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(54) **Title**: METHOD FOR CONTROLLING pH, OSMOLALITY AND DISSOLVED CARBON DIOXIDE LEVELS IN A MAMMALIAN CELL CULTURE PROCESS TO ENHANCE CELL VIABILITY AND BIOLOGIC PRODUCT YIELD

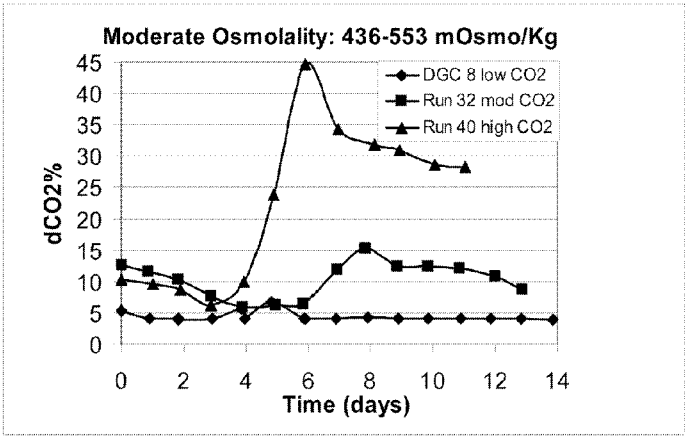

FIG. 1

(57) **Abstract**: Methods for controlling the level of dissolved carbon dioxide and limiting osmolality in a mammalian cell culture process to enhance cell growth, viability and density, and increase biologic product concentration and yield are provided. Such control of the level of dissolved carbon dioxide and pH as well as the resulting ability to limit osmolality in a mammalian cell culture process is achieved by adopting alternative pH control strategies and CO_2 stripping techniques during a mammalian cell culture process. Such pH control techniques and carbon dioxide stripping occur with little or no damage to the mammalian cells.

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METHOD FOR CONTROLLING pH, OSMOLALITY AND DISSOLVED CARBON DIOXIDE LEVELS IN A MAMMALIAN CELL CULTURE PROCESS TO ENHANCE CELL VIABILITY AND BIOLOGIC PRODUCT YIELD

Field of the Invention

[0001] The present invention relates to mammalian cell culture processes, and more particularly to methods for enhancing cell growth, cell density, cell viability, product concentration and product yield through improved control of process parameters including pH, osmolality and dissolved carbon dioxide level of the cell culture medium.

Background

[0002] Commercial production of protein therapeutics and other biological products such as monoclonal antibodies is presently carried out generally in bioreactors adapted for culturing suspensions of genetically optimized mammalian, insect or other cell types. Mammalian cell culture bioreactors typically have several hundred to several thousand liters in working volume. Most common full scale manufacturing plants have bioreactors with working volumes ranging from approximately 1,000 liters up to 25,000 liters. Drug candidates for clinical trials are produced in laboratory scale bioreactors having five (5) liters to several hundred liters of working volume.

[0003] The optimization to achieve the highest biological product yields possible in the smallest amount of time and the related challenges of bioreactor scale-up have focused on the control of recognized critical process parameters such as pH, dissolved oxygen (DO), temperature, nutrient composition and by-product profiles, agitation profile, gas sparging method, nutrient feed and product harvest profiles. The importance of other process parameters such as dissolved carbon dioxide (dCO_2) and osmolality (i.e. concentration of dissolved particles per kilogram of solution) is just recently being documented in the literature. As a matter of fact, many commercial bioreactors do not even have the means installed to measure dissolved carbon dioxide levels and/or osmolality in-situ, let alone a means to control and optimize those parameters. Depending on the scale of the

commercial operation – ranging from hundreds up to 25,000 liters of bioreactor volume – scale-up, optimization and control of the process pose different challenges. At commercial scales above about 1,000 liters, simultaneous and independent control of dissolved carbon dioxide levels and osmolality becomes difficult if not impossible with current best available technologies and methodologies.

[0004] Before a manufacturing-scale mammalian cell cultivation process starts in a bioreactor, a seed culture inoculum is typically prepared. This involves culturing production cells in a series of flasks in incubators and/or smaller bioreactors of increasing volume until enough cells are available for inoculation into the production bioreactor. The process involves transferring a cell population from one culture vessel to a larger one. Generally, a 20% dilution of the cell population is used for each transfer or subculture. In the incubator, the flasks with culture medium are clamped to a rotating platform to swirl the culture and facilitate gas transfer between the culture medium and the atmosphere in the incubators. Typically, the incubator for a mammalian cell culture process is set at 37°C with 5% carbon dioxide (CO_2) and a humidity level higher than about 80%. Similar temperatures and CO_2 levels are used for seed cultures grown in bioreactors. When the seed culture reaches a sufficient volume and cell density, it is inoculated into the production bioreactor.

[0005] After seed culture is inoculated into the bioreactor medium, parameters such as pH, temperature, and level of dissolved oxygen are controlled to the prescribed levels during the cell cultivation process. pH is typically controlled by adding basic or acidic solutions when necessary during the process. Commonly used base solutions include sodium bicarbonate, sodium carbonate and sodium hydroxide solutions. Dissolution of carbon dioxide (CO_2) is commonly used to achieve a more acidic pH. Although other acids are available for controlling pH, the dissolved CO_2 and sodium bicarbonate combination forms a most stable and favorable buffer system for the cell culture. The preferred temperature of the culture medium or solution for mammalian cell cultivation processes is about 37◦C. The desired level of dissolved oxygen in the culture

medium or solution is typically achieved through air sparging using sparger installed on the bottom of the bioreactor, along with agitation of the culture medium or solution using impellers which breakup the large air/oxygen bubbles to enhance the transfer of oxygen to the cell medium from the sparged air bubbles. Purging the bioreactor headspace with a cover gas provides a limited degree of surface gas exchange. Disadvantageously, air-sparging and agitation of the culture medium or solution may result in foaming and shear damage to the mammalian cells which adversely impacts cell viability. Accumulations of foam on the surface of the culture medium also serve to further limit surface gas exchange and to reduce the available working volume of the bioreactor.

[0006] Commercial-scale mammalian cell cultivation processes may be conducted in three different operation modes: batch mode or fed-batch mode for suspended cell cultures, and perfusion mode for immobilized cells. The majority of the commercial-scale mammalian cell cultivation processes are operated in fed-batch mode. In fed-batch mode, additional media and nutrients are added to the bioreactor at different times during the cell cultivation process to supplement the carbon source and other nutrients after initial bioreactor setup.

[0007] Before any bioreactor is used for mammalian cell cultivation, it typically must be sterilized and equipped with various probes as well as connections for supplemental gas supply and introduction of additional feeds. Temperature probes, pH detectors, dissolved oxygen probes and dissolved CO_2 probes or sensors are used to monitor the temperature, pH, dissolved oxygen and dissolved CO_2 levels of the cell medium or solution in real time. In addition, cell culture medium or solution samples can be withdrawn from the bioreactor at selected intervals to determine cell density and cell viability, as well as to analyze other characteristics such as metabolites and osmolality. Based on such analytical results, additional feed or other additives can be added to the cell culture medium or solution in an effort to prolong the cell viability and increase production of biological products. When cell viability reaches a prescribed lower threshold, the cell cultivation process can be stopped or shut down. The prescribed lower

threshold is often determined empirically based on the results of down-stream recovery and purification of the harvested biological products.

[0008] During the cultivation process, the mammalian cells exhibit three phases, namely the lag phase, the exponential growth phase, and the stationary or production phase. The lag phase occurs immediately after inoculation and is generally a period of physiological adaptation of mammalian cells to the new environment. After the lag phase, the mammalian cells are considered in the exponential growth phase. In the exponential growth phase, the mammalian cells multiply and cell density increases exponentially with time. Many cells actually start to produce the desired protein, antibody or biological product during some point in the exponential growth phase. Cell density refers to the total number of cells in culture, usually indicated in the density of viable and non-viable cells. When the mammalian cells reach the stationary or production phase, the viable cells are actively producing the biological products for downstream harvesting. During this phase, the total cell density may remain generally constant, but the cell viability (i.e. the percentage of viable cells) tends to decrease rapidly over time.

[0009] Mammalian cells are known to be sensitive to the amount of dissolved carbon dioxide in the cell culture media or solution. Mammalian cell cultures exposed to excess carbon dioxide levels during the exponential growth phase may demonstrate reduced production of monoclonal antibodies or other desired biological products. Before inoculation, the pH of the slightly alkaline culture media has to be lowered with carbon dioxide adjusted to an optimum value. This often leads to elevated levels of dissolved carbon dioxide at the beginning of the lag phase of many mammalian cell culture processes.

[0010] Dissolved carbon dioxide in mammalian cell culture bioreactors originates from chemical and biological sources. The chemical source of carbon dioxide is equilibrium chemical reactions occurring within the cell culture medium or solution that includes a selected amount of a buffer solution containing sodium bicarbonate and/or sodium carbonate. Additionally, carbon dioxide may be directly sparged into the slightly alkaline culture medium or solution to reduce the pH of the broth to a prescribed level, usually around 7.0, resulting in more

dissolved carbon dioxide. The biological source of carbon dioxide is a product of the respiration of the mammalian cells within the bioreactor. This biological source of carbon dioxide increases with cell density and generally reaches its maximum value at about the same time that cell density within the bioreactor is maximized. However, as more carbon dioxide is produced, the pH of the cell culture medium trends toward acidic such that additional bicarbonate is needed to keep the pH of the cell culture medium or solution within the desired range.

[0011] To offset the effects of increased dissolved carbon dioxide which depresses the pH, one may add sodium bicarbonate so as to maintain the pH of the solution within the prescribed range. Both of these means to offset the effects of increased carbon dioxide have other negative consequences on the mammalian cell culture process. First, any increase in dissolved carbon dioxide levels contributes to an increase in osmolality of the cell culture medium or solution. Similarly, the addition of sodium bicarbonate, needed to adjust the pH of the solution to offset the carbon dioxide, also increases osmolality. (Osmolality represents the number of dissolved particles per kilogram of solution and is commonly reported as mOsm/kg by freeze-point depression.) The addition of sodium bicarbonate will also increase the equilibrium saturation level of dissolved carbon dioxide allowed in the solution, making carbon dioxide more difficult to be removed during the aeration process. It is known in the art that increased levels of either dissolved carbon dioxide or increased osmolality have adverse or negative impacts on cell density or yield. However, the combined or synergistic effects of carbon dioxide levels and osmolality are not well understood.

[0012] Carbon dioxide dissociates into bicarbonate ions at a pH of 7 in water. Only a fraction of the carbon dioxide remains as free CO_2 in an un-dissociated state. Removing the dissolved carbon dioxide from a cell culture thus becomes difficult as most mammalian cell cultures take place at pH in the range of 6.5 to 7.5. The dissociated bicarbonate ions are not easily removed and generally must be recombined into free carbon dioxide before they can be stripped out of the solution. Any addition of sodium bicarbonate to balance the pH will also increase the equilibrium dissolved carbon dioxide concentration or saturation

level in the solution, making it more difficult to remove the carbon dioxide physically.

[0013] The conventional method of removing or stripping dissolved carbon dioxide from a mammalian cell culture solution is by sparging the cell culture solution with air or a gas mixture of air/oxygen/nitrogen in agitated tanks. However, gas sparging in agitated tanks results in adverse effects to the cell culture process. In particular, the gas-bubble breakage at the tip of the rotating agitator is a source of high shear rate that damages mammalian cell membranes, often sufficiently to cause cell death. Even when damage is sub-lethal, cell productivity is compromised in the period that the damaged membrane is repaired.

[0014] Also, sparging air or nitrogen into the bioreactor creates gas bubbles rising to the surface of the solution within the bioreactor where the gas is released into the headspace. Gas bubble breakage at the top surface of the cell culture solution is often more damaging to the mammalian cells than the damage caused by the agitator. Restraining the agitator speed and limiting the gas sparging rate are currently viewed as the best means to avoid such damage and increase cell viability. However, these measures reduce the amount of carbon dioxide that can be removed and the excess that cannot be removed also inhibits cell growth and viability. These disadvantages are particularly challenging to overcome in large, commercial-scale bioreactors where the shear rate goes up substantially with the diameter of the impellers. Also, the greater hydrostatic head of large scale bioreactors tends to increase the solubility of carbon dioxide, meaning that more needs to be removed to maintain dissolved CO_2 levels within an optimal range.

Summary of the Invention

[0015] The present invention may be characterized as a method for enhancing cell growth, cell viability, cell density, product yield and product concentration in a mammalian cell culture process comprising the steps of: (a) maintaining the dissolved carbon dioxide in a cell culture medium at a generally stable level of less than 10% concentration of dissolved carbon dioxide during a growth phase or production phase of the mammalian cell culture process; and maintaining the

osmolality in the cell culture medium at a value of between about 300 mOsmol/kg and 700 mOsmol/kg during the growth phase of the mammalian cell culture process.

Brief Description of the Drawings

[0016] The above and other aspects, features, and advantages of the present invention will be more apparent from the following, more detailed description thereof, presented in conjunction with the following drawings, wherein:

[0017] Fig. 1 is a graph that depicts percentage of dissolved carbon dioxide for three different runs of a mammalian cell line in a process having a moderate level of osmolality, wherein the three runs include one having a high peak level of dissolved carbon dioxide, another having moderate peak level of dissolved carbon dioxide, and the third having a low peak level of dissolved carbon dioxide;

[0018] Fig. 2A is a graph that depicts viable cell density in a mammalian cell culture process as a function of time in days for the three different runs of a mammalian cell line in the process from Fig. 1 having a moderate osmolality;

[0019] Fig. 2B is a graph that depicts cell viability as a percentage as a function of time in days for the three different runs of a mammalian cell line in the process from Fig. 1 having a moderate osmolality;

[0020] Fig. 2C is a graph that depicts total cell density cell in a mammalian cell culture process as a function of time in days for the three different runs of a mammalian cell line in the process from Fig. 1 having a moderate osmolality;

[0021] Fig. 3 is a graph that depicts biologic product concentration in a mammalian cell culture process as a function of time in days for the three different runs of a mammalian cell line in the process from Fig. 1 having a moderate osmolality;

[0022] Fig. 4 is a graph that depicts percentage of dissolved carbon dioxide in a mammalian cell culture process as a function of time in days for two different runs of a mammalian cell line in a process having generally constant or stable osmolalities wherein the first run includes a low peak level of dissolved carbon

dioxide and the second run includes a moderate overall peak level of dissolved carbon dioxide;

[0023] Fig. 5 is a graph that depicts viable cell density in a mammalian cell culture process as a function of time in days for the two different runs of a mammalian cell line in the process from Fig. 4 having a moderate osmolality and generally constant or stable levels of dissolved carbon dioxide;

[0024] Fig. 6 is a graph that depicts biologic product titer or concentration in a mammalian cell culture process as a function of time in days for the two different runs of a mammalian cell line in the process from Fig. 4 having a moderate osmolality and generally constant or stable levels of dissolved carbon dioxide;

[0025] Fig. 7 is a graph that depicts the dissolved carbon dioxide profile during the growth and production phases of a mammalian cell culture process;

[0026] Fig. 8 is a graph that depicts viable cell density in a mammalian cell culture process as a function of time in days for yet another two different runs of a mammalian cell line in which different but generally constant levels of dissolved carbon dioxide are maintained;

[0027] Fig. 9 is a graph that depicts osmolality in a mammalian cell culture process as a function of time in days for the two different runs of the mammalian cell line in the process from Fig. 8 having different but generally constant levels of dissolved carbon dioxide;

[0028] Fig. 10 is a graph that depicts percentage of viable cells in a mammalian cell culture process as a function of time in days for the two different runs of the mammalian cell line in the process from Fig. 8 having different but generally constant levels of dissolved carbon dioxide;

[0029] Fig. 11 is a graph that depicts biologic product yield or titer in a mammalian cell culture process as a function of time in days for the two different runs of a mammalian cell line in the process from Fig. 8 having different but generally constant levels of dissolved carbon dioxide;

[0030] Fig. 12 is a graph that depicts dissolved carbon dioxide in a mammalian cell culture process as a function of time in days for the two different

runs using the present Dynamic Gas Control (DGC) process compared against a standard run;

[0031] Fig. 13 is a graph that depicts cell viability in a mammalian cell culture process as a function of time in days for the two different runs using the present Dynamic Gas Control (DGC) process compared against a standard run;

[0032] Fig. 14 is a graph that depicts viable cell density in a mammalian cell culture process as a function of time in days for the two different runs using the present Dynamic Gas Control (DGC) process compared against a standard run;

[0033] Fig. 15 is a graph that depicts biologic product yield or titer in a mammalian cell culture process as a function of time in days for the two different runs using the present Dynamic Gas Control (DGC) process compared against a standard run;

[0034] Fig. 16 is a chart that depicts the trend of IgG titer versus peaked dCO_2 in the cell culture process with varying levels of osmolality;

[0035] Fig. 17 is a chart that depicts the trend of IgG titer versus maximum osmolality in the DGC type cell culture process with low levels of dissolved carbon dioxide; and

[0036] Fig. 18 is a table that provides the data collected during various cell culture process runs at various combinations of osmolality and dissolved carbon dioxide.

Detailed Description

Dissolved Carbon Dioxide, pH and Osmolality Relationship

[0037] With the majority of the commercial-scale mammalian cell culture manufacturing shifting to fed-batch processes, controlling to maintain a relatively constant osmolality, pH and dissolved carbon dioxide level is nearly impossible. Addition of nutrients and cell boosters during the fed-batch process will always tend to increase the cell culture osmolality, while pH and dissolved carbon dioxide levels are constantly changing throughout the process.

