

USE OF PHENOLIC ANTIOXIDANTS IN CELL CULTURE FOR THE PRODUCTION OF PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial No.
5 62/114,635, filed February 11, 2015; the entire content of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to new methods and processes for culturing
10 mammalian cells with the addition of antioxidants. Performance of the cell culturing methods and processes in their various aspects result in a higher viable cell density and higher protein titer.

INTRODUCTION

15 Current ongoing efforts to maximize bioreactor productivity in both time and volume directly affect the scale and capital investment required for a bioreactor suite. As cells reach higher concentrations more quickly, yield is increased; therefore, the number and scale of bioreactors can be reduced. To that end, not only cell engineering, but also culture media and related chemical and physical environments are used to assist cells in
20 reaching peak performance quickly and maintaining a high level as long as possible.

Removing or reducing reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, or singlet oxygen has been shown to improve productivity, presumably through reduction of apoptosis. Yun et al. showed with Chinese Hamster Ovary (CHO) culture that a combination of glutathione and iron chelators
25 decreased intracellular ROS levels and increased the number of viable cells ("Combined Addition of Glutathione and Iron Chelators for Decrease of Intracellular Level of Reactive Oxygen Species and Death of Chinese Hamster Ovary Cells", *J. Biosci. Bioeng.*, 95:124-127 (2003)). Addition of specific iron chelator combinations also reduced ROS and yielded improved viability. Adding several together was more effective than adding
30 single chelators separately. Similar results were reported upon addition of ascorbic acid and glutathione as antioxidants to CHO culture by Yun et al. ("Effect of Antioxidants on

the Apoptosis of CHO Cells and Production of Tissue Plasminogen Activator in Suspension Culture", *J. Biosci. Bioeng.*, 91:581-585 (2001)).

Antioxidants commonly used during cell culture include tocopherol, transferrin, selenium, ascorbate and reduced glutathione. Tocopherol is a membrane antioxidant with low solubility in water that functions to neutralize lipid peroxides. Transferrin is a chelator that binds iron with such high affinity leaving no iron available to generate free radicals. Transferrin serves as an extracellular iron transporter and storage molecule. Glutathione is a water-soluble antioxidant tripeptide, which contains a reducing thiol group. It is ubiquitously produced in all cell types and is important for cell proliferation and viability. Selenium is a component of the antioxidant enzymes glutathione peroxidase and thioredoxin reductase which reduce glutathione and thioredoxin, respectively. Ascorbate is a water-soluble antioxidant that regenerates reduced tocopherol. Most commercially-available basal mediums do not contain antioxidants as they were designed for use in conjunction with serum. The serum would typically provide the required antioxidants.

Recombinantly produced protein products are increasingly becoming medically and clinically important for use as therapeutics, treatments and prophylactics. Therefore, the development of reliable cell culture processes that economically and efficiently achieve an increased viable cell density thereby resulting in increased final protein product concentration fulfills both a desired and needed goal in the art.

SUMMARY OF THE INVENTION

The present invention provides new processes for the production of proteins by animal or mammalian cell cultures. These new processes achieve increased viable cell density, cell viability and productivity.

The inventors have identified suitable phenolic antioxidants over a range of concentrations, in different chemically defined basal and feed media, in small and large scale cell culture using different transfected cell lines that produce antibody or fusion proteins.

On aspect of the invention concerns the growth of cells in antioxidant supplemented culture media. More specifically, the cells are Chinese hamster Ovary cells and the antioxidant is added to the basal or feed media, or both the basal and feed media.

Another aspect of the method of this invention concerns the selection of phenolic antioxidant from the group consisting of apigenin, catechin, chlorogenic acid, daidzein, genistein, hesperetin, melatonin, naringenin, pelargonidin, quercetin, resveratrol, rosmarinic acid and silibinin. More specifically, one or more antioxidant is selected from catechin, chlorogenic acid, pelargonidin, quercetin, resveratrol, rosmarinic acid or silibinin. More specifically, the antioxidant utilized in the method of this invention is catechin or rosmarinic acid.

Another aspect of the method of this invention concerns the amount of antioxidant added to the cell culture. An amount of antioxidant suitable for use in the basal and/or feed medium comprises from about 0.0625 mM to about 1 mM. More specifically, the amount of antioxidant suitable for use in the basal media comprises the lower end of the suitable range, for example, from about 0.0625 mM to 0.25 mM. The amount of antioxidant suitable for the feed media comprises the upper end of the suitable range, for example, from about 0.25 mM to 1 mM.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the impact of 0.0625 mM, 0.25 mM and 1 mM of different antioxidants added to the feed media M154A1B on aCD137 viable cell density (VCD) at day 8 (peak VCD). The basal media M17IB was not supplemented with antioxidant. The cell density for each condition was normalized to the control condition with no additional antioxidants in the feed.

Figure 2 shows the impact of 0.0625 mM, 0.25 mM and 1 mM of different antioxidants added to the feed media M154A1B on productivity of the cultured aCD137 cells. The basal media M17IB was not supplemented with antioxidant. The final titer (harvested on days 14) for each condition was normalized to the control condition with no additional antioxidants in the feed.

Figure 3A-C show the impact of catechin, chlorogenic acid, quercetin, resveratrol and rosmarinic acid on aCD137 cell culture (A) viable cell density, (B) normalized protein titer, and (C) lactate concentration. The antioxidants were added to basal media M17IB at 0.07 mM and feed media M154A1B at 1 mM. The media were then used in a standard shaker flask fed-batch culture with aCD137 cells.

Figure 4A-D show the impact of catechin(□) on aCD137 cell culture (A) viable cell density, (B) cell viability, (C) normalized protein titer, and (D) ammonium concentration. Catechin was added to feed media F3.2 at 1 mM. The media was then used in a standard shaker flask fed-batch culture with aCD137 cells together with basal media B1

Figure 5A-E shows the impact of rosmarinic acid(◆) and catechin(▲) on aCD137 cell culture (A) viable cell density, (B) cell viability, (C) normalized protein titer, (D) lactate concentration and (E) ammonium concentration. Rosmarinic acid or catechin were added to basal media M17IB at 0.1 mM. The media was then used in a standard 7-liter fed-batch culture together with feed media M154A1B.

Figure 6A-D shows the impact of rosmarinic acid (▲) on aCD137 cell culture (A) viable cell density, (B) cell viability, (C) normalized protein titer, and (D) lactate concentration. Rosmarinic acid was added to basal media M17IB at 0.1 mM. The media was then used in a standard fed-batch culture together with feed media M154A1B.

Figure 7A-D shows the impact of rosmarinic acid addition to either basal media B1 at 0.1 mM (RA-Basal) or feed media F3.2 at 1 mM (RA-Feed), or both (RA-Basal+Feed) on (A) viable cell density, (B) cell viability, (C) normalized protein titer and (D) ammonium concentration. The media was then used in a standard shake flask with myostatin cells.

Figure 8A-C shows the impact of rosmarinic acid (RA-Basal) or catechin (Cat-Basal) addition to basal media B1 at 0.1 mM on (A) viable cell density, (B) cell viability, (C) normalized protein titer and (D) ammonium concentration. The media was used in a 20-liter reactor with myostatin cells together with feed media F3.2.

Figure 9A-C shows the impact of catechin addition to feed media 154A1 at 0.34 μM, 3.45 μM, 34.45 μM or 344.52 μM on (A) viable cell density, (B) cell viability and (C) normalized protein titer. Basal media M17IB without catechin was used for the culture. The media was used in standard shake flasks with aCD40L cells.

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to an enhanced process (method) for the preparation of recombinant proteins by mammalian cell cultures, specifically CHO cell cultures, in which the viable cell density and protein titer are increased by the use of a newly-

described media strategy in which antioxidants are added to the cell cultures. The antioxidants are provided to the cell cultures in an amount that is effective for attaining and maintaining viable cell density and therefore maximizing protein production until the end of a culturing run. As described herein, a variety of antioxidant supplementation
5 regimens are encompassed by the present invention.

Culturing Processes Involving Antioxidant Addition

In accordance with this invention, the addition of antioxidants to the cell culture increases and/or maintains viable cell density during the production run. The presence of
10 an effective amount of antioxidants in the basal media and/or over the course of the culturing process was found to result in an increased protein titer. To maintain and/or sustain the viable cell density antioxidants are added to the basal and or feed media. The processes and methods of the present invention are suited to both small (*e.g.*, 50 L-100 L) and large scale (*e.g.*, 500 L and greater) cell cultures. In addition, the methods of the
15 present invention are particularly suited to cells grown and maintained as fed-batch cultures, of both small and large scale. In addition, a variety of culture media as known in the art can be used in the culturing methods of this invention.

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to
20 an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or
25 polyphenols.

A polyphenol antioxidant is a type of antioxidant containing a polyphenolic or natural phenol substructure. Although all polyphenols have similar chemical structures, there are some distinctive differences. Based on these differences polyphenols are subdivided into several major subclasses including: phenolic acids (such as rosmarinic
30 acid and chlorogenic acid), stilbens (such as resveratrol), tannins, flavonolignans (such as silibinin) and flavonoids.

Flavonoids are the largest family of polyphenolic antioxidants. Flavonoids are further divided in several subclasses including: anthocyanins (such as pelargonidin), flavanols (such as catechin), flavanones (such as naringenin and hesperetin), flavonols (such as quercetin), flavones (such as apigenin), and isoflavones (such as genistein and
5 daidzein).

An advantage of the present invention is that protein production costs are reduced by the increase in protein production as achieved by the culturing methods described herein. In a preferred embodiment, higher viable cell density was maintained throughout the culture run when antioxidants were included in the basal and/or feeding medium
10 supplied to the cell culture as shown in Figures 1-9.

One embodiment of the invention involves the addition of antioxidant to the basal media to allow for the production of large amounts of protein independent of the reactor scale as shown in Figures 5, 6, 7 and 8.

One embodiment of the invention involves the maintenance of the antioxidant
15 feeding strategy throughout a production run, preferably, with daily feeding, to allow for the production of large amounts of protein independent of the reactor scale as shown in Figures 1, 2, 4, 7 and 9.

One embodiment of the invention involves the addition of antioxidant to the basal and feed media to allow for the production of large amounts of protein independent of the
20 reactor scale as shown in Figures 3 and 7.

An effective concentration of antioxidants as a component in a basal or feed media, increases, maintains and/or sustains viable cell density throughout the production run. The type and amount of antioxidant suitable for use in the basal or feed medium can be determined by the skilled practitioner based on the reactor size and volume of the
25 culture. The methods of the present invention are suitable for all reactor scales at which protein production occur, including, but not limited to, large and small production scale, and reactor scale, *e.g.*, large scale cultures or commercial scale cultures, *e.g.*, over 50 L, more preferably over 500 L. For example, the antioxidant supplementation strategy is applicable for production scale cultures, *e.g.*, having a volume of about 50 liters (50 L) or
30 less, as well as for reactor scale cultures, which can have a volume of several hundreds or thousands of liters.

In accordance with the methods of this invention, one or more antioxidant is selected from the group consisting of apigenin, catechin, chlorogenic acid, daidzein, genistein, hesperetin, melatonin, naringenin, pelargonidin, quercetin, resveratrol, rosmarinic acid and silibinin; preferably, one or more antioxidant is selected from catechin, chlorogenic acid, pelargonidin, quercetin, resveratrol, rosmarinic acid and silibinin; more preferably, one or more antioxidant is selected from catechin, chlorogenic acid, quercetin, resveratrol and rosmarinic acid; more preferably, the antioxidant is catechin or rosmarinic acid.

In accordance with the methods of this invention, basal or feed medium concentration of one or more of the antioxidants selected from the list above is preferably provided in an amount which affords a higher sustained or maintained viable cell density in the culture, or reactor, during the culturing process. An amount of antioxidant suitable for use in the basal and/or feed medium comprises from about 0.0625 mM to about 1 mM, preferably about 0.1 mM to about 1 mM. More specifically, the amount of antioxidant suitable for use in the basal media comprises the lower end of the suitable range, for example, from about 0.0625 mM to 0.25 mM, and the amount of antioxidant suitable for the feed media comprises the upper end of the suitable range, for example, from about 0.25 mM to 1 mM.

As specific yet nonlimiting examples, 1 mM catechin in the feed medium is suitable for use in the culturing method of the invention; or 0.1 mM rosmarinic acid or catechin in the basal media is suitable for use in the culturing method of the invention; or 0.1 mM rosmarinic acid in the basal media and 1 mM rosmarinic acid in the feed media is suitable for use in the culturing method of the invention.

Cell cultures are fed with feeding medium containing antioxidants of the invention using a variety of feeding schedules or regimens to deliver and maintain the antioxidants in the cultures in amounts that sustain a viable cell density effective antioxidant concentration thereby increasing protein production. In general, the culturing methods of the present invention comprise the feeding of cell cultures with the antioxidants of the invention in the feeding medium more than one time during the culture run. It is to be understood that the culture volume contributed by the feeding medium at the end of a culture run typically comprises approximately 30-60% of the original culture volume.

The cell cultures can be fed the antioxidants of the invention on a daily basis, or on other than a daily basis, *e.g.*, less often than once per day, and at varying intervals, preferably timed intervals, including every other day, every third day, every fourth day, and the like. For example, the feeding of cell cultures with the antioxidants of the invention, preferably with antioxidant-containing feeding medium, can be performed once per day, more than once per day, or less than once per day, and can occur one time, two times, or more than two times, *e.g.*, three, four, five or more times, during the total culture run. In one embodiment, the cells are fed with antioxidants more than once.

Also encompassed by this invention is a continuous feeding schedule, for example, involving a continuous infusion of the antioxidants of the invention, preferably an antioxidant-containing feeding medium, into the cultures. In such a continuous feeding regimen, the cultures receive antioxidants, preferably in feeding medium, for example, as a continuously-supplied "drip", or infusion, or other automated addition to the culture, in a timed, regulated, and/or programmed fashion so as to achieve and maintain the appropriate amount of antioxidant in the culture. Most preferred is a feeding regimen comprising a one time per day bolus feed with antioxidant, preferably with feeding medium containing antioxidant on each day of the culture run, from the beginning of the culture run to the day of harvesting the cells.

In accordance with the invention, the antioxidants of the invention can be fed to the cell culture at any of the aforementioned intervals in some way other than in the feed medium. As non-limiting examples, the antioxidants can be fed to the culture in DMSO, ethanol or water. As a non-limiting example, the culture may be fed with antioxidants and also fed with a feed medium, *i.e.*, there may be more than one composition being fed.

As used herein, the term "feed" refers to any addition of any substance made to a culture after inoculation. Feeding can be one or more additions.

As used herein, the term "inoculation" refers to the addition of cells to starting medium to begin the culture.

As used herein, the terms "feed medium", "feed media" and "feeding medium" refer to a medium containing one or more nutrients that is added to the culture beginning at some time after inoculation.

As used herein, the term "basal medium" and "basal media" refers to starting medium to which cells are added to begin the culture.

In another of its embodiments, the present invention encompasses a cell culture method or process of increasing protein titer in a cell culture, comprising the addition of catechin to the culturing medium. A related embodiment involves a cell culture method in which catechin is supplied to the culture for the duration of the production run, and preferably is present in the feeding medium. A preferred embodiment relates to processes of culturing cells comprising a daily feeding of the culture with medium containing catechin. The addition of catechin to the cells in culture as provided by this method, preferably supplied more than one time during the culture run, more preferably, on a daily basis, increases, maintains viable cell density in the culture, allowing the cells to continue to produce protein, thereby maximizing protein production. Another related embodiment involves a cell culture method in which catechin is supplied to the culture in the basal media. The addition of catechin to the cells in the basal media as provided by this method increases and/or maintains viable cell density in the culture, allowing the cells to continue to produce protein, thereby maximizing protein production. Another related embodiment involves a cell culture method in which catechin is supplied to the culture in the basal media as well as for the duration of the production run, preferably in the feeding medium. A preferred embodiment relates to processes of culturing cells comprising cell inoculation in an antioxidant supplemented basal media and a daily feeding of the culture with medium containing catechin. The addition of catechin to the cells in culture as provided by this method increases and/or maintains viable cell density in the culture, allowing the cells to continue to produce protein, thereby maximizing protein production.

In another of its embodiments, the present invention encompasses a cell culture method or process of increasing protein titer in a cell culture, comprising the addition of rosmarinic acid to the culturing medium. A related embodiment involves a cell culture method in which rosmarinic acid is supplied to the culture for the duration of the production run, and preferably is present in the feeding medium. A preferred embodiment relates to processes of culturing cells comprising a daily feeding of the culture with medium containing rosmarinic acid. The addition of rosmarinic acid to the cells in culture as provided by this method, preferably supplied more than one time during the culture run, more preferably, on a daily basis, increases, maintains viable cell density in the culture, allowing the cells to continue to produce protein, thereby maximizing protein production. Another related embodiment involves a cell culture method in which

rosmarinic acid is supplied to the culture in the basal media. The addition of rosmarinic acid to the cells in basal media as provided by this method increases and/or maintains viable cell density in the culture, allowing the cells to continue to produce protein, thereby maximizing protein production. Another related embodiment involves a cell culture method in which rosmarinic acid is supplied to the culture in the basal media as well as for the duration of the production run, preferably in the feeding medium. A preferred embodiment relates to processes of culturing cells comprising cell inoculation in an antioxidant supplemented basal media and a daily feeding of the culture with medium containing rosmarinic acid. The addition of rosmarinic acid to the cells in culture as provided by this method increases and/or maintains viable cell density in the culture, allowing the cells to continue to produce protein, thereby maximizing protein production.

In accordance with this invention, viable cell density is increased about 10-100% when the cell culture is supplemented with antioxidants, as compared with cell cultures in the absence of antioxidant supplementation.

In accordance with this invention, protein titer is increased, on average, about 30-130% when cell cultures are supplemented with antioxidants, as compared with cell cultures in the absence of antioxidant supplementation.

Screening Natural Phenolic Antioxidants for Bioproduction

Following the cell culture process described in Example 1, thirteen novel antioxidants (Table 1) were added to the feed media utilized in aCD137 cell culture. Three antioxidant concentrations were evaluated: 1 mM (Run#1-13), 0.25 mM (Run#17-29), and 0.0625 mM (Run#33-45). The antioxidants were dissolved in either pure ethanol or dimethyl sulfoxide (DMSO) at 50 mM stock concentration and added to feed media M154A1B at the designed final concentrations. The corresponding solvent negative controls were run number 16, 32, and 48. Two traditional antioxidants, ascorbic acid (Run#14, 30, and 46) and reduced glutathione (Run#15, 31, and 47), served as positive controls. The basal media M17IB was not supplemented with antioxidant.

Table 1: List of Antioxidants and Concentrations Evaluated

Run#	Component	Concentration (mM)	Run#	Component	Concentration (mM)
1	Catechin	1	25	Resveratrol	0.25
2	Apigenin	1	26	Melatonin	0.25
3	Naringenin	1	27	Chlorogenic acid	0.25
4	Hesperetin	1	28	Rosmarinic acid	0.25
5	Quercetin	1	29	Silibinin	0.25
6	Pelargonidin	1	30	Ascorbic Acid	0.25
7	Genistein	1	31	L-Glutathione reduced	0.25
8	Daidzein	1	32	Solvent-Ethanol	1% v/v
9	Resveratrol	1	33	Catechin	0.0625
10	Melatonin	1	34	Apigenin	0.0625
11	Chlorogenic acid	1	35	Naringenin	0.0625
12	Rosmarinic acid	1	36	Hesperetin	0.0625
13	Silibinin	1	37	Quercetin	0.0625
14	Ascorbic Acid	1	38	Pelargonidin	0.0625
15	L-Glutathione reduced	1	39	Genistein	0.0625
16	Solvent-DMSO	1% v / v	40	Daidzein	0.0625
17	Catechin	0.25	41	Resveratrol	0.0625
18	Apigenin	0.25	42	Melatonin	0.0625
19	Naringenin	0.25	43	Chlorogenic acid	0.0625
20	Hesperetin	0.25	44	Rosmarinic acid	0.0625
21	Quercetin	0.25	45	Silibinin	0.0625
22	Pelargonidin	0.25	46	Ascorbic Acid	0.0625
23	Genistein	0.25	47	L-Glutathione reduced	0.0625
24	Daidzein	0.25	48	Solvent-H ₂ O	N/A

As shown in Figure 1, addition of the antioxidants to the feed media affected viable cell density (VCD) on day 8 (peak VCD). In some instances, viable cell density was increased by as much as 30% by the addition of antioxidant compared to the controls (DMSO, ethanol, or water).

