.

EXAMPLE 2 – Generation of *T. reesei* strains expressing STT3 from *T. vaginalis*, *L. infantums* or *E. histolytica*

The coding sequences of the *Trichomonas vaginalis*, *Leishmania infantum* and *Entamoeba histolytica* oligosaccharyl transferase (STT3; amino acid sequences *T. vaginalis* SEQ ID NO: 7, *L. infantum* SEQ ID NO: 8, and *E. histolytica* SEQ ID NO: 10) were codon optimized for *Trichoderma reesei* expression (codon optimized *L. infantum* nucleic acid SEQ ID NO: 9). The optimized coding sequences were synthesized along with *T. reesei cbh1* terminator flanking sequence (SEQ ID NO:

11). Plasmids containing the STT3 genes under the constitutive *cDNA1* promoter, with *cbh1* terminator, *pyr4* loopout marker and *alg3* flanking regions (SEQ ID NO: 12 and SEQ ID NO: 13) were cloned by yeast homologous recombination as described in WO2012/069593. *Not*I fragment of plasmid pTTv38 was used as vector backbone. This vector contains *alg3* (tre104121) 5' and 3' flanks of the gene to allow the expression cassette to integrate into the *alg3* locus via homologous recombination in *T. reesei* and the plasmid has been described in WO2012/069593. The *STT3* genes were excised from the cloning vectors using *Sfi*I restriction enzyme digestion. The *cdna1* promoter and *cbh1* terminator fragments were created by PCR, using plasmids pTTv163 and pTTv166 as templates, respectively. The *pyr4* loopout marker was extracted from plasmid pTTv142 by *Not*I digestion (the plasmid pTTv142 having a human GNT2 catalytic domain fused with T. reesei MNT1/KRE2 targeting peptide has been described in WO2012/069593). The *pyr4* gene encodes the orotidine-5'-monophosphate (OMP) decarboxylase of *T. reesei* (Smith, J.L., et al., 1991, Current Genetics 19:27-33) and is needed for uridine synthesis. Strains deficient for OMP decarboxylase activity are unable to grow on minimal medium without uridine supplementation (i.e. are uridine auxotrophs). The primers used for cloning are listed in Table 7. The digested fragments and PCR products were separated with agarose gel electrophoresis and correct fragments were isolated from the gel with a gel extraction kit (Qiagen) according to manufacturer's protocol. The plasmids were constructed using the yeast homologous recombination method, using overlapping oligonucleotides for the recombination of the gap between the *pyr4* marker and *alg3* 3' flank as described in WO2012/069593. The plasmid DNA were rescued from yeast and transformed into electrocompetent TOP10 *E. coli* that were grown on ampicillin (100 µg/ml) selection plates. Miniprep plasmid preparations were made from several colonies. The presence of the *Trichomonas vaginalis* and *Leishmania infantum* STT3 genes was confirmed by digesting the prepared plasmids with *Bgl*II-*Kpn*I whereas the *Entamoeba histolytica* plasmid was digested with *Hind*III-*Kpn*I. Positive clones were sequenced to verify the plasmid sequences. One correct *Trichomonas vaginalis* clone was chosen to be the final vector pTTv321, and correct clones of *Leishmania infantum* and *Entamoeba histolytica* were chosen to be the pTTv322 and pTTv323 vectors, respectively. The primers used for sequencing the vectors are listed in Table 8.

Table 7: List of primers used for cloning vectors pTTv321, pTTv322 and pTTv323.

Fragment	Primer	Primer sequence
cDNA1 promoter, pTTv321	T1177_pTTv321_1	AGATTTCAGTCTCTCACCACTCACCTGAGTTGCCT CTCTCGGTCTGAAGGACGTGGAATGATG (SEQ ID NO:54)
	T1178_pTTv321_2	GCAGGGTGATGAGCTGGATCACCTTGACGGTGTT

