Title: IMPROVED CELL CULTURE MEDIUM

[Technical Field of the Invention]

This invention relates to the general field of biotechnology, particularly the cultivation of cells and their use for the production of polypeptides at industrial scale.

The present invention provides cell culture media which are suitable for the cultivation of cells with high cell viabilities, preferably mammalian cells like CHO cells, and which are characterized by their molar ratio of sodium to potassium ions. The cell culture media according to the present invention allow obtaining high polypeptide productivities when used for the production of a polypeptide, in particular by recombinant expression of polypeptides in mammalian cell culture systems, in particular at industrial scale.

[Technical Background of the Invention]

The preparation of polypeptides using recombinant technology has developed into a standard procedure during the last couple of decades. The access to recombinant polypeptides by cloning the genes encoding the respective polypeptide followed by subsequent transformation of suitable expression hosts with the gene to be expressed and final production and purification of the obtained recombinant polypeptide product has provided access to a whole new class of biologically designed and produced therapeutics.

Pharmaceutically active compounds have been prepared in increasing numbers in the pharmaceutical industry using recombinant DNA technology followed by production processes developed in the field of bioengineering.

Such biological products include monoclonal antibodies, which have been developed into important treatment options in various medical fields including

autoimmune diseases, inflammatory disorders, immunosuppression, oncology or the like.

Development of such therapeutics of biological origin requires production at industrial scale thereby providing access to large amounts of recombinant polypeptide. Preferred expression systems are mammalian cell cultures which are superior to most other eukaryotic systems based on insect cells, yeast or the like, or even traditional prokaryotic expression systems.

However, mammalian cell culture includes tremendous challenges especially at the industrial scale. Production facilities for mammalian cell culture require thorough optimization of many process conditions.

One of the most important process parameters for controlling the overall production process is the medium in which cells are grown and polypeptide production takes place. Suitable cell culture media must provide cell cultures with all necessary nutrient substances, which is especially difficult if no components of animal origin like serum or proteins, e.g. growth factors, are added to the media.

Consequently, a great variety of different cell culture media have been developed. In some cases, the focus has been on the general composition and media with a large variety of different substances have been proposed (US 5 122 469, EP 0 481 791, EP 0 283 942). In other cases, particular ingredients have been suggested to improve cell culture. Major goals have been to improve either growth or survival of the cells, or the quantity and quality of recombinantly expressed polypeptides.

Specific aspects addressed in prior art documents are amongst others the contribution of particular trace ions (e.g. WO 02/066603, EP 0 872 487, EP 1 360 314 A2), vitamins such as ascorbic acid (e.g. US 6 838 284), carbohydrates (EP 1 543 106) or the content of specific amino acids in

combination with additional features (e.g. EP 0 501 435, US 5 830 761, US 7 294 484).

The major ions and their concentrations in cell culture media are largely kept constant and remain unconsidered and unchanged. All classical types of media such as e.g. DMEM, DMEM/F12, BME or RPMI 1640 use relatively narrow and fixed ranges for the concentrations of bulk ions in general and the monovalent cations Na^+ and K^+ in particular. This is in line with the fact that the ionic balance of the bulk ions in general and the monovalent cations Na^+ and K^+ in particular is a rather universal property of almost all mammalian cells.

In more detail, the transmembrane gradient of sodium and potassium ions is a basic property of mammalian cells with high concentration of potassium ions inside the cell and high concentration of sodium ions outside the cell. The sodium potassium pump is one of the major ion pumps of the cell membrane which is electrogenic and contributes in establishing and maintaining the respective sodium and potassium ionic gradient across the membrane (Kaplan, Membrane cation transport and the control of proliferation of mammalian cells. Annu Rev Physiol.; 40: 19-41 (1978)). The pump uses about 30% of the cells energy and is one of the major energy consuming processes of the cells. Many basic biochemical processes are coupled to the electrochemical gradient of sodium ions, such as e.g. the Na^+/Ca^+ exchanger or the amino acid transport into cells. The concentrations of sodium and potassium ions outside a cell are therefore parameters of paramount importance that influence the gradient of these ions across the membrane and the basic status of the cell.

In accordance with the typical concentration of sodium ions inside and outside a generic mammalian cell (Alberts et al., Molecular Biology of the Cell (1994)) mostly sodium concentrations of about 145 mM are chosen together with potassium ion concentrations of around 5 mM. For most media types this results in a ratio

between sodium and potassium ions that ranges between about 20-30 (see Table 1 below and e.g. US 5 135 866).

Only few prior art documents describe cell culture media suitable for mammalian cell culture or the production of recombinant proteins mentioning specific ratios of sodium to potassium ions. These documents suggest media compositions with specifically high ratios in the high range of about 30.7 in US 5 232 848, or in a range of between about 25 and 35 thus reaching even higher values (EP 0 283 942, EP 0 389 786). Other media such as HAM's-F12 or defined animal cell culture media as proposed in US 2008/0261259 also specifically suggest higher values (e.g. 27.9 to 57.5 in US 2008/0261259). Only very few documents disclose media having a ratio of sodium to potassium ions below 20, such as 11.5 – 30 (US 7 294 484) or a ratio of about 15 (US 6 180 401). These documents still use ratios of higher than 10 and also do not assign a particular advantage to changing this parameter to the values as mentioned.