5 As shown in Figure 2, addition of the antioxidants to the feed media also affected the productivity of the cultured cells. In some instances, protein titer was increased by as much as 80% by addition of antioxidant compared to the controls (DMSO, ethanol, or water).

10 The antioxidants with the greatest positive impact on protein titer are presented in Table 2 below. Addition of catechin, chlorogenic acid, resveratrol, and rosmarinic acid increased protein titer when feed at all three concentrations (0.0625-1 mM). Quercetin feed at 0.25 mM and 1 mM increased protein titer. Pelargonidin and silibinin feed increased protein titer when feed at 1 mM.

15 Table 2: List of Antioxidants and Concentrations that Improved Protein Titer

Component	VCD%	Titer%	Concentration (mM)
Catechin	102.01	116.67	1
Catechin	93.52	112.70	0.25
Catechin	98.66	111.90	0.0625
Chlorogenic acid	129.57	151.59	1
Chlorogenic acid	109.35	109.92	0.25
Chlorogenic acid	115.85	110.71	0.0625
Pelargonidin	104.47	118.25	1
Quercetin	123.11	128.97	1
Quercetin	119.28	111.90	0.25
Resveratrol	107.48	144.05	1
Resveratrol	92.24	111.90	0.25
Resveratrol	102.53	112.70	0.0625
Rosmarinic acid	124.08	180.16	1
Rosmarinic acid	125.43	146.83	0.25

Component	VCD%	Titer%	Concentration (mM)
Rosmarinic acid	129.25	132.14	0.0625
Silibinin	99.11	144.44	1

Cells, Proteins and Cell Culture

In the cell culture processes or methods of this invention, the cells can be maintained in a variety of cell culture media, *i.e.*, basal culture media, as conventionally known in the art. For example, the methods are applicable for use with large volumes of cells maintained in cell culture medium, which can be supplemented with nutrients and the like. Typically, "cell culturing medium" (also called "culture medium") is a term that is understood by the practitioner in the art and is known to refer to a nutrient solution in which cells, preferably animal or mammalian cells, are grown and which generally provides at least one or more components from the following: an energy source (usually in the form of a carbohydrate such as glucose); all essential amino acids, and generally the twenty basic amino acids, plus cysteine; vitamins and/or other organic compounds typically required at low concentrations; lipids or free fatty acids, *e.g.*, linoleic acid; and trace elements, *e.g.*, inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range. Cell culture medium can also be supplemented to contain a variety of optional components, such as hormones and other growth factors, *e.g.*, insulin, transferrin, epidermal growth factor, serum, and the like; salts, *e.g.*, calcium, magnesium and phosphate, and buffers, *e.g.*, HEPES; nucleosides and bases, *e.g.*, adenosine, thymidine, hypoxanthine; and protein and tissue hydrolysates, *e.g.*, hydrolyzed animal protein (peptone or peptone mixtures, which can be obtained from animal byproducts, purified gelatin or plant material); antibiotics, *e.g.*, gentamycin; and cell protective agents, *e.g.*, a PLURONIC® polyol (PLURONIC® F68). Preferred is a cell nutrition medium that is serum-free and free of products or ingredients of animal origin.

As is appreciated by the practitioner, animal or mammalian cells are cultured in a medium suitable for the particular cells being cultured and which can be determined by the person of skill in the art without undue experimentation. Commercially available media can be utilized and include, for example, Minimal Essential Medium (MEM, Sigma, St. Louis, MO); Ham's F10 Medium (Sigma); Dulbecco's Modified Eagles

Medium (DMEM, Sigma); RPMI-1640 Medium (Sigma); HYCLONE® cell culture medium (HyClone, Logan, UT); and chemically-defined (CD) media, which are formulated for particular cell types. To the foregoing, exemplary media can be added the above-described supplementary components or ingredients, including optional
5 components, in appropriate concentrations or amounts, as necessary or desired, and as would be known and practiced by those having in the art using routine skill.

In addition, cell culture conditions suitable for the methods of the present invention are those that are typically employed and known for batch, fed-batch, or continuous culturing of cells, with attention paid to pH (*e.g.*, about 6.5 to about 7.5),
10 dissolved oxygen (O₂) (*e.g.*, between about 5-90% of air saturation), carbon dioxide (CO₂) (*e.g.*, between about 10-150%), agitation (between about 50 to 200 rpm) and humidity, in addition to temperature (between about 30 °C to 37 °C). As an illustrative, yet nonlimiting, example, a suitable cell culturing medium for the fed-batch processes of the present invention comprises a chemically defined basal and feed medium, preferably
15 one or both containing the antioxidants of the invention (*e.g.*, Example1).

Animal cells, mammalian cells, cultured cells, animal or mammalian host cells, host cells, recombinant cells, recombinant host cells, and the like, are all terms for the cells that can be cultured according to the processes of this invention. Such cells are typically cell lines obtained or derived from mammals and are able to grow and survive
20 when placed in either monolayer culture or suspension culture in medium containing appropriate nutrients and/or growth factors. Growth factors and nutrients that are necessary for the growth and maintenance of particular cell cultures are able to be readily determined empirically by those having skill in the pertinent art, such as is described, for example, by Barnes et al. (*Cell*, 22:649 (1980)); in Mather, J.P., ed., *Mammalian Cell*
25 *Culture*, Plenum Press, NY (1984); and in U.S. Patent No. 5,721,121.

Numerous types of cells can be cultured according to the methods of the present invention. The cells are typically animal or mammalian cells that can express and secrete, or that can be molecularly engineered to express and secrete, large quantities of a particular protein into the culture medium. It will be understood that the protein produced
30 by a host cell can be endogenous or homologous to the host cell. Alternatively, and preferably, the protein is heterologous, *i.e.*, foreign, to the host cell, for example, a human protein produced and secreted by a Chinese hamster ovary (CHO) host cell.

Examples of mammalian proteins that can be advantageously produced by the methods of this invention include, without limitation, cytokines, cytokine receptors, growth factors (*e.g.*, EGF, HER-2, FGF- α , FGF- β , TGF- α , TGF- β , PDGF, IGF-1, IGF-2, NGF, NGF- β); growth factor receptors, including fusion or chimeric proteins. Other nonlimiting examples include growth hormones (*e.g.*, human growth hormone, bovine growth hormone); insulin (*e.g.*, insulin A chain and insulin B chain), proinsulin; erythropoietin (EPO); colony stimulating factors (*e.g.*, G-CSF, GM-CSF, M-CSF); interleukins (*e.g.*, IL-1 through IL-12); vascular endothelial growth factor (VEGF) and its receptor (VEGF-R); interferons (*e.g.*, IFN- α , β , or γ); tumor necrosis factor (*e.g.*, TNF- α and TNF- β) and their receptors, TNFR-1 and TNFR-2; thrombopoietin (TPO); thrombin; brain natriuretic peptide (BNP); clotting factors (*e.g.*, Factor VIII, Factor IX, von Willebrands factor, and the like); anti-clotting factors; tissue plasminogen activator (TPA), *e.g.*, urokinase or human urine or tissue type TPA; follicle stimulating hormone (FSH); luteinizing hormone (LH); calcitonin; CD proteins (*e.g.*, CD3, CD4, CD8, CD28, CD19, etc.); CTLA proteins (*e.g.*, CTLA4); T-cell and B-cell receptor proteins; bone morphogenic proteins (BNPs, *e.g.*, BMP-1, BMP-2, BMP-3, etc.); neurotrophic factors, *e.g.*, bone derived neurotrophic factor (BDNF); neurotrophins, *e.g.*, 3-6; renin; rheumatoid factor; RANTES; albumin; relaxin; macrophage inhibitory protein (*e.g.*, MIP-1, MIP-2); viral proteins or antigens; surface membrane proteins; ion channel proteins; enzymes; regulatory proteins; antibodies; immunomodulatory proteins, (*e.g.*, HLA, MHC, the B7 family); homing receptors; transport proteins; superoxide dismutase (SOD); G-protein coupled receptor proteins (GPCRs); neuromodulatory proteins; Alzheimer's Disease associated proteins and peptides, (*e.g.*, A-beta), and others as known in the art. Fusion proteins and polypeptides, chimeric proteins and polypeptides, as well as fragments or portions, or mutants, variants, or analogs of any of the aforementioned proteins and polypeptides are also included among the suitable proteins, polypeptides and peptides that can be produced by the methods of the present invention.

Nonlimiting examples of animal or mammalian host cells suitable for harboring, expressing, and producing proteins for subsequent isolation and/or purification include Chinese hamster ovary cells (CHO), such as CHO-K1 (ATCC® CCL-61), DG44 (Chasin et al., *Som. Cell Molec. Genet.*, 12:555-556 (1986); Kolkekar et al., *Biochemistry*, 36:10901-10909 (1997); and WO 01/92337 A2), dihydrofolate reductase negative CHO

cells (CHO/-DHFR, Urlaub et al., *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)), and dp12.CHO cells (U.S. Patent No. 5,721,121); monkey kidney CV1 cells transformed by SV40 (COS cells, COS-7, ATCC® CRL-1651); human embryonic kidney cells (*e.g.*, 293 cells, or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen.* 5 *Virol.*, 36:59 (1977)); baby hamster kidney cells (BHK, ATCC® CCL-10); monkey kidney cells (CV1, ATCC® CCL-70); African green monkey kidney cells (VERO-76, ATCC® CRL-1587; VERO, ATCC® CCL-81); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 (1980)); human cervical carcinoma cells (HELA, ATCC® CCL-2); canine kidney cells (MDCK, ATCC® CCL-34); human lung cells (W138, ATCC® CCL-10 75); human hepatoma cells (HEP-G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC® CCL-51); buffalo rat liver cells (BRL 3A, ATCC® CRL-1442); TRI cells (Mather, *Annals NY Acad. Sci.*, 383:44-68 (1982)); MCR 5 cells; FS4 cells.

Preferred are CHO cells, particularly, CHO/-DHFR cells.

The cells suitable for culturing in the methods and processes of the present invention can contain introduced (*e.g.*, via transformation, transfection, infection, or injection) expression vectors (constructs), such as plasmids and the like, that harbor coding sequences, or portions thereof, encoding the proteins for expression and production in the culturing process. Such expression vectors contain the necessary elements for the transcription and translation of the inserted coding sequence. Methods 20 which are well known to and practiced by those skilled in the art can be used to construct expression vectors containing sequences encoding the produced proteins and polypeptides, as well as the appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described in Sambrook, J. et al., 25 *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Plainview, NY (1989) and in Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY (1989).

Control elements, or regulatory sequences, are those non-translated regions of the vector (*e.g.*, enhancers, promoters, 5' and 3' untranslated regions) that interact with host 30 cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. Depending on the vector system and host cell utilized, any number of suitable transcription and translation elements, including constitutive and

inducible promoters, can be used. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferred. The constructs for use in protein expression systems are designed to contain at least one promoter, an enhancer sequence (optional, for mammalian expression systems), and other sequences as
5 necessary or required for proper transcription and regulation of gene expression (*e.g.*, transcriptional initiation and termination sequences, origin of replication sites, polyadenylation sequences, *e.g.*, the Bovine Growth Hormone (BGH) poly A sequence).

As will be appreciated by those skilled in the art, the selection of the appropriate vector, components for proper transcription, expression, and isolation of proteins
10 produced in eukaryotic expression systems is known and routinely determined and practiced by those having skill in the art. The expression of proteins by the cells cultured in accordance with the methods of this invention can be placed under the control of promoters such as viral promoters, *e.g.*, cytomegalovirus (CMV), Rous sarcoma virus (RSV), phosphoglycerol kinase (PGK), thymidine kinase (TK), or the α -ACTIN®
15 promoter. Further, regulated promoters confer inducibility by particular compounds or molecules, *e.g.*, the glucocorticoid response element (GRE) of mouse mammary tumor virus (MMTV) is induced by glucocorticoids (Chandler, V. et al., *Cell*, 33:489-499 (1983)). Also, tissue-specific promoters or regulatory elements can be used (Swift, G. et al., *Cell*, 38:639-646 (1984)), if necessary or desired.

20 Expression constructs can be introduced into cells by a variety of gene transfer methods known to those skilled in the art, for example, conventional gene transfection methods, such as calcium phosphate co-precipitation, liposomal transfection, microinjection, electroporation, and infection or viral transduction. The choice of the method is within the competence of the skilled practitioner in the art. It will be apparent
25 to those skilled in the art that one or more constructs carrying DNA sequences for expression in cells can be transfected into the cells such that expression products are subsequently produced in and/or obtained from the cells.

In a particular aspect, mammalian expression systems containing appropriate control and regulatory sequences are preferred for use in protein expressing mammalian
30 cells of the present invention. Commonly used eukaryotic control sequences for generating mammalian expression vectors include promoters and control sequences compatible with mammalian cells such as, for example, the cytomegalovirus (CMV)

promoter (CDM8 vector) and avian sarcoma virus (ASV) π LN vector. Other commonly used promoters include the early and late promoters from Simian Virus 40 (SV40) (Fiers et al., *Nature*, 273:113 (1973)), or other viral promoters such as those derived from polyoma, Adenovirus 2, and bovine papilloma virus. An inducible promoter, such as
 5 hMTII (Karin et al., *Nature*, 299:797-802 (1982)) can also be used.

Examples of expression vectors suitable for eukaryotic host cells include, but are not limited to, vectors for mammalian host cells (*e.g.*, BPV-1, pHyg, pRSV, pSV2, pTK2 (Maniatis); pIRES (Clontech); pRc/CMV2, pRc/RSV, pSFV1 (Life Technologies); pVPakc Vectors, pCMV vectors, pSG5 vectors (Stratagene), retroviral vectors (*e.g.*, pFB
 10 vectors (Stratagene)), pcDNA-3 (Invitrogen), adenoviral vectors; Adeno-associated virus vectors, baculovirus vectors, yeast vectors (*e.g.*, pESC vectors (Stratagene)), or modified forms of any of the foregoing. Vectors can also contain enhancer sequences upstream or downstream of promoter region sequences for optimizing gene expression.

A selectable marker can also be used in a recombinant vector (*e.g.*, a plasmid) to
 15 confer resistance to the cells harboring (preferably, having stably integrated) the vector to allow their selection in appropriate selection medium. A number of selection systems can be used, including but not limited to, the Herpes Simplex Virus thymidine kinase (HSV TK), (Wigler et al., *Cell*, 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (HGPRT), (Szybalska et al., *Proc. Natl. Acad. Sci. USA*,
 20 48:202 (1992)), and adenine phosphoribosyltransferase (Lowy et al., *Cell*, 22:817 (1980)) genes, which can be employed in tk-, hgp^rt-, or ap^rt-cells (APRT), respectively.

Anti-metabolite resistance can also be used as the basis of selection for the following nonlimiting examples of marker genes: dhfr, which confers resistance to methotrexate (Wigler et al., *Proc. Natl. Acad. Sci. USA*, 77:357 (1980); and O'Hare et al.,
 25 *Proc. Natl. Acad. Sci. USA*, 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan et al., *Proc. Natl. Acad. Sci. USA*, 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G418 (*Clin. Pharmacy*, 12:488-505; Wu et al., *Biotherapy*, 3:87-95 (1991); Tolstoshev, *Ann. Rev. Pharmacol. Toxicol.*, 32:573-596 (1993); Mulligan, *Science*, 260:926-932 (1993); Anderson, *Ann. Rev. Biochem.*,
 30 62:191-21 (1993); *TIB TECH*, 11(5):155-215 (May 1993); and hyg^r, which confers resistance to hygromycin (Santerre et al., *Gene*, 30:147 (1984)). Methods commonly known in the art of recombinant DNA technology can be routinely applied to select the

desired recombinant cell clones, and such methods are described, for example, in Ausubel et al., eds., *Current Protocols in Molecular Biology*, John Wiley & Sons, NY (1993); Kriegler, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY (1990); in Dracopoli et al., eds., Chapters 12 and 13, *Current Protocols in Human*
5 *Genetics*, John Wiley & Sons, NY (1994); Colberre-Garapin et al., *J. Mol. Biol.*, 150:1 (1981), which are incorporated by reference herein in their entireties.

In addition, the expression levels of an expressed protein molecule can be increased by vector amplification (for a review, see Bebbington et al., Chapter 8: "The use of vectors based on gene amplification for the expression of cloned genes in
10 mammalian cells in DNA cloning", *DNA Cloning: A Practical Approach*, Vol. 3, pp. 163-188, IRL Press Limited (1987)). When a marker in the vector system expressing a protein is amplifiable, an increase in the level of inhibitor present in the host cell culture will increase the number of copies of the marker gene. Since the amplified region is associated with the protein-encoding gene, production of the protein will concomitantly
15 increase (Crouse et al., *Mol. Cell. Biol.*, 3:257 (1983)).

Vectors which harbor glutamine synthase (GS) or dihydrofolate reductase (DHFR) encoding nucleic acid as the selectable markers can be amplified in the presence of the drugs methionine sulfoximine or methotrexate, respectively. An advantage of glutamine synthase based vectors is the availability of cell lines (e.g., the murine
20 myeloma cell line, NSO) which are glutamine synthase negative. Glutamine synthase expression systems can also function in glutamine synthase expressing cells (e.g., CHO cells) by providing additional inhibitor to prevent the functioning of the endogenous gene.

Vectors that express DHFR as the selectable marker include, but are not limited to, the pSV2-dhfr plasmid (Subramani et al., *Mol. Cell. Biol.*, 1:854 (1981). Vectors that
25 express glutamine synthase as the selectable marker include, but are not limited to, the pEE6 expression vector described in Stephens et al., *Nucl. Acids. Res.*, 17:7110 (1989). A glutamine synthase expression system and components thereof are detailed in PCT publications: WO 87/04462; WO 86/05807; WO 89/01036; WO 89/10404; and WO 91/06657 which are incorporated by reference herein in their entireties. In addition,
30 glutamine synthase expression vectors that can be used in accordance with the present invention are commercially available from suppliers, including, for example, Lonza Biologics, Inc. (Portsmouth, NH).

Types of Cell Cultures

For the purposes of understanding, yet without limitation, it will be appreciated by the skilled practitioner that cell cultures and culturing runs for protein production can include three general types; namely, continuous culture, batch culture and fed-batch culture. In a continuous culture, for example, fresh culture medium supplement (*i.e.*, feeding medium) is provided to the cells during the culturing period, while old culture medium is removed daily and the product is harvested, for example, daily or continuously. In continuous culture, feeding medium can be added daily and can be added continuously, *i.e.*, as a drip or infusion. For continuous culturing, the cells can remain in culture as long as is desired, so long as the cells remain alive and the environmental and culturing conditions are maintained.

In batch culture, cells are initially cultured in medium and this medium is neither removed, replaced, nor supplemented, *i.e.*, the cells are not "fed" with new medium, during or before the end of the culturing run. The desired product is harvested at the end of the culturing run.

