(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 September 2020 (24.09.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/187420 A1

(51) International Patent Classification:
C12N 5/00 (2006.01)

(21) International Application Number:
PCT/EP2019/057105

(22) International Filing Date:
21 March 2019 (21.03.2019)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant: **FLORABIO TEKNOLOJI SANAYI VE TICARET A.S.** [TR/TR]; Gülbahce Mah., Gülbahce Cad., Teknopark Izmir A7 binasi 1 / 43 04, 35430 Urla/Izmir (TR).

(72) Inventors: **CAYLI, Aziz**; c/o Florabio Teknoloji Sanayi ve Ticaret A.S., Gülbahce Mah., Gülbahce Cad., Teknopark Izmir A7 binasi 1 / 43 04, 35430 Urla/Izmir (TR). **TONGUL, Burcu**; c/o Florabio Teknoloji Sanayi ve Ticaret A.S., Gülbahce Mah., Gülbahce Cad., Teknopark Izmir A7 binasi 1 / 43 04, 35430 Urla/Izmir (TR).

(74) Agent: **SCHWAHN, Hartmut** et al.; Gleiss Große Schrell und Partner mbB, Leitzstr. 45, 70469 Stuttgart (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

(54) **Title:** COMPLEX IRON CONTAINING CULTURE MEDIUM

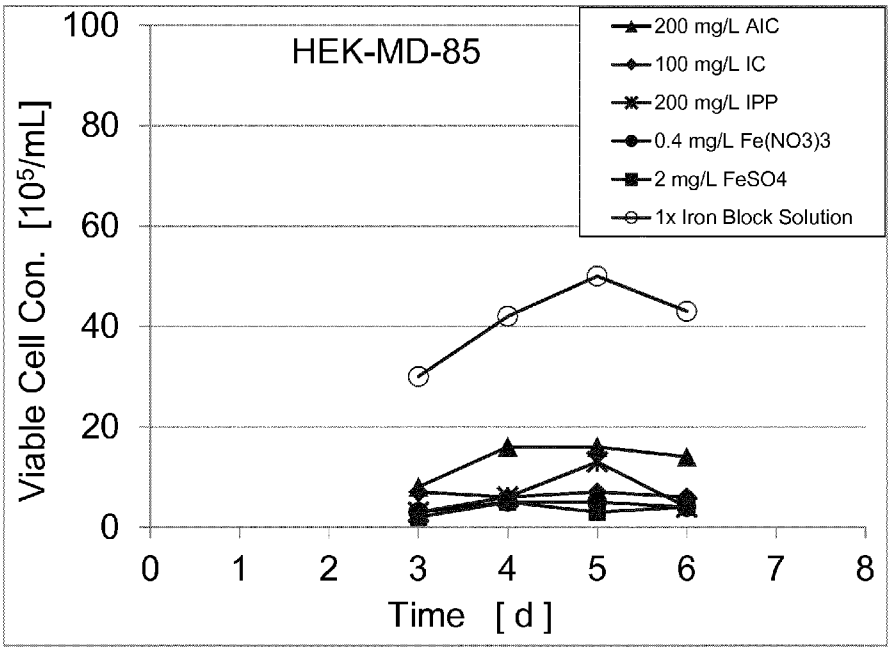

Figure 4

(57) **Abstract:** A cell culture medium for supporting the growth of cells in vitro and containing complex iron compounds. An "iron block solution" is defined containing multiple complex iron compounds. High concentration of iron is used and cells are enabled to transport more iron into the cell.

WO 2020/187420 A1

Complex iron containing culture medium

DESCRIPTION

The present invention provides a cell culture medium supporting the growth of cells in vitro and containing more than one complex iron compound. An “iron block solution” is defined containing multiple complex iron compounds. High concentration of iron is used and cells were forced to transport more iron into the cell. In the iron block solution no cell adaptation is necessary. Repetitive testing of each iron compound with each new cell line is not required anymore.

Mammalian cells have a requirement for iron which is supplied in the cell culture medium. In vivo, iron is presented to the mammalian cells by the iron binding protein transferrin. Transferrin works by binding iron and interacting with a transferrin receptor on the cell surface. The transferrin-iron complex is then taken into the cell by endocytosis. Once in the cell, the transferrin-iron complex is broken and the released iron is then complexed to an iron transporting protein (ferritin).

Transferrin typically used in cell culture media is of animal origin and in recent years there has been increasing regulatory pressure to remove proteins of animal origin from cell culture processes. The use of proteins of animal origin carries with it the risk of introducing contaminants and adventitious pathogens. In recent years recombinant transferrin is produced as well to overcome this regulatory bottleneck. Nevertheless, recombinant proteins are expensive to produce, therefore, if they are added into the culture medium, they increase the price of medium significantly. The industry is therefore seeking for a cheaper alternative to recombinant transferrin.

It is not clearly understood how transferrin-free iron is taken up into the cells. This is not surprising because it is even unknown which iron forms exists in aqueous solutions when iron is added in complex form. Alternative cellular iron transporters to transferrin have been sought and examined with varying degrees of success. The type and concentration

of any alternative iron transporter has often been found to be dependent on the type of mammalian cell being cultured.

Bertheussen (Cytotechnology 11: 219-231; 1993) has commented that iron cannot be effectively supplied to mammalian cells by adding simple iron salts to the cell culture medium, primarily due to the availability of iron to the cells being reduced by rapid oxidation and precipitation of iron. It is reported that the transferrin in cell culture media could be replaced by using aurintricarboxlic acid (ATA), a lipophilic iron chelator, and ferric ions added in the form of $FeCl_3$.

Neumannova et al. (In Vitro Cell. Dev. Biol. 31: 625-632; 1995) have tested 19 different cell types for long term growth in ferric citrate containing medium and only five cell lines were actually able to grow in this medium. It is concluded that different cell lines have different iron transport mechanism and one particular iron source may not be a universal solution for all cell types.

It is also reported that ferrous (II) or ferric (III) iron in culture medium may damage mitochondrial DNA of cells (Itoh et al.; Arch. Biochem. Biophys. 313: 120-125; 1994). The toxicity of iron is most likely related to the oxidative stress caused by Fenton reaction of free iron ions.

WO2006/047380 discloses a medium which comprises both, $Fe(NO_3)_3$ 9H2O and $FeSO_4$ $7H_2O$. Authors mention that ferric and ferrous ions can generate oxidative toxic compounds. These compounds may not only be toxic to the cells but may also generate precipitation with medium through reaction with other ions. Therefore, the authors propose to add to the medium a complex forming component, e.g. citrate to bind free iron and to reduce the oxidative potential of free iron.

The technical problem underlying the present invention is the provision of an improved transferrin-free iron supplementation of cells, which supports the growth of many types of mammalian cells or cell lines. It is a particular object of the present invention to provide iron supplementing medium without the need of individual adaption of the composition of the iron supplementing medium to each particular cell type, which would require intensive testing before use.

It is a particular object of the present invention to provide iron supplementing medium which supports the growth of many types of mammalian cells or cell lines and can be prepared void of free iron ions that could damage the cells.

The invention solves this technical problem by providing an iron supplementing medium, specifically apt for supporting the growth of mammalian cells in vitro (by provision of iron), and means for the ready preparation of such a cell culture medium, such as a media concentrate or concentrated stock solution, where according to the invention the medium or its concentrated stock solution is comprising more than two, preferably more than three or more than four, different complex iron compounds as the iron source.

According to the invention, each of the at least two or the at least three different complex iron compounds is selected from the group of specific complex iron compounds which consists of: iron(III) ammonium citrate, iron(II) ammonium citrate, iron(III) ammonium oxalate, iron(II) ascorbate, iron(III) ascorbate, iron(III) citrate, iron(III) manganese(II) citrate, iron(II) citrate, iron(III) pyrophosphate, iron(III) sodium pyrophosphate, iron(III) choline citrate, iron(III) sodium citrate, iron(II) sodium citrate, iron(III) sodium EDTA, iron(III) sodium EDTA trihydrate, iron(II) sodium EDTA, iron(II) fumarate, iron(III) fumarate, iron(III) polymaltose complex, iron(II) gluconate, iron(II) gluconate 2-hydrate, iron(III) gluconate, iron(III) glycerophosphate, ferric polyisomaltose (iron(III)-hydroxide-dextrin), iron(III) saccharate, iron(III) tartrate, iron(II) tartrate, iron(III) tartrate monohydrate, iron(II) tartrate 2.5-hydrate, iron(II) bisglycinate, iron(II) sodium oxalate, iron(II) succinate, and any hydrated form thereof, salt thereof, micronized dispersible form thereof, and encapsulated form thereof.

In variants, the iron (III) pyrophosphate is micronized dispersible iron (III) pyrophosphate, particularly iron (III) pyrophosphate “micro 2”. In other preferred variants, the iron (III) pyrophosphate (tetrairon tris(pyrophosphate); CAS 10058-44-3) is in solubilized form, preferably having an iron content of 10.5% to 12.5%).

In variants, the iron (II) fumarate, is microencapsulated iron (II) fumarate, preferably iron (II) fumarate DC 90 S (in 10% corn starch).

In preferred embodiments of the invention, the cell culture medium and the concentrated stock solution thereof comprises the at least two different complex iron compounds as the only iron source in the medium, most preferably the concentrated stock solution consists of these at least two different complex iron compounds as the only iron compounds.

In preferred embodiments of the invention, the cell culture medium and the concentrated stock solution thereof comprises the at least three or at least four different complex iron compounds as the only iron source in the medium, most preferably the concentrated stock solution consists of these at least three or at least four different complex iron compounds as the only iron compounds.

In preferred embodiments of the invention, the cell culture medium and the concentrated stock solution thereof comprises two to five, particularly three to five, different complex iron compounds as the only iron source in the medium, most preferably the concentrated stock solution consists of these two to five or three to five different complex iron compounds as the only iron compounds.

A preferred embodiment of the cell culture medium or the concentrated stock solution thereof the complex iron compounds are selected from: ammonium iron citrate, iron citrate, iron choline citrate, and iron pyrophosphate. Preferably these complex iron compounds are the only iron source in the medium.

A preferred variant is a cell culture medium or concentrated stock solution thereof, which comprises as the only iron sources in the medium (based on the final concentration of the cell culture medium): 4 to 4000 mg/L iron (III) ammonium citrate (16-19 % iron), 2 to 2000 mg/L iron (III) citrate, tribasic (18-20 % iron), 4 to 4000 mg/L iron (III) pyrophosphate (10-13 % iron), 0.1 to 80 mg/L iron (III) nitrate ($Fe(NO_3)_3$), 0.4 to 400 mg/L iron (II) sulfate ($FeSO_4$), and 3 to 3000 mg/L iron (III) choline citrate (14 % iron).

A more preferred variant is a cell culture medium or concentrated stock solution thereof, which comprises as the only iron sources in the medium (based on the final concentration of the cell culture medium): 4 to 4000 mg/L iron (III) ammonium citrate (16-19 % iron), 2 to 2000 mg/L iron (III) citrate tribasic (18-20 % iron), 4 to 4000 mg/L iron (III) pyrophosphate (10-13 % iron), and 3 to 3000 mg/L iron (III) choline citrate (14 % iron).

The medium and stock solution thus is preferably free of any non-complex iron compound.

A more preferred variant is a cell culture medium or concentrated stock solution thereof, which comprises as the only iron sources in the medium (based on the final concentration of the cell culture medium): 20 to 1000 mg/L iron (III) ammonium citrate (16-19 % iron), 10 to 800 mg/L iron (III) citrate tribasic (18-20 % iron), 20 to 1000 mg/L iron (III) pyrophosphate (10-13 % iron), and 20 to 800 mg/L iron (III) choline citrate (14 % iron). The medium and stock solution thus is preferably free of any non-complex iron compound.

A preferred specific variant thereof is a cell culture medium or concentrated stock solution thereof, which comprises as the only iron sources in the medium (based on the final concentration of the cell culture medium): 200 mg/L iron (III) ammonium citrate (16-19 % iron), 100 mg/L iron (III) citrate, tribasic (18-20 % iron), 200 mg/L iron (III) pyrophosphate (10-13 % iron), and 200 mg/L iron (III) choline citrate (14 % iron).

In preferred embodiments of the invention, cell culture medium or the concentrated stock solution thereof is free of components of animal origin.

In preferred embodiments of the invention, cell culture medium or the concentrated stock solution thereof is chemically defined.

In preferred embodiments of the invention, the cell culture medium or the concentrated stock solution thereof is specifically apt for supporting the growth of mammalian cells in suspension in an agitated cell culture.

In preferred embodiments of the invention, the cell culture medium or the concentrated stock solution thereof is specifically apt for supporting the growth of mammalian cells wherein the mammalian cells are selected from CHO, HEK293, BHK21, MRC5, and VERO cells.