In addition to the effects related to the ionic balance between particular ions, also the contribution of the major ions to the overall osmolality of the medium has to be considered. Most conventional media such as e.g. DMEM, MEM alpha, or Fischer's medium are characterized by a high amount of sodium chloride.

WO 02/101019 addresses the high content of glucose in the medium in combination with usage of a higher osmolality. The high glucose concentration between about 2-40 g/l has been achieved by reducing or even completely eliminating agents such as sodium chloride thereby maintaining osmolality at a given level.

Considering the above challenges and existing disadvantages, there is a continued need in the field of industrial biotechnology for improved cell culture media which allow producing recombinant polypeptides at an industrial scale.

[Summary of the Invention]

The present invention provides cell culture media with a reduced Na^+/K^+ ratio, i.e. a Na^+/K^+ ratio below a value of about 10. This is achieved by means of decreasing the number of total sodium ions and increasing the total potassium ion content. It has been found that such low ratio exerts several beneficial effects, in particular improved viability, growth and productivity of mammalian cells.

Thus, the present invention provides an optimized cell culture medium for the growth of mammalian cells as well as for polypeptide production which is characterized by a ratio of sodium to potassium ions, measured as molar content, of between about 10 to 1 and about 1 to 1, alternatively between about 8 to 1 and about 6 to 1. Implementation of this feature may include concentrations of sodium ions in the range between about 50 and about 90 mM and of potassium ions between about 8 and about 12 mM.

In another aspect, optimization of the cell culture medium includes selecting a total amino acid content of between about 40 mM and about 100 mM, alternatively between about 50 mM and about 100 mM. This feature may be combined with a particular low molar ratio between the total ion and the total amino acid concentration of about 1.9 to about 4.

In a further aspect, the invention provides a process where the cell culture medium according to the invention is used for culturing mammalian cells for the production of a desired recombinant polypeptide. The process involves culturing mammalian cells in a medium according to the invention and expressing the recombinant polypeptide.

Some implementations of the process include culture conditions where the temperature and/or the pH of the medium are shifted at least once during culturing. As a further option feeding is accomplished by a fed batch process.

The desired polypeptide products include glycosylated polypeptides and in particular antibodies and antibody fragments.

The mammalian cells used in the process of the present invention are preferably selected from the group consisting of CHO cells, HEK cells and SP2/0 cells.

In a further aspect, the present invention relates to a process for the production of a cell culture medium according to the invention wherein the different components are admixed with each other. In particular, the concentration of sodium chloride added to the medium composition may be in the range between about 7 and about 15 mM. The concentration of potassium chloride may be added to the medium composition may be in the range between about 8 to about 12 mM.

[Brief Description of the Drawings]

The invention will be better understood by reference to the following examples and figures. The examples, however, are not intended to limit the scope of the invention.

Fig. 1 shows the viable cell density of a mAb1-producing CHO cell clone as function of culture time in shake flask cultures (see Example 1) using a medium with a reduced Na^+/K^+ ratio as described by the invention. In addition the effect of a constant temperature versus a temperature shift is depicted.

Fig. 2 shows the viability of a mAb1-producing CHO cell clone (see Example 1) using a medium with a reduced Na^+/K^+ ratio and the effect of a constant temperature versus a temperature shift on day 3.

Fig. 3 shows the product titer as function of cultivation time for shake flask cultures of a mAb1-producing the CHO cell clone with and without a temperature shift (see Example 1). Cells were cultivated in a medium with a reduced Na^+/K^+ ratio according to the invention.

Fig. 4 shows the lactate concentration over cultivation time in a mAb2-producing clone (see Example 2). Cells were cultivated in a medium with a reduced Na^+/K^+ ratio according to the invention.

Fig. 5 shows the viable cell density as a function of cultivation time in a 300-L bioreactor with a CHO cell clone. Culture conditions included a temperature step (day 5) and two pH shifts due to pH regulation with a setpoint and a deadband (also see Example 2). Cells were cultivated in a medium with a reduced Na^+/K^+ ratio according to the invention.

Fig. 6 shows the product titer as function of cultivation time in a 300-L bioreactor with a CHO cell clone. The process combined a temperature with two pH shifts (see also Fig. 5 and Example 2). Cells were cultivated in a medium with a reduced Na^+/K^+ ratio according to the invention.

[Detailed Description of the Invention]

The cell culture media according to the present invention are used for growing mammalian cells, preferably CHO cells, HEK cells and SP2/0 cells, and for the production of recombinant polypeptides using such cells. CHO cells are especially preferred. The term cell culture medium refers to an aqueous solution of nutrients which can be used for growing cells over a prolonged period of time. Typically, cell culture media include the following components: A source of energy, which will be usually a carbohydrate compound, preferably glucose, amino acids, preferably the basic set of amino acids, including all essential amino acids, vitamins and/ or other organic compounds which are required at low concentrations, free fatty acids, and inorganic compounds including trace elements, inorganic salts, buffering compounds and nucleosides and bases.

The cell culture medium according to the present invention can be used in various cell culture processes. Cultivation of cells can be carried out in adherent culture, for instance in monolayer culture or preferably in suspension culture.

