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(54) Title: PROCESS OF OBTAINING GLYCOPROTEIN COMPOSITION WITH INCREASED GALACTOSYLATION CONTENT

(57) Abstract: The invention describes a cell culture process for production of glycoprotein composition with increased galactosylation content while maintaining specific productivity. The process involves culturing cells at high $_pCO_2$ and subjecting cells to temperature downshift to obtain a glycoprotein with specific glycoprotein composition.

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PROCESS OF OBTAINING GLYCOPROTEIN COMPOSITION WITH INCREASED GALACTOSYLATION CONTENT

RELATED APPLICATION

This application is related to and takes priority from Indian Provisional Application 1729/CHE/2013 filed 18th April 2013 and is herein incorporated in its entirety.

FIELD OF THE INVENTION

The present invention generally relates to the field of cell culture. In particular, the invention describes a cell culture process for obtaining a glycoprotein composition with enhanced galactosylation while maintaining specific productivity. More specifically, the invention describes a cell culture process wherein cells are cultured at high pCO_2 and subjected to a temperature shift to obtain a glycoprotein with a particular glycoform composition.

BACKGROUND OF THE INVENTION

Protein glycosylation is one of the most important post-translational modifications associated with eukaryotic proteins. The two major types of glycosylation in eukaryotic cells are N-linked glycosylation, in which glycans are attached to the asparagine of the recognition sequence Asn-X-Thr/Ser, where "X" is any amino acids except proline, and O-linked glycosylation in which glycans are attached to serine or threonine. N-linked glycans further are of two types - high mannose type consisting of two N-acetylglucosamines plus a large number of mannose residues (more than 4), and complex type that contain more than two N-acetylglucosamines plus any number of other types of sugars. In both N- and O-glycosylation, there is usually a range of glycan structures associated with each site, which is known as microheterogeneity. Macroheterogeneity results from the fact that not all N-glycan or O- glycan consensus sequences (Asn-X-Ser/Thr for N-glycan and serine or threonine for O-glycan present in the glycoproteins) are actually glycosylated. This may be a consequence of the competitive action of diverse enzymes during biosynthesis and is key to the understanding of glycoprotein heterogeneity *(Mariño, K., Nature Chemical Biology 6: 713–723, 2010).*

The process of N-linked glycosylation begins co-translationally in the endoplasmic reticulum where a complex set of reactions result in the attachment of $Glc_3NAc_2Man_9$ (3 glucose, 2 N-acetylglucosamine and 9 mannose) to a carrier molecule called dolichol, that is then transferred to the appropriate point on the polypeptide chain as it is trans-located into the ER lumen *(Schwarz, F. and Aebi M., Current Opinion in Structural Biology, 21:576–582, 2011 and Burda, P. and Aebi M., Biochimica et Biophysica Acta 1426(2): 239-257*, 1999). The glycan complex so formed in the ER lumen is modified by action of enzymes in the Golgi apparatus. If the saccharide is relatively inaccessible, it will most likely stay in the original high-mannose form. If it is accessible, then many of the mannose residues will be cleaved off and the saccharide will be further modified, resulting in the complex type N-glycan structure. For example, in *cis*-Golgi, mannosidase-1 may act to cleave or hydrolyze a high mannose glycan, while further on,fucosyltransferase FUT-8 fucosylates the glycan in the medial-Golgi *(Hanrue Imai-Nishiya, BMC Biotechnology, 7:84, 2007).*

Thus the sugar composition as well as the structural configuration of a glycan structure depends on the protein being glycosylated, the cells/cell lines, the glycosylation machinery in the Endoplasmic Reticulum and the Golgi apparatus, the accessibility of the machinery enzymes to the glycan structure, the order of action of each enzyme and the stage at which the protein is released from the glycosylation machinery.

The structure and composition of the glycan moieties of a glycoprotein can have a profound effect on the safety and efficacy of therapeutic proteins, including its immunogenicity, solubility and half-life. Pacis *et al* have shown that higher osmolality may result in increase in the number of Man5 residues on recombinant antibodies, with a simultaneous reduction in G_0F and G_1F glycoforms, resulting in its faster clearance from the body and thereby reducing its efficacy *(Pacis E., Yu, M., Autsen, J., Bayer, R., Li F., Biotechnol. Bioeng. 108(10) 2348-2358, 2011).* Further, absence of fucose in the glycan structure of the Fc region of the antibodies has been associated with higher antibody dependent cell mediated cytotoxicity (ADCC) activity (*Werner, R. G., Kopp, K. and Schlueter, M. Acta Paediatrica 96: 17–22, 2007*).