Surprisingly, it has been found out that when multiple iron compounds are mixed and offered to the cells, they grow better than in a single iron compound. Surprisingly, each iron compound seems to transport iron into the cells, at least in part, independently from

each other. Results give the indication that there are multiple iron transport mechanisms in the cells which work at least in part independently from each other. Surprisingly, for every iron compound an independent transport mechanism seems to exist. This is not reported before. The medium of the present invention can saturate each iron transporter independently from each other and thus supplies the cells with more iron.

According to the present invention a combination of at least two different complex iron compounds is a significantly better iron source than non-complex iron compounds and even a single complex iron compound in combination with one or more non-complex iron compounds.

According to the present invention there is defined an "iron block solution" containing at least two complex iron compounds. Contrary to the literature and to our surprise our iron block solution supports effectively the growth of four totally different cells including two types of different organ human cell lines and two animal cell lines. According to the invention, repetitive testing of each iron compound with each new cell line is not required anymore. The optimal concentration of the iron block solution of the invention is found for one of the cell lines was transferable to all other cell lines without any restriction.

The iron block solution of the present invention contains high concentration of iron. According to our invention, contrary to the literature and to our surprise our iron block solution seems to be harmless to the cells, so that no cell adaptation is necessary in such high iron containing media anymore. We assume that this is achieved because our iron block solution contains complex iron compound and non-complex iron compounds (for example iron sulfate, iron nitrate, iron chloride) are omitted.

In the context of this invention, we use high concentration of iron, let the cells transport more iron into the cells. Contrary to the literature and to our surprise, such high iron concentration is not toxic to the cells. Contrary, it supports the cell growth. The iron block solution contains at least two complex iron compounds. We don't add non-complex iron compounds like iron sulfate, iron nitrate or iron chloride, so that little free iron is available in medium for chemical reaction. Thus, free iron toxicity is reduced to a minimum.

According to the invention and contrary to the literature, we found that non-complex iron compounds are not beneficial to the cells. Even though they are commonly used in culture media non-complex iron compounds cannot transfer iron efficiently into the cells, they can even damage cells. Some examples but not limited of non-complex iron compounds in culture media are iron sulfate ($FeSO_4$), iron nitrate ($Fe(NO_3)_3$) and iron chloride ($FeCl_3$).

The present invention provides a cell culture medium for supporting the growth of cells in vitro and containing more than two complex iron compounds. The invention applies preferably to suspension adapted cells, preferably to agitated cell suspensions. The invention further applies preferably to cells grown in animal component free culture medium, preferably cells grown in chemically defined culture media. The invention further applies preferably to a batch, fed-batch, perfusion or continuous process.

The invention further applies preferably to cells expressing a recombinant protein, preferred cells produce a virus, even more preferred cells produce a vaccine.

In a second aspect, the invention provides an in vitro process for the production of a recombinant protein or a virus, comprising the steps of:

- providing mammalian cells or a cell line capable of producing of a recombinant protein or a virus,

- culturing the mammalian cells or a cell line in a cell culture medium of any on the preceding claims to produce said recombinant protein or a virus.

In a preferred variant of the process according to the invention the mammalian cells are selected from CHO, HEK293, BHK21, MRC5, and VERO cells.

In a preferred variant of the process according to the invention the culturing of the mammalian cells or cell line takes places in agitated cell suspensions. In a preferred variant of the process according to the invention the culturing of the mammalian cells or cell line takes places in animal component free culture medium, preferably the cells are cultured in chemically defined culture media. In a preferred variant of the process according to the invention the culturing of the mammalian cells or cell line takes places in

a discontinuous batch or fed-batch process. An alternative preferred variant of the process is continuous process. In a preferred variant of the process according to the invention the culturing of the mammalian cells or cell line takes places in a perfusion system.

In a third aspect, the invention provides a kit for the preparation of a cell culture medium for supporting the growth of mammalian cells in vitro by provision of iron, comprising:

- a concentrated stock solution, as characterized in any one of claims 1 to 11,
- a chemically defined basal medium, and
- set of instructions to dilute said stock solution in the basal medium to reach final concentration of ion compounds in the basal medium.

In the context of the present invention the term "cell cultivation" or "cell culture" means cell cultivation or fermentation processes in all scales, e.g. from micro titer plates to large-scale industrial bioreactors, in all different process modes, e.g. batch, fed-batch, perfusion of continuous cultivation. In a preferred embodiment of the present invention the cell culture is a mammalian cell culture and is a batch, fed-batch, perfusion or continuous culture.

The term "cell culture medium" as used herein is a medium to culture mammalian cells comprising essential nutrients and components such as vitamins, trace elements, salts, bulk salts, amino acids, lipids, carbohydrates in a preferably buffered aqueous solution (preferably pH about pH=7.6 - 6.6, most preferred pH=7.3 to 6.8) as well as proprietary media from various sources. The cell culture medium may be a basal cell culture medium. The cell culture medium can be supplemented with any medium additive, for example but not limited it can be a kit which is added to a cell culture medium.

The term "basal medium" or "basal cell culture medium" as used herein is a cell culture medium to culture mammalian cells. It refers to the medium in which the cells are cultured from the start of a cell culture run and is not used as an additive to another medium. The basal cell culture medium is provided from the beginning of a cell cultivation process. The basal medium is in general the first cell culture medium to which cells are contacted.

The term "feed" or "feed medium" as used herein relates to a concentrate of nutrients or concentrated nutrient composition which is specifically designed for use as a feed in the culture of mammalian cells. A feed medium can contain all the nutrients which a basal medium contains. Preferably, a feed medium can contain a smaller number of different nutrients than a basal medium or a perfusion medium contains. A feed medium preferably contains less salt as compared to a basal medium. A feed medium contains in general less sodium ions as compared to a basal medium. A feed medium preferably has higher osmolality compared to a basal medium. A feed medium is typically added to a basal cell culture medium or fermentation broth in fed-batch mode. A feed medium may also be added onto a perfusion medium, where cells are cultured in perfusion mode. Preferably, a feed medium is added daily, but may also be added more or less frequently, such as twice a day or every second day. A feed medium may also be added continuously during the fermentation process. Generally, cells are contacted with another medium, for example with a basal medium first. Thereafter, cells are contacted with a feed medium. Feeding can start and stop any time during the fermentation process.

The term "chemically defined medium" as used herein refers to a cell culture medium suitable for in vitro cell culture, in which all components are known and all components can be characterized. Chemically defined medium may contain hydrolysates. Preferably, chemically defined medium is free of hydrolysates. More specifically it does not comprise any supplements derived from animals, plants or yeast. It may comprise hydrolysates only if all components have been analyzed and the composition thereof is characterized and the medium can be reproducibly prepared. Characterization of hydrolysates of a chemically defined medium can be performed by any method, for example but not limited by a chromatographic method. Chemically defined media are preferably free of any hydrolysate (sometimes called peptone). Thus, chemically defined media most preferably consist of single identifiable chemicals. Chemically defined media are sometimes supplemented with proteins, growth factors or peptides. Those agents can be produced recombinantly or synthetically. Most preferred, chemically defined media are free of proteins, growth factors and peptides.

The term “animal component free” refers to a cell culture medium suitable for in vitro cell culture, in which the medium does not contain any substance gained from animals or

animal products. Preferably, animal component free medium does not contain a substance which has an animal as source of that substance. Preferably, animal component free medium does not contain a substance which is isolated directly from animals. More preferably, animal component free medium does not contain a substance which is isolated indirectly from an animal. Sometimes components will be added into the culture media which are not of animal source. However, in the preparation of that component some agents (for example enzymes) can be used which are isolated from animals. Animal component free medium do not include even those components of which preparation is made with animal derived materials.

The terms "iron compound" or "iron containing compound" refer to a chemical, i.e. molecule where iron is present or bound to, and which provides iron, specifically in its ferrous (II, 2+) or ferric (III, 3+) form, to a solution or medium, in particular to a cell culture medium, under chemical and physical conditions, specifically in terms pH, temperature and ionic strength, which are present in a cell culture medium adapted for supporting the growth of biological cells, in particular under chemical and physical conditions for the culturing and growth of mammalian cells. An "iron compound" or "iron containing compound" is considered the only iron source in such a medium.

The term "non-complex iron compound" refers to a chemical, i.e. a molecule in which iron is bound in ionic form in a soluble salt such that it easily dissociates in solutions and thus release free iron ions into the medium. Examples of non-complex iron compounds in culture media are iron sulfate ($FeSO_4$), iron nitrate ($Fe(NO_3)_3$), and iron chloride ($FeCl_3$).

The term "complex iron compound" means a molecule in which at least one iron ion is bound in complex form and thus the complex iron compound does not readily release the iron ion as free iron ion into the medium. In accordance with the present invention, complex iron compounds are: iron(III) ammonium citrate, iron(II) ammonium citrate, iron(III) ammonium oxalate, iron(II) ascorbate, iron(III) ascorbate, iron(III) citrate, iron(III) manganese(II) citrate, iron(II) citrate, iron(III) pyrophosphate, iron(III) sodium pyrophosphate, iron(III) choline citrate, iron(III) sodium citrate, iron(II) sodium citrate, iron(III) sodium EDTA, iron(III) sodium EDTA trihydrate, iron(II) sodium EDTA, iron(II) fumarate, iron(III) fumarate, iron(III) polymaltose complex, iron(II) gluconate,

iron(II) gluconate 2-hydrate, iron(III) gluconate, iron(III) glycerophosphate, ferric polyisomaltose (iron(III)-hydroxide-dextrin), iron(III) saccharate, iron(III) tartrate, iron(II) tartrate, iron(III) tartrate monohydrate, iron(II) tartrate 2.5-hydrate, iron(II) bisglycinate, iron(II) sodium oxalate, iron(II) succinate, and any hydrated form thereof, salt thereof, micronized dispersible form thereof, and encapsulated form thereof.

In the context of the present invention, the term "iron block solution" refers to the following: A solution consisting of many different iron compounds. An iron block solution thus contains, for example, two or three or four or five or even more different iron compounds i.e. complex iron compounds. There is generally no limit about how many iron compounds can be mixed together to form an iron block solution. Also, the concentration of each iron compound can be different.

The invention is further illustrated by the following non-limiting examples and accompanying figures:

FIGURES

Figure 1 Effect of single iron compounds to growth the BHK21 cell line.

Figure 2a Concentration titration of AIC.

Figure 2b Concentration titration of IC.

Figure 2c Concentration titration of IPP.

Figure 2d Concentration titration of Fe(NO3)3.

Figure 2e Concentration titration of FeSO4.

Figure 3 Testing of iron block solution in BHK cell line against single iron compounds.

Figure 4 Comparison of single iron compounds with the iron block solution in HEK293 cell line.

Figure 5 The effect of omitting iron compounds from the iron block solution.

Figure 6 Testing of the iron block solution and single irons in MRC5 cell line.

Figure 7 Testing of the iron block solution and single iron compounds with an antibody producing CHO DG44 cell line.

Figure 8 Testing the iron block solution in basal medium and feed medium in a fed-batch process with BHK21 cells.

Figure 9 Testing the iron block solution in basal medium and feed medium in a fed-batch process with CHO DG44 cells expressing an antibody.

EXAMPLES

The following cell lines were used in the examples: HEK293 GTP-AC Free, BHK21, MRC5, CHO DG44.

All cell lines were ordered from their original source, e.g. ECACC, Columbia University etc. All cell lines were growing adherently and in serum contain cell culture media when they have arrived by us. All cell lines were adapted in suspension growth conditions. All cell lines were cultured in chemically defined culture medium. All cell lines were cultured in animal component free medium. From all cell lines animal component free cell banks were prepared to continuously supply the experiments with cells. In all experiments only, the cells were used which were adapted to suspension growth, growing in animal component free and chemically defined media.

Even though during the experiments only four cell lines were used, the invention is not limited to those cell lines. Any mammalian cell would be suitable to use in this invention. Some other suitable cell lines are given in table 1.

Cell line	Reference
BHK21	ATCC CCL-10
2254-62.2 (BHK21 derivative)	ATCC CRL-8544
HaK	ATCC CCL-15
BHK TK	ECACC No.85011423
HEK293	ATCC CRL-1573
CHO	ECACC No. 8505302
CHO wild type	ECACC 00102307
CHO DG44	Urlaub et al., Cell 33(2), 405-412, 1983; Life Technologies A1091 101
CHO-K1	ATCC CCL-61, ECACC 85051005
CHOZN GS	GS deficient cells derived from CHO-K1 (SAFC ECACC 85051005)
CHO-K1 GS	Glutamine synthase (GS) deficient cells derived from CHO-K1
CHO-K1/SF	ECACC 93061607
HuNS1	ATCC CRL-8644
V79	ATCC CCC-93
Per. C6	Fallaux, F.J et al, Human Gene Therapy 9 (13), 1909-1917,1998
NS0	ECACC NO.85110503
COS7	ATCC CRL-1651
Lec13	Stanley P. Et al, Ann. Rev. Genetics 18, 525-552, 1984
SP2/0-Ag14	ATCC CRL-1581
CHO-DUKX (=CHO dukx$^-$ CHO/dhfr$^-$)	ATCC CRL-9096
CHO DUKX B11	ATCC CRL-9010
CHO-S	Life Technologies A1136401
CHO Pro-5	ATCC CRL-1781

Table 1: Examples of suitable mammalian cell lines

Routine stock cultures were established from all cell lines in parallel. Stock cultures were kept growing in shake flasks and cells were split into fresh culture medium every two to four days. That means, a small amount of cell culture was used as inoculum and that

inoculum was transferred into a new flask and supplemented with fresh culture medium. Cells were cultured at 37 °C in 7% CO_2 atmosphere in shake flasks under shaking conditions. The seeding density was set to 0.3 to 0.5 x 10^6 cells/ml depending on the cell line. In this way cells were cultured several passages. One passage is defined as a culture duration of 2-4 days. At different time points inoculum cells were taken from the stock culture and experiments were performed in various scales, for examples in 6-well plates, shake flasks, T-flasks, spinner flasks and bioreactors.