[0038] For example, carbon dioxide generated during the exponential growth phase can outpace the carbon dioxide stripping capacity of most current

bioreactors, resulting in a continuing increase in dissolved carbon dioxide levels. This continuing rise in dissolved carbon dioxide levels often requires the addition of an alkali to neutralize the effect of the dissolved carbon dioxide on pH, since controlling the pH of the cell culture medium is viewed as one of the most critical parameters to manage in any mammalian cell culture process. Increasing dissolved carbon dioxide and addition of alkali both further increase the osmolality of the cell culture medium or solution. In short, the pH, osmolality and dissolved carbon dioxide level in the cell culture medium or solution are all closely interrelated. Many of those skilled in the art believe lowest levels of dissolved carbon dioxide and osmolality should provide the best operating conditions of a mammalian cell culture process. However, recent studies and some empirical data disclosed herein suggest otherwise and that the optimum level of dissolved carbon dioxide and optimum osmolality still need to be determined for each separate mammalian cell line and cell culture process.

[0039] When carbon dioxide is dissolved in a mammalian cell culture medium, it forms HCO_3^-, an essential ion for growing cells. As the dissolved carbon dioxide establishes equilibrium with HCO_3^- ions, pH is lowered. The requirement for HCO_3^- is independent of its buffering action, but since carbon dioxide, HCO_3^- and pH are intimately interrelated, it has been difficult to define the optimum level and direct effects of dissolved carbon dioxide on cell growth. When incubating cells in open containers, gas mixtures of 95% air and 5% carbon dioxide are typically used. The concentration of carbon dioxide was selected originally on the basis of its being that found in the alveolar spaces of the lung. This carbon dioxide concentration was intended for studies on lung fibroblasts but has now become the typical carbon dioxide concentration in mammalian cell culture processes.

[0040] The gas phase carbon dioxide tension will regulate the concentration of dissolved carbon dioxide directly, as a function of temperature. This regulation in turn produces H_2CO_3, which dissociates according to the reaction:

$$H_2O + CO_2 \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^- \quad \text{.....(1)}$$

[0041] HCO_3^- has a fairly low dissociation constant, producing only low concentrations of hydrogen ions and achieving only a moderate lowering the solution pH. The net result of increasing atmospheric carbon dioxide is to depress pH by shifting the series of equilibria shown in (1) above to the right. To maintain a fixed pH, an alkali such as sodium bicarbonate is used to neutralize the effect of elevated carbon dioxide tension:

$$NaHCO_3 \Leftrightarrow Na^+ + HCO_3^- \quad \ldots\ldots\ldots\ldots (2)$$

[0042] The increased HCO_3^- concentration counteracts the effect of higher dissolved carbon dioxide levels, pushing the equilibria in (1) above leftwards until equilibrium can be established at pH 7.4 for the bicarbonate system.

[0043] In summary, cell cultures in open vessels need to be incubated in an atmosphere of carbon dioxide, the concentration of which is in equilibrium with the sodium bicarbonate in the medium. Cells grown in sealed flasks to moderately high concentrations (1×10^5 cells/ml) may not need carbon dioxide added to the gas phase provided that the bicarbonate concentration is kept low (~4 mM), particularly if the cells are high acid producers. At lower cell concentrations, however (e.g., during cloning or inoculation), and with some primary cultures, it is necessary to add carbon dioxide to the gas phase of sealed flasks. When venting is required to allow either the equilibration of carbon dioxide or its escape (as with high acid producers), it is necessary to leave the cap slack or to use a carbon dioxide-permeable cap. The majority of incubators are purged with mixtures of 95% air and 5% carbon dioxide.

[0044] In well controlled bioreactors, carbon dioxide will be needed at least at the start to adjust the medium pH to the proper value. Additional carbon dioxide will be needed to neutralize inoculants grown in small containers in incubators since these tend to have a higher pH than bioreactor set points. These initial pH adjustments with carbon dioxide will raise the osmolality of the starting batch.

[0045] As the cells cultured in a batch process reach the exponential growth phase, they become maximally metabolically active and each cell produces its

maximum carbon dioxide output. When the cell density is low, most of carbon dioxide can be removed by sparging the broth with air or sweeping the headspace of the bioreactor with a cover gas or air. A few days into the batch cycle, however, the carbon dioxide generation will exceed the normal carbon dioxide removal capacity of a typical bioreactor. The excess carbon dioxide generated by the cells will increase the dissolved carbon dioxide level and decrease the solution pH. In order to maintain the preferred pH, additional base has to be added, resulting in excessive dissolved carbon dioxide and undesirably high osmolality in the bioreactor broth.

[0046] The sub-optimal conditions due to imbalance between carbon dioxide generation and stripping rates become more severe with scale up to larger sized bioreactors. First, the surface-area-to-volume ratio decreases as conventional bioreactors increase in size. For the same cover gas to reactor volume, the effectiveness of carbon dioxide removal at the liquid surface is largely diminished. Examples of preferred carbon dioxide stripping systems and methods are disclosed in United States provisional patent application serial number 61/086665.

pH Optimization in Mammalian Cell Culture

[0047] The pH set-point in a mammalian cell culture process can significantly affect the cell-culture performance. Cell culture medium pH is known to affect intracellular enzymatic activity of many mammalian cell types. Lowering pH reduces specific glucose consumption and lactate production rates, reducing the risk of glucose depletion or toxic levels of lactate. The lower pH set point in typical mammalian cell cultures is about 7.0; a pH below about 6.8 is known to inhibit cell growth. Medium or moderate pH values also are known to affect the specific growth rate and specific production rate of mammalian cells, which ultimately affects the overall culture productivity. Excessively low or high pH can kill the cells.

[0048] A pH range of about 7.0 to 7.4 is commonly used in mammalian cell culture processes. The wide fluctuations in pH that often occur during the process

as, for example, when medium is replenished have an adverse effect on the cells. Controlling pH in mammalian cell culture processes is particularly important nowadays because high cell densities (>1x10^6 cells/ml) are routinely achieved. Without proper pH control, the cell culture broth can rapidly become acidic when cells are so concentrated.

[0049] Different types of mammalian cells may have different pH optima for growth. In general, human fibroblasts are grown at a higher pH (7.6-7.8) than established cells (pH 7.0-7.4), and it is usual to culture primary cells at a pH of 7.2-7.4. The optimum pH for growth of human foreskin fibroblasts (e.g. FS-4) at low culture densities is more alkaline than the optimum pH for growth of human lung fibroblasts (e.g. MRC-5). When culturing these cells during the growth phase at a density of about 10^5 cells/ml or less, the pH should be about 7.7 to 7.8 for FS-4 cells and about 7.5 to 7.6 for MRC-5 cells. For CHO cells, it is normally advantageous to cultivate the cells at a pH of about 7.0 during attachment. After several hours, the pH in CHO cell culture processes can be increased to slightly higher values.

[0050] Maintaining the cell culture broth at a pH of about 7.0 or higher presents another challenge to efforts to control dissolved carbon dioxide levels. As carbon dioxide can react with water, it may exist in the liquid phase in any of four forms: CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}.

[0051] The equilibrium relations as in equation (1), above, indicated that at a pH of about 5.0 or below, nearly all dissolved carbon dioxide is in the form of CO_2. At a pH of between about 7.0 to 9.0, bicarbonate is the dominant form of carbon. Finally, at a pH of about 11.0 or greater, nearly all is carbonate. Since the pH of most mammalian cell cultures is generally controlled between about pH7.0 to pH7.4, carbon dioxide removal is generally more difficult when compared to microbial fermentation processes where the pH can be much lower.

[0052] To remove dissolved carbon dioxide from a cell culture broth at pH between about 7.0 and 7.4, the limiting step can be either chemical or physical. Since only the dissolved carbon dioxide molecule is transported across the gas-liquid interface, the bicarbonate must be re-associated to form carbon dioxide

molecules. Separating equation (1) above into its two sections, it is noted that the reverse reaction set forth below as equations (3) and (4) is generally fast, whereas the first part of the reaction, represented by equation (5), is much slower.

$$H_2CO_3 \Leftrightarrow HCO_3^- + H^+ \quad \text{.....(3)}$$

$$K_{eq}(T = 28^oC) = \frac{[H^+][HCO_3^-]}{[H_2CO_3]} = 2.5x10^{-4}\ mol/L \quad \text{.....(4)}$$

$$H_2CO_3 \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} CO_2 + H_2O \quad \text{.....(5)}$$

where $k_1 = 20\ s^{-1}$ and $k_{-1} = 0.03\ s^{-1}$

[0053] Control of pH is a key operating condition as many types of mammalian cells die when the pH is substantially outside the range between pH7.0 and pH7.4. With the limitations inherent in current cell culture process controls, the primary target is pH regulation, with dissolved carbon dioxide/bicarbonate levels and osmolality largely uncontrolled and varying significantly during the culture cycle. Few data are available demonstrating the benefits of simultaneously maintaining constant pH, dissolved carbon dioxide and osmolality.

[0054] Culture media must be buffered under two sets of cell growth conditions: (1) in small open containers (e.g., inside an incubator), wherein the carbon dioxide can be lost to the atmosphere, causing the pH to rise, and (2) in a bioreactor when maximal production of carbon dioxide and lactic acid by high cell concentrations causes pH to fall. A buffer may be incorporated into the medium to stabilize the pH, but additional gaseous carbon dioxide is still required by some cell lines, particularly at low cell concentrations, to prevent the total loss of dissolved carbon dioxide and bicarbonate from the medium.

[0055] Despite its poor buffering capacity at physiological pH, bicarbonate buffer is still used more frequently than any other buffer because of its low toxicity, low cost, and nutritional benefits to the culture. Therefore, the role of carbon dioxide in controlling pH is still the most important aspect to consider when optimizing conditions for high cell yields and high cell viability.

[0056] If another alkali (e.g., NaOH) is used instead, the net result is similar to bicarbonate:

$$NaOH + H_2CO_3 \Leftrightarrow NaHCO_3 + H_2O \Leftrightarrow Na^+ + HCO_3^- + H_2O \quad \ldots (6)$$

[0057] Because many cell culture media components are made up in acid solution and may incorporate a buffer, it is difficult to predict how much bicarbonate to use when other bases may also indirectly contribute to bicarbonate levels as in equation (6) above.

[0058] With the introduction of Good's buffers (e.g. HEPES, Tricine) into tissue culture, there is speculation that carbon dioxide would no longer be necessary to stabilize the pH, and thus could be omitted. This speculation proved to be untrue, at least for a large number of mammalian cell types, particularly at low cell concentrations. Although 20 mM HEPES has been shown to control pH within the normal physiological range, the absence of atmospheric carbon dioxide allows equation (1) to move to the left, eventually eliminating dissolved carbon dioxide, and ultimately HCO_3^-, from the cell culture medium. This chain of events appears to limit cell growth, although it is not clear whether the limited cell growth is a result of lack of dissolved carbon dioxide or the lack of HCO_3^-, or both.

[0059] Another example is the Leibovitz L-15 cell culture medium that does not utilize carbon dioxide for buffering or to control pH. Leibovitz L-15 cell culture medium is preferably used when low tensions of carbon dioxide are required. Leibovitz L-15 contains a higher concentration of sodium pyruvate (550 mg/L) but lacks $NaHCO_3$ and does not require carbon dioxide in the gas phase. The inclusion of pyruvate in the medium enables mammalian cells to increase their production of carbon dioxide, making them independent of external supplied carbon dioxide, as well as HCO_3^-. Buffering in the Leibovitz L-15 cell culture medium is achieved via the relatively high amino acid concentrations. However, elimination of bicarbonate from the cell culture medium has a similar negative impact on cell growth as that seen with Good's buffers described above. These types of buffer systems may work well for small open dishes with low cell densities, but would be very detrimental in high cell density bioreactors.

[0060] At present, most cell culture media utilize a CO_2/HCO_3^- buffer system, but its capacity is often not sufficient to prevent pH decreasing towards the end of the cell culture cycle in small batch processes

[0061] In larger scale mammalian cell cultures in bioreactors, small changes in pH can be controlled by adding HCO_3 or increasing the carbon dioxide tension. Adding NaOH or HCl will control larger changes, but localized cell damage can result from addition of strong base or acid. The constant monitoring and control opportunities afforded by large-scale systems mean that HEPES is no longer essential for high cell yields. Cell culture pH can also be controlled when replenishing with fresh medium. Care should be taken not to significantly change the osmolality of the cell culture medium when adding buffers for pH control.

[0062] Medium osmolality significantly affects cell-culture productivity. Increased medium osmolality has been shown to decrease specific cell-growth rate and increase specific production rate. The initial medium osmolality can be predicted from the medium formulation. The amount of interaction between medium components typically does not make the osmolality significantly different from the sum of each component's contribution. Individual osmolalities for components of a typical medium are shown in the following table.

Medium Compositions	Osmotic Contribution(mOsm/Kg)
$CaCl_2$	19.42
$CuSO_4.5H_2O$	7.23
KCl	25.15
MgCl	15.00
$MgSO_4$	5.62
NaCl	34.74
NaH_2PO_4	18.23
$NaHCO_3$	23.27
ZnS_4-$7H_2O$	8.63
Glucose	6.49
L-glutamine	6.84
Amino acid pools	8.59
NaOH	50.00
Pluronic F-68	0.00
FBS	2.64

[0063] The growth and function of cells in culture depends on maintaining an appropriate osmolality in the medium. Some cells (e.g. HeLa and other established cell lines) can tolerate wide fluctuations in osmolality. In contrast, primary cells and normal diploid strains are very sensitive to changes in osmolality, and high yields can only be obtained if it is kept within a narrow range.

[0064] Controlling osmolality is reported to give more reproducible cultures. Whenever the source of a particular culture medium is changed, osmolality should be checked. Osmolality of cell culture media produced by commercial suppliers may differ, probably because of differences in interpretation of original formulations. However, high-yield cultures often require various additions to the medium during the culture cycle. These can include buffers (HEPES), acid (HCl), base (NaOH), growth hormone and nutrients. If it is necessary to raise osmolality, NaCl can be added, the correct amount required to achieve a particular osmolality is calculated as follows:

For example: 1 mg NaCl/ml = 1 ml stock (mOsm) = 32 mOsm increase.

$$\frac{D_{osm} - M_{osm}}{32} = X \quad \text{.....(7)}$$

where D_{osm} = desired osmolality (mOsm)
M_{osm} = measured osmolality (mOsm); and
X = ml of stock of NaCl (mOsm) to be added per milliliter of medium.

[0065] The osmolality of the medium is measured and the amount of stock NaCl (1 mg/ml) that must be added to achieve the desired osmolality is calculated. Measuring osmolality by freezing point depression is the most practical method, since it does not require diluting the nutrients in the medium or adding large volumes of buffers or saline solutions. Vapor pressure depression is another popular method of measuring osmolality.

pH Control

[0066] The most common procedure to maintain pH in mammalian cell culture is to use sodium bicarbonate/carbon dioxide, a gentle buffer that gives very good protection against pH fluctuations in the bioreactor. However, the

bicarbonate level dictates the equilibrium dissolved carbon dioxide level at the start of the cell culture cycle as the concentration ratio of bicarbonate to dissolved carbon dioxide is set by the rapid acid-base equilibrium. The pH in the bioreactor is thereafter controlled with further additions of bicarbonate or carbon dioxide. For example, lactic acid generation by the cell culture process would prompt further bicarbonate addition until a pH of about 7.0 is attained when the bicarbonate partially decomposes into carbon dioxide. Ammonia generated by cells during the cell culture process would prompt further carbon dioxide addition. Continually adding bicarbonate or carbon dioxide typically results in excessive osmolality in the cell culture medium as well as continual fluctuations in the dissolved carbon dioxide levels during the cell culture process.

[0067] The system and method disclosed herein for controlling pH in a mammalian cell culture process comprises ascertaining the desired pH range and desired level of dissolved carbon dioxide for the selected cell culture medium; providing an initial minimum amount of bicarbonate to adjust the pH of the cell culture medium to fall within the desired pH range and produce the desired level of dissolved carbon dioxide within the cell culture media. It was found that this initial equilibrium between dissolved carbon dioxide level and bicarbonate level has a significant impact on final cell viability and product level and yield. Enough sodium bicarbonate is added into the medium before inoculation sufficient to allow an equilibrium of dissolved carbon dioxide to attain only a low level, less than 10% and more preferably about 5%.

[0068] Thereafter, pH is maintained by adding sodium hydroxide as required to maintain pH within the desired range to avoid further increase in bicarbonate and an associated increase in dissolved carbon dioxide. The sodium hydroxide – a strong base - also maintains pH within the desired range without significantly increasing the osmolality and maintains the levels of dissolved carbon dioxide relatively stably at or near the desired levels.

Controlling Dissolved Carbon Dioxide Levels to Enhance Cell Culture Process

[0069] Some prior art references suggest that the level of dissolved level carbon dioxide in the cell culture solution has no effect on specific growth rate and cell density during the exponential growth phase or the production phase of the cell culture process.

[0070] The present system and method provides for tight control of the dissolved carbon dioxide level in the cell culture media both at start-up and during the exponential growth phase which provides a beneficial effect on cell viability during the production phase. Thus, the accumulated product yield is also influenced by the exposure of the cells to prescribed levels of dissolved carbon dioxide during the growth phase. As described herein, various test runs or test batches demonstrate that tightly controlling the level of dissolved carbon dioxide during the exponential growth phase yields higher accumulated product yield during production phase and also results in a slower degradation or reduction in cell viability during the production phase.