The use of cell culture media in the field of pharmaceutical industry, for instance for the production of therapeutically active recombinant polypeptides, does generally not allow the use of any material of biological origin due to safety and contamination issues. Therefore, the cell culture medium according to the present invention is preferably a serum- and/or protein-free medium. The term "serum- and/or protein-free medium" represents a fully chemically defined medium, containing no additives from animal source like tissue hydrolysates, e.g. fetal bovine serum or the like. Further, proteins, especially growth factors like insulin, transferrin or the like are also preferably not added to the cell culture according to the present invention. Preferably, the cell culture medium according to the present invention is also not supplemented with a hydrolysed protein source like soybean, wheat or rice peptone or yeast hydrolysate or the like.

The osmolality and pH of the media are adjusted to values that allow the growth of the cells, e.g. at values between about pH 6.8 and about pH 7.2. The osmolality of the media at the beginning of culturing is typically between about 280 and about 365 mOsm, but may also gradually increase during culturing and the addition of feeding solutions to values of less than or about 600 mOsm/kg. Preferably the media according to the present invention have an initial osmolality of between about 285 and about 365 mOsm/kg.

The temperature of the cell culture is selected in a range where cells are viable and grow. A typical temperature for cell culturing is in the range between about 30 °C and about 38 °C. For instance, the cells are initially grown at temperatures of about 36 to about 37 °C which is optimal for CHO cells. However, the exact temperature can be adapted to the needs of the cells and also changed during culturing to allow their optimal viability, growth or production.

The first aspect of the invention is related to the ionic balance between sodium and potassium ions in the cell culture. The present invention describes a molar ratio of sodium to potassium ions which is between about 10 to 1 and about 1 to 1. In further implementations of the invention the ratio is selected between about 9 to 1 and about 5 to 1. Alternatively the ratio is between about 8 to 1 and about 6 to 1.

The concentration of the sodium and potassium ions and the respective ratio is here defined via their molar content. The concentration of sodium and potassium ions is determined by calculating the total number of these ions in the growth medium, after the respective salts have been added and dissolved in the medium solution.

To reach the required sodium concentration usually different salts are added to the medium. Commonly used sodium salts are e.g. NaCl, mono- or dibasic sodium phosphate salts, sodium carbonate, sodium citrate, trace ions such e.g. sodium selenite, but are not limited to these examples. Also the base sodium hydroxide (NaOH) that may be added to the media for pH adjustment contributes to the total content of sodium ions. The term ion in this respect refers to the dissociated state. Calculating the molar content of ions thus means taking into account the valence of the ions. 1 mM sodium chloride (NaCl) added to a medium would therefore contribute 1 mM of sodium ions, while 1 mM dibasic sodium phosphate (Na_2HPO_4) would accordingly contribute 2 mM of sodium ions. According to the invention the sodium concentration used in the media is between about 50 and 90 mM. Alternatively the sodium ion concentration is selected to be about 65 to about 85 mM.

The potassium salt that is used for media typically is KCl but also includes e.g. K_2SO_4 or potassium dihydyrogen phosphate (KH_2PO_4). The potassium salt is not limited to these particular examples. Alternatively, the potassium concentration used in the media is between about 8 and about 12 mM, or about 10.7 mM.

Table 1 shows examples for media that are traditionally used for the growth of mammalian cells. These classic media such as DMEM, DMEM/F12, BGJ and others have a particularly high ratio of Na/K ions. The media according to the present invention are characterized by a particularly low Na/K ratio of less than about 10 to 1 (see Table 2). The media according to the present invention are suitable for culturing CHO and other mammalian cells and show an improved growth of the cells and/or allow for an improved production of polypeptides. Two examples for such media are shown in Table 3, which depicts the composition of two media examples and how a low Na/K ratio can be achieved. Synergistic effects between the low ratio of sodium to potassium ions and the other media features have even an additional advantageous effect on growth of cells and production of recombinant proteins.

In addition to the particular concentration of sodium and potassium ions and their specific ratio, the present media are also characterized by a particularly low concentration of sodium chloride (NaCl) that is added to the mixture of media components. Preferably concentrations of about 7 to about 15 mM are used.. This low amount of sodium chloride is unusual. In some implementations of the present invention, the sodium chloride concentration (in mM) is even lower than the concentration (in mM) of the respective potassium salt that is added. Furthermore, media according to the present invention often show a low total content of chloride ion. As shown by the examples of Table 2, this results in initial chloride concentrations of between about 36 and 46 mM. Mostly inorganic salts such as NaCl or $CaCl_2$ contribute to this value, but also media components such as choline chloride, or amino acids such as for example L-histidine hydrochloride or L-lysine hydrochloride may add on to the total concentration.

A further advantage of the media compositions according to the present invention is the combination of a low molar Na/K ratio together with a starting concentration of amino acids that ranges between about 40 mM, alternatively about 50 mM and about 100 mM. Classical media use comparatively low concentrations of amino

acids and/or high Na/K ratios. The combination of both features may provide additional effects that are advantageous for the growth of the cells and the production of polypeptides.

Table 2 shows different implementations of these parameters according to the invention. In addition to their total amino acid content suitable media according to the present invention optimized for cell growth preferably contain initial amino acid concentrations according to the following ranges.

