METHODS TO CONTROL PROTEIN HETEROGENEITY

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Application No. 61/696,219, filed on September 2, 2012, the disclosure of which is incorporated by reference herein in its entirety.

1. INTRODUCTION

The instant invention relates to the field of protein production, and in particular to compositions and processes for controlling and limiting the heterogeneity of proteins expressed in host cells.

2. BACKGROUND OF THE INVENTION

The production of proteins for biopharmaceutical applications typically involves the use of cell cultures that are known to produce proteins exhibiting varying levels of heterogeneity. The basis for such heterogeneity includes, but is not limited to, the presence of distinct glycosylation substitution patterns. For example, such heterogeneity can be observed in increases in the fraction of proteins substituted with agalactosyl fucosylated biantennary oligosaccharides NGA2F+NGA2F-GlcNAc and decreases in the fraction of proteins substituted with galactose-containing fucosylated biantennary oligosaccharides NA1F+NA2F. Such heterogeneity can be assayed by releasing oligosaccharides present on the protein of interest via enzymatic digestion with N-glycanase. Once the glycans are released, the free reducing end of each glycan can be labeled by reductive amination with a fluorescent tag. The resulting labeled glycans are separated by normal-phase HPLC (NP-HPLC) and detected by a fluorescence detector for quantitation.

Technological advances in recombinant protein production analysis have provided unique opportunities for identifying the extent of heterogeneity exhibited by a particular protein population, particularly in the context of large-scale production of recombinant proteins. Although such advances have allowed for the robust characterization of protein heterogeneity, there remains a need in the art to

identify culture conditions and production methods that allow for control over the development of such heterogeneity. Control of protein heterogeneity is particularly advantageous in the context of cell culture processes used for commercially produced recombinant bio-therapeutics as such heterogeneity has the potential to impact therapeutic utility. The instant invention addresses this need by providing compositions and processes to control protein heterogeneity.

3. SUMMARY OF THE INVENTION

The present invention is directed to compositions and methods that control (modulate or limit) protein heterogeneity arising in a population of proteins, e.g., in the context of recombinant protein production.

In certain embodiments, the heterogeneity corresponds to the glycosylation state of individual members of a population of proteins. In certain embodiments, control is exerted over the type of glycosylation substitutions present on individual members of a population of proteins. In certain embodiments, control is exerted over the extent of glycosylation substitutions present on individual members of a population of proteins. In certain embodiments, control is exerted over both the type and extent of glycosylation substitutions present on individual members of a population of proteins. In certain embodiments, such control results in a decrease in the amount of NGA2F+NGA2F-GlcNac oligosaccharides and/or an increase in the amount of NA1F+NA2F oligosaccharides linked to the protein of interest. In certain embodiments, such control results in an increase in the amount of NGA2F+NGA2F-GlcNac oligosaccharides and/or a decrease in the amount of NA1F+NA2F oligosaccharides linked to the protein of interest.

In certain embodiments, control over protein glycosylation heterogeneity is exerted by employing specific hydrolysates during production of the protein of interest, for example, but not by way of limitation, in adaptation cultures performed in media supplemented with hydrolysates. In certain embodiments, control over protein glycosylation heterogeneity is exerted by maintaining certain yeastolate to phytone ratios during production of the protein of interest. In certain embodiments, control over protein glycosylation heterogeneity is exerted by the addition of asparagine during the production of the protein of interest. In certain embodiments

the amount of asparagine present in the cell culture media will range from about 0mM to about 26mM.

In certain embodiments, control over the heterogeneity of the protein compositions described herein is exerted by employing one or more of the foregoing methods during the production and purification of the desired proteins, such as antibodies or antigen-binding portions thereof, described herein.

The heterogeneity of the proteins of interest in the resultant sample product can be analyzed using methods well known to those skilled in the art, e.g., weak cation exchange chromatography (WCX), capillary isoelectric focusing (cIEF), size-exclusion chromatography, Poros™ A HPLC Assay, Host Cell Protein ELISA, Protein A ELISA, and western blot analysis.

In yet another embodiment, the invention is directed to one or more pharmaceutical compositions comprising an isolated protein, such as an antibody or antigen-binding portion thereof, and an acceptable carrier. In another aspect, the compositions further comprise one or more pharmaceutically acceptable carriers, diluents, and/or pharmaceutical agents.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in adalimumab-producing CHO cell line #1 on **(a)** Culture growth, **(b)** Culture viability, and **(c)** Harvest titer.

Figure 2 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in adalimumab-producing CHO cell line #1 on **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 3 depicts the effect of combined supplementation of yeast and soy hydrolysates to CD media from multiple suppliers in adalimumab-producing CHO cell line #1 on **(a)** Culture growth, **(b)** Culture viability, and **(c)** Harvest titer.

Figure 4 depicts the effect of combined supplementation of yeast and soy hydrolysates to CD media from multiple suppliers in adalimumab-producing CHO cell line #1 on **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 5 depicts the effect of supplementing **(a)** yeast, **(b)** soy, or **(c)** wheat hydrolysate from multiple vendors to CDM GIA-1 on culture growth in CHO cell line #1.

Figure 6 depicts the effect of supplementing **(a)** yeast, **(b)** soy, or **(c)** wheat hydrolysate from multiple vendors to CDM GIA-1 on culture viability in CHO cell line #1.

Figure 7 depicts the effect of supplementing yeast, soy, or wheat hydrolysate from multiple vendors to CDM GIA-1 on harvest titer in CHO cell line #1.

Figure 8 depicts the effect of supplementing yeast, soy, or wheat hydrolysate from multiple vendors to CDM GIA-1 in CHO cell line #1 on **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 9 depicts viable cell density and viability in Example 4: Hydrolysate study #1 using distinct ratios of yeast to soy hydrolysate in adalimumab-producing CHO cell line #1.

Figure 10 depicts viable cell density and viability in Example 4: Hydrolysate study #2 using distinct ratios of yeast to soy hydrolysate in adalimumab-producing CHO cell line #1.

Figure 11 depicts the glycosylation profile in Example 4: Hydrolysate Study #1 in adalimumab-producing CHO cell line #1.

Figure 12 depicts the glycosylation profile in Example 4: Hydrolysate Study #2 in adalimumab-producing CHO cell line #1.

Figure 13 depicts the effect of supplementation of asparagine and/or glutamine on day 6 to hydrolysate based media in CHO cell line #1 on culture growth **(a)**, culture viability **(b)** and product titer **(c)**.

Figure 14 depicts the effect of supplementation of asparagine and/or glutamine on Day 6 to hydrolysate based media in adalimumab-producing CHO cell

line #1 on NGA2F and NGA2F-GlcNac glycans **(a)** and on NA1F and NA2F glycans **(b)**.

Figure 15 depicts the dose dependent effect of supplementation of asparagine on Day 7 to hydrolysate based media in adalimumab-producing CHO cell line #1 on culture growth **(a)** and culture viability **(b)** and product titer **(c)**.

Figure 16 depicts the dose dependent effect of supplementation of asparagine on Day 7 to hydrolysate based media in adalimumab-producing CHO cell line #1 on NGA2F and NGA2F-GlcNac glycans **(a)** and on NA1F and NA2F glycans **(b)**.

Figure 17 depicts the dose dependent effect of supplementation of asparagine on Day 0 to hydrolysate based media in adalimumab-producing CHO cell line #1 on culture growth **(a)** and culture viability **(b)** and product titer **(c)**.

Figure 18 depicts the dose dependent effect of supplementation of asparagine on Day 0 to hydrolysate based media in adalimumab-producing CHO cell line #1 on NGA2F and NGA2F-GlcNac glycans **(a)** and on NA1F and NA2F glycans **(b)**.

Figure 19 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM Irvine IS CHO-CD in adalimumab-producing CHO cell line #1 on **(a)** Culture growth, **(b)** Culture viability, and **(c)** Harvest titer.

Figure 20 depicts the effect of yeast, soy, or wheat hydrolysates addition to CDM Irvine IS CHO-CD in adalimumab-producing CHO cell line #1 on oligosaccharides profile **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 21 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in adalimumab-producing CHO cell line #2 on **(a)** Culture growth, **(b)** Culture viability, and **(c)** Harvest titer.

Figure 22 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in adalimumab-producing CHO cell line #2 on **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 23 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in adalimumab-producing CHO cell line #3 on **(a)** Culture growth, **(b)** Culture viability, and **(c)** Harvest titer.

Figure 24 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in adalimumab-producing CHO cell line #3 on **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 25 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in CHO cell line producing mAb #1 **(a)** Culture growth, **(b)** Culture viability, and **(c)** Harvest titer.

Figure 26 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in CHO cell line producing mAb #1 on **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 27 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in CHO cell line producing mAb #2 on **(a)** Culture growth, **(b)** Culture viability, and **(c)** Harvest titer.

Figure 28 depicts the effect of yeast, soy, or wheat hydrolysate addition to CDM GIA-1 in CHO cell line producing mAb #2 on **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 29 depicts the effect of combined supplementation of yeast, soy and/or wheat hydrolysates to CDM GIA-1 in adalimumab-producing CHO cell line #1 on **(a)** Culture growth, **(b)** Culture viability, and **(c)** Harvest titer.

Figure 30 depicts the effect of combined supplementation of yeast, soy, and/or wheat hydrolysates to CDM GIA-1 in adalimumab-producing CHO cell line #1 on **(a)** NGA2F+NGA2F-GlcNac and **(b)** NA1F+NA2F.

Figure 31 depicts the dose dependent effect of supplementation of asparagine on Day 6 to CDM GIA-1 in adalimumab-producing CHO cell line #1 on culture growth **(a)** and culture viability **(b)** and product titer **(c).**

Figure 32 depicts the dose dependent effect of supplementation of asparagine on Day 6 to CDM GIA-1 in adalimumab-producing CHO cell line #1 on NGA2F and NGA2F-GlcNac glycans **(a)** and on NA1F and NA2F glycans **(b)**.

Figure 33 depicts the dose dependent effect of supplementation of asparagine on Day 6 to CDM GIA-1 in adalimumab-producing CHO cell line #2 on culture growth **(a)** and culture viability **(b)** and product titer **(c)**.

Figure 34 depicts the dose dependent effect of supplementation of asparagine on Day 6 to CDM GIA-1 in adalimumab-producing CHO cell line #2 on NGA2F and NGA2F-GlcNac glycans **(a)** and on NA1F and NA2F glycans **(b)**.

Figure 35 depicts the dose dependent effect of supplementation of asparagine during medium preparation to CDM GIA-1 in CHO cell line producing mAb #2 on culture growth **(a)** and culture viability **(b)** and product titer **(c)**.

Figure 36 depicts the dose dependent effect of supplementation of asparagine during medium preparation to CDM GIA-1 in CHO cell line producing mAb #2 on NGA2F and NGA2F-GlcNac glycans **(a)** and on NA1F and NA2F glycans **(b)**.

