

O-mannosyltransferase Deficient Filamentous Fungal Cells and Methods of Use Thereof

FIELD OF THE INVENTION

The present disclosure relates to compositions and methods useful for the production of
5 heterologous proteins 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
10 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
15 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).

20 However, posttranslational modifications occurring in yeast and fungi may still be a concern for the production of recombinant therapeutic protein. In particular, O-mannosylation is one of the biggest hurdles to overcome in the production of biopharmaceuticals for human applications in fungi. More specifically, yeasts like *Pichia pastoris* and *Saccharomyces cerevisiae* tend to hyper-mannosylate heterologously
25 expressed biopharmaceuticals, thereby triggering adverse effects when applied to humans.

O-mannosylation to Serine and Threonine residues includes in mammals GalNAc based oligosaccharides or GlcNAc/N-acetylglucosamine comprising O-linked mannose glycans. In fungi O-mannosylation occurs as hexose monomers or oligomers. In yeasts, there are
30 typically several protein(/polypeptide) O-mannosyltransferases, which often function as complexes. Part of the knock-outs are harmful, at least for cell structures and stability and

not all yeast knock-outs or combinations are tolerated (for a review, see Goto 2007, *Biosci. Biotechnol. Biochem.* 71(6), 1415-1427).

There have been reports of knock-outs of yeast O-mannosyltransferase genes, aiming to reduce the O-mannosylation levels, and even multiple knock-out mutants involving two or
5 three *pmt* genes in *S. cerevisiae* (WO/1994/004687). *Pmt1* or *pmt2* knock-out of *S. cerevisiae* reduced the level of O-mannosylation of antifreeze glycoprotein III to about 30 % of the proteins and the residual mannosylated protein contains numerous mannose residues per protein, apparently also oligosaccharides (WO/2004/057007).

WO/2010/034708 reports no significant level of O-mannosylation of recombinant
10 hydrophobin *Trichoderma* protein when expressed in *pmt1* knock-out of *S. cerevisiae* host cell. Such O-mannosylation appears to be artificial yeast glycosylation of the original non-mannosylated filamentous fungal protein.

WO/2010/128143 further reports single chain antibody-albumin fusion construct in yeast *S. cerevisiae pmt1* and/or *pmt4* knock-out strains.

15 *Pmt1*, *pmt2*, and *pmt3* single gene knock-outs, double, and triple knock-outs of *Aspergillus* species (*Aspergillus nidulans*, *Aspergillus fumigatus*, and/or *Aspergillus awamori*) are described in Goto et al, 2009 (*Eukaryotic cell* 2009, 8(10):1465); Mouyna et al, 2010 (*Molecular Microbiology* 2010, 76(5), 1205-1221); Zhou et al, 2007 (*Eukaryotic cell* 2007, 6(12):2260); Oka et al, 2004 (*Microbiology* 2004, 150, 1973-1982);
20 Kriangkripipat et al, 2009; Fang et al, 2010 (*Glycobiology*, 2010, vol. 20 pp 542-552); and Oka et al, 2005 (*Microbiology* 2005, 151, 3657-3667).

Despite numerous reports on knock out of *pmt* homologues in filamentous fungi, there is no description of a filamentous fungal cell with reduced O-mannosylation and useful as a host cell for the production of recombinant glycoprotein.

25 In particular, Gorka-Niec et al (2008, *Acta Biochimica Polonica*, Vol. 55 No 2/2008, 251-259) reported the deletion of *pmt1* gene in *Trichoderma reesei*. PMT1 protein showed the highest identity to Pmt4p of *S. cerevisiae* (51%) but functionally complement *pmt2Δ S. cerevisiae* mutant (Gorka-Niec et al, 2007, *Biochimica et Biophysica Acta* 1770, 2007, 774-780). However, the authors reported that disruption of the *pmt1* gene caused a
30 decrease of protein secretion but did not alter O- and N-glycosylation of secreted protein.

Zakrzewska et al (Curr Genet 2003 43: 11-16) further reported that *Trichoderma reesei* *pmt1* gene did not functionally complement *pmt4Δ S. cerevisiae* mutant.

In fact, deletions of the PMT genes in yeasts or filamentous fungi appears to either result in no phenotype at all or lethality or severely impaired vital functions of the cells, which would not be suitable for recombinant production of heterologous proteins, especially mammalian glycoproteins. For this reason, alternative methods such as the use of pmt inhibitors have been proposed as an alternative to pmt knock out strains (WO2009/143041).

Thus, a need remains for improved filamentous fungal cells, such as *Trichoderma* fungus cells, that can stably produce heterologous proteins with no or reduced O-mannosylation, such as immunoglobulins, preferably at high levels of expression.

SUMMARY

The present disclosure relates to improved methods for producing proteins with no or reduced O-mannosylation in filamentous fungal expression systems, and more specifically, glycoproteins, such as antibodies or related immunoglobulins or fusion proteins which may be O-mannosylated when produced in filamentous fungal expression systems.

The present disclosure is based in part on the surprising discovery that filamentous fungal cells, such as *Trichoderma* cells, can be genetically modified to reduce or suppress O-mannosylation activity, without adversely affecting viability and yield of produced glycoproteins.

Accordingly, in a first aspect, the disclosure relates to a PMT-deficient filamentous fungal cell comprising:

- a) a first mutation that reduces or eliminates an endogenous protease activity compared to a parental filamentous fungal cell which does not have said first mutation, said endogenous proteases being selected among aspartic proteases, trypsin-like serine proteases, subtilisin proteases, glutamic proteases and sedolisin proteases, and,
- b) a second mutation in a protein O-mannosyltransferase (*PMT*) gene that reduces endogenous O-mannosyltransferase activity compared to a parental filamentous fungal cell which does not have said second mutation,

wherein said filamentous fungal cell is selected from the group consisting of *Trichoderma*, *Neurospora*, *Myceliophthora* and *Chrysosporium* cell.

In one embodiment, said PMT-deficient cell further expresses a heterologous protein containing serine and/or threonine residues. The expressed heterologous protein with serine and/or threonine residues has reduced O-mannosylation due to said mutation in
5 said PMT gene. For example, the O-mannosylation level of the heterologous protein expressed in a PMT-deficient cell of the disclosure is at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or at least 90% lower as compared to the O-mannosylation level of the heterologous protein when expressed in the parental filamentous fungal cell which does not have said second PMT-deficient mutation.

10 In another embodiment, said second mutation that reduces endogenous O-mannosyltransferase activity is a deletion or a disruption of a *PMT* gene encoding an endogenous protein O-mannosyltransferase activity.

In another embodiment, said second PMT-deficient mutation in a PMT gene may be a mutation (such as a deletion or disruption) in either:

- 15 a) *PMT1* gene comprising the polynucleotide of SEQ ID NO:1 ,
b) a functional homologous gene of *PMT1* gene, which functional gene is capable of restoring parental O-mannosylation level by functional complementation when introduced into a *T. reesei* strain having a disruption in said *PMT1* gene, or,
c) a polynucleotide encoding a polypeptide having at least 50%, at least 60%, at least
20 70%, at least 90%, or at least 95% identity with SEQ ID NO:2, said polypeptide having O-mannosyltransferase activity.

In another embodiment that may be combined with the precedent embodiments, said PMT-deficient cell has a third mutation that reduces or eliminates the level of expression of an *ALG3* gene compared to the level of expression in a parental cell which does not
25 have such third mutation. In a specific embodiment, said PMT-deficient cell further comprises a first polynucleotide encoding N-acetylglucosaminyltransferase I catalytic domain and a second polynucleotide encoding N-acetylglucosaminyltransferase II catalytic domain.

In another embodiment that may be combined with the preceding embodiments, said
30 PMT-deficient cell further comprises one or more polynucleotides encoding a polypeptide selected from the group consisting of:

- a) α 1,2 mannosidase,

- b) N-acetylglucosaminyltransferase I catalytic domain,
- c) α mannosidase II, and
- d) N-acetylglucosaminyltransferase II catalytic domain.

5 In another embodiment that may be combined with the preceding embodiments, said PMT-deficient cell further comprises one or more polynucleotides encoding a β 1,4 galactosyltransferase and/or a fucosyltransferase.

In one specific embodiment, said PMT-deficient cell is a *Trichoderma* cell comprising at least a mutation that reduces or eliminates the protein O-mannosyltransferase activity of
 10 *Trichoderma* pmt1 , and, optionally, further comprising mutations in at least one or more other PMT genes that reduces or eliminates the protein O-mannosyltransferase activity selected from the group consisting of pmt2 and pmt3.

In one embodiment that may be combined with the preceding embodiments, the PMT deficient cells comprise mutations that reduce or eliminate the activity of at least two, or at
 15 least three endogenous proteases. Typically, said cell may be a *Trichoderma* cell and may comprise mutations that reduce or eliminate the activity of

- a) the three endogenous proteases pep1 , tsp1 and slp1 ,
- b) the three endogenous proteases gap1 , slp1 and pep1 ,
- c) three endogenous proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep8, pep11, pep12, tsp1, slp1, slp2, slp3, slp7, gap1 and gap2,
 20
- d) three to six proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, tsp1, slp1, slp2, slp3, gap1 and gap2, or,
- e) 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.

25 In one embodiment that may be combined with the precedent embodiments, the filamentous fungal cell of the disclosure does not comprise a deletion or disruption of an endogenous gene encoding a chaperone protein. In particular, said filamentous fungal cell of the invention expresses functional endogenous chaperone protein Protein Disulphide Isomerase (PDI).

30 In another aspect, the disclosure relates to a method for producing a protein having reduced O-mannosylation, comprising:

- a) providing a PMT-deficient filamentous fungal cell, having a mutation in a PMT gene that reduces endogenous O-mannosyltransferase activity as compared to parental strain which does not have such mutation, and further comprising a polynucleotide encoding a protein with serine or threonine residue,
- 5 b) culturing said PMT-deficient filamentous fungal cell to produce said protein with reduced O-mannosylation,

wherein said filamentous fungal cell is selected from the group consisting of *Trichoderma*, *Neurospora*, *Myceliophthora* and *Chrysosporium* cell.

According to one specific embodiment of the method, said mutation in a *PMT* gene is a
10 mutation, such as a deletion or disruption, in either:

- a) *PMT1* gene comprising the polynucleotide of SEQ ID NO:1 ,
- b) a functional homologous gene of *PMT1* gene, which gene is capable of restoring parental O-mannosylation level by functional complementation when introduced into a *T. reesei* strain having a disruption in said *PMT1* gene, or,
- 15 c) a polynucleotide encoding a polypeptide having at least 50%, at least 60%, at least 70%, at least 90%, or at least 95% identity with SEQ ID NO:2, said polypeptide having protein O-mannosyltransferase activity.

In another embodiment of the method, said PMT-deficient cell is a *Trichoderma reesei* cell and said mutation is a deletion or a disruption of *T. reesei* *PMT1* gene.

20 In other embodiments of the method, said PMT-deficient cell is a PMT-deficient cell of the disclosure as described above.

In a specific embodiment, said polynucleotide encoding a protein is a recombinant polynucleotide encoding a heterologous protein. Typically, said heterologous protein may be a mammalian protein selected from the group consisting of

- 25 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,
- 30 f) a monomeric or multimeric single domain antibody,
g) a FAb-fragment, a FAb2-fragment, and,

h) their antigen-binding fragments.

In one embodiment of the method, that may be combined with the preceding embodiments, said polynucleotide encoding said protein further comprises a polynucleotide encoding CBH1 catalytic domain and linker as a carrier protein and/or *cbh 1* promoter.

In another embodiment, said polynucleotide encodes a protein with serine or threonine, which may be O-mannosylated in a PMT functional parental strain, and further comprising at least one N-glycan.

In another aspect, the disclosure also relates to a method for producing an antibody having reduced O-mannosylation, comprising:

a) providing a PMT-deficient filamentous fungal cell having

i. a mutation that reduces endogenous protein O-mannosyltransferase activity as compared to parental strain which does not have such mutation and

ii. a polynucleotide encoding a light chain antibody and a polynucleotide encoding a heavy chain antibody,

b) culturing the cell to produce said antibody, consisting of heavy and light chains, having reduced O-mannosylation,

wherein said filamentous fungal cell is selected from the group consisting of *Trichoderma*, *Neurospora*, *Myceliophthora* and *Chrysosporium* cell.

In a specific method for producing antibody, said PMT-deficient cell is a *Trichoderma reesei* cell and said mutation is a deletion or a disruption of *T. reesei* *PMT1* gene.

In one embodiment of the method for producing antibody, at least 70%, 80%, 90%, 95%, or 100% of the produced antibody is not O-mannosylated.

The disclosure also relates to the protein composition or antibody composition obtainable or obtained by the methods of the invention as described above. In one embodiment, at least 70%, 80%, 90%, 95%, or 100% of the antibodies as obtained or obtainable the methods of the invention are not O-mannosylated.

In one specific embodiment, such protein (e.g. a glycoprotein) or antibody composition with reduced O-mannosylation comprises, as a major glycoform, either,

- Man α 3[Man α 6(Man α 3)Man α 6]Man β 4GlcNA β 4GlcNAc (Man5 glycoform);
 - Man α 6(Man α 3)Man β 4GlcNA β 4GlcNAc (Man3 glycoform);
- 5 • hybrid or complex type N-glycans such as glycoforms selected from the subgroup consisting of GlcNAcMan3, G0, hybrid glycan, or GlcNAcMan5, or galactosylated derivatives, such as GalGlcNAcMan3, G1, G2; or, GalGlcNAcMan5 glycoform.

10 In one specific embodiment, when the core of the glycan consists of Man3, then the composition essentially lacks Man5 glycoforms.

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 the N-glycans and/or O-glycans of the protein composition comprises Neu5Gc and/or Gal α - 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 antibody composition comprises Neu5Gc and/or Gal α - structure.

15

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 the N-glycan of the glycoprotein composition comprises core fucose 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-glycan of the antibody composition comprises core fucose structures.

20

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 glycoprotein composition comprises terminal galactose epitopes Gal β 3/4GlcNAc. 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 antibody composition comprises terminal galactose epitopes Gal β 3/4GlcNAc.

