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

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

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

## SUMMARY

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

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

Accordingly, in a first aspect, ~~the invention relates to~~there is provided a filamentous fungal cell comprising

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In another aspect, there is provided a method of producing a heterologous glycoprotein, or antibody composition, with increased N-glycosylation site occupancy, comprising

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In ~~one~~ another aspect, ~~the invention also relates to~~ there is provided a glycoprotein composition obtainable by the method described above.

In ~~one~~ yet another aspect, ~~the invention relates to~~there is provided an antibody composition obtainable by the method described above.

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

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

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

## DESCRIPTION OF THE FIGURES

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

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

**Figure 3.** Schematic map of the STT3 expression cassettes.

**Figure 4.** Glycan structures produced in Δalg3 strains.

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