For fed-batch cultures, the culturing run time is increased by supplementing the culture medium one or more times daily (or continuously) with fresh medium during the run, *i.e.*, the cells are "fed" with new medium ("feeding medium") during the culturing period. Fed-batch cultures can include the various feeding regimens and times as described above, for example, daily, every other day, every two days, etc., more than once per day, or less than once per day, and so on. Further, fed-batch cultures can be fed continuously with feeding medium. The desired product is then harvested at the end of the culturing/production run.

Phases of Cell Culture and Associated Parameters

The term "inoculation" refers to the addition of cells to starting medium to begin the culture.

The growth phase of a culture is the phase during which the viable cell density at any time point is higher than at any previous time point.

The stationary phase of a culture is the phase during which the viable cell density is approximately constant (*i.e.*, within measuring error) over a time period of any length.

The death phase of a culture is the phase that comes after the growth phase or after the growth phase and the stationary phase, and during which the viable cell density at any time point is lower than at any previous time point during that phase.

In a growth-associated culture process, the production phase may start during the
5 extended growth phase.

In a non-growth associated culture process, the production phase of cell culture may be the stationary phase.

Preferably, the culture medium is supplemented ("fed") during the production phase to support continued protein production, particularly in an extended production
10 phase, and to attain ample quantities of protein product. Feeding can occur on a daily basis, or according to other schedules to support cell viability and protein production.

Antioxidants Improved Process Performance for Multiple CHO Clones aCD137 Cells

15 In another embodiment, the *dhfr*-negative Chinese Hamster Ovary (CHO) cell line DG44 (Invitrogen Corp. Carlsbad, CA) were transfected in order to establish a stable cell line expressing a human IgG4 antibody.

The transfected CHO DG44 cells expressing the antibody was grown in the presence of antioxidants according to the methods of the invention.

20 As shown in Figure 3, catechin, chlorogenic acid, quercetin, resveratrol or rosmarinic acid increased viable cell density and protein titer when added to both the basal media at 0.07 mM and the feed media at 1 mM.

Additionally, lactate production was reduced. Lactate produced in the glucose metabolism process is a major inhibitory waste product in mammalian cell cultures. High
25 lactate concentrations may be an indication of malfunction of cell energy metabolism. Additionally, high concentrations of lactate may also alter the pH of the cell culture. Therefore, it is preferred to maintain a lower lactate level (<5 g/L) during the course of production.

As shown in Figure 4, catechin addition to the cell culture maintained viable cell
30 density and increased protein titer when added to the feed media at 1 mM.

Further, ammonium formation was reduced. Like lactate, ammonium is a major inhibitory waste product in mammalian cell cultures. High ammonium concentrations

may alter the pH of the cell culture. Additionally, it is believed that high ammonium level may alter the quality attributes of the glycoproteins produced (N-link, etc.). Therefore, it is preferred to maintain a lower ammonium level for a cell culture process (<10 mM; <5 mM mostly preferred).

5 As shown in Figure 5, rosmarinic acid or catechin addition to the cell culture increased viable cell density and protein titer; and reduced ammonium and lactate formation. Rosmarinic acid or catechin was added to basal media at 0.1 mM.

 As shown in Figure 6, the positive rosmarinic effect is maintained when the cell culture is scaled up to 500-liter production run. Adding rosmarinic acid to the basal
10 media at 0.1 mM increased viable cell density and protein titer, and reduced lactate formation.

Myostatin Cells

 In another embodiment, the *dhfr*-negative Chinese Hamster Ovary (CHO) cell line
15 DG44 (Invitrogen Corp. Carlsbad, CA) was transfected in order to establish a stable cell line expressing a recombinant human fusion protein.

 The transfected CHO DG44 cells expressing the fusion protein was grown in the presence of antioxidants according to the methods of the invention.

 As shown in Figure 7, rosmarinic acid addition to the cell culture either in the
20 basal media at 0.1 mM or feed media at 1 mM or both, the feed and basal media, increased the viable cell density and protein titer, and reduced ammonium formation.

 Figure 8 shows that when the cell culture was scaled up to a 20-liter bioreactor, the positive rosmarinic acid and catechin effect on protein titer was maintained while the viable cell density was not changed by the addition of rosmarinic acid or catechin to the
25 basal media at 0.1 mM.

CD40L Cells

 In another embodiment, the *dhfr*-negative Chinese Hamster Ovary (CHO) cell line
30 DG44 (Invitrogen Corp. Carlsbad, CA) was transfected in order to establish a stable cell line expressing a recombinant human fusion protein.

 The transfected CHO DG44 cells expressing the fusion protein was grown in the presence of antioxidants according to the methods of the invention.

As shown in Figure 9, the increase in viable cell density and protein titer corresponds to an increase in catechin concentration in the feed media (0.34 μ M, 3.45 μ M, 34.45 μ M and 344.52 μ M).

5

Example 1

Cell Culture

Cells were cultured either in 50 ml spin tubes with an initial volume of 25 ml and a shaking speed of 300 rpm; or 250 ml shake flasks with an initial volume of 80~100 ml and a shaking speed of 150 rpm on an orbital shaker with 25 mm throw distance.

- 10 Temperature for the culture was controlled at constant 37 °C from the beginning of the culture and was shifted to 34 °C when viable cell density reached 10^6 cells/ml (usually on day 6) and CO₂ was controlled at 6%.

- A standard fed-batch culture process involved culturing the cells for 14 days with feeding beginning on day 3 at a feeding volume of 3.64% of initial culture volume. All
15 the media used for cultures were chemically defined. Chemically defined feed media M154A1B is an enriched version of feed media F3.2. While the ingredient lists are the same between the two feed media, the concentration of a few of the ingredients is increased. Likewise, chemically defined basal media M171B is an enriched version of basal media B1. For cultures in 7-liter or 500-liter reactors, dissolved oxygen (DO) was
20 maintained at 50% and pH was controlled between 6.8 and 7.4.

Analyses

- Viable cell density (VCD) and cell viability were measured off-line using a CEDEX® automated cell counter (Innovatis AG). Culture samples were also analyzed
25 off-line using a BIOPROFILE® 400 Analyzer to monitor pH, pCO₂, pO₂, glucose, glutamine, glutamate, lactate, and ammonium (Nova Biomedical Corporation). A Protein A HPLC method was used to measure protein titer, which were reported as normalized values.

30

WE CLAIM:

1. A cell culture process for the production of protein, comprising:
 - a) culturing host cells which produce a protein of interest in cell culture under
5 conditions that allow for protein production; and
 - b) adding one or more antioxidant selected from the group consisting of apigenin, catechin, chlorogenic acid, daidzein, genistein, hesperetin, melatonin, naringenin, pelargonidin, quercetin, resveratrol, rosmarinic acid and silibinin to the cell
10 culture.
2. The process according to claim 1, wherein the antioxidant is selected from the group consisting of catechin, chlorogenic acid, pelargonidin, quercetin, resveratrol, rosmarinic acid and silibinin.
- 15 3. The process according to claim 1, wherein the antioxidant is selected from the group consisting of catechin, chlorogenic acid, quercetin, resveratrol and rosmarinic acid.
4. The process according to claim 1, wherein the antioxidant is catechin or
20 rosmarinic acid.
5. The process according to claim 1-4, wherein the antioxidant is added to the basal media, feed media or both.
- 25 6. The process according to claim 5, wherein the antioxidant is added to the basal media.
7. The process according to claim 5, wherein the antioxidant is added to the feed media.
30
8. The process according to claim 5, wherein the antioxidant is added to the both the basal and feed media.

9. The process according to claim 7, wherein the feed media is added to the cell culture on a daily basis.

5 10. The process according to claim 1, wherein the antioxidant is added to the cells in an amount of about 0.0625 mM to 1 mM.

11. The process according to claim 10, wherein antioxidant is added to the cells in an amount of about 0.1 mM to 1 mM.

10

12. The process according to claim 1, wherein the cells are mammalian cells.

13. The process according to claim 12, wherein the mammalian cells are CHO cells.

15

14. The process according to claim 1, wherein viable cell density is increased or maintained compared to the same cell culture without antioxidant supplementation.

15. The process according to claim 1, wherein protein titer is increased
20 compared to the same cell culture without antioxidant supplementation.

16. A cell culture process for the production of protein, comprising:
a) culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production; and

25 b) feeding the cell culture with 1 mM rosmarinic acid added to the feed media.

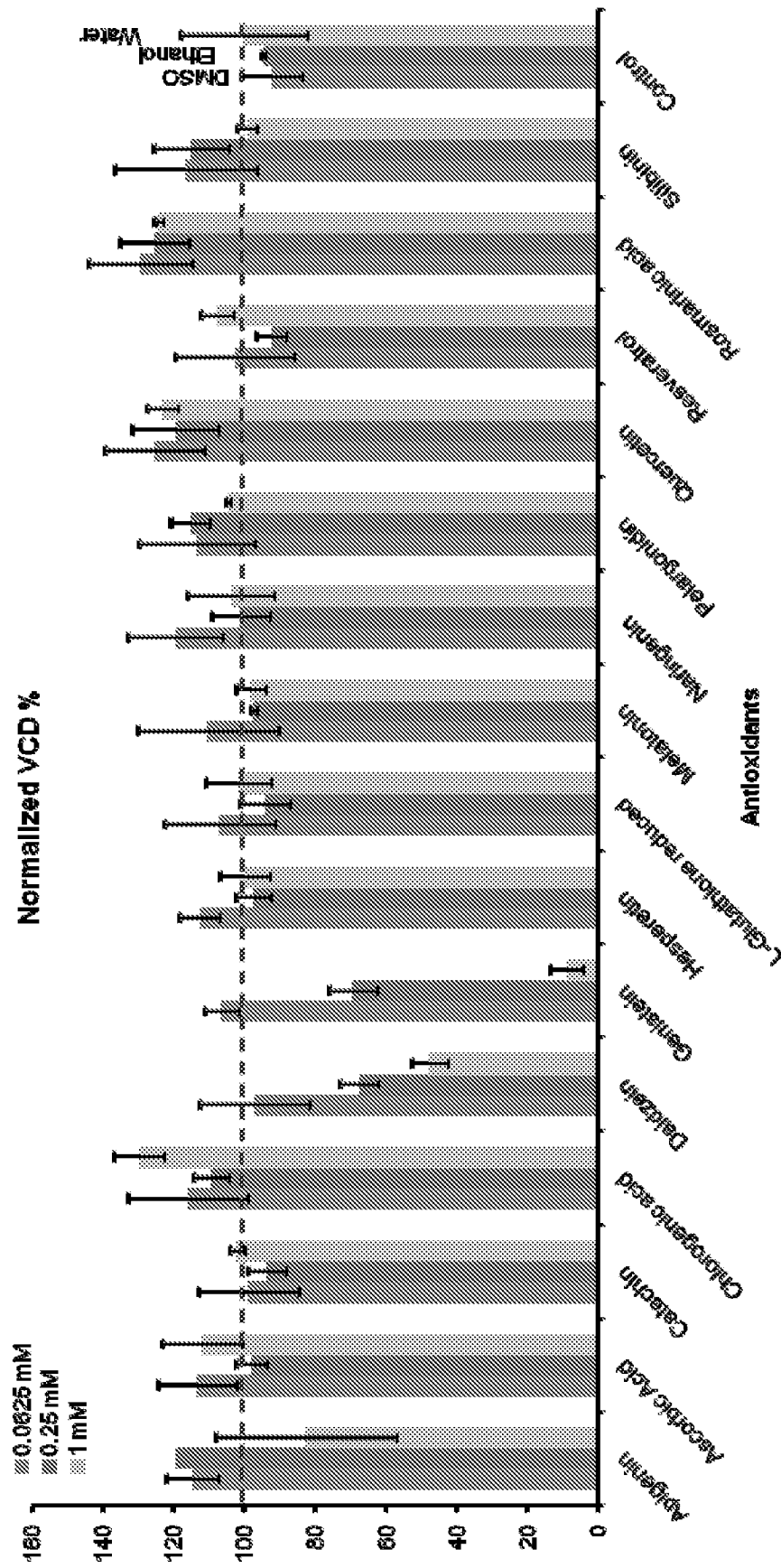
17. A cell culture process for the production of protein, comprising culturing CHO cells which produce a protein of interest in cell culture under conditions that allow
30 for protein production, wherein 0.1 mM rosmarinic acid is added to the basal media.

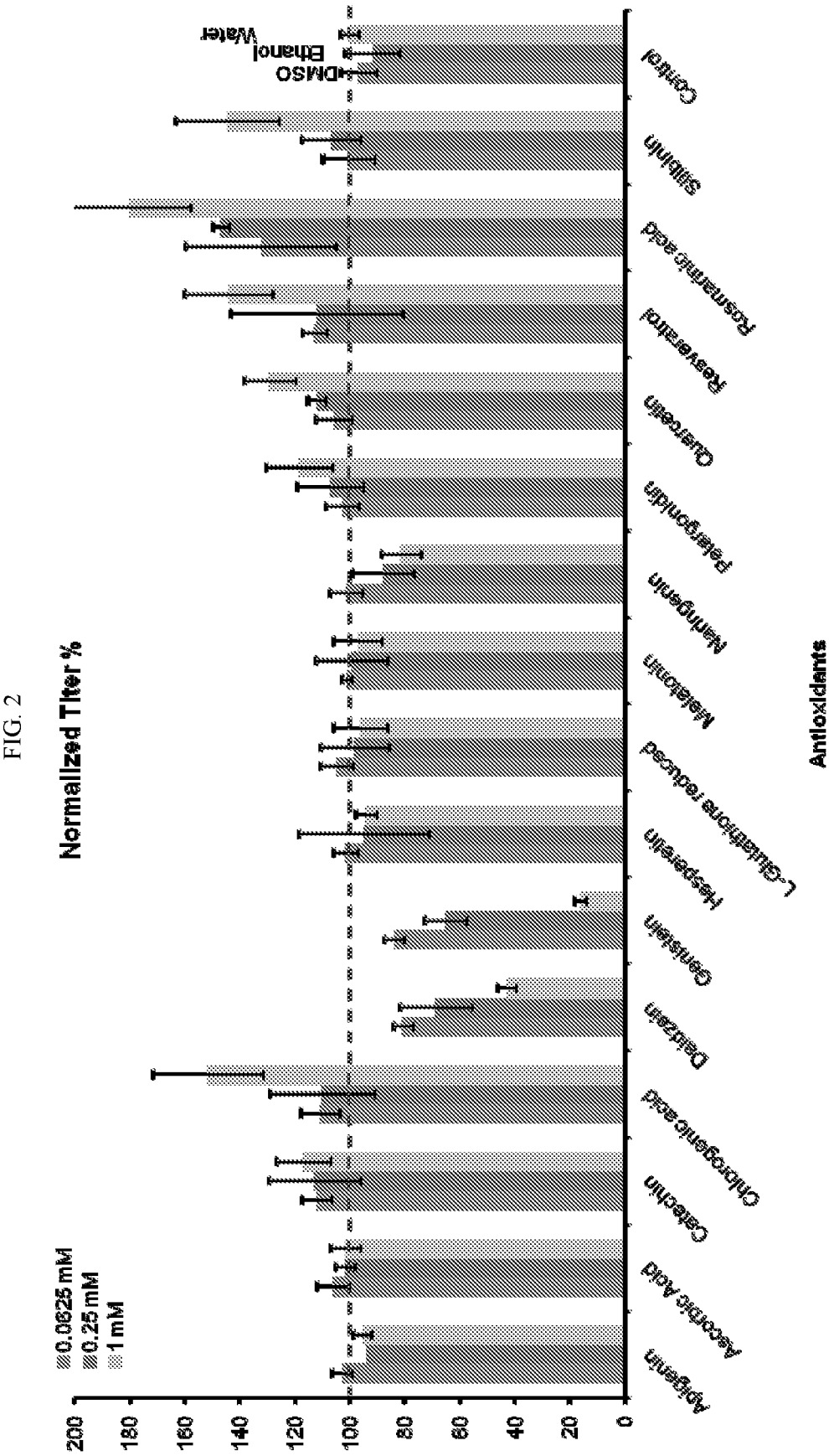
18. A cell culture process for the production of protein, comprising culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production, wherein 0.1 mM catechin is added to the basal media.

- 5 19. A cell culture process for the production of protein, comprising:
- a) culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
 - b) feeding the cell culture with 1 mM catechin added to the feed media.

- 10 20. A cell culture process for the production of protein, comprising:
- a) culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production wherein 0.1 mM rosmarinic acid is added to the basal media; and
 - b) feeding the cell culture with 1 mM rosmarinic acid added to the feed
- 15 media.

FIG. 1





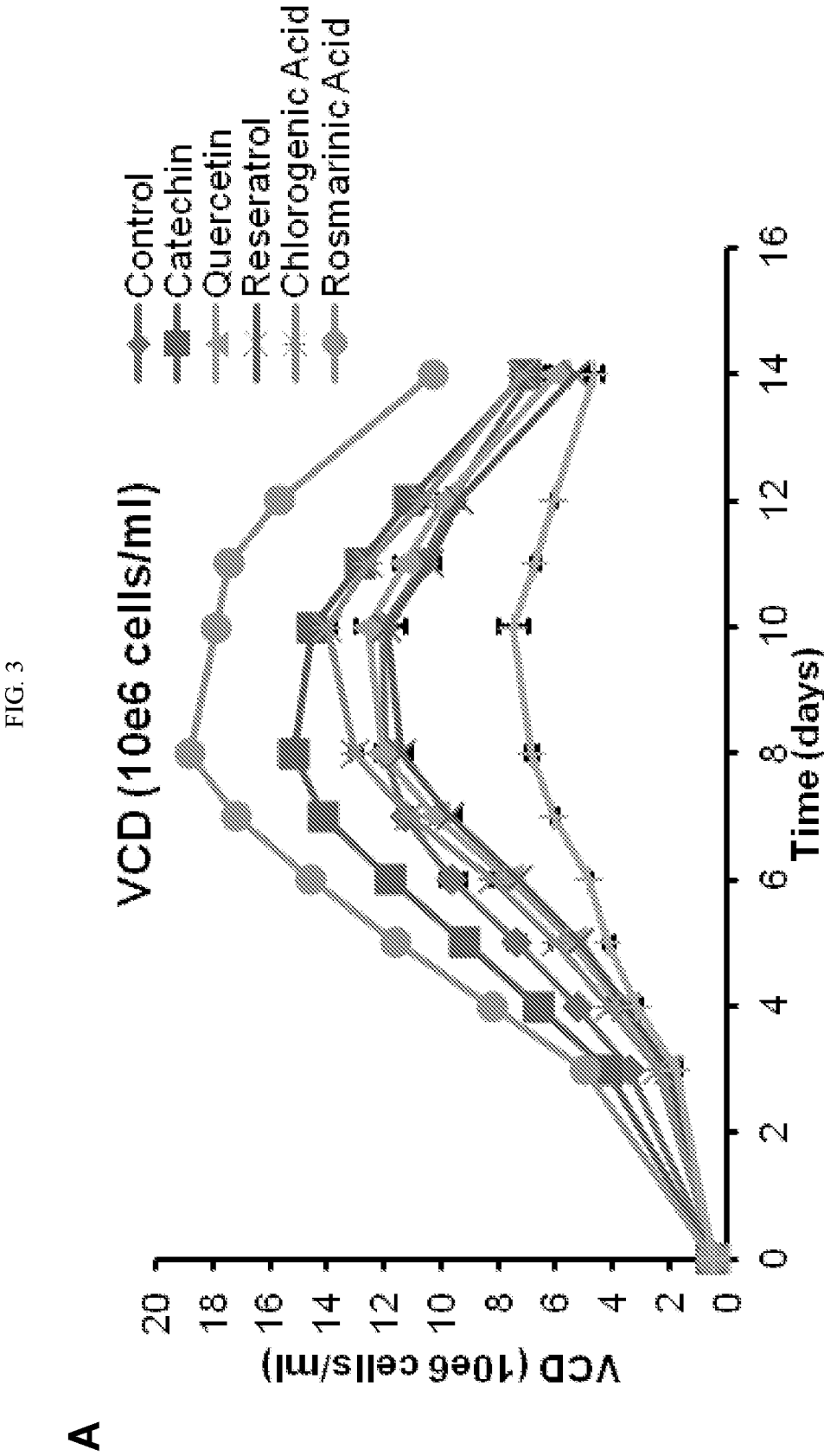


FIG. 3 (continued)