All experiments were performed in shake flasks with suspension cells under shaking conditions with 25 mL working volume. In order to initiate an experiment, the inoculum cells were taken from their stock culture. Cell suspension was centrifuged, supernatant removed and cell pellet was transferred into the test medium. Thus, the carry-over effect of substances was avoided. Especially, the iron was removed from inoculums medium.

Proprietary culture media was used for cultivation of all cells in all steps. The proprietary culture media were a basal medium and feed medium. Basal medium recipe was varying by some cells depending on selective pressure requirement of the particular cells. The proprietary basal medium was an animal component free, chemically defined medium. By some cells, the basal medium was free of protein, hydrolysate, peptone, peptide and growth factors.

The proprietary basal medium contains all necessary substances for cell growth, viability and cellular productivity. Some examples of ingredients of proprietary basal medium are glucose at 2 - 10 g/L, NaCl 2-4 g/L. KCl 200 - 400 mg/L, $CaCl_2$ 50-200 mg/L, $MgCl_2$ 50-300 mg/L, NaH_2PO_4 and Na_2HPO_4 200 - 1000 mg/L, Arginine 100-600 mg/L, Asparagine 200-900 mg/L, Aspartic acid 30-500 mg/L, Cysteine 30-150 mg/L, Glutamic acid 50-1000 mg/L, Glutamine 100 - 3200 mg/L, Glycine 30 - 100 mg/L, Histidine 50-150 mg/L, Isoleucine 100-900 mg/L, Leucine 100 - 1000 mg/L, Lysine 100 - 800 mg/L, Methionine 30 - 150 mg/L, Phenylalanine 200 - 600 mg/L, Proline 100 - 800 mg/L, Serine 100 - 800 mg/L, Threonine 100 - 800 mg/L, Tryptophan 30 - 150 mg/L, Tyrosine 100 - 600 mg/L, Valine 100 - 600 mg/L, $NaHCO_3$ 2 -3 g/L. The basal medium further comprises trace elements, vitamins, nucleotide and fatty acid precursors. Feed medium was a concentrated form of basal medium. It contained amino acids, vitamins, trace

elements, iron components. Feed medium had less sodium ions, but higher osmolality then the basal medium.

Example 1: Preparation of iron block solution according to the present invention

Basal medium was prepared without iron compound.

Iron stock solutions were prepared as below:

Ammonium Iron(III) Citrate (abbreviated as "AIC"). 50 g ammonium iron (III) citrate (Cas Number: 1185-57-5, with 16.5 - 18.5 % iron content) was solved in highly purified water with the final volume of 1 L, stirred 3 hours at 37 °C. The pH was measured as a control parameter and found as 4.5±0.2 and then the solution was sterile filtered with 0.1 µm pore size filter within 30 min.

Iron(III) Citrate Tribasic (abbreviated as "IC"). 15 g iron citrate tribasic monohydrate (CAS Number: 2338-05-8, MW: 262.96 g/mol, with 18 – 20 % iron content) was solved in highly purified water with the final volume of 1 L, stirred 3 hours at 90 °C. The pH was measured as a control parameter and found as 2.0±0.2 and then the solution was sterile filtered with 0.1 µm pore size filter within 30 min.

Iron(III) Pyrophosphate (abbreviated as "IPP"): 100 g iron (III) pyrophosphate (CAS Number: 10058-44-3, MW: 754.21 g/mol, with 10.5 - 12.5 % iron content) was dissolved in highly purified water with the final volume of 1 L and stirred 3 hours at 40 °C. The pH was measured as a control parameter and found as 6.2±0.2 and then the solution was sterile filtered with 0.1 µm pore size filter within 30 min.

Iron(III) Nitrate Nanohydrate (abbreviated as Fe(NO3)3): 0.4 g iron (III) nitrate nanohydrate (CAS Number: 7782-61-8, MW: 404.00 g/mol) was solved in highly purified water with the final volume of 1 L and stirred 3 hours at 37 °C. The pH was measured as a control parameter and found as 2.8±0.3 and then the solution was sterile filtered with 0.1 µm pore size filter within 30 min.

Iron(III) Sulfate Heptahydrate (abbreviated as "FeSO4"): 2 g iron (III) sulfate heptahydrate (CAS Number 7782-63-0 , MW: 278.01 g/mol) was solved in highly

purified water with the final volume of 1 L and stirred 3 hours at 37 °C. The pH was measured as a control parameter and found as 4.2±0.3 and then the solution was sterile filtered with 0.1 µm pore size filter within 30 min.

Iron(III) Choline Citrate: 13 g iron (III) choline citrate (CAS Number 1336-80-7, MW: 997.554 g/mol, with 14 % iron content) was solved in highly purified water with the final volume of 1 L and stirred 3 hours at 37 °C. The pH was measured as a control parameter and found as 2.9±0.2 and then the solution was sterile filtered with 0.1 µm pore size filter within 30 min.

Preparation of iron block solution according to the invention: The iron block solution of the invention contains at least two or more than two different iron compounds. Iron block solution can contain, for example 2 or 3 or 4 or 5, 6, 7, 8 different iron compounds. A stock solution of each iron compound is prepared separately as described above. Thereafter, single iron stock solutions will be mixed together to form an iron block solution as a concentrated stock solution.

As an example an iron block solution consisting of 5 different iron compounds is prepared as follows:

For 1 L iron block solution:

272 mL	ammonium iron(III) citrate (50 g/L),
457 mL	iron (III) citrate tribasic (15 g/L),
135 mL	iron (III) pyrophosphate (100 g/L),
68 mL	iron (III) nitrate nanohydrate, and
68 mL	iron (III) sulfate heptahydrate (2 g/L)

are mixed, stirred 15 min at room temperature; pH of solution is measured as pH 4.4±0.2.

This iron block solution is stable up to one year.

In order to reach one-time final concentration (1x) of each iron compound in culture media 14.7 mL of this iron block solution was added into the iron free culture medium.

Example 2: Screening of iron compounds in BHK-21 cells (BHK-MD-38)

This example demonstrates the effect of single iron compounds in BHK-21 cell line. The tested iron compounds are 200 mg/L ammonium iron(III) citrate (AIC), 33 mg/L iron(III) citrate tribasic (IC), 67 mg/L iron (III) pyrophosphate (IPP), 0.13 mg/L iron (III) nitrate ($Fe(NO_3)_3$) , 2 mg/L iron (II) sulfate ($FeSO_4$) and negative control without any iron source. A cell culture medium is prepared without any iron sources. Stock solutions of each iron compound are prepared as described in example 1, and the culture medium is supplemented with stock solutions to adjust the desired final concentration of each iron compound. The experiment is performed in batch modus in shake flask. BHK21 cell line used was adapted to suspension growth in animal component free, chemically defined medium.

Figure 1 demonstrates that cell growth is dependent on the sort of iron compound. BHK21 cell line could grow with complex iron compounds (AIC, IC and IPP) better than non-complex iron compounds ($Fe(NO_3)_3$ and $FeSO_4$).

Example 3: Optimal concentration of single iron compounds with BHK21 cells (BHK-MD-35)

This example serves to find out the optimal concentration of each iron compound for BHK21 cell line. The experiment is performed in batch modus in shake flask. BHK21 cell line is used, which was adapted to suspension growth conditions in animal component free, chemically defined medium. A cell culture medium is prepared without iron sources. Stock solutions of each iron compound are prepared as described in example 1 and supplemented into the culture medium to adjust the desired final concentration of iron compounds.

As seen in Figure 2, the optimum concentrations of AIC, IC, IPP, $Fe(NO_3)_3$ and $FeSO_4$ were found as 200 mg/L, 100 mg/L and 200 mg/L, 0.4 mg/L and 2 mg/L, respectively. At the same time, it is obvious that $FeSO_4$ and $Fe(NO_3)_3$ don't serve as an appropriate iron source for this cell line.

Example 4: Combining several iron compounds in one medium at their optimal concentrations with BHK-21 cells (BHK-MD-49)

The aim was to demonstrate the synergistic effect of single iron compounds combined in one medium. All growth promoting iron compounds were combined in one medium at their optimal concentration. The experiment is performed in batch modus in shake flask. BHK21 cell line is used, which was adapted to suspension growth conditions in animal component free, chemically defined medium

An iron block solution is prepared consisting of AIC, IC, IPP, $Fe(NO_3)_3$ and $FeSO_4$ as described in example 1. This solution is added into the iron free basal medium to have final concentration of 200 mg/L AIC, 100 mg/L IC , 200 mg/L IPP, 0.4 mg/L $Fe(NO_3)_3$ and 4 mg/L $FeSO_4$. This final iron concentration of each component is defined as 1x (one time concentrate) in basal medium. At the same time 0.5x, 1.5x, 2x, 2.5x and 3x concentrates were tested as well. If it is called 0.5x, then the concentration of all iron compounds was the half of the 1x concentrations in the basal medium, as following 100 mg/L AIC, 50 mg/L IC, 100 mg/L IPP, 0.2 mg/L $Fe(NO_3)_3$ and 1 mg/L $FeSO_4$. If it is called 2x, then the concentrations of all iron compounds are doubled in basal medium compared to 1x, as following 400 mg/L AIC, 200 mg/L IC, 400 mg/L IPP , 0.8 mg/L $Fe(NO_3)_3$ and 4 mg/L $FeSO_4$. The other concentrations (1.5x, 2.5x, 3x, etc.) are calculated accordingly. Negative control did not have any iron source in medium.

To our surprise, as seen in figure 3, the iron block solution of combined complex iron compounds promotes cell growth much better than the use of each single iron component as iron supplement. Moreover, the total iron added to the cell culture medium through the 1x iron block solution is high. Contrary to an expected toxicity to the cells, it is found not toxic. Even more, the opposite was found as it promotes the cell growth better then single iron compounds in non-toxic (i.e. lower) concentrations. Even if the concentration of iron block solution in the cell culture medium is increased to threefold (3x), still there is no significant toxicity of iron. This experiment shows further that the iron block solution combined complex iron compounds releases only very little free iron ions, which were reported to damage cells.

Example 5: Testing iron block solution with HEK293 cell line (HEK-MD-85)

Optimal iron block solution was found by using BHK21 cells. The question here was whether this iron block solution of combined complex iron compounds is suitable for human cells as well. More specifically, the question was whether the findings with BHK-21 cells is a cell specific finding or a universal finding. The experiment is performed in batch modus in shake flask. HEK293 cell line is used, which was adapted to suspension growth conditions, in an animal component free, chemically defined medium. A cell culture medium is prepared without an iron source. Stock solutions of each iron compound are prepared as described in example 1 and added into the culture medium to adjust the desired final concentration of each iron compound.

In order to examine this question, first all the single iron compounds were tested again in their optimal concentrations using HEK293 cells. Each iron compound promoted HEK293 cell growth in certain extent. To our surprise, the 1x iron block solution of combined complex iron compounds previously defined for BHK21 cells (see example 4) also promotes HEK293 cell growth as well (Figure 4).. This is evidence that the findings from BHK21 (animal) cells can be transferable to HEK293 (human) cells.

Example 6: Effect of single iron compounds within the iron block solution with HEK-293 cells (HEK-MD-92)

The experiment is performed in batch modus in shake flask. HEK293 cell line is used, which was adapted to suspension growth conditions in animal component free, chemically defined medium. A cell culture medium is prepared without iron sources. Stock solutions of each iron compound are prepared as described in example 1 and added into the culture medium to adjust the desired final concentration of each iron compound.

In previous experiments, an iron block solution of combined complex iron compounds is defined which supports growth of multiple types of cells. However, this iron block solution contains complex iron compounds and non-complex iron compounds as well. According to our theory, the complex iron compounds shall promote cell growth while the non-complex iron compounds shall be detrimental for the cells due to free iron ions

generated from non-complex iron compounds. The aim in this experiment is to find out the roles of each iron groups, the complex and non-complex iron compounds.