[0071] Exchange between gas in the bioreactor vessel headspace and that dissolved in the liquid/solution occurs at the surface of the cell culture solution. Carbon dioxide removal by this means is attractive as compared to stripping via sparged gas since it minimizes shear and bubble damage to cells and reduces or eliminates foaming. Surface gas exchange in commercial scale bioreactors is not presently exploited for carbon dioxide removal, however, since under current process conditions it is far too limited to have practical use. This is a direct consequence of the limited surface to volume ratio of typical conventional bioreactor vessels and the slow rates of culture surface renewal achieved by current agitator designs. These problems become worse in bioreactors with tall and narrow configurations.

[0072] Another disadvantage of surface gas exchange in commercial scale bioreactors occurs with the use of rotating shaft agitators. These cause the surface liquid to swirl around in a circle with little tendency for solution from deeper within the vessel to replace it. This has at least two consequences affecting

surface gas exchange: first, the surface liquid layer rapidly becomes depleted of dissolved carbon dioxide, lowering the driving force for subsequent CO_2 removal to the headspace; second, liquid from the bottom of the bioreactor (where the concentration of dissolved CO_2 is greatest thanks to the higher hydrostatic pressures in this region) is only rarely driven to the surface where it can donate dissolved gas to the headspace. The overall effect is that removal of dissolved CO_2 is slow and that there is a gradient of dissolved CO_2 concentration in the bioreactor, from very low at the surface to high at the bottom where it can easily reach levels that reduce cell productivity and viability.

[0073] The present method of controlling the dissolved CO_2 removal employs a bioreactor system having an upward flow impeller disposed within a draft tube disposed in the bioreactor vessel. The upward pumping impeller is driven via shaft by a motor outside the bioreactor vessel. The upward flow of the impeller provides a top surface renewal method that enhances surface gas exchange in a highly controllable manner. The upward pumping impeller moves cell culture medium and suspended mammalian cells from the bottom of the bioreactor vessel toward the liquid/headspace gas interface in the upper part of the reactor. In doing so, dissolved carbon dioxide in the cell culture solution or medium is continuously and rapidly brought to the surface of the liquid in the bioreactor where gas-liquid exchange is occurring. A high turnover in the surface liquid allows rapid removal of dissolved carbon dioxide to the headspace. The upward flow impeller allows a higher pumping velocity without creating sufficient shear to damage or kill the mammalian cells. A sweeping gas consisting of oxygen, nitrogen, air, carbon dioxide or other suitable gases and mixtures thereof that is introduced to the headspace in the bioreactor vessel, where it interacts with the top surface of the solution to achieve the desired liquid gas exchange, and is subsequently exhausted from the headspace in the bioreactor vessel.

[0074] The preferred bioreactor system also may include a plurality of sensors and analyzers including a pH sensor, a dCO_2 sensor, a temperature indicator, a dissolved oxygen analyzer, and a vent gas analyzer. Such sensors and analyzers are coupled as inputs to a system controller (not shown) that controls or

adjusts the gas supply of oxygen, nitrogen, and carbon dioxide to the bioreactor vessel. The system may also include an exhaust subsystem, a plurality of biological filters as well as a means for sterilizing the bioreactor vessel with water and steam, as needed. .

[0075] The upward pumping impeller is preferably located near the middle of the main bioreactor vessel so that the impeller is submerged for low liquid medium or solution starting levels. The impeller speed is adjustable and may be varied throughout the cell culture process to maintain the desired level of dissolved carbon dioxide at all times for the particular mammalian cell culture process. Preferably, the impeller speed is maintained at very low speeds when the liquid or solution level within the bioreactor vessel is low and should be increased as the liquid or solution level rises. Preferably, a draft tube is to be added to increase the upward flowing velocity, resulting in a higher gas exchange rate. The impeller speed is preferably highest during the end of the exponential growth phase of the cell culture process, when the liquid or solution level in the bioreactor vessel is also highest. Normally, surface gas exchange is an inefficient process as the available surface area is very limited. Any gas exchange occurring between the headspace and the liquid surface will quickly result in gas concentrations on either side of the gas/liquid interface quickly approaching saturation levels. Without proper concentration driving force at the interface, surface aeration is impractical unless measures are implemented to greatly increase the surface area available for gas exchange. Unfortunately, such measures (e.g., atomization of some of the liquid) create excessive shear that would damage and kill fragile mammalian cells. This invention overcomes those limitations, however, by rapidly sweeping the headspace gases to avoid carbon dioxide build up in the gas phase boundary layer. The limitation of the liquid phase boundary layer is also eliminated by the upward pumping action of the submerged impeller.

[0076] It was observed that a number of vertical baffles added on top of the impeller make very large improvements to the gas exchange rate. These vertical baffles translate the rotational velocity into virtually pure vertically oriented flows.

To compare the effect of the draft tube and vertical baffles on the dissolved CO_2 removal rate through the liquid surface, a carbon dioxide removal test was conducted in a 300 L vessel using the method described in this invention. The solution in the vessel was maintained at a pH of 7 and headspace swept with air. The helical impeller was set to run at two different speeds with a frequency inverter. Dissolved CO_2 level was measured continuously during the experiment. The results were reported in terms of volumetric mass transfer coefficient (K_La) in Table (1).

Frequency inverter (Hz)	Without Draft Tube or Baffles	Draft Tube + Baffles	Improvement in Mass Transfer Coefficient
	K_La (1/hr)	K_La (1/hr)	%
40	0.85	6.61	678%
30	1.29	4.2	226%

Table 1

[0077] Depending on the speed of the helical impeller, the results showed that the mass transfer coefficient improved between 226% and 678% when a draft tube with baffle was used. Further tests were conducted to show the importance of the vertical baffles on the surface gas exchange phenomena in these experiments, the helical impeller was installed in the bottom of the 300 L vessel with the vertical baffles removed. From the experimental work of this invention, it was concluded that it is critical to eliminate the swirling movement of the surface liquid (see Table 2). By eliminating swirling motion at the surface, the upward flowing liquid from the impeller emerges quickly from the impeller shaft and spreads across the entire vessel surface, re-submerging into the body of the liquid near the edge of the vessel. With the vertical baffles installed, the carbon dioxide removal rate was improved by 28% to 128%, depending on the rotational speed of the helical impeller. These experimental results show that liquid from the lower part of the bioreactor rapidly replaces the surface liquid, resulting in substantially higher rates of dissolved carbon dioxide removal and oxygen dissolution. Without the vertical baffle, the swirling surface liquid is not significantly replaced by fresh liquid from deeper within the bioreactor.

Frequency inverter (Hz)	Draft Tube, w/o Baffles	Draft Tube with Baffles	Improvement in Mass Transfer Coefficient
	K_La (1/hr)	K_La (1/hr)	%
40	1.37	3.12	128%
30	1.24	1.98	60%
20	0.8	1.02	28%

Table 2

[0078] As discussed above, the liquid or solution in the bottom of a large bioreactor vessel is exposed to significant hydrostatic pressures, and the dissolved carbon dioxide trapped inside the mammalian cells will be slow to equilibrate. The presently disclosed upward pumping impeller mitigates this problem. By recirculating liquid solution and mammalian cells from the bottom of the bioreactor vessel upward to the top surface, the mammalian cells are exposed to a lower overall average hydrostatic pressure regime and thus achieve a better equilibrium level of dissolved carbon dioxide. The continuous axial or upward recirculating of the cell culture medium or solution provides a varying level hydrostatic pressure on the mammalian cells which is believed to enhance the ability of the cells to expel excess dissolved carbon dioxide deep inside the plasma of the cells

[0079] Since there are no deflecting walls or dividers in the bioreactor the upward flowing liquid can reach the top surface very rapidly before rolling outward towards the bioreactor wall. This provides a very rapid renewal of the liquid surface which promotes rapid removal of dissolved carbon dioxide. Alternate forms of impellers can be used to provide the upward recirculating flow with or without the draft tube. Preferably, the upward pumping impeller is a screw impeller or propeller. However, other propellers may also be used so long as the lateral or radial flow from the propeller is minimized which, in turn reduces shearing and other damage to the mammalian cells.

[0080] Rapid gas-liquid surface renewal is also useful for dissolving gases into the liquid. For example, the presently disclosed gas-liquid surface renewal method can be used to dissolve the prescribed amount of oxygen needed for the growing cells. When the demand for oxygen is high, the oxygen composition in

the sweeping gas in the headspace is increased, resulting in increased transfer of oxygen to the top surface of the recirculating liquid. When the oxygen dissolution requirement is low, the oxygen composition in the sweeping gas in the headspace is reduced and replaced with air or nitrogen. The variation in oxygen composition of the sweeping gas has little or no impact on the carbon dioxide removal rate. The dissolved oxygen concentration is preferably maintained at about 50% in many mammalian cell culture processes. In some cases, such as recombinant protein production from virus infected sf-9 insect cell culture, very low oxygen concentrations (e.g. less than 5% oxygen concentration) are used in the cell culture solution to enhance protein production by the cells.

[0081] The dissolved carbon dioxide level can be adjusted or maintained at any desirable level. To decrease the dissolved carbon dioxide level at any time during the cell culture process, the flow rate of the sweeping gas going into the headspace of the bioreactor can be increased to more rapidly eliminate CO_2 from the liquid near the surface. The impeller rotational speed can also be increased to speed up the surface liquid renewal rate. To increase the dissolved carbon dioxide level, one would reduce the sweeping gas flow rate and/or decrease rotational speed of the upward pumping impeller. If additional carbon dioxide is needed as, for example, may be the case in the earliest stages of the process shortly after inoculation of the production bioreactor, it can be added to the sweeping gas mixture in the headspace as required. In typical mammalian cell culture processes, the dissolved oxygen requirement increases as the batch proceeds from the initial lag phase to the end of the exponential growth phase, while the dissolved carbon dioxide concentration increases due to cell respiration, reaches a maximum concentration towards the end of the exponential growth phase, and then is gradually reduced during the production phase. Therefore, gaseous carbon dioxide is added mostly during the lag phase to regulate and maintain pH. Also, some prescribed level of dissolved oxygen needs to be maintained during the cell production phase.

[0082] In addition to independently adjusting or controlling the nitrogen, oxygen and carbon dioxide concentrations in the sweeping gas mixture, increasing

the total headspace gas flow will also avoid accumulation of the stripped gases in the headspace.

[0083] In the preferred embodiment, the gas supply of nitrogen, oxygen and carbon dioxide to the bioreactor vessel is introduced above the top surface of the liquid in the headspace and preferably closely adjacent to the rolling surface of the liquid solution in the bioreactor vessel. Such gas introduction can be achieved by making the gas injectors movable so as to always inject the gases at or near the top surface as the liquid level in the bioreactor vessel rises. Impingement of the gas at the rolling top surface reduces the momentum boundary layer on the gas side and improves the total mass transfer rate between the liquid and gas. Alternatively, the gas supply may be delivered using fixed gas injectors disposed so as to introduce the gas at a location near the maximum liquid height that will be attained in the bioreactor vessel. In most mammalian cell culture processes, the maximum liquid height in the bioreactor vessel occurs during the peak of the exponential growth phase where removal of dCO_2 is most necessary.

[0084] Although not preferred, controlled introduction of the gas supply of nitrogen, air, oxygen and carbon dioxide to the bioreactor vessel may be done by sparging the gases within the solution using one or more spargers disposed within the bioreactor vessel. The sparger used to dissolve oxygen can have finer nozzles (or holes) to generate small oxygen bubbles that dissolve or are absorbed before breaking the liquid surface. The sparger for the stripping gas, typically introduced at considerably higher flow rates, can have much larger nozzles to provide large diameter gas bubbles. Large gas bubbles are less damaging when they break at the surface of the liquid and have less tendency to produce foam. Such submerged gas spargers can assist with the independent control of both oxygen and dissolved carbon dioxide levels in combination with the headspace gas exchange method. When used, the gas spargers are preferably located apart from the upward flow impeller to maximize their residence time in the cell culture medium. With this method, the stripping gas bubbles are much bigger than those injected into axial flow impellers and the potential for foaming is greatly diminished. Gas exchange now occurs both on the surface and in the bulk of the

liquid. Sparging small volumes of gases intermittently for short periods of time allows oxygen uptake and carbon dioxide removal to be maximized without resorting to very high flows of sweeping gas or employing the fastest impeller speeds. It is important that such sparging be done only at peak demand for oxygen dissolution and carbon dioxide removal in order to minimize cell damage.

[0085] The preferred upward pumping device is a helical impeller that can move large volumes of liquid upward with minimal radial flow. Using a helical impeller, carbon dioxide removal rate was measured from a simulated broth and reported as Volumetric Mass Coefficient. The higher the mass transfer coefficient, the better the gas exchange efficiency. Even with an upward pumping impeller, the moving liquid stream is going to be rotated by the rotation of the agitator. As a result, the surface liquid is going to swirl, greatly reducing liquid surface renewal as the surface liquid rotates in the plane of the surface. To stop the swirling, a vertical baffle system is also used on top of the impeller to break the rotation of the liquid and redirect the flow straight to the surface. Hence, the surface liquid radiates outwards from the shaft at the center of the vessel, spreading and thinning towards the edge of the vessel where it submerges. As a result, the surface gas exchange and carbon dioxide stripping is greatly improved.

[0086] Turning to Figs. 1-3 and in particular Fig. 1, there are shown test data in graphical form for three different runs of a mammalian cell culture process wherein the osmolality of the cell culture solution was maintained at a moderate value (i.e. 436 to 553 mOsm/kg). Of the three samples tested, one of the samples incorporating the presently disclosed Dynamic Gas Control (DGC) technology and identified as DGC 8, has a level of dissolved carbon dioxide maintained at about 4% throughout the process with a small increase to about 5.7% dissolved carbon dioxide on Day 5.. A second sample (identified as Run 32) has a starting level of dissolved carbon dioxide of about 12 % and then decreased to about 6% in the early stages of the growth phase, followed with increasing level of dissolved carbon dioxide to a maximum of about 15%. The level of dissolved carbon dioxide then gradually went down to about 10% in the production phase. A third sample (identified as Run 40) has a starting level of dissolved carbon

dioxide of about 6% to 10% during the lag phase and a high level of variability in dissolved carbon dioxide level ranging from about 5% to about 44% throughout days 4 through 11 of the cell culture process.

[0087] As seen in Fig. 2A DGC 8 maintained a higher viable cell density (MC/ml) during the production phase of the mammalian cell culture process than Run 30 and a significantly higher viable cell density than Run 40. The data in Figs. 2B and 2C depict similar benefits associated with the DGC technology disclosed in this application.

[0088] Similarly, as seen in Fig. 3, DGC 8 maintained a higher product yield (mg/l) of IgG than the product yield of Run 30 and corresponding product yield of Run 40. Also, the specific productivity (pg/viable cell .day) in DGC 8 with low dCO2 was increased significantly. Specific productivity for the sample processes were about 40 pg/viable cell.day (DGC 8) , 20 pg/viable cell.day (Run 32) and 16 pg/viable cell.day (Run 40), respectively. As evidenced by the DGC 8 data in Figs. 1, 2A, 2B, 2C, and 3, maintaining a stable and low level of dissolved carbon dioxide throughout the cell culture process can enhance cell viability, increase product yield and specific productivity.

[0089] Referring now to Figs. 4-6, there are shown graphs depicting the characteristics and results of two additional test runs of a mammalian cell culture process. As seen therein, the sample runs maintained a generally constant or stable level of dissolved carbon dioxide and moderate osmolality of the cell culture medium. Specifically, as shown in Fig. 4, Run 50 maintained a moderate level of dissolved carbon dioxide between about 13% and 18% during the exponential growth phase and production phase of the cell culture process whereas Run 55 maintained a low level of dissolved carbon dioxide between about 2% and 6% during the exponential growth phase and production phase of the cell culture process. As seen in Fig. 5 sample Run 55, with the generally stable but low level of dissolved carbon dioxide and moderate osmolality, demonstrated a higher percentage of cell viability during the production phase that Run 50 having a generally stable but moderate level of dissolved carbon dioxide and moderate osmolality. As seen in Fig. 6 sample Run 55, with the generally

stable but low level of dissolved carbon dioxide and moderate osmolality, demonstrated a higher product yield during the production phase that Run 50 having a generally stable but moderate level of dissolved carbon dioxide and moderate osmolality. The results of these charts further confirm the conclusions drawn from Figs 1-3 that maintaining a stable and low level of dissolved carbon dioxide throughout the cell culture process enhances cell viability, increase product yield and specific productivity

Optimization of Dissolved Carbon Dioxide Levels and Osmolality

[0090] The present system and method preferably maintains a generally constant or stable and low level of dissolved carbon dioxide of less than 10% during the lag and exponential growth phase, and more preferably around 3% to 5% while maintaining a moderate osmolality of between about 300 and 700 mmole/kg, and more preferably between about 350 and 560 mmole/kg during the lag phase and exponential growth phase (See Fig. 1, Fig 4 and Fig. 9). This combined dissolved carbon dioxide level and osmolality process condition provides longer cell viability and highest biological product yield during the production phase for given mammalian cell culture processes (see Figs 2, 3, 5, 6, 8, 10 and 11).

[0091] Turning now to Figs. 7-11 there are shown sample data obtained from yet two additional mammalian cell culture process runs. Fig. 7 depicts the dissolved carbon dioxide levels during the growth and production phases of Run 62 which has a low level of dissolved carbon dioxide of about 5% during the lag and exponential growth phases and Run 63 which has a moderate level of dissolved carbon dioxide of about 10% during the lag and exponential growth phases of the cell culture process. Fig. 8 further shows that the viable cell density for sample Run 62, with the low level of dissolved carbon dioxide demonstrated a higher degree of cell viability during the production phase than Run 63 having a moderate level of dissolved carbon dioxide. Figs. 10 and 11 shows that sample Run 62, with the low level of dissolved carbon dioxide demonstrated a higher

percentage of cell viability and higher product yield during the production phase that Run 63 having a moderate level of dissolved carbon dioxide.