B

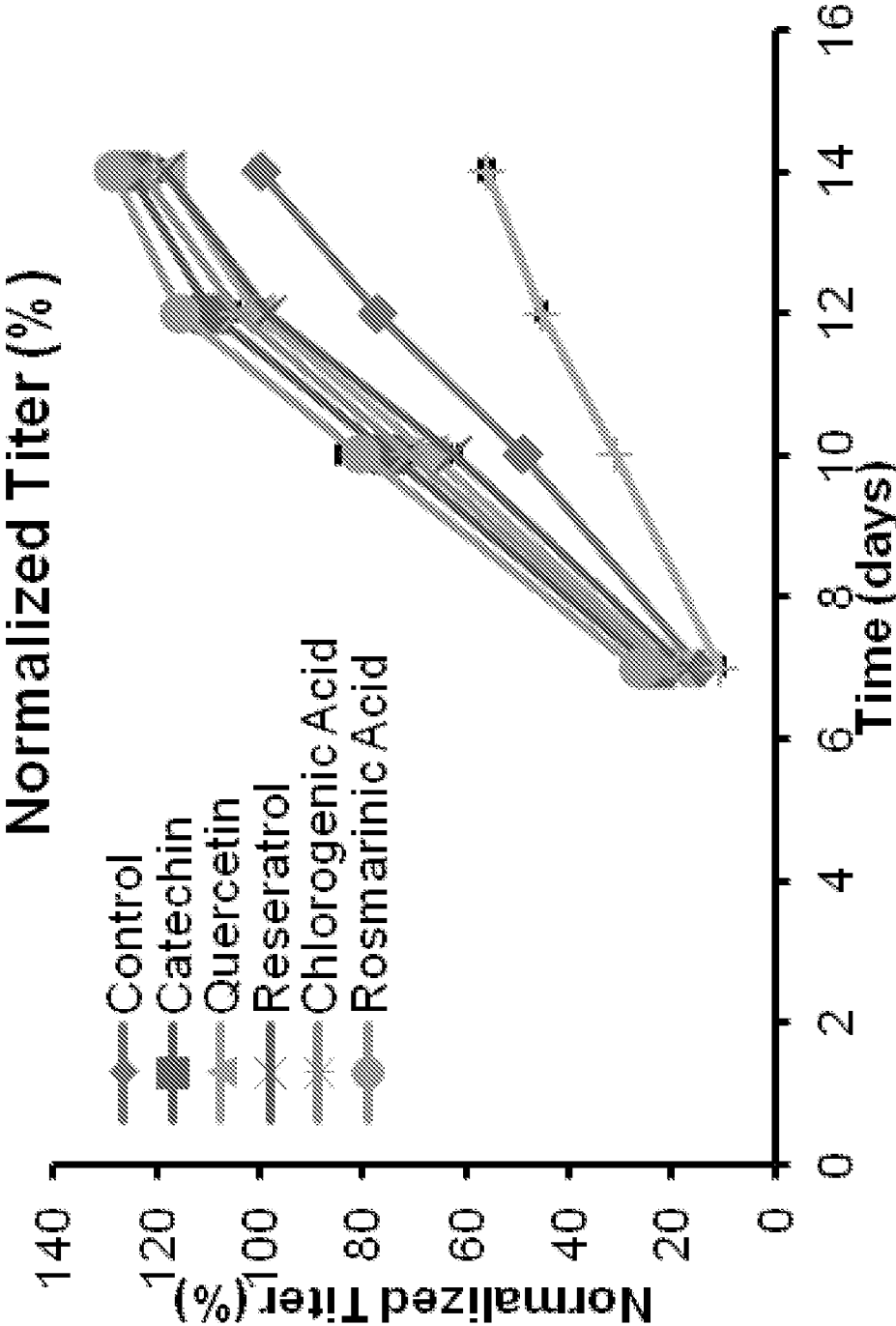


FIG. 3 (continued)

C

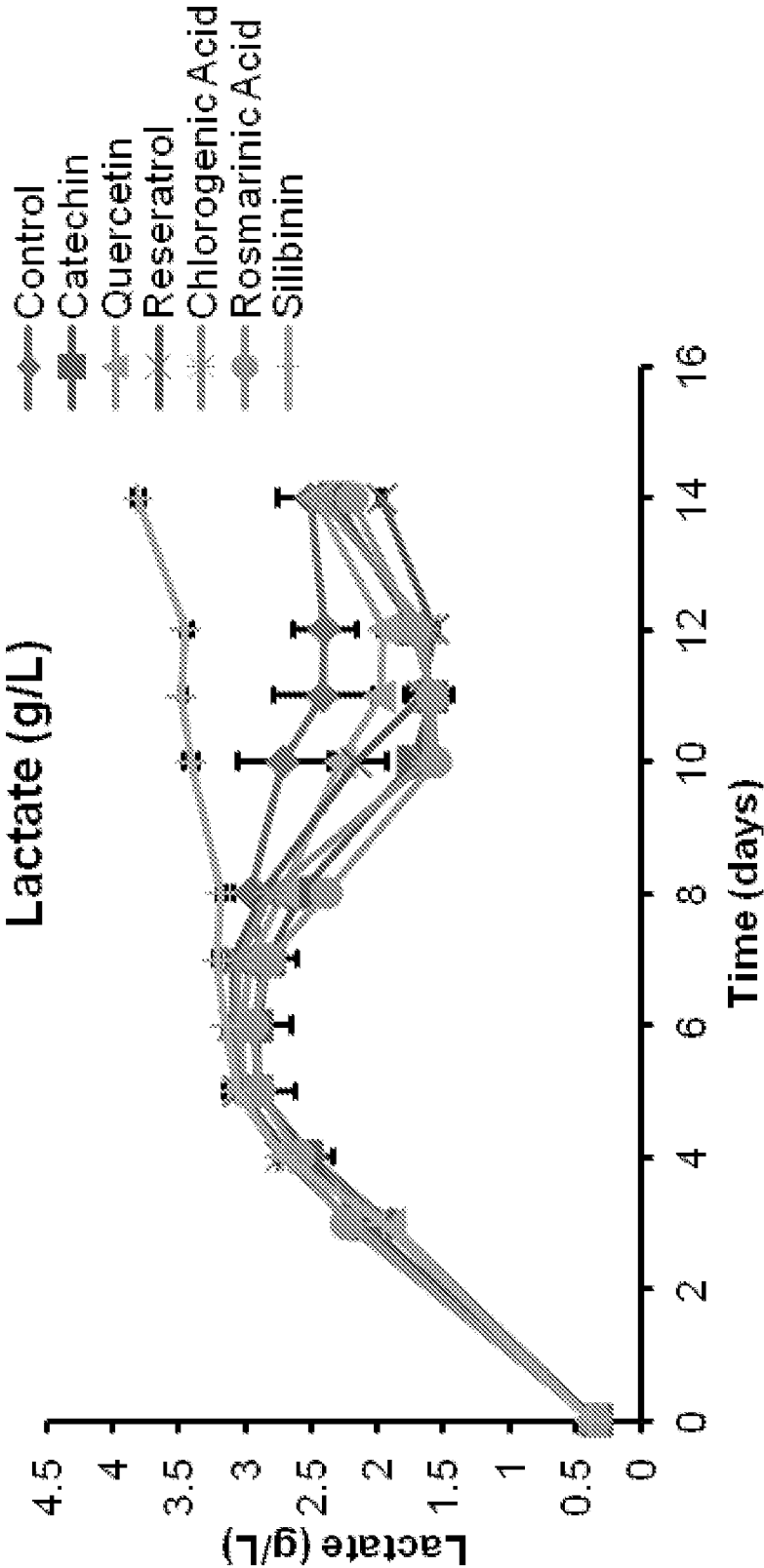


FIG. 4

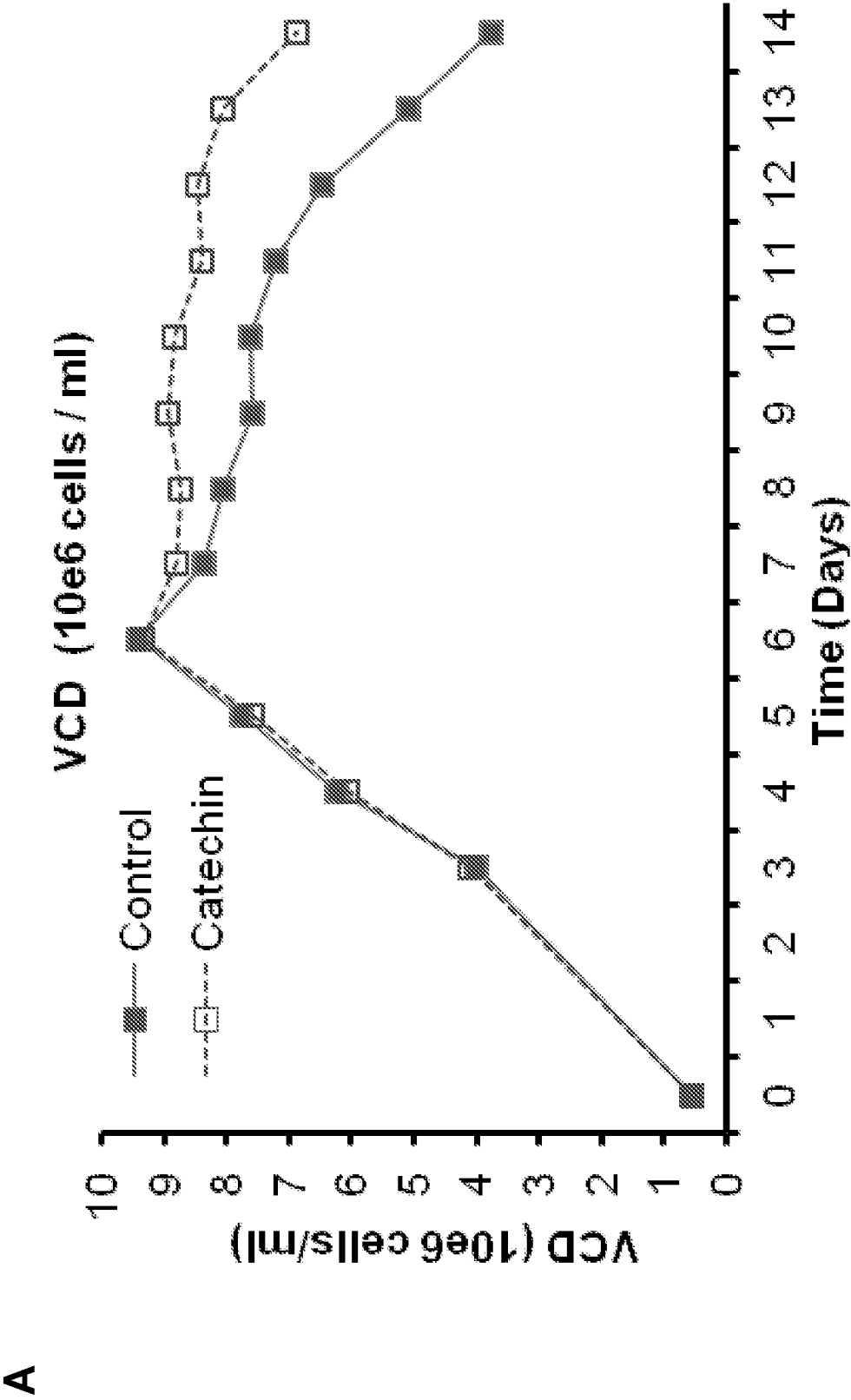


FIG. 4 (continued)

B

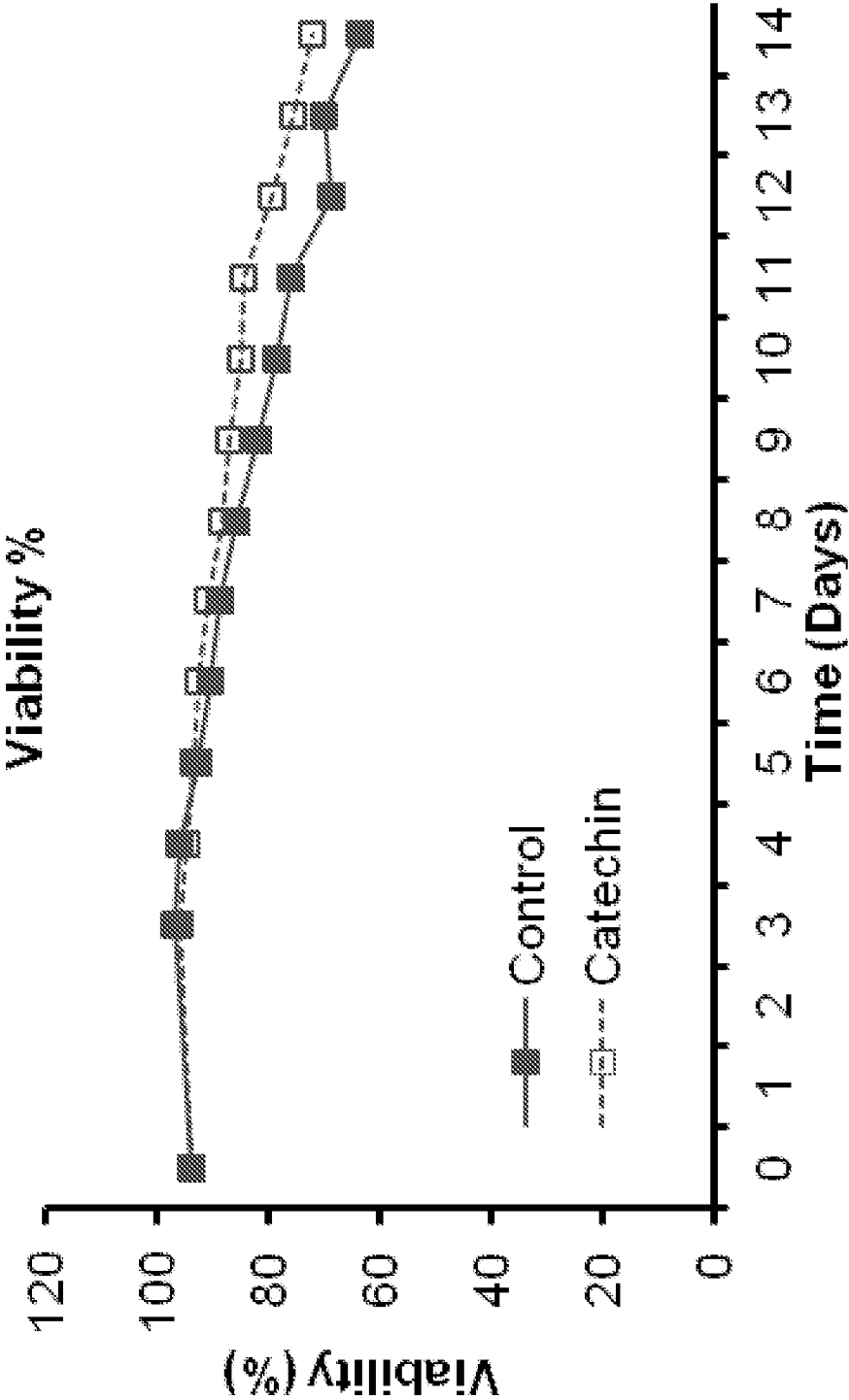
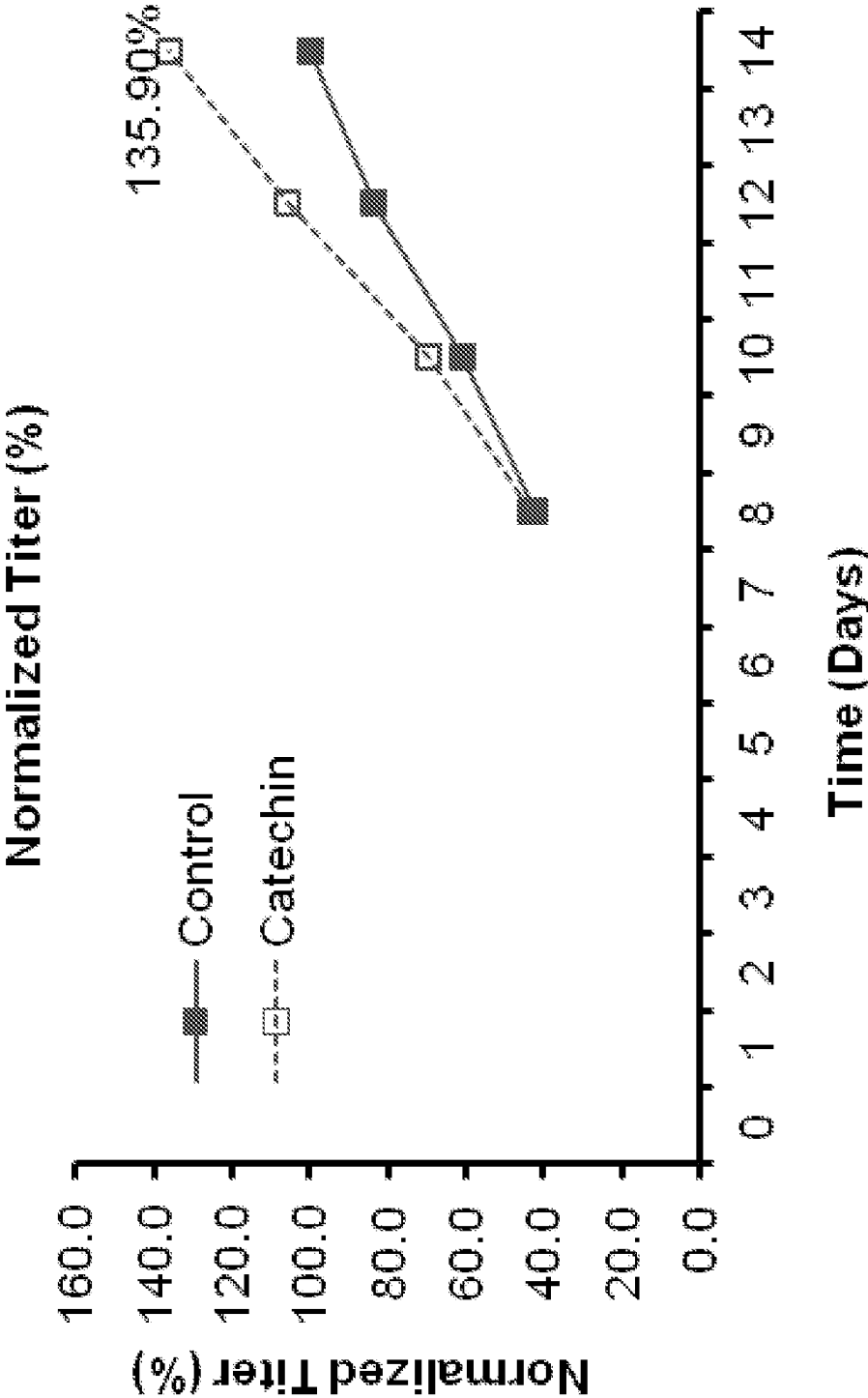