So far tested iron block solution consist of five different iron compounds as follows: 200 mg/L AIC, 100 mg/L IC, 200 mg/L IPP, 0.4 mg/L $Fe(NO_3)_3$ and 4 mg/L $FeSO_4$. In this iron block solution one particular iron component was omitted each time and was marked as minus (-). Specifically following combinations were tested:

"1x iron block" means: 200 mg/L AIC, 100 mg/L IC, 200 mg/L IPP, 0.4 mg/L $Fe(NO_3)_3$, 4 mg/L $FeSO_4$.

"-AIC" means: **No** AIC, 100 mg/L IC, 200 mg/L IPP, 0.4 mg/L $Fe(NO_3)_3$, 2 mg/L $FeSO_4$.

"-IC" means: 200 mg/L AIC, **No** IC, 200 mg/L IPP, 0.4 mg/L $Fe(NO_3)_3$, 2 mg/L $FeSO_4$.

"-IPP" means: 200 mg/L AIC, 100 mg/L IC, **No** IPP, 0.4 mg/L $Fe(NO_3)_3$, 2 mg/L $FeSO_4$.

"-$Fe(NO_3)_3$" means: 200 mg/L AIC, 100 mg/L IC, 200 mg/L IPP, **No** $Fe(NO_3)_3$, 2 mg/L $FeSO_4$.

"-$FeSO_4$" means: 200 mg/L AIC, 100 mg/L IC, 200 mg/L IPP, 0.4 mg/L $Fe(NO_3)_3$, **No** $FeSO_4$.

"-$Fe(NO_3)_3$ - FeSO4" means: 200 mg/L AIC, 100 mg/L IC, 200 mg/L IPP, **No** $Fe(NO_3)_3$, **No** $FeSO_4$.

Results show that when complex iron compounds (-AIC, -IC, -IPP) are removed from the iron block solution, the cell concentration decreases. This shows that the complex iron compounds have an additive effect in iron transport and cell growth.

When non-complex iron compounds ($Fe(NO_3)_3$, $FeSO_4$) are removed from the iron block solution, the cell concentration increases (Figure 5). This shows that non-complex iron compounds are not beneficial in cell culture media. In opposite, the free iron forming compounds have a negative effect to cell growth as postulated in this invention. This is surprising and contrary to the literature because still today it is common that cell culture media contain non-complex iron compounds like $Fe(NO_3)_3$, $FeSO_4$, $FeCl_3$. In this case the

most effective new iron block solution would consist of 200 mg/L AIC, 100 mg/L IC, 200 mg/L IPP.

Example 7: Testing Iron Block Solution with MRC5 cell line (MRC-MD-16)

Goal was again to test the single iron compounds and their combination with another cell line, Aim was to prove the universality of the iron block solution. The experiment is performed in batch modus in shake flask. MRC5 cell line is used, which was adapted to suspension growth conditions in animal component free, chemically defined medium. Single iron components were tested by their optimal concentration against the iron block solution. A cell culture medium is prepared without any iron source. Stock solutions of each iron compound are prepared as described in example 1 and added into the culture medium to adjust the desired final concentration of each iron compound in culture medium. The content and the concentration of 1x iron block solution is the same as described in example 4.

Results demonstrate the same findings as in other cell lines: each iron compound promotes the cell growth to certain extend, whereby non-complex iron compounds can hardly support cell growth. However, when complex iron compounds are combined in one medium, cell grow promoting effect is higher (Figure 6).

Example 8: Testing Iron Block Solution with CHO DG44 cell line (AFL-MD-32)

Aim is to prove the universality of the iron block solution whether the results can be transferred to another cell line. A CHO DG44 cell line was used expressing a recombinant antibody. This cell line and the culture medium were used later for large scale production of that recombinant antibody. The experiment is performed in batch modus in shake flask. The CHO DG44 cell line was adapted to suspension growth conditions in animal component free, chemically defined medium. A cell culture medium is prepared without any iron source. Stock solutions of each iron compound are prepared as described in example 1 and added into the culture medium to adjust the desired concentration of each iron compound in culture medium. The content and the concentration of 1x iron block solution is the same as described in example 4.

Results demonstrate the same findings as in all other cell lines; each iron compound promote the cell growth to a certain extend. However, when complex iron compounds are combined in one culture medium grow promoting effect became higher (Figure 7).

Another interesting fact is that if several complex iron compounds are combined in one medium, toxicity of high iron concentration is not visible. Further, cell don't need any adaptation time to this iron containing medium. Skilled person in the filed knows, that if a new iron is going to be applied by cells, normally the cells need to be adapted to the new iron compound which takes long time.

Example 9: Testing iron block solution in fed-batch process with BHK21 cells (BHK-MD-191)

The goal was to test iron block solution also in a feed medium. Cells reach in fed-batch processes higher concentration and that high number of cells need to be supplied with iron for longer process time compared to batch process. During fed-batch process cells might get iron limitation. In order to test this theory, feed medium was supplied with the iron block solution as well and tested in fed-batch process. The experiment is performed in shake flask. The BHK21 cell line was adapted to suspension growth in an animal component free, chemically defined basal medium and feed medium. Both media are prepared without any iron sources. Stock solution of each iron compound is prepared as described in example 1 and added into the basal medium and feed medium to adjust the desired concentration of each iron compound.

BHK21 cells were chosen as a representative cell line for virus production. Also, HEK293 or MRC5 cell lines were cultured in fed-batch processes with the same feed medium containing the same iron block solution.

The basal medium was supplemented with 1x iron block solution as described in example 4. The feed medium was supplemented with varying concentrations of iron block solution as 1x, 2x, 3x, 4x or 5x. Cells were cultured first in batch in the basal medium and the feeding have started at day 2 of the fed-batch process. A feed medium was defined consisting of amino acids, vitamins, trace elements, and other nutrients for cell growth. Cells were fed with the feeding medium daily until the end of the fed-batch process.

Results show that the fed-batch process promotes cell growth better than batch process whereby basal medium and feed medium both contain iron block solution (Figure 8).

Example 10: Testing iron block solution in fed-batch process with CHO cells (AFF-MD-71)

Goal was to test the iron block solution in fed-batch process with another cell line, in this case with CHO DG44 cell line expressing an antibody. The CHO DG44 cell line was adapted to suspension growth conditions in animal component free, chemically defined medium. Basal medium and feed medium are prepared without any iron sources. Stock solution of each iron compound is prepared as described in example 1 and added into the culture medium to adjust the final concentration of each iron compound in media.

The basal medium was supplemented with 1x iron block solution as described in example 4. The feed medium was supplemented with varying concentrations of iron block solution as 1x, 2x, 3x, 4x or 5x. Cells were cultured first in batch in basal medium and the feeding have started at day 2 of the fed-batch process. A feed medium was defined consisting of amino acids, vitamins, trace elements and other nutrients for cell growth. Cells were fed with the feeding medium daily until the end of the fed-batch process. Results show that a fed-batch process promotes the cell growth better than batch process whereby basal medium and feed medium both contain iron block solution (Figure 9).

Example 11: Generation of new iron block solution with iron choline citrate

According to our theory addition of more complex iron compounds into culture media shall not be detrimental. In opposite, iron choline citrate might be internalized by cells by a different mechanism, so that additional iron choline citrate might promote cell growth even more. Alternatively, iron choline citrate increases the variability of iron sources and thus the culture media containing iron choline citrate might even be more suitable for many different cell lines. Each new cell line might not need time to adapt into the new culture medium because the specific iron source what that cell line needs is already present in medium.

This theory was tested by generating an iron block solution including iron choline citrate. A cell culture medium is prepared without any iron source. Stock solutions of each iron compound are prepared as described in example 1 and the new iron block solution is prepared by mixing iron compounds together into one iron block solution. An adequate amount of iron block solution is then added into the medium and following final concentrations are achieved in basal medium: 200 mg/L Ammonium Iron Citrate; 100 mg/L Iron (III) Citrate tribasic; 200 mg/L Iron (III) Pyrophosphate; 0.4 mg/L Iron (III) Nitrate ($Fe(NO_3)_3$); 2 mg/L Iron (II) Sulfate ($FeSO_4$); 200 mg/L Iron Choline Citrate.

The CHO DG44 cell line is used for the experiment. The cell line grows in animal component free, chemically defined medium in suspension and expresses an antibody. Cells grow better in medium with above described iron compounds including iron choline citrate as expected (data not shown). Even if the growth benefit was not significantly higher, the medium with more complex iron compounds serves to more different cell lines to growth in this medium without adaptation. Therefore, this medium represents another preferred embodiment of the invention.

In a further test, the non-complex iron compounds were removed from the iron block solution because of their detrimental effect. The new iron block solution contained only complex-iron compounds including iron choline citrate. Specifically, the basal medium contained following iron sources: 200 mg/L Ammonium Iron Citrate; 100 mg/L Iron (III) Citrate tribasic; 200 mg/L Iron (III) Pyrophosphate; 200 mg/L Iron Choline Citrate. As expected cells grow better and kept the cell viability higher in this iron composition in the basal medium. There was no precipitation in medium observed.

CLAIMS

1. An iron providing cell culture medium or a concentrated stock solution thereof, for supporting the growth of mammalian cells in vitro by provision of iron, comprising more than two different complex iron compounds, selected from:

iron (III) ammonium citrate,
iron (II) ammonium citrate,
iron (III) ammonium oxalate,
iron (II) ascorbate,
iron (III) ascorbate,
iron (III) citrate,
iron (III) manganese(II) citrate,
iron (II) citrate,
iron (III) pyrophosphate,
iron (III) sodium pyrophosphate,
iron (III) choline citrate,
iron (III) sodium citrate,
iron (II) sodium citrate,
iron (III) sodium EDTA,
iron (III) sodium EDTA trihydrate,
iron (II) sodium EDTA,
iron (II) fumarate,
iron (III) fumarate,
iron (III) polymaltose complex,
iron (II) gluconate,
iron (II) gluconate 2-hydrate,
iron (III) gluconate,
iron (III) glycerophosphate,
ferri polyisomaltose,
iron (III) saccharate,

iron (III) tartrate,
iron (II) tartrate,
iron (III) tartrate monohydrate,
iron (II) tartrate 2.5-hydrate,
iron (II) bisglycinate,
iron (II) sodium oxalate,
iron (II) succinate, and
hydrated forms, salts, micronized dispersible forms, or encapsulated forms thereof.

2. The cell culture medium according to claim 1 or a concentrated stock solution thereof comprising the at least two different complex iron compounds as the only iron source in the medium.

3. The cell culture medium according to claim 2 or a concentrated stock solution thereof comprising at least three different complex iron compounds as the only iron source in the medium.

4. The cell culture medium according to any one of the preceding claims or a concentrated stock solution thereof, comprising at least three different complex iron compounds selected from: ammonium iron citrate, iron citrate, iron choline citrate, and iron pyrophosphate.

5. The cell culture medium according to any one of the preceding claims or a concentrated stock solution thereof, comprising as the only iron source in the medium (based on the final concentration of the iron compound in the cell culture medium):

4 to 4000 mg/L iron (III) ammonium citrate (16-19 % iron),
2 to 2000 mg/L iron (III) citrate, tribasic (18-20 % iron),
4 to 4000 mg/L iron (III) pyrophosphate (10-13 % iron),
0.1 to 80 mg/L iron (III) nitrate ($Fe(NO_3)_3$),
0.4 to 400 mg/L iron (II) sulfate ($FeSO_4$), and
3 to 3000 mg/L iron (III) choline citrate (14 % iron).

6. The cell culture medium according to any one of the preceding claims or a concentrated stock solution thereof, comprising as the only iron source in the medium (based on the final concentration of the iron compound in the cell culture medium):

4 to 4000 mg/L iron (III) ammonium citrate (16-19 % iron),
2 to 2000 mg/L iron (III) citrate, tribasic (18-20 % iron),
4 to 4000 mg/L iron (III) pyrophosphate (10-13 % iron), and
3 to 3000 mg/L iron (III) choline citrate (14 % iron).

7. The cell culture medium according to claim 6 or a concentrated stock solution thereof, comprising as the only iron source in the medium (based on the final concentration of the iron compound in the cell culture medium):

20 to 1000 mg/L iron (III) ammonium citrate (16-19 % iron),
10 to 800 mg/L iron (III) citrate, tribasic (18-20 % iron),
20 to 1000 mg/L iron (III) pyrophosphate (10-13 % iron), and
20 to 800 mg/L iron (III) choline citrate (14 % iron).

8. The cell culture medium according to claim 7 or a concentrated stock solution thereof, comprising as the only iron source in the medium (based on the final concentration of the iron compound in the cell culture medium):

200 mg/L iron (III) ammonium citrate (16-19 % iron),
100 mg/L iron (III) citrate, tribasic (18-20 % iron),
200 mg/L iron (III) pyrophosphate (10-13 % iron), and
200 mg/L iron (III) choline citrate (14 % iron).

9. The cell culture medium according to any one of the preceding claims or a concentrated stock solution thereof, which is free of components of animal origin and/or is chemically defined.

