activates mannosyl-oligosaccharide 1,2-α-mannosidase and simultaneously inhibits α-1,3-mannosyl-glycoprotein 2-β-N-acetylglucosaminyl-transferase). Information on *in vitro* metal cofactors required for activation, or for structural arrangements of the protein, and metal inhibitors of a number of enzymes involved in glycosylation can be obtained from the BRENDA database (http://www.brenda-enzymes.org). This dual activity of various metal co-factors highlights the need for tight regulation of trace element usage in glycoprotein production.

Glycoengineering, i.e. changing protein-associated carbohydrate by recombinant co-expression of endogenous or heterologous glycoenzymes, has been shown to affect pharmacokinetic properties of proteins, such as molecular stability, solubility, *in* vivo biological activity and immunogenicity. In antibodies in particular, increasing the amount of galactosylation has been shown to affect antibody effector functions, such as the CDC (Raju, Curr. Opin. Immunol. **20.4** (2008) 471-8) or the antibody-dependent cellular toxicity (ADCC), for example galactosylation of human IgG monoclonal anti-D affects Fc receptor-mediated functional activity (Kumpel et al., Hum. Antib. Hyb. **5.3-4** (1994) 143-51); or an increase in the amount of galactosylation has been shown to enhance the blocking of complement-mediated inflammation by IgG immune complexes (Karsten et al., Nat. Med. **18.9** (2012) 1401-6). N-glycosylation of antibody Fc regions is essential for binding to the Fc receptors, which engage the antibody effector functions. Fucose is a core residue in the N-glycans of many antibodies, but non-fucosylated forms have been shown to have an enhanced ADCC over their fucosylated counterparts. Methods to increase production of non-fucosylated proteins and/or to influence fucosylation of antibodies are thus also desirable.

Accordingly, there remains a need in the art for methods to enhance cell culture strategies, including to improve cell growth and biomass generation and/or to direct glycosylation and hence maturation of glycoproteins.

SUMMARY OF THE INVENTION

The inventors have determined the role of bioactive trace elements in cell culture performance and in particular on cell growth and glycan formation in glycoproteins and hence in glycoprotein maturation. Accordingly, the present disclosure ~~invention~~ relates to methods for selecting between conditions which affect cell growth or biomass generation and conditions which affect N-glycan maturity in expressed glycoprotein produced by the cells. Thus, in the

methods of the present disclosure ~~invention~~, the glycoprotein producing cells are cultured in a medium which is tailored to a desired end result.

In one aspect, the ~~The~~ present invention is a method for production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell, the method comprising adjusting the concentrations of each of iron, copper, zinc and manganese in the culture medium during the culture to affect biomass generation and/or N-glycan maturity in the expressed glycoprotein.

The present method encompasses adjusting concentrations of iron, copper, zinc and manganese to affect biomass generation and/or N-glycan maturity of the expressed glycoprotein. The adjustment of the concentrations of iron, copper, zinc and manganese will mean that the conditions in the cell will favour biomass generation or will have an effect on the maturity of the expressed glycoprotein. By "favour" as used herein is meant that the activity of the cell will be in the stated direction as compared to a cell grown under the same conditions but in which the concentrations of each of iron, copper, zinc and manganese have not been so adjusted. Thus, when biomass generation is favoured, the cell will be directed more towards multiplying than production of glycoprotein. Accordingly, there will be an enhancement or increase of biomass generation. Likewise, when the adjustment of the concentrations of iron, copper, zinc and manganese, or of zinc and manganese alone, affects N-glycan maturity of the expressed glycoprotein, the cell will be directed more towards production of such glycoproteins than towards e.g. biomass generation or production of other glycoprotein types. Thus the production of the desired glycoprotein type will be enhanced or increased.

The methods of the disclosure ~~invention~~ affect the N-glycan maturity of the expressed glycoprotein, for example to result in production of a glycoprotein with a desired maturity level. Thus, the methods affect the production of mature N-glycosylated glycoproteins and/or immature N-glycosylated glycoproteins, mature non-fucosylated glycoproteins and/or immature non-fucosylated glycoproteins. The methods of the present disclosure ~~invention~~ include increasing the maturity of expressed N-glycosylated glycoproteins, increasing production of mature N-glycosylated glycoproteins, increasing production of mature non-fucosylated glycoproteins and/or increasing production of immature non-fucosylated glycoproteins. Concomitant with increasing production of mature N-glycosylated glycoproteins may be a decrease in production of mature and/or immature non-fucosylated glycoprotein species and immature N-glycosylated glycoproteins. Concomitant with increasing production of immature non-

fucosylated glycoproteins may be a decrease in production of mature non-fucosylated glycoproteins and/or mature or immature N-glycosylated glycoproteins.

