

Cell culture processes

Field of invention

The present invention belongs to the field of the manufacture of recombinant proteins, particularly proteins, such as antibodies.

Background of the invention

The development of recombinant proteins as therapeutic proteins, such as therapeutic antibodies, requires production of the recombinant proteins at an industrial scale. In order to achieve this, different expression systems, such as prokaryotic and eukaryotic systems, may be employed. Over the past two decades, however, the majority of the approved therapeutic proteins have been manufactured through mammalian cell cultures and such systems remain the preferred expression system for producing a large quantity of recombinant proteins for use in humans.

Cell culture conditions, such as the composition of the medium (Kshirsagar *et al.*, 2012; US20130281355; WO2013158275) and the growing conditions, including pH and temperature (WO2011134919) have been shown to impact the yield and the quality attributes of therapeutic proteins. Over the last 30 years, much effort has been dedicated to establishing the basic parameters of cell culture, media and recombinant protein expression with much focus of the research dedicated to reaching optimal cell growth through changes of the composition of the cell culture media (see e.g. Hecklau *et al.*, 2016; Zang *et al.*, 2011), operating conditions and development of large bioreactors.

Some components present at high concentration in feed media tend to precipitate during storage (until said media are added in the bioreactor), especially when the pH of said media is around neutrality. Such a precipitation prior to use is not desirable: indeed, it may have an impact on the exact composition of the medium (as the amount of components in solution / in the precipitate will be unknown). WO2008013809, which relates to chemically concentrated feed media (between 10X to 100X), discloses that salts typically precipitate when dissolved together at certain pH values, such as at pH above 5.8, or yet that other components such as folic acid require a pH of 8.6 for solubilization. WO2008141207 provides stable feed media containing cysteine, tyrosine and optionally cystine, and further containing pyruvate as a stability agent towards components that are difficult to solubilise at high concentration (such as tyrosine or cysteine). WO2011133902 proposes to supplement the concentrated feed media with small peptides having two to six amino acids (such as alanyl tyrosine and/or alanyl cysteine and/or alanyl cystine dimer) in order to limit the risk of precipitation of said feeds. To solve the issue of precipitations in the feed tank, WO2022038250 proposes to reduce the pH of the main feed to a pH between 5.00 and 6.30.

Yet, there remains the need to provide further improved feed media to be used in the context of cell culture processes for the production of therapeutic proteins, while having a minimal impact on yield and protein heterogeneity.

Summary of the invention

In a first aspect, the invention provides a process for producing a recombinant protein in a bioreactor, wherein the process comprises the steps of (a) culturing mammalian cells expressing said recombinant protein in a culture medium in the bioreactor and (b) supplementing the cell culture with at least one feed medium, wherein the pH of this at least one feed medium is from 5.10±0.2 to 5.20±0.2.

In a second aspect, the invention provides a process for culturing mammalian cells expressing a recombinant protein in a bioreactor, wherein the process comprises the steps of (a) culturing the mammalian cells in a culture medium in the bioreactor and (b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of this at least one feed medium is from 5.10±0.2 to 5.20±0.2.

In a third aspect, the invention provides a process for reducing or preventing CaHPO₄ and or CaHPO₄·2H₂O precipitation in a feed medium in a container, during storage and/or during cultivation of mammalian cells and/or during production of a recombinant protein, wherein the process comprises the steps of: a) preparing the at least one feed medium in a container, b) adjusting the pH of the feed of step a) so that its pH is from about 5.10 to about 5.20.

In a fourth aspect, the invention relates to a feed medium for use in any of the processes herein described, wherein its pH is from 5.10±0.2 to 5.20±0.2.

In a fifth aspect the invention describes a process for producing a recombinant protein in a bioreactor, wherein the process comprises the steps of:

- (a) culturing mammalian cells expressing said recombinant protein in a culture medium (typically a basal culture medium) in the bioreactor, and
- (b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of this at least one feed medium is:
 - i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca²⁺ ions in said feed medium is at or lower than 85 µg/mL ±10%, or
 - ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca²⁺ ions in said feed medium is at or above 95 µg/mL ±10%.

In a sixth of this second embodiment, the invention provides a process for culturing mammalian cells expressing a recombinant protein in a bioreactor, wherein the process comprises the steps of:

- (a) culturing the mammalian cells in a culture medium (typically a basal culture medium) in the bioreactor, and
- (b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of this at least one feed medium is:
 - i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca²⁺ ions in said feed medium is at or lower than 85 µg/mL ±10%, or

ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

In a seventh, herein described is a process for reducing or preventing CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation in a feed medium (alternatively named main feed medium as defined above) in a container, during storage or during production of a recombinant protein, wherein the process comprises the steps of:

(a) preparing the at least one feed medium in a container,

(b) adjusting the pH of the feed of step (a) so that:

- i) its pH is from 5.10 to 5.50 if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in the at least one feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or
- ii) its pH is from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in the at least one feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

Definitions

In the case of conflict, the present specification, including definitions, will control. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one skilled in art to which the subject matter herein belongs. As used herein, the following definitions are supplied in order to facilitate the understanding of the present invention.

As used in the specification and claims, the term "and/or" used in a phrase such as "A and/or B" herein is intended to include "A and B", "A or B", "A", and "B".

As used herein, the term "invention as a whole" relates to aspects that apply to any and all embodiments herein described.

The forms "a", "an", and "the" include both single and plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a precipitation" includes both "a precipitation" and "precipitations".

The following terms are interchangeable: 1) " CaHPO_4 " and " CaHPO_4 ", 2) " H_2O " and " H_2O " (including in " $2\text{H}_2\text{O}$ " and " $2\text{H}_2\text{O}$ "), and 3) " Ca^{2+} " and " Ca^{2+} ". As used in the specification and claims, the term "cell culture" or "culture" is meant the growth, propagation and/or maintenance of cells in vitro, i.e. outside of an organism or tissue. Suitable culture conditions for mammalian cells are known in the art, such as taught in Cell Culture Technology for Pharmaceutical and Cell-Based Therapies (2005). Mammalian cells may be cultivated in suspension or while attached to a solid substrate.

The terms "cell culture medium," "culture medium," "medium," and any plural thereof, refer to any medium in which cells of any type can be cultivated. A "basal medium" refers to a cell culture medium that contains all of the essential ingredients useful for cell metabolism. This includes for instance amino acids, lipids, carbon source, vitamins and mineral salts. DMEM (Dulbeccos' Modified Eagles Medium), RPMI (Roswell Park Memorial Institute Medium) or medium F12 (Ham's F12 medium) are examples of commercially available basal media. Other suitable media have e.g.

been described in WO9808934 and US20060148074 (both incorporated herein in their entirety). Further suitable commercially available media include, but are not limited to, AmpliCHO CD medium, Dynamis™ Medium, EX-CELL® Advanced™ CHO Fed-batch System, CD FortiCHO™ medium, CP OptiCHO™ medium, Minimum Essential Media (MEM), BalanCD® CHO Growth A Medium, ActiPro™ medium, DMEM–Dulbecco's Modified Eagle Medium and RPMI-1640 medium. Alternatively, said basal medium can be a proprietary medium, also herein called "chemically defined medium" or "chemically defined culture medium", in which all of the components can be described in terms of the chemical formulas and are present in specific concentrations. The culture medium is preferably free of proteins and free of serum, and can be supplemented by any additional compound(s) such as amino acids, salts, lipids, sugars, vitamins, hormones, growth factors, depending on the needs of the cells in culture.