10. The cell culture medium according to any one of the preceding claims or a concentrated stock solution thereof for supporting the growth of mammalian cells in suspension in an agitated cell culture.

11. The cell culture medium according to any one of the preceding claims or a concentrated stock solution thereof, wherein the mammalian cells are selected from CHO, HEK293, BHK21, MRC5, and VERO cells.

12. An in vitro process for the production of a recombinant protein or a virus, comprising the steps of:

- providing mammalian cells or cell line capable of producing of a recombinant protein or a virus,
- culturing the mammalian cells or cell line in a cell culture medium of any on the preceding claims to produce said recombinant protein or a virus.

13. The process according to claim 12, wherein the mammalian cells are selected from CHO, HEK293, BHK21, MRC5, and VERO cells.

14. A kit for the preparation of a cell culture medium for supporting the growth of mammalian cells in vitro by provision of iron, comprising:

- a concentrated stock solution, as characterized in any one of claims 1 to 11, and
- set of instructions to dilute said stock solution in a basal medium to reach final concentration of ion compounds in the basal medium.

15. The kit according to claim 14, further comprising:

- a chemically defined basal medium.

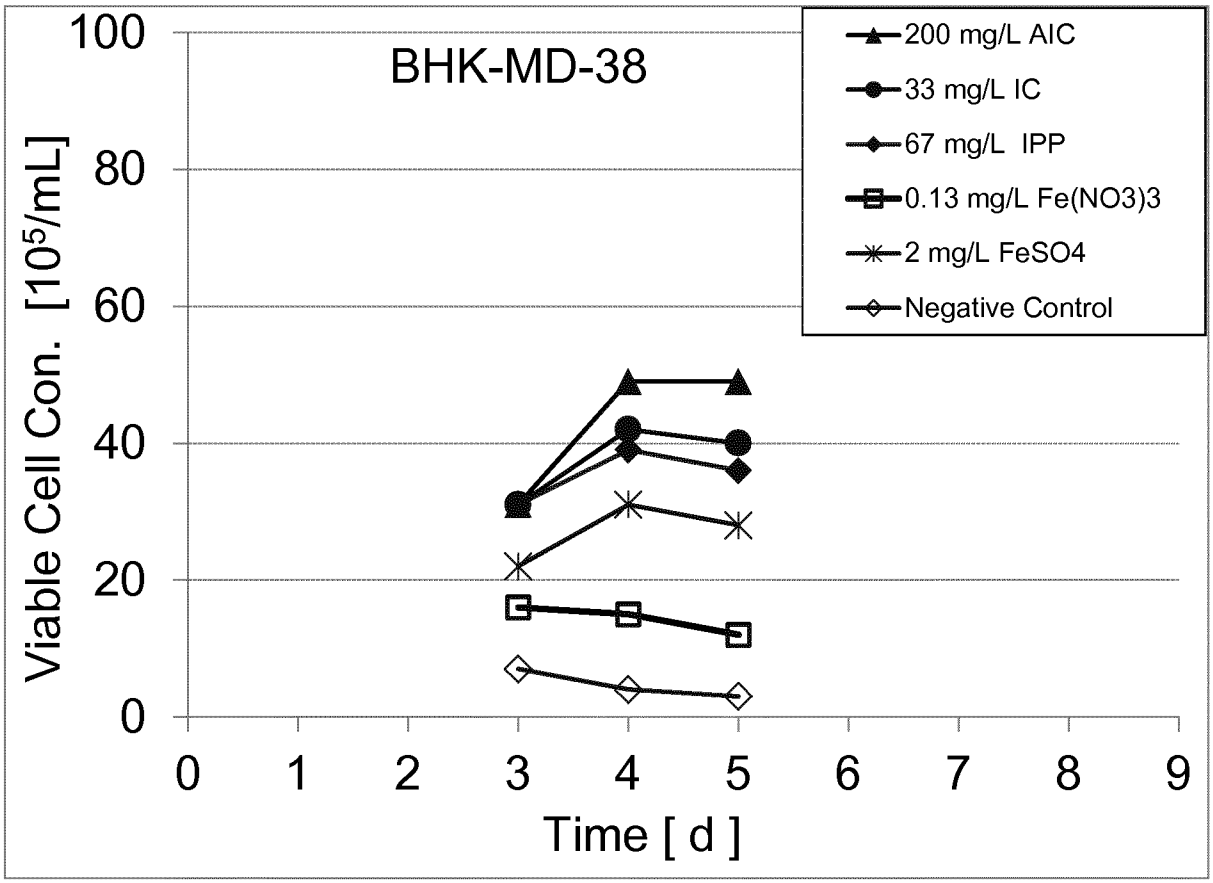
BHK-MD-38
Viable Cell Con. [10^5/mL]
Time [d]
200 mg/L AIC
33 mg/L IC
67 mg/L IPP
0.13 mg/L Fe(NO3)3
2 mg/L FeSO4
Negative Control
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9

Figure 1

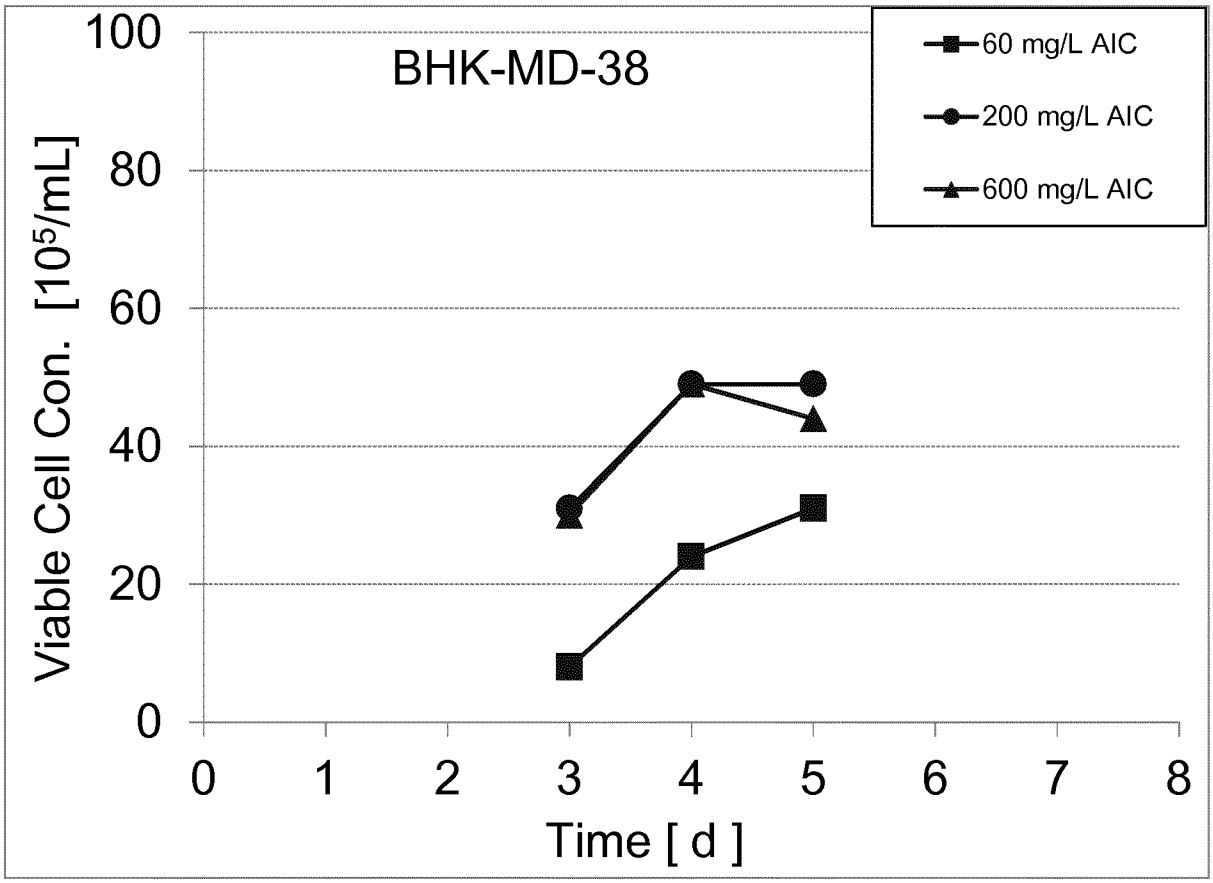
BHK-MD-38
60 mg/L AIC
200 mg/L AIC
600 mg/L AIC
Viable Cell Con. [10^5/mL]
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time [d]

Figure 2a

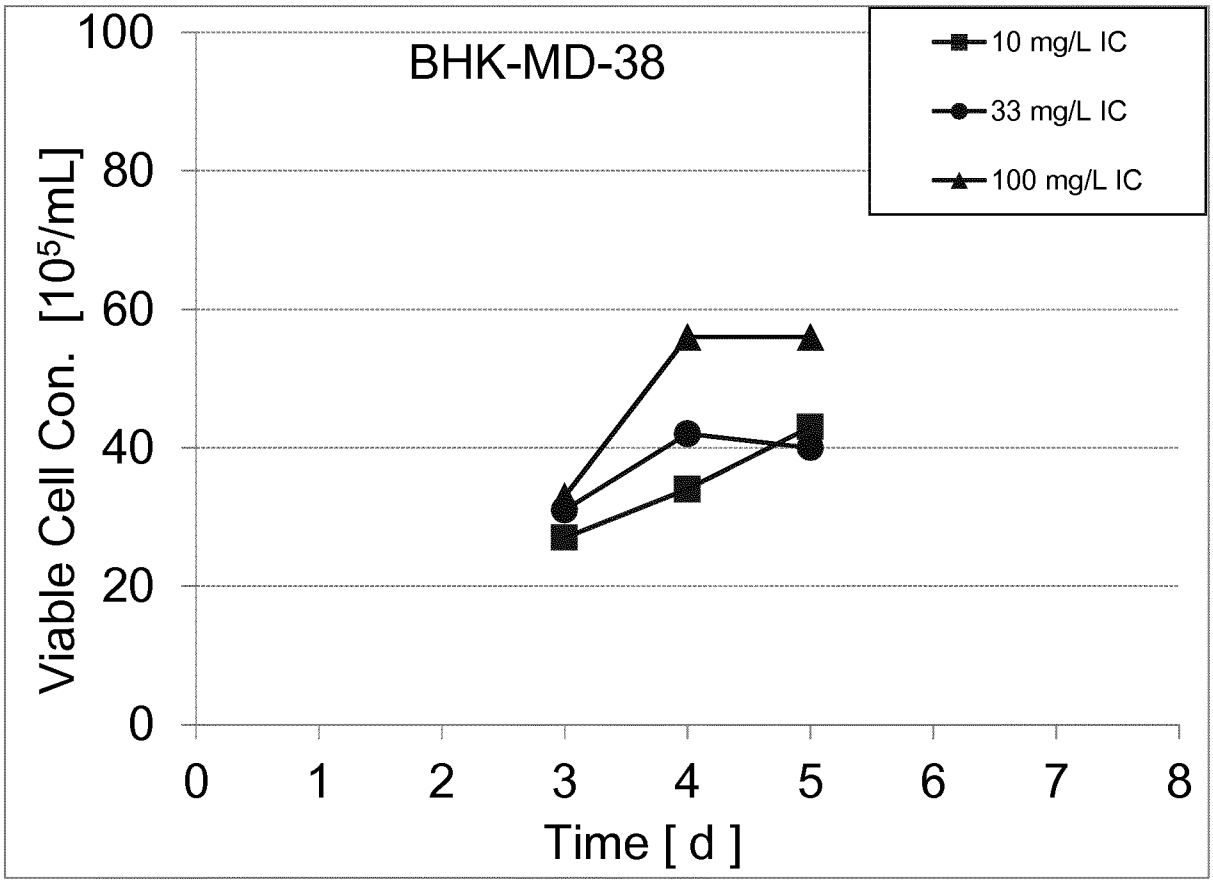
BHK-MD-38
10 mg/L IC
33 mg/L IC
100 mg/L IC
Viable Cell Con. [10^5/mL]
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time [d]