Figure 37 depicts the dose dependent effect of supplementation of asparagine on Day 5 to CDM GIA-1 in CHO cell line producing mAb #2 on culture growth **(a)** and culture viability **(b)** and product titer **(c)**.

Figure 38 depicts the dose dependent effect of supplementation of asparagine on Day 5 to CDM GIA-1 in CHO cell line producing mAb #2 on NGA2F and NGA2F-GlcNac glycans **(a)** and on NA1F and NA2F glycans **(b)**.

Figure 39 depicts the experimental design for Example 1.

Figure 40 depicts the experimental design for Example 2.

Figure 41 depicts the experimental design for Example 3.

Figure 42 depicts the experimental design for Example 6.

Figure 43 depicts the experimental design for Example 7.

Figure 44 depicts the experimental design for Example 8.

Figure 45 depicts the experimental design for Example 9.

Figure 46 depicts the experimental design for Example 10.

Figure 47 depicts the experimental design for Example 11 (adaptation stage).

Figure 48 depicts the experimental design for Example 11 (production stage).

5. DETAILED DESCRIPTION

For clarity and not by way of limitation, this detailed description is divided into the following sub-portions:

5.1 Definitions; and

5.2 Control of Heterogeneity:

5.2.1 Supplementation of CD Media with Yeast and/or Plant Hydrolysates

5.2.2 Changing Yeast to Plant Hydrolysate Ratio in Cell Culture Medium

5.2.3 Supplementation with Asparagine

5.1 Definitions

In order that the present invention may be more readily understood, certain terms are first defined.

As used herein, the term "glycosylation" refers to the addition of a carbohydrate to an amino acid. Such addition commonly, although not exclusively, occurs via a nitrogen of asparagine or arginine ("N-linked" glycosylation) or to the hydroxy oxygen of serine, threonine, tyrosine, hydroxylysine, or hydroxyproline side-chains ("O-linked" glycosylation). In eukaryotes, N-linked glycosylation occurs on

the asparagine of the consensus sequence Asn-Xaa-Ser/Thr, in which Xaa is any amino acid except proline (Komfeld et al., Ann Rev Biochem 54: 631-664 (1985); Kukuruzinska et al, Proc. Natl. Acad. Sci. USA 84: 2145-2149 (1987); Herscovics et al, FASEB J. 7:540-550 (1993); and Orlean, Saccharomyces Vol. 3 (1996)). O-linked glycosylation also takes place at serine or threonine residues (Tanner et al., Biochim. Biophys. Acta. 906: 81-91 (1987); and Hounsell et al, Glycoconj. J. 13: 19-26 (1996)). However, other glycosylation patterns can be formed, e.g., by linking glycosylphosphatidyl-inositol to the carboxyl-terminal carboxyl group of a protein.

The term "antibody" includes an immunoglobulin molecule comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region (CH). The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The term "antigen-binding portion" of an antibody (or "antibody portion") includes fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., in the case of Adalimumab, hTNFα). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment comprising the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment comprising the VH and CH1 domains; (iv) a Fv fragment comprising the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546, the entire teaching of which

is incorporated herein by reference), which comprises a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see, e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883, the entire teachings of which are incorporated herein by reference). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see, e.g., Holliger, P., et al. (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak, R. J., et al. (1994) Structure 2:1121-1123, the entire teachings of which are incorporated herein by reference). Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecule, formed by covalent or non-covalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S. M., et al. (1995) Human Antibodies and Hybridomas 6:93-101, the entire teaching of which is incorporated herein by reference) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S. M., et al. (1994) Mol. Immunol. 31:1047-1058, the entire teaching of which is incorporated herein by reference). Antibody portions, such as Fab and F(ab')2 fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein. In one aspect, the antigen binding portions are complete domains or pairs of complete domains.

As used herein, the term "recombinant host cell" (or simply "host cell") refers to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. In certain embodiments the host cell is employed in the context of a cell culture.

As used herein, the term "cell culture" refers to methods and techniques employed to generate and maintain a population of host cells capable of producing a recombinant protein of interest, as well as the methods and techniques for optimizing the production and collection of the protein of interest. For example, once an expression vector has been incorporated into an appropriate host, the host can be maintained under conditions suitable for high level expression of the relevant nucleotide coding sequences, and the collection and purification of the desired recombinant protein. Mammalian cells are preferred for expression and production of the recombinant of the present invention; however other eukaryotic cell types can also be employed in the context of the instant invention. See, e.g., Winnacker, From Genes to Clones, VCH Publishers, N.Y., N.Y. (1987). Suitable mammalian host cells for expressing recombinant proteins according to the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) PNAS USA 77:4216-4220, used with a DHFR selectable marker, e.g., as described in Kaufman and Sharp (1982) Mol. Biol. 159:601-621, the entire teachings of which are incorporated herein by reference), NS0 myeloma cells, COS cells and SP2 cells. Other, non-limiting, examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol. 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells

(BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2), the entire teachings of which are incorporated herein by reference.

When using the cell culture techniques of the instant invention, the protein of interest can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. In embodiments where the protein of interest is produced intracellularly, the particulate debris, either host cells or lysed cells (e.g., resulting from homogenization), can be removed by a variety of means, including but not limited to, by centrifugation or ultrafiltration. Where the protein of interest is secreted into the medium, supernatants from such expression systems can be first concentrated using a commercially available protein concentration filter, e.g., an Amicon™ or Millipore Pellicon™ ultrafiltration unit, which can then be subjected to one or more additional purification techniques, including but not limited to affinity chromatography, including protein A affinity chromatography, ion exchange chromatography, such as anion or cation exchange chromatography, and hydrophobic interaction chromatography.

As used herein a "recombinant expression vector" can be any suitable recombinant expression vector, and can be used to transform or transfect any suitable host. For example, one of ordinary skill in the art would appreciate that transformation or transfection is a process by which exogenous nucleic acid such as DNA is introduced into a cell wherein the transformation or transfection process involves contacting the cell with the exogenous nucleic acid such as the recombinant expression vector as described herein. Non-limiting examples of such expression vectors are the pUC series of vectors (Fermentas Life Sciences), the pBluescript series of vectors (Stratagene, LaJolla, Calif.), the pET series of vectors (Novagen, Madison, Wis.), the pGEX series of vectors (Pharmacia Biotech, Uppsala, Sweden), and the pEX series vectors (Clontech, Palo Alto, Calif.).

As used herein, the term "recombinant protein" refers to a protein produced as the result of the transcription and translation of a gene carried on a recombinant expression vector that has been introduced into a host cell. In certain

embodiments the recombinant protein is an antibody, preferably a chimeric, humanized, or fully human antibody. In certain embodiments the recombinant protein is an antibody of an isotype selected from group consisting of: IgG (e.g., IgG1, IgG2, IgG3, IgG4), IgM, IgA1, IgA2, IgD, or IgE. In certain embodiments the antibody molecule is a full-length antibody (e.g., an IgG1 or IgG4 immunoglobulin) or alternatively the antibody can be a fragment (e.g., a Fc fragment or a Fab fragment).

As used herein, the term "Adalimumab", also known by its trade name Humira® (Abbott Laboratories) refers to a human IgG antibody that binds the human form of tumor necrosis factor alpha. In general, the heavy chain constant domain 2 (C_H2) of the Adalimumab IgG-Fc region is glycosylated through covalent attachment of oligosaccharide at asparagine 297 (Asn-297). Adalimumab produced by Chinese hamster ovary (CHO) cells exists in 6 oligosaccharide forms, designated as NGA2F, NGA2F-GlcNAc, NA1F, NA2F, M5 and M6. Weak cation-exchange chromatography (WCX) analysis of the antibody has shown that it has three main charged-variants (i.e. Lys 0, Lys 1, and Lys 2). These variants, or "charged isomers," are the result of incomplete posttranslational cleavage of the C-terminal lysine residues. In addition, WCX analysis has show that production of the antibody can result in the accumulation of two acidic species, identified herein as AR1 and AR2.

The term "about", as used herein, is intended to refer to ranges of approximately 10-20% greater than or less than the referenced value. In certain circumstances, one of skill in the art will recognize that, due to the nature of the referenced value, the term "about" can mean more or less than a 10-20% deviation from that value.

The term "control", as used herein, is intended to refer to both limitation as well as to modulation. For example, in certain embodiments, the instant invention provides methods for controlling diversity that decrease the diversity of certain characteristics of protein populations, including, but not limited to, glycosylation patterns. Such decreases in diversity can occur by: (1) promotion of a desired characteristic, such as a favored glycosylation pattern; (2) inhibition of an unwanted characteristic, such as a disfavored glycosylation pattern; or (3) a combination of the foregoing. As used herein, the term "control" also embraces

contexts where heterogeneity is modulated, i.e., shifted, from one diverse population to a second population of equal or even greater diversity, where the second population exhibits a distinct profile of the characteristic of interest. For example, in certain embodiments, the methods of the instant invention can be used to modulate the types of oligosaccharide substitutions present on proteins from a first population of substitutions to a second equally diverse, but distinct, population of substitutions.

5.2 Control of Protein Heterogeneity

5.2.1 Supplementation of CD Media with Yeast and/or Plant Hydrolysates

It is well known that the pattern of glycoforms that arise in recombinant proteins, including monoclonal antibodies, can be affected by culture conditions during production. (Nam et al., The effects of culture conditions on the glycosylation of secreted human placental alkaline phosphatase produced in Chinese hamster ovary cells. Biotechnol Bioeng. 2008 Aug 15; 100(6): 1178-92). Consistency in the quality of the glycoproteins is important because glycosylation may impact protein solubility, activity, and circulatory half-life. (Gawlitzek et al., Effect of Different Cell Culture Conditions on the Polypeptide Integrity and N-glycosylation of a Recombinant Model Glycoprotein. Biotechnol. Bioeng. 1995; 46:536-544; and Hayter et al., Glucose-limited Chemostat Culture of Chinese Hamster Ovary Cells Producing Recombinant Human Interferon-γ. Biotechnol. Bioeng. 1992; 39:327-335).

In certain instances, such glycosylation-based heterogeneity can take the form of differences in the galactose composition of N-linked oligosaccharides. For example, a terminal galactose is added to NGA2F by β-galactosyltransferase enzyme in the presence of manganese chloride, to produce NA1F (in the case of an addition of a single terminal galactose) or NA2F (in the case of an addition of two terminal galactose molecules). This galactosyltransferase-mediated reaction employs UDP-galactose as the sugar substrate and Mn^{2+} as a cofactor for galactosyltransferase. Thus, without being bound by theory, it is believed that a change in protein homogeneity taking the form of an increase in the fraction of N-linked oligosaccharide NGA2F and a decrease in the fraction of NA1F+NA2F N-linked

oligosaccharides could be caused by either an insufficient amount of the substrate (UDP-galactose), the cofactor for galactosyltransferase (Mn^{2+}), or both.