The term "feed medium" or "feed" (and plural thereof) refers to a medium which is added during culture to replenish the nutrients which are consumed. The feed medium can be a commercially available feed medium or a proprietary feed medium (herein alternatively named "defined feed medium" or "chemically defined feed medium"). Suitable commercially available feed media include, but are not limited to, Cell Boost™ supplements, EfficientFeed™ supplements, ExpiCHO™ Feeds. Alternatively, said feed medium can be a proprietary feed medium in which all of the components can be described in terms of the chemical formulas and are present in specific concentrations. A feed medium is typically concentrated, compared to a basal medium, in order not to increase to a high level the final volume of the culture. Such a feed medium can contain most of the components of the cell culture medium at, for example, about 1.5X, 2X, 5X, 6X, 7X, 8X, 9X, 10X, 12X, 14X, 16X, 20X, 30X, 50X, 100X, 200X or even 500X of their normal amount in a basal medium. Proprietary feed media are typically in powder. Commercial feeds are either liquid or in powder. When feeds are already in liquid form, they are used as such, according to the leaflet. Feeds which are in powder need to be solubilised, in water for instance, before use. They are supposed to be solubilised in a given amount of water (e.g. 100g in 1L of water, see Figure 1A). However, feeds in powder can be further concentrated, beyond the instructions in the leaflet or beyond original protocol. In such a case, they will be solubilised using less liquid than normally needed for said quantity (e.g. 200g in 1L of water as shown in Figure 1B). Liquid feeds or feeds in powder that are prepared according to the standard protocol are herein also called "normal" feed (or alternatively "non-concentrated feed"). Liquid feeds or feeds in powder that are prepared according to a concentrated process are herein called "concentrated feed".

Different feed media of different compositions can be added throughout the culture process. For instance, three different feed media can be used during the same process: one feed medium consisting of the carbon source (e.g. glucose), one feed medium comprising most of the nutrients which are consumed (this feed is, herein, also referred to as "main feed", "main feed medium" or "at least one feed medium"), and a further feed medium comprising some further nutrients for

instance when this nutrients present aggregation/stability issues (such as for instance cysteine and/or cystine and/or tyrosine).

The term "bioreactor" refers to any system in which cells can be cultivated. It includes but is not limited to flasks, static flasks, spinner flasks, tubes, shake tubes, shake bottles, wave bags, bioreactors, fibre bioreactors, and stirred-tank bioreactors with or without microcarriers. Alternatively, this term also includes microtiter plates, capillaries or multi-well plates. Any size of bioreactor can be used, for instance from 1 millilitre (1 mL, very small scale) to 20000 litres (20000L or 20 KL, very large scale), such as 0.1 mL, 0.5 mL, 1 mL, 5 mL, 0.01 L, 0.1 L, 1 L, 2L, 5L, 10L, 50L, 100L, 500L, 1000L (1 KL), 2000L (2KL), 5000L (5KL), 10000L (10KL), 15000L (15KL) or 20000L (20KL).

The term "container" refers to any kind of containers that can contain the at least one main feed for storage or during a manufacturing process for instance. The term "container" includes, but is not limited to bags, vessels or tanks.

The term "fed-batch culture" refers to a process of growing cells, where there is a bolus (or several boli) or continuous addition of a feed medium (or feed media) (note that "addition" is also named "supplementation" in the context of this invention) to replenish the nutrients which are consumed, without removal of any medium. Feed(s) can be added according to a predetermined schedule of, for example, every day, once every two days, once every three days, etc. Alternatively, should the feeding be continuous, the feeding rate can be varied throughout the culture. This cell culture technique has the potential to obtain high cell densities in the order of greater than 10×10^6 to 30×10^6 cells/ml, depending on the media formulations, cell line, and other cell growth conditions. A biphasic culture condition can be created and sustained by a variety of feed strategies and media formulations.

The term "growth phase" according to the present invention corresponds to the stage just before the "production stage" during which cells are deemed to grow and multiply quickly (in a so-called growth bioreactor, seed bioreactor or N-1 bioreactor) in order to have enough material to inoculate the production bioreactor (for the production phase). Alternatively this stage is called pre-production phase/stage, N-1 phase/stage or seed phase/stage. In the growth phase, cells can typically be grown according to any techniques from the art, such as in perfusion mode, batch mode or fed-batch mode.

The term "production phase" according to the present invention comprises that stage of cell culturing during the process for manufacturing a recombinant protein when the cells express (i.e. produce) the recombinant polypeptide(s). The production phase typically begins when the titre of the desired recombinant protein increases and/or when cell growth has essentially ended and ends with harvest of the cells (or the cell culture fluid or supernatant) when the recombinant protein production has essentially ended. The cells may be maintained in production phase until a desired cell density or desired recombinant protein titre is reached. For instance, the cells are maintained in the subsequent production phase until the titre of the recombinant protein reaches a maximum.

Alternatively, the culture may be harvested prior to this point, depending on the production requirement of the skilled person or the needs of the cells themselves. Typically, at the beginning of the production phase, the cell culture is transferred from a growth bioreactor (N-1 bioreactor) to a production bioreactor (N bioreactor). Harvest is the step during which the cell culture fluid is removed from the production bioreactor, in order for the recombinant protein, e.g. the recombinant antibody, to be recovered and purified in subsequent steps. In the production phase, cells can typically be grown according to any techniques from the art, such as in perfusion mode, batch mode or fed-batch mode.

As used herein, "cell concentration" (also known as "cell density") refers to the number of cells in a given volume of culture medium. "Viable cell concentration" (or "VCC") refers to the number of living cells in a given volume of culture medium. This is determined by standard viability assays. The term "viability", or "cell viability" refers to the ratio between the total number of viable cells and the total number of cells in culture. Although the viability is typically acceptable as long as it does not go below a 60 % threshold compared to the start of the culture, the acceptable threshold can be determined on a case-by-case basis. Viability is often used to determine time for harvest. For instance, in fed-batch culture, harvest can be performed once viability reaches at 60% or after about 14 days (typically 14 days $\pm$ 1 day) in culture.

The wording "titre" refers to the concentration of the recombinant protein of interest in solution. This is determined by standard titre assays, such as serial dilutions combined with a detection method (colorimetric, chromatographic etc.), with a CEDEX® or protein A high-pressure liquid chromatography (HPLC), Biacore C® or ForteBIO Octet® methods, as used in the example section.

The term "higher titre" or "higher productivity", and equivalents thereof, means that the titre or the productivity is increased by at least 10% when compared to the control culture condition. The titre or specific productivity will be considered as maintained if it is in the range of -10% to 10% compared to the control culture condition. The terms "lower titre" or "lower productivity", and equivalents thereof, means that the titre or the productivity is decreased by at least 10% when compared to the control culture condition.

The term "reduction of precipitation" is to be understood as the decrease of sedimented precipitates or/and precipitates in feed medium, as assessed visually for instance, when compared with the precipitation observed under control conditions. The term "prevention of precipitation" is to be understood as the absence of sedimented precipitates or/and precipitates in feed medium, as assessed visually for instance (i.e. visual inspection). Precipitation of elements composing the feed medium (in the context of this invention it is also referred to precipitation of the feed) may occur during preparation, after preparation and/or storage step. Precipitation can be visually assessed as small solid particles in solution (in solution and/or sedimenting as particles at the bottom of the container). Said assessment is well within the knowledge of the skilled person.