[0092] The present system and method also provides for higher osmolality and higher levels of dissolved carbon dioxide in the production phase (See Figs. 7 and 9). Such higher osmolalities and higher levels of dissolved carbon dioxide in the production phase may actually enhance the overall bioreactor productivity. Specifically, Fig. 9 shows the osmolalities for Run 62 and Run 63 during the exponential growth phase and production phase of the cell culture process which were maintained in a moderate range of about 350 mOsmol/kg to about 400 mOsmol/kg during the growth phase and between about 400 mOsmol/kg to about 700 mOsmol/kg during the production phase. Fig. 7 shows the dissolved carbon dioxide levels during production phase of between about 20% to 50% for Run 62 and between about 20% and 30% for Run 63.

[0093] Turning now to Figs. 12 through 17, there are shown charts containing data comparing the cell culture process using Dynamic Gas Control (DGC) compared to a cell culture process without employing the Dynamic Gas Control (DGC). The data on the illustrated charts suggest that sample runs employing the Dynamic Gas Control (DGC) process at moderate osmolality, namely samples DGC2 and DGC3,

[0094] In sample process DGC2, the dissolved carbon dioxide was started at about 8.45%, and was subsequently maintained in a range between about 7.0% to 7.5% throughout the remaining cell culture process. In sample process DGC3, the dissolved carbon dioxide was started at about 5.5%, and was maintained in a range between about 5.5% to 6.3% for Day 1 and Day 2, and subsequently decreased to about 4.5% at Day 3 and Day 4, and further reduced to about 4.0% from Day 4 to Day 15. Finally, Run 32: had a dissolved carbon dioxide profile very typical cell culture process where the average dCO2 was maintained about 6% in the growth phase, followed with increasing dCO2 to about 15%, then gradually lowered to about 10% in the production phase.

[0095] The data contained in Figs 12-17 shown that the dissolved carbon dioxide levels can be well maintained at desired low level through the process with

Dynamic gas Control (DGC) process Both the DGC2 and DGC3 sample runs had higher viable cell density and viability during later stages of protein production. Sample run DGC3 had the highest product titer among these three runs, and reached maximum product titer much earlier than either DGC2 or Run 32.

[0096] Fig. 18 is a table that provides the cell culture process data collected during various sample runs at various combinations of osmolality and dissolved carbon dioxide.

[0097] The present system and method also provides for a low level of dissolved carbon dioxide of less than 10%, and more preferably around 5% or less while diluting the mammalian cell culture batch with water during the in the production phase while adding additional nutrient and cell booster during the production phase. This dilution and nutrient supplementation procedure provides higher mammalian cell culture bioreactor product yields and appears to dilute some of the critical toxic waste buildup.

[0098] All three of the above process optimization techniques, alone or in combination, enhance typical mammalian cell culture bioreactor product yields by controlling a plurality of critical process parameters, including the level of dissolved carbon dioxide and osmolality in addition to the previously recognized process parameters of pH, dissolved oxygen level, temperature, pressure, nutrient and waste product profiles in the media, agitation, gas sparging, nutrient feed and product harvest

[0100] The impact on the process yields are initially established either under scaled-down conditions in a smaller scale bioreactor or at full commercial bioreactor scale. After establishing the optimal dissolved carbon dioxide, osmolality, pH, dissolved oxygen, temperature and nutrient and waste product levels in the media, agitation profile, gas sparging method, nutrient feed and product harvest profiles optimum for commercial production, we propose to implement these conditions by measuring and controlling such parameters.

[0101] Broadly described, the present cell culture process optimization and control method comprises: (1) process optimization phase; and (2) active control phase. The process optimization phase involves empirically determining the

desired pH, osmolality and dissolved carbon dioxide levels for a given mammalian cell culture process, cell line and bioreactor configuration. A microprocessor-based controller is then programmed to establish the initial settings as well as permissible values or ranges for overlay gas composition, overlay gas flow rate, sparged gas composition, sparged gas flow rate, acid addition, base addition, nutrient addition, media harvest, etc. to achieve the desired dissolved carbon dioxide and osmolality in the bioreactor while maintaining pH within the desired range and maintaining one or more of the other process parameters such as dissolved oxygen level, agitator speed, temperature, pressure, nutrient content, waste product content, etc. within specifications Individual gases or gas mixtures relevant for cell culture bioreactor sparging and overlaying may include the addition and removal of oxygen, nitrogen, air, argon, carbon dioxide, or combinations thereof. The empirical determination of desired pH, osmolality and dissolved carbon dioxide level for a given mammalian cell culture process is preferably accomplished in laboratory scale bioreactors running scaled-down process conditions and may be supplemented with appropriate model-based studies.

[0102] The active control phase involves monitoring or measuring a plurality of parameters to be used as inputs to the microprocessor-based controller. Such inputs include the dissolved carbon dioxide level, osmolality and pH, as well as optional inputs of dissolved oxygen level, temperature, nutrient and waste product concentration, agitation, gas sparging or overlay gas flow rate and composition, nutrient feed volume and composition and product harvest volume and composition. Such inputs are fed to the controller at a regular interval or a continuous basis throughout the production and growth phase of the cell culture process. The microprocessor based controller receives the inputs and produces one or more output signals representing the value and setting of at least one parameter selected from the group of overlay gas composition, overlay gas flow rate, sparged gas composition, sparged gas flow rate, agitator speed, acid addition, base addition, nutrient addition, media harvest, etc. The output signals are used to control or adjust the overlay gas composition, overlay gas flow rate, sparged gas

composition, sparged gas flow rate, agitator speed, acid addition, base addition, nutrient addition, media harvest, etc which maintains the dissolved carbon dioxide level, osmolality, or pH at the desired values or ranges. As the production phase progresses, the monitoring and measuring of parameters and corresponding adjustment or control of such parameters continues until the bioreactor process is complete.

[0103] This proposed process control scheme is applicable for nearly constant physiological temperature and also hypothermic cell culture processes. Hypothermic cell culture processes run at least part of the time at less than the typical approx. 37°C process temperature. This proposed process control scheme is also applicable to nearly any configuration of bioreactor and operating in any mode, including batch mode, fed-batch mode, or a continuous mode of operation.

[0104] From the foregoing, it should be appreciated that the present invention thus provides various methods and systems for controlling the dissolved carbon dioxide level, pH and osmolality during a mammalian cell culture process to enhance cell viability and biologic product yield. Numerous modifications, changes, and variations of the present methods and systems will be apparent to a person skilled in the art and it is to be understood that such modifications, changes, and variations are to be included within the purview of this application.

Claims

What is claimed is:

1. A method for enhancing cell growth, cell viability, cell density, product yield and product concentration in a mammalian cell culture process comprising the steps of:

maintaining the dissolved carbon dioxide in a cell culture medium at a generally stable level of less than 10% concentration of dissolved carbon dioxide during a growth phase or production phase of the mammalian cell culture process; and

maintaining the osmolality in the cell culture medium at a value of between about 300 mOsmol/kg and 700 mOsmol/kg during the growth phase of the mammalian cell culture process.

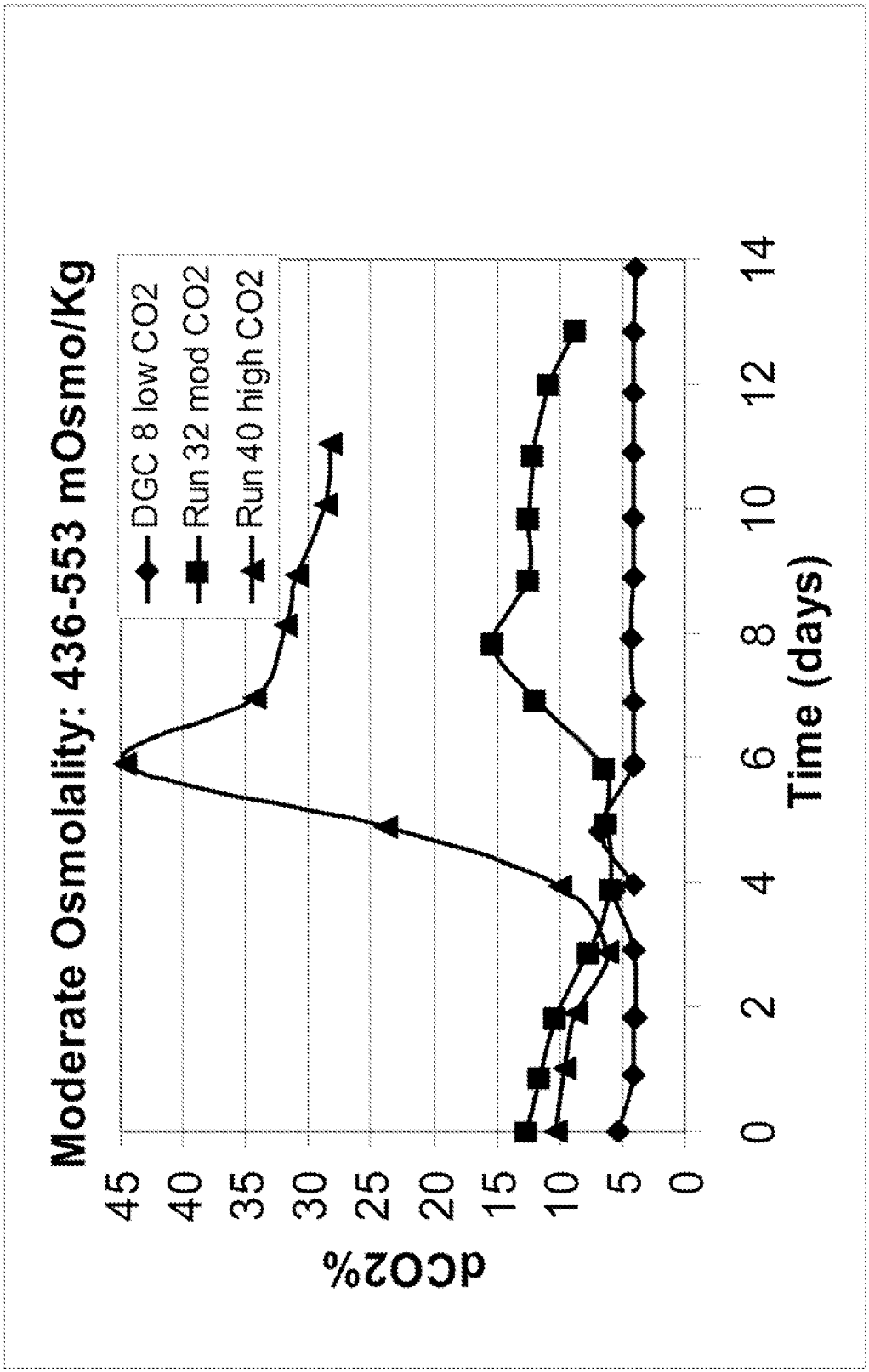
Moderate Osmolality: 436-553 mOsmo/Kg
DGC 8 low CO2
Run 32 mod CO2
Run 40 high CO2
dCO2%
Time (days)
0
5
10
15
20
25
30
35
40
45
0
2
4
6
8
10
12
14

FIG. 1

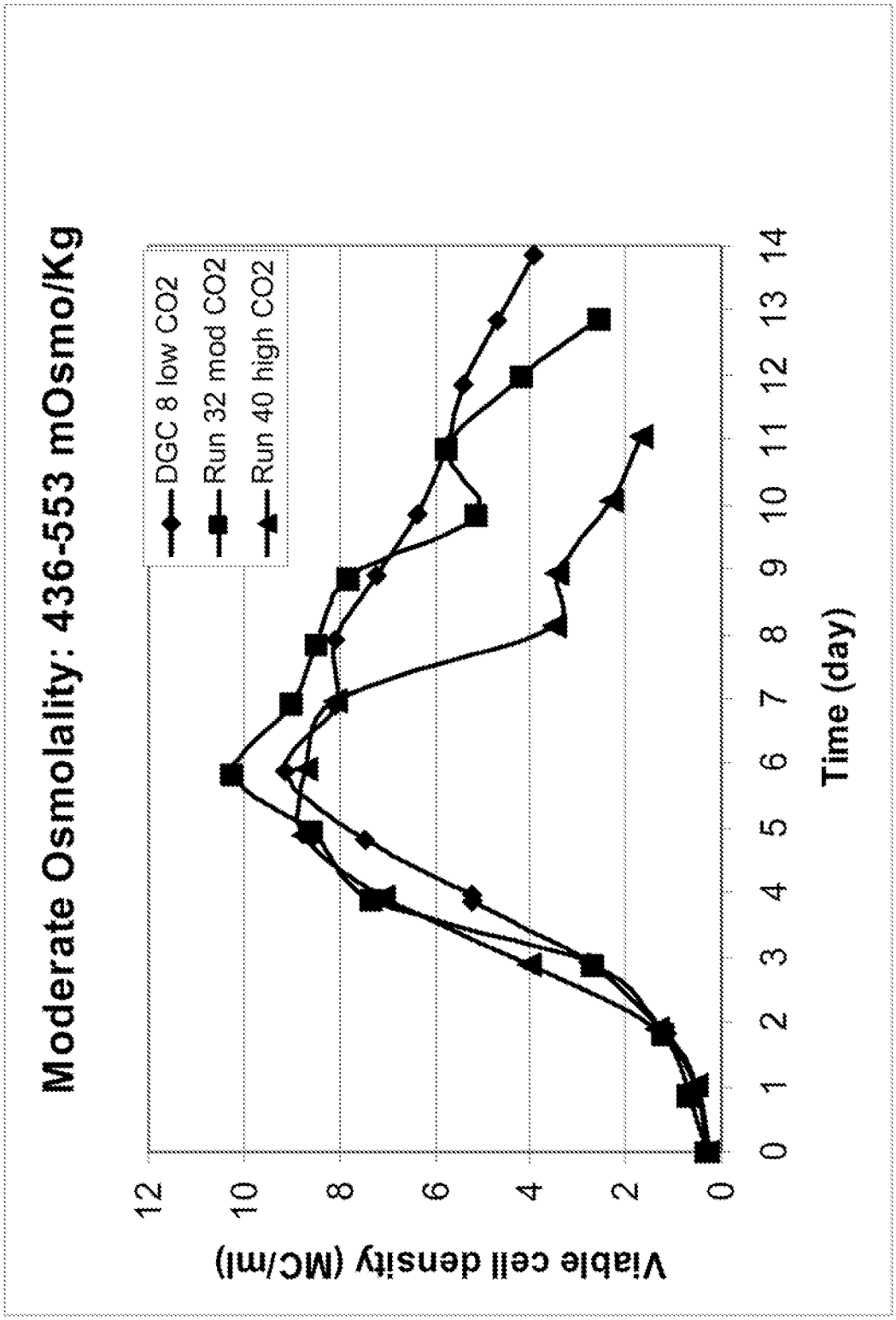
Moderate Osmolality: 436-553 mOsmo/Kg
DGC 8 low CO2
Run 32 mod CO2
Run 40 high CO2
Viable cell density (MC/ml)
0
2
4
6
8
10
12
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (day)

FIG. 2A

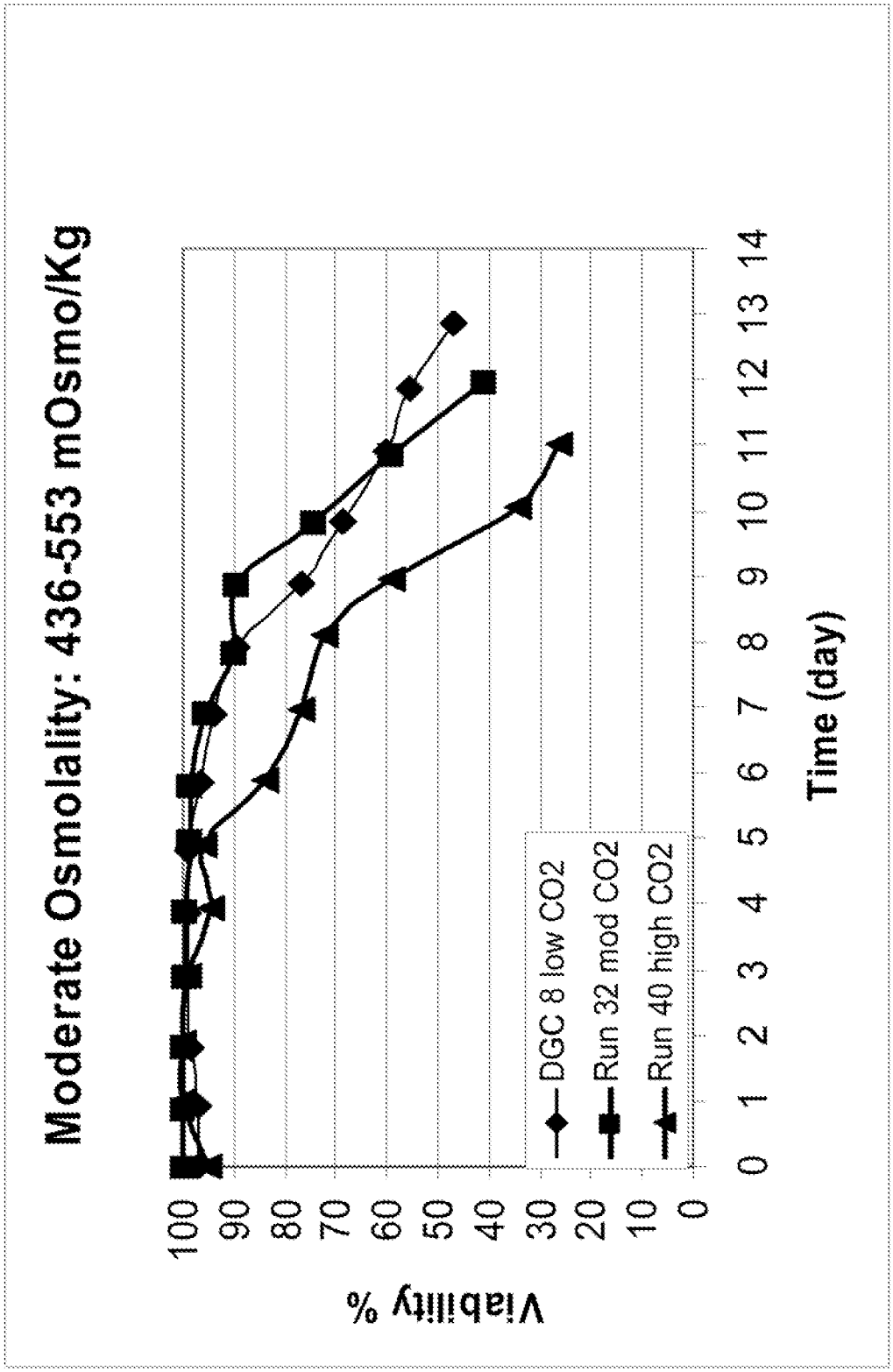
Moderate Osmolality: 436-553 mOsmo/Kg
Viability %
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (day)
DGC 8 low CO2
Run 32 mod CO2
Run 40 high CO2