		GCCCATGTTGAGAGAAGTTGTTGGATTGATCA (SEQ ID NO:55)
cDNA1 promoter, pTTv322	T1177_pTTv321_1	AGATTTCAGTCTCTCACCACTCACCTGAGTTGCCT CTCTCGGTCTGAAGGACGTGGAATGATG (SEQ ID NO:56)
	T1183_pTTv322_1	CAGAGCCGCTATCGCCGAGGAGGTTGCCCTTCTT GCCCATGTTGAGAGAAGTTGTTGGATTGATCA (SEQ ID NO:57)
cDNA1 promoter, pTTv323	T1177_pTTv321_1	AGATTTCAGTCTCTCACCACTCACCTGAGTTGCCT CTCTCGGTCTGAAGGACGTGGAATGATG (SEQ ID NO:58)
	T1184_pTTv323_1	TCTTGAGGATGAGCTGGACGAGGGTCTTGAAAAA GCCCATGTTGAGAGAAGTTGTTGGATTGATCA (SEQ ID NO:59)
cbh1 terminator	T1179_pTTv321_3	AGCTCCGTGGCGAAAGCCTGA (SEQ ID NO:60)
	T1180_pTTv321_4	CAGCCGCAGCCTCAGCCTCTCTCAGCCTCATCAG CCGCGGCCGCCAACTTTGCGTCCCTTGTGACG (SEQ ID NO:61)
pyr4-alg3 3' flank overlapping oligos	T1181_pTTv321_5	GCAACGAGAGCAGAGCAGCAGTAGTCGATGCTA GGCGGCCGCGGGCAGTATGCCGGATGGCTGGCT TATACAGGCA (SEQ ID NO:62)
	T1182_pTTv321_6	TGCCTGTATAAGCCAGCCATCCGGCATACTGCCC GCGGCCGCCTAGCATCGACTACTGCTGCTCTGCT CTCGTTGC (SEQ ID NO:63)

Table 8: List of primers used for sequencing vectors pTTv321, pTTv322 and pTTv323.

Primer	Sequence
T027_Pyr4_orf_start_rev	TGCGTCGCCGTCTCGCTCCT (SEQ ID NO:64)
T061_pyr4_orf_screen_2F	TTAGGCGACCTCTTTTTCCA (SEQ ID NO:65)
T143_cDNA1promoter_seqF3	CGAGGAAGTCTCGTGAGGAT

	(SEQ ID NO:66)
T410_alg3_5-flank_F	CAGCTAAACCGACGGGCCA (SEQ ID NO:67)
T1153_cbh1_term_start_rev	GACCGTATATTTGAAAAGGG (SEQ ID NO:68)

To prepare the vectors for transformation, the vectors were cut with *Pme*I to release the expression cassettes (Fig. 3). The fragments were separated with agarose gel electrophoresis and the correct fragment was isolated from the gel with a gel extraction kit (Qiagen) according to manufacturer's protocol. The purified expression cassette DNA was then transformed into protoplasts of the *Trichoderma reesei* M317. Preparation of protoplasts and transformation were carried out essentially according to methods in Penttilä et al. (1987, Gene 61:155-164) and Gruber et al (1990, Curr. Genet. 18:71-76) for *pyr4* selection. The transformed protoplasts were plated onto *Trichoderma* minimal media (TrMM) plates containing sorbitol.

Transformants were then streaked onto TrMM plates with 0.1% TritonX-100. Transformants growing fast as selective streaks were screened by PCR using the primers listed in Table 9. DNA from mycelia was purified and analyzed by PCR to look at the integration of the 5' and 3' flanks of cassette and the existence of the *alg3* ORF. The cassette was targeted into the *alg3* locus; therefore the open reading frame was not present in the positively integrated transformants, purified to single cell clones. To screen for 5' integration, sequence outside of the 5' integration flank was used to create a forward primer that would amplify genomic DNA flanking *alg3* and the reverse primer was made from sequence in the *cDNA1* promoter of the cassette. To check for proper integration of the cassette in the 3' flank, a reverse primer was made from sequence outside of the 3' integration flank that would amplify genomic DNA flanking *alg3* and the forward primer was made from sequence in the *pyr4* marker. Thus, one primer would amplify sequence from genomic DNA outside of the cassette and the other would amplify sequence from DNA in the cassette.

Table 9: List of primers used for PCR screening of *T. reesei* transformants.