- The term "heterogeneity" as used herein refers to differences between individual molecules, e.g. recombinant proteins, in a population of molecules produced by the same manufacturing process, or within the same manufacturing batch. Heterogeneity can result from incomplete or inhomogeneous modifications of the recombinant polypeptides, e.g. due to post-translational modifications of the polypeptide or to misincorporation during transcription or translation. Post-translational modifications can e.g. be the result of deamination reactions and/or oxidation reactions and/or covalent addition of small molecules such as glycation reactions and/or isomerization reactions and/or fragmentation reactions and/or other reactions and also include variation on the glycation patterns. The chemo-physical manifestation of such heterogeneity leads to various characteristics in the resulting recombinant polypeptide preparations which include, but are not limited to, charge variant profile, colour or colour intensity and molecular weight profile. When measuring the isoforms of the recombination proteins, besides the main charge species are also measured the acidic isoforms (APG) and the basic isoforms (BPG). The main charge species represents the isoform of the recombinant protein that one wishes to obtain.
- 15 The term "recombinant protein" means a protein produced by recombinant techniques. Recombinant techniques are well within the knowledge of the skilled person (see for instance Sambrook et al., 1989, and updates). The term "protein" can be for instance a cytokine, a growth factor, a hormone, an antibody or a fusion protein comprising a domain or other fragments of an antibody.
- 20 The term "antibody" as used herein includes, but is not limited to, monoclonal antibodies, polyclonal antibodies and recombinant antibodies that are generated by recombinant technologies as known in the art. "Antibody" include antibodies of any species, in particular of mammalian species; such as human antibodies of any isotype, including IgG1, IgG2a, IgG2b, IgG3, IgG4, IgE, IgD and antibodies that are produced as dimers of this basic structure including IgGA1, IgGA2, or pentamers such as IgM and modified variants thereof; non-human primate antibodies, e.g. from chimpanzee, baboon, rhesus or cynomolgus monkey; rodent antibodies, e.g. from mouse, or rat; rabbit, goat or horse antibodies; camelid antibodies (e.g. from camels or llamas such as NanobodiesTM) and derivatives thereof; antibodies of bird species such as chicken antibodies; or antibodies of fish species such as shark antibodies. The term "antibody" also refers to "chimeric" antibodies in which a first portion of at least one heavy and/or light chain antibody sequence is from a first species and a second portion of the heavy and/or light chain antibody sequence is from a second species. Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences. "Humanized" antibodies are chimeric antibodies that contain a sequence derived from non-human antibodies. For the most part, humanized antibodies are human antibodies (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region [or complementarity determining region (CDR)] of a non-human
- 35

species (donor antibody) such as mouse, rat, rabbit, chicken or non-human primate, having the desired specificity, affinity, and activity. In most instances residues of the human (recipient) antibody outside of the CDR; i.e. in the framework region (FR), are additionally replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues

5 that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody properties. Humanization reduces the immunogenicity of non-human antibodies in humans, thus facilitating the application of antibodies to the treatment of human disease. Humanized antibodies and several different technologies to generate them are well known in the art. The term "antibody" also refers to human antibodies, which can be generated as

10 an alternative to humanization. For example, it is possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of production of endogenous murine antibodies. Other methods for obtaining human antibodies/antibody fragments in vitro are based on display technologies such as phage display or ribosome display technology, wherein recombinant DNA libraries are used that are either

15 generated at least in part artificially or from immunoglobulin variable (V) domain gene repertoires of donors. Phage and ribosome display technologies for generating human antibodies are well known in the art. Human antibodies may also be generated from isolated human B cells that are ex vivo immunized with an antigen of interest and subsequently fused to generate hybridomas which can then be screened for the optimal human antibody. The term "antibody" refers to both

20 glycosylated and aglycosylated antibodies. Furthermore, the term "antibody" as used herein not only refers to full-length antibodies, but also refers to antibody fragments and in particular to antigen-binding fragments. A fragment of an antibody comprises at least one heavy or light chain immunoglobulin domain as known in the art and binds to one or more antigen(s). Examples of antibody fragments according to the invention include a Fab, modified Fab, Fab', modified Fab',

25 F(ab')₂, Fv, Fab-Fv, Fab-dsFv, Fab-Fv-Fv, scFv and Bis-scFv fragment. Said fragment can also be a diabody, tribody, triabody, tetrabody, minibody, single domain antibody (dAb) such as sdAb, VL, VH, VHH or camelid antibody (e.g. from camels or llamas such as a NanobodyTM) and VNAR fragment. An antigen-binding fragment according to the invention can also comprise a Fab linked to one or two scFvs or dsscFvs, each scFv or dsscFv binding the same or a different target (e.g.,

30 one scFv or dsscFv binding a therapeutic target and one scFv or dsscFv that increases half-life by binding, for instance, albumin). Exemplary of such antibody fragments are FabdsscFv (also referred to as BYbe®) or Fab-(dsscFv)₂ (also referred to as TrYbe®, see WO2015197772 for instance). Antibody fragments as defined above are known in the art.

- The terms "about" or "approximately" denote an interval of accuracy that the person skilled in the

35 art will understand to still ensure the technical effect of the feature in question. For instance, for pH, the term typically indicates deviation from the indicated numerical value of no more than ± 0.2 . Where the term "about" is used the application also discloses employing the exact value specified. Where point values are referred to, the application also discloses about such values being

employed, the same being the case for endpoints of ranges. For example should the description specify "about 5.1 to about 5.4", that would mean " 5.1 ± 0.2 to 5.4 ± 0.2 " as well as "5.1 to 5.5".

Detailed description of the invention

5 Feed solutions are typically prepared in advance of a manufacturing run and/or in parallel of a manufacturing run. They are then stored in a container until said container is connected to the bioreactor containing the cells in culture, and/or at least maintained/stored in a container connected to the bioreactor containing the cells in culture as long as feed solutions are needed for the process. Depending on the feeding strategy and/or length of the manufacturing run, the feed solutions can be added at different time points of the culture, in bolus or continuous mode. This timing of addition and the facility constraints will drive the storage duration in the container. The solution can also be split in multiple containers connected at different time points to the bioreactor. It typically happens that feed solutions are maintained in their container for up to 2 to 4 weeks. During storage and/or during addition, precipitation of said feed media in their container and/or addition line can be observed, typically by visual inspection (e.g. visual observation). This precipitation can be mainly observed when the storage temperature of the container is at room temperature (typically 15-25 °C), however it is not excluded that they can happen whatever the storage temperature of the container (including lower temperature such as 2-8°C). These precipitations may also occur during preparation or shortly after preparation in certain conditions. A study of these precipitates by the inventors has revealed that the main species leading to precipitates is calcium hydrogen phosphate (CaHPO_4 ; also called dicalcium phosphate), and more specifically its hydrated form calcium hydrogen phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$). More particularly, it was shown that the solubility of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ in the feed solution is driven by the concentration of calcium ions (Ca^{2+}), not by the concentration of phosphates which are usually at higher concentration in cell culture media, and as such are not limiting components. Based on these findings and taking into account the calcium solubility curves obtained with two types of main feed (see Figure 2), a new preferred range of pH has been identified for the main feed. Following the teaching of the present invention, the skilled one could easily drastically reduce or prevent precipitation, such as CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation, of the at least one main feed medium in its container. The processes and methods described herein can be applied to any feed solutions, whatever the temperature they are maintained at.

The present invention generally relates to processes for the production of recombinant proteins in mammalian cells. In particular, the invention is based on the finding from the inventors that by lowering the pH of the main feed to be added in the frame of a fed-batch process to a specific range, i.e. from about 5.10 to about 5.50 (alternative writing " 5.10 ± 0.2 to 5.50 ± 0.2 "), it was possible to avoid the CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation of said main feed throughout the cultivation process without impacting the overall process performances (e.g. as assessed in term of VCC or titre). More particularly, it would be advantageous to use a main feed having a pH of

from 5.10 ± 0.2 to 5.20 ± 0.2 . In an alternative, it could be advantageous to use a main feed having a pH that is a function of the type of feed that is used: i) from 5.10 ± 0.2 to 5.50 ± 0.2 , if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or ii) from 5.10 ± 0.2 to 5.20 ± 0.2 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$. It is anticipated that the present invention could also be used for media to be used in perfusion process, should the perfusion rate be preferably below 5 bv (bv = bioreactor volume). Indeed, should the perfusion rate be above this limit, it is likely that the low pH of the feed will impact the overall pH of the culture and thus that it would be critical to monitor and correct pH in real time. To be clear, it would not be impossible to use such pH, should the perfusion rate be above 5bv, it would simply add further constraints that can be acceptable depending on the level of online monitoring one is ready to apply to their processes. In case of perfusion, the skilled person will understand that the term "feed medium" will mean the "perfusion medium". It is noted that for a concentration of Ca^{2+} ions in the feed medium between $85\text{-}95 \mu\text{g/mL}$, and in light of the calcium solubility curve (see Figure 2), the risk of precipitation would be very low. Therefore, in the context of the invention as a whole, and when it comes to perfusion, although to be on the safe side it would be recommended to go for a pH from about 5.10 to about 5.20, the skilled person can decide what pH they prefer to apply.