The experiments disclosed herein demonstrate that, in certain embodiments, supplementation of CD cell culture media with yeast and/or plant hydrolysates can modulate product quality of a mAb by, in certain embodiments, decreasing the NGA2F+NGA2F-GlcNac and, in certain embodiments, increasing the NA1F+NA2F oligosaccharides. These results were achieved in multiple CD media available from multiple vendors (Life Sciences Gibco, HyClone, and Irvine Scientific), using yeast and/or plant hydrolysates (for example, but not by way of limitation, soy, wheat, rice, cotton seed, pea, corn, and potato) from multiple vendors (BD Biosciences, Organotechnie, Sheffield/Kerry Biosciences, Irvine Scientific, and DMV International). In experiments where yeast or plant hydrolysates were added individually, a dose-dependent effect in the extent of reduction of NGA2F+NGA2F-GlcNac oligosaccharides (and a corresponding increase in the NA1F+NA2F oligosaccharides) with increasing yeast or plant hydrolysates concentration in culture CD media was observed. For example, but not by way of limitation, yeast hydrolysates can be used to supplement a CD cell culture media at concentrations ranging from about 2 g/L to about 11 g/L to achieve the desired reduction of NGA2F+NGA2F-GlcNac oligosaccharides and a corresponding increase in the NA1F+NA2F oligosaccahrides. In certain non-limiting embodiments, yeast hydrolysates can be used to supplement a CD cell culture media at concentrations of about 2 g/L, about 5 g/L, or about 11 g/L. In certain non-limiting embodiments, plant hydrolysates can be used to supplement a CD cell culture media at concentrations ranging from about 2 g/L to about 15 g/L to achieve the desired reduction of NGA2F+NGA2F-GlcNac oligosaccahrides and a corresponding increase in the NA1F+NA2F oligosaccharides. In certain non-limiting embodiments, plant hydrolysates can be used to supplement a CD cell culture media at concentrations of about 2 g/L, about 4 g/L, 7 g/L, 10 g/L, or about 15 g/L.

In certain embodiments, the concentration of yeast and/or plant hydrolysates is maintained in such a manner as to reduce the NGA2F+NGA2F-GlcNac sum in a protein or antibody sample by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%,

45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, and ranges within one or more of the preceding. In certain embodiments, the concentration of yeast and/or plant hydrolysates is maintained in such a manner as to increase the NA1F+NA2F sum in a protein or antibody sample by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, and ranges within one or more of the preceding.

In certain embodiments, control over the glycosylation distribution of proteins produced by cell culture can be exerted by maintaining the appropriate yeast hydrolysate concentration in the cell culture expressing the protein of interest as described herein. Specific culture conditions can be used in various cultivation methods including, but not limited to, batch, fed-batch, chemostat and perfusion, and with various cell culture equipment including, but not limited to, shake flasks with or without suitable agitation, spinner flasks, stirred bioreactors, airlift bioreactors, membrane bioreactors, reactors with cells retained on a solid support or immobilized/entrapped as in microporous beads, and any other configuration appropriate for optimal growth and productivity of the desired cell line

5.2.2 Changing Yeast to Plant Hydrolysate Ratio in Cell Culture Medium

The instant disclosure relates to control of the glycosylation distribution in mammalian cell culture processes, including where specific components, such as hydrolyzed yeast and soy-based supplements, are commonly used and are typical constituents of suspension culture media in such processes. These nutrients are important for ensuring both robust cell growth and production of glycoproteins. However, the present invention utilizes these components in such a way to affect the critical quality attributes of the glycoprotein. For example, but not by way of limitation, by adjusting the concentration ratio of these two hydrolysates, yeast and soy (phytone), within the range of about 0.25 to about 1.55, the resultant glycosylation distribution can be modified. As outlined in Example 1, non-limiting embodiments of the present invention include supplements comprising 100% yeast hydrolysate as well as those that are 100% plant hydrolysate. Thus, this disclosure provides a means to modulate glycosylation variations introduced by process inputs,

such as raw materials, and other variability inherent in dynamic manufacturing operations. Ultimately, the disclosure enables in-process control of protein glycosylation with respect to desired product specifications.

In certain embodiments, the ratio of these two hydrolysates, yeast and soy (phytone), is maintained in such a manner as to reduce the NGA2F+NGA2F-GlcNac sum in a protein or antibody sample by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, and ranges within one or more of the preceding. In certain embodiments, the ratio of these two hydrolysates, yeast and soy (phytone), is maintained in such a manner as to increase the NA1F+NA2F sum in a protein or antibody sample by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, and ranges within one or more of the preceding.

In certain embodiments, control over the glycosylation distribution of protein produced by cell culture can be exerted by maintaining the appropriate yeast to plant hydrolysate ratio in the cell culture expressing the protein of interest as described herein. Specific culture conditions can be used in various cultivation methods including, but not limited to, batch, fed-batch, chemostat and perfusion, and with various cell culture equipment including, but not limited to, shake flasks with or without suitable agitation, spinner flasks, stirred bioreactors, airlift bioreactors, membrane bioreactors, reactors with cells retained on a solid support or immobilized/entrapped as in microporous beads, and any other configuration appropriate for optimal growth and productivity of the desired cell line

5.2.3 Supplementation with Asparagine

The instant disclosure relates to control of the glycosylation distribution in mammalian cell culture processes, including where specific components, such as amino acids and amino acid-based supplements, are commonly used and are typical constituents of suspension culture media. These nutrients are important for ensuring both robust cell growth and production of glycoproteins. However, this present invention utilizes these components, and in particular

asparagine and/or glutamine in such a way to affect the critical quality attributes of the glycoprotein. For example, but not by way of limitation, by adjusting the concentration of one or both of these two amino acids the resultant glycosylation distribution can be modified. Thus, this disclosure provides a means to modulate glycosylation variations introduced by process inputs, such as raw materials, and other variability inherent in dynamic manufacturing operations. Ultimately, the disclosure enables in-process control of protein glycosylation with respect to desired product specifications.

The experiments disclosed herein demonstrate that, in certain embodiments, supplementation of cell culture media with asparagine and/or glutamine can modulate product quality of a mAb by, in certain embodiments, increasing the NGA2F+NGA2F-GlcNac and, in certain embodiments, decreasing the NA1F+NA2F oligosaccharides. For example, but not by way of limitation, the percentage of NGA2F+NGA2F-GlcNac can be increased by 2-4% and the percentage of NA1F+NA2F was decreased by 2-5% when 0.4 to 1.6 g/L asparagine is added on either day 0 or days 6 or 7, as outlined in Example 5, below. Similarly, addition of 0.4 g/L glutamine, to the culture run described in Example 5, below, increased the percentage of NGA2F+NGA2F-GlcNac by 1% and lowered the percentage of NA1F+NA2F by 1%. Finally, adding both asparagine and glutamine (0.4 g/L of each), to the cell culture run described in Example 5, below, increased the percentage of NGA2F+NGA2F-GlcNac by 3% and decreased the percentage of NA1F+NA2F by 4%. In addition, the cell growth profile is the same when 0.8 and 1.6 g/L of asparagine was added, but a dose dependent effect on oligosaccharide distribution was observed, indicating that the effect on oligosaccharide distribution was due to the addition of asparagine and not the increased maximum viable cell density or delayed drop in viability. In certain embodiments, the total amount of asparagine in the cell culture media will range from about 0 mM to about 26 mM. In certain embodiments, for example those embodiments where a hydrolysate media is employed, the range of asparagine in the cell culture media will range from about 1.3 mM to about 14.6 mM. In certain embodiments, for example, but not limited to, those embodiments where GIA1 media is employed, the range of asparagine in the cell culture media will range from about 12.3 mM to about 25.7 mM.

In certain embodiments, the concentration of asparagine and/or glutamine is maintained in such a manner as to reduce the NA1F+NA2F sum in a protein or antibody sample by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, and ranges within one or more of the preceding. In certain embodiments, the concentration of asparagine and/or glutamine is maintained in such a manner as to increase the NGA2F+NGA2F-GlcNac sum in a protein or antibody sample by about 1%, 1.2%, 1.5%, 2%, 2.2%, 2.5%, 3%, 3.2%, 3.5%, 4%, 4.2%, 4.5%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, and ranges within one or more of the preceding.

In certain embodiments, control over the glycosylation distribution of protein produced by cell culture can be exerted by maintaining the appropriate asparagine and/or glutamine concentration in the cell culture expressing the protein of interest as described herein. Specific culture conditions can be used in various cultivation methods including, but not limited to, batch, fed-batch, chemostat and perfusion, and with various cell culture equipment including, but not limited to, shake flasks with or without suitable agitation, spinner flasks, stirred bioreactors, airlift bioreactors, membrane bioreactors, reactors with cells retained on a solid support or immobilized/entrapped as in microporous beads, and any other configuration appropriate for optimal growth and productivity of the desired cell line.

EXAMPLES

Example 1: Control of Heterogeneity by Addition of Hydrolysates to CD Media GIA-1 for Culture of an Adalimumab-Producing CHO Cell Line #1

Control of heterogeneity of therapeutic monoclonal antibodies (mAbs) can aid in ensuring their efficacy, stability, immunogenicity, and biological activity. Media composition has been shown to play a role in product quality of mAbs together with process conditions and choice of cell line. In certain embodiments, the present invention provides methods for fine-tuning the product quality profile of a mAb produced in various Chinese hamster ovary (CHO) cell lines by supplementation of yeast and/or plant hydrolysates to chemically defined (CD) media. In certain

embodiments, the resulting mAb product is characterized by having a decreased content of complex agalactosylated glycans NGA2F and NGA2F-GlcNac and increased levels of terminally galactosylated glycans NA1F and NA2F. In certain embodiments, addition of increasing amounts of yeast, soy or wheat hydrolysates from several suppliers to a CD medium resulted in altered product quality profiles in a concentration-dependent manner.

In the studies summarized in this example, the effects on glycosylation resulting from the addition of yeast (Bacto TC Yeastolate: 2, 5, 11 g/L), soy (BBL Phytone Peptone: 2, 4, 7, 10, 15 g/L), or wheat (Wheat Peptone E1: 2, 4, 7, 10, 15 g/L) hydrolysates to CD medium GIA-1 (Life Technologies Gibco; proprietary formulation) in the adalimumab-producing CHO cell line #1 were investigated.

1.1 Materials and methods

Adaptation and production media were supplemented with Bacto TC Yeastolate, BBL Phytone Peptone, or Wheat Peptone E1 according to the experimental design in Figure 39. The control cultures were not supplemented with hydrolysates. In addition to hydrolysates, adaptation media was supplemented with 0.876 g/kg L-glutamine and 2.0 mL/kg methotrexate solution, and production media was supplemented with 0.584 g/L L-glutamine. The experiment was designed into two blocks. All media pH was adjusted to approximately 7.1 using 6N hydrochloric acid/5N sodium hydroxide. The media osmolality was adjusted to 290 - 300 mOsmol/kg with sodium chloride.