Amino acids	Conc. (mmol/L)
Arginine, free base	4.0 – 6.0, preferably 4.5 – 5.5
Asparagine monohydrate	3.0 – 6.0, preferably 4.0 – 5.5
Aspartic acid	2.5 – 4.0, preferably 3.0 – 3.6
Glycine	0.3 – 0.8, preferably 0.5 – 0.7
Histidine, HCl H_2O	0.6 – 1.0, preferably 0.7 – 0.9
Isoleucine	2.0 – 5.0, preferably 2.9 – 4.0
Leucine	3.0 – 7.0, preferably 3.5 – 6.0
Lysine HCl	2.0 – 4.0, preferably 2.5 – 3.5
Methionine	1.0 – 1.5, preferably 1.2 – 1.4
Phenylalanine	1.0 – 2.0, preferably 1.3 – 1.8
Proline	2.5 – 6.0, preferably 3.0 – 5.5
Serine	3.0 – 8.0, preferably 4.0 – 7.0
Threonine	2.0 – 3.5, preferably 2.5 – 3.1
Tryptophane	0.4 – 1.0, preferably 0.5 – 0.8
Valine	2.5 – 5.0, preferably 3.0 – 4.5
Tyrosine	1.0 – 2.0, preferably 1.2 – 1.8
Cystine	0.5 – 1.0, preferably 0.6 – 0.9
Glutamine	5.5 – 9.5, preferably 6.2 – 8.2

The media of the present invention further specified by amino acids as defined in the above table can be favourably used in the improved cell culture processes according to the present invention.

In a particularly preferred embodiment media according to the present invention are optimized for production and preferably contain initial amino acid concentrations according to the following ranges.

Amino acids	Conc. (mmol/L)
Arginine, free base	4.0 – 6.0, preferably 4.5 – 5.5
Asparagine monohydrate	9.0 – 11.0, preferably 9.5 – 10.5
Aspartic acid	2.5 – 4.0, preferably 3.0 – 3.6
Glycine	0.3 – 0.8, preferably 0.5 – 0.7
Histidine, HCl H_2O	1.0 – 1.5, preferably 1.1 – 1.3
Isoleucine	5.5 – 7.0, preferably 6.0 – 6.9
Leucine	8.0 – 10.0, preferably 9 – 9.2
Lysine HCl	3.0 – 6.0, preferably 4.0 – 5.0
Methionine	1.5 – 2.5, preferably 1.5 – 2.0
Phenylalanine	2.0 – 3.5, preferably 2.5 – 3.0
Proline	7.5 – 9.0, preferably 8.0 – 8.5
Serine	10.5 – 13.0, preferably 11.0 – 11.9
Threonine	3.5 – 5.5, preferably 4.0 – 5.0
Tryptophane	0.9 – 2.0 preferably 1.0 – 1.4
Valine	5.5 – 7.5, preferably 6.0 – 6.8
Tyrosine	1.0 – 3.0, preferably 2.0 – 2.5
Cystine	0.5 – 2.0, preferably 1.0 – 1.3
Glutamine	5.5 – 9.5, preferably 6.2 – 8.2
Glutamic acid	0.5 – 2.5, preferably 1.0 – 1.2

Production media containing amino acids as defined in the above table can be favourably used in the improved cell culture processes according to the present invention.

In a further aspect of the invention, in addition to the specific ratio between sodium and potassium ions, also the overall balance between total ion concentrations (contributing to the overall ionic strength of the medium), and the amino acids in the medium is important. The ratio between the total ion concentration and the amino acids in the nutrient growth medium is mostly dominated by bulk inorganic salts such as e.g. sodium chloride, potassium chloride, sodium hydrogen carbonate, and others, that are major ingredients of most types of cell culture media for animal cells. Also salts of trace ions, amino acids or vitamins contribute to this value (e.g. cupric sulphate, L-arginine hydrochloride, L-histidine hydrochloride, choline chloride, D-calcium pantothenate and others). It may be advantageous for the cells to also adjust the concentrations of these ions. We therefore define the total concentration of ions here as the sum of all major organic

and inorganic salts added to the medium which are ionizable in an aqueous medium solution plus the base NaOH and acid HCl. Trace elements are not included. Thus, 1 mM of NaCl, NaOH or the organic salts such as Lysine-HCl or choline chloride would contribute each 2 mM of ions. 1 mM of $MgCl_2$ would accordingly add 3 mM of ions while 1 mM of the organic salt trisodium citrate contributes with 4 mM.

According to the present invention the molar ratio between ions and amino acids is thus selected to be between about 1.9 and 4. In some implementation the ratio is selected to range between about 2.0 and 3.9. These particular low ratios are not only reached by a relatively high content of amino acids but also by a relatively low molar content of ions in the medium. The content of ions in the medium generally is less than 250 mM. For instance, the values are selected to be between about 150 and 220 mM, or alternatively between about 170 and 200 mM.

In summary, the described particular media features have important effects on cellular metabolism and physiology while at the same time also affecting general parameters such as osmolality or e.g. availability of nutritional components. The balance of the different media features thus lead to unique properties that lead to unexpected synergistic effects for the cells.

The media with a low Na/K ratio are generally suitable for growth of different mammalian cells and the manufacturing of recombinant polypeptides/proteins in large scale production. The polypeptides and proteins as used herein refer to recombinant polypeptides that are expressed by the respective mammalian cell after transfection of the cells with the DNA construct or constructs coding for the product of interest. Any polypeptide that can be expressed in a host cell may be produced according with the present invention. After the polypeptide(s) has/have been produced by the process of the present invention it is either extracellularly secreted, bound to the cells or remains in the cells, depending on the specific product and cell line used. The polypeptide product can be recovered from culture supernatant directly or after lysis of the cells by standard procedures. Also further

isolation and purification is done by the standard techniques known to the skilled person. The polypeptide of the invention can also be included in a pharmaceutical composition.