In a first embodiment, the invention provides processes and feeds when the particulars of the feed are unknown or the concentration of Ca^{2+} ions of a feed is unknown / not taken into consideration. In a first aspect of this first embodiment, the invention provides a process for producing a recombinant protein in a bioreactor, wherein the process comprises the steps of:

- (a) culturing mammalian cells expressing said recombinant protein in a culture medium (typically a basal culture medium) in the bioreactor, and
- (b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of this at least one feed medium is from 5.10 to 5.20.

One of the advantages of such process is that CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitations of the at least one feed medium in the container are prevented or at least deeply reduced.

In a second aspect of this first embodiment, the invention provides a process for culturing mammalian cells expressing a recombinant protein in a bioreactor, wherein the process comprises the steps of:

- (a) culturing the mammalian cells in a culture medium (typically a basal culture medium) in the bioreactor, and
- (b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of this at least one feed medium is from 5.10 to 5.20.

One of the advantages of such process is that CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitations of the at least one feed medium in the container are prevented or at least deeply reduced.

In a further aspect of this first embodiment, herein described is a process for reducing or preventing CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation in a feed medium (alternatively named main feed medium as defined above) in a container, during storage or during production of a recombinant protein, wherein the process comprises the steps of:

- 5 (a) preparing the at least one feed medium in a container,
 (b) adjusting the pH of the feed of step (a) so that its pH is from 5.10 to 5.20.

In the context of this first embodiment as a whole, the processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein comprise an initial step of inoculating the mammalian cells in a culture medium (such as a basal medium) in a bioreactor
10 (such as a growth bioreactor or a production bioreactor). Therefore in a non-limiting example, the processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein herein described comprise the steps of: (a) inoculating the mammalian cells in a culture medium in a production bioreactor, (b) culturing mammalian cells expressing said recombinant protein in a culture medium (typically a basal culture medium) in the bioreactor, and
15 (c) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of this at least one feed medium is from 5.10 to 5.20. In another non-limiting example, the processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein herein described, comprise the steps of: (a) inoculating the mammalian cells in a culture medium in a growth bioreactor, (b) progressing the cell culture obtained from step (a)
20 through a growth phase wherein the mammalian cells can grow, (c) inoculating the mammalian cells in a culture medium in a production bioreactor with cells grown during step (b), (d) progressing the cell culture obtained from step (c) through a production phase wherein the recombinant protein is produced by the mammalian cells, and wherein, during said growth phase and/or production phase, the cell culture is supplemented with at least one feed medium having a pH of from 5.10 to
25 5.20.

In the context of this first embodiment, also herein described are processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein herein described comprise the steps of:

- (a) inoculating the mammalian cells in a culture medium in a production bioreactor,
30 (b) progressing the cell culture obtained from step (a) through a production phase wherein the recombinant protein is produced by the mammalian cells,

and wherein, during said production phase, the cell culture is supplemented with at least one feed medium having a pH of from 5.10 to 5.20.

Alternatively, also herein described are processes for producing a recombinant protein or for
35 culturing mammalian cells expressing a recombinant protein herein described, comprise the steps of:

- (a) inoculating the mammalian cells in a culture medium in a growth bioreactor,

(b) progressing the cell culture obtained from step (a) through a growth phase wherein the mammalian cells can grow,

(c) inoculating the mammalian cells in a culture medium in a production bioreactor with cells grown during step (b),

5 (d) progressing the cell culture obtained from step (c) through a production phase wherein the recombinant protein is produced by the mammalian cells,

and wherein, during said growth phase and/or production phase, the cell culture is supplemented with at least one feed medium having a pH of from 5.10 to 5.20.

10 In another aspect of this first embodiment, the invention relates to a feed medium for use in any of the processes herein described, wherein the pH of this at least one feed medium is from 5.10 to 5.20.

In the context of this first embodiment as a whole, the feed medium is preferably the main feed medium and the pH of the (at least one) feed medium is from 5.10 to 5.20, such as 5.10, 5.15, 5.20 and any intermediate values (e.g. 5.11, 5.12, 5.13, 5.14, 5.16, 5.17, 5.18 and 5.19). Due to batch-to-batch variabilities and/or naturally occurring variations of the pH during production process, the pH may be let vary between certain thresholds (also called dead bands). In addition, due to variations linked to human errors, variability from one pH meter to another pH meter and other variations that cannot be controlled, a margin around the ranges of pH should be allowed. Thus, in the context of this embodiment as a whole, when it is mentioned that "the pH of the (at least one) feed medium is from 5.10 to 5.20", it is intended to include the variability: the pH of the (at least one) feed medium is from about 5.10 to about 5.20, such as about 5.10, 5.15, 5.20 and any intermediate values (e.g. 5.11, 5.12, 5.13, 5.14, 5.16, 5.17, 5.18 and 5.19). Depending on the variation that the skilled person will accept for their specific project (therefore the variation the skilled person will accept for the concept of "about"), in the context of this embodiment as a whole, the pH of the (at least one) feed medium is from 5.10 ± 0.2 to 5.20 ± 0.2 , from 5.10 ± 0.15 to 5.20 ± 0.15 , from 5.10 ± 0.10 to 5.20 ± 0.10 or yet from 5.10 ± 0.05 to 5.20 ± 0.05 . In a non-limiting example, the pH of said feed medium according to the invention can for instance be about 5.15, 5.15 ± 0.20 , 5.15 ± 0.15 or yet 5.15 ± 0.10 .

30 In a second embodiment, the invention provides processes and feeds when the particulars of the feed are known and/or the concentration of Ca^{2+} ions of a feed is known and taken into account.

In a first aspect of this second embodiment, the invention provides a process for producing a recombinant protein in a bioreactor, wherein the process comprises the steps of:

35 (a) culturing mammalian cells expressing said recombinant protein in a culture medium (typically a basal culture medium) in the bioreactor, and

(b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of this at least one feed medium is:

- i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or
- ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

5 One of the advantages of such process is that CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitations of the at least one feed medium in the container are reduced or prevented.

In a second aspect of this second embodiment, the invention provides a process for culturing mammalian cells expressing a recombinant protein in a bioreactor, wherein the process comprises the steps of:

- 10 (a) culturing the mammalian cells in a culture medium (typically a basal culture medium) in the bioreactor, and
- (b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of this at least one feed medium is:
 - i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or
 - 15 ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

One of the advantages of such process is that CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitations of the at least one feed medium in the container are reduced or prevented.

20 In a further aspect of this second embodiment, herein described is a process for reducing or preventing CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation in a feed medium (alternatively named main feed medium as defined above) in a container, during storage or during production of a recombinant protein, wherein the process comprises the steps of:

- (a) preparing the at least one feed medium in a container,
- 25 (b) adjusting the pH of the feed of step (a) so that:
 - i) its pH is from 5.10 to 5.50 if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in the at least one feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or
 - ii) its pH is from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in the at least one feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

30 In the context of the second embodiment as a whole, the processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein comprise the initial step of inoculating the mammalian cells in a culture medium (such as a basal medium) in a bioreactor (such as a growth bioreactor or a production bioreactor). Therefore in a non-limiting example, the processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein herein described comprise the steps of: (a) inoculating the mammalian cells in a culture medium in a production bioreactor, (b) culturing mammalian cells expressing said recombinant protein in a culture medium (typically a basal culture medium) in the bioreactor, and

35 (c) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the

pH of this at least one feed medium is: i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$. In another non-limiting example, the processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein herein described, comprise the steps of: (a) inoculating the mammalian cells in a culture medium in a growth bioreactor, (b) progressing the cell culture obtained from step (a) through a growth phase wherein the mammalian cells can grow, (c) inoculating the mammalian cells in a culture medium in a production bioreactor with cells grown during step (b), (d) progressing the cell culture obtained from step (c) through a production phase wherein the recombinant protein is produced by the mammalian cells, and wherein, during said growth phase and/or production phase, the cell culture is supplemented with at least one feed medium having a pH of i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