In certain examples ~~embodiments~~, the concentrations of iron, copper, zinc and manganese may be adjusted in the culture medium to favour biomass generation, that is to enhance growth of the cells, and thereby increase biomass. In other examples ~~embodiments~~, the concentrations of iron, copper, zinc and manganese may be adjusted in the culture medium to increase the maturity of the expressed glycoprotein. In one example ~~embodiment~~, the concentrations of iron, copper, zinc and manganese, or of only zinc and manganese, may be adjusted in the culture medium to favour, i.e. increase production of, mature N-glycosylated glycoproteins. In another example ~~embodiment~~, the concentrations of iron, copper, zinc and manganese may be adjusted to favour, i.e. increase the production of, immature non-fucosylated glycoproteins. In yet another example ~~embodiment~~, the concentrations of iron, copper, zinc and manganese in the culture medium may be adjusted first to enhance growth and thereby increase biomass and then again to affect glycoprotein maturity, be that increasing maturity in expressed glycoproteins, increasing production of mature N-glycosylated glycoproteins or increasing production of mature or immature non-fucosylated glycoproteins.

Thus, the disclosure ~~invention~~ includes:

- favouring biomass generation by increasing the concentration of each of iron, copper, zinc and manganese in the culture medium;
- favouring increased maturity in expressed N-glycosylated glycoproteins by increasing the concentration of each of zinc and manganese and, optionally, reducing the concentration of iron and copper in the culture medium; and
- favouring production of immature non-fucosylated glycoproteins by either (i) decreasing the concentrations of each of iron, copper, zinc and manganese in the culture medium, or (ii) increasing the concentrations of each of copper and iron and decreasing the concentration of each of zinc and manganese in the culture medium.

In one aspect, the present invention provides a method for production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell, the method comprising increasing the concentration of zinc and manganese in the culture medium during the culture to increase N-glycan maturity in the expressed glycoprotein.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein in order to favour biomass generation the concentrations of iron, copper, zinc and manganese are adjusted in the culture medium to:

- (a) iron - from 15 µM to more than 80 µM;
- (b) copper - from 0.3 µM to more than 2.5 µM;
- (c) zinc - from 20 µM to more than 50 µM; and

- (d) manganese - from 0.01 μM to more than 3 μM.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein in order to favour increased maturity in expressed N-glycosylated glycoproteins, the concentrations of iron, copper, zinc and manganese [in (i)] or the concentrations of zinc and manganese [in (ii)], are adjusted in the culture medium to:

(i)

- (a) iron - from 0 μM to 25 μM;
- (b) copper - from 0 μM to 0.1 μM;
- (c) zinc - from 20 μM to more than 50 μM; and
- (d) manganese - from 0.01 μM to more than 3 μM; or

(ii)

- (a) zinc - from 20 μM to more than 50 μM; and
- (b) manganese - from 0.01 μM to more than 3 μM.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein in order to favour production of immature non-fucosylated glycoproteins either the concentrations of iron, copper, zinc and manganese are adjusted in the culture medium to either:

(i)

- (a) iron - from 0 μM to 35 μM;
- (b) copper - from 0 μM to 1 μM;
- (c) zinc - from 0 μM to 20 μM; and
- (d) manganese - from 0 μM to 0.01 μM; or

(ii)

(a) iron - from 15 μM to more than 80 μM;

- (b) copper - from 0.3 μM to more than 2.5 μM;
- (c) zinc - from 0 μM to 20 μM; and
- (d) manganese - from 0 μM to 0.01 μM.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein the glycoprotein is exogenous or endogenous to the eukaryotic cell, optionally wherein the glycoprotein is a structural glycoprotein, hormone, antibody or enzyme.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein the glycoprotein is an antibody, optionally wherein the antibody is a therapeutic or diagnostic antibody, optionally a chimeric, humanized or human antibody.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein the eukaryotic cell is a mammalian cell, a yeast cell or an insect cell.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein the concentrations of iron, copper, zinc and manganese are adjusted during the growth and/or productions culture phases.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein an increase in the concentration of any or all of iron, copper, zinc and manganese in the culture medium is achieved by supplementing the medium in which the cells are cultured with any or all of iron, copper, zinc and manganese and/or by splitting the cells into a fresh medium supplemented with any or all of iron, copper, zinc and manganese.

Also disclosed are In ~~one aspect, the invention includes~~ methods as described above wherein a decrease in the bioavailable concentration of any or all of iron, copper, zinc and manganese in the culture medium is achieved by complexing the iron, copper, zinc and manganese with a chelator and/or by seeding the cells into a fresh medium containing a reduced concentration of any or all of iron, copper, zinc and manganese compared to the medium of the immediately preceding culture phase.

Also disclosed are ~~In one aspect, the invention includes~~ methods as described above wherein the concentrations of each of iron, copper, zinc and manganese in the culture medium are adjusted during the culture first to favour biomass generation and then to favour either production of mature N-glycosylated glycoproteins or production of immature non-fucosylated glycoproteins.