Another aspect of the invention relates to a process for the production of a recombinant polypeptide comprising culturing mammalian cells in a medium according to the present invention wherein the culturing conditions comprise at least one temperature shift and/or at least one pH shift.

Accordingly, in another aspect of the invention it may be advantageous to change the temperature during the course of culturing and include one or more temperature shifts that are initiated at certain time points. A change/shift in the temperature does not refer to spontaneous fluctuations in the temperature, but to changes in temperature of at least 1 °C or alternatively at least 2 °C that are intended, and where the second temperature is being maintained for at least one day. A change / shift can be implemented by altering the temperature setpoint of the culture. The timing is dependent on either the growth state of the culture, a predetermined number of days after the start of the culture or the metabolic needs of the cells. Thus, the temperature may be shifted in a period of about 1 to 10 days after starting the culture. Preferably a temperature shift is done during the growth phase of the cells or towards the end of this phase. Depending on the culture vessel volume, the change may occur rapidly or more slowly and lasts several hours. In one example such a shift in temperature is implemented during the growth phase of the culture when the density is between about 40 and about 90% of the maximal density. In one example the first temperature is between about 33 and about 38 °C, while in other examples the first temperature is between about 36 and about 38 °C. The second temperature is between about 30 and about 37°C, or alternatively between about 32 and about 34 °C.

In another aspect of the present invention it may be advantageous to change the pH during the course of the culturing by including one or more pH shifts. In further aspects of the invention shifts in temperature may also be combined with one or

more shifts in the pH. While the first pH (e.g. pH of 7.0) is chosen to be favourable for rapid expansion of the cells it is advantageous to modify the pH of the culture, once a certain cell density is reached. This change or shift in pH is accomplished by changing the pH setpoint of the bioreactor/culture vessel or by defining a pH setpoint in combination with a deadband. A pH change does not refer to small fluctuations in the pH, it rather refers to an intended change. The second pH value (e.g. 6.8) is selected to reduce cell death and to allow high cell-specific production rates of polypeptides of adequate quality. The second pH can be maintained until the end of culturing or additional pH shifts may be introduced. In one implementation it may be useful to change the pH by at least 0.2. In one embodiment the first pH is selected to be in the range between pH 6.8 and 7.5. In another embodiment the first pH is selected to be in the range between pH 6.8 and 7.2. The second pH value that is reached after the shift in pH may be in the range between pH 6.0 and pH 7.5, or alternatively between 6.5 and 6.8.

The cell culture medium according to the present invention can be used in various cell culture processes. Cultivation of cells can be carried out in adherent culture, for instance in monolayer culture or preferably in suspension culture.

Large scale cultivation of cells can be used for instance by the various fermentation processes established in industrial biotechnology. Continuous and discontinuous cell culture processes can be utilized using the cell culture media according to the present invention. Other known reactor technologies, e.g. perfusion technologies or the like can be also utilized. Batch processes are one preferred embodiment.

The batch cell culture includes fed-batch culture or simple batch culture. The term "fed batch cell culture" refers to cell culture wherein mammalian cells and cell culture medium are supplied to the culturing vessel initially and additional culture nutrients are fed continuously or in discrete increments to the culture during the culturing process with or without periodic cell and/or product harvest before termination of the culture. The term "simple batch culture" relates to a procedure in

which all components for cell culturing including the mammalian cells and the cell culture medium are supplied to the culturing vessel at the start of the culturing process.

According to one preferred embodiment of the present invention feeding of the cultures is done in a fed batch process. Such feeding is beneficial for the cells to replace media components and nutrients that are depleted in the media during the culture process. Typically feed solutions comprise amino acids, at least one carbohydrate as an energy source, trace elements, vitamins or specific ions. The feed solutions are added depending on the needs of the cells, which are either based on a predetermined schedule that has been determined for the particular cell line or cell clone and product or measured during the culture process. It is particularly advantageous to use concentrated feed solutions in order to avoid large volume increase and dilution of the media. In some embodiments it may also be useful to have at least two different feed solutions. This allows independent dosing of two or more different groups of nutrients and components to the cells and thus a better adjustment of the feeding conditions concerning optimal supply of certain nutrients.

In a further embodiment of the invention, one of the two feed solutions added to the cell culture medium is a feed comprising the dipeptide cystine and the amino acid tyrosine. Preferably, the feed contains the dipeptide cystine and the amino acid tyrosine at respective concentrations in the range of about 6.5 g/l and about 8.0 g/l and in the range of about 9 g/l and about 11 g/l in an aqueous solution at a basic pH of above 10. In a particular embodiment, the concentrated feed comprises the dipeptide cystine and the amino acid tyrosine at respective concentrations of 10.06 g/l L-tyrosine and 7.25 g/l cystine at a pH of above 10.

The feed medium comprising cystine and tyrosine as described above can be added either based on the measured consumption of the respective amino acids or according to a fixed schedule at e.g. about 0.2 to about 0.8 wt% of the initial cell

culture medium weight per day, preferably at about 0.4 wt% of the initial cell culture medium weight per day.

In some examples the other feed solution contains all other amino acids that are also present in the basic medium except tyrosine and cystine. In some examples this additional feed solution may consist of particular selected components such as e.g. amino acids or carbohydrates. In a further preferred embodiment of the invention this concentrated feed medium preferably contains selected amino acids according to the following concentration ranges.