In the context of this first embodiment, also herein described are processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein herein described, comprise the steps of:

- (a) inoculating the mammalian cells in a culture medium in a production bioreactor,
- (b) progressing the cell culture obtained from step (a) through a production phase wherein the recombinant protein is produced by the mammalian cells, and wherein, during said production phase, the cell culture is supplemented with at least one feed medium having a pH of i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

Alternatively, also herein described are processes for producing a recombinant protein or for culturing mammalian cells expressing a recombinant protein herein described, comprise the steps of:

- (a) inoculating the mammalian cells in a culture medium in a growth bioreactor,
- (b) progressing the cell culture obtained from step (a) through a growth phase wherein the mammalian cells can grow,
- (c) inoculating the mammalian cells in a culture medium in a production bioreactor with cells grown during step (b),
- (d) progressing the cell culture obtained from step (c) through a production phase wherein the recombinant protein is produced by the mammalian cells, and wherein, during said growth phase and/or production phase, the cell culture is supplemented with at least one feed medium having a pH of i) from 5.10 to 5.50, if the feed is

a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

- In the context of this embodiment as a whole, a preliminary step of measuring the concentration of Ca^{2+} ions in the feed medium of interest can be performed. Said preliminary step of measurement can be performed only once, should the feed being used have an unknown concentration of Ca^{2+} ions. In other word, once the concentration of Ca^{2+} ions is known for a given type of feed, the measurement does not need to be repeated each time. Any methods can be used for such measurement, including, but not limited to, the one disclosed in the Example section.
- In the context of the second embodiment as a whole, the feed medium is preferably the main feed medium and can be either a non-concentrated feed medium or a concentrated feed medium (e.g. a concentrated main feed medium). If the feed medium is i) a non-concentrated feed medium or ii) a feed medium comprising no more than $85 \mu\text{g/mL} \pm 10\% \text{Ca}^{2+}$ ions, the pH of this at least one feed medium is preferably from 5.10 to 5.50 (and any intermediate values), or from 5.25 to 5.50 (5.25 to 5.50) such as 5.25, 5.30, 5.35, 5.40, 5.45, 5.50, optionally from 5.30 to 5.40 such as 5.30, 5.31, 5.32, 5.33, 5.34, 5.35, 5.36, 5.37, 5.38, 5.39 or 5.40, optionally at 5.35. The reasoning to apply such range of pH for a non-concentrated feed, even if its concentration in Ca^{2+} ions is unknown, is that such a non-concentrated feed medium has intrinsically more chances of containing lower concentrations of calcium ions, i.e. more chances of containing no more than $85 \mu\text{g/mL} \pm 10\% \text{Ca}^{2+}$ ions. As shown in the example, using an even lower pH has no negative impact but may not be needed. In the other hand, if the feed medium is a) a concentrated feed medium or b) a feed comprising more than $95 \mu\text{g/mL} \pm 10\% \text{Ca}^{2+}$ ions, the pH of this at least one feed medium is preferably from 5.10 to 5.20 (and any intermediate values such as 5.11, 5.12, 5.13, 5.14, 5.15, 5.16, 5.17, 5.18, 5.19), such as 5.15. The reasoning to apply such narrow range of pH for a concentrated feed even if its concentration in Ca^{2+} ions is unknown is that such a concentrated feed medium has intrinsically more risks of containing high concentrations of calcium ions, such as higher than $95 \mu\text{g/mL} \pm 10\% \text{Ca}^{2+}$ ions. Although the invention can be put in practice based on the teaching herein, i.e. without knowing the concentration in Ca^{2+} ions, the skilled person, based on their common general knowledge and on the examples described herein, can also easily determine the concentration in calcium ions in their feed medium in order to determine the preferred pH. As already mentioned above, it is noted that for a concentration of Ca^{2+} ions in the feed medium between 85-95 $\mu\text{g/mL}$, and in light of the calcium solubility curve herein shown (see Figure 2), the risk of precipitation would be very low. Therefore, although to be on the safe side it would be recommended to go for a pH from 5.10 to 5.20 (such as 5.15), the skilled person can decide if they prefer to apply a pH from 5.10 to 5.20 (such as 5.15) or from 5.10 to 5.50 (such as 5.35).

In another aspect of the second embodiment, the invention relates to a feed medium for use in any of the processes herein described, wherein the pH of this at least one feed medium is i) from 5.10

to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

In the context of the second embodiment as a whole, the pH of the (at least one) feed medium is

- 5 (i) from 5.10 to 5.50 if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, such as 5.10, 5.15, 5.20, 5.25, 5.30, 5.35, 5.40, 5.45 or 5.50 and any intermediate values or (ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$, such as 5.10, 5.15, 5.20. Due to batch-to-batch variabilities and/or naturally occurring variations of the
- 10 pH during production process, the pH may be let vary between certain thresholds (also called deadbands). In addition, due to variations linked to human errors, variability from one pH meter to another pH meter and other variations that cannot be controlled, a margin around the ranges of pH should be allowed. Thus, in the context of the second embodiment as a whole, when it is mentioned that “the pH of the (at least one) feed medium is from 5.10 to 5.50” or “the pH of the (at
- 15 least one) feed medium is from 5.10 to 5.20”, it is intended to include the variability: the pH of the (at least one) feed medium is (i) from about 5.10 to about 5.50 (and any intermediate values) if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, such as about 5.10, 5.15, 5.20, 5.25, 5.30, 5.35, 5.40, 5.45 or 5.50 or (ii) from about 5.10 to about 5.20 (and any intermediate values) if the feed is a concentrated feed
- 20 or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$, such as about 5.10, 5.15, 5.20 and any intermediate values. Depending on the variation that the skilled person will accept for their specific project, (thus the variation the skilled person will accept for the concept of “about”), in the context of this embodiment as a whole, the pH of the (at least one) feed medium is (i) from 5.10 ± 0.2 to 5.50 ± 0.2 , from 5.10 ± 0.15 to 5.50 ± 0.15 , from 5.10 ± 0.10 to 5.50 ± 0.10
- 25 or yet from 5.10 ± 0.05 to 5.50 ± 0.05 if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$ or (ii) from 5.10 ± 0.2 to 5.20 ± 0.2 , from 5.10 ± 0.15 to 5.20 ± 0.15 , from 5.10 ± 0.10 to 5.20 ± 0.10 or yet from 5.10 ± 0.05 to 5.20 ± 0.05 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$. As a non-limited example, should the feed be a normal feed or a feed
- 30 medium comprising no more than $85 \mu\text{g/mL} \pm 10\%$ Ca^{2+} ions, the pH of said feed medium according to the invention can for instance be 5.35 ± 0.20 , 5.35 ± 0.15 or yet 5.35 ± 0.10 . In other non-limiting example, should the feed be a concentrated feed or a feed medium comprising at least $95 \mu\text{g/mL} \pm 10\%$ Ca^{2+} ions, the pH of said feed medium according to the invention can for instance be 5.15 ± 0.20 , 5.15 ± 0.15 or yet 5.15 ± 0.10 .

35

Depending on the overall strategy used for producing a recombinant protein, for culturing mammalian cells expressing a recombinant protein or for reducing or preventing CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation in a feed medium in a container, the feed medium according to the

invention as a whole (i.e. having a pH from 5.10 to 5.50 or having a pH from 5.10 to 5.20) can also be used during stages preceding the production phase, such as during the N-1 stage.

In the context of the processes according to the invention as a whole and as shown in the example section, thanks to the lowering of the pH of the (at least one) feed medium, it was possible to avoid the CaHPO₄ precipitation of the main feed throughout the cultivation process without impacting the overall process performances (cell culture performance and/or quality attributes).

In a further aspect, the invention as a whole describes a recombinant protein produced by any one of the processes according to the present invention.