FIG. 2B

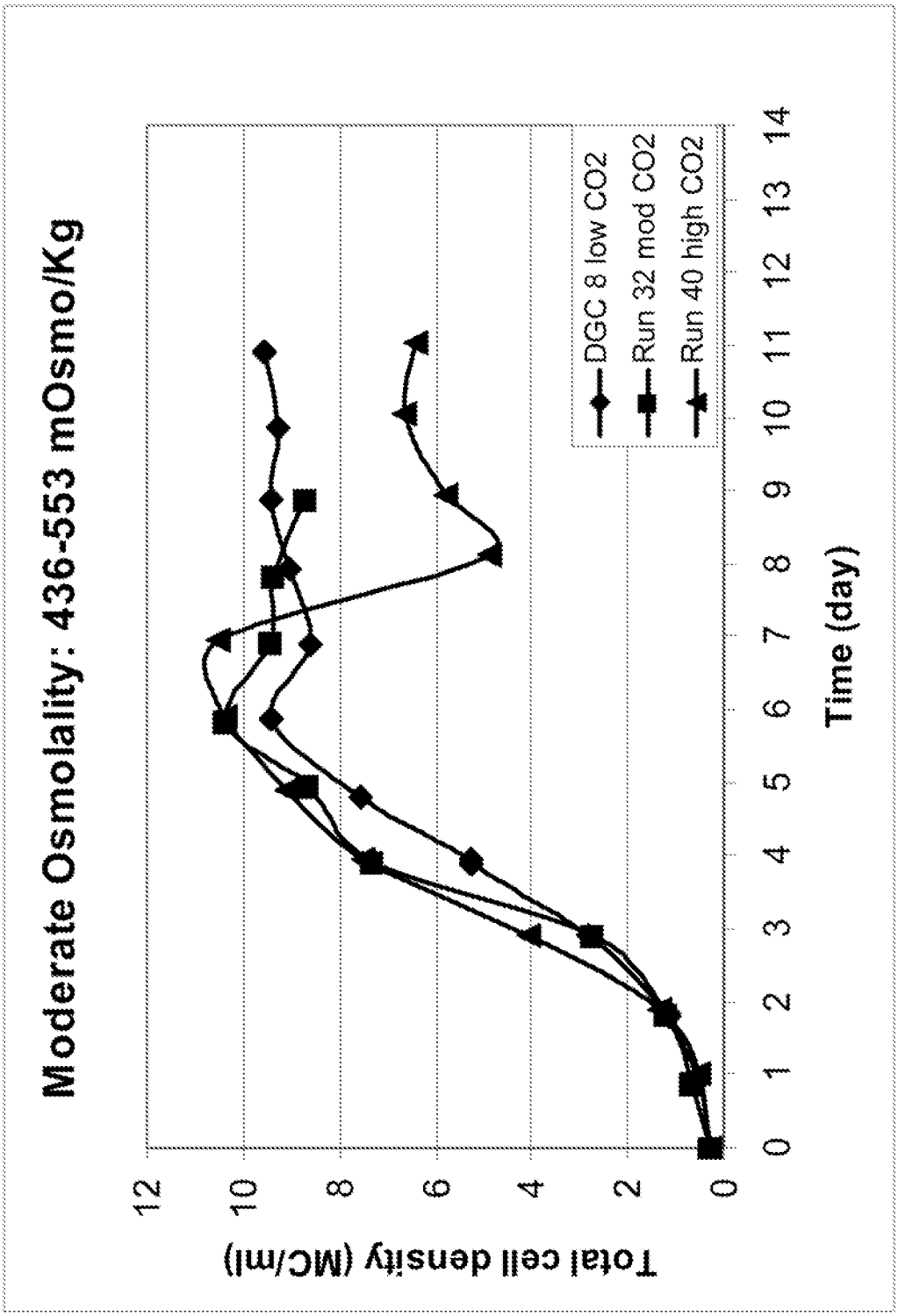
Moderate Osmolality: 436-553 mOsmo/Kg
Total cell density (MC/ml)
0
2
4
6
8
10
12
Time (day)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DGC 8 low CO2
Run 32 mod CO2
Run 40 high CO2

FIG. 2C

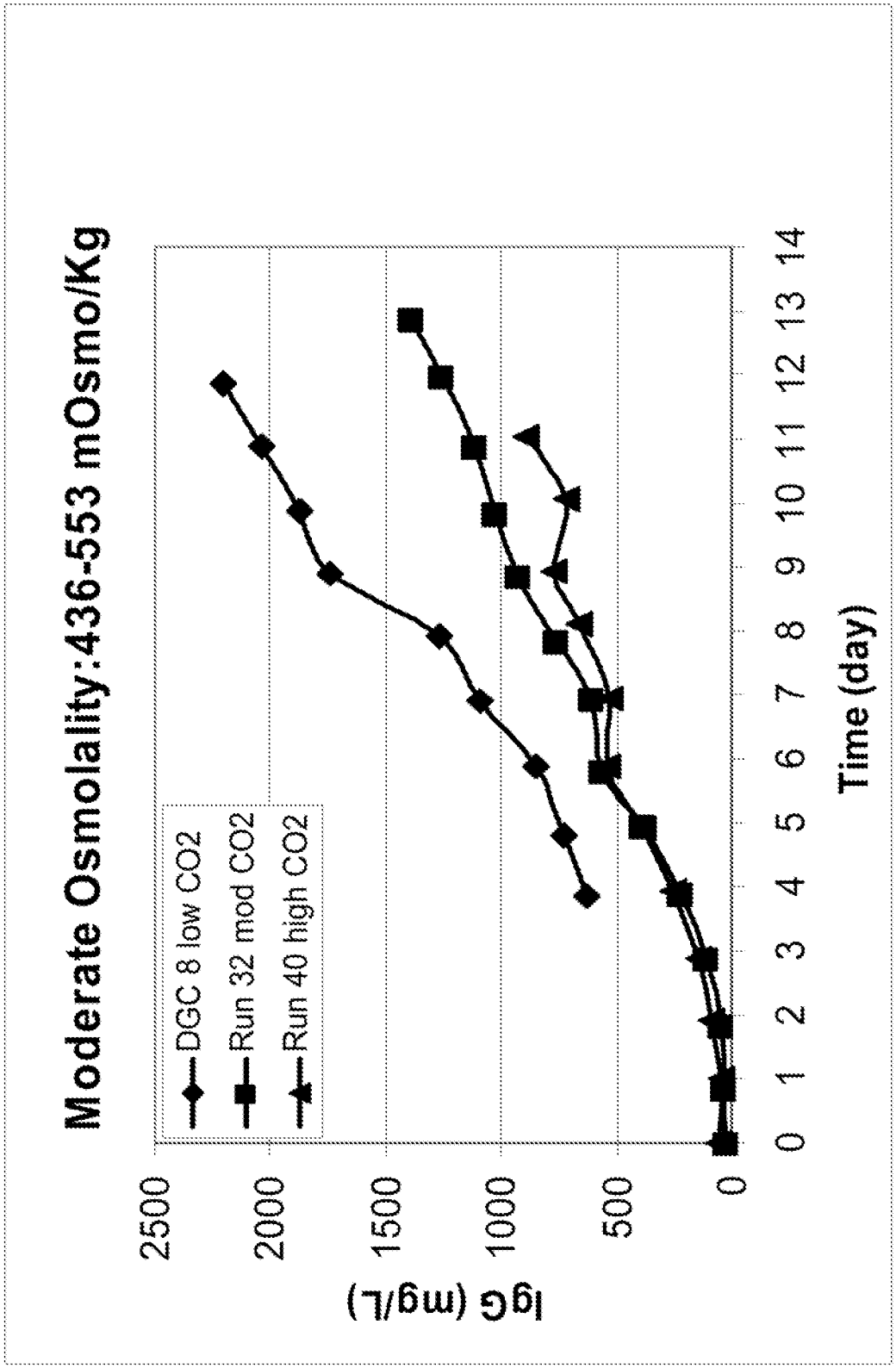
Moderate Osmolality:436-553 mOsmo/Kg
DGC 8 low CO2
Run 32 mod CO2
Run 40 high CO2
IgG (mg/L)
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (day)

FIG. 3

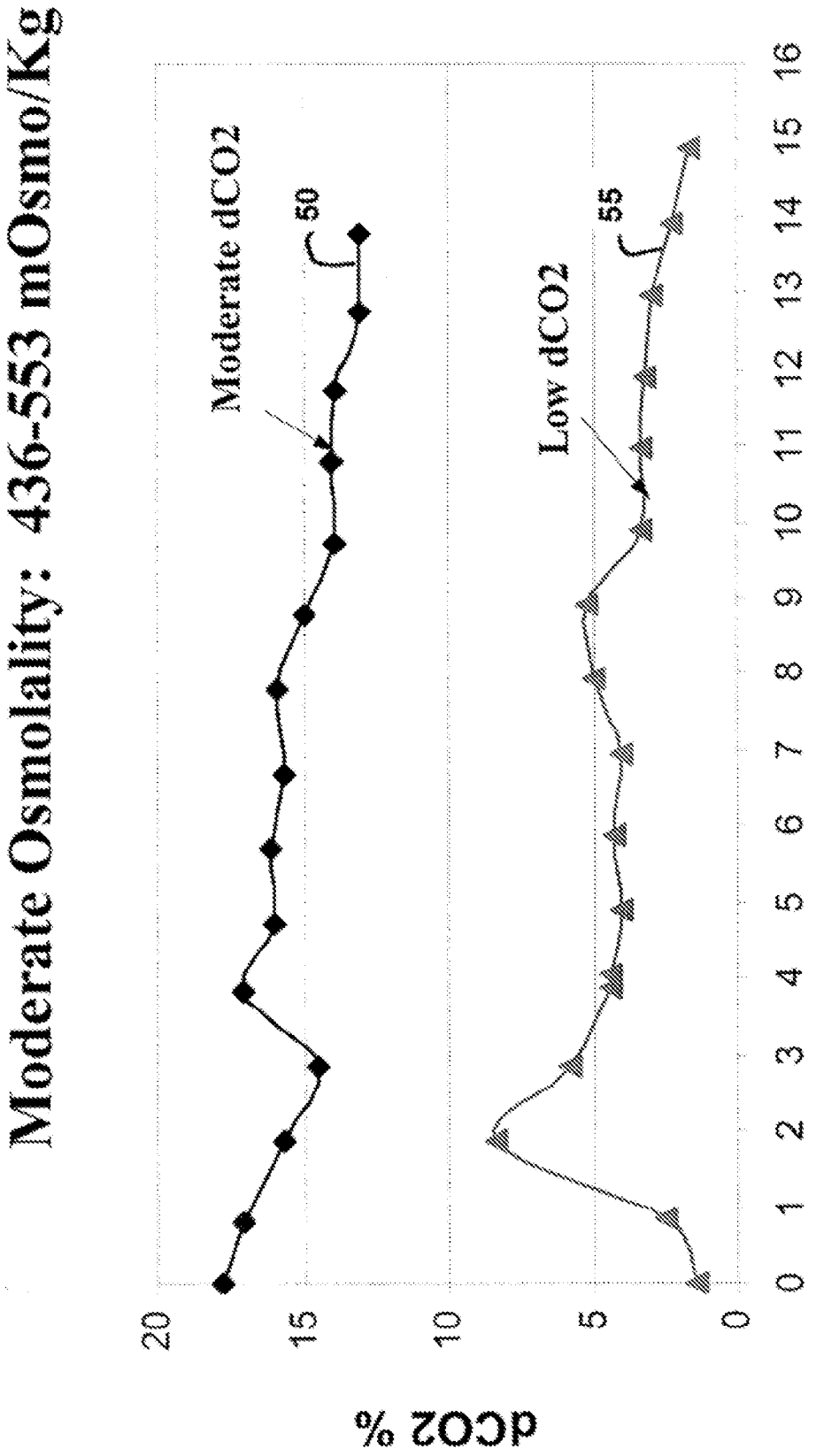
Moderate Osmolality: 436-553 mOsmo/Kg
Moderate dCO2
50
Low dCO2
55
dCO2 %
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (day)

FIG. 4

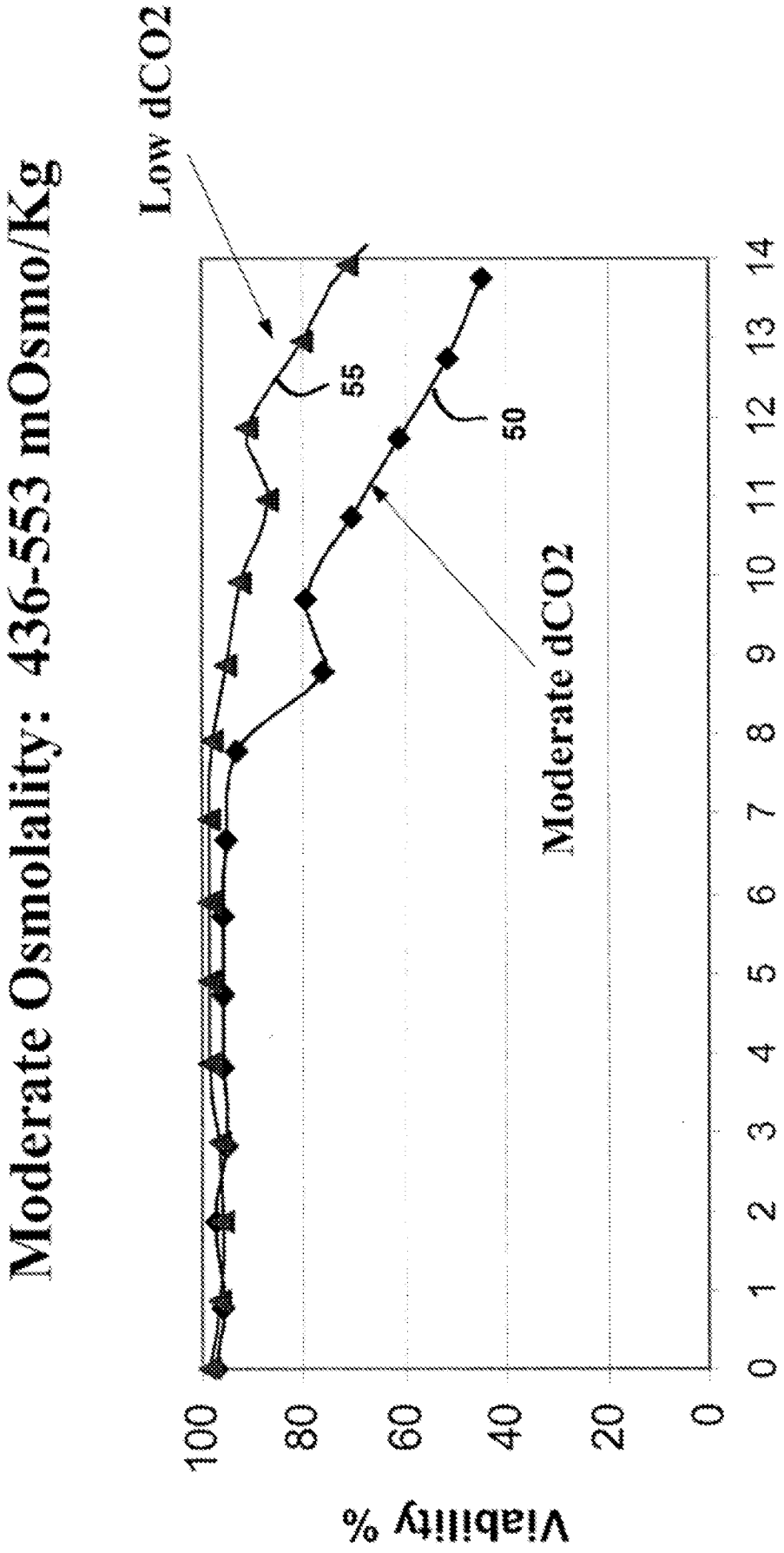
Moderate Osmolality: 436-553 mOsmo/Kg
Low dCO2
Moderate dCO2
55
50
Viability %
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (day)