5' flank screening primers:	1165 bp product
T066_104121_5int	GATGTTGCGCCTGGGTTGAC (SEQ ID NO:69)
T140_cDNA1promoter_seqR1	TAACTTGTACGCTCTCAGTTCGA (SEQ ID NO:70)
3' flank screening primers:	1469 bp product

T026_Pyr4_orf_5rev2	CCATGAGCTTGAACAGGTAA (SEQ ID NO:71)
T068_104121_3int	GATTGTCATGGTGTACGTGA (SEQ ID NO:72)
alg3 ORF primers:	689 bp product
T767_alg3_del_F	CAAGATGGAGGGCGGCACAG (SEQ ID NO:73)
T768_alg3_del_R	GCCAGTAGCGTGATAGAGAAGC (SEQ ID NO:74)
alg3 ORF primers:	1491 bp product
T069_104121_5orf_pcr	GCGTCACTCATCAAAACTGC (SEQ ID NO:75)
T070_104121_3orf_pcr	CTTCGGCTTCGATGTTTCA (SEQ ID NO:76)

Four final strains each showing proper integration and a deletion of *alg3* ORF were grown in large shake flasks in TrMM medium supplemented with 40 g/l lactose, 20 g/l spent grain extract, 9 g/l casamino acids and 100 mM PIPPS, pH 5.5. Growth for pTTv321 and pTTv323 strains was somewhat slower than parental strain M304 (Table 10). Three out of four *Leishmania infantum* pTTv322 clones grew somewhat better than the parental strain.

Table 10. Cell dry weight measurements (in g/L) of the parental strains M304 and STT3 expressing strains.

Strain	3 days	5 days	7 days
M304	3,06	3,34	4,08
pTTv321#18-9-2	2,54	2,89	2,52
pTTv321#18-9-10	2,44	3,03	2,65
pTTv321#18-12-1	2,43	3,12	2,86
pTTv321#18-12-2	2,84	3,49	3,39
pTTv322#60-2	3,02	3,42	3,63
pTTv322#60-6	3,37	4,45	4,68
pTTv322#60-12	3,30	4,15	4,29
pTTv322#60-14	2,92	3,90	4,39
pTTv323#37-4-1	2,29	2,27	2,59
pTTv323#37-4-14	1,88	2,08	2,69
pTTv323#37-11-3	2,15	2,27	2,62
pTTv323#37-11-8	1,92	2,25	2,62

Site occupancy and glycan analyses

From day 5 supernatant samples, MAB01 was purified using Protein G HP MultiTrap 96-well filter plate (GE Healthcare) according to manufacturer's instructions. Approx. 1.4 ml of culture supernatant was loaded and the elution volume was 230 µl. The antibody concentrations were determined via UV absorbance against MAB01 standard curve.

For site occupancy analysis 16-20 µg of purified MAB01 antibody was taken and antibodies were digested, purified, and analysed as described in example 1. The 100% site occupancy was achieved with *Leishmania infantum* STT3 clones 60-6, 60-12 and 60-14 (Table 11). In *T. vaginalis* and *E. histolytica* STT3 transformants the site occupancy was low and in the latter the antibodies appeared to be degraded resulting that no site occupancy analysis could be performed for one strain.

Table 11. N-glycosylation site occupancy of antibodies from STT3 variants and parental M304 at day 5.

M304				
Glycosylation state	**%**			
Non-glycosylated	8			
Glycosylated	92			
***Trichomonas vaginalis* STT3, Δalg3**	**18-9-2**	**18-9-10**	**18-12-1**	**18-12-2**
Glycosylation state	**%**	**%**	**%**	**%**
Non-glycosylated	75	71	69	64
Glycosylated	25	29	31	36
***Leishmania infantum* STT3, Δalg3**	**60-2**	**60-6**	**60-12**	**60-14**
Glycosylation state	**%**	**%**	**%**	**%**
Non-glycosylated	38	0	0	0
Glycosylated	62	100	100	100
***Entamoeba histolytica* STT3, Δalg3**	**37-4-1**	**37-4-14**	**37-11-3**	**37-11-8**
Glycosylation state	**%**	**%**	**%**	**%**
Non-glycosylated	82	n.d.	73	86
Glycosylated	18	n.d.	27	14

These results shows that overexpression of the catalytic subunit of *Leishmania infantum* is capable of increasing the N-glycosylation site occupancy in filamentous fungal cells, up to 100%.

In contrast, the STT3 genes from *Trichomonas vaginalis* or *Entamoeba histolytica* do not result in high N-glycosylation site occupancy.