25

30 In an embodiment that may be combined with one or more of the preceding embodiments, less than 1.0%, 0.5%, 0.1%, 0.01 %, 0.001 %, or 0 % of the glycoprotein composition comprises glycation structures. 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 antibody composition comprises glycation structures.

In another embodiment that may be combined with one or more of the preceding
5 embodiments, 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 Gal α 3Gal β 4GlcNAc, terminal Gal β 4GlcNAc, terminal Gal β 3GlcNAc, core linked fucose and glycation structures.

10 The disclosure also relates to a method of reducing O-mannosylation level of a recombinant glycoprotein composition produced in a filamentous fungal cell, for example, *Trichoderma* cell, typically, *Trichoderma reesei*, said method consisting of using a filamentous fungal cell having a mutation in a *PMT* gene wherein said *PMT* gene is either:

- i. *PMT1* gene comprising the polynucleotide of SEQ ID NO:1,
- 15 ii. a functional homologous gene of *PMT1* gene, which gene is capable of restoring parental O-mannosylation level by functional complementation when introduced into a *T. reesei* strain having a disruption in said *PMT1* gene, or,
- iii. a polynucleotide encoding a polypeptide having at least 50%, at least 60%, at least 70%, at least 90%, or at least 95% identity with SEQ ID NO:2, said
20 polypeptide having protein O-mannosyltransferase activity.

The disclosure also provides a protein or antibody composition obtainable by the method described herein.

DESCRIPTION OF THE FIGURES

Figure 1 depicts results for Southern analyses of *Trichoderma reesei pmt1* deletion strains expressing antibody MAB01. A) A 5.7 kb signal is expected from parental strains M124
25 and M304 with *pmt1* ORF probe after *SpeI* + *XbaI* digestion. No signal is expected from pure *pmt1* deletion strains. B) A 3.5 kb signal is expected for *pmt1* 5'flank probe from deletion strains after *SpeI* + *Ascl* digestion. C) A 1.7 kb signal is expected for *pmt1* 3'flank probe from deletion strains after *Ascl* + *XbaI* digestions. *Ascl* does not cut intact *pmt1*
30 locus in close distance, therefore signals of over 16 kb (B) and 10 kb (C) are expected from parental strains M124 or M304. A 4.1 kb signal is expected from *PmeI* digested plasmid pTTv185 used as a control in hybridisations with both flank probes (B, C).

Figure 2 depicts a spectra of light chain of flask cultured parental *T. reesei* strain M317 (pyr4⁻ of M304) (A) and Δ *pmt1* disruptant clone 26-8A (B), day 7.

Figure 3 depicts results for Western analyses of *Trichoderma reesei* *pmt1* deletion strain M403 from fed-batch fermentation. Upper panel: MAB01 light chain, lower panel: MAB01 heavy chain. 0.1 µl of supernatant was loaded on each lane.

Figure 4 depicts a spectrum of light chain of fermenter cultured *T. reesei* strain M403
5 (pmt1 deletion strain of MAB01 antibody producing strain, clone 26-8A), day 7.

Figure 5 depicts a phylogeny of PMTs of selected filamentous fungi.

Figure 6 depicts a partial sequence alignment of the results of the PMT BLAST searches.

DETAILED DESCRIPTION

Definitions

10 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
15 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
20 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
25 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
30 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.

The terms “O-mannosylation” or “O-mannosyltransferase activity” are used herein to refer to the covalent linkage of at least one mannose to one specific amino acid via one oxygen (typically from serine or threonine). O-mannosyltransferase protein typically adds mannose to hydroxyl groups such as hydroxyl of serine or threonine residues.

In particular, O-mannosyltransferase activity may refer to the specificity of O-mannosyltransferase activity of fungal PMT gene encoding enzymes, and more specifically with the same specificity of *T. reesei* PMT1.

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, α 3-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 $\alpha 3$ and $\alpha 6$ mean the same as $\alpha 2-3$ and $\alpha 2-6$, respectively, and for hexose monosaccharide residues $\alpha 1-3$, $\alpha 1-6$, $\beta 1-2$, $\beta 1-3$, $\beta 1-4$, and $\beta 1-6$ can be shortened as $\alpha 3$, $\alpha 6$, $\beta 2$, $\beta 3$, $\beta 4$, and $\beta 6$, respectively. Lactosamine refers to type II N-acetyllactosamine, Gal $\beta 4$ GlcNAc, and/or type I N-acetyllactosamine. Gal $\beta 3$ GlcNAc 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 Man₃GlcNAc₂ (Man $\alpha 6$ (Man $\alpha 3$)Man $\beta 4$ GlcNA $\beta 4$ GlcNAc, referred to as Man3). In some embodiments Man3 glycan or its derivative Man $\alpha 6$ (GlcNAc $\beta 2$ Man $\alpha 3$)Man $\beta 4$ GlcNA $\beta 4$ GlcNAc is the major glycoform. When a fucose is attached to the core structure, preferably $\alpha 6$ -linked to reducing end GlcNAc, the N-glycan or the core of N-glycan, may be represented as Man₃GlcNAc₂(Fuc). In an embodiment the major N-glycan is Man $\alpha 3$ [Man $\alpha 6$ (Man $\alpha 3$)Man $\alpha 6$]Man $\beta 4$ GlcNA $\beta 4$ GlcNAc (Man5).

Preferred hybrid type N-glycans comprise GlcNAc β 2Man α 3[Man α 6(Man α 3)Man α 6]Man β 4GlcNA β 4GlcNAc ("GlcNAcMan5"), or b4-galactosylated derivatives thereof Gal β 4GlcNAcMan3, G1, G2, or GalGlcNAcMan5 glycoform.

- 5 A "complex N-glycan" refers to a N-glycan which has at least one GlcNAc residue, optionally by GlcNAc β 2-residue, on terminal 1,3 mannose arm of the core structure and at least one GlcNAc residue, optionally by GlcNAc β 2-residue, on terminal 1,6 mannose arm of the core structure.

- Such complex N-glycans include, without limitation, GlcNAc₂Man₃GlcNAc₂ (also referred
10 as G0 glycoform), Gal₁GlcNAc₂Man₃GlcNAc₂ (also referred as G1 glycoform), and Gal₂GlcNAc₂Man₃GlcNAc₂ (also referred as G2 glycoform), and their core fucosylated glycoforms FG0, FG1 and FG2, respectively GlcNAc₂Man₃GlcNAc₂(Fuc), Gal₁GlcNAc₂Man₃GlcNAc₂(Fuc), and Gal₂GlcNAc₂Man₃GlcNAc₂(Fuc).

- "Increased" or "Reduced activity of an endogenous enzyme": The filamentous fungal cell
15 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
20 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.

- 25 "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
30 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
5 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
10 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
15 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
20 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
25 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
30 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)
35 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
5 or decreases (reduces) the activity of a given protein (e.g., an O-mannosyltransferase or
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%,
10 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., an
O-mannosyltransferase or protease) has an increase or decrease, respectively, in the
15 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.).

20 As used herein, the terms "identical" or percent "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%,
25 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
30 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.

5 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

10 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)].

15

20

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 wordlength (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 wordlength 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.

25

30

35

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
5 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
10 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,
15 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,
20 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)
25 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

30 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* 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
 5 *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
 10 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 PMT deficient filamentous fungal cell, for
 15 example selected from *Trichoderma*, *Neurospora*, *Myceliophthora* or a *Chrysosporium* cells, such as *Trichoderma reesei* fungal cell, comprising:

- a. at least a first mutation that reduces or eliminates an endogenous protease activity compared to the parental filamentous fungal cell which does not have said first mutation (i.e. a protease-deficient mutation), and,
- 20 b. at least a second mutation in a *PMT* gene that reduces or eliminates an endogenous O-mannosyltransferase activity compared to a parental filamentous fungal cell which does not have said second mutation (i.e. a PMT-deficient mutation).

Proteases with reduced activity

25 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
 30 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.

Nine aspartic proteases have been identified in *Trichoderma* fungal cells: *pep1* (tre74156); *pep2* (tre53961); *pep3* (tre121133); *pep4* (tre77579), *pep5* (tre81004), and *pep7* (tre58669), *pep8* (tre122076), *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:134), *Trichoderma reesei* EGR49498 *pep11* (SEQ

ID NO:135), *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.

5 *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*.

- 5 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.

10 *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
15 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
20 (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
25 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
 5 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.

10 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, pep11, pep12, tsp1, slp1, slp2, slp3, slp5, slp6, slp7, slp8, tpp1, gap1*
 15 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
 20 production of the protein with reduced O-mannosylation in said filamentous fungal cell, preferably in a PMT-deficient *Trichoderma* cell.

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
 25 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
 30 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 proteases, 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 GCTCCCTGGGGTCTTGCCCGTATCTCTACCGAGAGACGCTCAACTTTGGCTC CTTCAACAAGTACCTCTACACCGCTGATGGTGGTGAGGGTGTTGATGCCTATGT CATTGACACCGGCACCAACATCGAGCACGTCGACTTTGAGGGTCGTGCCAAGT GGGGCAAGACCATCCCTGCCGGCGATGAGGACGAGGACGGCAACGGCCACG GCACTCACTGCTCTGGTACCGTTGCTGGTAAGAAGTACGGTGTTGCCAAGAAG GCCACGTCTACCGCTCAAGGTGCTCCGATCCAACGGATCCGGCACCATGTC TGACGTCGTCAAGGGCGTCGAGTACG (SEQ ID NO:17)

- 10 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 PMT-deficient
15 *Trichoderma* cell, which has a first 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 at least a second mutation in a PMT gene that reduces endogenous protein O-mannosyltransferase activity compared to a parental *Trichoderma* cell which does not
20 have said second PMT-deficient mutation.

- 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
25 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.

5 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
10 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
15 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
20 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
25 limitation, site-directed mutagenesis and PCR generated mutagenesis (see, for example, Botstein and Shortle, 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. Biol. 57: 157; Ho et al., 1989, Gene 77: 61; Horton et al., 1989, Gene 77: 61; and Sarkar and Sommer, 1990, BioTechniques 8:
30 404).

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
5 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,
10 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
15 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
20 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
25 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
30 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 PCR 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'-nitrosoguanidine (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, 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 PMT-deficient 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 another mutation in a PMT gene that reduces endogenous protein O-mannosyltransferase activity compared to a parental *Trichoderma* cell which does not have said mutation.

In certain embodiments, the PMT-deficient filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in pep1, tsp1, and slp1. In other embodiments, the PMT-deficient filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in gap1, slp1, and pep1. In certain embodiments, the PMT-deficient filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1 and gap1. In certain embodiments, the PMT-deficient filamentous fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1 and pep4. In certain embodiments, the PMT-deficient filamentous

fungal cell or *Trichoderma* cell, has reduced or no detectable protease activity in slp2, pep1, gap1, pep4 and slp1. In certain embodiments, the PMT-deficient 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 PMT-deficient 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 PMT-deficient 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 PMT-deficient 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 PMT-deficient 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 PMT-deficient 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 PMT-deficient 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 PMT-deficient 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 PMT-deficient 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, pep11, pep12, tsp1, slp2, slp3, slp7, gap1 and gap2. In certain embodiments, the PMT-deficient 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 PMT-deficient 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.

In one embodiment that may be combined with the precedent embodiments, the filamentous fungal cell of the invention does not comprise a deletion or disruption of an endogenous gene encoding a chaperone protein. In particular, said filamentous fungal cell of the invention expresses functional endogenous chaperone protein Protein Disulphide Isomerase (PDI).

Endogenous O-mannosyltransferase in filamentous fungal cells

O-mannosyltransferases are encoded by *pmt* genes in yeasts and filamentous fungi, which can be divided into three subfamilies, based on sequence homologies: PMT1, PMT2 and PMT4.

- 5 For example, in yeast *S. cerevisiae*, 7 different PMTs have been characterized: ScPMT1, ScPMT5 and ScPMT7 belong to the PMT1 subfamily. ScPMT2, ScPMT3 and ScPMT6 belong to the PMT2 subfamily and ScPMT4 belongs to the PMT4 subfamily. Such O-mannosyltransferases and their coding sequences may be identified and isolated from filamentous fungal cells and tested to determine whether reduction in their activity enables
- 10 the reduction of O-mannosylation on secreted O-mannosylated recombinant protein preferably not affecting the production of such recombinant polypeptide from the filamentous fungal cell. Methods for identifying and isolating PMTs are well known in the art. An identified O-mannosyltransferase may then be tested by deleting the gene encoding the identified O-mannosyltransferase from a filamentous fungal cell that
- 15 expresses a recombinant O-mannosylated protein, such a heterologous or mammalian O-mannosylated protein, and determining whether the deletion results in a decrease in total O-mannosyltransferase activity of the cell, preferably not affecting the level of production of the expressed recombinant protein. Methods for deleting genes and measuring levels of produced protein are well known in the art and include the methods described herein.
- 20 Three O-mannosyltransferases have been identified in *Trichoderma* fungal cells: *pmt1*, *pmt2* and *pmt3*, belonging respectively based on sequence homologies to the PMT4, PMT1 and PMT2 subfamily.

- Examples of suitable O-mannosyltransferase include, without limitation, *Trichoderma reesei pmt1* (SEQ ID NO: 2), *Trichoderma reesei pmt2* (SEQ ID NO: 3), *Trichoderma*
- 25 *reesei pmt3* (SEQ ID NO: 4) and homologs thereof. Figure 5 shows phylogeny of *pmt1* homologs in selected filamentous fungi and figure 6 shows an alignment of *pmt1* conserved domains among different species.

- In a preferred embodiment, said PMT-deficient filamentous fungal cell, e.g., a *Trichoderma* cell, has at least one mutation in a *PMT* gene selected from the group
- 30 consisting of:

- a) *PMT1* gene comprising the polynucleotide of SEQ ID NO:1,

- b) a functional homologous gene of *PMT1* gene, which functional homologous gene is capable of restoring parental O-mannosylation level by functional complementation when introduced into a *T. reesei* strain having a disruption in said *PMT1* gene, and,
- 5 c) a polynucleotide encoding a polypeptide having at least 50%, at least 60%, at least 70%, at least 90%, or at least 95% identity with SEQ ID NO:2, said polypeptide having protein O-mannosyltransferase activity.