Antibodies and galactosylation

Studies by Gazzano-Santoro *et.al.* have demonstrated that an increase in G_0 glycan levels of antibodies is associated with a reduction in biological activity as measured by complement dependent activity (CDC) assay *(H. Gazzano-Santoro, P. Ralph, T. Ryskamp, A. Chen, V. Mukku, J. Immunol. Methods, 202: 163, 1997).* Removal of terminal galactose residues from the chimeric mouse–human IgG1 antibody (alemtuzumab) reduces CDC, without affecting FcγR-mediated functions (*Boyd, P. N., Lines, A. C. & Patel, A. K. Mol. Immunol. 32, 1311–1318, 1995*). Further, it has been reported that G_1F–G_1F glycoform of rituximab triggered a CDC response twice as large as that triggered by the G_0F–G_0F glycoform *(Nature Reviews Drug Discovery 8, 226-234, March 2009).* Thus, given the role of the galactosylation on the activity and efficacy of glycoproteins, monitoring and controlling the galactosylation level in the glycoprotein composition is critical.

The prior art discloses various methods for modulating galactosylation profile of a glycoprotein composition. Study by Gramer M J.*et al.* (*Biotechnol. Bioeng. 108(7): 1591-1602, Jul 2011*) discloses methods of modulating galactosylation by manipulating uridine, manganese chloride and galactose.

Similarly, US20120276631 provides a method for controlling galactosylation using manganese and/or galactose supplement.

Further, EP2511293 demonstrates a method to control the ratio of G_0 and G_1 by pCO_2 regulation. In particular, the process utilizes two pCO_2 set points wherein G_0 increases and G_1 decreases if the later pCO_2 set point is higher pCO_2 than the initial set point or vice versa.

However, these methods results in decreased glucose consumption, accumulation of free galactose or drop in antibody concentration upon addition of medium additives (Gramer M J *et.al*).

Further, as demonstrated by EP2511293, galactosylation decreases upon culturing cells at higher pCO_2 values. Also, elevated pCO_2 during cell culture process may result in depressed cell growth and protein production rate (*Kimura R., and Miller W.M. Biotechnol. Bioeng. 52: 152-160, 1996*).

Thus it can be stated that prior art lacks in describing a cell culture process for obtaining a glycoprotein composition with enhanced galactosylated glycans wherein the cell culture performance is not affected.

The present invention describes a process of obtaining an antibody composition comprising a higher percentage of galactosylated glycans while maintaining specific productivity. In particular the invention describes a cell culture process wherein the cells are cultured at high pCO_2, subjected to temperature downshift and harvested at suitable viability.

SUMMARY OF THE INVENTION

A method for obtaining glycoprotein composition with increased percentage of galactosylated glycans has been described here. The method involves i) culturing cells at a first temperature for a first period of time and ii) subjecting cells to a second temperature for a second period of time wherein pCO_2 at the time of harvest is greater than 70 mm of Hg.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an illustration of the pCO_2 profiles for various antibody batches as described in Example 1.

Fig. 2 is an illustration of pCO_2 at the time of harvest, specific productivity and percentage galactosylation for various antibody batches as described in Example 1.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term “glycan” refers to a monosaccharide or polysaccharide moiety.

The term “glycoprotein” refers to protein or polypeptide having at least one glycan moiety. Thus, any polypeptide attached to a saccharide moiety is termed as glycoprotein.

The term “glycoform” or “glycovariant” have been used interchangeably herein, and refers to various oligosaccharide entities or moieties linked in their entirety to the Asparagine 297 (as per Kabat numbering) of the human Fc region of

the glycoprotein in question, co translationally or post translationally within a host cell. The glycan moieties may be added during protein glycosylation include M_3, M_4, M_{5-8}, M_3NAG etc. Examples of such glycans and their structures are listed in Table 1. However, Table 1 may in no way be considered to limit the scope of this invention to these glycans.