The adalimumab-producing cultures were expanded for 3 passages (3 days each) in their respective adaptation media in a combination of 250 mL (50 mL or 100 mL working volume) and 500 mL (150 mL working volume) Corning vented non-baffled shake flasks and maintained on an orbital shaker at 110 RPM in a 35°C, 5% CO_2 dry incubator. At each passage, cultures were inoculated at an initial viable cell density (VCD) of approximately 0.5 x 10^6 cells/mL.

Production cultures were initiated in duplicate 500 mL Corning, vented, non-baffled shake flasks each containing 200 mL culture in dry incubators at 35°C, 5% CO_2 , and 110 RPM. Initial VCD was approximately 0.5 x 10^6 cells/mL. A

1.25% (v/v) 40% glucose stock solution was fed when the media glucose concentration was less than 3 g/L.

For all studies described throughout this application, samples were collected daily and measured for cell density and viability using a Cedex cell counter. Retention samples for titer analysis (2 x 1.5 mL per condition) via Poros A method were collected daily after culture viability fell below 90%. Samples were centrifuged at 12,000 RPM for 5 min and the supernatant was stored at -80°C until further analysis. The harvest procedure was performed by centrifugation of the culture sample at 3,000 RPM for 30 min followed by storage of the supernatant in 125 mL PETG bottles at -80°C until protein A purification, oligosaccharide, and WCX-10 analysis.

For the oligosaccharide assay, the oligosaccharides are released from the protein by enzymatic digestion with N-glycanase. Once the glycans are released, the free reducing end of each glycan is labeled by reductive amination with a fluorescent tag, 2-aminobenzamide (2-AB). The resulting labeled glycans are separated by normal-phase HPLC (NP-HPLC) in acetonitrile: 50 mM ammonium formate, pH 4.4, and detected by a fluorescence detector. Quantitation is based on the relative area percent of detected sugars. The relative area percents of the agalactosyl fucosylated biantennary oligosaccharides (NGA2F + [NGA2F-GlcNac]) and the galactose-containing fucosylated biantennary oligosaccharides NA1F+NA2F are reported and discussed.

1.2 Culture growth and productivity

The majority of cultures grew to a similar peak VCD in the range of 9-11 x 10^6 cells/mL. Cultures supplemented with 11 g/L yeast hydrolysate BD TC yeastolate experienced slight inhibition of growth (Figure 1A). Viability profiles were comparable to the control condition with cultures lasting 11 to 13 days (Figure 1B). Increasing the yeast hydrolysate concentration in CDM media GIA-1 resulted in decreased average productivity compared to the control condition. Cultures supplemented with soy or wheat hydrolysates lasted 12 to 13 days, and experienced slightly increased average titer compared to the control condition (Figure 1C).

1.3 Oligosaccharide analysis

Addition of yeast, soy, or wheat hydrolysates to CD media GIA-1 lowered the percentage of glycans NGA2F+NGA2F-GlcNac by 1-14 % and increased the percentage of NA1F+NA2F glycans by 2-12 % compared to control condition (NGA2F+NGA2F-GlcNac: 89%; NA1F+NA2F: 6%) (Figures 2A-B). A dose-dependent decrease in NGA2F+NGA2F-GlcNac and a corresponding increase in NA1F+NA2F glycans was observed with the addition of yeast, soy, or wheat hydrolysate over the tested range. The highest percentage decrease in NGA2F+NGA2F-GlcNac and corresponding highest increase in NA1F+NA2F glycans was recorded for the condition supplemented with 7 g/L BD BBL phytone peptone (NGA2F+NGA2F-GlcNac: 78%, and NA1F+NA2F: 18%) compared to control.

Example 2: Yeast and Soy Hydrolysates Combined Addition to Multiple Commercially Available CD Media for Culture of an Adalimumab-Producing CHO Cell Line #1

In the study summarized in this example, the effects of combined yeast and soy hydrolysates addition to CD media from multiple suppliers: Life Technologies Gibco (OptiCHO and GIA-1), Irvine Scientific (IS CHO-CD), and HyClone/Thermo Scientific (CDM4CHO) on product quality in the adalimumab-producing CHO cell line #1 utilized in Example 1 were evaluated.

2.1 Materials and methods

The liquid or powder formulation media were purchased from multiple vendors (Life Technologies Gibco - OptiCHO and GIA-1; Irvine Scientific – IS CHO-CD; and HyClone/Thermo Scientific - CDM4CHO), reconstituted per the manufacturers' recommendations, and supplemented with Bacto TC Yeastolate and BBL Phytone Peptone according to the experimental design in Figure 40. The control cultures for each condition were not supplemented with hydrolysates. All media pH was adjusted to approximately 7.1 using 6N hydrochloric acid/5N sodium hydroxide.

Cultures were expanded for 3 passages (3 days each) in their respective adaptation media in a combination of 250 mL (50 mL or 100 mL working volume) and 500 mL (150 mL working volume) Corning vented non-baffled shake flasks and maintained on an orbital shaker at 110 RPM in a 35°C, 5% CO_2 dry incubator. Production cultures were initiated in duplicate 500 mL (200 mL working volume) Corning vented non-baffled shake flasks at an initial VCD of approximately 0.5 x 10^6 cells/mL. The shake flask study was run in an extended batch-mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L.

2.2 Culture growth and productivity

Commercially available CD media supported markedly different culture growth profiles with maximum VCD of 2-9 x 10^6 cells/mL and culture duration ranging from 7 to 15 days (Figure 3A). Addition of yeast and soy hydrolysates to Life Technologies Gibco OptiCHO and GIA-1, and HyClone CDM4CHO media decreased peak VCD and increased culture length by 2 to 6 days. However, addition of hydrolysates to Irvine IS CHO-CD media increased peak VCD from 2.5 x 10^6 cells/mL to 5.4 x 10^6 cells/mL. Culture viability declined slower with addition of hydrolysates for all media tested (Figure 3B). Productivity also varied significantly among cultures; however, the addition of hydrolysates to CD media increased productivity in all cases (Figure 3C).

2.3 Oligosaccharide analysis

The combined addition of yeast and soy hydrolysates to various commercially available CD media lowered the percentage of NGA2F+NGA2F-GlcNac glycans by 2-10% compared to control (Figure 4A): from 81% to 79% (HyClone CDM4CHO); from 80% to 75% (Irvine IS CHO-CD); from 88% to 80% (Life Technologies OptiCHO); from 90% to 80% (Life Technologies GIA-1). The percentage of NA1F+NA2F glycans increased by 3-8% compared to control (Figure 4B): from 15% to 18% (HyClone CDM4CHO); from 6% to 12% (Life Technologies GIA-1); from 16% to 21% (Irvine IS CHO-CD); from 5% to 13% (Life Technologies OptiCHO).

Example 3: Supplementation of Yeast, Soy and Wheat Hydrolysates from Multiple Vendors to CD Media GIA-1 for Culture of an Adalimumab-Producing CHO Cell Line #1

In the study summarized in this example, we investigated the effects on glycosylation resulting from the addition of yeast (5, 11 g/L), soy (4, 7 g/L) or wheat (4, 7 g/L) hydrolysates from multiple vendors (BD Biosciences, Sheffield/Kerry Biosciences, DMV International, Irvine Scientific, and Organotechnie) to CDM GIA-1 in the adalimumab-producing CHO cell line #1.

3.1 Materials and methods

Adaptation and production media were supplemented with Bacto TC Yeastolate, BBL Phytone Peptone, or Wheat Peptone E1 according to the experimental design in Figure 41. The control cultures were not supplemented with hydrolysates. All media pH was adjusted to approximately 7.1 using 6N hydrochloric acid/5N sodium hydroxide. The media osmolality was adjusted to 290 - 300 mOsmol/kg with sodium chloride.

Cultures were expanded for 3 passages (3 days each) in their respective adaptation media in a combination of 250 mL (50 mL or 100 mL working volume) and 500 mL (150 mL working volume) Corning vented non-baffled shake flasks and maintained on an orbital shaker at 110 RPM in a 35°C, 5% CO_2 dry incubator. Production cultures were initiated in duplicate 500 mL (200 mL working volume) Corning, vented, non-baffled shake flasks at an initial VCD of approximately 0.5 x 10^6 cells/mL. The shake flask study was run in an extended-batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L.

3.2 Culture growth and productivity

Culture growth and viability profiles were comparable among all test conditions (Figures 5A-C, 6A-C) except for 11 g/L BD Bacto TC yeastolate, for which a slight decrease in the growth rate and maximum VCD was observed. Supplementation of CD media GIA-1 with yeast hydrolysates lowered the harvest

titer by up to 25% compared to the control, while the harvest titer increased up to 14% and 27% with the addition of soy or wheat hydrolysates, respectively (Figure 7).

3.3 Oligosaccharide analysis

Addition of yeast, soy or wheat hydrolysates to CD media GIA-1 decreased the NGA2F+NGA2F-GlcNac glycans in a dose-dependent manner for all hydrolysate vendors evaluated (Figures 8A-B). Addition of yeast hydrolysates to CD media GIA-1 lowered the percentage of NGA2F+NGA2F-GlcNac glycans by 4-9%, and increased the percentage of NA1F+NA2F glycans by 5-10% compared to control (NGA2F+NGA2F-GlcNac: 90%; NA1F+NA2F: 6%). Addition of soy hydrolysates to CD media GIA-1 decreased the NGA2F+NGA2F-GlcNac glycans by 9-14%, and increased the NA1F+NA2F glycans by 11-15% compared to control. Addition of wheat hydrolysates decreased the NGA2F+NGA2F-GlcNac glycans by 4-11%, and increased the NA1F+NA2F glycans by 6-12% compared to control.

Example 4: Control of Heterogeneity by Addition of Reduced Ratio of Yeast to Plant Hydrolysate

To identify the role which the ratio of yeast to plant hydrolysate plays in connection with the generation of protein heterogeneity, experiments employing a range of different hydrolysate ratios were undertaken. The cell culture medium employed in each experimental process contains both yeast and soy hydrolysate (phytone). The ratios of yeast to soy hydrolysate (by weight) are 1.55, 0.67 and 0.25. The total weight of yeastolate and soy hydrolysate were not changed in each experimental process. Two distinct yeastolate lots were used in connection with these experiments (see Figures 9 & 11 and 10 & 12, respectively). Culture growth, productivity and product quality were assessed. As outlined in Figures 9-12, reducing the yeast to soy hydrolysate ratio resulted in altered oligosaccharide profiles.