Figure 2b

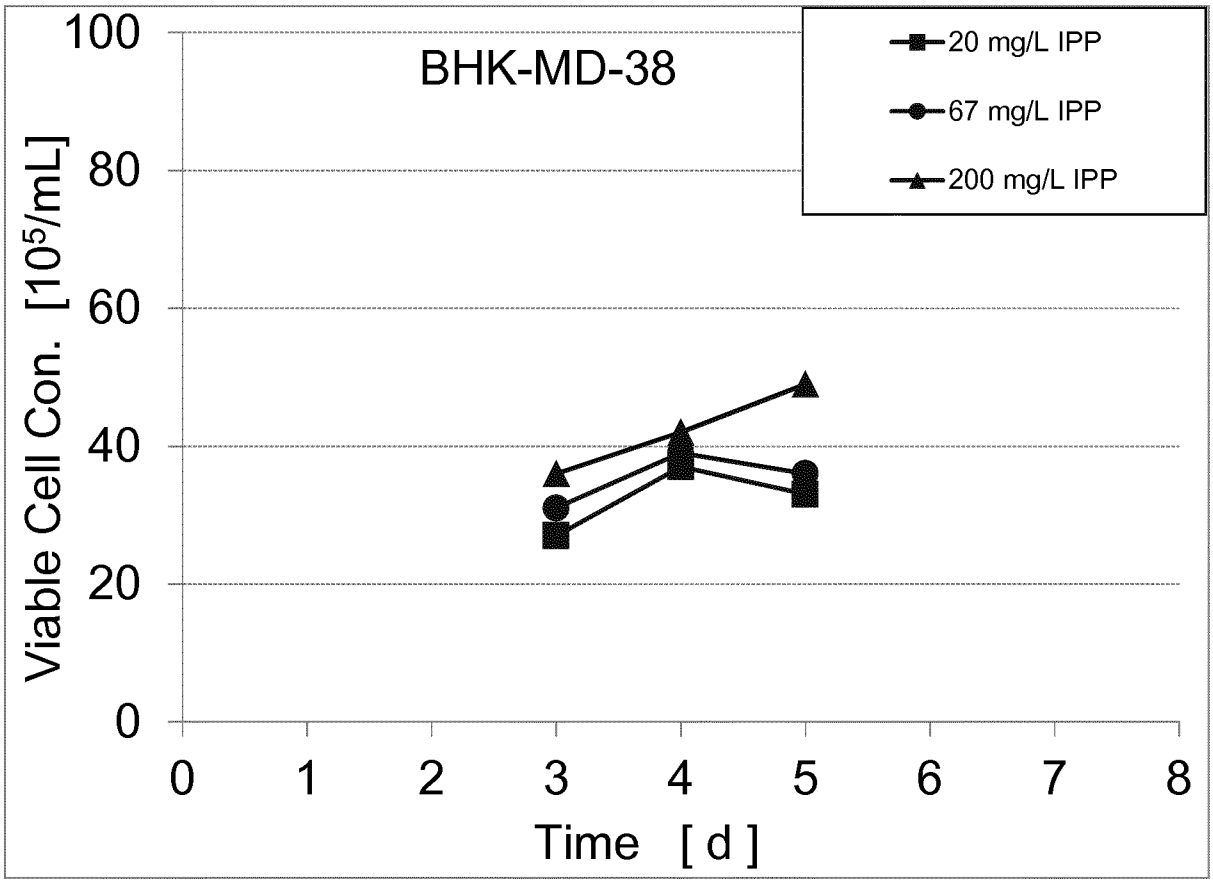
BHK-MD-38
20 mg/L IPP
67 mg/L IPP
200 mg/L IPP
Viable Cell Con. [10^5/mL]
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time [d]

Figure 2c

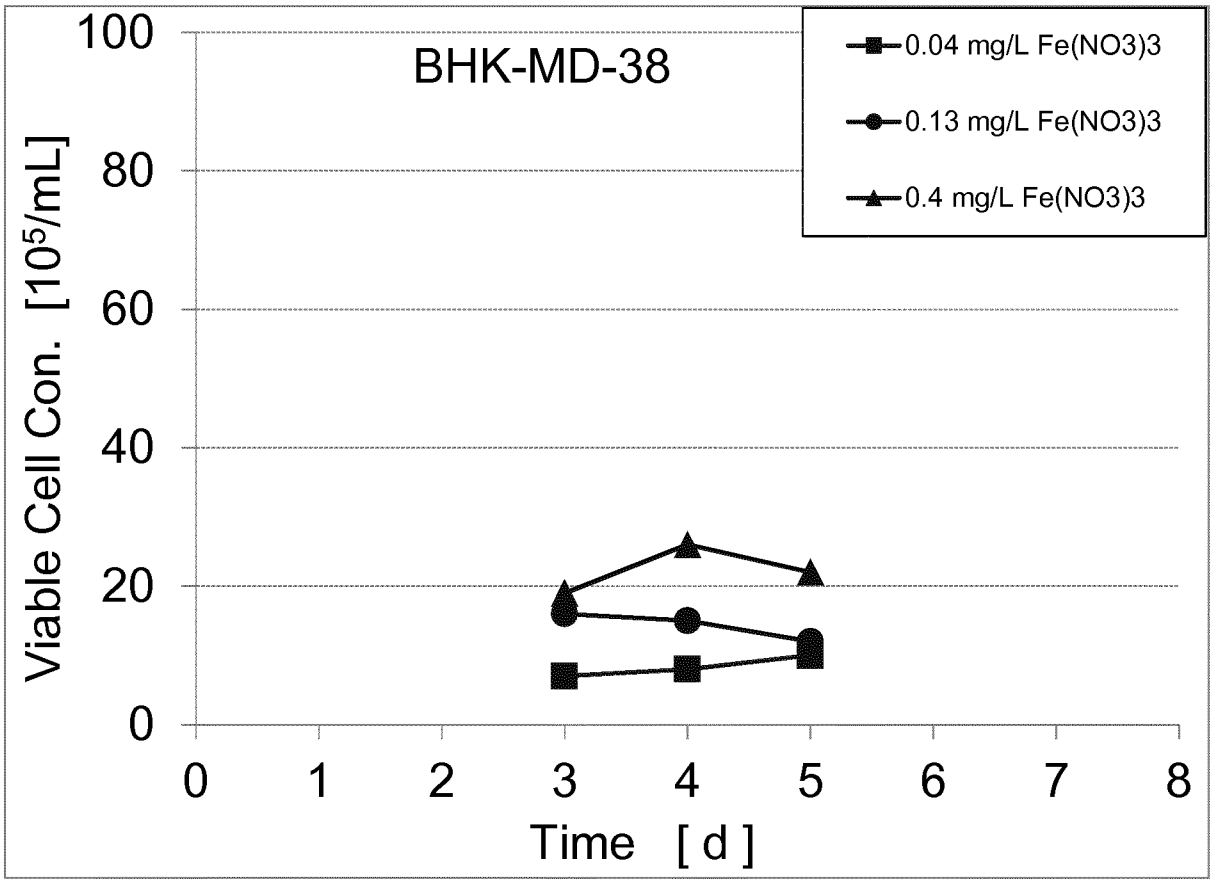
BHK-MD-38
0.04 mg/L Fe(NO3)3
0.13 mg/L Fe(NO3)3
0.4 mg/L Fe(NO3)3
Viable Cell Con. [10^5/mL]
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8
Time [d]

Figure 2d

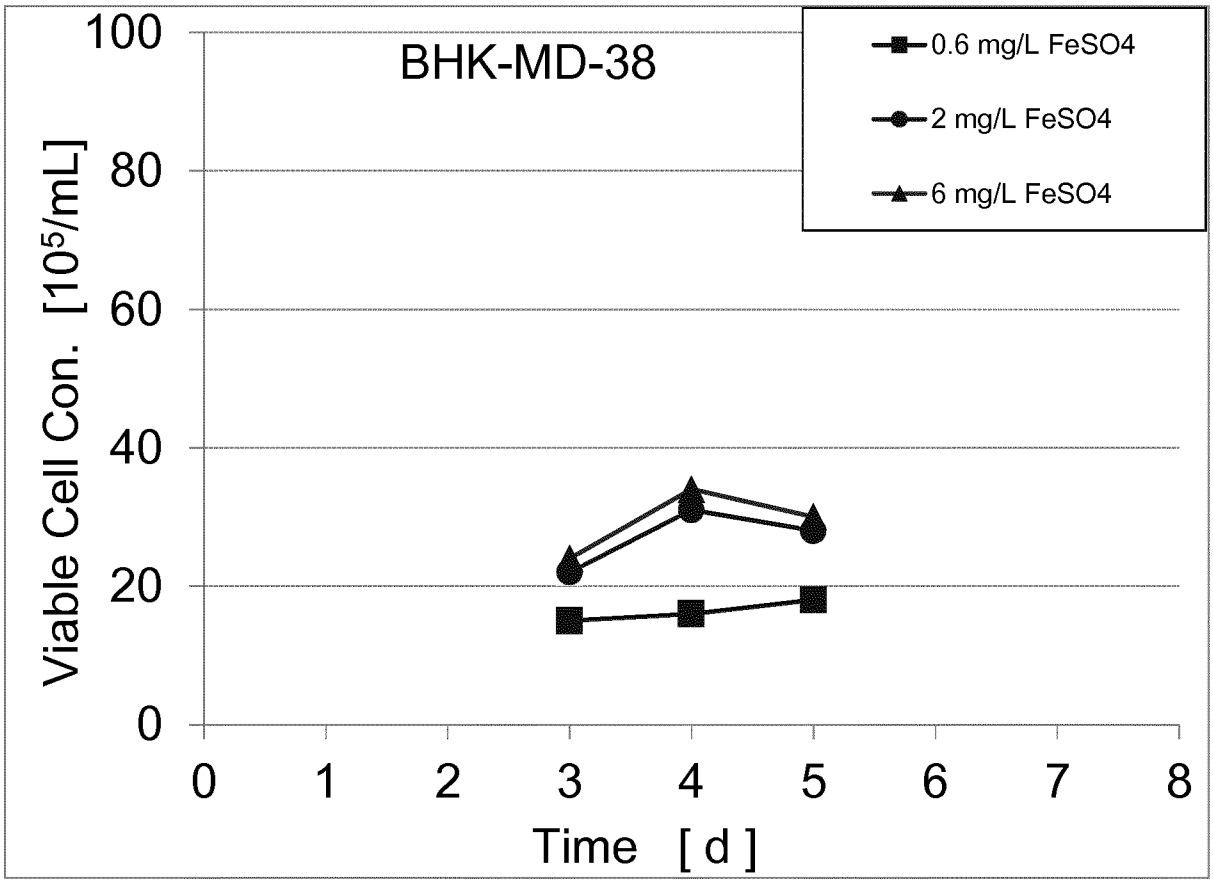
BHK-MD-38
0.6 mg/L FeSO4
2 mg/L FeSO4
6 mg/L FeSO4
Viable Cell Con. [10^5/mL]
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8
Time [d]

Figure 2e

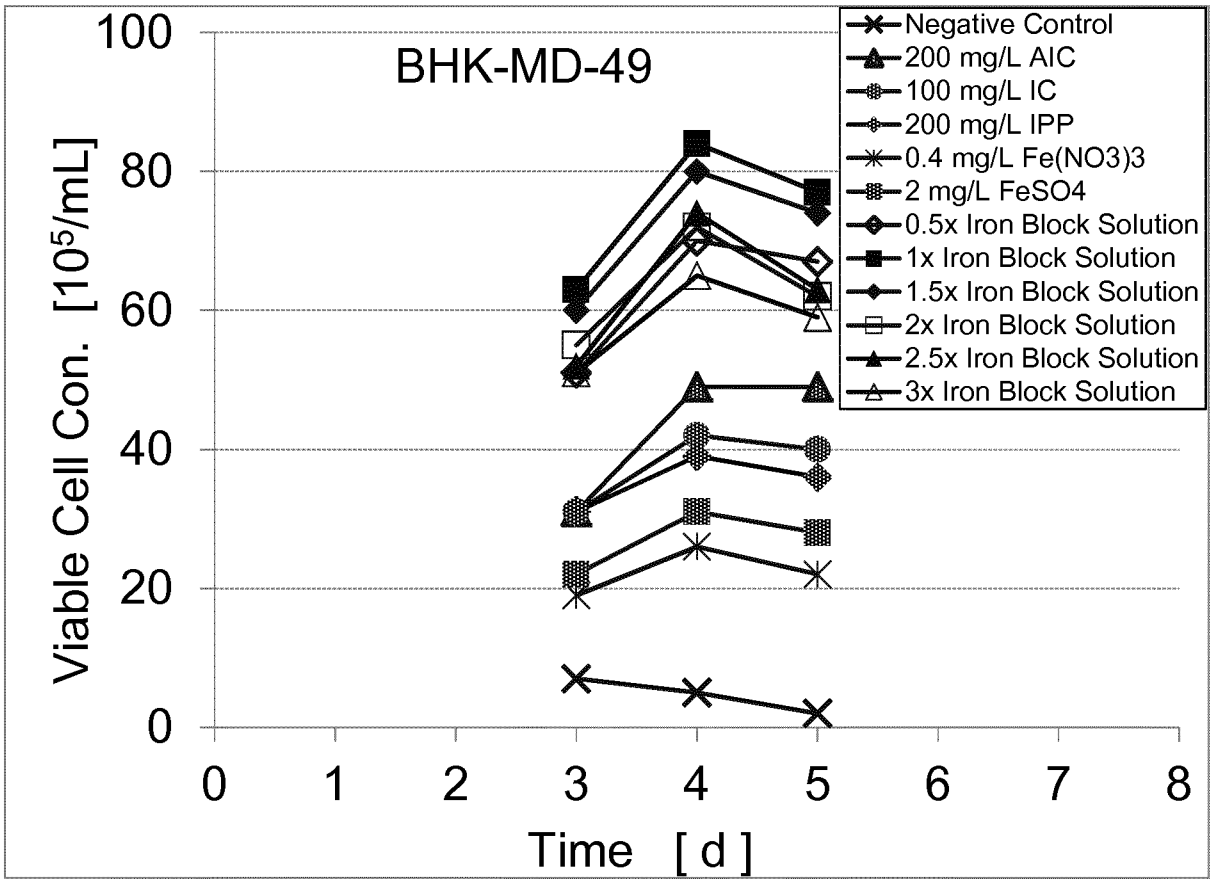
BHK-MD-49
Viable Cell Con. [10^5/mL]
Time [d]
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8
Negative Control
200 mg/L AIC
100 mg/L IC
200 mg/L IPP
0.4 mg/L Fe(NO3)3
2 mg/L FeSO4
0.5x Iron Block Solution
1x Iron Block Solution
1.5x Iron Block Solution
2x Iron Block Solution
2.5x Iron Block Solution
3x Iron Block Solution