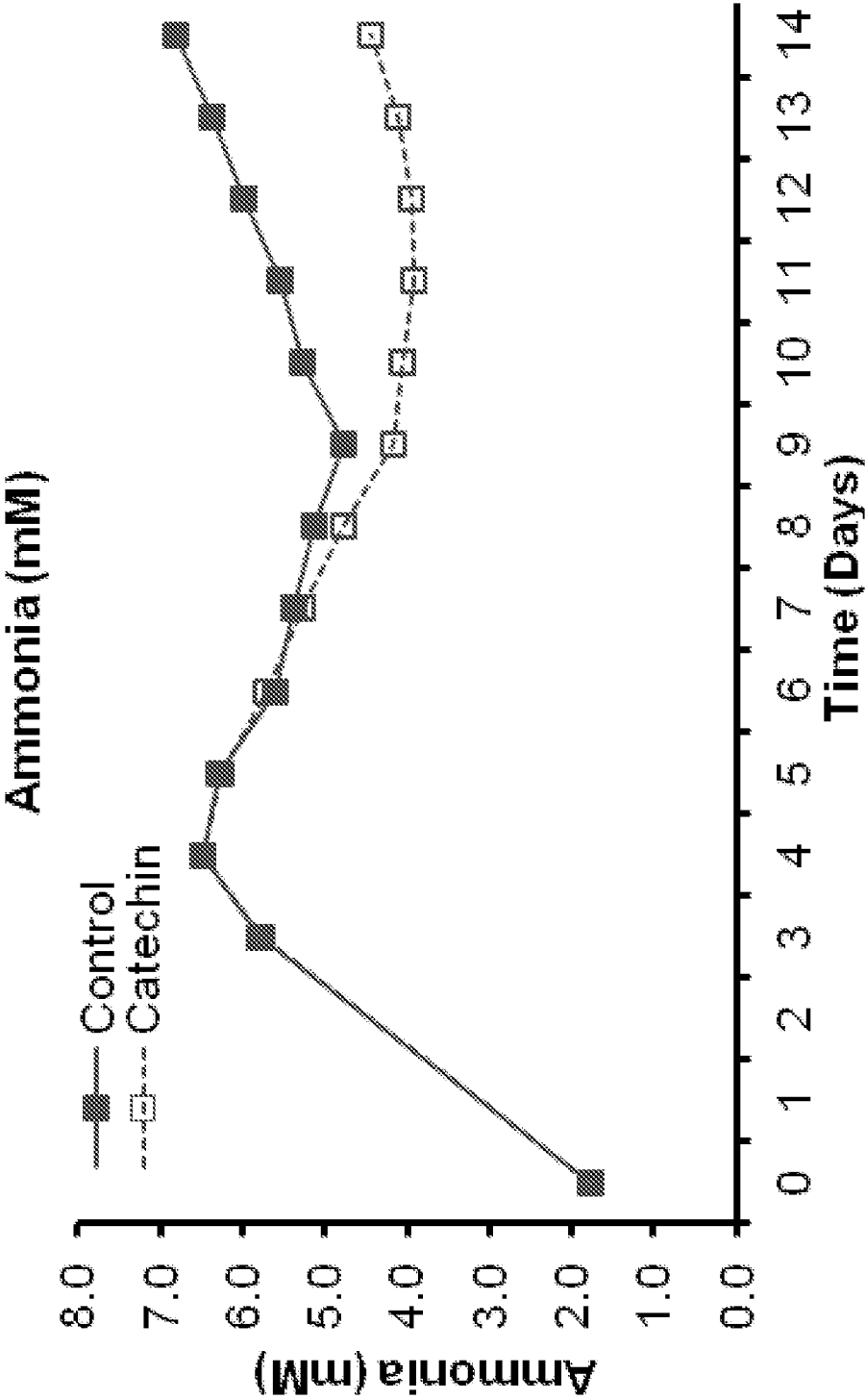
FIG. 4 (continued)



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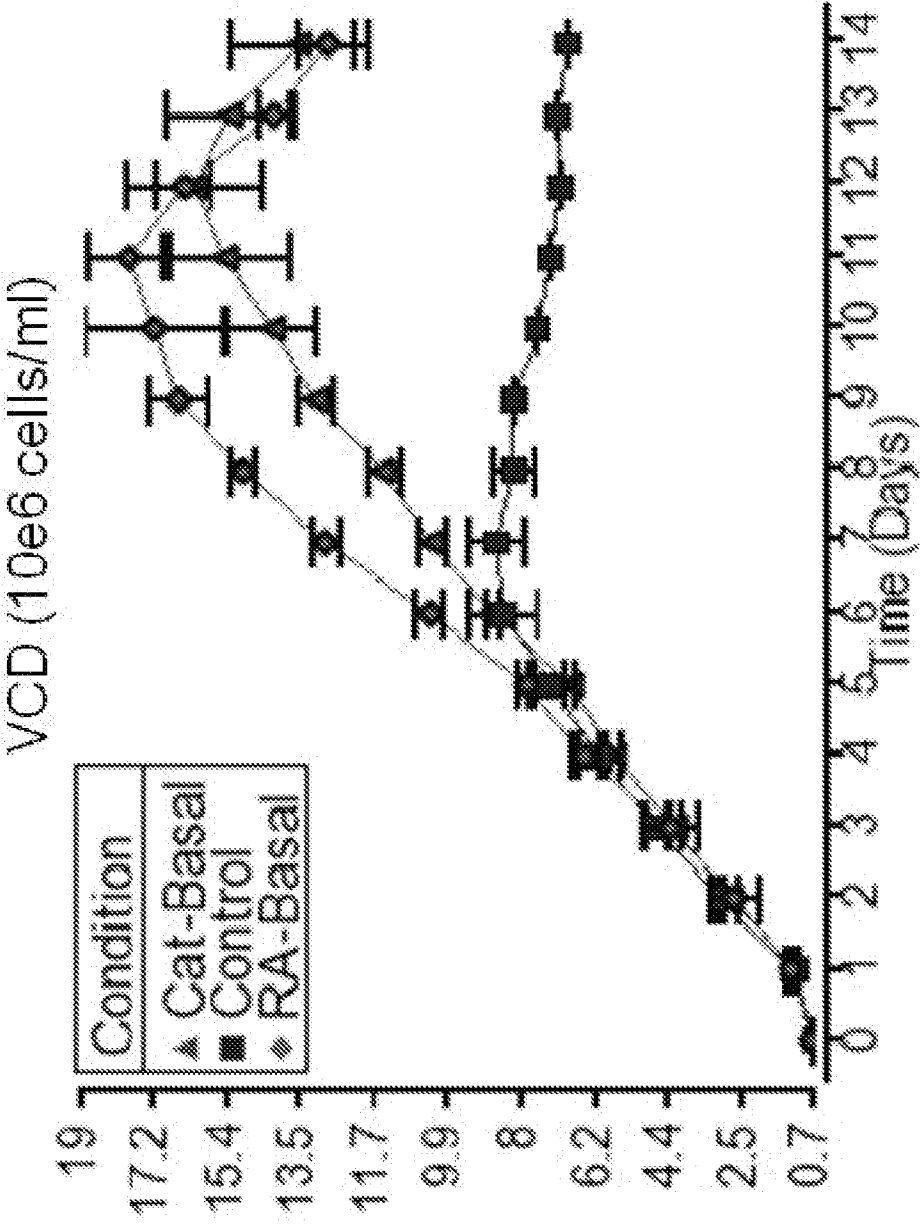
FIG. 4 (continued)

D



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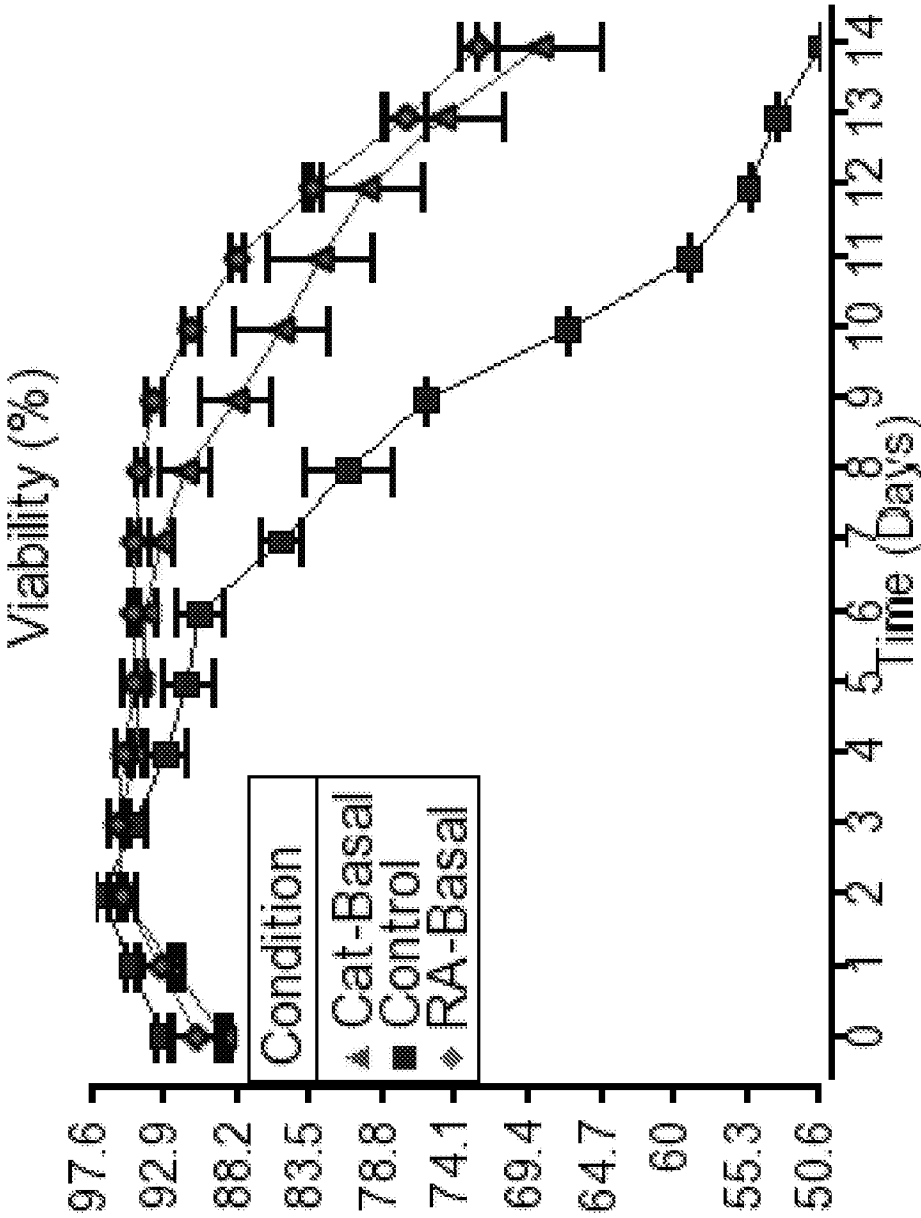
FIG. 5



A

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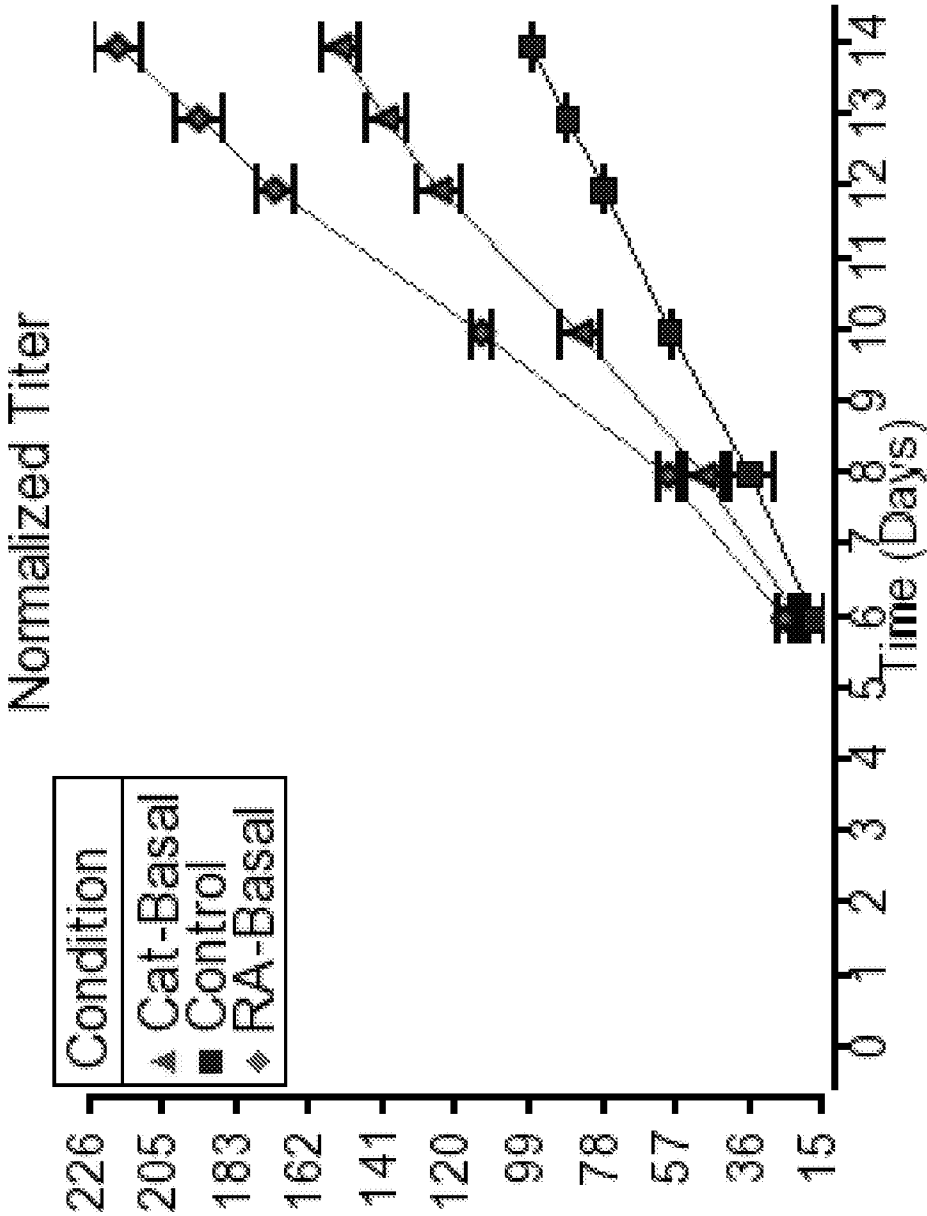
FIG. 5 (continued)



B

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FIG. 5 (continued)



C

FIG. 5 (continued)

D

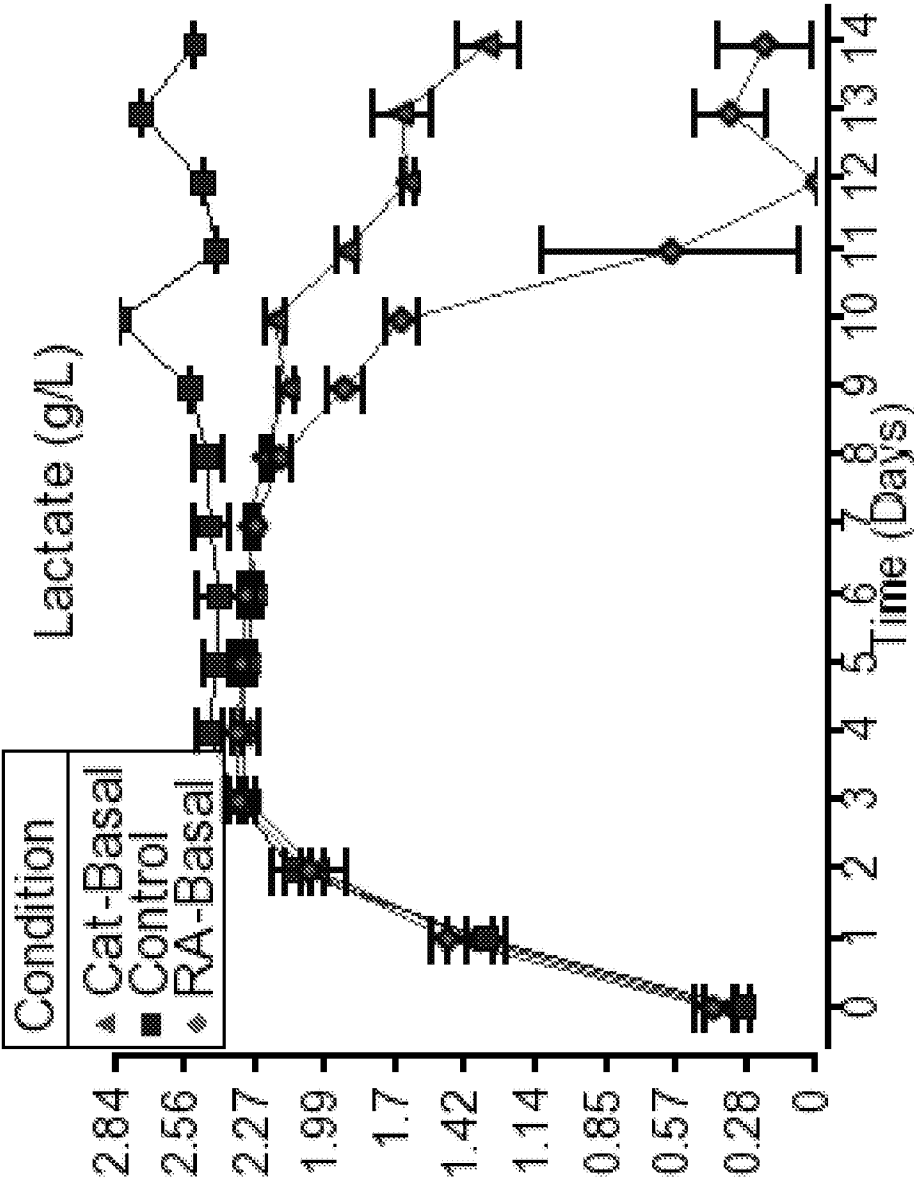
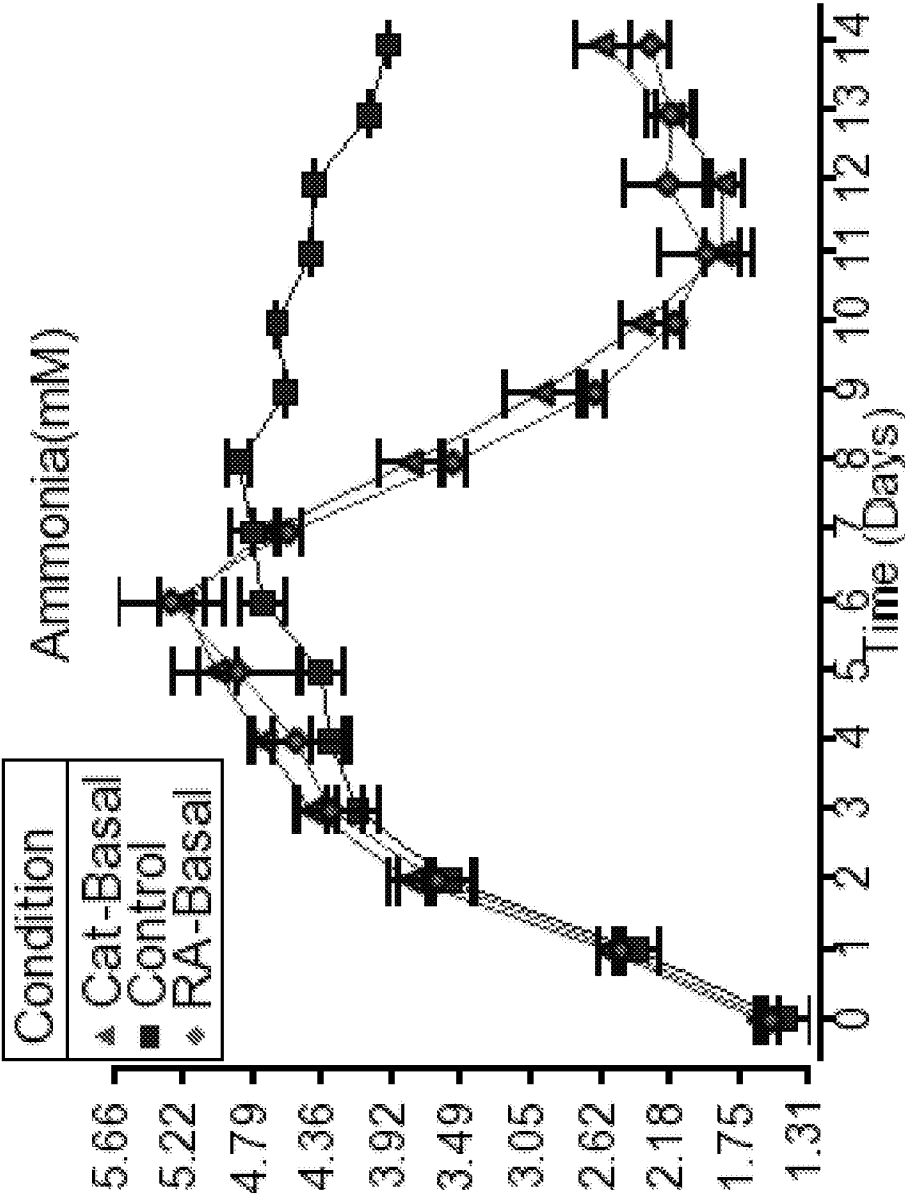