Amino acids	**Feed Medium** Conc. (mmol/L)
Arginine, free base	12.0 – 17, preferably 13.5 – 16.0
Histidine, HCl H_2O	5.5 – 7.5, preferably 5.9 – 7.0
Isoleucine	21 – 28.0, preferably 22.0 – 27
Leucine	32 – 42, preferably 34.5 – 40.0
Lysine HCl	17.0 – 22.0, preferably 17.5 – 21.5
Methionine	5.5 – 8.0, preferably 6.0 – 7.5
Phenylalanine	8.5 – 12.0, preferably 9.0 – 10.5
Proline	18.0 – 24, preferably 18.5 – 22.0
Serine	39.0 – 49.0, preferably 39.5 – 46.5
Threonine	14.5 – 19.0, preferably 15.0 – 18.5
Tryptophane	3.0 – 5.0, preferably 3.5 – 4.9
Valine	23.0 – 29.0, preferably 23.8 – 27.5
Glutamine	175.0 – 220.0, preferably 176.0 – 201

Preferably also carbohydrates such as glucose are added to this concentrated feed medium, preferred concentrations being between about 1200 and about 1400 mmol/l, or alternatively between about 1300 and about 1395 mmol/l.

The feed medium as just described, preferably including a carbohydrate, such as glucose, can be added either based on the measured consumption of the respective amino acids or according to a fixed schedule at e.g. about 1 to about 4 wt% of the initial cell culture medium weight per day, preferably at about 2 wt% of the initial cell culture medium weight per day.

The cells cultivated in the cell culture medium according to the present invention include mammalian and non-mammalian cells. Non-mammalian cells include insect cells or the like. However, mammalian cells are preferred. The terms cell, cell line and cell culture may be interchangeably used herein.

Examples of mammalian cells include human retinoblasts; human cervical carcinoma cells, human embryonic kidney line, human lung cells, human liver cells, PER.C6 cells (a human retinoblast-derived cell line), human hepatoma line and human cell lines such as AGE1.HN; monkey kidney CV1 line transformed by SV40; monkey kidney cells, African green monkey kidney cells, Chinese hamster ovary cells/-DHFR, baby hamster kidney cells; mouse sertoli cells; mouse mammary tumor, canine kidney cells; buffalo rat liver cells; TRI cells; MRC 5 cells; FS4 cells; CHO cells are a preferred cell line for practicing the invention.

In one preferred embodiment of the invention these cells can be different strains of CHO cells, such as wild type CHO K1, CHO dhfr- (Dux1) or CHO dhfr- (DG44), but also HEK cells, Sp2/0 cells. These cells are typically transfected with one or more DNA constructs that encode the polypeptide(s) of interests. Any polypeptide that can be expressed in these host cells may be produced according with the present invention.

Another class of cells that can be used with the cell culture media according to the present invention include hybridoma cells which are commonly used for the production of monoclonal or polyclonal antibodies.

The polypeptides that can be produced from the cell cultures and the cell culture media according to the present invention are not limited. The polypeptides can be recombinant or not recombinant. The term "polypeptide" as used herein encompasses molecules composed of a chain of more than two amino acids joined by peptide bonds; molecules containing two or more such chains; molecules comprising one or more such chains being additionally modified, e.g. by glycosylation. The term polypeptide is intended to encompass proteins.

The preferred class of polypeptides produced by cell cultures and the cell culture media according to the present invention are recombinant antibodies.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), nanobodies modified antibodies, subunits of antibodies, antibody derivatives, artificial antibodies, combinations of antibodies with proteins and antibody fragments sufficiently long to display the desired biological activity. The monoclonal antibodies as used herein may be human antibodies.

However, polypeptides other than antibodies can also be produced using cell cultures and the cell culture media according to the present invention, e.g. polypeptides like transmembrane proteins, receptors, hormones, growth factors, proteases, clotting and anti-clotting proteins, inhibitor proteins, interleukins, transport factors, fusion proteins and the like.

The products obtained from such cell culture processes can be used for the preparation of pharmaceutical preparations. The term "pharmaceutical preparation" indicates a composition suitable or adapted to for administration to a mammal, especially a human. In addition, the protein(s) according to the invention can be administered together with other components of biologically active agents such as pharmaceutically acceptable surfactants, recipients, carriers, diluents and vehicles.

Table 1 summarizes compositions of commercially available media and additional cell culture media from the prior art: values are based on published values; additions such as NaOH are thus not included in the values. Sodium concentration or the sodium to potassium ratio is thus rather underestimated and even further apart from the values of the present invention.

Table 1

Media	Na+ (mM)	NaCl (mM)	K+ (mM)	Ratio Na/K	Cl- (mM)	Total Ions (mM)	Total AA (mM)	Ratio Ions/AA
Commercial media								
DMEM/F12	123	120.6	4.2	29	129.1	290	7.0	41.4
Ham's F12	147.0	130.1	3.0	49	135.0	305	4.0	76.2
MEM alpha	145.4	117.2	5.3	27	127.0	310.2	9.0	34.5
RPMI 1640	138.7	103.4	5.3	26	109.0	283.6	6.6	42.9
BME	144.4	117.2	5.3	27	126.4	307.1	1.4	219
Fischer's	152.3	136.8	5.3	28	144.9	317	5.6	56.6
Other media								
US 7294484	48/125/86	19/95/64	4.2	12/30/21	not determined	ND	> 70	ND
WO 02/101019 Media A/B	10.9/8.1	0/0	20.2/19.9	0.5/0.4	48/31	122.8/78.9	78.1/59.5	1.6/1.3

Table 2 discloses cell culture media formulations according to the present invention characterized by their particular low ratio of sodium to potassium ions.