More preferably, said PMT-deficient filamentous fungal cell, e.g., a *Trichoderma* cell, has at least one mutation in a PMT gene which

- 10 a) has a polynucleotide encoding a polypeptide having at least 50%, at least 60%, at least 70%, at least 90%, or at least 95% identity with SEQ ID NO:2, and,
- b) is capable of restoring, at least 50%, preferably about 100% of parental O-mannosylation level by functional complementation when introduced into a *T. reesei* strain having a disruption in a *T. reesei PMT1* gene.

- 15 Methods for disrupting *PMT1* gene in *T. reesei* are disclosed in the Examples below.

Sequences of homologs of *pmt1* in filamentous fungi can be found in the databases using sequence alignment search tools, such as BLAST algorithm. It includes without limitation, *A. oryzae* gi391865791, EIT75070.1 (SEQ ID NO:5), *A. niger* gi317036343, XP_001398147.2 (SEQ ID NO:6), *A. nidulans* gi67522004, XP_659063.1 (SEQ ID NO:7),
 20 *T. virens* gi358379774, EHK17453.1 (SEQ ID NO:8), *T. atroviride* gi358400594, EHK49920.1 (SEQ ID NO:9), *F. oxysporum* gi342879728, EGU80965.1 (SEQ ID NO:10), *G. zeae* gi46107450, XP_380784.1 (SEQ ID NO:11), *M. thermophila* gi367020262, XP_003659416.1 (SEQ ID NO:12), *N. crassa* gi164423013, XP_963926.2 (SEQ ID NO:13), and *P. chrysogenum* gi255953619, XP_002567562.1 (SEQ ID NO:14).

25 **Reducing endogenous protein O-mannosyltransferase activity in filamentous fungal cell of the invention**

The PMT-deficient filamentous fungal cells according to the invention have reduced activity of at least one O-mannosyltransferase activity, in order to reduce or decrease O-mannosylation in said filamentous fungal cell, preferably *Trichoderma* cell.

- 30 The activity of said O-mannosyltransferases 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 O-mannosyltransferases is achieved by reducing the expression of the O-mannosyltransferases, for example, by promoter modification or RNAi.

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

Deletion or disruption mutation can be performed as described in the above sections, in particular in relation to deletion or disruption of genes encoding proteases. These includes without limitation knock-out mutation, a truncation mutation, a point mutation, a missense
10 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 O-mannosyltransferase activity.

In certain embodiments, the mutation or modification in an O-mannosyltransferase (PMT)
15 encoding gene of the present disclosure results in a modified O-mannosyltransferase that has no detectable O-mannosyltransferase activity. In other embodiments, the at least one modification in a O-mannosyltransferase encoding gene of the present disclosure results in a modified O-mannosyltransferase 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 O-mannosyltransferase
20 activity compared to a corresponding non-modified O-mannosyltransferase.

In preferred embodiment, a mutation that reduces endogenous protein O-mannosyltransferase activity in a PMT-deficient filamentous fungal cell, e.g. *Trichoderma* cell, is a PMT-deficient cell which has a deletion or disruption of a PMT gene encoding said O-mannosyltransferase activity, resulting in no detectable expression for such
25 deleted or disrupted PMT gene.

One specific embodiment of the present invention is a PMT-deficient *Trichoderma reesei* cell, comprising

- a. at least a first mutation that reduces an endogenous protease activity compared to a parental *Trichoderma* cell which does not have said first mutation, and,
- 30 b. at least a disruption or deletion of PMT1 gene of *T. reesei*.
- c. optionally, said cell further express a heterologous protein with serine or threonine, which has reduced O-mannosylation due to said mutation in said PMT gene.

The reduction (or decrease) of O-mannosyltransferase activity may be determined by comparing the O-mannosylation level of a heterologous protein in PMT-deficient filamentous fungal cell according to the invention, with the O-mannosylation level of a heterologous protein in the parental cell which does not have said PMT-deficient mutation.

- 5 In specific embodiments, the PMT-deficient filamentous fungal cell according to the invention expresses a heterologous protein which has reduced O-mannosylation due to said mutation in said PMT gene and the O-mannosylation level on the expressed heterologous protein is at least 20%, 40%, 50%, 60%, 70%, 80%, or 90% lower than the O-mannosylation level of the heterologous protein when expressed in the parental
10 filamentous fungal cell which does not have said second PMT-deficient mutation.

- O-mannosylation level may also be determined as mole % of O-mannosylated polypeptide per total polypeptide as produced by the host cell of the invention. Analytical methods, such as MALDI TOF MS analysis may be used to determine O-mannosylation level as described in detail in the Example 1 below, section entitled "Analyses of Dpmt1 strains
15 M403, M404, M406 and M407. In brief, a polypeptide as produced by the PMT-deficient filamentous fungal cell is purified to determine its O-mannosylation level. Non O-mannosylated, and O-mannosylated structure of the polypeptide are separated and quantified by MALDI-TOF MS analysis. For example, the quantification of O-mannosylation level may be performed by determining area values or intensity of the
20 different peaks of MALDI-TOF MS spectrum. An O-mannosylation level of 5% as determined by such method, using area values or intensity, reflects that about 95% (mol%) of the analysed polypeptides in the composition are not O-mannosylated. In specific embodiments, the PMT-deficient filamentous fungal cell expresses a heterologous protein which has reduced O-mannosylation due to said mutation in said PMT gene, and the O-mannosylation level on the expressed heterologous protein (for example, as defined
25 above by determining area or intensity values of MALDI TOF MS spectrum peaks) is reduced to less than 25 %, 20%, 17 %, 15%, 13%, 12%, 11%, 10%, 9 %, 8 %, 7 %, 6 %, 5 %, 4 %, 3%, 2 %, or 1%, or 0.5 % (as mole % of mannose residues per polypeptide chain).

- 30 In an embodiment, the heterologous protein with reduced O-mannosylation 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,
- 5 h) their antigen-binding fragments.

In a specific embodiment, a mutation that reduces endogenous O-mannosyltransferase activity is a deletion or a disruption of a PMT gene encoding said endogenous protein O-mannosyltransferase activity. For example in *Trichoderma* cell, a mutation that reduces
 10 endogenous O-mannosyltransferase activity is a deletion or a disruption of a PMT1 gene.

Filamentous fungal cell for producing glycoproteins with reduced O-mannosylation and mammalian-like N-glycans

The filamentous fungal cells according to the present invention may be useful in particular for producing heterologous glycoproteins with reduced O-mannosylation and mammalian-
 15 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 with no or reduced O-mannosylation and with mammalian-like N-glycan as major glycoforms.

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

In certain embodiment, the glycoprotein comprises, as a major glycoform, the mammalian-like N-glycan having the formula

$$[(\text{Gal}\beta 4)_x \text{GlcNAc}\beta 2]_z \text{Man}\alpha 3 [(\text{Gal}\beta 4)_y \text{GlcNAc}\beta 2]_w \text{Man}\alpha 6 \text{Man}\{\beta 4 \text{GlcNAc}\beta \text{GlcNAc}$$
 25) 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 x, y, z and w are 0 or 1, independently. In an embodiment w and z are 1.

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

- 30 i. $\text{Man}\alpha 3 [\text{Man}\alpha 6 (\text{Man}\alpha 3) \text{Man}\alpha 6] \text{Man}\beta 4 \text{GlcNAc}\beta 4 \text{GlcNAc}$ (Man5 glycoform);

- ii. GlcNAc β 2Man α 3[Man α 6(Man α 3)Man α 6]Man β 4GlcNA β 4GlcNAc (GlcNAcMan5 glycoform);
- iii. Man α 6(Man α 3)Man β 4GlcNA β 4GlcNAc (Man3 glycoform);
- iv. Man α 6(GlcNAc β 2Man α 3)Man β 4GlcNA β 4GlcNAc (GlcNAcMan3) or,
- 5 v. complex type N-glycans selected from the G0, G1, or G2 glycoform.

In an embodiment, the glycoprotein 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 α 3[Man α 6(Man α 3)Man α 6]Man β 4GlcNA β 4GlcNAc (Man5). In specific embodiments, the filamentous fungal cell produces glycoproteins with, as major glycoform, the trimannosyl N-glycan structure Man α 3[Man α 6]Man β 4GlcNA β 4GlcNAc. In other embodiments, the filamentous fungal cell produces glycoproteins with, as major glycoform, the G0 N-glycan structure GlcNAc β 2Man α 3[GlcNAc β 2Man α 6]Man β 4GlcNA β 4GlcNAc.

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

In some embodiments, Man3GlcNAc2 N-glycan (i.e. Man α 3[Man α 6]Man β 4GlcNA β 4GlcNAc) 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 protein with reduced O-mannosylation, as expressed in a filamentous fungal cells of the invention.

In other embodiments, GlcNAc2Man3 N-glycan (for example G0 GlcNAc β 2Man α 3[GlcNAc β 2Man α 6]Man β 4GlcNA β 4GlcNAc) 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 protein with reduced O-mannosylation, 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 a heterologous protein with reduced O-mannosylation, 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 a heterologous protein with reduced O-mannosylation, 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 protein with reduced O-mannosylation, as expressed in a filamentous fungal cells of the invention.

10 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 protein with reduced O-mannosylation, as expressed in a filamentous fungal cells of the invention.

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

The Neu5Gc and Gal α - (non-reducing end terminal Gal α 3Gal β 4GlcNAc) 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 Gal α - 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 composition comprises Neu5Gc and/or Gal α - structure. In an embodiment that may be combined with the preceding 25 embodiments, less than 0.1%, 0.01 %, 0.001 % or 0 % of the N-glycans and/or O-glycans of the antibody composition comprises Neu5Gc and/or Gal α - 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 30 embodiments, less than 0.1%, 0.01 %, 0.001 %, or 0 % of the N-glycan of the glycoprotein composition comprises core fucose 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-glycan of the antibody composition comprises core fucose structures.

The terminal Gal β 4GlcNAc structure of N-glycan of mammalian cell produced glycans
5 affects bioactivity of antibodies and Gal β 3GlcNAc 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
glycoprotein composition comprises terminal galactose epitopes Gal β 3/4GlcNAc. In an
embodiment that may be combined with one or more of the preceding embodiments, less
10 than 0.1%, 0.01 %, 0.001 %, or 0 % of the N-glycan of the antibody composition
comprises terminal galactose epitopes Gal β 3/4GlcNAc.

Glycation is a common post-translational modification of proteins, resulting from the
chemical reaction between reducing sugars such as glucose and the primary amino
15 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
20 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 glycoprotein
composition comprises glycation structures. 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
antibody composition comprises glycation structures.

25 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
Gal α 3Gal β 4GlcNAc, terminal Gal β 4GlcNAc, terminal Gal β 3GlcNAc, core linked fucose
and glycation structures.

30 In certain embodiments, such glycoprotein protein with mammalian-like N-glycan and
reduced O-mannosylation, 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,
35 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 and reduced O-mannosylation as produced by the filamentous fungal cell according to the invention may be a therapeutic glycoprotein such as rituximab.

- 5 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
10 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
15 the production of core Man₃GlcNAc₂ N-glycans, as acceptor substrate for GnTI and GnTII subsequent reactions and bypassing the need for mannosidase α 1,2 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 Man₅ 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
25 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.
30 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 PMT-deficient 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) α 1,2 mannosidase,
- ii) N-acetylglucosaminyltransferase I catalytic domain,
- iii) α mannosidase II,
- iv) N-acetylglucosaminyltransferase II catalytic domain,
- v) β 1,4 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
 5 embodiment the filamentous fungal cell is used for the production of a glycoprotein, wherein the glycan(s) comprise or consist of Man α 3[Man α 6]Man β 4GlcNAc β 4GlcNAc, 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-
 10 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
 15 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-
 20 acetylglucosamine to a terminal Man α 3 and a polynucleotide encoding an N-acetylglucosaminyltransferase II catalytic domain (GnTII), that catalyses N-acetylglucosamine to a terminal Man α 6 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
 25 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 $\rightleftharpoons$ 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
 30 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 5 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 15 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 20 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 25 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 $\text{UDP-N-acetyl-D-glucosamine} + 6\text{-(}\alpha\text{-D-mannosyl)-}\beta\text{-D-mannosyl-R} \rightleftharpoons \text{UDP} + 6\text{-(2-(N-acetyl-beta-D-glucosaminyl)-}\alpha\text{-D-mannosyl)-}\beta\text{-D-mannosyl-R}$, 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 35 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.*
5 *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
10 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-
15 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-
20 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
25 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
30 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.

- 5 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.
- 10 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
- 15 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
- 20 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
- 25 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 β 4-GalT1, β 4-GalT-II, -III, -IV, -V, and -
- 30 VI, such as mammalian or human β 4-GalT1 or β 4GalT-II, -III, -IV, -V, and -VI or any combinations thereof. β 4-GalT1, β 4-GalTII, or β 4-GalTIII are especially useful for galactosylation of terminal GlcNAc β 2-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 β 4GalT enzymes include β 4GalT1, 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); β 4GalTII enzymes such as human β 4GalTII BAA75819.1, Chinese hamster *Cricetulus griseus* AAM77195, *Mus musculus* enzyme BAA34385, and Japanese Medaka fish *Oryzias latipes* BAH36754; and β 4GalTIII enzymes such as human β 4GalTIII 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 Mn^{2+} , Ca^{2+} or Mg^{2+} 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* 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 protein to be produced,
- b) a mutation in a PMT gene, for example *T. reesei* pmt1 gene, that reduces or eliminates endogenous O-mannosyltransferase activity compared to a parental *Trichoderma* cell which does not have said second mutation,
 - c) a polynucleotide encoding a protein having at least one serine or threonine, 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 β 1,4 galactosyltransferase,
 - g) optionally, a polynucleotide or polynucleotides encoding UDP-Gal 4 epimerase and/or transporter.

Targeting Sequences

In certain embodiments, recombinant enzymes, such as α 1,2 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*,
 5 *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
 10 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
 15 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
 20 Mns1 fused to α 2-mannosidase (Martinet et al, Biotech Lett 1998, 1171), N-terminal portion of Kre2 linked to catalytic domain of GnTI or β 4GalT (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
 25 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).