4.1. Materials and Methods

The CHO cell line #1 was employed in the studies covered here. The production medium used in this experiment contains basal medium PFCHO, Bacto TC yeastolate and phytone peptone. The pH of all media was adjusted to 7.15; and media osmolality was adjusted to 373 – 403 mOsmol/kg with sodium chloride. For

each experiment, 500 mL shakers with 200 mL working volume were employed at the following conditions: 35°C constant temperature; 5% CO_2; and 110 RPM. Cultures were inoculated at an initial viable cell density (VCD) of approximately 0.5 x 10^6 cells/mL. Two mL of 40% w/w glucose solution was added to each shaker when the glucose concentration dropped below 2 g/L. The shakers were harvested when the viable cell density decreased to approximately 50%. The harvest broth was centrifuged at 3200 rpm for 30 min at 5 °C to remove cells and the supernatant was stored at –80 °C

Samples were taken daily from each shaker to monitor growth. The following equipment was used to analyze the samples: Cedex cell counter, Radiometer blood gas analyzer, YSI glucose analyzer, and osmometer. The harvest samples stored at –80 °C were later thawed and analyzed for titer with Poros A HPLC method. In addition, the thawed samples were filtered through a 0.2μm filter, purified by Protein A chromatography, and then oligosaccharide analysis was performed as described in Example 1.

4.2 Cell Growth and Productivity

In the first hydrolysate study, the viable cell densities for the reduced ratios of yeastolate to phytone (i.e. Y/P=0.67 and Y/P=0.25) were much lower than the viable cell density for the 1.55 ratio of yeastolate to phytone (Figure 9). As a result, the IVCC on day 13 (i.e. the harvest day) was significantly lower for the reduced ratio conditions compared to the 1.55 ratio condition, and the titer was also lower (but not statistically significantly – data not shown). The viability profiles were comparable until day 8 (Figure 9). After day 8, the viability declined faster for the reduced ratio conditions. In hydrolysate study 2, the viable cell density and viability for the 1.55 ratio were slightly lower than those with reduced ratio in the exponential phase, but higher in the decline phase (Figure 10). However, the titer for the 1.55 ratio shaker was 0.2 g/L lower than the reduced ratio (i.e. Y/P = 0.67) (data not shown).

4.3. Oligosaccharide Analysis

Glycosylation profiles for hydrolysate studies 1 and 2 are shown in Figures 11 and 12, respectively. Reducing the ratio of yeastolate to phytone reduced the percentage of NGA2F + (NGA2F-GlcNAc) glycan. In hydrolysate study 1, the

percentage of NGA2F + (NGA2F-GlcNAc) was significantly reduced for Y/P = 0.67 and Y/P = 0.25 as compared to Y/P = 1.55. The *p* values were 0.03 and 0.001 for Y/P = 0.67 and Y/P = 0.25, respectively. At the same time, the percentage of NA1F + NA2F was increased significantly as the ratio of yeastolate to phytone was reduced.

As shown in Figure 12 in hydrolysate study 2, the difference in the percentage of NGA2F + (NGA2F-GlcNAc) between Y/P = 0.67 and Y/P =1.55 was significant (i.e. $p = 0.000002$). The percentage of NGA2F + (NGA2F-GlcNAc) was lowered from 77.5% in the 1.55 ratio to approximately 75.4% with the reduced ratio.

Therefore, this study successfully demonstrated that reducing the ratio of yeastolate to phytone could alter oligosaccharide profile using two different lots of yeast hydrolysate.

Example 5: Control of Heterogeneity by Supplementation with Asparagine

The present invention relates to methods for modulating the glycosylation profile of a monoclonal antibody (mAb) by varying the concentration of asparagine in cell culture media. Cell culture medium components, such as asparagine, are commonly used and are typical constituents of suspension culture media. These nutrients are important for ensuring both robust cell growth and production of glycoproteins. It has been shown that the cell viability and product titer can be enhanced by the addition of asparagine to a glutamine-free production medium (Genentech, Inc. "Production of Proteins in Glutamine-Free Cell Culture Media" WO2011019619 (2010)). However, the present invention provides methods to modify glycosyaltion distribution by adjusting the concentration of asparagine. Without being bound by theory, it is thought that the effect of asparagine on glycosylation profile of an antibody is through its conversion to glutamine and/or aspartate. Asparagine is the amide donor for glutamine and can be converted to glutamine and/or aspartate (H Huang, Y Yu, X Yi, Y Zhang "Nitrogen metabolism of asparagine and glutamate in Vero cells studied by 1 H/15 N NMR spectroscopy" Applied microbiology and biotechnology 77 (2007) 427-436). Glutamine and aspartate are important intermediates in pyrimidine synthesis; and it is known that enhancing *de novo* pyrimidine biosynthesis could increase intracellular UTP concentration (Genentech,

Inc. "Galacosylation of Recombinant Glycoproteins" US20030211573 (2003)). In addition, studies have suggested that glutamine and aspartate limitation is expected to inhibit amino sugar synthesis (GB Nyberg, RR Balcarcel, BD Follstad, G Stephanopoulos, DI Wang "Metabolic effects on recombinant interferon-gamma glycosylation in continuous culture of Chinese hamster ovary cells" Biotechnology and Bioengineering 62 (1999) 336-47; DCF Wong, KTK Wong, LT Goh, CK Heng, MGS. Yap "Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures" Biotechnology and Bioengineering 89 (2005) 164-177). Both UTP and amino sugar are required for the synthesis of UDP-GlcNac, which is the substrate for protein glycosylation process. It is also possible that the effect of asparagine on glycosyaltion is via increasing ammonia concentration in the cell culture medium since it is showed that the addition of ammonia in CHO cultures could reduce the extent of glycosylation of synthesized EPO (M. Yang and M. Butler "Effect of Ammonia on the Glycosylation of Human Recombinant Erythropoietin in Culture" Biotechnol. Prog. 16 (2000) 751-759). We have found that ammonia concentration was increased after asparagine addition into the cell culture media.

In the studies summarized in Example 5, we investigated the effects on product quality attributes resulting from the addition of asparagine to hydrolysate based medium in an adalimumab-producing CHO cell line, generically named CHO cell line #1. Two experiments were performed in the instant Example. For the first experiment, glutamine and/or asparagine were added (at an individual concentration of 0.4 g/L) on day 6. For the second experiment, asparagine was added at different dosage (i.e. 0.4g/L, 0.8g/L or 1.6g/L) either on day 0 (before inoculation) or together with the first glucose shot (happened on day 7).

5.1 Materials and methods

The CHO cell line #1 was employed in the studies covered here. Upon thaw, cells were expanded in a 19-days seed train and then transferred into seed reactors for up to 7 days in growth medium. The cells were then brought to the laboratory and used in the small scale bioreactor experiments. The media used in

these experiments contains basal media PFCHO (proprietary formulation), Bacto TC Yeastolate and Phytone Peptone.

Three litter Applikon bioreactors were sterilized and then charged with production medium. At inoculation, cells were aseptically transferred into each bioreactor to reach an initial cell density of 0.5 x 10^6 viable cells/mL. After inoculation, the bioreactors were set to the following conditions: pH=7.1, T=35°C, DO=30%, and agitation=200 rpm. The pH was shifted from 7.1 to 6.9 over the first 2.5 days and held at 6.9 for the remainder of the run. The percentage of dissolved oxygen was controlled by sparging a mixture of air and oxygen. The addition of 0.5 N NaOH or sparging of CO_2 maintained the pH. When the glucose concentration fell below 2 g/L, approximately 1.25% (v/v) of glucose solution (400g/kg) was added to the cell culture.

For the first experiment, glutamine and/or asparagine were added (at an individual concentration of 0.4 g/L) together with the first glucose shot (happened on day 6). For the second experiment, asparagine was added at different dosage (i.e. 0.4g/L, 0.8g/L or 1.6g/L) either on day 0 (before inoculation) or together with the first glucose shot (happened on day 7).

Samples were taken daily from each reactor to monitor growth. The following equipment was used to analyze the samples: Cedex cell counter for cell density and viability; Radiometer ABL 5 blood gas analyzer for pH, pCO2 and pO2; YSI 7100 analyzer for glucose and lactate concentration. Some of the daily samples and the harvest samples were centrifuged at 3,000 RPM for 30min and then the supernatants were stored at –80 °C. Later, the thawed harvest samples were filtered through a 0.2μm filter, purified by Protein A chromatography, and then oligosaccharide analysis was performed and then oligosaccharide analysis was performed as described in Example 1.

5.2 Culture growth and productivity

In both of the experiments performed in 3L bioreactor in hydrolysate based media with CHO cell line #1 described in the instant Example, the

addition of glutamine and/or asparagine together with a glucose shot increased the maximum cell density (Figure 13A and 15A, respectively). The increase in cell density is started two days after the addition in both cases. Maximum viable cell density was consistent when 0.4 g/L of glutamine or asparagine was added. Increasing the concentration of asparagine to 0.8 g/L or adding both glutamine and asparagine at a concentration of 0.4 g/L each further increased the maximum viable cell density; however, adding asparagine at a higher concentration than 0.8 g/L (e.g., 1.6 g/L) did not continue to increase the maximum viable cell density. In contrast, when asparagine was added on day 0 (before inoculation), the maximum viable cell density increased in a dose dependent manner, with the maximum viable cell density being reached when 1.6 g/L of asparagine was added on day 0 (Figure 17A).

A drop in viability was delayed, as compared to control cultures, in both experiments described in the instant Example for approximately 3 days when glutamine and/or asparagine was added on day 6 or 7 (Figures 13B and 15B, respectively). However, the drop in viability accelerated on the last day of the cultures. In contrast, although the drop in viability was delayed when asparagine was added on day 0, the effect of delaying viability decay was not as efficient as when the amino acids were added later (e.g., on day 6 or day 7) as shown in Figure 17B.

5.3 Oligosaccharide analysis

The experiments described in the instant Example indicate that oligosaccharide distribution is altered with the addition of asparagine and/or glutamine. The addition of asparagine increased NGA2F+NGA2F-GlcNac in a dose dependent manner. Compared to control, the percentage of NGA2F+NGA2F-GlcNac was increased by 1.0-3.9% and the percentage of NA1F+NA2F was decreased by 1.1-4.3% when 0.4 to 1.6 g/L asparagine was added on either day 0 or days 6 or 7 (Figures 14A-14B, 16A-16B and 18A-18B). Addition of 0.4 g/L glutamine increased the percentage of NGA2F+NGA2F-GlcNac by 0.7% and lowered the percentage of NA1F+NA2F by 0.9%. Adding both asparagine and glutamine (0.4 g/L of each) increased the percentage of NGA2F+NGA2F-GlcNAc by 3.3% and decreased the percentage of NA1F+NA2F by 4.2%. In addition, the cell growth profile is the same when 0.8 and 1.6 g/L of asparagine was added on day7 (Figure 15A and 15B), but a dose dependent effect on oligosaccharide distribution was observed (Figures 16A and

16B), indicating that the effect on oligosaccharide distribution was due to the addition of asparagine and not the increased maximum viable cell density or delayed drop in viability.