N-glycans were analysed from three of the *Leishmania infantum* STT3 clones. The PNGase F reactions were carried out to 20 µg of MAB01 antibody as described in examples and the released N-glycans were analysed with MALDI-TOF MS. The three strains produced about 25% of Man3 N-glycan attached to MAB01 whereas Hex6 glycoform represents about 60% of N-glycans attached to MAB01 (Table 12).

Table 12: Neutral N-glycans and site occupancy analysis of MAB01 from *L. infantum* STT3 clones at day 5.

Leishmania infantum STT3, Δalg3				
Clones		60-6	60-12	60-14
Short	m\z	%	%	%
Man3	933.3	25.9	26.4	25.9
Man4	1095.4	9.4	9.3	9.0
Man5	1257.4	6.5	6.1	7.6
Hex6	1419.5	58.3	58.2	57.5
Fc		0	0	0
Fc + Gn		0	0	0
Glycosylated		100	100	100

This shows that the Man3, G0, G1 and/or G2 glycoforms represent at least 25% of the total neutral N-glycans of MAB01 in 3 different clones overexpressing STT3 from *L. infantum*. Figure 4 shows the glycan structures of Man3, Man4, Man5, and Hex6 produced in Δalg3 strains. "Fc" means an Fc fragment (without any N-glycans) and "Fc+Gn" means an Fc fragment with one attached N-acetylglucosamine (possible Endo T enzyme activity could cleave N-glycans of an Fc resulting Fc+Gn).

EXAMPLE 3 – Generation of Δalg3 strains of MAB01 expressing strains

The acetamide marker of the pTTv38 *alg3* deletion plasmid was changed to *pyr4* marker. The pTTv38 and pTTv142 vectors were digested with *Not*I and fragments separated with agarose gel electrophoresis. Correct fragments were isolated from the gel with a gel extraction kit (Qiagen) according to manufacturer's protocol. The purified *pyr4* loopout marker from pTTv142 was ligated into the pTTv38 plasmid with T4 DNA ligase. The ligation reaction was transformed into electrocompetent TOP10 *E. coli* and grown on ampicillin (100 µg/ml) selection plates. Miniprep plasmid preparations were made from four colonies. The orientation of the marker was confirmed by sequencing the clones with primers listed in Table 13. A clone with the marker in inverted direction was chosen to be the final vector pTTv324.

Table 13. List of primers used for sequencing vectors pTTv324.

Primer	Sequence
T027_Pyr4_orf_start_rev	TGCGTCGCCGTCTCGCTCCT (SEQ ID NO:77)
T060_pyr4_orf_screen_1F	TGACGTACCAGTTGGGATGA (SEQ ID NO:78)

A *pyr4*- strain of the *Leishmania major* STT3 expressing strain M420 was generated by looping out the *pyr4* marker by 5-FOA selection as described in the International Patent Application No. PCT/EP2013/050126. One *pyr4*- strains was designated with number M602.

To prepare the vectors for transformation, the pTTv324 vector was cut with *Pme*I to release the deletion cassette. The fragments were separated with agarose gel electrophoresis and the correct fragment was isolated from the gel with a gel extraction kit (Qiagen) according to manufacturer's protocol. The purified deletion cassette DNA was then transformed into protoplasts of the *Trichoderma reesei* M317 and M602. Preparation of protoplasts, transformation, and protoplast plating were carried out as described above.

Transformants were then streaked onto TrMM plates with 0.1% TritonX-100. Transformants growing fast as selective streaks were screened by PCR using the primers listed in Table 14. DNA from mycelia was purified and analyzed by PCR to look at the integration of the 5' and 3' flanks of cassette and the existence of the *alg3* ORF. The cassette was targeted into the *alg3* locus; therefore the open reading frame was not present in the positively integrated transformants, purified to single cell clones. To screen for 5' integration, sequence outside of the 5' integration flank was used to create a forward primer that would amplify genomic DNA flanking *alg3* and the reverse primer was made from sequence in the *pyr4* marker of the cassette. To check for proper integration of the cassette in the 3' flank, a reverse primer was made from sequence outside of the 3' integration flank that would amplify genomic DNA flanking *alg3* and the forward primer was made from sequence in the *pyr4* marker. Thus, one primer would amplify sequence from genomic DNA outside of the cassette and the other would amplify sequence from DNA in the cassette.

Table 14: List of primers used for PCR screening of *T. reesei* transformants.