30 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	TrelD	Amino acid sequence
human GNT2	-	MRFRITYKRKVLILTLVVAACGFVLWSSNGRQR KNEALAPPLDAEPARGAGGRGGDHP (SEQ ID NO:42)
KRE2	21576	MASTNARYVRYLLIAFFTILVFYFVSNSKYEGV DLNKGTFAPDSTKTTTPK (SEQ ID NO:43)
KRE2-like	69211	MAIARPVRALGGLAAILWCFFLYQLLRPSSSY NSPGDRYINFERDPNLDPSTG (SEQ ID NO:44)
Och1	65646	MLNPRRALIAAAFILTVFFLISRSHNSEASTS (SEQ ID NO:45)
Anp1	82551	MMPRHHSSGFSNGYPRADTFEISPHRFQPPRA TLPPHRKRKRRTAIRVGIAVVVLVLVWFGQPR SVASLISLGILSGYDDLKLE (SEQ ID NO:46)
Van1	81211	MLLPKGGLDWRSARAQIPTRALWNAVTRTR FILLVGITGLILLWLRGVSTSAE (SEQ ID NO:47)

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

- 5 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
- 10 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 protein having reduced O-mannosylation

- 15 The filamentous fungal cells as described above are useful in methods for producing a protein having reduced O-mannosylation.

Accordingly, in another aspect, the invention relates to a method for producing a protein having reduced O-mannosylation, comprising:

- a) providing a PMT-deficient *Trichoderma* cell having a mutation in a PMT gene that reduces endogenous protein O-mannosyltransferase activity as compared to parental strain which does not have such mutation, and further comprising a polynucleotide encoding a protein with serine or threonine, which may be O-mannosylated,
- 20

- b) culturing said PMT-deficient *Trichoderma* cell to produce said protein having reduced O-mannosylation.

In such method, the produced protein has reduced O-mannosylation due to said mutation in said PMT gene as described in the previous sections. The PMT-deficient *Trichoderma* cell may optionally have reduced endogenous protease activity as described in the previous sections.

The filamentous fungal cells and methods of the invention are useful for the production of protein with serine or threonine which may be O-mannosylated. For example, it is particularly useful for the production of protein which are O-mannosylated when produced in a parental PMT-functional filamentous fungal host cell, for example, in at least one *Trichoderma* cell which is wild type for PMT1 gene, such as SEQ ID NO:1.

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, the protein which may be O-mannosylated is a heterologous protein, preferably a mammalian protein. In other embodiments, the heterologous protein is a non-mammalian protein.

In certain embodiments, the protein which may be O-mannosylated is a glycoprotein with N-glycan posttranslational modifications.

In certain embodiments, a mammalian protein which may be O-mannosylated 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.

- 5 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
10 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,
15 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 is at least 0.5, at least 1,
20 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
25 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
30 acid residues.

In certain embodiments where the mammalian glycoprotein 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 certain embodiments, where the protein with reduced O-mannosylation, e.g. an antibody, is purified from cell culture, the culture contains at least 70%, 80%, 90%, 95% or 100% of the proteins that is not O-mannosylated (mol%, as determined for example by MALDI TOF MS analysis, and measuring area or intensity of peaks as described in the Example 1 below).

In certain embodiments where the protein with at least one serine or threonine residue which may be O-mannosylated is purified from cell culture, and where the strain is a *Trichoderma* cell genetically engineered to produce complex N-glycans, the culture further comprises at least 5%, 10%, 15%, 20%, 25%, 30% of secreted complex neutral N-glycans (mol%) compared to total secreted neutral N-glycans (as measured for example as described in WO2012069593).

In other embodiments, the heterologous protein with reduced O-mannosylation, for example, the antibody, comprises the trimannosyl N-glycan structure $\text{Man}\alpha 3[\text{Man}\alpha 6]\text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc}$. In some embodiments, the $\text{Man}\alpha 3[\text{Man}\alpha 6]\text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc}$ structure represents at least 20%, 30%; 40%, 50%; 60%, 70%, 80% (mol%) or more, of the total N-glycans of the heterologous protein with reduced O-mannosylation. In other embodiments, the heterologous protein with reduced O-mannosylation comprises the G0 N-glycan structure $\text{GlcNAc}\beta 2\text{Man}\alpha 3[\text{GlcNAc}\beta 2\text{Man}\alpha 6]\text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc}$. 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 protein with reduced O-mannosylation. 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 protein with reduced O-mannosylation, for example an antibody. In certain

embodiments, the culture or the heterologous protein, for example an antibody, comprises no galactosylated N-glycans.

In certain embodiments, the heterologous (purified) protein is an antibody, a light chain antibody, a heavy chain antibody or a Fab, that comprises Man3, GlcNAcMan3, Man5, GlcNAcMan5, G0, core G0, G1, or G2 N-glycan structure as major glycoform and less than 35%, 20%, 17%, 15%, 13%, 12%, 11%, 10%, 9 %, 8 %, 7 %, 6 %, 5 %, 4 %, 3%, 2 %, or 1%, or less than 0.5 % of O-mannosylation level (as mole% as determined for example by MALDI TOF MS analysis, and measuring area or intensity of peaks as described in Example 1).

10 In a specific embodiment, the invention therefore relates to a method for producing an antibody having reduced O-mannosylation, comprising:

- a. providing a PMT-deficient *Trichoderma* cell having
 - i. a mutation that reduces endogenous protein O-mannosyltransferase activity as compared to parental strain which does not have such mutation and
 - 15 ii. a polynucleotide encoding a light chain antibody and a polynucleotide encoding a heavy chain antibody,
- b. culturing the cell to produce said antibody, consisting of heavy and light chains, having reduced O-mannosylation.

20 In such specific embodiments of the methods related to the production of antibody, at least 70%, 80%, 90%, 95%, 97%, 98 %, 99% or 100% of the produced antibody is not O-mannosylated (mol%, as determined for example by MALDI TOF MS analysis, and measuring area or intensity of peaks as described in Example 1.

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 polypeptide. 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 certain embodiments, the filamentous fungal cell of the invention overexpress KEX2 protease. In an embodiment the heterologous protein 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 protein composition, for example the antibody composition, obtainable or obtained by the method as disclosed above.

In specific embodiment, such antibody composition obtainable or obtained by the methods of the invention, comprises at least 70%, 80%, 90%, 95%, or 100% of the antibodies that are not O-mannosylated (mol%, as determined for example by MALDI TOF MS analysis, and measuring area or intensity of peaks as described in Example 1). In other specific

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

- (i) $\text{Man}\alpha 3[\text{Man}\alpha 6(\text{Man}\alpha 3)\text{Man}\alpha 6]\text{Man}\beta 4\text{GlcNA}\beta 4\text{GlcNAc}$ (Man5 glycoform);
- (ii) $\text{GlcNAc}\beta 2\text{Man}\alpha 3[\text{Man}\alpha 6(\text{Man}\alpha 3)\text{Man}\alpha 6]\text{Man}\beta 4\text{GlcNA}\beta 4\text{GlcNAc}$, or $\beta 4$ -galactosylated variant thereof;
- (iii) $\text{Man}\alpha 6(\text{Man}\alpha 3)\text{Man}\beta 4\text{GlcNA}\beta 4\text{GlcNAc}$;
- (iv) $\text{Man}\alpha 6(\text{GlcNAc}\beta 2\text{Man}\alpha 3)\text{Man}\beta 4\text{GlcNA}\beta 4\text{GlcNAc}$, or $\beta 4$ -galactosylated variant thereof: or,
- (v) complex type N-glycans selected from the G0, G1 or G2 glycoform.

- 10 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.

The invention also relates to a method of reducing O-mannosylation level of a recombinant glycoprotein composition produced in a *Trichoderma* cell, said method consisting of using a *Trichoderma* cell having a mutation in a *PMT* gene wherein said

- 15 *PMT* gene is either:

- a. *PMT1* gene comprising the polynucleotide of SEQ ID NO:1,
- b. a functional homologous gene of *PMT1* gene, which gene is capable of restoring parental O-mannosylation level by functional complementation when introduced into a *T. reesei* strain having a disruption in said *PMT1* gene, or,
- c. a polynucleotide encoding a polypeptide having at least 50%, at least 60%, at least 70%, at least 90%, or at least 95% identity with SEQ ID NO:2, said polypeptide having protein O-mannosyltransferase activity.

In one specific embodiment of such method, said *Trichoderma* cell is *Trichoderma reesei*.

- 25 In another specific embodiment of such method, said recombinant glycoprotein comprises at least a light chain antibody or its fragments comprising at least one serine or threonine residue and with at least one N-glycan.

EXAMPLES

- 30 As more specifically exemplified in Example 2, after deletion of *pmt1*, almost 95% of purified mAb and 70% of Fab molecules no longer contained any O-mannose residues. In

contrast, as exemplified in Examples 3 to 4, O-mannosylation level analysis performed on *pmt2* and *pmt3* deletion strains did not exhibit any appreciable reduction in O-mannosylation. Together with the titer and growth analysis set forth in Example 2, these results demonstrate that filamentous fungal cells, such as *Trichoderma* cells, can be genetically modified to reduce or suppress O-mannosylation activity, without adversely affecting viability and yield of produced glycoproteins. As such, *pmt1* is identified a valuable target to reduce O-mannosylation of secreted proteins and to improve product quality of biopharmaceuticals produced by *Trichoderma reesei*.

Example 1: *pmt1* deletion in a *Trichoderma reesei* strain

This example demonstrates that *pmt1* is a valuable target to reduce O-mannosylation of secreted proteins and to improve product quality of biopharmaceuticals produced by *Trichoderma reesei*.

Generation of pmt1 deletion plasmids

Three different deletion plasmids (pTTv36, pTTv124, pTTv185) were constructed for deletion of the protein O-mannosyltransferase gene *pmt1* (TreID75421). All the plasmids contain the same 5' and 3' flanking regions for correct integration to the *pmt1* locus. The difference between the three plasmids is the marker used in the selection; pTTv36 contains a gene encoding acetamidase of *Aspergillus nidulans* (*amdS*), pTTv124 contains a loopout version (blaster cassette) of the *amdS* marker and pTTv185 a loopout version (blaster cassette) of a gene encoding orotidine-5'-monophosphate (OMP) decarboxylase of *T. reesei* (*pyr4*).

The third deletion construct, pTTv185, for the protein O-mannosyltransferase gene *pmt1* (TreID75421) was designed to enable removal of the selection marker from the *Trichoderma reesei* genome after successful integration and thereby recycling of the selection marker for subsequent transformations. In this approach, the recycling of the marker, i.e. removal of *pyr4* gene from the deletion construct, resembles so called blaster cassettes developed for yeasts (Hartl, L. and Seiboth, B., 2005, Curr Genet 48:204-211; and Alani, E. et al., 1987, Genetics 116:541-545). Similar blaster cassettes have also been developed for filamentous fungi including *Hypocrea jecorina* (anamorph: *T. reesei*) (Hartl, L. and Seiboth, B., 2005, Curr Genet 48:204-211).

The TreID number refers to the identification number of a particular protease gene from the Joint Genome Institute *Trichoderma reesei* v2.0 genome database. Primers for

construction of deletion plasmids were designed either manually or using Primer3 software (Primer3 website, Rozen and Skaletsky (2000) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386).

The principle of the blaster cassette using *pyr4* as the marker gene is as follows: *pyr4*,
5 encoding orotidine-5'-monophosphate (OMP) decarboxylase of *T. reesei* (Smith, J.L., et al., 1991, Current Genetics 19:27-33) 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 utilisation of 5-fluoroorotic acid (5-FOA) in generation of mutant strains lacking OMP decarboxylase activity (*pyr4*⁻ strains) is based
10 on the conversion of 5-FOA to a toxic intermediate 5-fluoro-UMP by OMP decarboxylase. Therefore, cells which have a mutated *pyr4* gene are resistant to 5-FOA, but in addition are also auxotrophic for uridine. The 5-FOA resistance can in principle result also from a mutation in another gene (*pyr2*, orotate phosphoribosyltransferase), and therefore the spontaneous mutants obtained with this selection need to be verified for the *pyr4*⁻
15 genotype by complementing the mutant with the *pyr4* gene. Once mutated, the *pyr4* gene can be used as an auxotrophic selection marker in *T. reesei*. In our blaster cassette *pyr4* is followed by a 310 bp direct repeat of *pyr4* 5' untranslated region (5'UTR) and surrounded by 5' and 3' flanking regions of the gene to be deleted. Integration of the deletion cassette is selected via the *pyr4* function. Removal of the *pyr4* marker is then
20 forced in the presence of 5-FOA by recombination between the two homologous regions (direct repeat of 5'UTR) resulting in looping out of the selection marker and enabling the utilisation of the same blaster cassette (*pyr4* loopout) in successive rounds of gene deletions. After looping out, only the 310 bp sequence of 5'UTR remains in the locus.

Thus, the *pyr4* selection marker and the 5' direct repeat (DR) fragment (310 bp of *pyr4*
25 5'UTR) were produced by PCR using plasmid containing a genomic copy of *T. reesei pyr4* as a template. Both fragments contained 40 bp overlapping sequences needed to clone the plasmid with the loopout cassette using homologous recombination in yeast (see below). To enable possible additional cloning steps, an *Ascl* digestion site was placed between the *pyr4* marker and the 5' direct repeat and *NotI* sites to surround the complete
30 blaster cassette.

1100 bp of 5' and 1000 bp of 3' flanking regions were selected as the basis of the *pmt1* deletion plasmids. The flanking region fragments were produced by PCR using a *T. reesei* wild type strain QM6a (ATCC13631) as the template. For the yeast homologous recombination system used in cloning (see below), overlapping sequences for the vector

and the selection marker were placed to the appropriate PCR-primers. To enable marker switch in the construct, *NotI* restriction sites were introduced between the flanking regions and the selection marker. *PmeI* restriction sites were placed between the vector and the flanking regions for removal of vector sequence prior to transformation into *T. reesei*.

5 Vector backbone pRS426 was digested with restriction enzymes (*EcoRI* and *XhoI*).

First deletion plasmid for *pmt1* (plasmid pTTv36, Table 2) used *amdS*, a gene encoding acetamidase of *Aspergillus nidulans*, as the marker. The marker cassette was digested from an existing plasmid pHHO1 with *NotI*. All fragments used in cloning were separated with agarose gel electrophoresis and correct fragments were isolated from the gel with a
10 gel extraction kit (Qiagen) using standard laboratory methods.