Also provided herein is a medium suitable for production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell, the medium comprising a concentration of each of iron, copper, zinc and manganese to favour biomass generation and/or to affect N-glycan maturity of the expressed glycoprotein.

In one aspect, the present invention provides a medium suitable for production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell, the medium comprising a concentration of each of iron, copper, zinc and manganese to affect biomass generation and/or N-glycan maturity in the expressed glycoprotein.

Also disclosed is ~~In one aspect,~~ the medium comprises concentrations of iron, copper, zinc and manganese of:

- (a) iron - from 15 µM to more than 80 µM;
- (b) copper - from 0.3 µM to more than 2.5 µM;

- (c) zinc - from 20 µM to more than 50 µM; and
- (d) manganese - from 0.01 µM to more than 3 µM.

Also disclosed is the ~~The~~ use of such a medium in the production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell favours biomass generation.

In one aspect, the present invention provides the use of the medium disclosed herein in the production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell to favour enhanced biomass generation.

Also disclosed is ~~In one aspect,~~ the medium comprises concentrations of iron, copper, zinc and manganese, or of zinc and manganese, of:

(i)

- (a) iron - from 0 µM to 25 µM;
- (b) copper - from 0 µM to 0.1 µM;
- (c) zinc - from 20 µM to more than 50 µM; and
- (d) manganese - from 0.01 µM to more than 3 µM; or

(ii)

- (a) zinc - from 20 µM to more than 50 µM; and
- (b) manganese - from 0.01 µM to more than 3 µM.

Also disclosed is use ~~Use~~ of a medium comprising iron, copper, zinc and manganese concentrations or of zinc and manganese of (i) or (ii) respectively, in the production of a recombinant glycoprotein *under fermentation culture conditions in a eukaryotic cell favours increased maturity in expressed* N-glycosylated glycoproteins.

In one aspect, the present invention provides the use of the medium disclosed herein in the production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell to increase maturity in expressed N-glycoproteins.

Also disclosed is ~~In one aspect,~~ the medium comprises concentrations of iron, copper, zinc and manganese of either:

(i)

- (a) iron - from 0 µM to 35 µM;
- (b) copper - from 0 µM to 1 µM;
- (c) zinc - from 0 µM to 20 µM; and
- (d) manganese - from 0 µM to 0.01 µM; or

(ii)

- (a) iron - from 15 µM to more than 80 µM;
- (b) copper - from 0.3 µM to more than 2.5 µM;
- (c) zinc - from 0 µM to 20 µM; and
- (d) manganese - from 0 µM to 0.01 µM.

Also disclosed is use ~~Use~~ of a medium comprising iron, copper, zinc and manganese concentrations of (i) or (ii), above in the production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell favours production of immature non-fucosylated glycoproteins.

In one aspect, the present invention provides use of a medium of claim 24 in the production of a recombinant glycoprotein under fermentation culture conditions in a eukaryotic cell to favour production of immature non-fucosylated glycoproteins.

DESCRIPTION OF THE FIGURES

Figure 1: **Process steps of a fermentation campaign.** Frozen vials e.g. of a Primary Seed Bank (PSB), are thawed in seed train medium. Here, cells are cultivated for roughly three weeks to recover form thawing stress (normalizing cell doubling times). The cells are subsequently passed to inoculation train n-1 and n-2 for the expansion of the culture. After approximately one week the cells are transferred to the production scale for a fed-batch fermentation process of two weeks.

Figure 2: **Fed-batch processes are characterized by two phenotypically distinct phases.** The production phase is separated by two segments where either cell growth or target protein production dominate.

Figure 3: **Different Approaches for DoE Experiments.** Dependent on desired experiment outcome, different DoE approaches can be used for process optimization. For example, optimization of three factors are normally varied between a minimum (-1), medium (0), and maximum level (+1) for optimization DoEs. (Picture adopted from http://www.gmpua.com and modified)

Figure 4: **Effect of trace elements on clone independent mAb galactosylation.** Aluminum, molybdenum, barium, chromium, bromine, iodine, copper, manganese, rubidium, silver, zinc, tin, and zirconium salt stocks were supplemented to a fed-batch culture of clone 1 (n=4) and clone 3 (n=3) using a proprietary cell culture platform A (medium and process) at the start of the experiment. The effect of the trace elements on mAb galactosylation at day 7, 12, and 13 was analyzed by modelling G1 (A, B) and G2 (C, D) levels with existing experimental data. Each point in the *actual* vs *predicted* graphs (A, C) represents a single cultivation experiment. The statistical significance of each trace element on G1 and G2 levels was centred by mean and scaled by range/2 for G1 (B) and G2 (D).

Figure 5: **Timing of regulation of mAb galactosylation by manganese.** The significant effect of manganese on G1 (A) and G2 (B) of clone 3 (plate 5, 6, and 7) glycan formation was analyzed by the prediction profiler tool of JMP software. The positive effect of