Figure 3

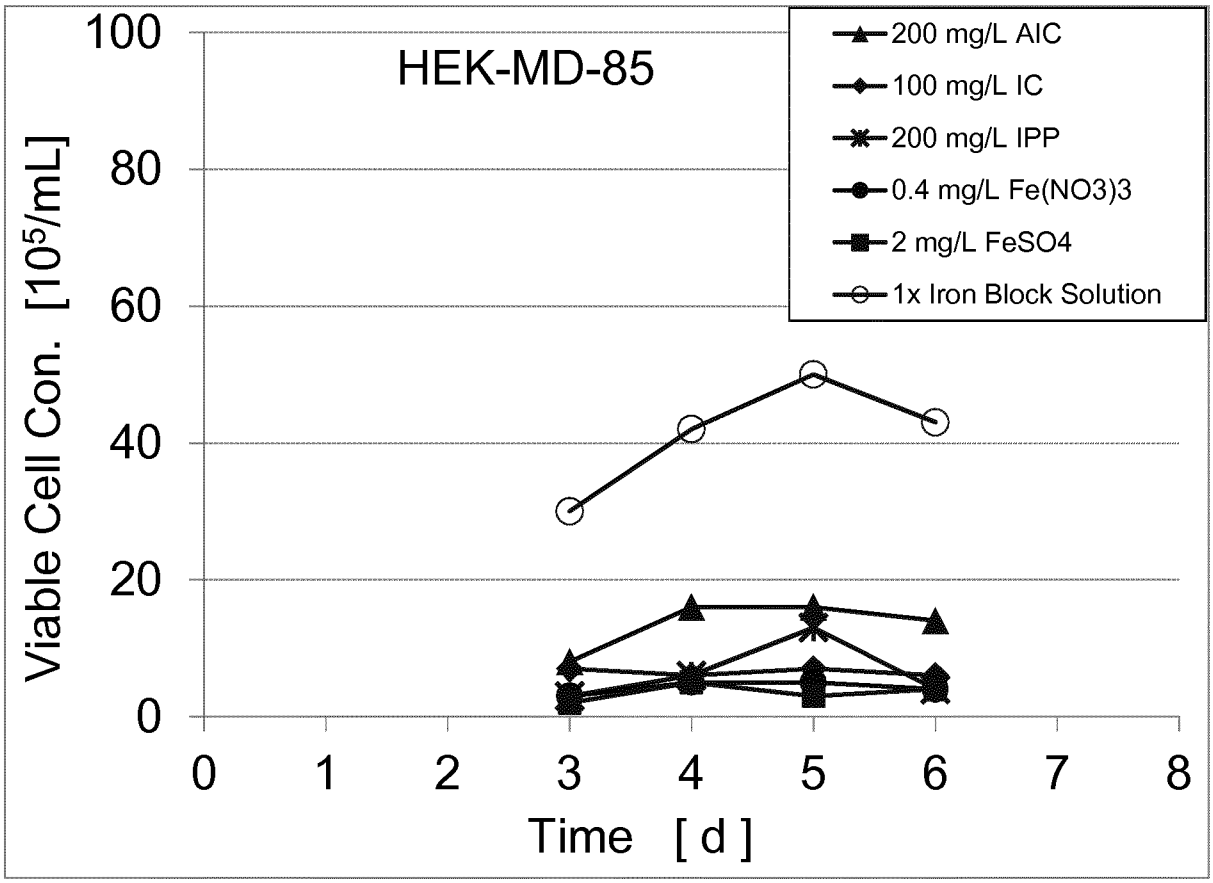
HEK-MD-85
Viable Cell Con. [10^5/mL]
Time [d]
200 mg/L AIC
100 mg/L IC
200 mg/L IPP
0.4 mg/L Fe(NO3)3
2 mg/L FeSO4
1x Iron Block Solution
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8

Figure 4

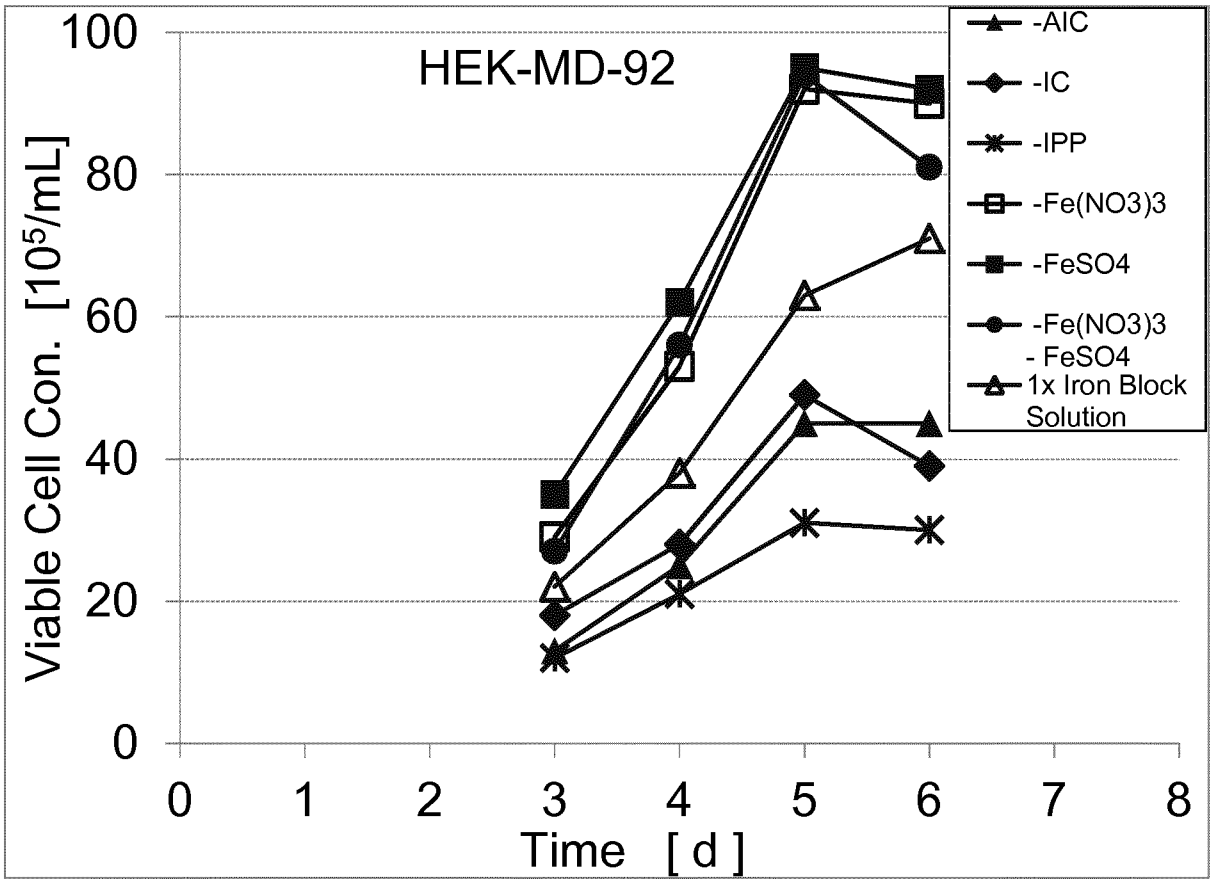
HEK-MD-92
Viable Cell Con. [10^5/mL]
Time [d]
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8
-AIC
-IC
-IPP
-Fe(NO3)3
-FeSO4
-Fe(NO3)3 - FeSO4
1x Iron Block Solution

Figure 5

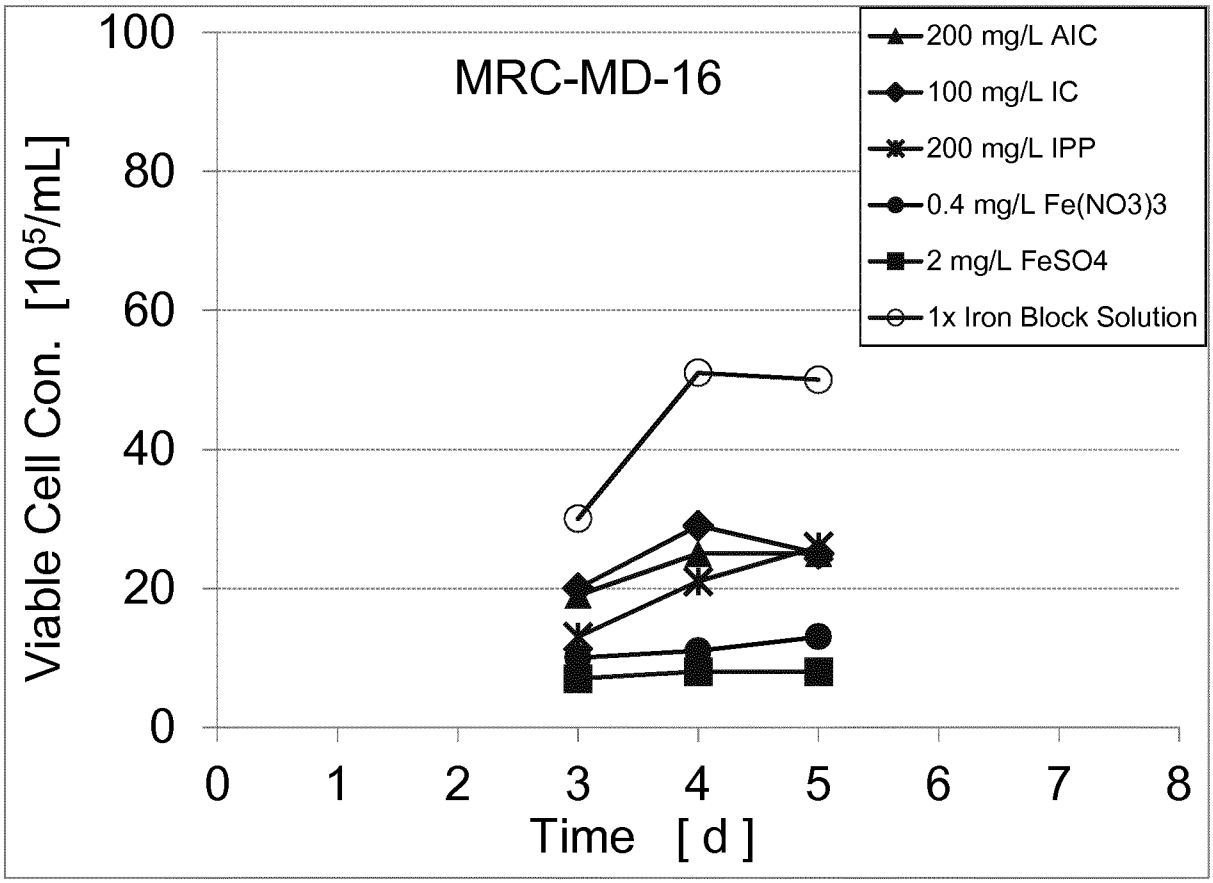
MRC-MD-16
Viable Cell Con. [10^5/mL]
Time [d]
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8
200 mg/L AIC
100 mg/L IC
200 mg/L IPP
0.4 mg/L Fe(NO3)3
2 mg/L FeSO4
1x Iron Block Solution