In the context of the invention as a whole, the culture medium at the start of the culture (step (a) and/or step (c) if applicable) is preferably a protein- and serum-free culture medium. Said protein- and serum-free culture medium can be commercially available (such as a commercial chemically defined medium) or a chemically defined in-house medium.

In the context of the invention as a whole, the at least one feed medium (also herein referred to as main feed medium, as per the definition section) is preferably a protein- and serum-free feed medium and comprises all or most of the essential elements. If not included in the at least one feed medium, the carbon source can be brought via an additional feed. The (at least one) feed medium can be a "normal feed", prepared and used according to standard protocols (e.g. as disclosed in Figure 1A) or can be a concentrated feed medium (e.g. a defined concentrated main feed medium, prepared for instance as disclosed in Figure 1B or a commercial concentrated feed medium). In a non-limiting example, the (at least one) feed medium (also herein referred to as main feed medium) is preferably a protein- and serum-free feed medium and comprises all or most of the essential elements, but does not comprise any of the following free amino acids: cysteine/cystine (both referred as Cys) and tyrosine (Tyr), as these amino acids are known to be difficult to solubilise and to stabilise at pH lower than about 8.0. The carbon source as well as Cys and Tyr can be brought via additional feeds such as one feed consisting of the carbon source and 1) one feed consisting of Cys and Tyr or 2) two different feeds consisting of Cys and Tyr respectively. In another non-limiting example the (at least one) feed medium (also herein referred to as main feed medium) is preferably a protein- and serum-free feed medium and comprises all or most of the essential elements, but does not comprise any of the following free amino acids: cysteine and/or cystine (both referred as Cys), tryptophan (Trp) and tyrosine (Tyr). The carbon source as well as Cys, Tyr and Trp can be brought via additional feeds such as one feed consisting of the carbon source and 1) one feed consisting of Cys, Tyr and Trp, 2) two feeds consisting of a combination of any two amino acids selected from of Cys, Tyr, Trp, the third amino acid being added in a separate feed or 3) three different feeds consisting of Cys, Tyr and Trp respectively.

In the context of the invention as a whole, the mammalian cells expressing the recombinant proteins can be cultivated in the production phase in a fed batch, batch or perfusion process. In a non-limiting example, the production phase is run as a fed batch. In an embodiment, the production phase has a duration of at least 7 days, preferably at least 10 days, more preferably at least 12

days, such as 12 days, 13 days, 14 or 15 days. The at least one feed medium (also herein referred to as main feed medium, as per the definition section) can be supplemented according to any standard processes or in-house processes, such as supplemented at regular interval (such as daily or every other day), continuously or on demand, typically until 1 or 2 days before the harvest. If
5 any additional feeds are needed (such as feeds comprising the carbone source and/or any missing elements), they can also be supplemented according to any standard processes or in-house processes, such as supplemented at regular interval (such as daily or every other day), continuously or on demand, typically until 1 or 2 days before the harvest.

In the context of the present invention, the production phase is carried out in a bioreactor (such as
10 a production bioreactor), preferably with a volume of equal or more than 50 L, equal or more than 100 L, equal or more than 200 L, equal or more than 500 L, equal or more than 1,000 L, equal or more than 2,000 L, equal or more than 5,000 L, equal or more than 10,000 L or equal or more than 20,000 L. In other words, the mammalian cells producing the recombinant proteins are cultivated in a bioreactor (such as a production bioreactor), preferably with a volume of equal or more than
15 50 L, equal or more than 100 L, equal or more than 500 L, equal or more than 1,000 L, equal or more than 2,000 L, equal or more than 5,000 L, equal or more than 10,000 L or equal or more than 20,000 L.

In the context of the invention as a whole, suitable mammalian host cells (also named mammalian cells) include Chinese Hamster Ovary (CHO cells), cells derived from human embryonic kidney
20 cells (such as HEK293 cells), lymphocytic cell lines, e.g., NSO myeloma cells and SP2 cells, COS cells, myeloma or hybridoma cells. In a preferred embodiment, the mammalian cell is a CHO. Suitable types of CHO cells may include CHO and CHO-K1 cells including dhfr- CHO cells, such as CHO-DG44 cells and CHO-DXB11 cells and which may be used with a DHFR selectable marker or CHOK1-SV cells which may be used with a glutamine synthetase selectable marker. The host
25 cells are preferably stably transformed or transfected with expression vectors encoding the recombinant protein of interest.

In the context of the invention as a whole, the recombinant protein can be a cytokine, a growth factor, a hormone, a fusion protein (such as a protein comprising a domain or other fragments of an antibody), or an antibody (as above defined). For instance, when the protein is an antibody, it
30 is preferably an IgG such as IgG1, IgG2, IgG3 or IgG4.

When using the processes and/or cell culture techniques of the present invention, in mammalian cells, the recombinant proteins are generally directly secreted into the culture medium. Supernatants from such expression systems can be first harvested and clarified, in order to start isolating the protein of interest and concentrate if before it is purified and formulated. Therefore,
35 the process of the invention optionally further comprises a step of recovering the recombinant protein from the cell culture medium, preferably at the end of the production (harvest step). Subsequently to the harvest, the recombinant protein may be purified, e.g. if the protein is an antibody, using Protein A chromatography. The process further optionally comprises a step of

formulating the purified recombinant protein, e.g. into a formulation with a high protein concentration, such as a concentration of 10 mg/ml or more, e.g. 50 mg/ml or more, such as 100 mg/ml or more, e.g. 150 mg/ml or more. Without any limitation, the formulation can be a liquid formulation, lyophilised formulation or a spray-dried formulation.

5

Further aspects

Aspect 1. A process for producing a recombinant protein in a bioreactor, wherein the process comprises the steps of:

- 10 a) culturing mammalian cells expressing said recombinant protein in a culture medium in the bioreactor, and
- b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of the at least one feed medium is:
 - i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or
 - 15 ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

Aspect 2. A process for culturing mammalian cells expressing a recombinant protein in a bioreactor, wherein the process comprises the steps of:

- 20 a) culturing the mammalian cells in a culture medium in the bioreactor and
- b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of the at least one feed medium is:
 - i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or
 - 25 ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

Aspect 3. The process according to any one of aspects 1 or 2, wherein the at least one feed medium is 1) stored in a container until being used and/or 2) is contained in a container connected to the bioreactor.

30 Aspect 4. The process according to any one of aspects 1 to 3, wherein the CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation of the at least one feed medium in the container is reduced or prevented.

Aspect 5. A process for reducing or preventing CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation in a feed medium in a container, during storage and/or during cultivation of mammalian cells and/or during production of a recombinant protein, wherein the process comprises the steps of:

- 35 a) preparing the at least one feed medium in a container,
- b) adjusting the pH of the feed of step a) so that its pH is:
 - i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or

ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

Aspect 6. A feed medium for use in the process according to any one of aspects 1 to 5, wherein its pH is i) from 5.10 to 5.50, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or ii) from 5.10 to 5.20 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

Aspect 7. The process according to any one of aspects 1 to 5, further comprising a preliminary step of measuring the concentration of Ca^{2+} ions in said feed medium, wherein said measurement can be performed only once, should the feed being used have an unknown concentration of Ca^{2+} ions.

Aspect 8. The process according to any one of aspects 1 to 5 or 7 or the feed medium according to aspect 6, wherein the pH of the at least one feed medium is i) at 5.35, if the feed is a non-concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or lower than $85 \mu\text{g/mL} \pm 10\%$, or ii) 5.15 if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.

Aspect 9. The process according to any one of aspects 1 to 5, 7 or 8 or the feed medium according to any one of aspects 6 or 8, wherein the feed medium does not comprise i) any one of the free amino acids cysteine and tyrosine or ii) any one of the free amino acids cysteine, tyrosine and tryptophan.

Aspect 10. The process according to aspect 9, wherein i) should the feed medium not comprise any one of the free amino acids cysteine and tyrosine, said cysteine and tyrosine are supplied separately or ii) should the feed medium not comprise any one of the free amino acids cysteine, tyrosine and tryptophan, said cysteine, tyrosine and tryptophan are supplied separately.