Example 6: Yeast, Soy, or Wheat Hydrolysate Addition to Commercially Available CD Media IS CHO-CD for Culture of an Adalimumab-Producing CHO Cell Line #1

In the study summarized in this example, the effects on glycosylation resulting from the addition of yeast, soy or wheat hydrolysates to CD media IS CHO-CD (Irvine Scientific) in the adalimumab-producing CHO cell line #1 utilized in Example 1 were evaluated.

6.1 Materials and methods

Adaptation and production media (Irvine Scientific IS CHO-CD 91119) were supplemented with Bacto TC Yeastolate, BBL Phytone Peptone, or Wheat Peptone E1 according to the experimental design in Figure 42. The control cultures were not supplemented with hydrolysates. All media pH was adjusted to approximately 7.1.

Cultures were expanded for 3 passages (3 days each) in their respective adaptation media in a combination of 250 mL (50 mL or 100 mL working volume) and 500 mL (150 mL working volume) Corning vented non-baffled shake flasks and maintained on an orbital shaker at 110 RPM in a 35°C, 5% CO_2 dry incubator. Production cultures were initiated in duplicate 500 mL (200 mL working volume) Corning vented non-baffled shake flasks at an initial VCD of approximately 0.5 x 10^6 cells/mL. The shake flask study was run in an extended-batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L.

6.2 Culture growth and productivity

Addition of yeast, soy or wheat hydrolysates to Irvine IS CHO-CD media increased the maximum VCD and culture length for most conditions studied compared to the control (Figure 19A). The largest increase in maximum VCD was

recorded for cultures supplemented with 5 g/L Bacto TC Yeastolate. A concentration-dependent increase in harvest titer was observed for all cultures supplemented with hydrolysates (Figure 19C).

6.3 Oligosaccharide analysis

Supplementation of Irvine IS CHO-CD media with yeast hydrolysates decreased the percentage of NGA2F+NGA2F-GlcNac glycans by 3-4%, and increased the percentage of NA1F+NA2F glycans by the same percentage compared to control (NGA2F+NGA2F-GlcNac: 73%; NA1F+NA2F: 25%) (Figures 20A-B). Addition of soy hydrolysates to Irvine IS CHO-CD media decreased the percentage of NGA2F+NGA2F-GlcNac glycans by 4%, and increased the percentage of NA1F+NA2F glycans by the same percentage compared to control. However, addition of wheat hydrolysates to Irvine IS CHO-CD media resulted in an opposite trend. A concentration-dependent increase in the percentage of NGA2F+NGA2F-GlcNac glycans by 1-3% and a corresponding decrease in the percentage of NA1F+NA2F glycans was observed.

Example 7: Yeast, Soy, or Wheat Hydrolysate Addition to CD Media GIA-1 for Culture of an Adalimumab-Producing CHO Cell Line #2

In the study summarized in this example, the effects on glycosylation resulting from the addition of yeast, soy or wheat hydrolysates to CD media GIA-1 in an adalimumab-producing CHO cell line, generically named CHO cell line #2 were evaluated.

7.1 Materials and methods

Adaptation and production media were supplemented with Bacto TC Yeastolate, BBL Phytone Peptone, or Wheat Peptone E1 according to the experimental design in Figure 43. The control cultures were not supplemented with hydrolysates. All media pH was adjusted to approximately 7.1 and the media osmolality was adjusted to 290 - 300 mOsmol/kg.

Cultures were expanded for 3 passages(3 days each) in their respective adaptation media in a combination of 250 mL (50 mL or 100 mL working volume)

and 500 mL (150 mL working volume) Corning vented non-baffled shake flasks and maintained on an orbital shaker at 180 RPM in a 35°C, 5% CO_2 dry incubator. Production cultures were initiated in duplicate 500 mL (200 mL working volume) Corning vented non-baffled shake flasks at an initial VCD of approximately 0.5 x 10^6 cells/mL. The shake flask study was run in an extended-batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L.

7.2 Culture growth and productivity

Supplementation of yeast, soy or wheat hydrolysates to CD media GIA-1 extended the culture length by 1 to 3 days and decreased the maximum VCD in a dose-dependent manner (Figures 21A-B). The addition of these hydrolysates at the highest concentrations significantly decreased maximum VCD, with wheat hydrolysates added at 10 g/L showing the most severe growth inhibition effects. However, an impact on harvest titer was only observed for the culture supplemented with 10 g/L wheat hydrolysates (65% reduction). An increase in the harvest titer compared to the control (Figure 21C) was found in most other cultures.

7.3 Oligosaccharide analysis

Addition of yeast hydrolysates decreased the percentage of NGA2F+NGA2F-GlcNac glycans by 3-5%, and increased the percentage of NA1F+NA2F glycans by 5-8% compared to control (NGA2F+NGA2F-GlcNac: 89%; NA1F+NA2F: 3%) (Figures 22A-B). Addition of soy hydrolysates to CD media GIA-1 decreased the NGA2F+NGA2F-GlcNac glycans by 8-12%, and increased the NA1F+NA2F glycans by 10-15% compared to control. Addition of wheat hydrolysates decreased the NGA2F+NGA2F-GlcNac glycans by 6-7%, and increased the NA1F+NA2F glycans by 9-10% compared to control.

Example 8: Yeast, Soy, or Wheat Hydrolysate Addition to CD Media GIA-1 for Culture of an Adalimumab-Producing CHO Cell Line #3

In the study summarized in this example, the effects on glycosylation resulting from the addition of yeast, soy or wheat hydrolysates to CD media GIA-1 in

an adalimumab-producing CHO cell line, generically named CHO cell line #3 were evaluated.

8.1 Materials and methods

Adaptation and production media were supplemented with Bacto TC Yeastolate, BBL Phytone Peptone, or Wheat Peptone E1 according to the experimental design in Figure 44. The control cultures were not supplemented with hydrolysates. All media pH was adjusted to approximately 7.1 and the media osmolality was adjusted to 290 - 300 mOsmol/kg.

Cultures were expanded for 3 passages(3 days each) in their respective adaptation media in a combination of 250 mL (50 mL or 100 mL working volume) and 500 mL (150 mL working volume) Corning vented non-baffled shake flasks and maintained on an orbital shaker at 140 RPM in a 36°C, 5% CO_2 dry incubator. Production cultures were initiated in duplicate 500 mL (200 mL working volume) Corning vented non-baffled shake flasks at an initial VCD of approximately 0.5 x 10^6 cells/mL. The shake flask study was run in an extended-batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L.

8.2 Culture growth and productivity

Supplementation of production CD media with high concentrations of hydrolysates – 11 g/L yeast, 15 g/L soy or 15 g/L wheat hydrolysates, decreased the culture growth rate and increased the culture length compared to the control (Figures 23A-B). Harvest titer increased with increasing hydrolysate concentrations in the production media, except for the condition supplemented with 15 g/L wheat hydrolysates, which experienced significant growth inhibition and harvest titer decrease compared to control (Figure 23C).

8.3 Oligosaccharide analysis

Supplementation of CD media GIA-1 with yeast, soy or wheat hydrolysates decreased the percentage of NGA2F+NGA2F-GlcNac glycans and increased the percentage of NA1F+NA2F glycans in a dose-dependent manner (Figures 24A-B). Addition of yeast hydrolysates decreased the percentage of NGA2F+NGA2F-GlcNac glycans by 5-12%, and increased the percentage of NA1F+NA2F glycans by 3-11% compared to control (NGA2F+NGA2F-GlcNac: 91%; NA1F+NA2F: 6%). Addition of soy hydrolysates to CD media GIA-1 decreased the NGA2F+NGA2F-GlcNac glycans by 13-25%, and increased the NA1F+NA2F glycans by 13-25% compared to control. Addition of wheat hydrolysates decreased the NGA2F+NGA2F-GlcNac glycans by 12-18%, and increased the NA1F+NA2F glycans by 12-18% compared to control.

Example 9: Yeast, Soy, or Wheat Hydrolysate Addition to CD Media GIA-1 for Culture of a CHO Cell Line Producing mAb #1

In the studies summarized in this example, the effects on glycosylation resulting from the addition of yeast, soy or wheat hydrolysates to CD media GIA-1 in a CHO cell line producing mAb #1 were evaluated.

9.1 Materials and methods

Adaptation and production media were supplemented with Bacto TC Yeastolate (BD Biosciences; catalog #255772), BBL Phytone Peptone (BD Biosciences; catalog #211096), or Wheat Peptone E1 (Organotechnie; catalog #19559) according to the experimental design in Figure 45. The control cultures were not supplemented with hydrolysates. All media pH was adjusted to approximately 7.2 and the media osmolality was adjusted to 290 - 330 mOsmol/kg.

Cultures were expanded for 4 passages (3 days each) in their respective adaptation media in a combination of 250 mL (50 mL or 100 mL working volume) and 500 mL (150 mL working volume) Corning vented non-baffled shake flasks and maintained on an Infors Multitron orbital shaker at 140 RPM in a 36°C, 5% CO_2 incubator. Production cultures were initiated in duplicate 500 mL (200 mL working volume) Corning vented non-baffled shake flasks at approximately 1.0 x 10^6 cells/mL initial VCD. The study was run in an extended-batch mode by feeding a glucose

solution (1.0% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L.

9.2 Culture growth and productivity

Supplementation of yeast, soy or wheat hydrolysates to the CD media GIA-1 did not affect culture growth profiles dramatically (Figures 25A-B). There was some dose-dependent reduction of the peak VCD compared to control as the hydrolysate concentrations increased, particularly in the case of soy hydrolysates, but overall the growth profiles were similar. However, the culture duration was extended to 11-14 days compared to 9 days for control. Cultures supplemented with 11 g/L yeast hydrolysate had a substantial increase in harvest titer (Figure 25C) that far exceeded the other conditions.

9.3 Oligosaccharide analysis

Addition of yeast hydrolysates to CD media GIA-1 lowered the percentage of NGA2F+NGA2F-GlcNac glycans by 3%, and increased the percentage of NA1F+NA2F glycans by 4% compared to control (NGA2F+NGA2F-GlcNac: 92%; NA1F+NA2F: 5%) (Figures 26A-B). Addition of soy hydrolysates lowered the percentage of NGA2F+NGA2F-GlcNac glycans by 7-13%, and increased the percentage of NA1F+NA2F glycans by 8-12% compared to control. Addition of wheat hydrolysates lowered the percentage of NGA2F+NGA2F-GlcNac glycans by 5-8%, and increased the percentage of NA1F+NA2F glycans by 6-9% compared to control.

