containing medium. Further, low concentrations of vitamin A were disclosed to be growth stimulating. However, iron concentrations are not discussed and no advantageous effect of vitamin A in cell culture is implied.

[007] Fischer et al. (The Journal of Cell biology (1981) 91: 373) report that retinol at concentrations of 10 to 50 µM had an inhibitory effect on growth and protein synthesis in CHO cultures and further that higher retinol concentrations were cytotoxic.

[008] Jetten AM, (Federation Proceedings (1984) 43(1):134-139) reports that retinoids can enhance or inhibit cell growth *in vitro*. Due to its growth promoting activity it is suggested to be included in tests to optimize cell culture conditions in serum-free medium. Inhibition of growth by retinoids was observed in the presence of serum, but retinoids in serum-fee medium containing transferrin, insulin and EFG enhanced proliferation.

[009] WO 2014/109858 A discloses the use of CDK4 inhibitor in cell culture such as batch, fed-batch and perfusion culture. A similar disclosure is found in Du et al. (Biotechnology and Bioengineering (2015) 112(1): 141-155). Du et al., further teaches that CDK4/6 inhibitors specifically inhibit the cell cycle without affecting other cellular targets. The application does not disclose vitamin A to improve cell culture performance.

[010] WO 2016/006479 A discloses that a low L-asparagine concentration of 5 mM or less arrests cell growth in mammalian cell cultures, particularly in perfusion cultures and results in increased protein production. However, vitamin A is not mentioned in the application.

[011] Vitamin A is a class of compounds comprising carotenoids and compounds having the biological activity of retinol (vitamin A alcohol). Of biological relevance are retinol and retinyl esters such as retinyl acetate and retinyl palmitate, which can be converted to the active metabolites retinal and retinoic acid *in vivo*. Vitamin A is classified as “generally regarded as safe” (GRAS) by the FDA and is routinely used in many chemically defined media. As such it has already been tested and hence no data regarding safety and clearance are required. 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. Thus, there is a need for controlling cell growth once an optimal VCD has been obtained and thus minimizing cell bleed to increase the product recovered per perfusion run and generating a more efficient method for operating perfusion processes.

[013] In the present disclosure a perfusion culture medium comprising a retinoid and Fe ions and methods of culturing mammalian cells using said medium are provided. It is shown that vitamin A induced cell growth arrest in the presence of Fe ions of perfusion Chinese hamster ovary (CHO) cell cultures. This medium can inhibit proliferation without negatively affecting viability and/or cell specific productivity (qP). 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.

[014] In one aspect a method of culturing mammalian cells expressing a heterologous protein in a perfusion cell culture is provided comprising: (a) culturing 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) maintaining the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein step (b) is optional. In one example of the method prior to step (c) the retinoid concentration in the mammalian cell culture is adjusted to the retinoid concentration in the serum-free perfusion medium of step (c), preferably by bolus addition.

[015] In a related aspect a method of reducing cell bleeding in a perfusion cell culture expressing a heterologous protein is provided comprising: (a) culturing 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) maintaining the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein step (b) is optional. In one example of the method prior to step (c) the retinoid concentration in the mammalian cell culture is adjusted to the retinoid concentration in the serum-free perfusion medium of step (c), preferably by bolus addition.

[016] In another related aspect a method of increasing protein production in a perfusion cell culture expressing a heterologous protein is provided comprising: (a) culturing 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) maintaining the mammalian cells during production phase by perfusion with a serum-free perfusion medium comprising Fe ions at a concentration of 230 µM to 3.5 mM and a retinoid at a concentration of 10 µM to 400 µM, wherein step (b) is optional. In one example of the method prior to step (c) the retinoid concentration in the mammalian cell culture is adjusted to the retinoid concentration in the serum-free perfusion medium of step (c), preferably by bolus addition.

[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/. 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 with Fe ions at a concentration of less than 230 µM and a retinoid at a concentration of less than 10 µM and cultured under the same conditions. In addition to the serum-free perfusion medium of step (c) also the serum-free perfusion medium in step (b), and optionally also the serum-free culture medium of step (a) may comprise Fe ions at a concentration of 230 µM to 3.5 mM.

[019] In some examples of the methods of the disclosure the retinoid concentration is about 60 µM to 400 µM, preferably about 100 µM to about 200 µM and more preferably about 150 µM to about 200 µM. In some examples the Fe ion concentration is about 350 µM to 3.5 mM, preferably about 1 mM to 3.5 mM, more preferably about 1.4 mM to 3.5 mM and even more preferably about 1.6 mM to 3.5 mM or the Fe ion concentration is about 350 µM to about 3.1 mM, preferably about 1 mM to about 3.1 mM, more preferably about 1.4 mM to about 3.1 mM and even more preferably about 1.6 mM to about 3.1 mM.

[020] The retinoid may be retinol, retinal, retinoic acid or a retinyl ester and is preferably retinol or a retinyl ester. The retinyl ester may be retinyl acetate or retinyl palmitate, preferably retinyl acetate. The Fe ion may be provided as $FeSO_4$, Fe-citrate, Fe-transferrin, iron choline citrate, Fe-EDTA, $Fe(NO_3)_3$, $FeCl_2$, $FeCl_3$, or combinations thereof, preferably as $FeSO_4$, Fe-citrate, $Fe(NO_3)_3$, Fe-transferrin, or combinations thereof, more preferably as Fe-citrate.

[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. This 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 Fe ions at a concentration ranging from 230 µM to 3.5 mM and a retinoid in a concentration ranging from 10 µM to 400 µM. In some examples the retinoid concentration is about 60 µM to 400 µM, preferably about 100 µM to about 200 µM and more preferably about 150 µM to about 200 µM. In some examples the Fe ion concentration is about 350 µM to 3.5 mM, preferably about 1 mM to 3.5 mM, more preferably about 1.4 mM to 3.5 mM and even more preferably about 1.6 mM to 3.5 mM or the Fe ion concentration is about 350 µM to about 3.1 mM, preferably about 1 mM to about 3.1 mM, more preferably about 1.4 mM to about 3.1 mM and even more preferably about 1.6 mM to about 3.1 mM.

[026] The retinoid may be retinol, retinal, retinoic acid or a retinyl ester and is preferably retinol or a retinyl ester. The retinyl ester may be retinyl acetate or retinyl palmitate, preferably retinyl acetate. The Fe ion may be provided as $FeSO_4$, Fe-citrate, Fe-transferrin, iron choline citrate, Fe-EDTA, $Fe(NO_3)_3$, $FeCl_2$, $FeCl_3$, or combinations thereof, preferably as $FeSO_4$, Fe-citrate, $Fe(NO_3)_3$, Fe-transferrin, or combinations thereof, more preferably as Fe-citrate.

[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 is provided. In yet another aspect a use of the serum-free perfusion medium of the disclosure for reducing the total cell bleed volume in a perfusion culture is provided. In yet another aspect a use of the serum-free perfusion medium of the disclosure for increasing protein production in a perfusion cell culture is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[030] Figure 1: CHO cells in small scale culture were exposed to control (diamonds), ethanol control (squares), 5 µM retinyl acetate (triangles) and 20 µM retinyl acetate (X) using a basal medium containing 3.1 mM ferric citrate. Shown in panel (A) is the viable cell density (e5 cells/mL, which is the technical spelling for "10^5 cells/mL") and in panel (B) the viability of CHO cells in the culture. Data represent average of triplicates; error bars represent standard deviation.