Aspect 11. The process according to any one of aspects 1 to 5 or 7 to 10 wherein the supplementation of the culture medium in the bioreactor is performed during a growth phase and/or a production phase.

Aspect 12. The process according to any one of aspects 1 to 5 or 7 to 11, wherein the mammalian cells are Chinese Hamster Ovary (CHO) cells.

Aspect 13. The process according to any one of aspects 1 to 5 or 7 to 12, wherein the recombinant protein is a cytokine, a growth factor, a hormone, an antibody or a fusion protein.

Aspect 14. The process according to aspects 13, wherein the antibody is a chimeric antibody, a humanised antibody or a fully human antibody.

Aspect 15. The process according to aspect 13 or aspect 14, wherein the antibody is selected from the group consisting of an IgG1, IgG2, IgG3 or IgG4.

Aspect 16. A recombinant protein produced by the process according to any of aspects 1 to 5 or 7 to 15.

Description of the figure:

Figure 1: A) Example of the preparation of a “normal” feed from powder. B) Example of the preparation of a “concentrated” feed from powder.

Figure 2: Solubility data and curve of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, expressed in terms of calcium (A) and phosphorus (B), as a function of pH of a normal main feed and a concentrated main feed, measured at 5°C and 25°C.

Figure 3: A) Cell growth profiles for mAb1 cells and mAb2 cells. B) Titre for mAb1 and mAb2.

Figure 4: A) Percentages of main charge variant species for mAb1 and mAb2. B) Percentages of monomers for mAb1 and mAb2.

Figure 5: A) Cell growth profiles for mAb3 cells. B) Titre for mAb3.

Figure 6: A) Percentages of main charge variant species for mAb3. B) Percentages of monomers for mAb3.

Examples**15 Cell line, cell culture and experimental procedure**

CHO-DG44 cell lines were used.

mAb1 is an IgG1 antibody having a pI of 8.0-8.80, mAb2 and mAb3 are IgG4 antibodies having respectively a pI of 8.3-8.7 and 7.70-7.90

The CHO-DG44 cells, expressing either mAb1, mAb2 or mAb3, were cultivated in stirred tank glass bioreactor (STR) with supply towers (B-DCU, Sartorius Stedim Biotech) controlled by a multi-fermentation control system (MFCS, Sartorius Stedim Biotech) or in shake flasks. The reactors were equipped with a 3-segment blade impeller. The cultivation start volume was adapted to ensure the cultivation end volume was optimal. The production bioreactors were seeded at target seeding density (TSD) in a basal chemically defined medium. The pH control of the production bioreactor was set to 7.0 with a dead band of 0.2 (pH at about 7.0, in other words $\text{pH } 7.0 \pm 0.2$). The pO₂ target was set to 40-60 % air saturation and controlled according to standard practice. The temperature was controlled at 36.8°C with a dead band of 0.2 ($36.8^\circ\text{C} \pm 0.2$).

The non-concentrated feed used in examples 1 and 2 was prepared by dissolution of the main feed powder according to the standard protocol for this powder (in order to reach a concentration of x1) and the pH was adjusted to the target pH. A concentrated main feed was used for examples 1 and 3. This main feed (aqueous) was prepared by dissolution of the main feed powder (same powder as for the normal feed) until the desired concentration (concentrated about x2 compared to the standard protocol for this powder) was reached in the liquid and the pH was adjusted to the target pH. The bags with the main feeds were kept at room temperature during all the cultivation process. 48 hours after inoculation, continuous nutrient feeding (with a concentrated feed, named “main feed” or “Feed 1”) was started with a predetermined rate. A glucose bolus feed was added to the culture on demand, i.e. when the glucose concentration dropped below a given threshold

(glucose concentrations were measured daily). As the main feed did not comprise any one of Cys, Tyr and Trp, these amino acids were added separately.

The production was operated in fed-experiment mode for 14 days, at room temperature. During this phase, the monoclonal antibody (mAb) was secreted into the medium. Samples were drawn daily to determine, among others, VCD, viability, off-line pH, pCO₂, osmolality, glucose-lactate concentration, amino acid concentration and mAb concentration. Samples for the amino acid analysis were taken before the feeds addition. At the end of the production process, samples were also analysed for quality attributes.

10 Cell culture performance related method and other analytical Methods

Cells were counted by using a VI-CELL® XR (Beckman-Coulter) automated cell counting device that operated based on trypan blue exclusion. Glucose and lactate levels in the culture medium were determined using a Cedex® Bio HT (Roche). A model 2020 freezing point osmometer (Advanced Instruments) was used for osmolality determination. Offline gas and pH measurements were performed with a model BioProfile pHox® blood gas analyzer (Nova Biomedical Corp.). Metabolites concentrations were also daily determined using a Cedex® BioHT system (Roche). Product titre analysis was performed with the Cedex® or protein A high-pressure liquid chromatography (HPLC) with cell culture supernatant samples which were stored at -80°C prior to analysis. The cell culture supernatant samples were purified with a Protein A purification on the ÄKTA Xpress system. The relative percentage of main isoform of the purified mAb was determined by Imaged Capillary Electrophoresis (ProteinSimple iCE3). Statistical analyses were performed using SAS software JMP 11 ©.

Calcium phosphate solubility

25 In a HPLC-type vial, about 15 mg of seeds (CaHPO₄·2H₂O) and 1.5 mL of Feed 1 were stirred at 250 rpm for three days at a given temperature in small scale crystallization set-up. The remaining solid was then let to settle. One mL of liquid phase was filtered through a syringe filter (0.2 µm) and the amount of calcium (Ca²⁺) and phosphorus (P) in the liquid phase was quantified by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). The pH of the liquid phase was also recorded. The remaining solid was analysed by X-Ray Powder Diffraction (XRPD) to confirm the identity of the crystalline phase.

Example 1 – Calcium Phosphate solubility understanding within cell culture media

35 The aim of this case study was to determine if the main feed (Feed 1) solutions were saturated in calcium and phosphate. In parallel the effect of the temperature and the pH on the saturation was also tested. CaHPO₄·2H₂O was identified as the unique species precipitating in the main Feed solutions. Solubility of CaHPO₄·2H₂O was successfully assessed in normal and concentrated Feed at different pH and at different temperatures. Normal feed 1 solution (1X) and concentrated Feed

1 solution (about 2X) were prepared at different pH. As the spontaneous crystallization was not always observed (data not shown), the crystallization by seeding approach was used in this study. The results showed that the solubility of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ was determined and characterized by concentration of calcium ions (Ca^{2+}) in solution and not by the concentration of phosphorus ions, likely due to a source of phosphorus (as phosphates) largely in excess of the stoichiometric quantities. Solubility curve of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, expressed as calcium concentration as a function of pH in feed solutions is shown in Figure 2A. From the data the solubility of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ appears not to depend on temperature in the investigated range, nor on the feed concentration or on phosphorus (See Figure 2B).

At pH 6.65 (standard pH), the comparison with the initial concentration in the main feed allowed to conclude that $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ was supersaturated in both the normal and concentrated feeds. Under these conditions, the precipitation of calcium phosphate could be triggered by any minor perturbation. These perturbations can be experienced more specifically at large scale and can include any event that would either lower the energy barrier for crystallization (particulates providing surface for spontaneous nucleation) or provide energy to the system (shear flow in the feeding line, peristaltic pump shear, shear at impeller tip).

Based on the results of example 1, to minimise as much as possible the risk of precipitations (taking into account the variability batch per batch) it was shown that a pH of 5.10 ± 0.15 to 5.50 ± 0.15 (such as 5.35 ± 0.15) for a main non-concentrated feed or a feed comprising no more than $85 \mu\text{g/mL} \pm 10\%$ Ca^{2+} ions would likely remove (or at least minimize) risk of precipitation, as such precipitation is mainly (if not only) made from $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$. For a main concentrated feed or a feed comprising at least $95 \mu\text{g/mL} \pm 10\%$ Ca^{2+} ions, a pH of 5.10 ± 0.15 to 5.20 ± 0.15 (such as 5.15 ± 0.15) would likely further minimize risk of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation in the feed.