To construct the first deletion plasmid pTTv36, the vector backbone and the appropriate marker and flanking region fragments were transformed into *Saccharomyces cerevisiae* (strain H3488/FY834). The yeast transformation protocol was based on the method for homologous yeast recombination described in the *Neurospora* knockouts workshop
15 material of Colot and Collopy, (Dartmouth Neurospora genome protocols website), and the Gietz laboratory protocol (University of Manitoba, Gietz laboratory website). The plasmid DNA from the yeast transformants was rescued by transformation into *Escherichia coli*. A few clones were cultivated, plasmid DNA was isolated and digested to screen for correct recombination using standard laboratory methods. A few clones with
20 correct insert sizes were sequenced and stored.

To clone the second *pmt1* deletion plasmid (pTTv124, Table 2), the *amdS* marker was removed from the deletion plasmid pTTv36 with *NotI* digestion and replaced by another variant of the blaster cassette, *amdS* loopout cassette containing the *amdS* selection marker gene, followed by *AscI* restriction site and a 300 bp direct repeat of *amdS* 5'UTR.
25 The *amdS* blaster cassette functions in a similar manner to the *pyr4* blaster cassette. The clones containing the *amdS* blaster cassette are able to grow on acetamide as sole nitrogen source. On medium containing 5-fluoroacetamide (5-FAA) a functional *amdS* gene will convert 5-FAA to a toxic fluoroacetate and therefore, in the presence of 5-FAA, removal of *amdS* gene is beneficial to the fungus. Removal of *amdS* blaster cassette is
30 enhanced via the 300 bp DRs in the cassette like in the *pyr4* blaster cassette, which enables the *amdS* gene to loop out via single crossover between the two DRs. Resulting clones are resistant to 5-FAA and unable to grow on acetamide as the sole nitrogen source.

The fragments needed for the *amdS* blaster cassette were produced by PCR using a plasmid p3SR2 (Hynes M.J. et al, 1983, Mol. Cell. Biol. 3:1430-1439) containing a genomic copy of the *amdS* gene as the template. For the yeast homologous recombination system used in cloning (see above), overlapping sequences were placed to the appropriate PCR-primers. To enable marker switch in the construct, *NotI* restriction sites were kept between the flanking regions and the blaster cassette. Additional restriction sites *FseI* and *AsiSI* were introduced to the 5' end of *amdS* and an *Ascl* site between *amdS* and *amdS* 5'DR. The plasmid pTTv124 was constructed using the yeast recombination system described above. The plasmid DNA from the yeast transformants was rescued by transformation into *Escherichia coli*. A few clones were cultivated, plasmid DNA was isolated and digested to screen for correct recombination using standard laboratory methods. A few clones with correct insert sizes were sequenced and stored.

To clone the third *pmt1* deletion plasmid (pTTv185, Table 2), the *amdS* marker was removed from the deletion plasmid pTTv36 with *NotI* digestion and replaced by the *pyr4* blaster cassette described above. The *pyr4* blaster cassette was obtained from another plasmid with *NotI* digestion, ligated to *NotI* cut pTTv36 and transformed into *E. coli* using standard laboratory methods. A few transformants were cultivated, plasmid DNA isolated and digested to screen for correct ligation and orientation of the *pyr4* blaster cassette using standard laboratory methods. One clone with correct insert size and orientation was sequenced and stored.

These deletion plasmids for *pmt1* (pTTv36, pTTv124 and pTTv185) result in 2465 bp deletion in the *pmt1* locus and cover the complete coding sequence of PMT1.

Table 2. Primers for generating deletion plasmids pTTv36, pTTv124 and pTTv185 for protein O-mannosyltransferase 1 (*pmt1*, TreID75421)

Deletion plasmid pTTv36 for <i>pmt1</i> (TreID75421), vector backbone pRS426	
Primer	Sequence
75421_5'F	CGATTAAGTTGGGTAACGCCAGGGTTTTCCAGTCACGACGGTTTAAACGCTGCAGGGCGTACAGAACT (SEQ ID NO:48)
75421_5'R	ATCTCTCAAAGGAAGAATCCCTTCAGGGTTGCGTTTCCAGTGCGGCCGCGGCTCTAAAATGCTTCACAG (SEQ ID NO:49)
75421_3'F	CGGTTCTCATCTGGGCTTGCTCGGTCCTGGCGTAGATCTAGCGGCCGCACGATGATGATGACAGCCAG (SEQ ID NO:50)
75421_3'R	GTGGAATTGTGAGCGGATAACAATTTACACAGGAAACAGCGTTTAAACCGTCCAGCTCCCGCAGCGCC (SEQ ID NO:51)
Deletion plasmid pTTv124 for <i>pmt1</i> (TreID75421), vector backbone pTTv36	

T282_75421_amds_5for	ATCGCTAACTGCTTTCTCTTCTGTGAAGCATTTTAGAGCCGCGGC CGCGGCCGGCCGCGATCGCCTAGATCTACGCCAGGACCG (SEQ ID NO:52)
T283_amds_3rev_loop	CGGTCCTGGCGTAGATCTAGGGCGCGCCACTGGAAACGCAACC CTGAA (SEQ ID NO:53)
T284_amds_loop_5for	TTCAGGGTTGCGTTTCCAGTGGCGCGCCCTAGATCTACGCCAGG ACCG (SEQ ID NO:54)
T287_75421_loop_3rev	AGCATCATGACCGCCCCCTTCTGGCTGTCATCATCATCGTGCGG CCGCGATTATTGCACAAGCAGCGA (SEQ ID NO:55)

Deletion plasmid pTTv185 for *pmt1* (TreID75421), vector backbone pTTv36

Primer	Sequence
no new primers, pTTv36 digested with NotI and ligated with <i>pyr4</i> -loopout fragment obtained from another plasmid	

Generation of pmt1 deletion strains M403, M404, M406 and M407

To generate a *pyr4* negative target strain suitable for the deletion of *pmt1* using plasmid pTTv185, the MAB01 antibody producing strain M304 was subjected to selection in the presence of 5-fluoro-orotic acid in order to select for strains containing impaired *pyr4* genes. The generation of the strain M304 is described in the International Patent Application No. PCT/EP2013/05012. *T. reesei* strain M304 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, $\Delta pep1\Delta tsp1\Delta s/p1$, and overexpresses *T. reesei* KEX2.

Spores of M304 were spread onto minimal medium plates containing 20 g/l glucose, 2 g/l proteose peptone, 5 mM uridine and 1.5 g/l 5-FOA, pH 4.8. Some 5-FOA resistant colonies were streaked after 5-7 days onto plates described above with 1 ml/l Triton X-100 supplementation. A few clones were further purified to single cell clones via consecutive purification platings: a small piece of mycelia was picked to 0.8% NaCl – 0.025% Tween 20 – 20% glycerol, suspended thoroughly by vortexing and filtrated through a cotton-filled pipette tip. Purified clones were sporulated on plates containing 39 g/l potato dextrose agarose. These clones were tested for uridine auxotrophy by plating spores onto minimal medium plates (20 g/l glucose, 1 ml/l Triton X-100, pH 4.8) with and without 5 mM uridine supplementation. No growth was observed on plates without uridine indicating the selected clones were putative *pyr4*⁻. Clones were stored for future use and one of them was designated with strain number M317.

Pmt1 was deleted from M317 (*pyr4*⁻ of the strain M304) using the *pmt1* deletion cassette from plasmid pTTv185 described above. To remove the vector sequence, plasmid pTTv185 ($\Delta pmt1$ -*pyr4*) was digested with *PmeI* + *XbaI* and the correct fragment was

purified from an agarose gel using QIAquick Gel Extraction Kit (Qiagen). Approximately 5 µg of the *pmt1* deletion cassette was used to transform strain M317. Preparation of protoplasts and transformation for *pyr4* selection 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).

100 colonies were picked as selective streaks. 40 transformants were screened by PCR using the primers in Table 3 for the correct integration of the deletion cassette using standard laboratory methods. 12 putative deletion clones were purified to single cell clones. Purified clones were rescreened for integration and for deletion of *pmt1* ORF using primers on Table 5. Four clones (in duplicate) were pure disruptants (i.e. no signal with ORF primers).

Table 3. Primers for screening integration of deletion cassette pTTv185 and for deletion of protein O-mannosyltransferase 1 (*pmt1*, TrlD75421) from M317.

Primer	Sequence
T296_75421_5int	TATGGCTTTAGATGGGGACA (SEQ ID NO:56)
T027_Pyr4_orf_start_rev	TGCGTCGCCGTCTCGCTCCT (SEQ ID NO:57)
T061_pyr4_orf_screen_2 F	TTAGGCGACCTCTTTTCCA (SEQ ID NO:58)
T297_75421_3int	CCTGTATCGTCCTGTTCC (SEQ ID NO:59)
T359_pmt1_orf_for	GCGCCTGTGCGAGTCGGCATT (SEQ ID NO:60)
T360_pmt1_orf_rev	CACCGGCCATGCTCTTGCCA (SEQ ID NO:61)
T756_pmt1_orf_for2	CAAGGTGCCCTATGTCGC (SEQ ID NO:62)
T757_pmt1_orf_rev2	GATCGGGTCAGGACGGAA (SEQ ID NO:63)

15 Deletion of *pmt1* was verified by Southern analyses. DNA for Southern analyses was extracted with Easy-DNA kit for genomic DNA isolation (Invitrogen) essentially according to the manufacturer's instructions.

Southern analyses were essentially performed according to the protocol for homologous hybridizations in Sambrook et al. (1989, *Molecular Cloning: A laboratory manual*. 2nd Ed., Cold Spring Harbor Laboratory Press) using radioactive labeling (³²P-dCTP) and DecaLabel Plus kit (Fermentas). Southern digestion schemes were designed using Geneious Pro software (Geneious website). Fragments for probes were produced by PCR using the primers listed in Table 4 using a *T. reesei* wild type strain QM6a (ATCC13631) as the template. PCR products were separated with agarose gel electrophoresis and correct fragments were isolated from the gel with a gel extraction kit (Qiagen) using standard laboratory methods.

Table 4. Primers for production of probe fragments used in Southern analyses of protein O-mannosyltransferase 1 (*pmt1*, TreID75421) deletion strains.

Primer	Sequence
T635_pmt1_5f_for	AGCCTGTCTGAGGGACGG (SEQ ID NO:64)
T636_pmt1_5f_rev	CAAGGTCGAGATTCGGCA (SEQ ID NO:65)
T637_pmt1_3f_for	CAGAAGGGGGCGGTCAT (SEQ ID NO:66)
T638_pmt1_3f_rev	GTCCCAGCTCCCGCTCT (SEQ ID NO:67)
T359_pmt1_orf_for	GCGCCTGTCGAGTCGGCATT (SEQ ID NO:68)
T360_pmt1_orf_rev	CACCGGCCATGCTCTTGCCA (SEQ ID NO:69)

None of the clones hybridised with *pmt1* ORF probe (Fig. 1A) indicating successful deletion of *pmt1*. Analyses using 5' and 3' flank probes revealed that four of the clones were single integrants (Fig. 1B and 1C; 26-8A and B, 26-21A and B). Four clones gave additional signals and thus indicated multiple integration of the deletion cassette. Four pure clones (with and without additional copies of the deletion cassette) have been stored for future use (M403; 26-8A, M404; 26-19A, M406; 26-16B and M407; 26-19B).

10 Example 2 Analyses of $\Delta pmt1$ strains M403, M404, M406 and M407

Shake flask cultivation of *T. reesei* M304 and eight *pmt1* deletion strains (26-8A (M403), 26-8B, 26-16A, 26-16B (M406), 26-19A (M404), 26-19B (M407), 26-21A, 26-21B) was carried out in *Trichoderma* minimal medium with 40 g/l lactose, 20 g/l spent grain extract, 100 mM PIPPS, 9 g/l casamino acids, pH 5.5 at +28°C, 200 rpm. Samples were collected on days 3, 5, 7 and 10 by vacuum filtration. Supernatant samples were stored to -20°C (antibody and glycan analyses) or used in pH determinations. Mycelia for cell dry weight determinations were rinsed once with DDIW and dried at +100°C for 20-24 h. Mycelia for genomic DNA extraction were rinsed once with DDIW and stored to -20°C.

O-mannosylation status analysis was performed to shake flask cultivations of *T. reesei* M304, eight *pmt1* disruptants (pTTv185: 26-8A, 26-8B, 26-16A, 26-16B, 26-19A, 26-19B, 26-21A, 26-21B). All were cultivated in TrMM – 40 g/l lactose – 20 g/l SGE – 100 mM PIPPS – 9 g/l casamino acids, pH 5.5 at +28°C and samples were taken on time point days 3, 5, 7 and 10.

MAB01 antibody from each sample from day 7 was purified from 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. For O-mannosylation analysis, 10 µg of protein was incubated in 6 M Guanidinium HCl for 30 minutes at +60°C after which 5 µl of fresh 0.1 M DTT was added and incubated again as above. The samples were purified using Poros R1 96-well plate and the resulting light chains were analysed using MALDI-
 5 TOF MS. All were made as duplicates.

In flask cultures the O-mannosylation status in *pmt1* disruptants was remarkably changed; all $\Delta pmt1$ disruptants looked the same – nearly complete loss of O-mannosylation in MAB01 LC (Fig. 2: Spectra of light chain of flask cultured parental *T. reesei* strain M317 (pyr4⁻ of M304) (A) and $\Delta pmt1$ disruptant clone 26-8A (B), day 7).

10 Fermentation of $\Delta pmt1$ strain M403

Fermentation was carried out with $\Delta pmt1$ strain M403 (clone 26-8A; pTTv185 in M317). Fermentation culture medium contained 30 g/l glucose, 60 g/l lactose, 60 g/l whole spent grain at pH 5.5. Lactose feed was started after glucose exhaustion. Growth temperature was shifted from +28°C to +22°C after glucose exhaustion. Samples were collected by
 15 vacuum filtration. Supernatant samples were stored to -20°C.