5' flank screening primers:	1455 bp product
T066_104121_5int	GATGTTGCGCCTGGGTTGAC (SEQ ID NO:79)
T060_pyr4_orf_screen_1F	TGACGTACCAGTTGGGATGA (SEQ ID NO:80)

3' flank screening primers:	1433 bp product
T027_Pyr4_orf_start_rev	TGCGTCGCCGTCTCGCTCCT (SEQ ID NO:81)
T068_104121_3int	GATTGTCATGGTGTACGTGA (SEQ ID NO:82)
alg3 ORF primers:	689 bp product
T767_alg3_del_F	CAAGATGGAGGGCGGCACAG (SEQ ID NO:83)
T768_alg3_del_R	GCCAGTAGCGTGATAGAGAAGC (SEQ ID NO:84)

Two M602 strains and seven M317 strains showing proper integration and a deletion of *alg3* ORF were grown in large shake flasks in TrMM medium supplemented with 40 g/l lactose, 20 g/l spent grain extract, 9 g/l casamino acids and 100 mM PIPPS, pH 5.5 (Table 15). The M317 strain 19.13 and 19.20 were designated the numbers M697 and M698, respectively, and the M602 strains 1.22 and 11.18 were designated the numbers M699 and M700, respectively.

Table 15 Cell dry weight measurements (in g/l) of the parental strains M304 and STT3 expressing strain M420 and *alg3* deletion transformants.

Strain	3 days	5 days	7 days
M602 1.22	3,63	3,23	3,79
M602 11.18	3,52	3,74	4,12
M317 19.1	3,64	3,84	4,22
M317 19.5	3,54	3,87	4,31
M317 19.6	3,72	3,66	4,78
M317 19.13	3,63	3,21	4,06
M317 19.20	3,97	4,28	5,09
M317 19.43	3,77	4,02	4,18
M317 19.44	3,58	3,78	4,17
M420	3,31	3,69	5,57
M304	2,55	2,99	4,09

Site occupancy and glycan analyses

Two transformants from overexpression of STT3 from *Leishmania major* in *alg3* deletion strain [pTTv324; 1.22 (M699) and 11.18 (M700)] and seven transformants with *alg3* deletion [M317, *pyr4*- of M304; clones 19.1, 19.5, 19.6, 19.13 (M697), 19.20 (M698), 19.43 and 19.44], and their parental

strains M420 and M304 were cultivated in shake flasks in TrMM, 4% lactose, 2% spent grain extract, 0.9% casamino acids, 100 mM PIPPS, pH 5.5. MAB01 antibody was purified and analysed from culture supernatants from day 5 as described in Example 1 except that 30 µg of antibody was digested with 80.4 U of FabRICATOR (Genovis), +37°C, overnight, to produce F(ab')2 and Fc fragments.

In both clones with *alg3* deletion and overexpression of LmSTT3 the site occupancy was 100% (Table 16). Without LmSTT3 the site coverage varied between 56 – 71% in *alg3* deletion clones. The improved site occupancy was shown also in parental strain M420 compared to M304, both with wild type glycosylation.

Table 16: The site occupancy of the shake flask samples. The analysis failed in M317 clones 19.5 and 19.6.

Strain	Clone	Explanation	Site occupancy %
M602	1.22	M304 LmSTT3 Δalg3	100
M602	11.18	M304 LmSTT3 Δalg3	100
M317	19.1	M304 Δalg3	71
M317	19.13	M304 Δalg3	62
M317	19.2	M304 Δalg3	56
M317	19.43	M304 Δalg3	63
M317	19.44	M304 Δalg3	60
M420		Parental strain M304 LmSTT3	100
M304		Parental strain	89

For N-glycan analysis MAB01 was purified from day 7 culture supernatants as described above and N-glycans were released from EtOH precipitated and SDS denatured antibody using PNGase F (Prozyme) in 20 mM sodium phosphate buffer, pH 7.3, in overnight reaction at +37ºC. The released N-glycans were purified with Hypersep C18 and Hypersep Hypercarb (Thermo Scientific) and analysed with MALDI-TOF MS.

Man3 levels were in range of 21 to 49% whereas the main glycoform in clones of M602 and M317 was Hex6 (Table 17). Man5 levels were about 73% in the strains expressing wild type glycosylation (M304) and LmSTT3 (M420).

Table 17. Relative proportions of neutral N-glycans from purified antibody from M602 and M317 clones and parental strains M420 and M304.