FIG. 5

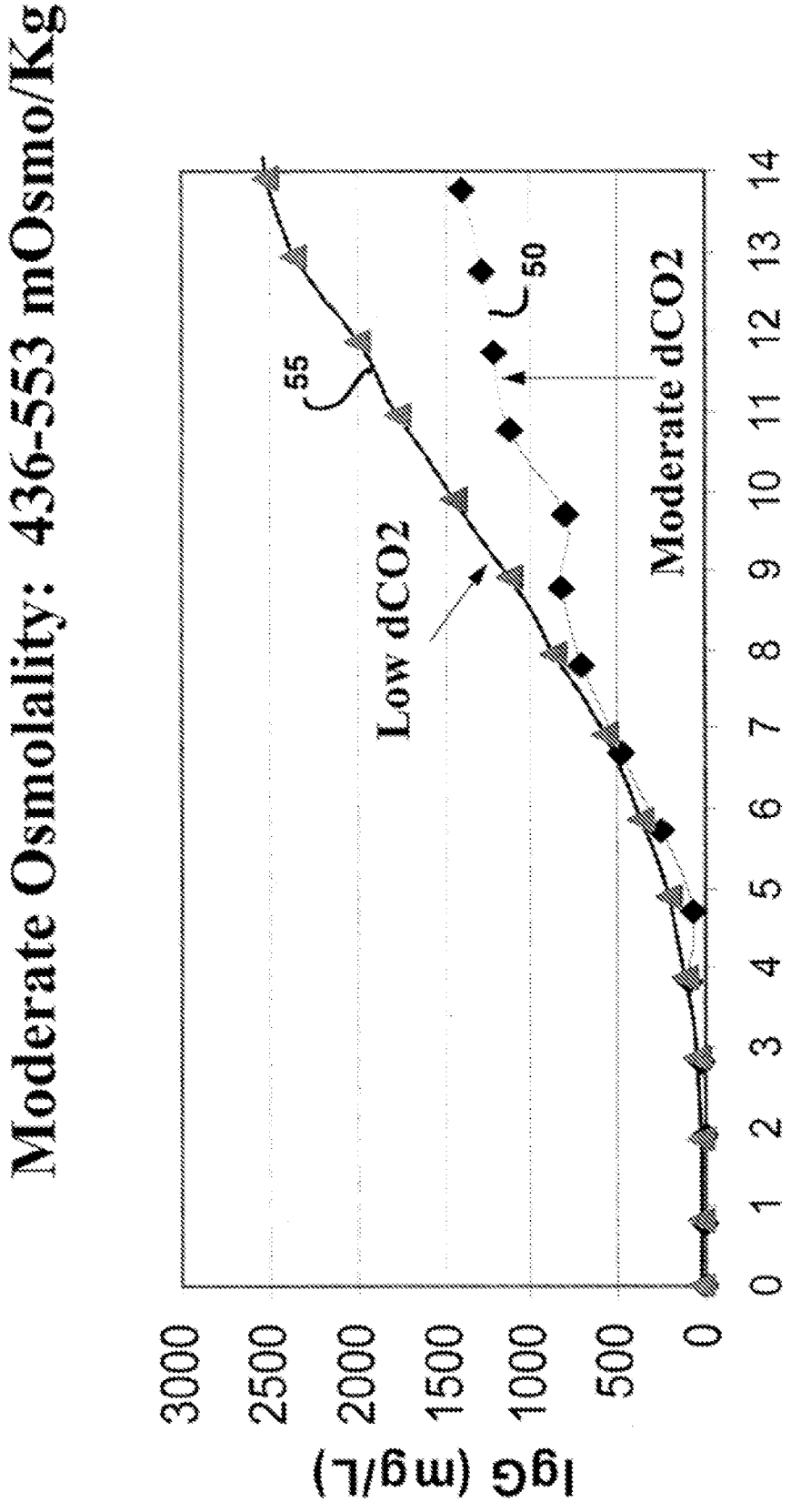
Moderate Osmolality: 436-553 mOsmo/Kg
IgG (mg/L)
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (day)
55
50
Low dCO2
Moderate dCO2

FIG. 6

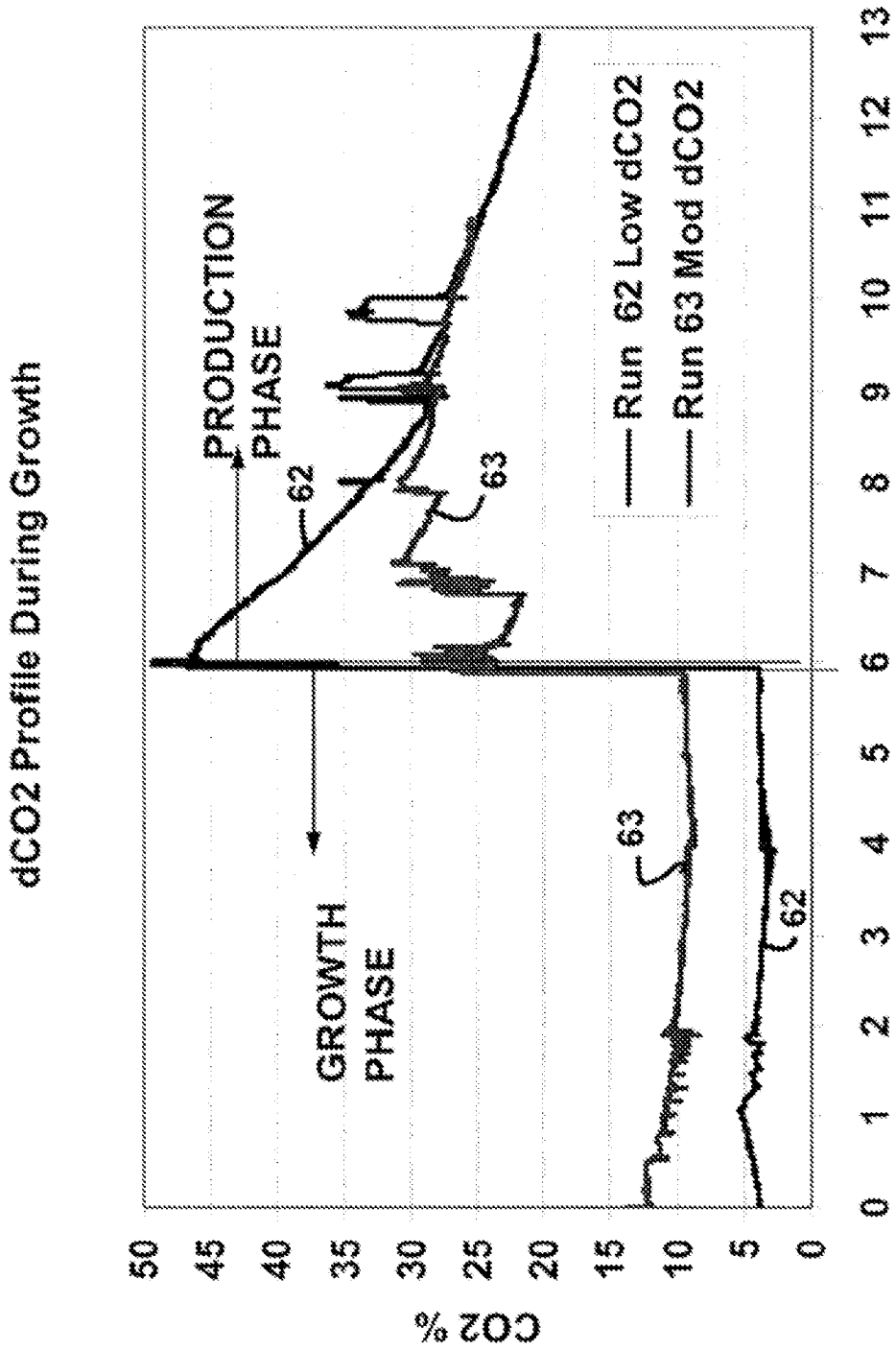
dCO2 Profile During Growth
PRODUCTION PHASE
GROWTH PHASE
62
63
63
62
Run 62 Low dCO2
Run 63 Mod dCO2
CO2 %
Time (day)
0
5
10
15
20
25
30
35
40
45
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13

FIG. 7

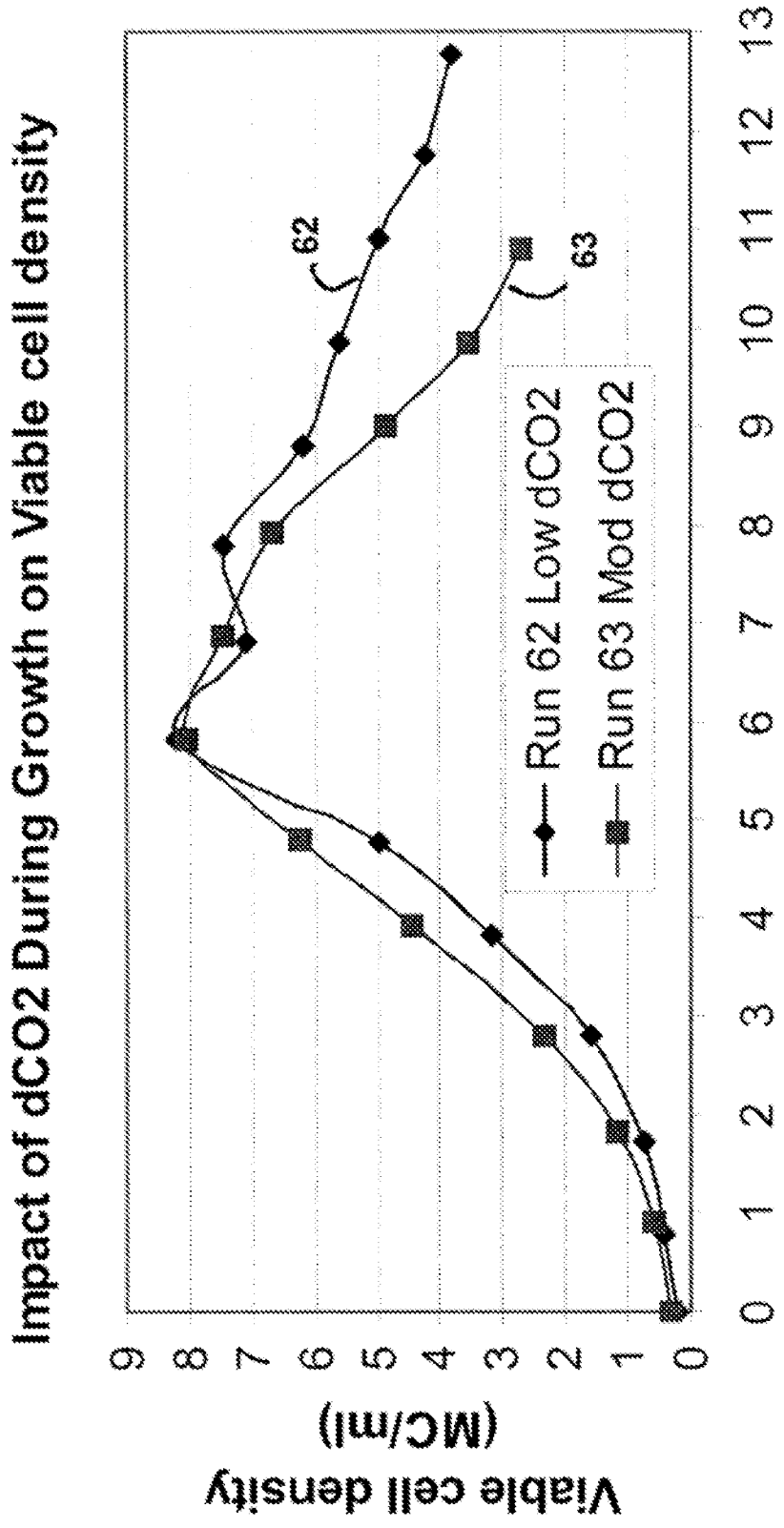
Impact of dCO2 During Growth on Viable cell density
Viable cell density (MC/ml)
Time (day)
Run 62 Low dCO2
Run 63 Mod dCO2
62
63
0
1
2
3
4
5
6
7
8
9
10
11
12
13

FIG. 8

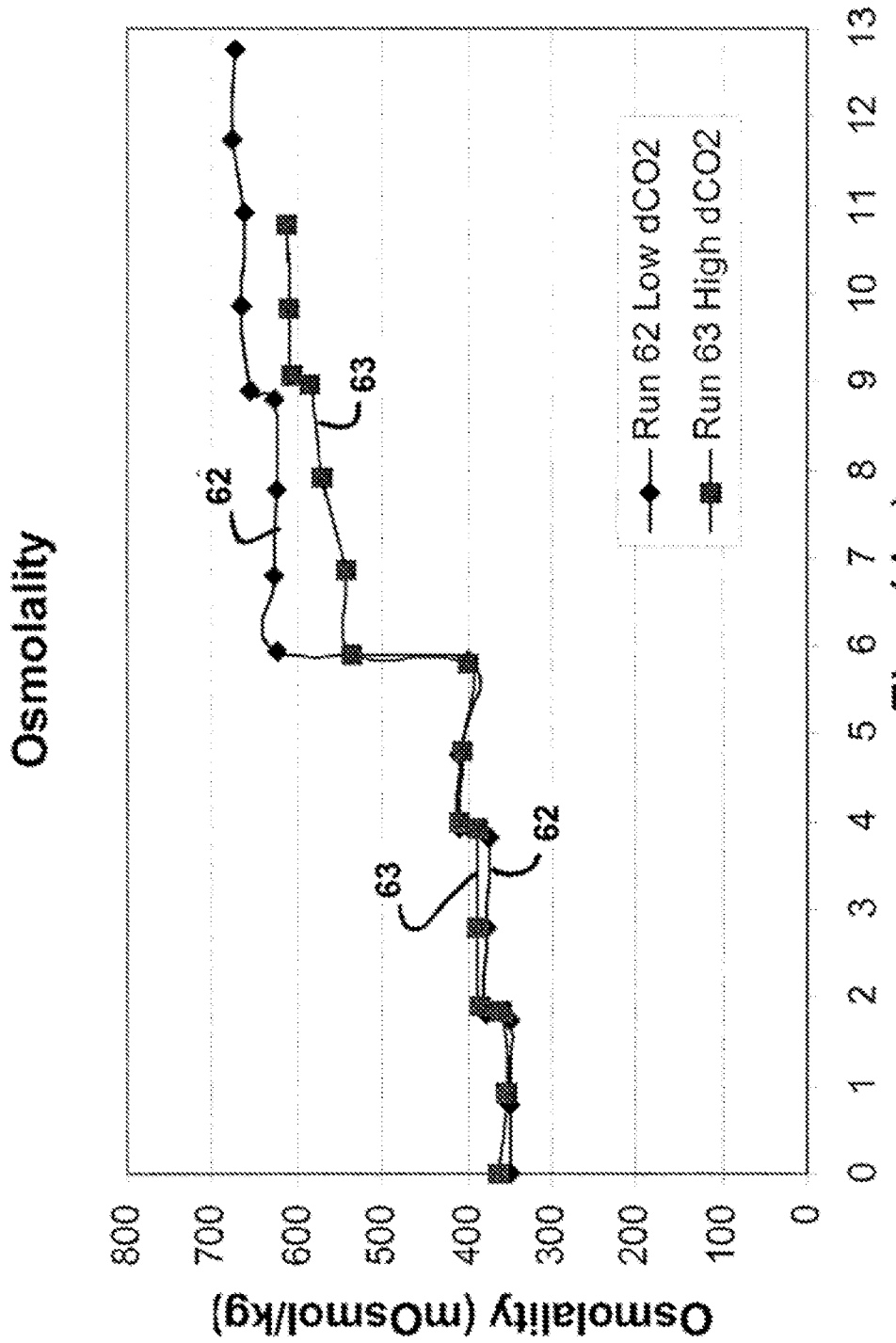
Osmolality
Osmolality (mOsmol/kg)
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (day)
Run 62 Low dCO2
Run 63 High dCO2
62
63
62
63