In Figure 3 is shown the Western analyses of supernatant samples. MAB01 heavy and light chains were detected from supernatant after day three. Despite the deletion of *pmt1*, that could also reduce O-mannosylation of the linker and thus aid KEX2 cleavage, substantial amount of light chain remains attached to the carrier in the early days of the
 20 fermentation. At later stages, the cleavage is more complete but the yield may be affected by the degradation of the heavy chain. Results on antibody titres (Table 7 below) indicate fairly steady expression between days 7 to 10. In this fermentation the *pmt1* deletion strain produced approximately equal antibody levels as the parental strain. Higher titres were obtained when the same strain was fermented using a different fermenter.

25 M403 (clone 26-8A) was cultivated in fermenter in TrMM, 30 g/l glucose, 60 g/l lactose, 60 g/l spent grain, pH 5.5 with lactose feed. Samples were harvested on days 2, 3 and 5 - 11. O-mannosylation level analysis was performed as to flask cultures. The O-mannosylation status was greatly decreased also in fermenter culture (Figure 4, Table 5).

The O-mannosylation level was calculated from average of area and intensity (Table 5).
 30 Area (Table 6) seems to give more commonly higher rate of non-O-glycosylated LC than intensity (Table 7). In all time points the O-mannosylation level was below 5%.

Table 5. O-mannosylation status of *T. reesei* strain M403 (pmt1 deletion strain of MAB01 antibody producing strain, clone 26-8A) from fermenter culture.

Percentages calculated from area and intensity of single charged signals. In time point d 9 both samples gave 100% to LC, LC + Hex1 being practically absent.

	3 d	5 d		6 d		7 d		d 8	d 9	d 10		d 11	
	Average	Average	Std	Average	Std	Average	Std	Average	Average	Average	Std	Average	Std
LC	95,8	96,8	0,30	97,5	0,29	97,4	0,36	97,3	100,0	96,6	0,2	95,5	0,11
LC+Hex	4,2	3,2	0,30	2,5	0,29	2,6	0,36	2,7	0,0	3,4	0,2	4,5	0,11

Table 6. The percentages of area values of three parallel samples from fermenter cultured M403 from day 7.

	Area average	Std
LC	98,5	0,15
LC+Hex	1,5	0,15

Table 7. The percentages of intensity values of three parallel samples from fermenter cultured M403 from day 7.

	Intensity average	Std
LC	96,3	0,57
LC+Hex	3,7	0,57

No negative effects of strain growth characteristic and secretion capacity were observed. The strain M403 grew well and produced increased amount of antibody in function of time in fermenter culture. The best titer was obtained from day 10 (Table 8). On day 11 the titer is decreased.

Table 8: Titers from fermenter cultured MAB01 producing strain M403. The antibody was purified using Protein G 96-well plate.

Time point	Days cultured	Titer g/l
54:30 hours	2	0,04
71:50 hours	3	0,04
77:45 hours	3	0,07
126:20 hours	5	0,91
148:20 hours	6	1,23
168:20 hours	7	1,47
192:00 hours	8	1,50
217:15 hours	9	1,35
241:00 hours	10	1,52
275:20 hours	11	1,06

Deletion of *pmt1* diminished dramatically MAB01 O-mannosylation; the amount of O-mannosylated LC was ~ 61% in parental strain, 3% in the best $\Delta pmt1$ clone in shake flask culture and practically 0% in fermenter culture in time point day 9.

5 Deletion of *pmt1* in a Fab expressing *Trichoderma reesei* Strain

The *pmt1* disruption cassette (*pmt1 amdS*) was released from its backbone vector pTTv124 described above by restriction digestion and purified through gel extraction. Using protoplast transformation the deletion cassette was introduced to *T. reesei* strains M304 (3-fold protease deletion strain expressing MAB01) and M307 (4-fold protease deletion strain $\Delta pep1 \Delta tsp1 \Delta slp1 \Delta gap1$, also described in PCT/EP2013/050126 that has been transformed to express a Fab). Transformants were plated to acetamidase selective medium (minimal medium containing acetamide as the sole carbon source).

Transformants were screened by PCR for homologous integration of the acetamidase marker to the *pmt1* locus using a forward primer outside the 5' flanking region fragment of the construct and the reverse primer inside the AmdS selection marker (5' integration) as well as a forward primer inside the AmdS selection marker and a reverse primer outside the 3' flanking region fragment (3' integration). Three independent transformants of each transformation (MAB01 and Fab expressing strains), which gave PCR results displaying correct integration of the construct to the *pmt1* locus were selected for single spore purification to obtain uninuclear clones. Proper integration of the disruption cassette was reconfirmed by PCR using the same primer combinations as described above and the absence of the *pmt1* gene was verified by using a primer combination targeted to the *pmt1* open reading frame. Correct integration of the disruption cassette was additionally verified for all clones applying Southern hybridization. Digested genomic DNA of the three clones as well as the parental strain were probed against the 5' and 3' flanks of the *pmt1*

gene to confirm modification of the *pmt1* locus as expected. Furthermore, the blotted DNA was hybridized with a probe specific to the *pmt1* open reading frame in order to substantiate the absence of *pmt1*.

MAB01 and Fab expression for O-mannosylation Analysis

- 5 To evaluate the impact of *pmt1* deletion on O-mannosylation levels of mAb and Fab molecules, strains 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 NH₄OH). The starting temperature was 30 °C, which was shifted to 22 °C after 48 hours . mAb fermentations
- 10 (strains M304, M403, M406 and M407) were carried out in 4 parallel 2L glas reactor vessels (DASGIP) with a culture volume of 1L and the Fab fermentation (TR090#5) was done in a 15L steel tank reactor (Infors) with a culture volume of 6L. Fab strains (TR090#5, TR090#3, TR090#17) were additionally cultured in shake flasks for 4 days at 28 °C. Main media components were 1% yeast extract, 2% cellobiose, 1% sorbose, 15g/L
- 15 KH₂PO₄ and 5g/L (NH₄)₂SO₄ and the pH was uncontrolled (pH drops from 5.5 to <3 during a time course of cultivation). Culture supernatant samples were taken during the course of the runs and stored at -20 °C. Samples were collected daily from the whole course of these cultivations, and production levels were analyzed by affinity liquid chromatography. Samples with maximum production levels were subject to purification
- 20 and further O-mannosylation analysis.

Analysis of O-mannosylation on Fab and mAb

- O-mannosylation was analyzed on mAb and Fab molecules expressed from both, the *pmt1* deletion and parental strains. The mAb and Fab was purified from culture supernatants using Lambda Select Sure and CaptureSelect Fab Lambda (BAC) affinity
- 25 chromatography resin, respectively, applying conditions as described by the manufactures protocols. Both purified molecules including, the purified mAb and Fab were subjected to RP-LC-QTOF-MS either as intact and/or reduced/alkylated samples.

- For intact analysis, an equivalent of 20 µg protein was injected onto the column. For reduced/alkylated analyses of mAb, an equivalent of 100 µg protein was deglycosylated
- 30 using PNGase-F enzyme, reduced using DTT and alkylated using iodoacetamide prior to LC-MS analysis. For reduced/alkylated analyses of Fab, an equivalent of 100 µg protein was reduced with DTT and alkylated with iodoacetamide prior to LC-MS analysis. 6 µg of the reduced/alkylated sample were injected onto the column. Reversed-phase

chromatography separation was carried out on a 2.1 x 150 mm Zorbax C3 column packed with 5 µm particles, 300 Å pore size the eluents were: eluent A 0.1% TFA in water and eluent B 0.1% TFA in 70% IPA, 20% ACN, 10% water. The column was heated at 75 °C and the flow rate was 200 µL/min. The gradient used for the sample separation is shown in

5 Table 9.

Table 9 : HPLC gradient used for intact and reduced/alkylated samples

Time	% B	Flow (mL/min)
0	10	0.1
0.1	10	0.2
2	10	0.2
4	28	0.2
30	36.4	0.2
31	100	0.2
34	100	0.2
35	10	0.2
40	10	0.2

The HPLC was directly coupled with a Q-TOF Ultima mass spectrometer (Waters, Manchester, UK). The ESI-TOF mass spectrometer was set to run in positive ion mode.

10 The data evaluation of intact and reduced/alkylated analyses was performed using MassLynx analysis software (Waters, Manchester, UK). The deconvolution of the averaged mass spectra from the main UV signals was carried out using the MaxEnt algorithm, a part of the MassLynx analysis software (Waters, Manchester, UK). The deconvolution parameters were the following: “max numbers of iterations” are 8;
 15 resolution is 0.1 Da/channel; Uniform Gaussian – width at half height is 1 Da for intact and 0.5 for the reduced chains and minimum intensity ratios are left 30% and right 30%. The estimated level of O-mannosylation (%) was determined using the peak signal height after deconvolution. The observed O-mannosylation levels (%) of mAbs and Fabs from independent pmt1 deletion strains are compared to the ones of the respective parental
 20 wild-type strains in Tables 10 and 11.

Table 10: O-mannosylation level [%] of Fabs from different strains

Sample	Strain			
	Parental M307	TR090#5	TR090#3	TR090#17
Intact Fab	70.1	34.2	34.3	34.7

LC	58.8	10.4	10.1	10.8
HC	42.9	26.1	25.9	25.8

5

Table 11: O-mannosylation level [%] of MAB01 from different *pmt1* deficient strains M403, M406 and M407. Parental strain is M304

10

Sample	Strain in yeast extract medium			
	Parental	M403	M406	M407
LC	50.7	5.7	5.8	5.8
HC	4.8	Not detected	Not detected	Not detected

The O-mannosylation level was found to be 70% on intact Fab derived from the parental strain and reduced to ~34% in all three *pmt1* deletion strains. The transfer of mannoses was more efficiently diminished on the Fab light chains (10% of residual O-mannosylation on light chains obtained from *pmt1* deletion strains vs. 59% for the parental strain), as compared to the heavy chains, for which it decreased from 43% to ~26%.

The O-mannosylation level was found to be 50% on the light chain of mAb derived from parental strains and reduced to 5.7-5.8% in all three *pmt1* deletion strains. The O-mannosylation level was found to be 4.8% on the heavy chain of mAb derived from parental strains and was completely reduced (below the limit of detection by LC-MS) in all three *pmt1* deletion strains.

In conclusion, after deletion of *pmt1*, almost 95% of purified mAb and 70% of Fab molecules did no longer contain any O-mannose residues. Therefore, *pmt1* is a valuable target to reduce O-mannosylation of secreted proteins and to improve product quality of biopharmaceuticals produced by *Trichoderma reesei*.

Example 3: *pmt2* deletion in a *Trichoderma reesei* strain

*Generation of *pmt2* deletion plasmids*

Three different deletion plasmids (pTTv34, pTTv122, pTTv186) were constructed for deletion of the protein O-mannosyltransferase gene *pmt2* (TrelD22005). All the plasmids

contain the same 5' and 3' flanking regions for correct integration to the *pmt2* locus. The difference between the three plasmids is the marker used in the selection; pTTv34 contains a gene encoding acetamidase of *Aspergillus nidulans* (*amdS*), pTTv122 contains a loopout version (blaster cassette) of the *amdS* marker and pTTv186 a loopout version
 5 (blaster cassette) of a gene encoding orotidine-5'-monophosphate (OMP) decarboxylase of *T. reesei* (*pyr4*).

1100 bp of 5' and 1000 bp of 3' flanking regions were selected as the basis of the second protein O-mannosyltransferase gene, *pmt2* (TrelD22005), deletion plasmids. The construction of the first plasmid for this gene was carried out essentially as described for
 10 *pmt1* in Example 1. As for *pmt1*, the first deletion plasmid for *pmt2* (plasmid pTTv34, Table 12) used *amdS*, a gene encoding acetamidase of *Aspergillus nidulans*, as the selection marker.

Like for *pmt1* in Example 1, to clone the second deletion plasmid, pTTv122 (Table 12), the *amdS* marker was removed from the deletion plasmid pTTv34 with *NotI* digestion and
 15 replaced by *amdS* blaster cassette for which the fragments were produced by PCR (see Example 1 above for details). The plasmid pTTv122 was constructed using the yeast recombination system described in Example 1. The plasmid DNA from the yeast transformants was rescued by transformation into *Escherichia coli*. A few clones were cultivated, plasmid DNA was isolated and digested to screen for correct recombination
 20 using standard laboratory methods. A few clones with correct insert sizes were sequenced and stored.

The third deletion plasmid for *pmt2*, pTTv186 (Table 12) was cloned like the third plasmid for *pmt1*; the *amdS* blaster cassette was removed from the deletion plasmid pTTv122 with *NotI* digestion and replaced by the *pyr4* blaster cassette described in Example 1. The *pyr4*
 25 blaster cassette was obtained from another plasmid with *NotI* digestion, ligated to *NotI* cut pTTv122 and transformed into *E. coli* using standard laboratory methods. A few transformants were cultivated, plasmid DNA isolated and digested to screen for correct ligation and orientation of the *pyr4* blaster cassette using standard laboratory methods. One clone with correct insert size and orientation was sequenced and stored. These
 30 deletion plasmids for *pmt2* (pTTv34, pTTv122 and pTTv186, Table 12) result in 3186 bp deletion in the *pmt2* locus and cover the complete coding sequence of PMT2.

Table 12. Primers for generating deletion plasmids pTTv34, pTTv122 and pTTv186 for protein O-mannosyltransferase 2 (*pmt2*, TrelD22005).