Table 2

Media	Na+ (mM)	NaCl (mM)	K+ (mM)	Ratio Na/K	Cl- (mM)	Total Ions (mM)	Total AA (mM)	Ratio Ions/AA
Medium 1	83.6	14.6	10.7	7.8	36.2	195.7	51.1	3.8
Medium 2	69.5	8.6	10.7	6.5	45.5	191.2	90.5	2.1
Medium 3	79.7	8.6	10.7	7.4	41.4	190.6	90.7	2.1
Medium 4	73.4	8.6	10.7	6.9	39.9	177.5	80.2	2.2
Medium 5	67.2	8.6	10.7	6.3	38.3	164.3	69.7	2.4
Medium 6	60.9	8.6	10.7	5.7	36.8	151.1	59.2	2.6

Table 3 discloses the compositions of examples for chemically defined cell culture media according to the present invention. The individual components of these cell culture media are available from standard commercial sources.

Table 3

Components	Medium 1 Final conc. (mg/l)	Medium 2 Final conc. (mg/l)	Medium 3 Final conc. (mg/l)	Medium 4 Final conc. (mg/l)	Medium 5 Final conc. (mg/l)	Medium 6 Final conc. (mg/l)
$CaCl_2$, anhydr.	131	133.2	130.6	130.6	130.6	130.6
KCl, anhydr.	800	800	800	800	800	800
$MgCl_2$, anhydr.	155	250.4	250.2	226.4	202.6	178.9
NaCl	850.6	500	500	500	500	500
Di-Sodium hydrogenphosphate, anhydr.	710	1065	1775	1508.8	1242.5	976.3
Sodium hydrogen carbonate, anhydr.	2500	2000	2000	2000	2000	2000
L-Arginine, free base	871	871	-	-	-	-
L-Arginin x HCl	-	-	1053	1053	1053	1053
L-Asparagine, H_2O	616	1501	1501	1279.8	1058.5	837.3
L-Aspartic acid	461	461	461	461	461	461
L-Cystine	200.1	304.5	304.5	228.4	152.3	76.1
L-Glutamic acid Na salt Hydrate	-	182	-	-	-	-
L-Glutamic acid	-	-	182	136.5	91	45.5
L-Histidine, HCl-H_2O	168	268	268	243	218	193
L-Isoleucine	394	894	894	769	644	519
L-Leucine	499	1199	1199	1025	850	675
L-Lysine, HCl	621	821	821	772	722	672
L-Methionine	179	279	279	255	230	205
L-Phenylalanine	264	464	464	414	364	314
L-Proline	368	968	968	818	668	518
L-Serine	432	1232	1232	1032	832	632
L-Threonin	333	533	533	484	434	384
L-Tryptophan	102	252	252	214.5	177	139.5
L-Valine	375	775	775	676	576	476
L-Tyrosine	277.7	422.5	422.5	316.9	211.3	105.6
Glycine	38	38	38	38	38	38
L-Glutamine	1169.2	1169.2	1169.2	1169.2	1169.2	1169.2
Biotin	0.4	0.4	0.4	0.4	0.4	0.4
D-Ca- Pantothenate	4	4	4	4	4	4
Folic acid	5	5	5	5	5	5
myo-Inositol	40	140	140	115	90	65
Nicotinamide	4	4	4	4	4	4
Pyridoxine, HCl	2	2	2	2	2	2
Riboflavin	0.4	0.4	0.4	0.4	0.4	0.4
Vitamine B12	2	2	2	2	2	2
Thiamine, HCl	4	4	4	4	4	4
Putrescine, 2HCl	10	110	110	85	60	35

Cholin chloride	40	240	240	190	140	90
Sodium selenit (Na_2SeO_3)	0.03	0.03	-	-	-	-
Sodium selenit Heptahydr. (Na_2SeO_3x $5H_2O$)	-	-	0.02	0.02	0.02	0.02
Manganese chloride tetrahydrate	0.3	0.3	0.3	0.3	0.3	0.3
Ammonium molybdate tetrahydrate	0.3	0.3	0.3	0.3	0.3	0.3
Zinc chlorid, anhydr.	3	3	3	3	3	3
Cupric chloride dihydrate	0.3	0.3	0.3	0.3	0.3	0.3
Cobalt chloride hexahydrate	0.3	0.3	0.3	0.3	0.3	0.3
Ethanolamine	10	100	100	77.5	55	32.5
Monothioglycerol	2	-	-	-	-	-
HEPES, acid form	17870	4766	4766	3574.5	2383	1191.5
Tri-Sodiumcitrate dihydrate	911.7	1235.2	911.2	911.2	911.2	911.2
$FeCl_3$x$6H_2O$	54.1	54.1	54.1	54.1	54.1	54.1
Pluronic F68	1000	1000	1000	1000	1000	1000
D-Glucose, anhydr.	10000	10000	10000	10000	10000	10000
HCl	-	327.6	-	-	-	-
NaOH	799.2	339.9	519.9	419.9	319.9	220

Examples

In the examples described below, chemically defined cell culture media 1 and 2 having the composition as detailed in Table 3 above are used. The individual components of these cell culture media are available from standard commercial sources.

