effect could have been due to an increase in osmolarity or the acetate. The medium used was RPMI 1640 medium with 10 % serum, comprising > 100 mM sodium and hence the culture was not a serum-free culture and performed in a molar excess of sodium over potassium.

[011] Yoon et al., (Biotechnology and Bioprocess Engineering (2007), 12: 399-403) discloses that high potassium ion concentrations (≥ 60 mM) increase protein production and inhibits cell growth in CHO cells in a repeated batch mode using serum-free culture medium. Potassium concentrations up to 40 mM were reported not to influence cell growth significantly and concentrations at 60 mM or higher were shown to affect cell viability. Also this document is silent on the sodium concentration.

Potassium and sodium are present in all cell culture media and altering their concentration is advantageous over having to introduce other additives, since there are no concerns regarding safety and clearance. Also when developing a culture media it is desired to keep the medium as simple as possible and limited to the essential compounds. This avoids unnecessary compounds and reduces osmolarity issues. While culture media are constantly altered and improved, there is still a need to improve perfusion culture by reducing waste and increasing productivity.

SUMMARY

[012] A wasteful cell bleed can be utilized to maintain a sustainable VCD and preserve viability. In continuous processes a large proportion of the culture medium and hence product is lost due to the cell “bleed” which siphons off proliferating cells and medium in order to maintain a constant, sustainable viable cell density within the bioreactor. Up to one-third of harvestable material can be lost due to cell bleed. Using a cell bleed therefore decreases the product yield per run as the product within the cell bleed is not harvested. In order to decrease the cell bleed volume and thus retain more supernatant for harvest it is therefore advantageous to inhibit cell proliferation once the desired viable cell density is reached in the production phase without affecting viability or specific protein productivity. By reducing or eliminating cell bleed, protein production is increased as compared to conventional perfusion methods. Thus, there is a need for controlling cell growth once an optimal VCD has been obtained, thus minimizing cell bleed to increase the product recovered per perfusion run, and at the same time to increase the cell specific productivity to generate a more efficient method for operating perfusion processes.

[013] In the present disclosure a perfusion culture medium comprising sodium and potassium at a Na:K ratio of less than 1 and a potassium concentration of > 30 mM and methods of culturing mammalian cells using said medium are provided. It is shown that the medium inhibits proliferation and increases cell specific productivity (qp) without negatively affecting viability. In particular a high potassium concentration induced cell growth arrest and a low sodium concentration increases cell specific productivity of Chinese hamster ovary (CHO) in perfusion cell culture. By controlling cell growth and thus minimizing cell bleed,

the product recovered per perfusion run is greatly increased, generating a more efficient method for operating perfusion processes. This is further enhanced by the positive effect on cell specific productivity.

[014] In one aspect a method of culturing mammalian cells expressing a heterologous protein in a perfusion cell culture is provided comprising: (a) inoculating mammalian cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the mammalian cells during growth phase by perfusion with a serum-free perfusion medium; and (c) culturing the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising potassium ions at a concentration of 30 mM to 250 mM and a molar ratio of sodium to potassium ions of less than 1, wherein step (b) is optional.

[015] In one related aspect a method of reducing cell bleeding in a perfusion cell culture expressing a heterologous protein in a perfusion cell culture is provided comprising: (a) inoculating mammalian cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the mammalian cells during growth phase by perfusion with a serum-free perfusion medium; and (c) culturing the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising potassium ions at a concentration of 30 mM to 250 mM and a molar ratio of sodium to potassium ions of less than 1, wherein step (b) is optional.

[016] In another related aspect a method of increasing protein production in a perfusion cell culture expressing a heterologous protein comprising: (a) inoculating mammalian cells expressing a heterologous protein in a serum-free culture medium; (b) culturing the mammalian cells during growth phase by perfusion with a serum-free perfusion medium; and (c) culturing the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising potassium ions at a concentration of 30 mM to 250 mM and a molar ratio of sodium to potassium ions of less than 1, wherein step (b) is optional.

[017] Step (c) is started once the target cell density is reached. Thus, it may be started at a cell density of 10 x 10^6 cells/ml to about 120 x 10^6 cells/ml or even higher. Preferably step (c) is initiated at a cell density of at least 10 x 10^6 cells/ml, at least 20 x 10^6 cells/ml, at least 30 x 10^6 cells/ml, at least 40 x 10^6 cells/ml or at least 50 x 10^6 cells/ml. Most preferably step (c) is initiated at a cell density of about 30 to about 50 x 10^6 cells/ml.

[018] Step (c) of the methods of the disclosure may further comprise maintaining a cell density by cell bleeding. Using the methods of the present disclosure the cell bleeding is reduced compared to a perfusion cell culture using the same serum-free perfusion medium comprising potassium ions at a concentration of less than 30 mM and a molar ratio of sodium to potassium of more than 2 and cultured under the same conditions.

[019] In an example of the methods of the disclosure the potassium ion concentration is about 40 mM to about 200 mM, preferably about 60 mM to about 150 mM and more preferably about 80 mM to

about 100 mM. In an example the molar ratio of sodium to potassium is between about 0.9 and 0.1, between about 0.8 and 0.2, between about 0.7 and 0.2, preferably between about 0.6 and 0.3 and more preferably between about 0.5 and 0.4.