The term "glycoform composition" or "glycoform distribution" as used herein pertains to the quantity or percentage of different glycoforms present in a glycoprotein.

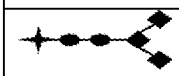
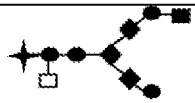
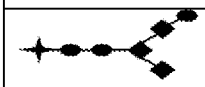
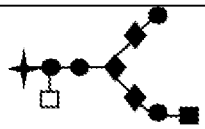
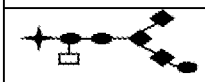
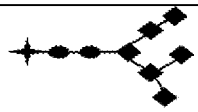
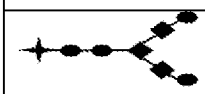
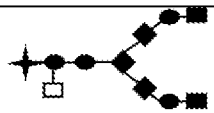
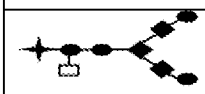
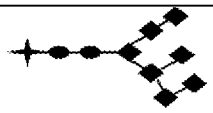
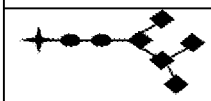
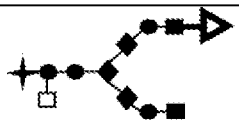
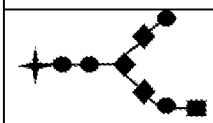
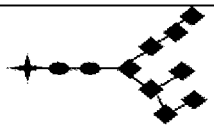
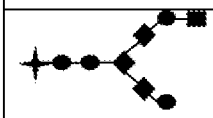
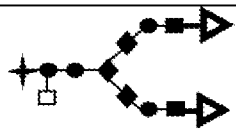
"Galactosylated glycans" refers to glycans containing terminal galactose residues such as G_{1A}, G_{1B}, $G_{1A}F$, $G_{1B}F$, G_2, G_2F and G_2SF.

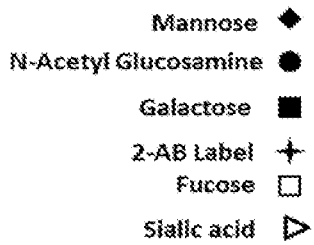
The term "Specific productivity" refers to the amount of protein produced per cell per time. Further, specific productivity is determined by calculating the ratio of 'titre' to 'IVCC' at the time of harvest.

The term "pCO_2" refers to partial pressure or tension of carbon dioxide as measured in millimeter (mm) of mercury (Hg).

The term "osmolality" as used herein is defined as a measure of the milliosmoles of solute per kilogram of solvent (mOsm/kg) and may include ionized or non-ionized molecules and may change during the cell culture process for e.g. by addition of feed, salts, additives or metabolites.

Table I: Representative table of various glycans

Glycan Structure	Code	Glycan Structure	Code
	M3		$G_{1A}F$
	M3NAG		$G_{1B}F$
	M3NAGF		M6
	G_0		G_2F
	G_0F		M7
	M5		G_2SF
	G_{1A}		M8
	G_{1B}		G_2S_2F



DETAILED DESCRIPTION OF THE EMBODIMENTS

The present invention provides a method for obtaining a glycoprotein composition with increased galactosylation content.

In an embodiment, the invention provides a cell culture process for obtaining a glycoprotein composition with increased percentage of galactosylated glycans comprising

a) culturing cells at a first temperature for a first period of time

b) subjecting cells to a second temperature for a second period of time

wherein the pCO_2 at the time of harvest is greater than 70mm of Hg.

The shift in temperature may be further accompanied by addition of feed. Further, the cells may be harvested on day 12 or at a viability less than or equal to 40% whichever was earlier.

In one embodiment, the invention provides a method of increasing galactosylated glycans by about 5% to about 18%.

In another embodiment, the invention provides a method of increasing galactosylated glycans by about 5%.

In yet another embodiment, the invention provides a method of increasing galactosylated glycans by about 11%.

In a further embodiment, the invention provides a method of increasing galactosylated glycans by about 16%.

In a further embodiment, the invention provides a method of increasing galactosylated glycans by about 18%.