Example 10: Yeast, Soy, or Wheat Hydrolysate Addition to CD Media GIA-1 for Culture of a CHO Cell Line Producing mAb #2

In the study summarized in this example, the effects on glycosylation resulting from the addition of yeast, soy or wheat hydrolysates to CD media GIA-1 in a CHO cell line producing mAb #2 were evaluated.

10.1 Materials and methods

Adaptation and production media were supplemented with Bacto TC, BBL Phytone Peptone, or Wheat Peptone E1 according to the experimental design in Figure 46. The control cultures were not supplemented with hydrolysates. All media

pH was adjusted to approximately 7.2 and the media osmolality was adjusted to 280 - 330 mOsmol/kg.

Upon thaw, cells were cultured in CD media GIA-1 growth media in a combination of Corning vented non-baffled shake flasks and maintained on a shaker platform at 140 RPM and 20 L cell bags. Cultures were propagated in a 35°C, 5% CO_2 dry incubator. Production cultures were initiated in duplicate 500 mL (200 mL working volume) Corning vented non-baffled shake flasks at an initial VCD of approximately 0.5 x 10^6 cells/mL. The shake flask study was run in an extended-batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L. For this study, samples were collected daily and measured for cell density and viability using a NOVA cell counter.

10.2 Culture growth and productivity

Supplementation of yeast, soy or wheat hydrolysates to CD media GIA-1 did not impact culture growth for most conditions studied compared to control (Figure 27A). Supplementation with hydrolysates led to higher viability profiles compared to control (Figure 27B). The addition of wheat hydrolysates increased harvest titer compared to the control (Figure 27C).

10.3 Oligosaccharide analysis

Addition of yeast hydrolysates to CD media GIA-1 lowered the percentage of NGA2F+NGA2F-GlcNac glycans by 3% (Figure 28A), and increased the percentage of NA1F+NA2F glycans by 7% (Figure 28B) in a dose-dependent manner compared to control (NGA2F+NGA2F-GlcNac: 75%; NA1F+NA2F: 8%). Addition of soy hydrolysates lowered the percentage of NGA2F+NGA2F-GlcNac by 2-12%, and increased the percentage of NA1F+NA2F by 4-16% compared to control (NGA2F+NGA2F-GlcNac: 76%; NA1F+NA2F: 11%). For this cell line, there was no significant difference in the percentage of NGA2F+NGA2F-GlcNac glycans between the control condition and the cultures supplemented with wheat hydrolysates at the concentration range evaluated. Furthermore, only a minor increase in the percentage of NA1F+NA2F glycans was observed.

Example 11: Combined Yeast, Soy, and/or Wheat Hydrolysate Addition to CD Media GIA-1 for Culture of an Adalimumab-Producing CHO Cell Line #1

In the study summarized in this example, the effects on glycosylation resulting from the individual or combined addition of yeast, soy, and/or wheat hydrolysates to CD media GIA-1 in the adalimumab-producing CHO cell line #1 utilized in Example 1 were evaluated.

11.1 Materials and methods

Adaptation and production media were supplemented with Bacto TC Yeastolate, BBL Phytone Peptone, and/or Wheat Peptone E1 according to the experimental design in Figures 47 and 48. The control cultures were not supplemented with hydrolysates. All media pH was adjusted to approximately 7.1 and the media osmolality was adjusted to 290 - 300 mOsmol/kg.

Cultures were expanded for 3 passages (3 days each) in their respective adaptation media in a combination of 250 mL (50 mL or 100 mL working volume) and 500 mL (150 mL working volume) Corning vented non-baffled shake flasks and maintained on an orbital shaker at 110 RPM in a 35°C, 5% CO_2 dry incubator. Production cultures were initiated in duplicate 500 mL (200 mL working volume) Corning vented non-baffled shake flasks at an initial VCD of approximately 0.5 x 10^6 cells/mL. The shake flask study was run in an extended-batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L.

11.2 Culture growth and productivity

Supplementation of yeast, soy, and/or wheat hydrolysates to CD media GIA-1 resulted in slight growth inhibition and reduced maximum VCD compared to the control (Figure 29A). Culture viability profiles and harvest titer were comparable for all cultures (Figures 29B-C).

11.3 Oligosaccharide analysis

Supplementation of yeast hydrolysates only to CD media GIA-1 decreased the percentage of NGA2F+NGA2F-GlcNac glycans by 4% and increased the percentage of NA1F+NA2F glycans by 6% compared to control (NGA2F+NGA2F-GlcNac: 90%; NA1F+NA2F: 4%) (Figures 30A-B). Supplementation of soy hydrolysates only decreased the percentage of NGA2F+NGA2F-GlcNac glycans by 7%, and increased the percentage of NA1F+NA2F glycans by 9% compared to control. Supplementation of wheat hydrolysates only decreased the percentage of NGA2F+NGA2F-GlcNac glycans by 5% and increased the percentage of NA1F+NA2F glycans by 8% compared to control.

The addition of two hydrolysates (yeast and soy; yeast and wheat; soy and wheat) further decreased the percentage of NGA2F+NGA2F-GlcNac glycans and increased the percentage of NA1F+NA2F glycans by a couple of percentages compared to the addition of each component individually (Figures 30A-B). Supplementing CD media GIA-1 with all three hydrolysates did not result in any further changes in the glycosylation profile, indicating a saturation state being reached.

Example 12: Effect of Asparagine in CD Media GIA-1 for Culture of Adalimumab-Producing CHO Cell Line #1

In the study summarized in this Example, the effects on product quality attributes resulting from the addition of asparagine to CD media GIA-1 in an adalimumab-producing CHO cell line, generically named CHO cell line #1 were investigated.

12.1 Materials and methods

The CHO cell line #1 was employed in the study covered here. Upon thaw, cells were expanded in a 19-days seed train and then transferred into seed reactors for up to 7 days in growth medium. The cells were then brought to the

laboratory and adapted in 500-mL shaker flasks with 200 mL working volume in CD media GIA1 medium for 13 days with 3 passages. The shaker flasks were placed on a shaker platform at 110 RPM in a 35°C, 5% CO_2 incubator.

The chemical defined growth or production media, was prepared from basal IVGN CD media GIA1. For preparation of the IVGN CD media formulation, the proprietary media was supplemented with L-glutamine, insulin, sodium bicarbonate, sodium chloride, and methotrexate solution. Production media consisted of all the components in the growth medium, excluding methotrexate. In addition, 5mM of Galactose (Sigma, G5388) and 10μM of Manganese (Sigma, M1787) were supplemented into production medium. Osmolality was adjusted by the concentration of sodium chloride. All media was filtered through filter systems (0.22 μm PES) and stored at 4°C until usage.

Production cultures were initiated in duplicate 500 mL Corning, vented, non-baffled shaker flasks each containing 200 mL culture in dry incubators with 5% CO_2 at 35°C and 110 RPM. Initial VCD was approximately 0.5 x 10^6 cells/ml. The shake flask study was run in an extended batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L. Asparagine stock solution (20g/L) was fed to culture on Day 6 to increase Asparagine concentration by 0, 0.4, 1.2 and 2.0 g/L.

Samples were taken daily from each reactor to monitor growth. The following equipment was used to analyze the samples: Cedex cell counter for cell density and viability; YSI 7100 analyzer for glucose and lactate concentration.

Some of the daily samples and the harvest samples were centrifuged at 3,000rpm for 30min and then supernatants were stored at -80°C. The thawed harvest samples were subsequently filtered through a 0.2μm filter, purified by Protein A chromatography, and then oligosaccharide analysis was performed as described in Example 1.

12.2 Culture growth and productivity

Feeding of asparagine to CD media GIA-1 did not impact culture growth for most conditions studied as compared to the control (Figure 31A). The

cultures showed similar growth rates and reached maximum VCD of ~12 x 10^6 cells/mL. Culture viabilities were also very similar to that of the controls (Figure 31B). Similarly, all the cultures examined here resulted in comparable harvest titers of approximately 1.7 g/L (Figure 31C).

12.3 Oligosaccharide analysis

The effect of asparagine addition on oligosaccharide distribution was consistent with the experiments performed in hydrolysate based media described above. The addition of asparagine increased NGA2F+NGA2F-GlcNac glycans in a dose dependent manner (Figure 32A). The percentage of NGA2F+NGA2F-GlcNac in the control sample (without Asparagine addition) was as low as 74.7%. In the sample with the addition of asparagine the percentage of NGA2F+NGA2F-GlcNAc was increased to 76.1% (0.4g/L of asparagine), 79.2% (1.2g/L of asparagine), and 79.0% (2.0g/L of asparagine), for a total increase of 4.5%.

The percentage of NA1F+NA2F in the control sample (without asparagine addition) was as high as 22.3% (Figure 32B). In the sample with the addition of asparagine the percentage of NA1F+NA2F was decreased to 21.1% (0.4g/L of asparagine), 17.8% (1.2g/L of asparagine), and 17.8% (2.0g/L of asparagine), for a total reduction of 4.5%.

Example 13: Effect of Asparagine in CD Media GIA-1 for Culture of Adalimumab-Producing CHO Cell Line #3

In the study summarized in Example 13, the effects on product quality attributes resulting from the addition of asparagine to CD media GIA-1 in an adalimumab-producing CHO cell line, generically named CHO cell line #3 were investigated.

13.1 Materials and methods

The CHO cell line #3 was employed in the study covered here. Upon thaw, adalimumab producing cell line #3 was cultured in CD media GIA-1 in a combination of vented shake flasks on a shaker platform @140 rpm and 20L wave

bags. Cultures were propagated in a 36°C, 5% CO_2 incubator to obtain the required number of cells to be able to initiate production stage cultures.

The chemical defined growth or production media was prepared from basal IVGN CD media GIA1. For preparation of the IVGN CD media formulation, the proprietary media was supplemented with L-glutamine, sodium bicarbonate, sodium chloride, and methotrexate solution. Production media consisted of all the components in the growth medium, excluding methotrexate. In addition, 10mM of Galactose (Sigma, G5388) and 0.2μM of Manganese (Sigma, M1787) were supplemented into production medium. Osmolality was adjusted by the concentration of sodium chloride. All media was filtered through filter systems (0.22 μm PES) and stored at 4°C until usage.

Production cultures were initiated in duplicate 500 mL Corning, vented, non-baffled shaker flasks each containing 200 mL culture in dry incubators with 5% CO_2 at 36°C and 140 RPM. Initial VCD was approximately 0.5 x 10^6 cells/ml. The shake flask study was run in an extended batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L. Asparagine stock solution (20g/L) was fed to culture on Day 6 to increase asparagine concentration by 0, 0.4, 0.8, 1.2, 1.6, and 2.0 g/L.

Samples were taken daily from each reactor to monitor growth. The following equipment was used to analyze the samples: Cedex cell counter for cell density and viability; YSI 7100 analyzer for glucose and lactate concentration.