FIG. 5 (continued)



E

FIG. 6

A

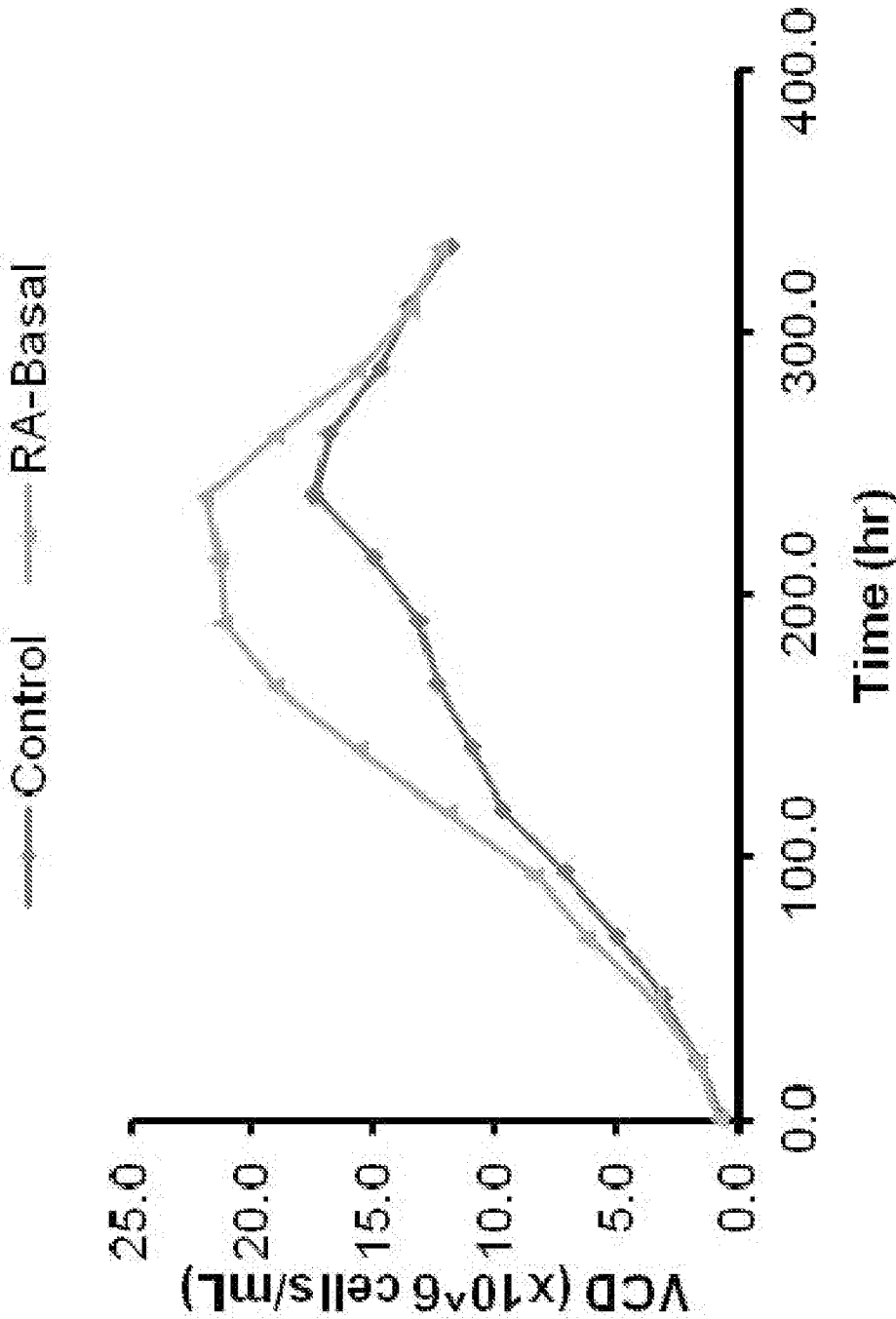


FIG. 6 (continued)

B

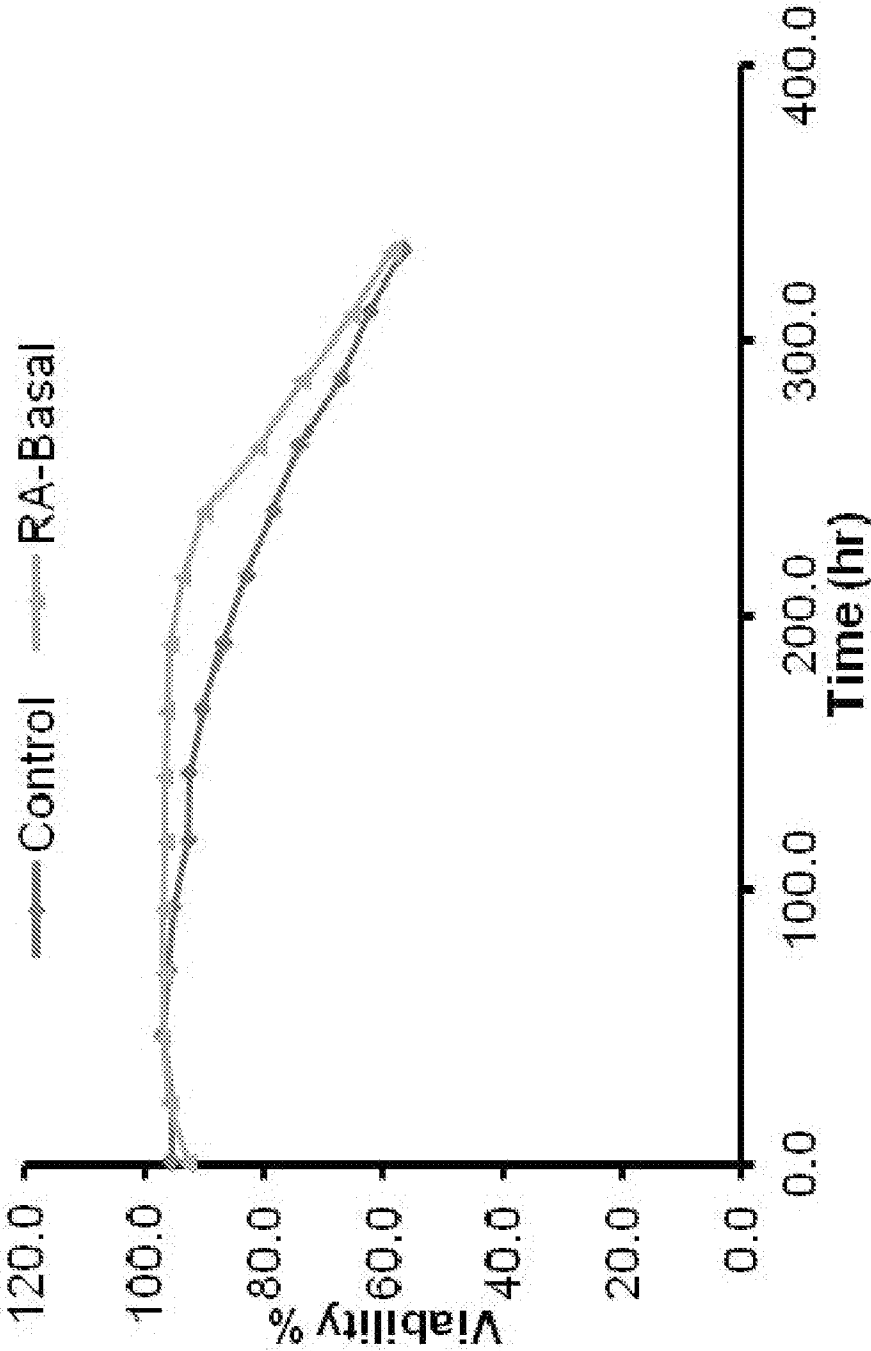
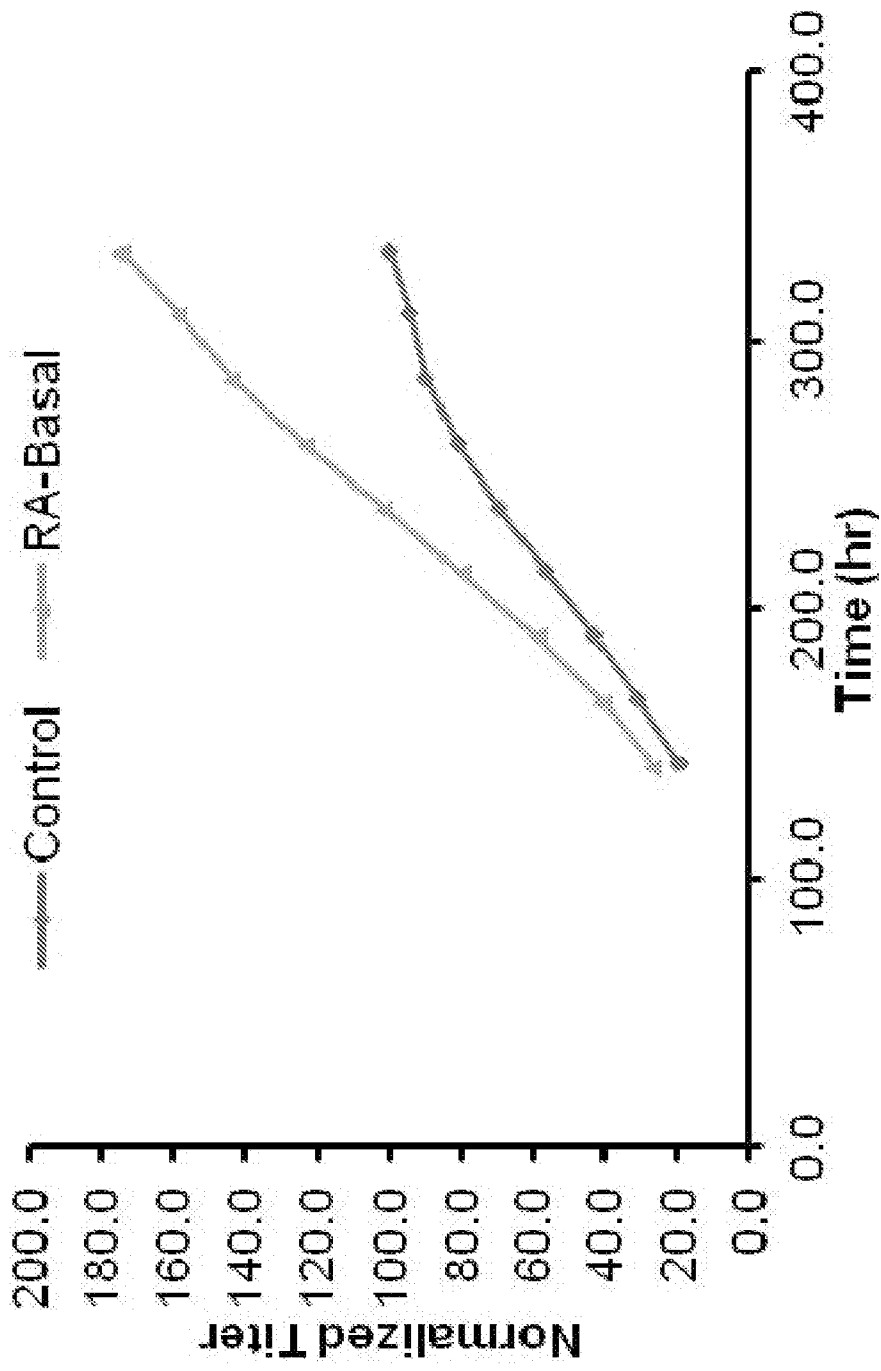


FIG. 6 (continued)



C

FIG. 6 (continued)

D

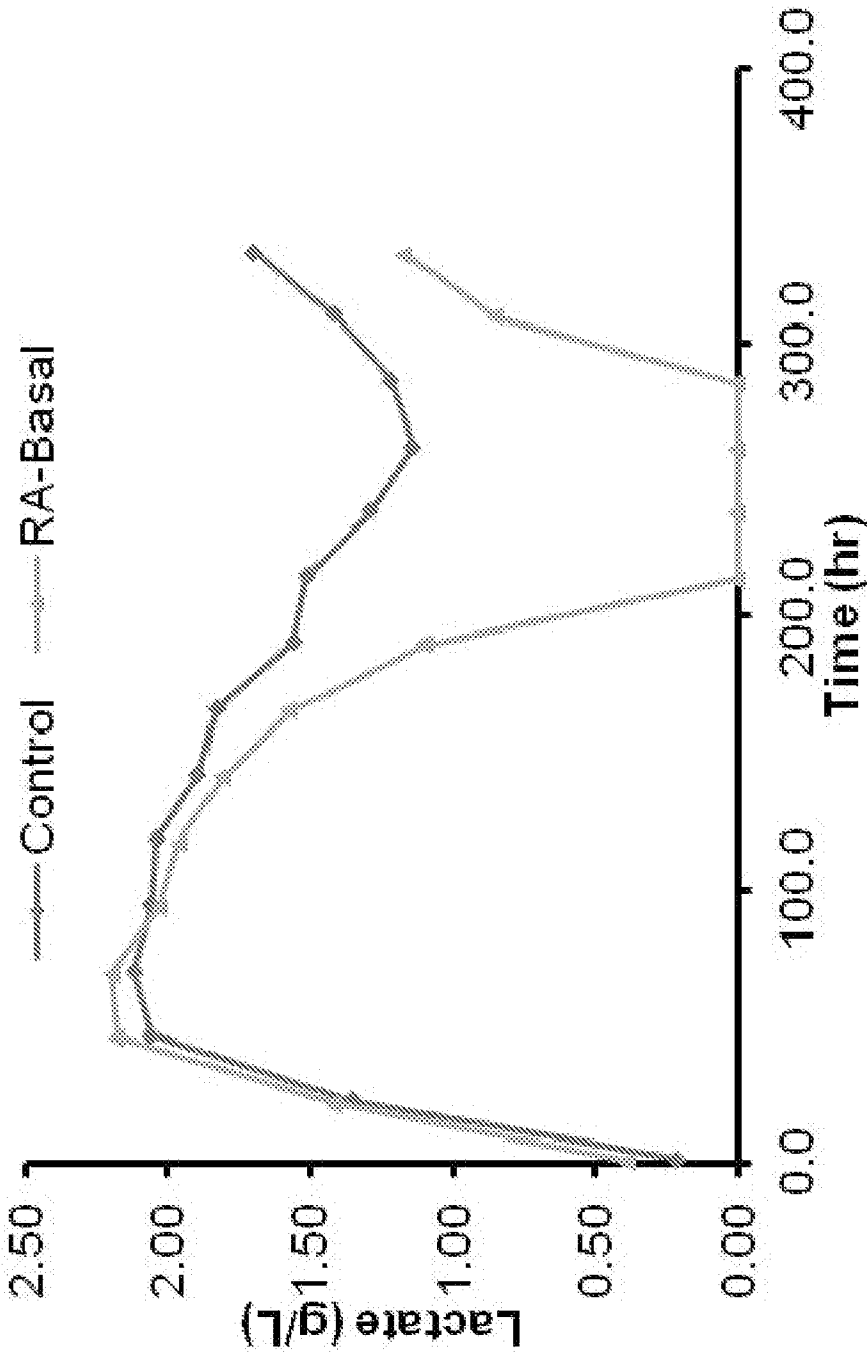


FIG. 7

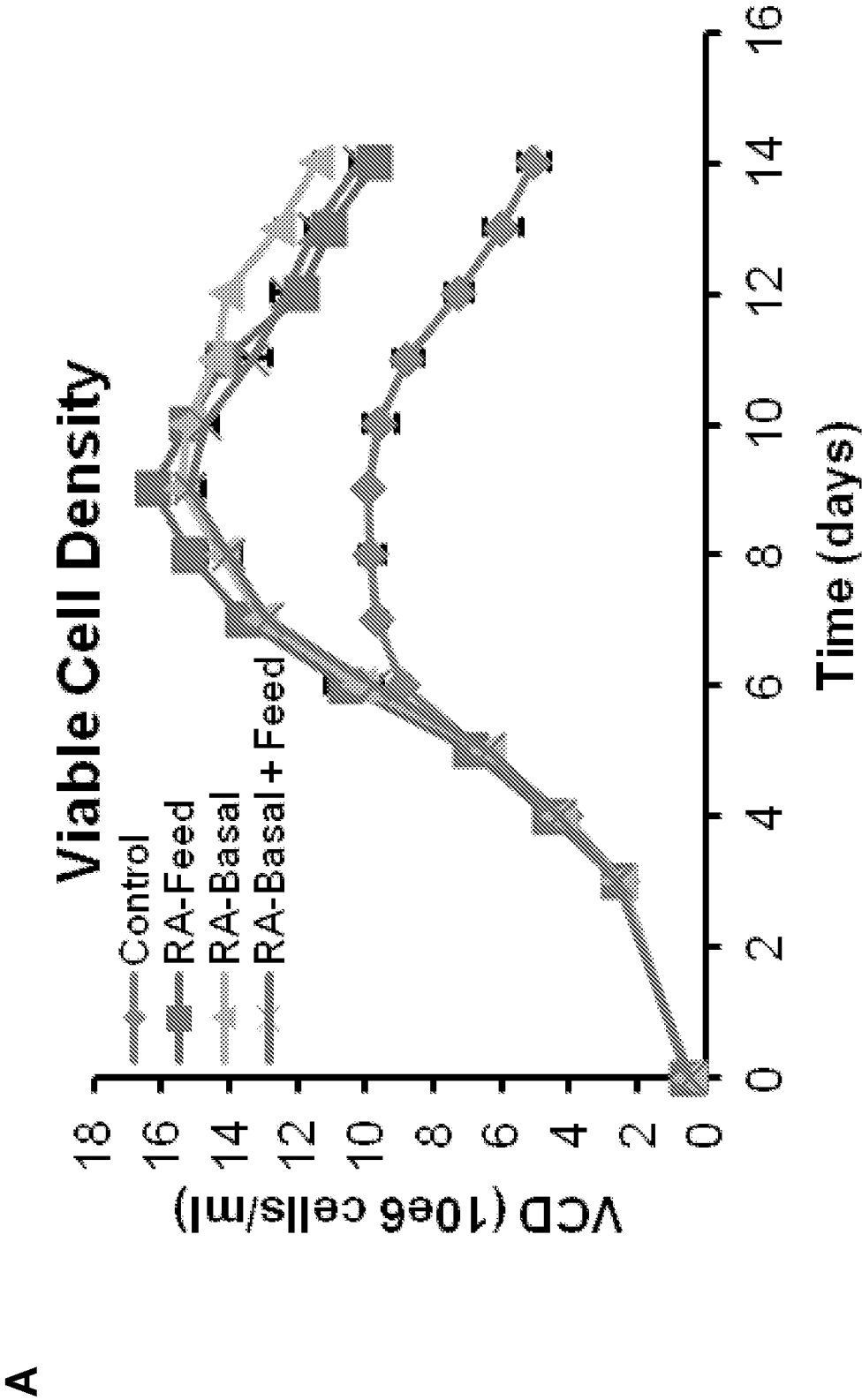


FIG. 7 (continued)