In another embodiment, the invention provides a method of obtaining a glycoprotein composition comprising about 44% to about 49% galactosylated glycans.

In another embodiment cells may first be cultured at a temperature of about 35°C – 37°C followed by lowering of temperatures by about 2-7°C.

In yet another embodiment, cells are first cultured at about 37°C followed by shifting the temperature to 35°C.

In a further embodiment, cells are cultured at a pCO_2 greater than 70 mm of Hg for at leastfive days.

Various methods described in the art such as Wuhreret. al., Ruhaak L.R., and Geoffrey et. al. can be used for assessing glycovariants present in a glycoprotein composition (*Wuhrer M. et al., Journal of Chromatography B, 2005, Vol.825, Issue 2, pages 124-133, Ruhaak L.R., Anal BioanalChem, 2010, Vol. 397:3457-3481, Geoffrey, R. G. et. al. Analytical Biochemistry 1996, Vol. 240, pages 210-226*).

The feed medium may be concentrated culture media, single or combination of several different commercial media, either of which may be supplemented with additive component or feed can also be a combination of individual components chosen. The feeds are usually given in the form of concentrated solution so as to reduce the volume increase in the cell culture. Also the feed may or may not increase the culture osmolality, depending upon the feed composition.

The feed medium may include, but are not limited to: PowerCHO-2, HYQ 15.4 (Cell Boost 2, HyClone®), Sodium chloride, Potassium chloride, HYQ 15.4 (Cell Boost 2), MEM Amino acids (50x). It may include nutrient(s) which have been depleted during the cell culture process or are added to attain a particular cell culture or product characteristic. One or more supplements/additives may be added together or separately, independent of time at which other is added.

Further, the feed(s) in the present invention may be added in a continuous, profile or a bolus manner. Also it may be that one or more feeds are in one manner (e.g. profile mode) and others are in second mode (e.g. bolus or continuous mode).

The cell culture media refers to composition which provides cell with all necessary nutrients such as carbon, nitrogen sources, growth factors, vitamins and minerals etc. Such a composition may be made of just one propriety media or a combination of several such media. The cell culture media that are useful in the invention include but are not limited to, are commercially available products such as PF CHO (HyClone®), PowerCHO® 2 (Lonza), Zap-CHO (Invitria), CD CHO, CDOptiCHO™ and CHO-S-SFMII (Invitrogen), ProCHO™ (Lonza), CDM4CHO™ (Hyclone), DMEM (Invitrogen), DMEM/F12 (Invitrogen), Ham's F10 (Sigma), Minimal Essential Media (Sigma), and RPMI -1640 (Sigma).

Certain aspects and embodiments of the invention are more fully defined by reference to the following examples. These examples should not, however, be construed as limiting the scope of the invention.

EXAMPLES

Example I

A CD20-antibody was cloned and expressed in a CHO cell line as described in U.S. Patent No. 7381560 which is incorporated herein by reference. rCHO cells expressing antibody were seeded at density of 0.75±0.1 million cells/ml in PowerCHO® 2 (Lonza, Catalog no: 12-771Q) at 37 ^{0}C and pH 7.05. The cells were cultured till an IVCC of 4.0-5.2 was attained following which temperature was shifted to 35°C and feed (4X PowerCHO-2) was added. The cells were further cultured and harvested on day 12 or at a viability less than or equal to 40% whichever was earlier. The pCO_2 during the cell culture process (I) and at the time of harvest was less than 70 mm Hg.

In an alternate, the pCO_2 during the cell culture process (II-V) was greater than 70 mm Hg for at least five days. Further, pCO_2 at the time of harvest was greater than 70 mm Hg. The pCO_2 profiles during the cell culture batches (I-V) are depicted in Figure 1 and Table II shows the percentage galactosylation. Figure 2 depicts pCO_2 at the time of harvest, specific productivity, and percentage galactosylation.

Table II: Galactosylation content of different antibody batches

Antibody	Galactosylation (%)
I	41.6
II	43.6
III	46.4
IV	48.9
V	48.3

5

CLAIMS

We claim:

1. A cell culture process for obtaining a glycoprotein composition with increased percentage of galactosylated glycans comprising the steps of:

a) culturing cells at a first temperature for a first period of time,

b) subjecting cells to a second temperature for a second period of time,

c) followed by harvesting the cells, wherein

the pCO_2 level during harvest is about 70 mm to about 110 mm of Hg.