			M602		M317					Parental strains	
			1.22	11.18	19.1	19.13	19.2	19.43	19.44	M420	M304
Composition	Short	m\z	%	%	%	%	%	%	%	%	%
Hex3HexNAc2	Man3	933.3	21.1	27.3	45.4	37.5	34.9	24.6	48.6	0.0	0.0
Hex4HexNAc2	Man4	1095.4	9.5	8.7	6.2	7.6	7.1	7.5	9.4	0.8	0.0
Hex5HexNAc2	Man5	1257.4	5.8	7.0	8.1	7.6	6.7	5.6	6.6	72.5	72.8
Hex6HexNAc2	Man6/Hex6	1419.5	63.1	56.6	39.7	45.8	51.4	61.8	34.6	15.6	16.4
Hex7HexNAc2	Man7/Hex7	1581.5	0.5	0.5	0.6	0.8	0.0	0.5	0.7	7.2	7.9
Hex8HexNAc2	Man8/Hex8	1743.6	0.0	0.0	0.0	0.6	0.0	0.0	0.0	3.2	2.4
Hex9HexNAc2	Man9/Hex9	1905.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.5

Fermentation and site occupancy

L. major STT3 *alg3* deletion strain M699 (pTTv324; clone 1.22) and strain M698 with *alg3* deletion [M317, *pyr4*- of M304; clone 19.20], and the parental strain M304 were fermented in 2% YE, 4% cellulose, 8% cellobiose, 4% sorbose. The samples were harvested on day 3, 4, 5 and 6. MAB01 antibody was purified and analysed from culture supernatants from day 5 as described in Example 1 except that 30 µg of antibody was digested with 80.4 U of FabRICATOR (Genovis), +37°C, overnight, to produce F(ab')2 and Fc fragments.

Results

In the strain M699 site occupancy was more than 90% in all time points (Table 18). Without LmSTT3 the site coverage varied between 29-37% in the strain M698. In the parental strain M304 the site coverage varied between 45-57%. At day 6 MAB01 titers were 1.2 and 1.3 g/L for strains M699 and M698, respectively, and 1.8 g/L in the parental strain M304.

Table 18. MAB01 antibody titers and site occupancy analysis results of fermented strains M699 and M698 and the parental strain M304.

M699	**d3**	**d4**	**d5**	**d6**
Titer g/l	0.206	0.361	0.685	1.22
Glycosylation state	**%**	**%**	**%**	**%**
Non-glycosylated	2.4	6.8	8.0	8.5
Glycosylated	97.6	93.2	92.0	91.5
Fc + Gn	0.0	0.0	0.0	0.0
M698	**d3**	**d4**	**d5**	**d6**
Titer g/l	0.252	0.423	0.8	1.317
Glycosylation state	**%**	**%**	**%**	**%**
Non-glycosylated	63.0	70.8	64.3	65.8
Glycosylated	37.0	29.2	35.7	34.2
Fc + Gn	0.0	0.0	0.0	0.0
M304	**d3**	**d4**	**d5**	**d6**
Titer g/l	0.589	0.964	1.41	1.79
Glycosylation state	**%**	**%**	**%**	**%**
Non-glycosylated	45.9	43.3	n.d.	54.9
Glycosylated	54.1	56.7	n.d.	45.1
Fc + Gn	0.0	0.0	n.d.	0.0

In conclusion, overexpression of the catalytic subunit of *Leishmania* STT3 is capable of increasing the N-glycosylation site occupancy in Δalg3 filamentous fungal cells up to 91.5- 100%.

Table 19 below recapitulates the different strains used in the Examples:

Database	Vector	Clone	Strain transformed	Locus, random or K/o	Proteases k/o	Description	Selection of tr.	Markers in strain
M44					None	Base strain		None
M124				K/o mus53	None	mus53 deletion of M44		pyr4
M127				pyr4- of M124	None	pyr4 negative strain of M124		pyr4-
M181	pTTv71	9-20A-1	M127	K/o pep1	pep1	pep1 deletion	pyr4	pyr4
M194	pTTv42	13-172D	M181	K/o tsp1	pep1 tsp1	pep1 tsp1 deletion	bar	bar/pyr4
M252	pTTv99/67	6.14A	M194	cbh1 egl1 loci	pep1 tsp1	MAB01 LC NVISKR/HC AXE1	AmdS/HygR	AmdS/HygR/bar/pyr4
M284	5-FOA of M252	3A	pyr4- of M252	Spontaneous mutation	pep1 tsp1	pyr4 negative strain of M252	none	AmdS/HygR/bar/pyr4-
M304	pTTv128	12A	M284	K/o slp1, Kex2 o/e	pep1 tsp1 slp1	Overexpression of native Kex2, slp1 del	pyr4	AmdS/HygR/bar/pyr4
M317	5-FOA of M304	1A	pyr4- of M304	pyr4 loopout	pep1 tsp1 slp1	pyr4 negative strain of M304	None	AmdS/HygR/bar/pyr4-
M420	pTTv201	17A-a	M317	xylanase 1	pep1 tsp1 slp1	*Leishmania major* stt3 Oligosaccharyl transferase	pyr4	AmdS/HygR/bar/pyr4
M421	pTTv201	26B-a	M317	xylanase 1	pep1 tsp1 slp1	*Leishmania major* stt3 Oligosaccharyl transferase	pyr4	AmdS/HygR/bar/pyr4
M422	pTTv201	65B-a	M317	xylanase 1	pep1 tsp1 slp1	*Leishmania major* stt3 Oligosaccharyl transferase	pyr4	AmdS/HygR/bar/pyr4
M423	pTTv201	97A-a	M317	xylanase 1	pep1 tsp1 slp1	*Leishmania major* stt3 Oligosaccharyl transferase	pyr4	AmdS/HygR/bar/pyr4
M602	5-FOA of M420	2A	pyr4- of M420	pyr4 loopout	pep1 tsp1 slp1	pyr4 negative strain of M420	none	AmdS/HygR/bar/pyr4-
M698	pTTv324	19.20	M317	alg3	pep1 tsp1 slp1	Deletion of alg3	pyr4	AmdS/HygR/bar/pyr4
M699	pTTv324	1.22	M602	alg3	pep1 tsp1 slp1	Deletion of alg3	pyr4	AmdS/HygR/bar/pyr4
M800	pTTv322	60-6	M317	alg3	pep1 tsp1 slp1	Leishmania infantum STT3, cDNA1p cbh1t	pyr4	AmdS/HygR/bar/pyr4
M801	pTTv322	60-12	M317	alg3	pep1 tsp1 slp1	Leishmania infantum STT3,	pyr4	AmdS/HygR/bar/pyr4

						cDNA1p cbh1t		
M802	pTTv322	60-14	M317	alg3	pep1 tsp1 slp1	Leishmania infantum STT3, cDNA1p cbh1t	pyr4	AmdS/HygR/bar/pyr4

Trichoderma strains having STT3 (M420-M423) are triple protease deficient (pep1, tsp1, slp1) as well as deficient of xylanase1, cbh1, and egl1.

Embodiments include also higher order protease deficient strains.

CLAIMS

1. A filamentous fungal cell comprising

   i. one or more mutations that reduces or eliminates one or more endogenous protease activity compared to a parental filamentous fungal cell which does not have said mutation(s),

   ii. a polynucleotide encoding a heterologous catalytic subunit of oligosaccharyl transferase, and

   iii. a polynucleotide encoding a heterologous glycoprotein,

   wherein said catalytic subunit of oligosaccharyl transferase is selected from *Leishmania* oligosaccharyl transferase catalytic subunits.

2. The filamentous fungal cell of Claim 1, wherein the filamentous fungal cell is a *Trichoderma*, *Neurospora*, *Myceliophtora*, *Chrysosporium*, *Aspergillus*, or *Fusarium* cell.

3. The filamentous fungal cell of any one of the preceding claims, wherein said polynucleotide encoding the heterologous catalytic subunit of oliogaccharyl transferase comprises a nucleic acid selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 9, SEQ ID NO: 88 and SEQ ID NO: 90 or a polynucleotide encoding a functional variant polypeptide having at least 50%, at least 60%, at least 70% identity, at least 80% identity, at least 90% identity, or at least 95% identity with SEQ ID NO: 1, SEQ ID NO: 8, SEQ ID NO: 89 or SEQ ID NO:91, said functional variant polypeptide having oligosaccharyltransferase activity.