B

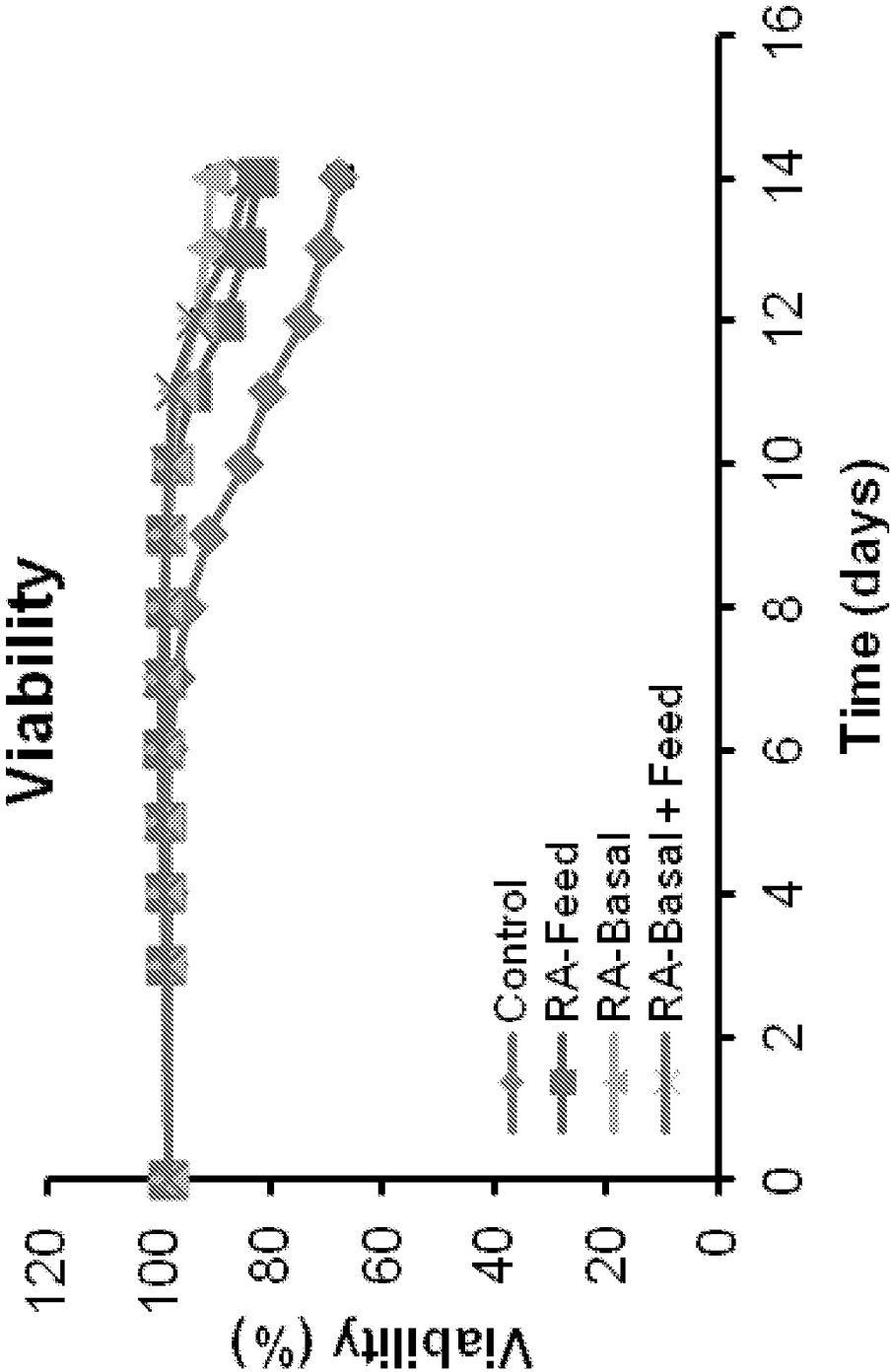


FIG. 7 (continued)

C

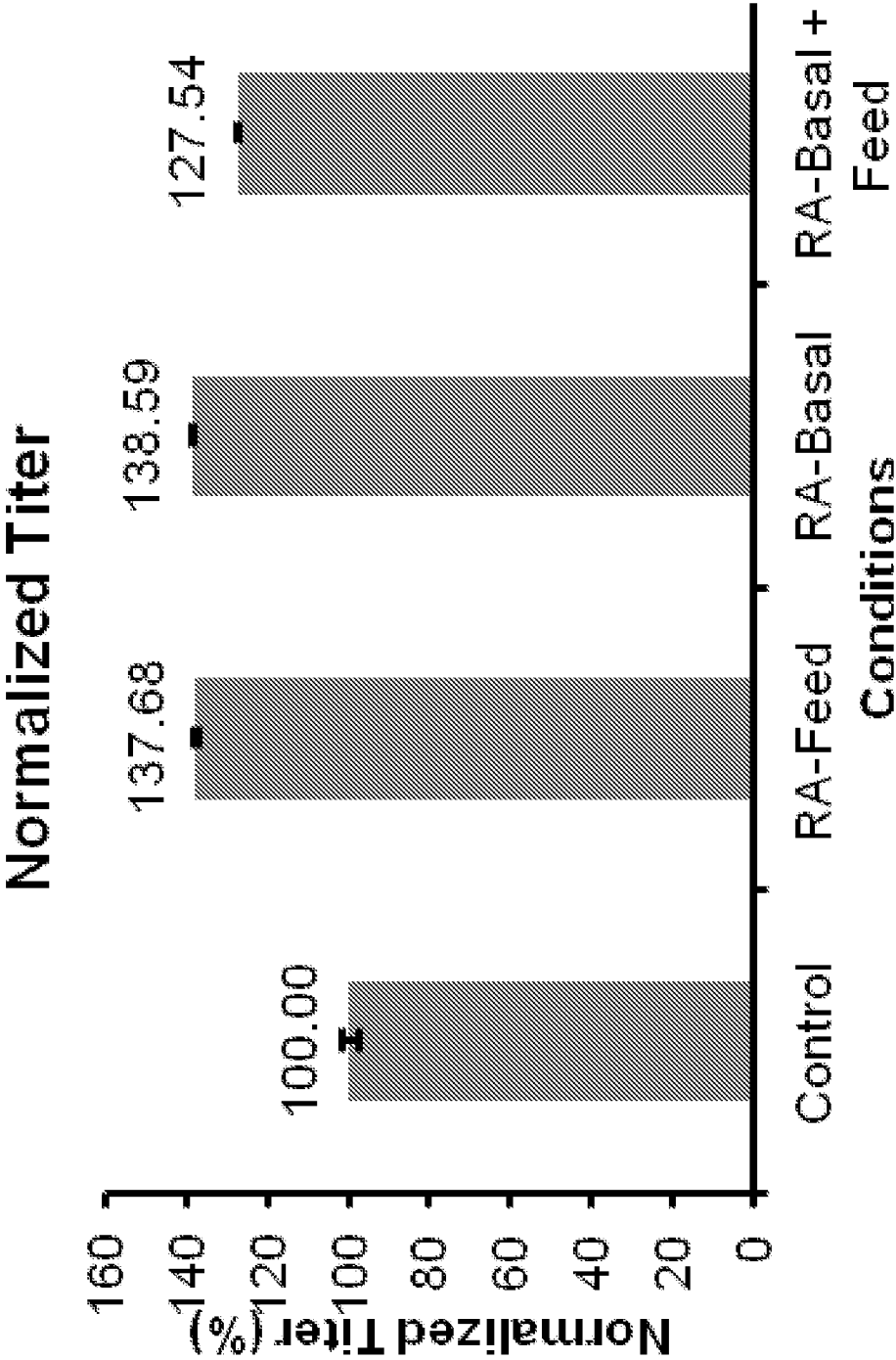


FIG. 7 (continued)

D

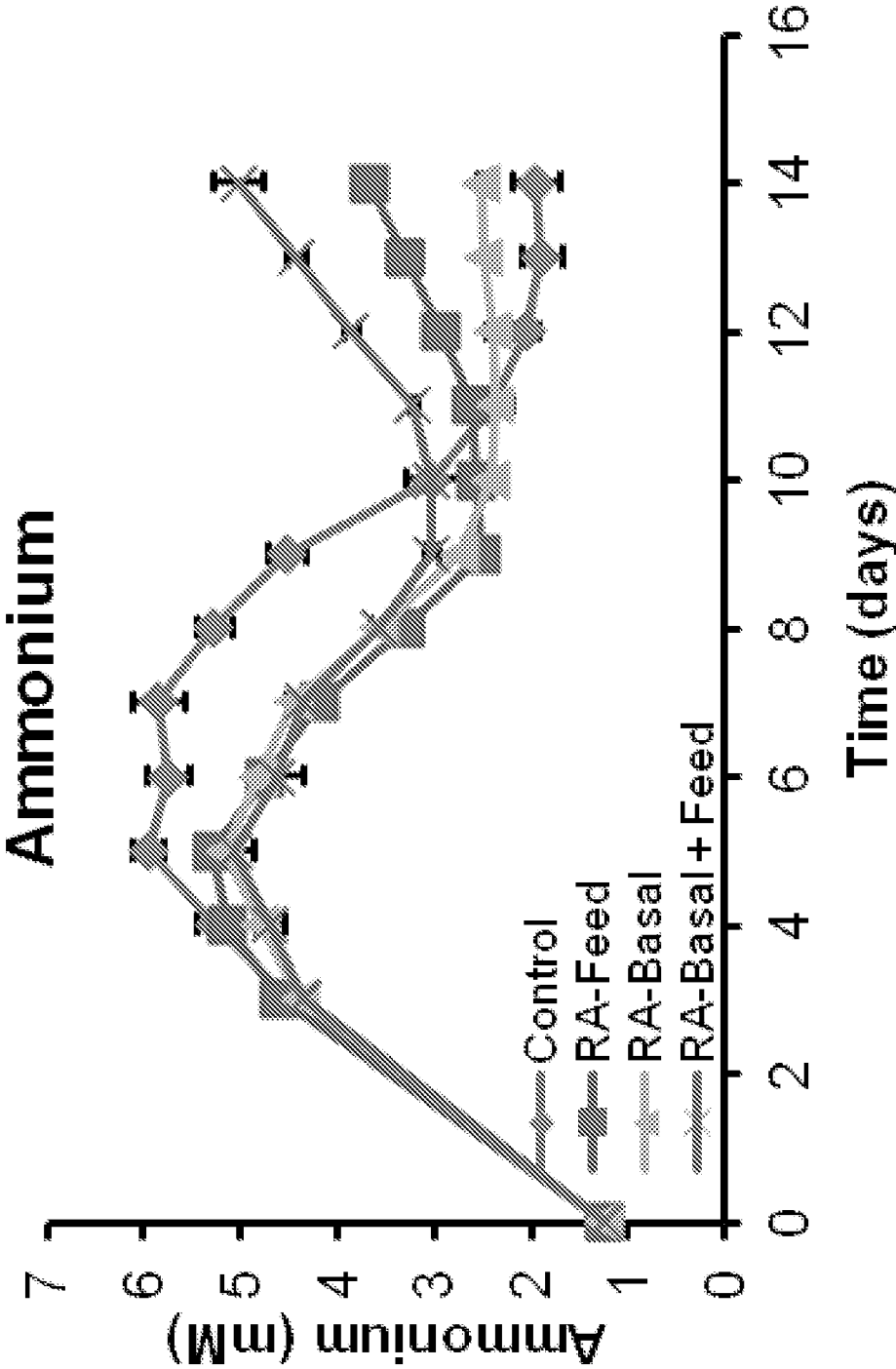


FIG. 8

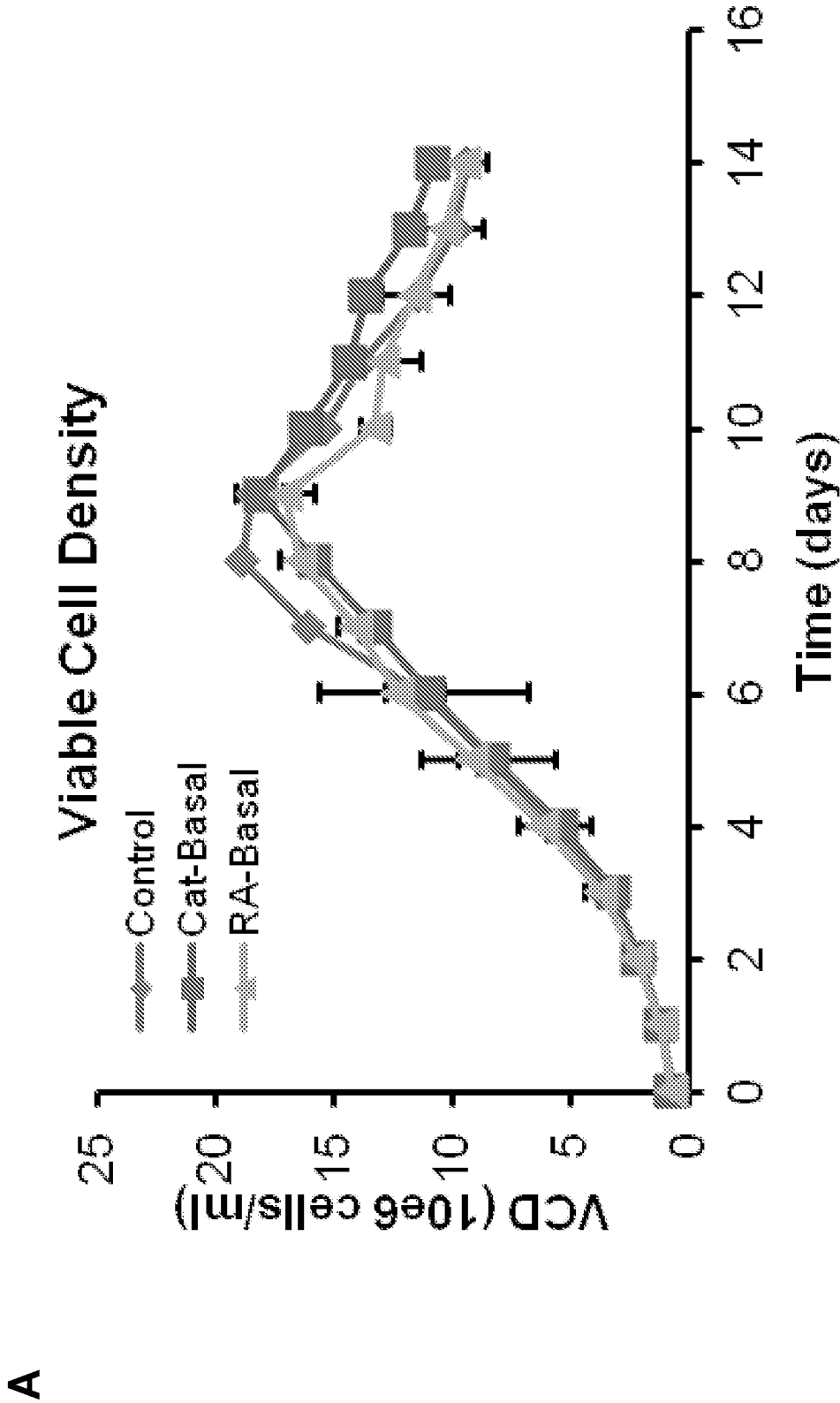


FIG. 8 (continued)

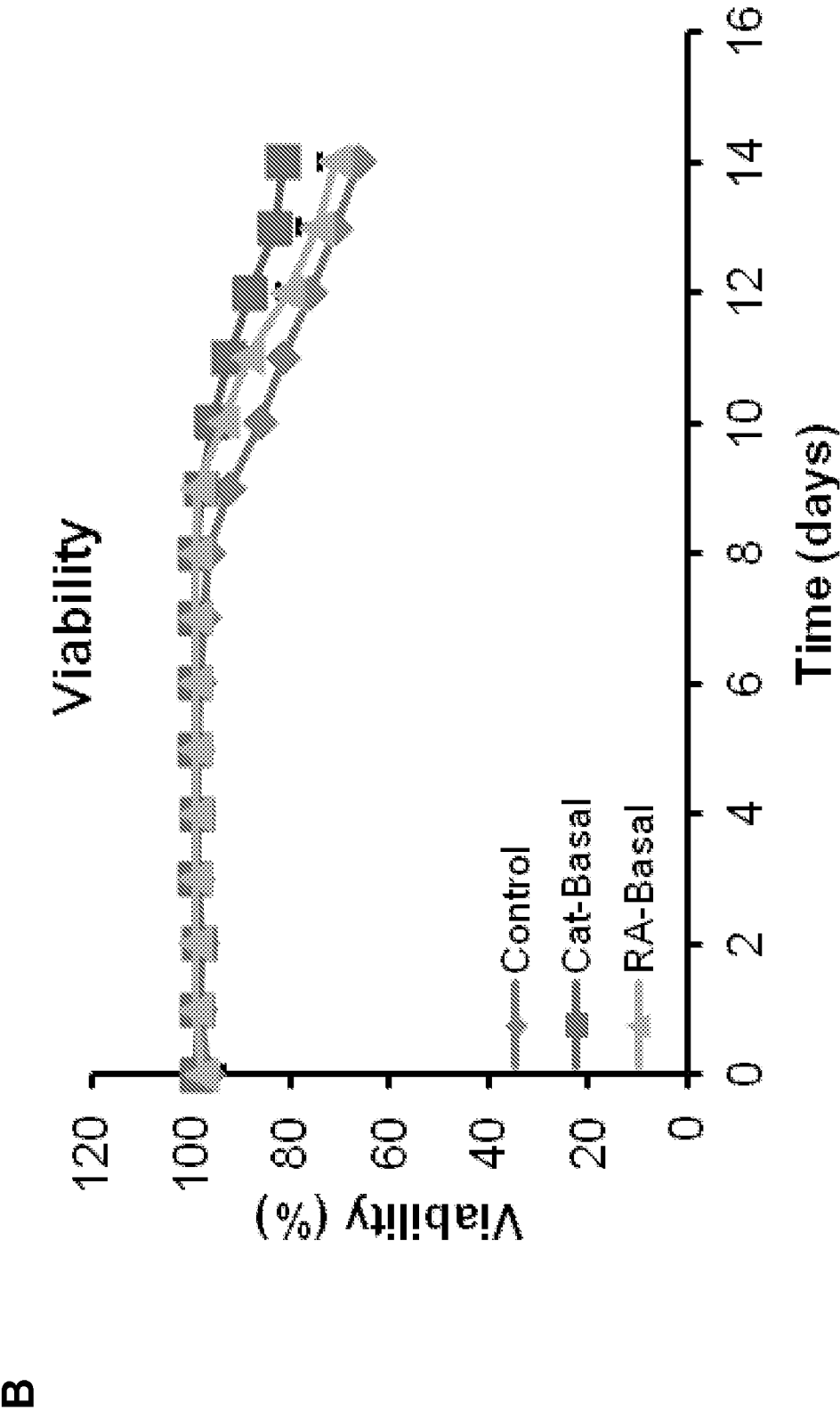


FIG. 8 (continued)