FIG. 9

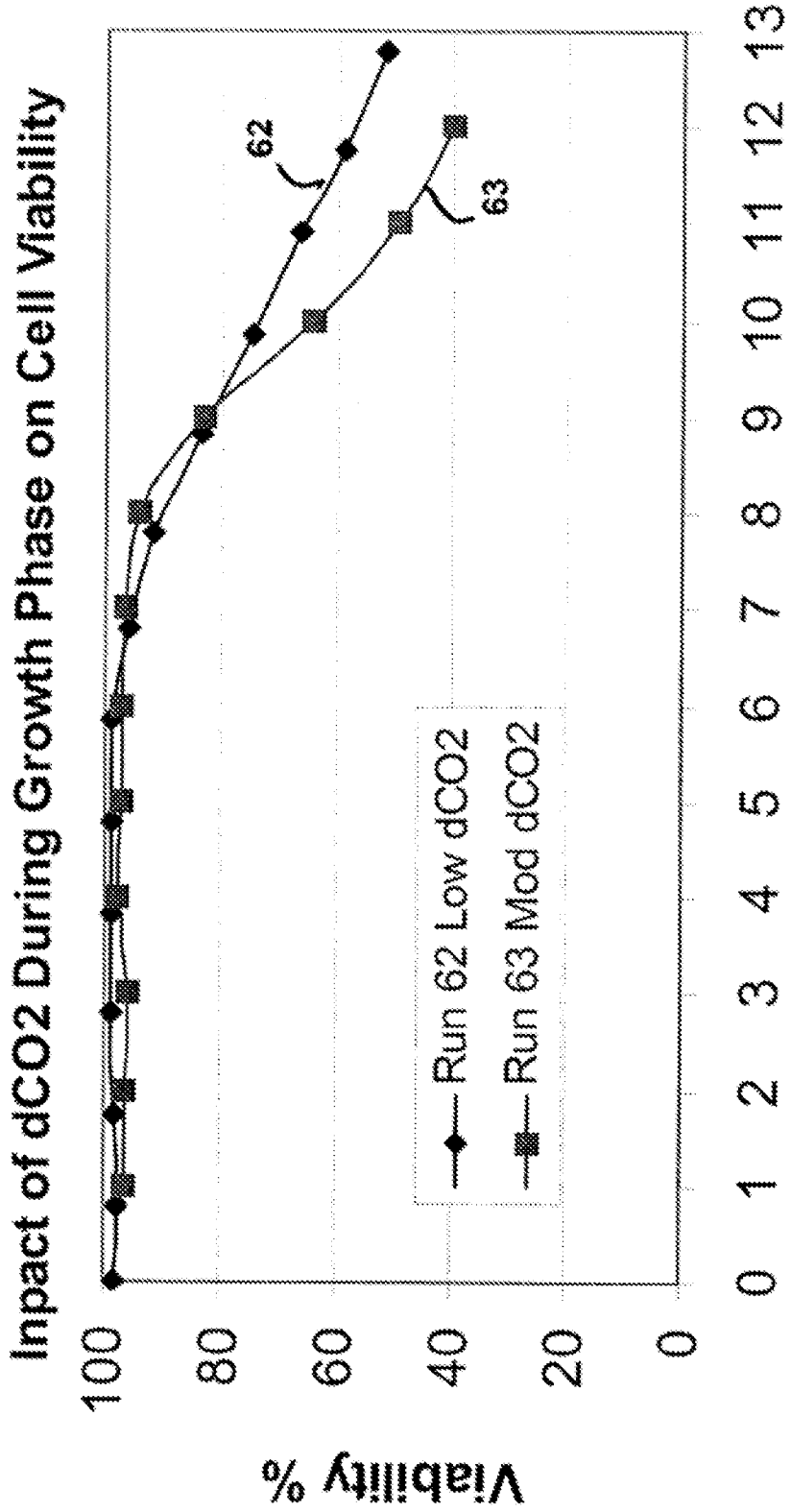
Inpact of dCO2 During Growth Phase on Cell Viability
Viability %
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (day)
62
63
Run 62 Low dCO2
Run 63 Mod dCO2

FIG. 10

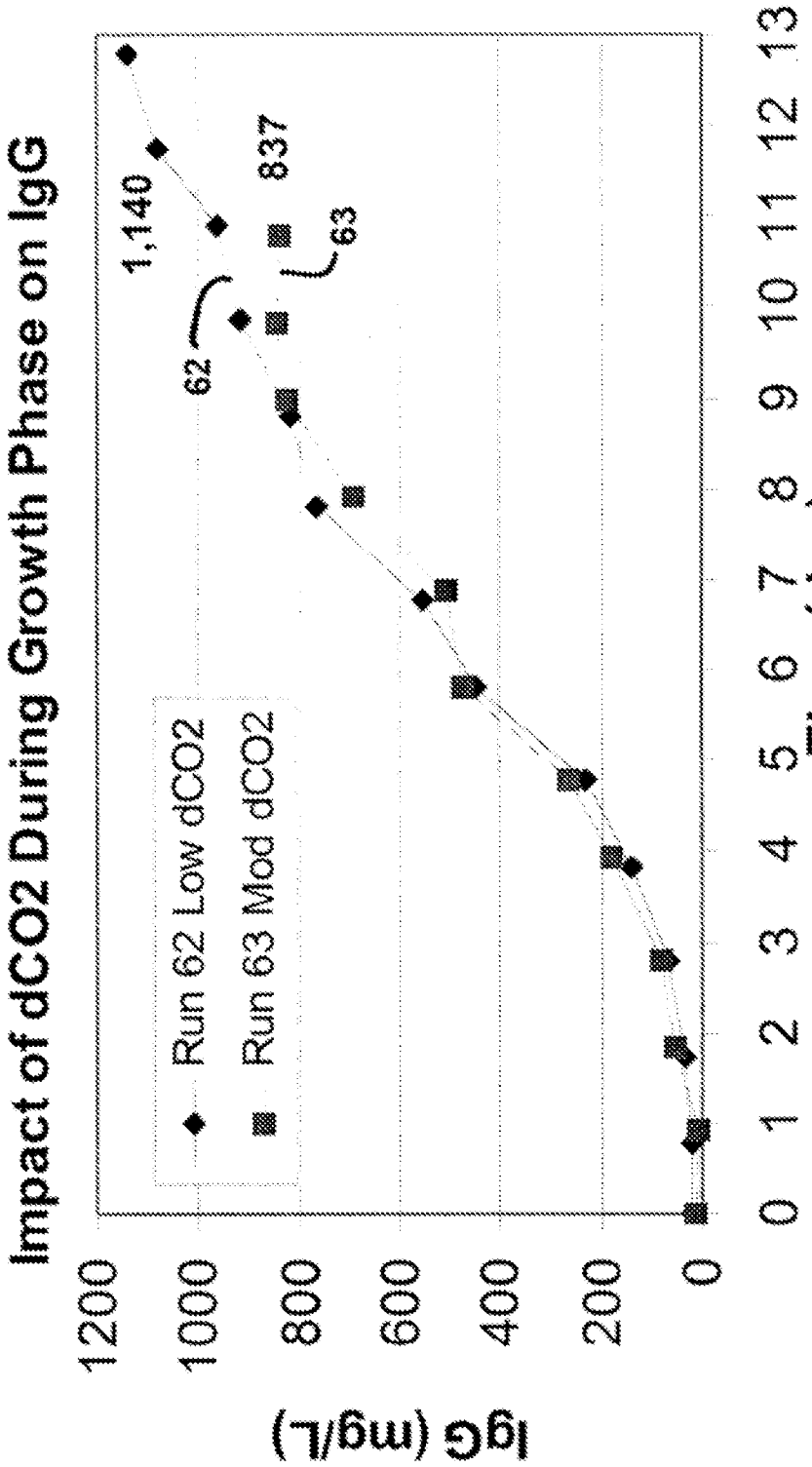
Impact of dCO2 During Growth Phase on IgG
IgG (mg/L)
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Time (day)
Run 62 Low dCO2
Run 63 Mod dCO2
1,140
837
62
63

FIG. 11

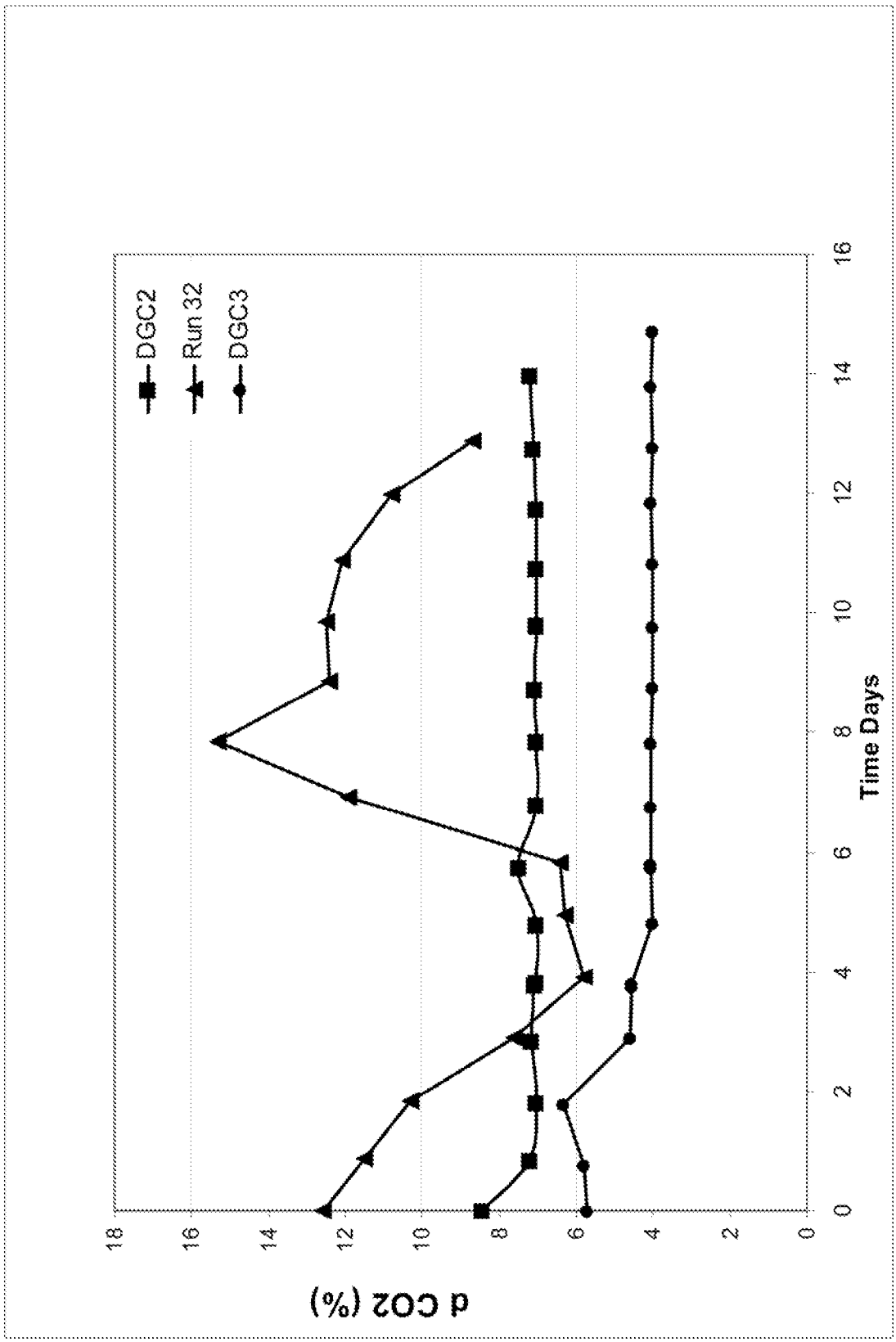

FIG. 12

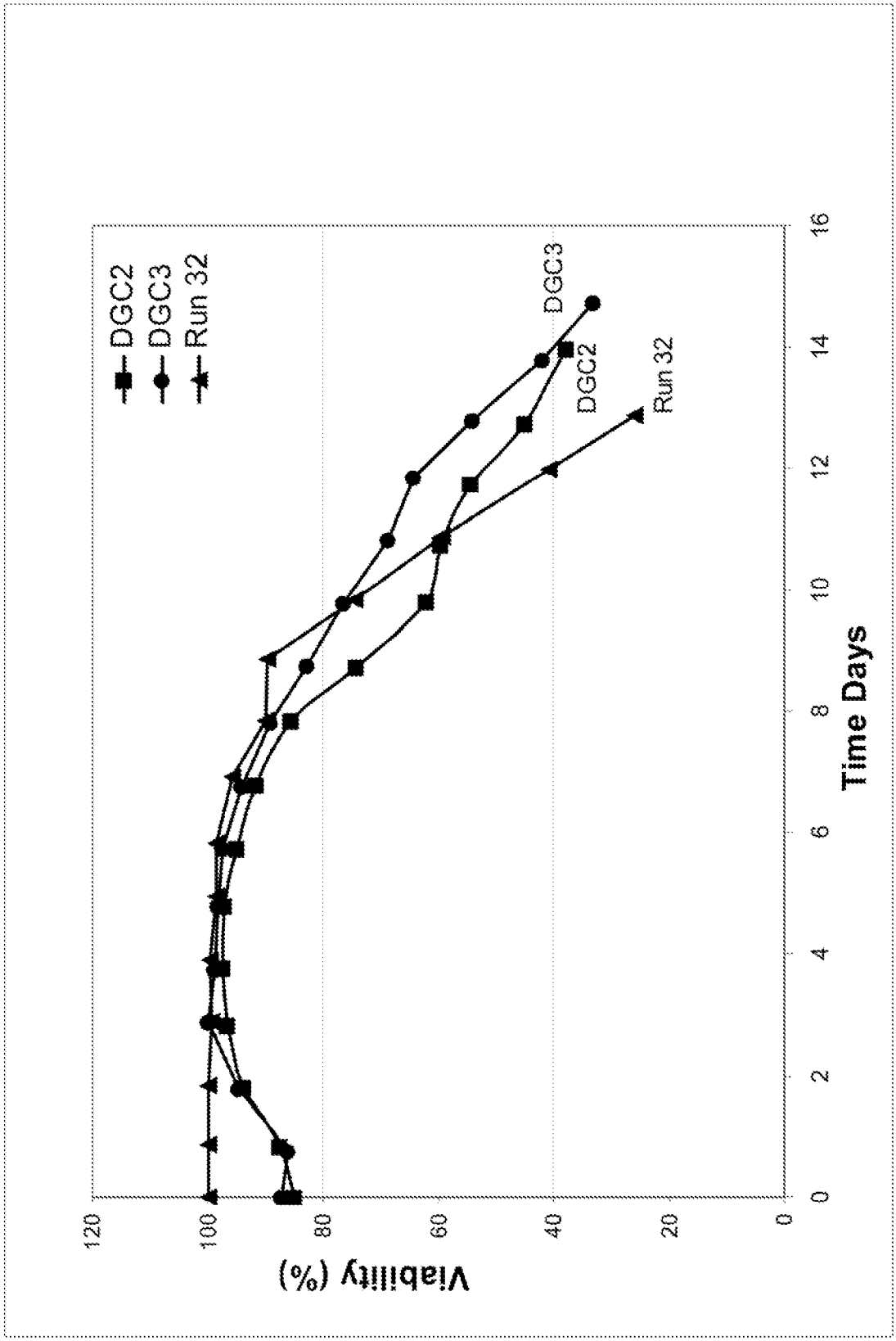
DGC2
DGC3
Run 32
DGC3
DGC2
Run 32
Viability (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Time Days

FIG. 13

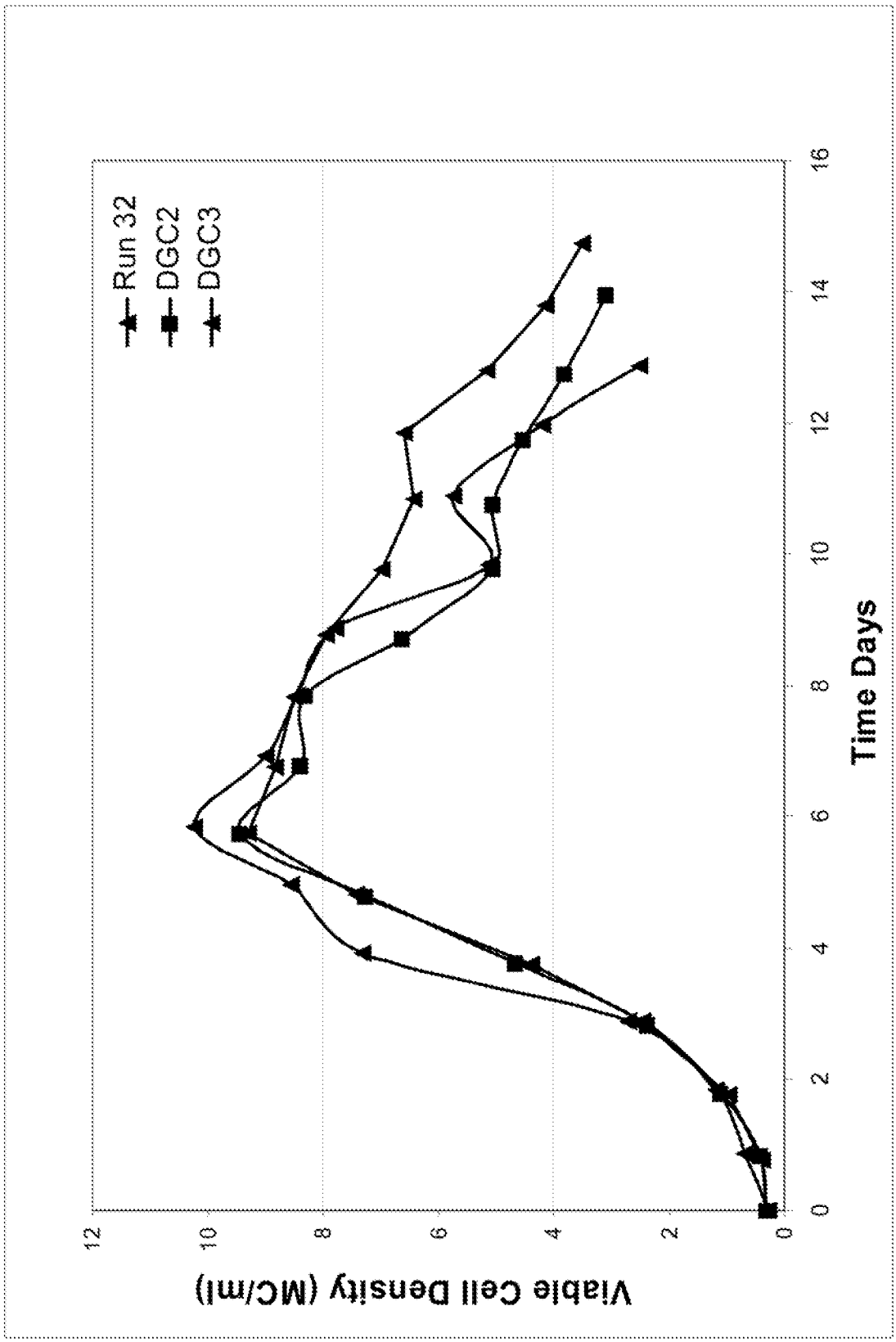
Viable Cell Density (MC/ml)
Time Days
Run 32
DGC2
DGC3
0
2
4
6
8
10
12
14
16