Figure 6

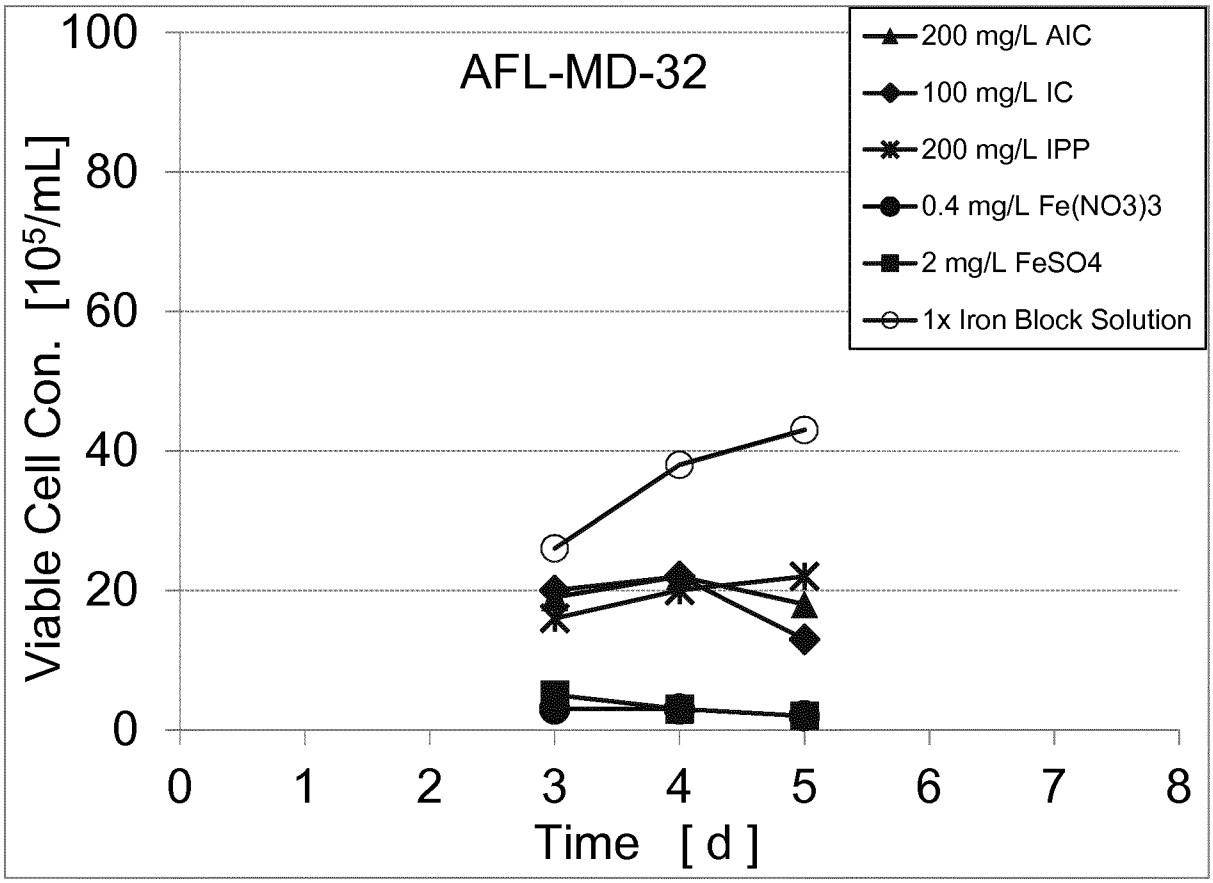
AFL-MD-32
Viable Cell Con. [10^5/mL]
Time [d]
200 mg/L AIC
100 mg/L IC
200 mg/L IPP
0.4 mg/L Fe(NO3)3
2 mg/L FeSO4
1x Iron Block Solution
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8

Figure 7

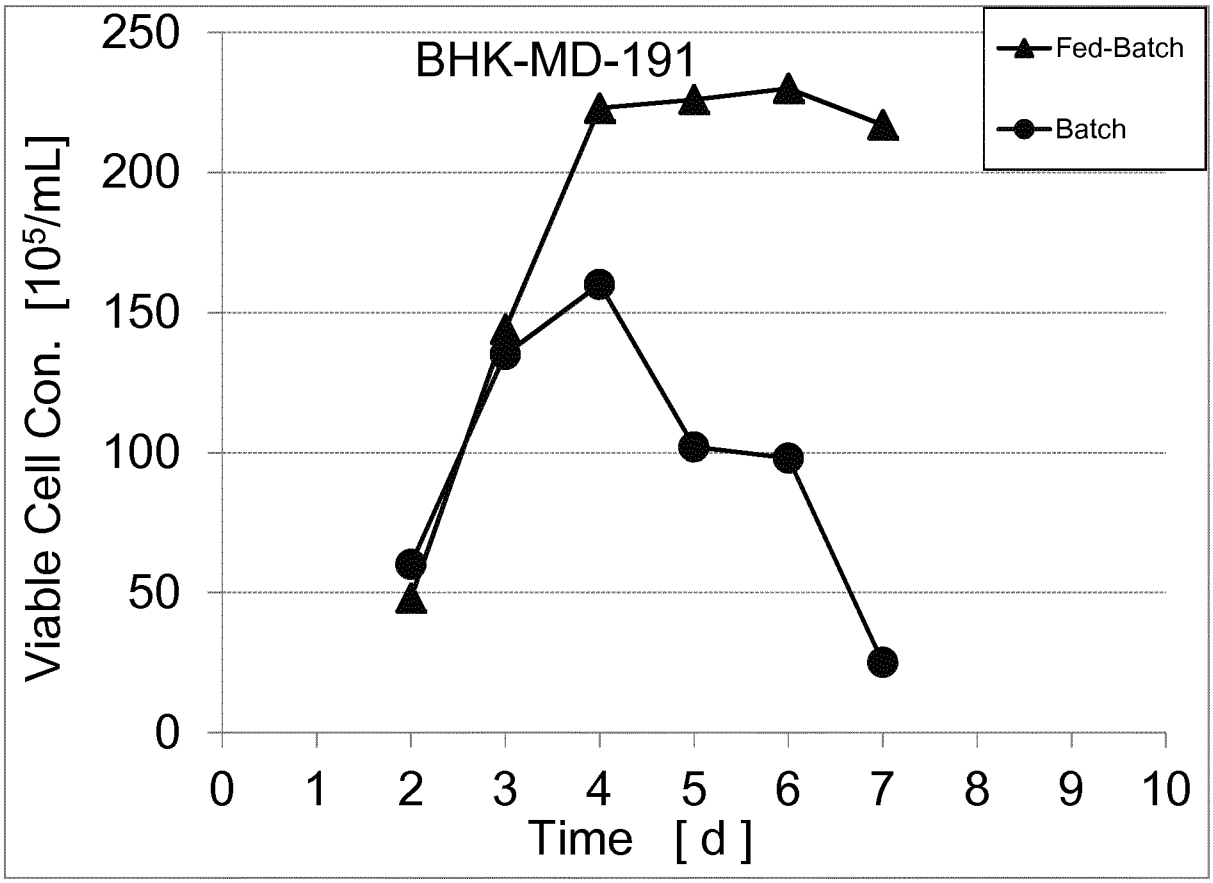
BHK-MD-191
Fed-Batch
Batch
Viable Cell Con. [10^5/mL]
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10
Time [d]

Figure 8

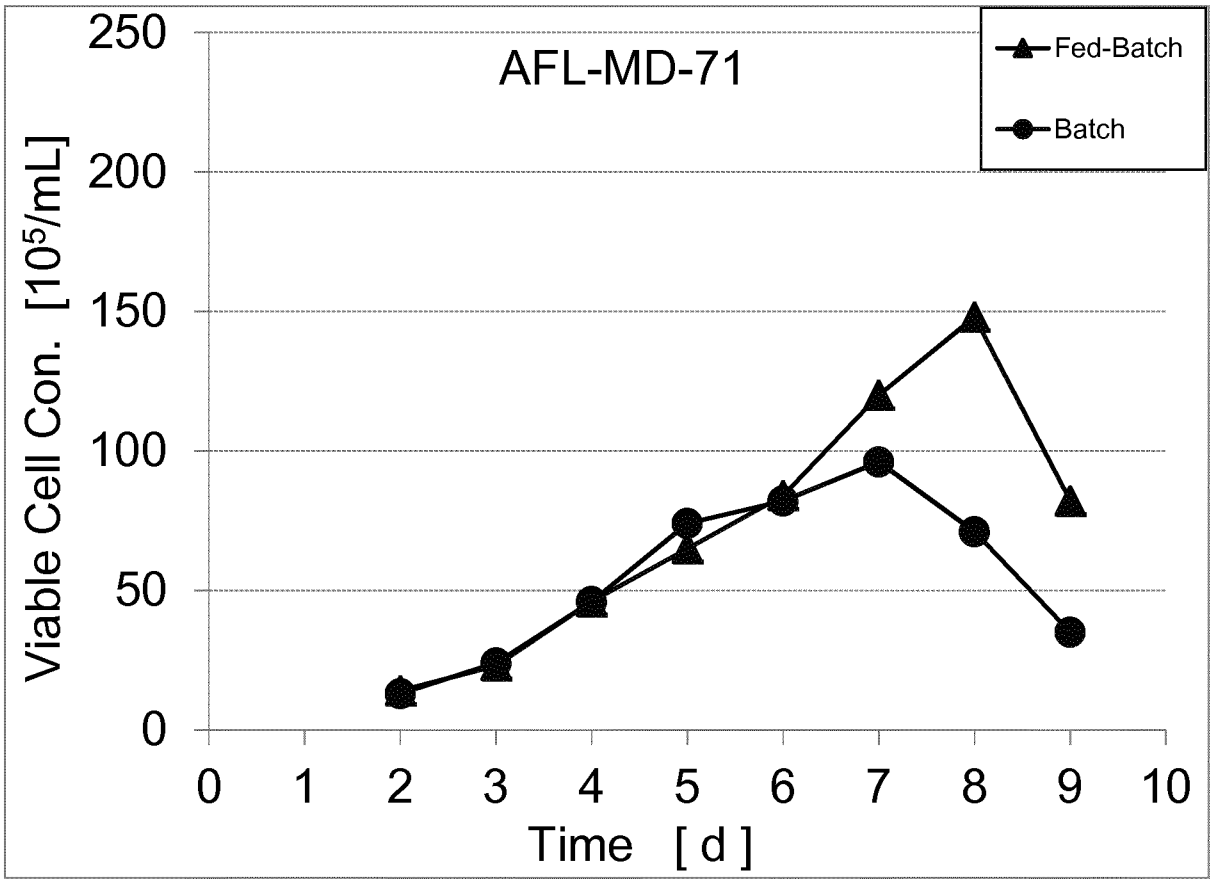
AFL-MD-71
Fed-Batch
Batch
Viable Cell Con. [10^5/mL]
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10
Time [d]

Figure 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/057105

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BERHEUSSEN K ED - KAWAHARA HIROHARU ET AL: "GROWTH OF CELLS IN A NEW DEFINED PROTEIN-FREE MEDIUM", CYTOTECHNOLOGY, KLUWER ACADEMIC PUBLISHERS, DORDRECHT, NL, vol. 11, no. 3, 1 January 1993 (1993-01-01), pages 219-231, XP008004070, ISSN: 0920-9069, DOI: 10.1007/BF00749873 page 225, left-hand column, paragraph 1; tables 1-3, 5 ----- -/--	1-15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2019	05/12/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Petri, Bernhard

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KEENAN J ET AL: "Replacement of transferrin by simple iron compounds for MDCK cells grown and subcultured in serum-free medium", IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY. ANIMAL, SPRINGER US, NEW YORK, vol. 32, no. 8, 1 September 1996 (1996-09-01), pages 451-453, XP009186846, ISSN: 1071-2690, DOI: 10.1007/BF02723044 page 451, left-hand column, paragraph 5 -----	1-15
Y	ILL C R ET AL: "Species specificity of iron delivery in hybridomas", IN VITRO CELLULAR AND DEVELOPMENTAL BIOLOGY, THE ASSOCIATION, GAITHERSBURG, MD, US, vol. 24, no. 5, 1 May 1988 (1988-05-01), pages 413-419, XP008159805, ISSN: 0883-8364, DOI: 10.1007/BF02628492 [retrieved on 2007-05-09] page 413, right-hand column, paragraph 1 page 416, left-hand column, paragraph 2 -----	1-15
Y	YUNLING BAI ET AL: "Role of Iron and Sodium Citrate in Animal Protein-Free CHO Cell Culture Medium on Cell Growth and Monoclonal Antibody Production", 20110101 , vol. 27, no. 1 1 January 2011 (2011-01-01), pages 209-219, XP002678838, DOI: 10.1002/BTPR.513 Retrieved from the Internet: URL:http://onlinelibrary.wiley.com/doi/10.1002/btpr.513/abstract [retrieved on 2010-11-23] figure 1 -----	1-15
Y	US 2018/346881 A1 (CLEMENS CHRISTOPH [DE] ET AL) 6 December 2018 (2018-12-06) paragraphs [0017] - [0019] -----	1-15

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/057105

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2018346881 A1	06-12-2018	AU 2016239662 A1	27-07-2017
		CA 2996643 A1	06-10-2016
		CN 107429227 A	01-12-2017
		EP 3277797 A1	07-02-2018
		JP 2018509920 A	12-04-2018
		KR 20170140251 A	20-12-2017
		SG 11201705743Y A	30-08-2017
		US 2018346881 A1	06-12-2018
		WO 2016156476 A1	06-10-2016