Deletion plasmid pTTv34 for *pmt2* (TreID22005), vector backbone pRS426

Primer	Sequence
22005_5'F	CGATTAAGTTGGGTAACGCCAGGGTTTTCCAGTCACGACGGT TTAAACGTTTCAGGTACCAACACCTG (SEQ ID NO:70)
22005_5'R	ATCTCTCAAAGGAAGAATCCCTTCAGGGTTGCGTTTCCAGTGC GGCCGCGGCGAAGAGTCTGGCGGGGA (SEQ ID NO:71)
22005_3'F	CGGTTCTCATCTGGGCTTGCTCGGTCTGGCGTAGATCTAGCG GCCGCAAGAGGATGGGGGTAAAGCT (SEQ ID NO:72)
22005_3'R	GTGGAATTGTGAGCGGATAACAATTTACACAGGAAACAGCGT TTAAACGAGGAGGACTCGTGAGTTAT (SEQ ID NO:73)

Deletion plasmid pTTv122 for *pmt2* (TreID22005), vector backbone pTTv34

T280_22005_amds_5for	GCGCCCTTCGCGCTCGACAATCCCCGCCAGACTCTTCGCCGC GGCCGCGGCCGCGCGGATCGCCTAGATCTACGCCAGGACC G (SEQ ID NO:74)
T283_amds_3rev_loop	CGGTCCTGGCGTAGATCTAGGGCGCGCCACTGGAAACGCAAC CCTGAA (SEQ ID NO:75)
T284_amds_loop_5for	TTCAGGGTTGCGTTTCCAGTGGCGCGCCCTAGATCTACGCCAG GACCG (SEQ ID NO:76)
T285_22005_loop_3rev	GAGCTGGCCAGAAAAGACCAAGCTTTACCCCATCCTCTTGCG GCCGCGATTATTGCACAAGCAGCGA (SEQ ID NO:77)

Deletion plasmid pTTv186 for *pmt2* (TreID22005), vector backbone pTTv122

Primer	Sequence
no new primers, pTTv122 digested with NotI and ligated with <i>pyr4</i> -loopout fragment from another plasmid	

*Generation of *pmt2* deletion strains M338, M339 and M340*

- To remove vector sequence plasmid pTTv122 ($\Delta pmt2$ -*amdS*) was digested with *PmeI*+*XbaI* and the 5.2 kb fragment purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen). Approximately 5 μ g of the *pmt2* deletion cassette was used to transform the strain M124 (M124 strain is described in WO2012/069593). Protoplast preparation and transformation were carried out essentially according to Penttilä *et al.*, 1987, Gene 61:155-164 and Gruber *et al.*, 1990, Current Genetics 18:71-76 for *amdS* selection.
- 120 colonies were picked as selective streaks. 10 transformants were screened by PCR using the primers in Table 13 for the correct integration of the deletion cassette using standard laboratory methods. Five putative deletion clones were purified to single cell clones. Purified clones (two parallel from each) were rescreened for correct integration and for deletion of *pmt2* ORF (primers on Table 13). Five clones were selected for Southern analyses.

Table 13. Primers for screening integration of deletion cassette pTTv122 and for deletion of protein O-mannosyltransferase 2 (*pmt2*, TreID22005) from M124.

Primer	Sequence
T288_22005_5int	ACGAGTTGTTTCGTGTACCG (SEQ ID NO:78)
T020_Amds_rev2	CTTCCATTCATCAGGGATGG (SEQ ID NO:79)
T021_amds_end_fwd	GGAGACTCAGTGAAGAGAGG (SEQ ID NO:80)
T289_22005_3int	ATGTTGCAGTTGCGAAAG (SEQ ID NO:81)
T290_22005_5orf	CCCTCGTCGCAGAAAAGATG (SEQ ID NO:82)
T291_22005_3orf	AGCCTCCTTGGGAACCTCAG (SEQ ID NO:83)

Deletion of *pmt2* was verified by Southern analyses. DNA for Southern analyses was
 5 extracted with Easy-DNA kit for genomic DNA isolation (Invitrogen) essentially according to the manufacturer's instructions.

Southern analyses were essentially performed as described in Example 1. Fragments for probes were produced by PCR using the primers listed in Table 14 using a *T. reesei* strain M124 as the template for the ORF probe and plasmid pTTv122 for the 5' and 3' flank
 10 probes. PCR products were separated with agarose gel electrophoresis and correct fragments were isolated from the gel with a gel extraction kit (Qiagen) using standard laboratory methods.

Table 14. Primers for production of probe fragments used in Southern analyses of protein O-mannosyltransferase 2 (*pmt2*, TreID22005) deletion strains.

Primer	Sequence
T639_22005_5' flank probe F	CTTAGTGCGCTGGAGGGCG (SEQ ID NO:84)
T640_22005_5' flank probe R	GGCCGGTTTCGTGCAACTGGA (SEQ ID NO:85)
T641_22005_3' flank probe F	GGCCGCAAGAGGATGGGGGT (SEQ ID NO:86)
T642_22005_3' flank probe R	TCGGGCCAGCTGAAGCACAAC (SEQ ID NO:87)
T643_22005 orf 5' probe	TTGAGGAACGGCTGCCTGCG (SEQ ID NO:88)
T644_22005 orf 3' probe	CGATGGCTCCGTCATCCGCC (SEQ ID NO:89)

15

Three of the clones did not hybridise with *pmt2* ORF probe (Data not shown) indicating successful deletion of *pmt2*. Analyses using 5' and 3' flank probes revealed that the same three clones were single integrants (Data not shown). The two other clones (19-35A and 19-40B) gave signals corresponding to parental strain M124. Three pure clones have
 20 been stored for future use (M338; 19-7B, M339; 19-22B and M340; 19-39B).

Analyses of *Δpmt2* strains M338, M339 and M340

Shake flask cultivation of *T. reesei* strain M124 and the *pmt2* deletion strains (19-7B/M338, 19-22B/M339 and 19-39B/M340) was carried out in *Trichoderma* minimal medium with 40 g/l lactose, 20 g/l spent grain extract, 100 mM PIPPS, pH 5.5 with and without 1 M sorbitol as osmotic stabiliser at +28°C, 200 rpm. Samples were collected on days 3, 5 and 7 by vacuum filtration. Supernatant samples were stored to -20°C (antibody and glycan analyses) or used in pH determinations. Mycelia for cell dry weight determinations were rinsed once with DDIW and dried at +100°C for 20-24 h. Mycelia for genomic DNA extraction were rinsed once with DDIW and stored to -20°C.

10 Generation of *pmt2* deletion strains M452, M453 and M454

Generation of M317 is described in Example 1 above.

To remove vector sequence plasmid pTTv186 (*Δpmt2-pyr4*) was digested with *PmeI*+*XbaI* and the 4.1 kb fragment purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen). Approximately 5 µg of the *pmt2* deletion cassette was used to transform M317.

15 Protoplast preparation and transformation were carried out essentially according to Penttilä *et al.*, 1987, Gene 61:155-164 and Gruber *et al.*, 1990, Current Genetics 18:71-76 for *pyr4* selection.

100 colonies were picked as selective streaks. 20 transformants were screened by PCR using the primers in Table 15 for the correct integration of the deletion cassette using standard laboratory methods. Nine putative deletion clones were purified to single cell clones. Purified clones were rescreened for 5' integration and for deletion of *pmt2* ORF (primers on Table 14). Three clones were pure deletants (i.e. no signal with ORF primers).

Table 15. Primers for screening integration of deletion cassette pTTv186 and for deletion of protein O-mannosyltransferase 2 (*pmt2*, TrlD22005) from M317.

Primer	Sequence
T288_22005_5int	ACGAGTTGTTTCGTGTACCG (SEQ ID NO:90)
T027_Pyr4_orf_start_rev	TGCGTCGCCGTCTCGCTCCT (SEQ ID NO:91)
T061_pyr4_orf_screen_2 F	TTAGGCGACCTCTTTTCCA (SEQ ID NO:92)
T289_22005_3int	ATGTTGCAGTTGCGAAAG (SEQ ID NO:93)
T290_22005_5orf	CCCTCGTCGCAGAAAAGATG (SEQ ID NO:94)
T291_22005_3orf	AGCCTCCTTGGAACCTCAG (SEQ ID NO:95)

Deletion of *pmt2* was verified by Southern analyses. DNA for Southern analyses was extracted with Easy-DNA kit for genomic DNA isolation (Invitrogen) essentially according to the manufacturer's instructions.

Southern analyses were essentially performed as described in Example 1. Fragments for probes were produced by PCR using the primers listed in Table 16 using a *T. reesei* wild type strain QM6a (ATCC13631) as the template for *pmt2* ORF probe and plasmid pTTv186 for 5' and 3' flank probes. PCR products were separated with agarose gel electrophoresis and correct fragments were isolated from the gel with a gel extraction kit (Qiagen) using standard laboratory methods.

10 **Table 16. Primers for production of probe fragments used in Southern analyses of protein O-mannosyltransferase 2 (*pmt2*, TreID22005) deletion clones.**

Primer	Sequence	
T639_22005 probe F	5' flank	CTTAGTGCGGCTGGAGGGCG (SEQ ID NO: 96)
T640_22005 probe R	5' flank	GGCCGGTTTCGTGCAACTGGA (SEQ ID NO: 97)
T641_22005 probe F	3' flank	GGCCGCAAGAGGATGGGGGT (SEQ ID NO: 98)
T642_22005 probe R	3' flank	TCGGGCCAGCTGAAGCACAAC (SEQ ID NO: 99)
T290_22005_5orf		CCCTCGTCGCAGAAAAGATG (SEQ ID NO: 100)
T291_22005_3orf		AGCCTCCTTGGAACCTCAG (SEQ ID NO: 101)

None of the clones hybridised with *pmt2* ORF probe (Data not shown) indicating successful deletion of *pmt2*. Analyses using 5' and 3' flank probes revealed that two of the clones were single integrants (Data not shown). One clone gave additional signal from the 3' flank probing (Data not shown) and thus indicated partial or multiple integration of the deletion cassette. Three pure clones (with and without additional copies of the deletion cassette) have been stored for future use (M452; 27-10A, M453; 27-17A and M454; 27-18B).

20 *Analyses of $\Delta pmt2$ strains M452, M453 and M454*

Shake flask cultivation of *T. reesei* strain M304 and three *pmt2* deletion strains (27-10A/M452, 27-17A/M453 and 27-18B/M454) was carried out in *Trichoderma* minimal medium with 40 g/l lactose, 20 g/l spent grain extract, 100 mM PIPPS, 9 g/l casamino acids, pH 5.5 at +28°C, 200 rpm. Samples were collected on days 3, 5, 7 and 10 by vacuum filtration. Supernatant samples were stored to -20°C (antibody and glycan

analyses) or used in pH determinations. Mycelia for cell dry weight determinations were rinsed once with DDIW and dried at +100°C for 20-24 h. Mycelia for genomic DNA extraction were rinsed once with DDIW and stored to -20°C.

O-mannosylation level analysis was performed to *pmt2* deletion strains as to flask cultures
5 of *pmt1* deletion strains. No difference was observed in O-mannosylation compared to parental strain M304.

Example 4: *pmt3* deletion in a *Trichoderma reesei* strain

Generation of pmt3 deletion plasmids

Three different deletion plasmids (pTTv35, pTTv123, pTTv187) were constructed for
10 deletion of the protein O-mannosyltransferase gene *pmt3* (TrelD22527). All the plasmids contain the same 5' and 3' flanking regions for correct integration to the *pmt3* locus. The difference between the three plasmids is the marker used in the selection; pTTv35 contains a gene encoding acetamidase of *Aspergillus nidulans* (*amdS*), pTTv123 contains a loopout version (blaster cassette) of the *amdS* marker and pTTv187 a loopout version
15 (blaster cassette) of a gene encoding orotidine-5'-monophosphate (OMP) decarboxylase of *T. reesei* (*pyr4*).

1100 bp of 5' and 1000 bp of 3' flanking regions were selected as the basis of the third protein O-mannosyltransferase gene, *pmt3* (TrelD22527), deletion plasmids. The construction of the first plasmid for this gene was carried out essentially as described for
20 *pmt1* in Example 1. As for *pmt1*, the first deletion plasmid for *pmt3* (plasmid pTTv35, Table 17) used *amdS*, a gene encoding acetamidase of *Aspergillus nidulans*, as the selection marker.

Like for *pmt1* in Example 1, to clone the second deletion plasmid, pTTv123 (Table 16), the *amdS* marker was removed from the deletion plasmid pTTv35 with *NotI* digestion and
25 replaced by *amdS* blaster cassette for which the fragments were produced by PCR (see Example 1 above for details). The plasmid pTTv123 was constructed using the yeast recombination system described in Example 1. The plasmid DNA from the yeast transformants was rescued by transformation into *Escherichia coli*. A few clones were cultivated, plasmid DNA was isolated and digested to screen for correct recombination
30 using standard laboratory methods. A few clones with correct insert sizes were sequenced and stored.

The third deletion plasmid for *pmt3*, pTTv187 (Table 17) was cloned like the third plasmid for *pmt1*; the *amdS* blaster cassette was removed from the deletion plasmid pTTv123 with *NotI* digestion and replaced by the *pyr4* blaster cassette described in Example 1. The *pyr4* blaster cassette was obtained from another plasmid with *NotI* digestion, ligated to *NotI* cut pTTv123 and transformed into *E. coli* using standard laboratory methods. A few transformants were cultivated, plasmid DNA isolated and digested to screen for correct ligation and orientation of the *pyr4* blaster cassette using standard laboratory methods. One clone with correct insert size and orientation was sequenced and stored. These deletion plasmids for *pmt3* (pTTv35, pTTv123 and pTTv187, Table 17) result in 2495 bp deletion in the *pmt3* locus and cover the complete coding sequence of PMT3.

Table 17. Primers for generating deletion plasmids pTTv35, pTTv123 and pTTv187 for protein O-mannosyltransferase 3 (*pmt3*, TreID22527).

Deletion plasmid pTTv35 for <i>pmt3</i> (TreID22527), vector backbone pRS426	
Primer	Sequence
22527_5'F	CGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGAC GGTTTAAACGTGTTTAAATTTGATGAGGC (SEQ ID NO:102)
22527_5'R	ATCTCTCAAAGGAAGAATCCCTTCAGGGTTGCGTTTCCAGT GCGGCCGCGGTCTCAGAGACAGCCTTCT (SEQ ID NO:103)
22527_3'F	CGGTTCTCATCTGGGCTTGCTCGGTCCTGGCGTAGATCTA GCGGCCGCACTCGGCTTCTTTGTCCGAG (SEQ ID NO:104)
22527_3'R	GTGGAATTGTGAGCGGATAACAATTTACACAGGAAACAG CGTTTAAACTCCTCGTCGGCAACAAGGCC (SEQ ID NO:105)
Deletion plasmid pTTv123 for <i>pmt3</i> (TreID22527), vector backbone pTTv35	
T281_22527_amds_5f or	GCAGATCTGGGGGAGGAATCAGAAGGCTGTCTCTGAGACC GCGGCCGCGGCCGCGCGATCGCCTAGATCTACGCCAG GACCG (SEQ ID NO:106)
T283_amds_3rev_loop	CGGTCCTGGCGTAGATCTAGGGCGCGCCACTGGAAACGC AACCCTGAA (SEQ ID NO:107)
T284_amds_loop_5for	TTCAGGGTTGCGTTTCCAGTGGCGCGCCCTAGATCTACGC CAGGACCG (SEQ ID NO:108)
T286_22527_loop_3rev	AAAGTGGGCGAGCTGAGATACTCGGACAAAGAAGCCGAGT GCGGCCGCGATTATTGCACAAGCAGCGA (SEQ ID NO:109)
Deletion plasmid pTTv187 for <i>pmt3</i> (TreID22527), vector backbone pTTv123	
Primer	Sequence
no new primers, pTTv123 digested with <i>NotI</i> and ligated with <i>pyr4</i> -loopout fragment from another plasmid.	