FIG. 14

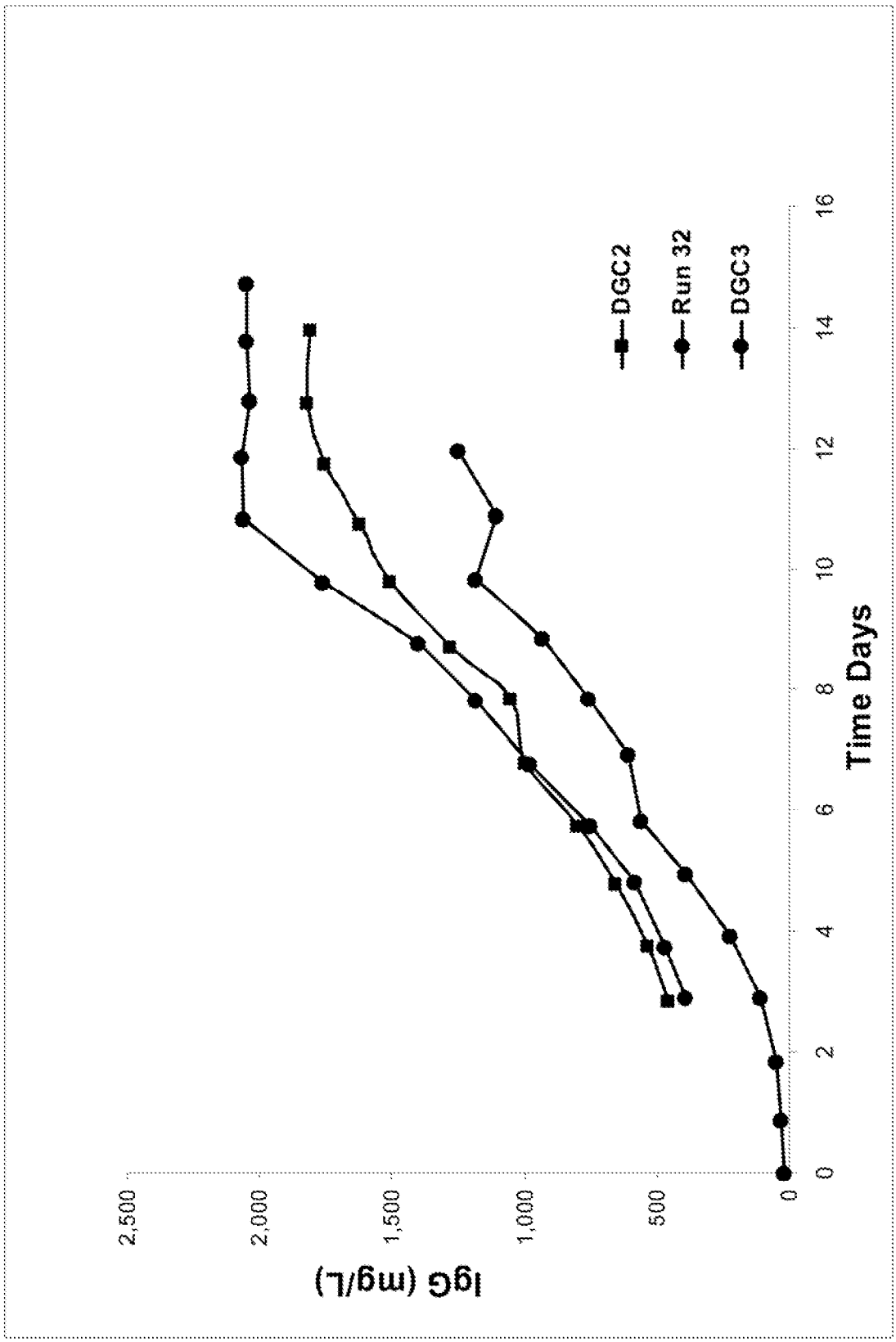
IgG (mg/L)
2,500
2,000
1,500
1,000
500
0
0
2
4
6
8
10
12
14
16
Time Days
DGC2
Run 32
DGC3

FIG. 15

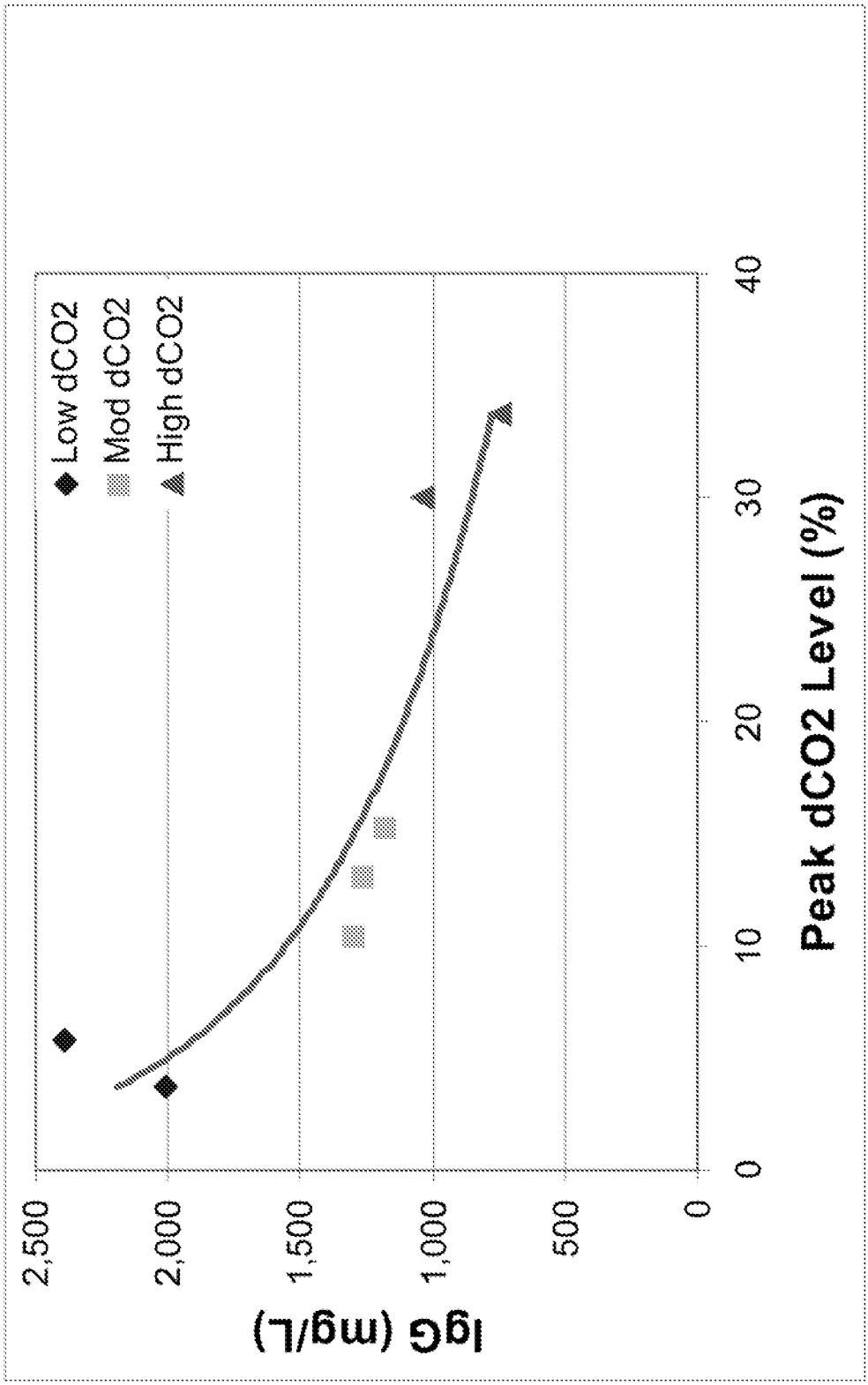
Low dCO2
Mod dCO2
High dCO2
IgG (mg/L)
2,500
2,000
1,500
1,000
500
0
0
10
20
30
40
Peak dCO2 Level (%)

FIG. 16

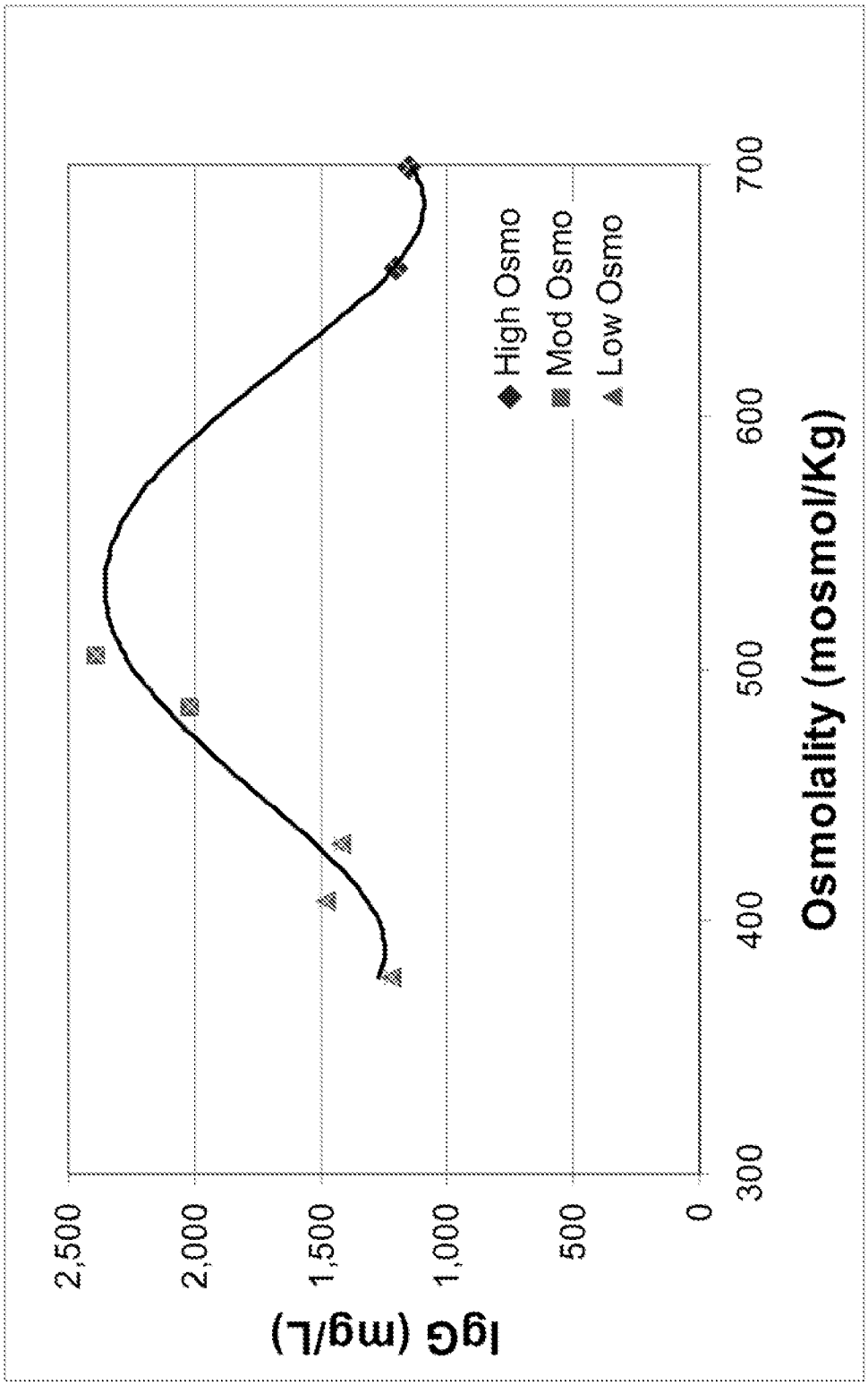
IgG (mg/L)
2,500
2,000
1,500
1,000
500
0
300
400
500
600
700
Osmolality (mosmol/Kg)
High Osmo
Mod Osmo
Low Osmo

FIG. 17

Low dCO2 & High Osmolality						
Run	dCO2	SdCO2	Osmo	Cell D	IgG	SP
48	6.00	4.71	659	3.0	1,200	42.7
55	4.50	2.92	699	2.5	1,149	63.7
Low dCO2 & Moderate Osmolality						
Run	dCO2	SdCO2	Osmo	Cell D	IgG	SP
42	5.80	2.54	506	9.3	2,388	32.2
52	3.70	2.51	485	9	2,012	48.3
Low dCO2 & Low Osmolality						
Run	dCO2	SdCO2	Osmo	Cell-D	IgG	SP
44	2.70	2.25	408	9.4	1,476	26.0
47	3.58	2.42	431	9.3	1,418	34.3
Moderate dCO2 & High Osmolality						
Run	dCO2m	SdCO2	Osmo	Cell D	IgG	SP
37	9	11.11	559	5.6	607	27.0
38	9.3	8.6	656	7.1	654	23.9
Moderate dCO2 & Moderate Osmolality						
Run	dCO2m	SdCO2	Osmo	Cell D	IgG	SP
29	10.5	12.2	436	11.0	1,294	22.1
30	13.1	10.46	439	13.9	1,266	18.9
32	15.3	12.6	503	10.2	1,183	19.2
Moderate dCO2 & Low Osmolality						
Run	dCO2m	SdCO2	Osmo	Cell D	IgG	SP
43	15.5	6.49	394	9.8	1,476	29.8
High dCO2 & High Osmolality						
Run	dCO2m	SdCO2	Osmo	Cell D	IgG	SP
34	34	8.51	535	4.7	566	20.7
35	34	10.76	559	5.6	688	22.3
36	30	12.16	601	4.5	632	28.8
56	35	12.92	660	2.5	646	65.3
High dCO2 & Moderate Osmolality						
Run	dCO2m	SdCO2	Osmo	Cell D	IgG	SP
40	33.7	7.53	503	8.7	745	16.2
59	30	14.32	553	3	1,040	55.9
High dCO2 & Low Osmolality						
Run	dCO2m	SdCO2	Osmo	Cell D	IgG	SP
33	20	11.1	426	2.3	426	28.8

FIG. 18

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/052912

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/06 C12N5/00

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 C12M

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 practical, search terms used)
EPO-Internal, COMPENDEX, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHU MARIE M ET AL: "Effects of elevated pCO2 and osmolality on growth of CHO cells and production of antibody-fusion protein B1: a case study" BIOTECHNOLOGY PROGRESS 2005 JAN-FEB,, vol. 21, no. 1, 1 January 2005 (2005-01-01), pages 70-77, XP002536931 [retrieved on 2004-12-18] the whole document -/--	1

[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 document 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
28 October 2009	05/11/2009

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	Westphal-Daniel, K

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/052912

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VIVIAN M DEZENGOTITA ET AL: "Effects of CO2 and osmolality on hybridoma cells: growth, metabolism and monoclonal antibody production" CYTOTECHNOLOGY, KLUWER ACADEMIC PUBLISHERS, DO, vol. 28, no. 1-3, 1 September 1998 (1998-09-01), pages 213-227, XP019236586 ISSN: 1573-0778 the whole document	1
X	KIMURA R ET AL: "Effects of elevated pCO2 and/or osmolality on the growth and recombinant tPA production of CHO cells" BIOTECHNOLOGY AND BIOENGINEERING, WILEY & SONS, HOBOKEN, NJ, US, vol. 52, no. 1, 5 October 1996 (1996-10-05), pages 152-160, XP002536930 ISSN: 0006-3592 the whole document	1
A	JOLICOEUR M ET AL: "Development of a helical-ribbon impeller bioreactor for high-density plant cell suspension culture" BIOTECHNOLOGY AND BIOENGINEERING 1992 MAR 5 US,, vol. 39, no. 5, 5 March 1992 (1992-03-05), pages 511-521, XP002548862 the whole document	
A	WO 2007/071072 A (ECOLE POLYTECH [CA]; JOLICOEUR MARIO [CA]; LEGROS ROBERT [CA]; DE DOBB) 28 June 2007 (2007-06-28) page 13, line 14 - page 14, line 2; figure 2a page 19, line 7 - page 36, line 23	
A	DE DOBBELEER C ET AL: "A high-rate perfusion bioreactor for plant cells" BIOTECHNOLOGY AND BIOENGINEERING, WILEY & SONS, HOBOKEN, NJ, US, vol. 95, no. 6, 20 December 2006 (2006-12-20), pages 1126-1137, XP003015870 ISSN: 0006-3592 the whole document	
A	US 2 530 814 A (DE BECZE GEORGE ET AL) 21 November 1950 (1950-11-21) the whole document	

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/052912

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NIENOW A W: "Reactor engineering in large scale animal cell culture" CYTOTECHNOLOGY 200603 NL,, vol. 50, no. 1-3, 1 March 2006 (2006-03-01), pages 9-33, XP019393910 the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2009/052912

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007071072 A	28-06-2007	NONE	
US 2530814 A	21-11-1950	NONE	