[020] According to the present disclosure the potassium ion is provided as one or more potassium salt. In an example the one or more potassium salt is selected from the group consisting of potassium bicarbonate, potassium chloride, potassium hydroxide, L-tyrosine dipotassium salt, potassium phosphate dibasic, potassium phosphate monobasic, potassium selenite, potassium pyruvic acid, potassium glutathione, potassium D-gluconate, potassium succinate and potassium ascorbate. Preferably the one or more potassium salt substitutes the corresponding sodium salt in the serum-free perfusion culture medium. More specifically the one or more potassium salt preferably substitutes the corresponding sodium salt in the serum-free perfusion medium of step b).

[021] The osmolarity of the serum-free perfusion medium should be in the range of between 300 and 1400 mOsmol/kg, preferably between 300 and 500 mOsmol/kg, more preferably between 330 and 450 mOsmol/kg and even more preferably between 360 and 390 mOsmol/kg.

[022] The mammalian cell may be a Chinese hamster ovary (CHO) cell, preferably a CHO-DG44 cell, a CHO-K1 cell, a CHO DXB11 cell, a CHO-S cell, a CHO GS deficient cell or a derivative of any of these cells.

[023] The serum-free perfusion medium may be chemically defined and/or hydrolysate free. Preferably the serum-free perfusion medium is protein-free or protein-free except for recombinant insulin and/or insulin-like growth factor, more preferably the serum-free perfusion medium is chemically defined and protein-free or protein-free except for recombinant insulin and/or insulin-like growth factor.

[024] In a further aspect a method of producing a therapeutic protein using the methods of the disclosure is provided. The method optionally comprises a further step of purifying and formulating the therapeutic protein into a pharmaceutically acceptable formulation.

[025] In another aspect a serum-free perfusion medium is provided comprising potassium ions at a concentration of 30 mM to 250 mM and a molar ratio of sodium to potassium ions of less than 1. In an example, the potassium ion concentration is about 40 mM to about 200 mM, preferably about 60 mM to about 150 mM and more preferably about 80 mM to about 100 mM. In an example, the molar ratio of sodium to potassium is between about 0.9 and 0.1, between about 0.8 and 0.2, between about 0.7 and 0.2, preferably between about 0.6 and 0.3 and more preferably between about 0.5 and 0.4.

[026] According to the present disclosure the potassium ion is provided as one or more potassium salt. In an example, the one or more potassium salt is selected from the group consisting of potassium bicarbonate, potassium chloride, potassium hydroxide, L-tyrosine dipotassium salt, potassium phosphate dibasic, potassium phosphate monobasic, potassium selenite, potassium pyruvic acid, potassium glutathione, potassium D-gluconate, potassium succinate and potassium ascorbate. Preferably the one or

more potassium salt substitutes the corresponding sodium salt in the serum-free perfusion culture medium.

[027] The osmolarity of the serum-free perfusion medium should be in the range of between 300 and 1400 mOsmol/kg, preferably between 300 and 500 mOsmol/kg, more preferably between 330 and 450 mOsmol/kg and even more preferably between 360 and 390 mOsmol/kg.

[028] The serum-free perfusion medium may be chemically defined and/or hydrolysate free. Preferably the serum-free perfusion medium is protein-free or protein-free except for recombinant insulin and/or insulin-like growth factor, more preferably the serum-free perfusion medium is chemically defined and protein-free or protein-free except for recombinant insulin and/or insulin-like growth factor.

[029] In yet another aspect a use of the serum-free perfusion medium of the disclosure for culturing mammalian cells in a perfusion culture during production phase or for reducing the total cell bleed volume in a perfusion culture is provided. In another aspect a use of the serum-free perfusion medium of the disclosure for increasing protein production in a perfusion cell culture is provided. In another aspect, a use of the serum-free perfusion medium of the disclosure for increasing cell specific protein production in a perfusion cell culture is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[030] Figure 1: Effect of cell bleed on total productivity. A CHO cell line producing monoclonal antibody mAb1 was cultured in 3L glass perfusion bioreactors at a 2L working volume utilizing two different recirculation devices as indicated: ATF#1 and ATF#2 were replicates using an ATF2 device (Repligen, Waltham, MA) and the TFF bioreactor used a centrifugal pump (Levitronix, Zurich, Switzerland). Titer was measured for the harvest (permeate) stream (recovered product) and bioreactor contents (reactor titer) daily and the total grams produced (total produced) and product lost in the bleed and the reactor at the end of cell culture (day 29) were calculate.

[031] Figure 2: Cell growth and productivity response to change in Na+/K+ concentrations in a deep-well model of perfusion. Degree of response is expressed as proportion of difference from the control condition Na=78 mM, K=37 mM (Na:K 2) where a score of 0.4 corresponds to an area where anticipated response at the indicated Na+ and K+ levels is 40% of the control (Na:K 2) condition. Contour plots of cumulative specific growth rate (SGR) (A) and cumulative cell specific productivity (qp) (B) are shown for the range of concentrations tested in a CHO cell line producing monoclonal antibody mAb1. Actual data points for conditions tested are shown as markers in both contour plots. Representative viable cell density (VCD) and viability plots are shown in panels (C) and (D) respectively for the entire time course of cell culture. The viable cell density (VCD) is given in e5 cells/mL, which is the technical spelling for "10^5 cells/mL". Differences between different conditions were observed in viable cell density over time while viability remained consistently high (>85%) throughout the entire 7 days of culture.

[032] Figure 3: Cell cycle analysis of a CHO cell line producing monoclonal antibody mAb1 treated with different Na+/K+ concentrations in a deep-well model of perfusion. Cell cycle analysis was performed