2. A cell culture process for obtaining a glycoprotein composition with increased percentage of galactosylated glycans comprising

a) culturing cells at a first temperature for a first period of time

b) subjecting cells to a second temperature for a second period of time

c) followed by harvesting the cells, wherein

the pCO_2 level reaches in the range of about 70 mm Hg to about 110 mm Hg on or after about day 4 of culture initiation.

3. A process according to claim 1 or claim 2, wherein the first temperature of the culture is maintained at about 35°C to about 37°C.

4. A process according to claim 1 or claim 2, wherein the second temperature of the culture is reduced by about 2°C to about 7°C from the first temperature.

5. A process according to claim 1 or claim 2, wherein the increase in galactosylation content of the glycoprotein of interest is about 5% to about 18%.

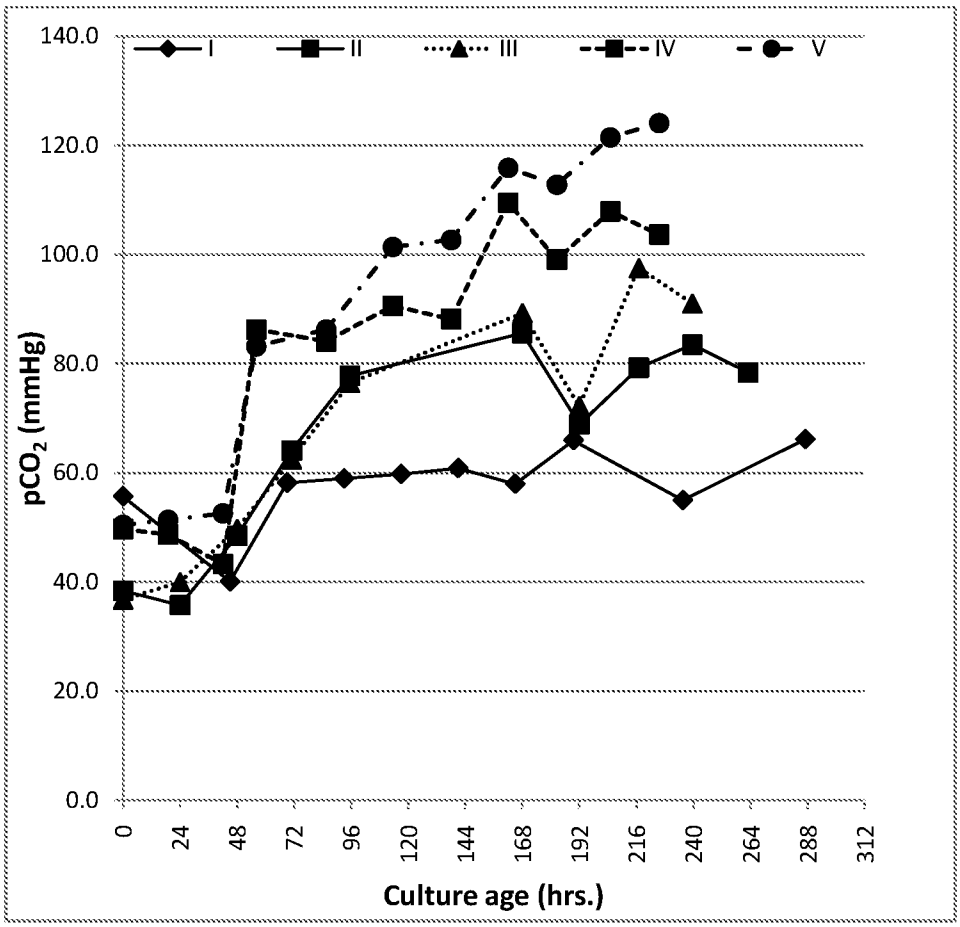

140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
I
II
III
IV
V
pCO2 (mmHg)
0
24
48
72
96
120
144
168
192
216
240
264
288
312
Culture age (hrs.)