Generation of pmt3 deletion strains M341 and M342

To remove vector sequence plasmid pTTv123 ($\Delta pmt3$ -*amdS*) was digested with *PmeI*+*XbaI* and the 5.2 kb fragment purified from agarose gel using QIAquick Gel

Extraction Kit (Qiagen). Approximately 5 µg of the *pmt3* deletion cassette was used to transform the strain M124. Protoplast preparation and transformation were carried out essentially according to Penttilä *et al.*, 1987, Gene 61:155-164 and Gruber *et al.*, 1990, Current Genetics 18:71-76 for *amdS* selection.

- 5 120 colonies were picked as selective streaks. 10 transformants were screened by PCR using the primers in Table 18 for the correct integration of the deletion cassette using standard laboratory methods. Three putative deletion clones were purified to single cell clones. Purified clones (three parallel from each) were rescreened for correct integration and for deletion of *pmt3* ORF (primers on Table 18). Three clones were selected for
10 Southern analyses.

Table 18. Primers for screening integration of deletion cassette pTTv123 and for deletion of protein O-mannosyltransferase 3 (*pmt3*, TreID22527) from M124.

Primer	Sequence
T292_22527_5int	ACGGGAGATCTCGGAAAA (SEQ ID NO:110)
T020_Amds_rev2	CTTTCCATTCATCAGGGATGG (SEQ ID NO:111)
T021_amds_end_fwd	GGAGACTCAGTGAAGAGAGG (SEQ ID NO:112)
T293_22527_3int	ATGAAGCTCAGCCTGTGG (SEQ ID NO:113)
T294_22527_5orf	GGGGACGGCTTGAGGAAG (SEQ ID NO:114)
T295_22527_3orf	CTGCTTGCTGCTTCCAGTCA (SEQ ID NO:115)

- Deletion of *pmt3* was verified by Southern analyses. DNA for Southern analyses was
15 extracted with Easy-DNA kit for genomic DNA isolation (Invitrogen) essentially according to the manufacturer's instructions.

- Southern analyses were essentially performed as described in Example 1. Fragments for probes were produced by PCR using the primers listed in Table 19 using a *T. reesei* strain M124 as the template for the ORF probe and plasmid pTTv123 for the 5' and 3' flank
20 probes. PCR products were separated with agarose gel electrophoresis and correct fragments were isolated from the gel with a gel extraction kit (Qiagen) using standard laboratory methods.

Table 19. Primers for production of probe fragments used in Southern analyses of protein O-mannosyltransferase 3 (*pmt3*, TreID22527) deletion strains.

Primer	Sequence
T645_22527_5' flank probe F	TGGCAGATGCCGAAAGGCGG (SEQ ID NO:116)

T646_22527	5'	flank	TGGCAACCAGCTGTGGCTCC (SEQ ID NO:117)
probe R			
T647_22527	3'	flank	CGGCCGCACTCGGCTTCTTT (SEQ ID NO:118)
probe F			
T648_22527	3'	flank	GAGTGGGCTAGGCGCAACGG (SEQ ID NO:119)
probe R			
T649_22527	orf 5'	probe	GGATCGGCCACTGCCACCAC (SEQ ID NO:120)
T650_22527	orf 3'	probe	GCCCACTTCTCTGCGCGTGT (SEQ ID NO:121)

Two of the clones did not hybridise with *pmt3* ORF probe (Data not shown) indicating successful deletion of *pmt3*. Analyses using 5' and 3' flank probes revealed that the same two clones were single integrants (Data not shown). One clone (20-32C) gave signals
 5 corresponding to parental strain M124. Two clones have been stored for future use (M341; 20-34C and M342; 20-35B).

Analyses of $\Delta pmt3$ strains M341 and M342

Shake flask cultivation of *T. reesei* strain M124 and the *pmt3* deletion strains (20-34C/M341 and 20-35B/M342) was carried out in *Trichoderma* minimal medium with 40 g/l
 10 lactose, 20 g/l spent grain extract, 100 mM PIPPS, pH 5.5 with and without 1 M sorbitol as osmotic stabiliser at +28°C, 200 rpm. Samples were collected on days 3, 5 and 7 by vacuum filtration. Supernatant samples were stored to -20°C (antibody and glycan analyses) or used in pH determinations. Mycelia for cell dry weight determinations were rinsed once with DDIW and dried at +100°C for 20-24 h. Mycelia for genomic DNA
 15 extraction were rinsed once with DDIW and stored to -20°C.

*Generation of *pmt3* deletion strains M522 and M523*

Generation of M317 is described in Example 1 above.

To remove vector sequence plasmid pTTv187 ($\Delta pmt3$ -*pyr4*) was digested with *PmeI*+*XbaI* and the 4.1 kb fragment purified from agarose gel using QIAquick Gel Extraction Kit
 20 (Qiagen). Approximately 5 µg of the *pmt3* deletion cassette was used to transform M317. Protoplast preparation and transformation were carried out essentially according to Penttilä *et al.*, 1987, Gene 61:155-164 and Gruber *et al.*, 1990, Current Genetics 18:71-76 for *pyr4* selection.

200 colonies were picked as selective streaks. 59 transformants were screened by PCR
 25 using the primers in Table 20 for the correct integration of the deletion cassette using standard laboratory methods. Three putative deletion clones were purified to single cell

clones. Purified clones were rescreened for correct integration and for deletion of *pmt3* ORF (primers on Table 19). Two clones (several parallels) were pure deletants (i.e. no signal with ORF primers).

Table 20. Primers for screening integration of deletion cassette pTTv187 and for deletion of protein O-mannosyltransferase 3 (*pmt3*, TreID22527) from M317.

Primer	Sequence
T292_22527_5int	ACGGGAGATCTCGGAAAA (SEQ ID NO:122)
T026_Pyr4_orf_5rev2	CCATGAGCTTGAACAGGTAA (SEQ ID NO:123)
T061_pyr4_orf_screen_2F	TTAGGCGACCTCTTTTCCA (SEQ ID NO:124)
T293_22527_3int	ATGAAGCTCAGCCTGTGG (SEQ ID NO:125)
T649_22527_orf_5' probe	GGATCGGCCACTGCCACCAC (SEQ ID NO:126)
T650_22527_orf_3' probe	GCCCACTTCTCTGCGCGTGT (SEQ ID NO:127)

Deletion of *pmt3* was verified by Southern analyses. DNA for Southern analyses was extracted with Easy-DNA kit for genomic DNA isolation (Invitrogen) essentially according to the manufacturer's instructions.

- 10 Southern analyses were essentially performed as described in Example 1. Fragments for probes were produced by PCR using the primers listed in Table 21 using a *T. reesei* wild type strain QM6a (ATCC13631) as the template for the ORF probe and plasmid pTTv187 for the 5' and 3' flank probes. PCR products were separated with agarose gel electrophoresis and correct fragments were isolated from the gel with a gel extraction kit
- 15 (Qiagen) using standard laboratory methods.

Table 21. Primers for production of probe fragments used in Southern analyses of protein O-mannosyltransferase 3 (*pmt3*, TreID22527) deletion strains.

Primer	Sequence
T645_22527_5' flank probe F	TGGCAGATGCCGAAAGGCGG (SEQ ID NO:128)
T646_22527_5' flank probe R	TGGCAACCAGCTGTGGCTCC (SEQ ID NO:129)
T647_22527_3' flank probe F	CGGCCGCACTCGGCTTCTTT (SEQ ID NO:130)
T648_22527_3' flank probe R	GAGTGGGCTAGGCGCAACGG (SEQ ID NO:131)
T874_pmt3_orf_f3	CTCTGCGCGTGTGTGG (SEQ ID NO:132)
T875_pmt3_orf_r3	TAAGGGTGCGGATTCGG (SEQ ID NO:133)

Eight of the clones did not hybridise with *pmt3* ORF probe (Data not shown) indicating successful deletion of *pmt3*. One clone (33-37K) hybridised with *pmt3* ORF probe even though the signal size did not correspond to those from parental strains suggesting rearrangement in the *pmt3* locus. Analyses using 5' and 3' flank probes revealed that the eight $\Delta pmt3$ clones were single integrants (Data not shown). One clone (33-37K) gave incorrect or additional signals suggesting rearrangements in the *pmt3* locus and multiple integrations of the deletion cassette. Two pure clones have been stored for future use (M522; 33-34A and M523; 33-188A-a).

10 Analyses of $\Delta pmt3$ strains M522 and M523

24-well plate cultivation of *T. reesei* strain M304 and eight *pmt3* deletion strains (33-34S/M522, 33-34T, 33-34U, 33-34Ö, 33-188A-a/M523, 33-188B-a, 33-188C-a and 33-188D-a) was carried out in *Trichoderma* minimal medium with 40 g/l lactose, 20 g/l spent grain extract, 100 mM PIPPS, 9 g/l casamino acids, pH 5.5 at +28°C, 800 rpm with humidity control. Samples were collected on days 3, 5 and 6 by centrifugation. Supernatant samples were stored to -20°C. Mycelia for cell dry weight determinations were rinsed once with DDIW and dried at +100°C for 20-24 h. Mycelia for genomic DNA extraction were rinsed twice with DDIW and stored to -20°C.

O-mannosylation level analysis was performed to *pmt3* deletion strains as to flask cultures of *pmt1* deletion strains. No difference was observed in O-mannosylation compared to parental strain M304.

Example 5 –pmt Homologs

T. reesei pmt homologs were identified from other organisms.

BLAST searches were conducted using the National Center for Biotechnology Information (NCBI) non-redundant amino acid database using the *Trichoderma reesei* PMT amino acid sequences as queries. Sequence hits from the BLAST searches were aligned using the ClustalW2 alignment tool provided by EBI. Phylogenetic trees were generated using average distance with BLOSUM62 after aligning the sequences in the Clustal Omega alignment tool.

A phylogenetic tree and a partial sequence alignment of the results of the PMT BLAST searches are depicted in Figures 5 and 6, respectively.

CLAIMS

1. A PMT-deficient filamentous fungal cell comprising:
 - a) a first mutation that reduces or eliminates an endogenous protease activity compared to a parental filamentous fungal cell which does not have said first mutation, said endogenous proteases being selected among aspartic proteases, trypsin-like serine proteases, subtilisin proteases, glutamic proteases and sedolisin proteases, and
 - b) a second mutation in a protein O-mannosyltransferase (*PMT*) gene that reduces endogenous O-mannosyltransferase activity compared to a parental filamentous fungal cell which does not have said second mutation, wherein said filamentous fungal cell is selected from the group consisting of *Trichoderma*, *Neurospora*, *Myceliophthora* and *Chrysosporium* cell.
2. The PMT-deficient filamentous fungal cell of claim 1, wherein said second mutation that reduces the endogenous O-mannosyltransferase activity is a deletion or a disruption of a *PMT* gene encoding an endogenous protein O-mannosyltransferase activity.
3. The PMT-deficient filamentous fungal cell of claim 1 or 2, wherein said second mutation in a PMT gene is a mutation in either :
 - a) *PMT1* gene comprising the polynucleotide of SEQ ID NO:1,
 - b) a functional homologous gene of *PMT1* gene, which functional homologous gene is capable of restoring parental O-mannosylation level by functional complementation when introduced into a *T. reesei* strain having a disruption in said *PMT1* gene, or;
 - c) a polynucleotide encoding a polypeptide having at least 50% identity with SEQ ID NO:2, said polypeptide having O-mannosyltransferase activity.
4. The PMT-deficient filamentous fungal cell of any one of claims 1-3, wherein said cell has a third mutation that reduces or eliminates the level of expression of an *ALG3* gene compared to the level of expression in a parental cell which does not have such third mutation.
5. The PMT-deficient filamentous fungal cell of Claim 4, further comprising a first polynucleotide encoding a N-acetylglucosaminyltransferase I catalytic domain and a

second polynucleotide encoding a N-acetylglucosaminyltransferase II catalytic domain.

6. The PMT-deficient filamentous fungal cell of any one of claims 1-5, further comprising one or more polynucleotides encoding a polypeptide selected from the group consisting of:

- a) α 1,2 mannosidase;
- b) N-acetylglucosaminyltransferase I catalytic domain;
- c) α mannosidase II;
- d) N-acetylglucosaminyltransferase II catalytic domain;
- e) β 1,4 galactosyltransferase; and,
- f) fucosyltransferase.

7. The PMT-deficient filamentous fungal cell of any one of claims 1-6, wherein said cell is a *Trichoderma* cell comprising a mutation that reduces or eliminates the protein O-mannosyltransferase activity of *Trichoderma* pmt1.

8. The PMT-deficient filamentous fungal cell of any one of claims 1-7, wherein said cell is a *Trichoderma* cell, for example *Trichoderma reesei*, and said cell comprises mutations that reduce or eliminate the activity of

- a) the three endogenous proteases pep1, tsp1 and slp1;
- b) the three endogenous proteases gap1, slp1 and pep1;
- c) the three endogenous proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, pep8, pep11, pep12, tsp1, slp1, slp2, slp3, slp7, gap1 and gap2;
- d) three to six proteases selected from the group consisting of pep1, pep2, pep3, pep4, pep5, tsp1, slp1, slp2, slp3, gap1 and gap2; or
- e) 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.