Table 4 below shows the composition of a concentrated feed medium containing L-tyrosine and cystine. The feed medium can be added either based on the measured consumption of the respective amino acids or according to a fixed schedule at e.g. 0.4% wt per day.

Table 4

Components	Feed Medium (g/l)
NaOH 32%	18.7 mL
L-Tyrosine	10.06
Cystine	7.25

Table 5 below shows the composition of an exemplary concentrated feed medium. The feed medium can be added either based on the measured consumption of amino acids or according to a fixed schedule at e.g. 2% wt per day.

Table 5

Components	Feed Medium (g/l)
L-Arginine, free base	2.72
L-Histidine, HCl-H_2O	1.44
L-Isoleucine	3.44
L-Leucine	5.20
L-Lysine, HCl	3.72
L-Methionine	1.08
L-Phenylalanine	1.72
L-Proline	2.44
L-Serine	4.76
L-Threonin	2.08
L-Tryptophan	0.88
L-Valine	3.16
L-Glutamine	29.23
D-Glucose-monohydrate	275.00
25 % HCl	8.25 ml
32 % NaOH	5.6 ml

For the experiments of the examples a parental CHO cell line is used which is derived from the dhfr (+) CHO-K1 cell line ATCC CCL-61 (Kao et. al., Genetics, 1967, 55, 513-524; Kao et. al., PNAS, 1968, 60, 1275-1281; Puck et. al., J. Exp. Med., 1958, 108, 945-959) by adaptation to serum-free, protein-free media conditions. Two aliquots of this parental cell line are transfected to express two different monoclonal antibodies mAB1 and mAB2, respectively.

Example 1

In Example 1, two shake flask cultures containing medium 1 are inoculated in parallel with a mAb1-producing CHO clone. The shake flask cultures are incubated in a carbon dioxide incubator at 37°C. On day 3, one shake flask is transferred to a carbon dioxide incubator set at 33°C. Both shake flasks are similarly fed with two

feed solutions. Feed was supplemented according to a fixed schedule, with addition of 0.4 % of the first feed solution (Table 2) and 2 % of the second feed (Table 3) per day starting on day 5 and lasting until the end of the culture.

The temperature shift to 33°C enables longer maintenance of the viable cell density and viability of the culture with time (Fig. 1 and 2) and the achievement of a higher product titer (Fig. 3), compared to the culture that is maintained at 37°C for the whole duration of the experiment. This example illustrates the benefit of implementing a temperature shift to 33°C during a cell culture production process based on a CHO cell line.

Example 2

In this example, a 300-L bioreactor containing medium 2 is inoculated with a mAb2-producing CHO clone. On day 5, the temperature of the bioreactor is shifted from 36.5°C to 33°C. The pH setpoint is 6.90 and the deadband is 0.10. As a result, the culture starts at pH 7.00, the pH drifts down to 6.80 between day 2 and day 4, and then progressively returns to 7.00 due to lactic acid consumption by the cells (Fig. 4). The shift to pH 6.80 enables to reduce the addition of base compared to a scenario with a constant pH 7.00. The return to pH 7.00 enables to reduce the concentration of CO_2 in the medium compared to a scenario where the pH is left at 6.80 after the first shift. In this process that combines temperature and pH shifts, a high viable cell density is reached and the decrease in viable cell density over time is minimized (Fig. 5), allowing to reach on day 14 a high titer (Fig. 6) of product of adequate quality. Feeding is applied similarly as in Example 1.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A serum-free cell culture medium for growing mammalian cells which is characterized by a molar ratio of sodium to potassium ions of between about 10 to 1 and about 1 to 1.

2. The cell culture medium of claim 1 wherein the medium is a protein-free medium.

3. The cell culture medium of claim 1 or 2 wherein the molar ratio of sodium to potassium ions is between about 8 to 1 and about 6 to 1.

4. The cell culture medium of any one of claims 1 to 3 wherein the concentration of sodium ions is between about 50 and about 90 mM.

5. The cell culture medium of any one of claims 1 to 4 wherein the concentration of potassium ions is between about 8 and about 12 mM.

6. The cell culture medium of any one of claims 1 to 5 wherein the total amino acid concentration is between about 40 and about 100 mM.

7. The cell culture medium of any one of claims 1 to 6 wherein the molar ratio between the total ion concentration to the total amino acid concentration is between about 1.9 and about 4.

8. A process for the production of a recombinant polypeptide comprising culturing mammalian cells in a medium as defined in any one of claims 1 to 7 and expressing the recombinant polypeptide.

9. The process according to claim 8 wherein the culturing conditions comprise at least one temperature shift and/or at least one pH shift.

10. The process according to claim 8 or 9, wherein culturing is done by a fed batch process.

11. The process according to any one of claims 8 to 10 wherein the produced polypeptides are glycosylated.

12. The process according to any one of claims 8 to 11, wherein the polypeptide is an antibody or antibody fragment.

13. The process according to any one of claims 8 to 12, wherein the mammalian cells are selected from the group consisting of CHO cells, HEK cells and SP2/0 cells.

14. A process for the production of a cell culture medium according to claim 1 wherein the different components are admixed with each other.

15. The process according to claim 14 wherein sodium chloride is added at a concentration between about 7 and about 15 mM.

16. The process according to claim 14 or 15 wherein potassium chloride is added at a concentration between about 8 and about 12 mM.