C

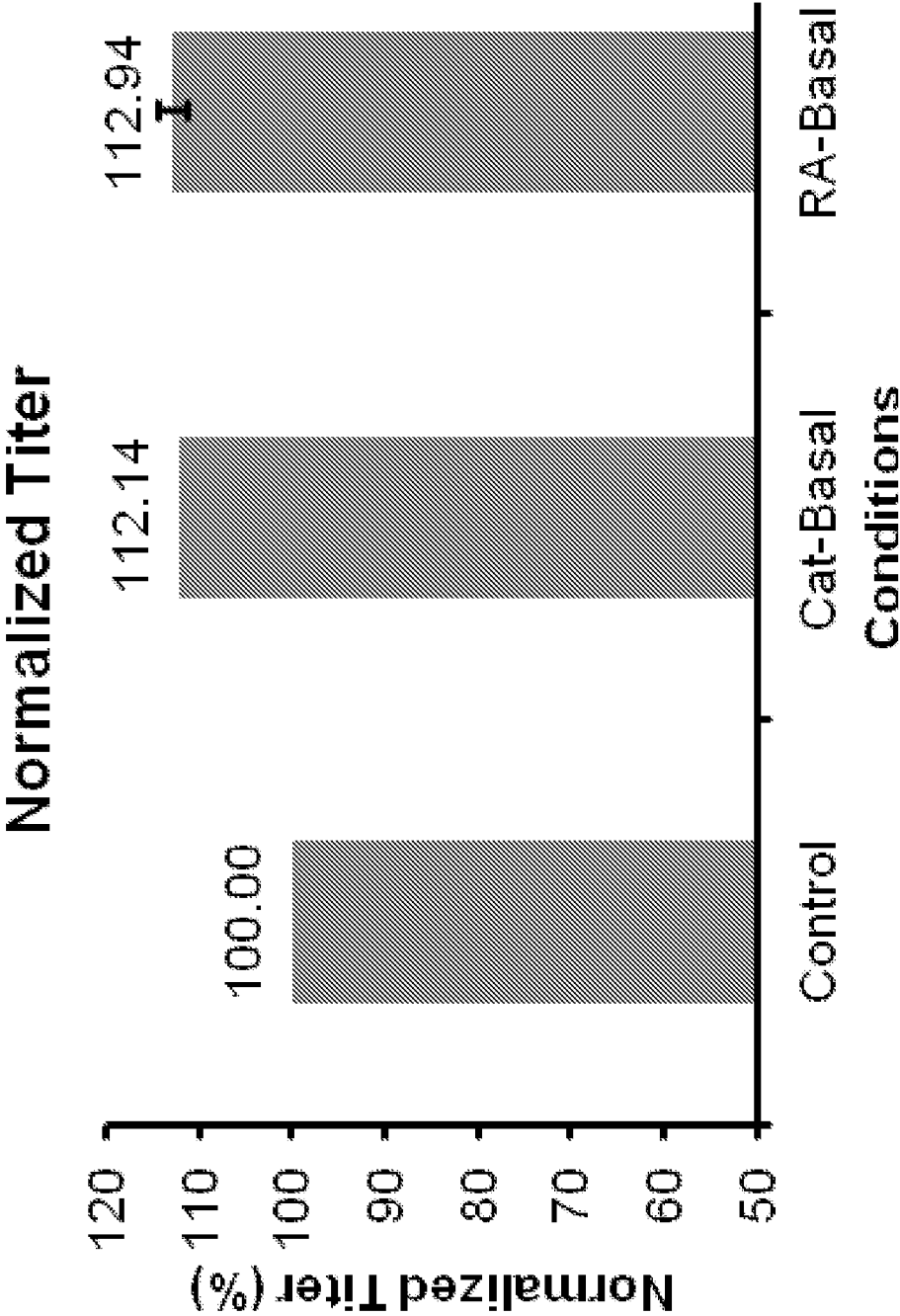


FIG. 9

A

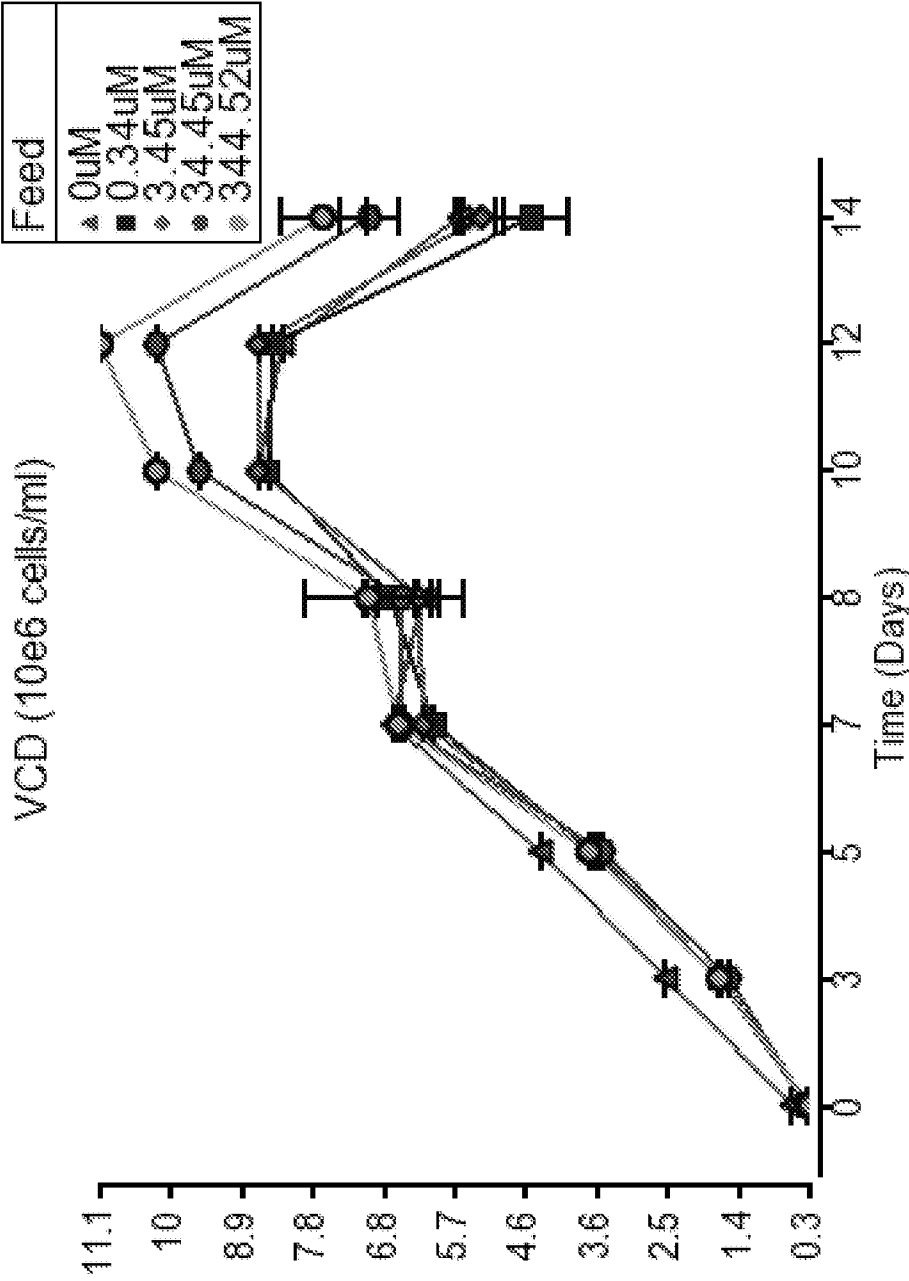


FIG. 9 (continued)

B

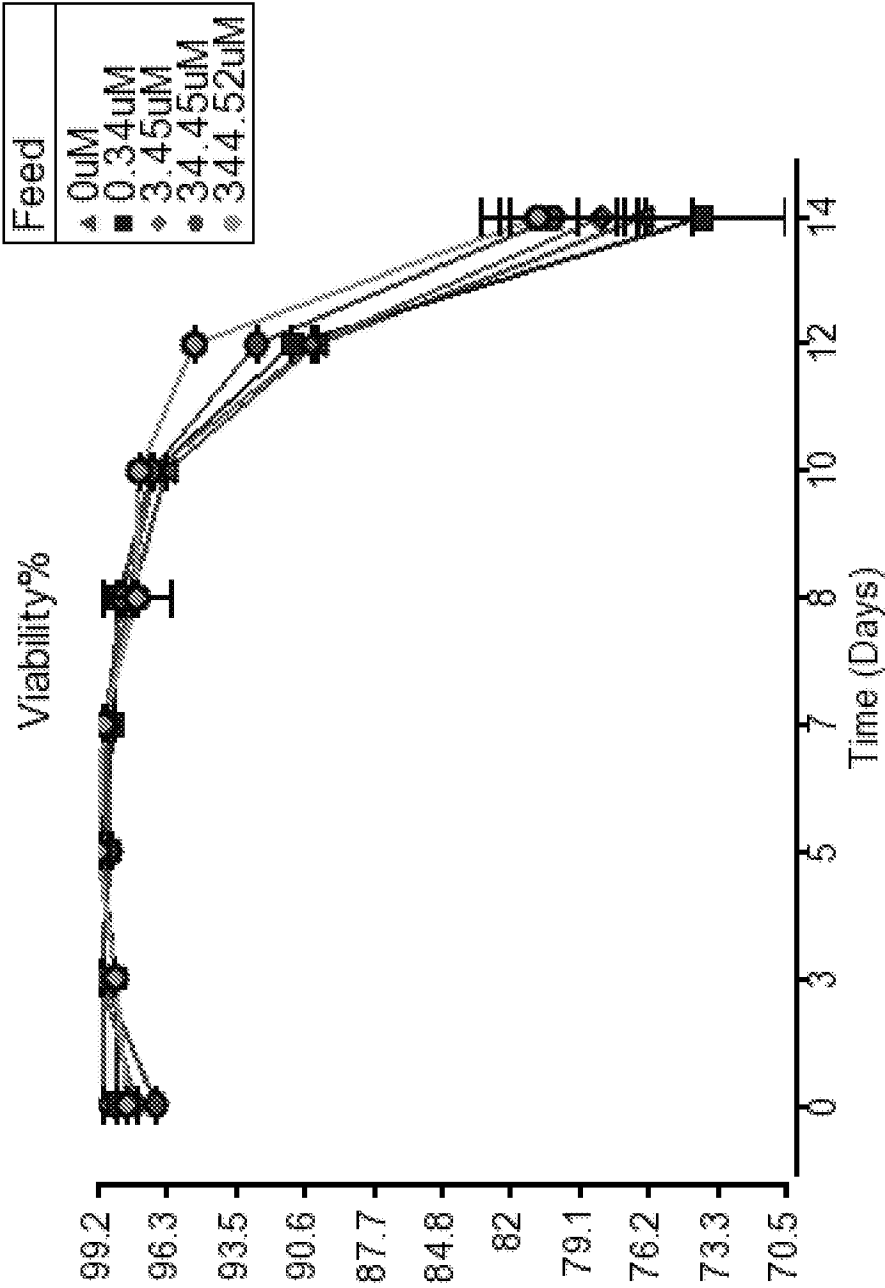
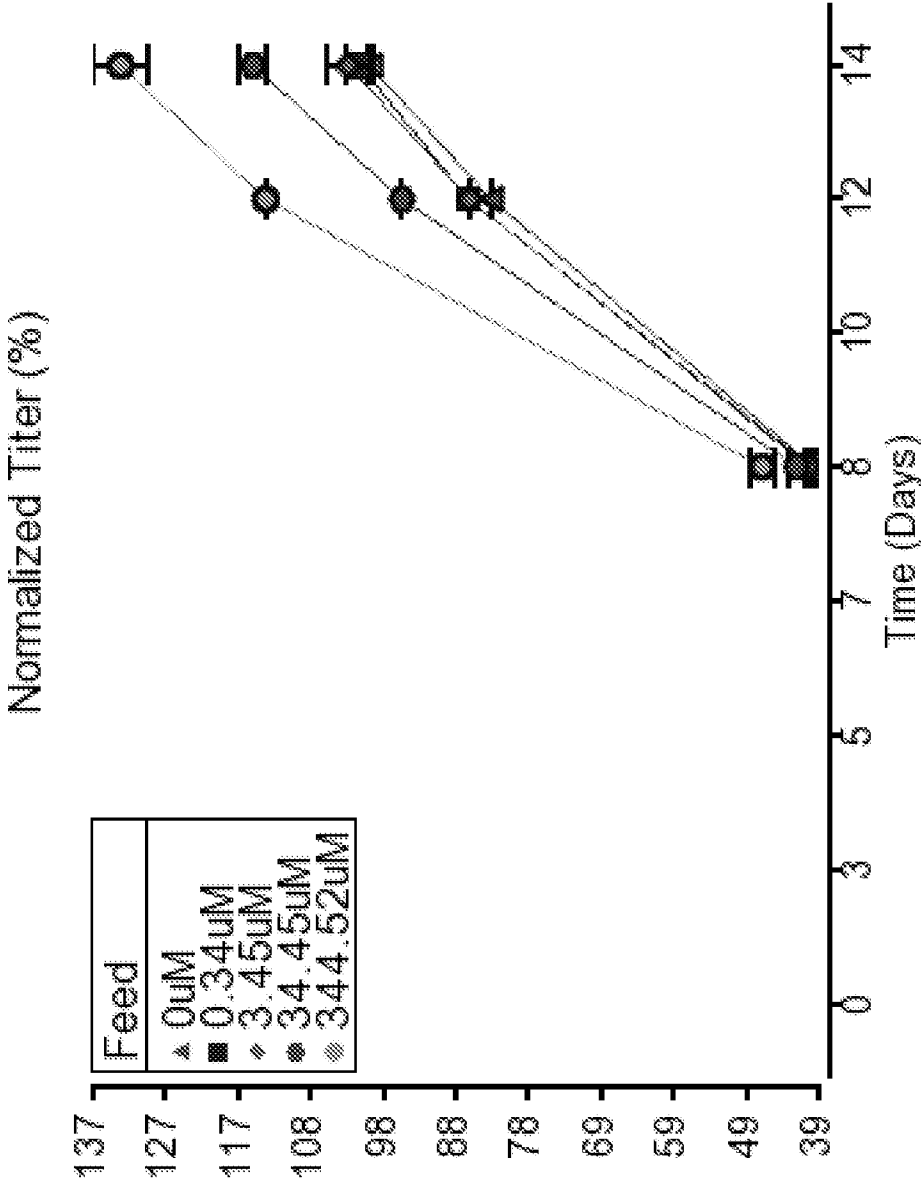


FIG. 9 (continued)



C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/017432

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
16-20(completely); 1-15(partially)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/017432

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P21/02
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)
C12P

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, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/182658 A1 (ABBVIE INC [US]) 13 November 2014 (2014-11-13) [0008] - [0009], [0011], [0039] - [0040], [0054]; WO2014182658 relates to invention 1; claims 14-16	1,6-9, 12-15
A	WO 2010/071800 A1 (SCHERING CORP [US]; LING WAI LAM WONG [US]; OUYANG ANLI [US]; MANAHAN) 24 June 2010 (2010-06-24) [0005] - [0007]; WO2010071800 relates to invention 1; example 2	1,5-15
	----- -/--	



Further documents are listed in the continuation of Box C.



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

20 July 2016

Date of mailing of the international search report

26/07/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Schönwasser, D

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/017432

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2011/050286 A1 (UNIV ARKANSAS STATE [US]; DOLAN MAUREEN C [US]; LORENCE ARGELIA [US];) 28 April 2011 (2011-04-28) WO2011050286 relates to invention 1; page 4, line 13 - page 7, line 16 page 12, line 31 - page 13, line 13 page 14, line 23 - page 15, line 2 -----	1,5-15
A	KIM KYUNG-CHAN ET AL: "Axl receptor tyrosine kinase is a novel target of apigenin for the inhibition of cell proliferation", INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE, vol. 34, no. 2, August 2014 (2014-08), pages 592-598, XP002756641, this article relates to invention 1; page 594, column 1, paragraph 3 - page 595, column 1, paragraph 2; figures 1,4 -----	1,5-15
A	YOON MAN-SOO ET AL: "Apigenin inhibits immunostimulatory function of dendritic cells: Implication of immunotherapeutic adjuvant", MOLECULAR PHARMACOLOGY, vol. 70, no. 3, September 2006 (2006-09), pages 1033-1044, XP002756642, ISSN: 0026-895X this article relates to invention 1; abstract; figure 1; table 1 -----	1,5-15
X	KR 2014 0096534 A (DONG A SOCIO HOLDINGS CO LTD [KR]) 6 August 2014 (2014-08-06) [0007]-[0010],[0028],[0058], [0063]-[0070]; WO20140096534 relates to invention 2 -----	1-15,18, 19
X	WO 2007/067564 A2 (AMGEN INC [US]; CROWELL CHRISTOPHER K [US]) 14 June 2007 (2007-06-14) WO2007067564 relates to invention 2; page 11, paragraph 3 -----	1-4,14, 15
X	AU 2010 236 145 A1 (FORSYTH DENTAL IN RY FOR CHILDREN FA) 10 November 2011 (2011-11-10)	1-15
A	AU2010236145 relates to invention 2 and invention 12; page 28, line 21 - line 23 -----	16,17,20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2016/017432

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014182658 A1	13-11-2014	TW 201446961 A US 2014329279 A1 WO 2014182658 A1	16-12-2014 06-11-2014 13-11-2014
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant apigenin to the cell culture.

2. claims: 18, 19(completely); 1-15(partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant catechin to the cell culture; a cell culture process for the production of protein, comprising culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production, wherein 0.1 mM catechin is added to the basal media and a cell culture process for the production of protein, comprising:
a) culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) feeding the cell culture with 1 mM catechin added to the feed media.

3. claims: 1-3, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant chlorogenic acid to the cell culture.

4. claims: 1, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant daidzein to the cell culture.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. claims: 1, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant genistein to the cell culture.

6. claims: 1, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant hesperetin to the cell culture.

7. claims: 1, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant melatonin to the cell culture.

8. claims: 1, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant naringenin to the cell culture.

9. claims: 1, 2, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
b) adding the antioxidant pelargonidin to the cell culture.

10. claims: 1-3, 5-15(all partially)

A cell culture process for the production of protein, comprising:
a) culturing host cells which produce a protein of interest

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

in cell culture under conditions that allow for protein production; and
 b) adding the antioxidant quercetin to the cell culture.

11. claims: 1-3, 5-15(all partially)

A cell culture process for the production of protein, comprising:
 a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
 b) adding the antioxidant resveratrol to the cell culture.

12. claims: 16, 17, 20(completely); 1-15(partially)

A cell culture process for the production of protein, comprising:
 a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
 b) adding the antioxidant rosmarinic acid to the cell culture, a cell culture process for the production of protein, comprising:
 a) culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
 b) feeding the cell culture with 1 mM rosmarinic acid added to the feed media, a cell culture process for the production of protein, comprising culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production, wherein 0.1 mM rosmarinic acid is added to the basal media and a cell culture process for the production of protein, comprising:
 a) culturing CHO cells which produce a protein of interest in cell culture under conditions that allow for protein production wherein 0.1 mM rosmarinic acid is added to the basal media; and
 b) feeding the cell culture with 1 mM rosmarinic acid added to the feed media.

13. claims: 1, 2, 5-15(all partially)

A cell culture process for the production of protein, comprising:
 a) culturing host cells which produce a protein of interest in cell culture under conditions that allow for protein production; and
 b) adding the antioxidant silibinin to the cell culture.
