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 ~~invention~~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 ~~invention~~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 ~~invention~~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, and,

b) a second mutation in a *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 said PMT gene. For example, the O-mannosylation level of the heterologous protein expressed in a PMT-deficient cell of the ~~invention~~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.

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:

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 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 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 PMT-deficient cell further comprises one or more polynucleotides encoding a polypeptide selected from the group consisting of:

a) α1,2 mannosidase,

b) N-acetylglucosaminyltransferase I catalytic domain,

c) α mannosidase II, and

d) N-acetylglucosaminyltransferase II catalytic domain.

In another embodiment that may be combined with the preceding embodiments, said PMT-deficient cell further comprises one or more polynucleotides encoding a β1,4 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 *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 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,

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.

In one embodiment that may be combined with the precedent embodiments, the filamentous fungal cell of the ~~invention~~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).

In another aspect, the ~~invention~~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,

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 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,

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.

In other embodiments of the method, said PMT-deficient cell is a PMT-deficient cell of the ~~invention~~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

a) an immunoglubulin, such as IgG,

b) a light chain or heavy chain of an immunoglobulin,

c) a heavy chain or a light chain of an antibody,

d) a single chain antibody,

e) a camelid antibody,

f) a monomeric or multimeric single domain antibody,

g) a FAb-fragment, a FAb2-fragment, and,

h) their antigen-binding fragments.

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~~The~~ ~~invention~~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 ~~invention~~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.

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 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 Galα3Galβ4GlcNAc, terminal Galβ4GlcNAc, terminal Galβ3GlcNAc, core linked fucose and glycation structures.

The ~~invention~~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,
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 polypeptide having protein O-mannosyltransferase activity.

In another aspect, there is provided 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 and M304 with *pmt1* ORF probe after *Spe*I + *Xba*I 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 *Spe*I + *Asc*I digestion. C) A 1.7 kb signal is expected for *pmt1* 3'flank probe from deletion strains after *Asc*I + *Xba*I digestions. *Asc*I does not cut intact *pmt1* 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 *Pme*I 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 Δpmt1 disruptant clone 26-8A (B), day 7.