Some of the daily samples and the harvest samples were centrifuged at 3,000rpm for 30min and then supernatants were stored at -80°C. The thawed harvest samples were subsequently filtered through a 0.2μm filter, purified by Protein A chromatography, and then oligosaccharide analysis was performed as described in Example 1.

13.2 Culture growth and productivity

The experiment described in the instant Example used a different cell line (i.e., CHO cell line #3) in CD media GIA-1. Culture growth and viability profiles were comparable among all test conditions with different dosage of asparagine added

on day6 (Figures 33A and 33B). All cultures reached maximum VCD of ~18-19 x 10^6 cells/mL. The product titer (~1.5-1.6 g/L) was slightly reduced when higher dosage of asparagine was added (Figure 33C).

13.3 Oligosaccharide analysis

Again, the addition of asparagine increased NGA2F+NGA2F-GlcNac (Figure 34A). The percentage of NGA2F+NGA2F-GlcNac in the control sample (without asparagine addition) was as low as 68.7%. In the sample with the addition of asparagine, the percentage of NGA2F+NGA2F-GlcNac was increased by 4.1-5.1% when 0.4 to 2.0 g/L asparagine was added on day 6 (Figure 34A). The percentage of NA1F+NA2F in the control sample (without asparagine addition) was as high as 25.6% (Figure 34B). In the sample with the addition of asparagine the percentage of NA1F+NA2F was decreased by 3.8-4.6% when 0.4 to 2.0 g/L asparagine was added on day 6 (Figure 34B).

Example 14: Effect of Asparagine in a Shaker Flask Batch Culture in CD Media GIA- 1 with a CHO Cell LineProducing mAb #2

In the studies summarized in Example 14, the effects on product quality attributes resulting from the addition of asparagine to CD media GIA-1 from Life Technologies Gibco in a CHO cell line producing monoclonal antibody #2 were investigated. In this instant Example, asparagine was either supplemented into culture media during media preparation or added on day 5 of the cell culture process.

14.1 Materials and methods

mAb #2 producing cell line was employed in the study covered here. Upon thaw, cells were cultured in chemically defined growth media in a combination of vented baffled shake flasks (Corning) on a shaker platform at 140 RPM. All media pH was adjusted to approximately 7.2 and the media osmolality was adjusted to 280 - 330 mOsmol/kg.

Cultures were propagated in a 35°C, 5% CO_2 incubator to obtain the required number of cells to be able to initiate production stage cultures. Production cultures were initiated in duplicate 500mL vented non-baffled Corning shake flasks

(200mL working volume) at an initial viable cell density (VCD) of approximately 0.5 x 10^6 cells/mL. The shake flask study was run in an extended batch mode by feeding a glucose solution (1.25% (v/v) of 40% solution) when the media glucose concentration fell below 3 g/L. Asparagine (Sigma, Catalog Number A4284) were solubilized in Milli-Q water to make a 30g/L stock solution. All media was filtered through Corning or Millipore 1L filter systems (0.22 μm PES) and stored at 4°C until usage.

For asparagine supplemented into culture media during media preparation, asparagine stock solution was supplemented to production media to increase asparagine concentration by 0, 0.4, 0.8 and 1.6 g/L. After addition of asparagine, media was brought to a pH similar to non-supplemented (control) media using 5N hydrochloric acid/5N NaOH, and it was brought to an osmolality similar to non-supplemented (control) media by adjusting the concentration of sodium chloride. For asparagine addition study, asparagine stock solution was added to culture on Day 5 to increase Asparagine concentration by 0, 0.4, 0.8 and 1.6 g/L.

For all studies described throughout this invention, samples were collected daily and measured for cell density and viability using a NOVA cell counter. Retention samples for titer analysis via Poros A method were collected by centrifugation at 12,000 RPM for 5 min when the culture viability began declining. The cultures were harvested by collecting 125 mL aliquots and centrifuging at 3,000 RPM for 30 min when culture viability was near or below 50%. All supernatants were stored at -80°C until analysis. The harvest samples were Protein A purified and then oligosaccharide analysis was performed as described in Example 1.

14.2 Culture growth and productivity

Adding asparagine to CD media GIA-1 during medium preparation or on day 5 of the cell culture did not impact culture growth for most conditions studied as compared to the non-supplemented 0 g/L controls (Figures 45A and 47A). The cultures showed similar growth rates and reached maximum VCD of 22-24 x 10^6 cells/mL. Culture viabilities were also very similar to that of the controls (Figures 35B and 37B). Similarly, all the cultures examined here resulted in comparable harvest titers of approximately 0.9 g/L of mAb #2 (Figures 35C and 37C).

14.3 Oligosaccharide analysis

The addition of asparagine during medium preparation increased NGA2F+NGA2F-GlcNac glycans in a dose dependent manner (Figure 36A). The percentage of NGA2F+NGA2F-GlcNac in the control sample (without asparagine addition) was as low as 76.3%. In the sample with the addition of asparagine the percentage of NGA2F+NGA2F-GlcNac was increased to 81.5% (0.4g/L of asparagine), 85.5% (0.8g/L of asparagine), and 85.9% (1.6g/L of asparagine), for a total increase of 9.6%. The percentage of NA1F+NA2F in the control sample (without asparagine addition) was as high as 11.5% (Figure 36B). In the sample with the addition of asparagine the percentage of NA1F+NA2F was decreased to 9.8% (0.4g/L of asparagine), 7.8% (0.8g/L of asparagine), and 7.0% (1.6g/L of asparagine), for a total reduction of 4.5%. With mAb #2 cell line used in the study, the percentage of Mannose type glycans was also decreased with the supplementation of asparagine. The percentage of Mannoses in the control sample (without asparagine addition) was as high as 12.2% (Figure 36B). In the sample with the addition of asparagine the percentage of Mannoses was decreased to 8.6% (0.4g/L of asparagine), 6.7% (0.8g/L of asparagine), and 7.1% (1.6g/L of asparagine), for a total reduction of 5.5%.

The addition of asparagine on day 5 of the culture also increased NGA2F+NGA2F-GlcNac glycans in a dose dependent manner (Figure 38A). The percentage of NGA2F+NGA2F-GlcNac in the control sample (without asparagine addition) was as low as 79.7%. In the sample with the addition of asparagine the percentage of NGA2F+NGA2F-GlcNac was increased to 80.5% (0.4g/L of asparagine), 82.1% (0.8g/L of asparagine), and 84.1% (1.6g/L of asparagine), for a total increase of 4.4%. The percentage of NA1F+NA2F in the control sample (without asparagine addition) was as high as 9.7% (Figure 38B). In the sample with the addition of asparagine the percentage of NA1F+NA2F was decreased to 9.4% (0.4g/L of asparagine), 9.6% (0.8g/L of asparagine), and 8.5% (1.6g/L of asparagine), for a total reduction of 1.2%. Again, the percentage of Mannose type glycans was also decreased with the supplementation of asparagine. The percentage of Mannoses in the control sample (without asparagine addition) was as high as 10.6% (Figure 38B). In the sample with the addition of asparagine the percentage of Mannoses was decreased

to 10.1% (0.4g/L of asparagine), 8.3% (0.8g/L of asparagine), and 7.4% (1.6g/L of asparagine), for a total reduction of 3.2%.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Furthermore, the strategies described herein can be easily implemented either in-process or ad hoc to control the oligosaccharide distribution, thus reducing the potential impact of raw material changes. For example, Adalimumab production strategies can use these techniques to achieve maximized cell growth and specific productivity without compromising product quality.

Patents, patent applications, publications, product descriptions, GenBank Accession Numbers, and protocols that may be cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes. For example, but not by way of limitation, patent applications designated by the following attorney docket numbers are incorporated herein by reference in their entireties for all purposes: 082254.0104; 082254.0235; 082254.0236; 082254.0238; and 082254.0242.

What is claimed is:

1. A method for controlling the oligosaccharide distribution of a recombinantly-expressed protein comprising supplementing a cell culture media used in the recombinant expression of said protein with a yeast hydrolysate supplement and/or a plant hydrolysate supplement.

2. The method of claim 1 wherein the recombinantly-expressed protein is an antibody.

3. The method of claim 2 wherein the antibody is an anti-TNFα antibody.

4. The method of claim 3 wherein the anti-TNFα antibody is adalimumab.

5. The method of claim 1 wherein the yeast hydrolysate supplement is selected from the group consisting of: Bacto TC Yeastolate, HyPep Yeast Extract, and UF Yeast Hydrolysate.

6. The method of claim 1 wherein the plant hydrolysate supplement is a soy hydrolysate or a wheat, rice, cotton seed, pead, corn or potato hydrolysate.

7. The method of claim 6 wherein plant hydrolysate supplement is selected from the group consisting of: BBL Phytone Peptone; HyPep 1510; SE50 MAF-UF; UF Soy Hydrolysate; Wheat Peptone E1; HyPep 4601; and Proyield WGE80M Wheat.

8. The method of claim 1 wherein the cell culture media is supplemented with a sufficient amount of the yeast hydrolysate supplement to achieve a yeast hydrolysate concentration selected from about 2 g/L to about 11 g/L.

9. The method of claim 8 wherein the cell culture media is supplemented with a sufficient amount of the yeast hydrolysate supplement to achieve a yeast hydrolysate concentration selected from the following: 2 g/L, 5 g/L, and 11 g/L.

10. The method of claim 1 wherein the cell culture media is supplemented with a sufficient amount of the plant hydrolysate supplement to achieve a plant hydrolysate concentration selected from about 2 g/L to about 15 g/L.

11. The method of claim 10 wherein the production media is supplemented with a sufficient amount of the plant hydrolysate supplement to achieve a plant hydrolysate concentration selected from the following: 2 g/L, 4 g/L, 7 g/L, 10 g/L, and 15 g/L.

12. The method of claim 1 wherein the cell culture media is supplemented with a sufficient amount of the yeast hydrolysate supplement and the plant hydrolysate supplement to achieve the yeast hydrolysate to the plant hydrolysate ratio of about 0.25 to about 1.55.

13. The method of claim 12 wherein the cell culture media is supplemented with a sufficient amount of the yeast hydrolysate supplement and plant hydrolysate supplement to achieve a yeast hydrolysate and plant hydrolysate concentration selected from the following presented as yeast hydrolysate (g/L)/ plant hydrolysate (g/L): 0/2; 0/4; 0/7; 2/0; 5/0; 11/0; 4/2.58; and 10.7/6.92.

14. A method for controlling the oligosaccharide distribution of a recombinantly-expressed protein comprising modulating the asparagine concentration of the cell culture media used in the recombinant expression of said protein.

15. The method of claim 14 wherein the recombinantly-expressed protein is an antibody.

16. The method of claim 15 wherein the antibody is an anti-TNFα antibody.

17. The method of claim 16 wherein the anti-TNFα antibody is adalimumab.

18. The method of claim 14 wherein the total concentration of asparagine in the cell culture media is from about 0 mM to about 26 mM.