4. The filamentous fungal cell of anyone of the preceding claims, wherein the N-glycosylation site occupancy of the heterologous glycoprotein is at least 95% and Man3, Man5, G0, G1 and/or G2 glycoforms represent at least 50% of total neutral N-glycans of the heterologous glycoprotein.

5. The filamentous fungal cell of any one of the preceding claims, wherein said cell is a *Trichoderma* cell, for example, *Trichoderma reesei*, and said cell comprises mutations that reduce or eliminate the activity of

- the three endogenous proteases pep1, tsp1, and slp1;
- the three endogenous proteases gap1, slp1, and pep1;
- the three endogenous proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep8, pep9, pep11, pep12, tsp1, slp1, slp2, slp3, slp7, gap1 and gap2;

- three to six proteases selected from the goup consisting of pep1, pep2, pep3, pep4, pep5, tsp1, slp1, slp2, slp3, gap1 and gap2; or,
- seven to ten proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep7, pep8, tsp1, slp1, slp2, slp3, slp5, slp6, slp7, slp8, tpp1, gap1 and gap2.

6. The filamentous fungal cell of anyone of the preceding claims, wherein the fungal cell further comprises a mutation in the gene encoding ALG3 that reduces or eliminates the corresponding ALG3 expression compared to the level of expression of ALG3 gene in a parental cell which does not have such mutation.

7. The filamentous fungal cell of any one of the preceding claims, further comprising a polynucleotide encoding an N-acetylglucosaminyltransferase I catalytic domain and an N-acetylglucosaminyltransferase II catalytic domain.

8. The filamentous fungal cell of any one of the preceding claims, further comprising one or more polynucleotides encoding a polypeptide selected from the group consisting of:

i. α1, 2 mannosidase;
ii. N-acetylglucosaminyltransferase I catalytic domain;
iii. α-mannosidase II;
iv. N-acetylglucosaminyltransferase II catalytic domain;
v. β1,4 galactosyltransferase; and,
vi. fucosyltransferase.

9. A method of producing a heterologous glycoprotein, or antibody composition, with increased N-glycosylation site occupancy, comprising

a) providing a filamentous fungal cell, for example a Trichoderma cell, having a *Leishmania* STT3D gene encoding a catalytic subunit of oligosaccharyl transferase, or a functional variant thereof, and a polynucleotide encoding said heterologous glycoprotein or antibody,
b) culturing the cell under appropriate conditions for expression of the STT3D gene or its functional variant, or said functional variant, and the production of the heterologous glycoprotein; and,
c) recovering and, optionally, purifying the heterologous glycoprotein.

10. The method of claim 9, wherein said filamentous fungal host cell comprises one or more mutation(s) that reduces or eliminates one or more endogenous protease activity compared to a parental filamentous fungal cell which does not have said mutation.

11. The method of any one of claims 9 to 10, wherein said filamentous fungal host cell is the cell as defined in any of claims 1-8.

12. The method of any one of claims 9 to 11, wherein said *Leishmania* STT3D gene encoding a catalytic subunit of oligosaccharyl transferase comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:9, SEQ ID NO: 88 and SEQ ID NO: 90, or a polynucleotide encoding a functional variant polypeptide having at least 50%, at least 60%, at least 70% identity, at least 80% identity, at least 90% identity, or at least 95% identity with SEQ ID NO:1, SEQ ID NO:8, SEQ ID NO: 89 or SEQ ID NO: 91, said functional variant polypeptide having oligosaccharyltransferase activity.

13. The method of any one of claims 9-12, wherein N-glycosylation site occupancy of the produced glycoprotein composition is at least 80%.

14. A glycoprotein or antibody composition obtainable by the method of any one of claims 9-13.

15. The glycoprotein or antibody composition according to claim 14, wherein said antibody composition further comprises, as a major glycoform, either:

i. Manα3[Manα6(Manα3)Manα6]Manβ4GlcNAβ4GlcNAc (Man5 glycoform);
ii. GlcNAcβ2Manα3[Manα6(Manα3)Manα6]Manβ4GlcNAβ4GlcNAc (GlcNAcMan5 glycoform);
iii. Manα6(Manα3)Manβ4GlcNAβ4GlcNAc (Man3 glycoform);
iv. Manα6(GlcNAcβ2Manα3)Manβ4GlcNAβ4GlcNAc (GlcNAcMan3 glycoform); or,
v. complex type N-glycans selected from the G0, G1, or G2 glycoform.