FIG. 1

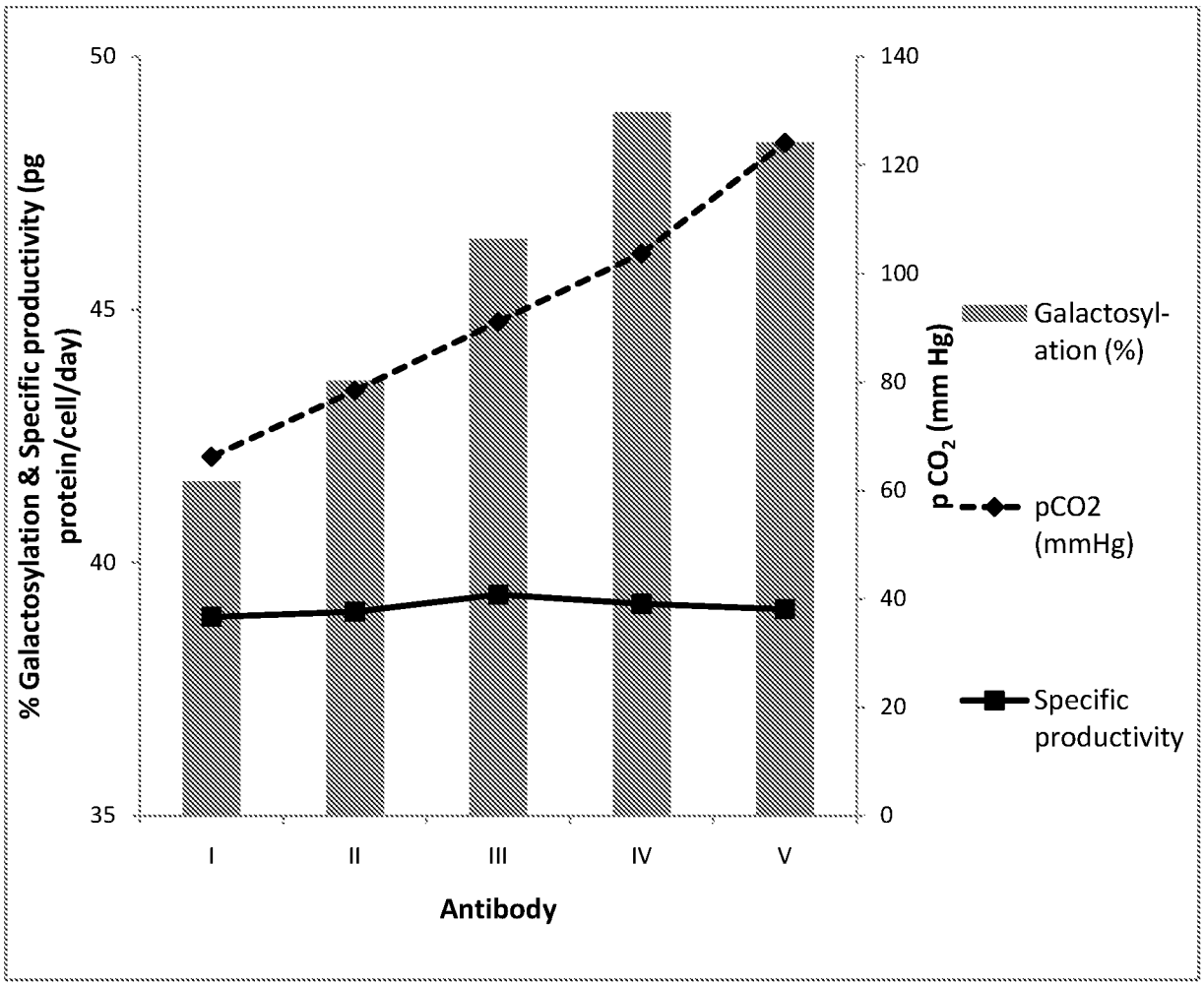

% Galactosylation & Specific productivity (pg protein/cell/day)
50
45
40
35
140
120
100
80
60
40
20
0
p CO2 (mm Hg)
I
II
III
IV
V
Antibody
Galactosyl-ation (%)
pCO2 (mmHg)
Specific productivity

FIG. 2