For a concentration of Ca^{2+} ions in the feed medium between $85\text{--}95 \mu\text{g/mL}$, and in light of the calcium solubility curve herein shown (see Figure 2), the risk of precipitation would be very low. Therefore, although to be on the safe side it could be recommended to go for a pH from about 5.10 to about 5.20, the skilled person can decide if they prefer to apply a pH from about 5.10 to 5.20 or from 5.10 to 5.50.

Example 2 – pH adaptation of the Feed 1 to very low pH to produce two different mAb

Based on the results of Example 1, it was decided to assess the effect of a pH of 5.35 when a normal main Feed (1X, corresponding to a Ca^{2+} ion concentration of about $80 \mu\text{g/mL}$) was used, on the quality attribute a cell culture performance of two mammalian cell lines producing respectively mAb1 and mAb2.

For this experiment, 2L bioreactors had been inoculated with CHO cells producing respectively mAb1 or mAb2 at a seeding density of 0.35×10^6 cells /mL. In this experiment, three experimental conditions were tested in fed-batch process as described in the above experimental procedures. Different feeding strategy were used to produce mAb1 and mAb2. However the same feeding

strategy has been applied for each cell line whatever the feed pH used (see Table 1). The main feed used for this Example was diluted according to the original instruction (i.e. “normal feed”)

5 Table 1: Experimental conditions for example 2

Conditions	mAb1 cells			mAb2 cells		
	Main Feed pH			Main Feed pH		
	5.35	5.50	6.65	5.35	5.50	6.65
Replicates	2	6	4	3	6	4
Precipitation	No	No	Yes	No	No	Yes

10 Cell culture performance: Cell growth profiles are shown in Figure 3A. Cell growth for all these conditions presented similar trend over the 14-days of culture. For each cell line the titre at day 14 was comparable between the different Feed pH conditions tested. The results confirm that there was no negative impact of adding a main Feed 1 at lower pH on cell culture performance, more especially on the overall mAbs production (see Figure 3B), while having the advantage of at least mitigating calcium phosphate precipitation risk of the main feed.

15 Quality attributes: As shown in Figures 4A and 4B, the lower pH of 5.35 and 5.50 did not impact negatively the quality attributes of the two mAbs, as exemplified by the percentage of Main species and monomer.

20 Conclusion: Example 2 highlights that a normal main feed at a pH of 5.35 and a pH of 5.50 compared to its control pH (i.e. pH 6.65 ± 0.2), can be added to cell cultures without having adverse effects on the overall process performances/quality attributes and could avoid the precipitation risk of the main feed during the overall cultivation process.

Example 3 - Supplementation of main concentrated Feed 1 at very low pH to produce mAb3

25 For this experiment, different bioreactors had been inoculated with CHO cells producing mAb3 at a seeding density of 3.40x10⁶ cells /mL. Two experimental conditions were tested in fed-batch process, as described in the above experimental procedures, at different scales. Bioreactor ID 1 and bioreactor 2/3 had the same feeding strategy but were fed with main feeds having two different pH: respectively 5.50 and 5.15 (see Table 2). The same main Feed as Example 1 was used but this time concentrated about 2x compared to the original instructions (about 160 µg/mL of Ca²⁺ ions).

Table 2: Experimental conditions

Conditions	pH Main feed	Scale	Precipitations	Replicates
Bioreactor ID1	5.5	2L	No	2
Bioreactor ID2	5.15	2L	No	1
Bioreactor ID3	5.15	10L	No	1

Cell culture performance: As shown in Figure 5A, cell growth for the different tested conditions and on both scales presented similar trend over the 14-days of culture. Bioreactor ID1 also shows a titre (on days 13 and 14), comparable to the ones of bioreactors ID2 and ID3 (See Figure 5B). The results confirm that there was no negative impact of adding a main Feed 1 at a pH of 5.15 compared to a pH of 5.50 on overall mAb3 production, while having the advantage of at least mitigating precipitation risk of the concentrated main feed, although not observed at this scale.

Quality attributes: As shown in Figure 6A and 6B, main charge species and monomer species presented similar trends whatever the pH of the feed added during the manufacturing process. The results confirm that there was no negative impact of adding a main Feed 1 at a pH of 5.15 compared to a main Feed 1 at a pH of 5.50 on mAb3 quality attributes.

Conclusion pf Example 3: This example confirms that a main concentrated Feed 1 at a pH of 5.15 compared to a pH 5.5, can be added to cell cultures without having adverse effects on the overall process performances/quality attributes and could avoid the precipitation risk of the main feed during the overall cultivation process.

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Claims

1. A process for producing a recombinant protein in a bioreactor, wherein the process comprises
5 the steps of:
- a) culturing mammalian cells expressing said recombinant protein in a culture medium in the bioreactor, and
 - b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of the at least one feed medium is from 5.10 ± 0.2 to 5.20 ± 0.2 .
- 10 2. A process for culturing mammalian cells expressing a recombinant protein in a bioreactor, wherein the process comprises the steps of:
- a) culturing the mammalian cells in a culture medium in the bioreactor and
 - b) supplementing the culture medium in the bioreactor with at least one feed medium, wherein the pH of the at least one feed medium is from 5.10 ± 0.2 to 5.20 ± 0.2 .
- 15 3. The process according to any one of claims 1 or 2, wherein the at least one feed medium is 1) stored in a container until being used and/or 2) is contained in a container connected to the bioreactor.
4. The process according to any one of claims 1 to 3, wherein the CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation of the at least one feed medium in the container is reduced or prevented.
- 20 5. A process for reducing or preventing CaHPO_4 and/or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ precipitation in a feed medium in a container, during storage and/or during cultivation of mammalian cells and/or during production of a recombinant protein, wherein the process comprises the steps of:
- a) preparing the at least one feed medium in a container,
 - b) adjusting the pH of the feed of step a) so that its pH is from 5.10 ± 0.2 to 5.20 ± 0.2 .
- 25 6. A feed medium for use in the process according to any one of claims 1 to 5, wherein its pH is from 5.10 ± 0.2 to about 5.20 ± 0.2 .
7. The process according to any one of claims 1 to 5, further comprising a preliminary step of measuring the concentration of Ca^{2+} ions in said feed medium, wherein said measurement can be performed only once, should the feed being used have an unknown concentration of Ca^{2+} ions.
- 30 8. The process according to any one of claims 1 to 5 or 7 or the feed medium according to claim 6, wherein the pH of the at least one feed medium is from 5.10 ± 0.2 to 5.20 ± 0.2 , if the feed is a concentrated feed or if the concentration of Ca^{2+} ions in said feed medium is at or above $95 \mu\text{g/mL} \pm 10\%$.
9. The process according to any one of claims 1 to 5, 7 or 8 or the feed medium according to any one of claims 6 or 8, wherein the feed medium does not comprise i) any one of the free amino acids cysteine and Tyrosine or ii) any one of the free amino acids cysteine, Tyrosine and Tryptophan and wherein said free amino acids are supplied separately.
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10. The process according to any one of claims 1 to 5 or 7 to 9 wherein the supplementation of the culture medium in the bioreactor is performed during a growth phase and/or a production phase.
11. The process according to any one of claims 1 to 5 or 7 to 10, wherein the mammalian cells are Chinese Hamster Ovary (CHO) cells.
- 5 12. The process according to any one of claims 1 to 5 or 7 to 11, wherein the recombinant protein is a cytokine, a growth factor, a hormone, an antibody or a fusion protein.
13. The process according to claim 12, wherein the antibody is a chimeric antibody, a humanised antibody or a fully human antibody
14. The process according to claim 12 or claim 13, wherein the antibody is selected from the group
- 10 consisting of an IgG1, IgG2, IgG3 or IgG4.
15. A recombinant protein produced by the process according to any of claims 1 to 5 or 7 to 14.