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(54) Title: CELL CULTURE MEDIA AND METHODS OF ANTIBODY PRODUCTION

FIG. 1A

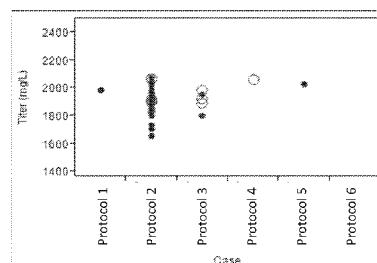
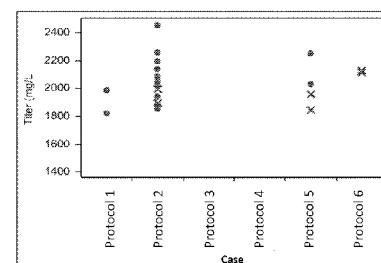


FIG. 1B



(57) Abstract: Cell culture media are provided herein as are methods of using the media for cell culture and antibody production from cells. Compositions comprising antibodies and fragments thereof, produced by the methods herein are also provided.

CELL CULTURE MEDIA AND METHODS OF ANTIBODY PRODUCTION

CROSS REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application Serial No. 61/801,247, filed March 15, 2013, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to cell culture media for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, and to methods of using the media in bevacizumab production as well as compositions and kits comprising the bevacizumab, or a fragment thereof, produced by the methods provided herein.

BACKGROUND OF THE INVENTION

[0003] Cell culture manufacturing technology is widely used for the production of protein-based therapeutics, such as antibodies, for use in pharmaceutical formulations. Commercial production of protein-based products, such as an antibody product, requires optimization of cell culture parameters in order for the cell to produce enough of the protein product to meet manufacturing demands. However, when cell culture parameters are optimized for improving productivity of the protein product it is also necessary to maintain the desired quality attributes of the product such as the glycosylation profile, aggregate levels, charge heterogeneity, and amino acid sequence integrity (Li, et al., 2010, *mAbs.*, 2(5):466-477).

[0004] Bevacizumab, also known as “Avastin®”, is a recombinant humanized monoclonal antibody that binds vascular endothelial growth factor in *in vitro* and *in vivo* assay systems (US 7227004; US 6884879; US 7060269; US 7169901; US 7297334) and is used in the treatment of cancer, where it inhibits tumor growth by blocking the formation of new blood vessels. Bevacizumab has an approximate molecular weight of 149,000 daltons, is glycosylated, and is produced in a mammalian cell (Chinese Hamster Ovary) expression system in a nutrient cell culture medium.

[0005] Improved and cost-effective methods of producing bevacizumab, or a fragment thereof, are desirable. Cell culture media comprising components that enable a cell to produce a desired amount of bevacizumab, or a fragment thereof, while maintaining acceptable product quality attributes of bevacizumab, or a fragment thereof, would be beneficial. Cell culture media for use in producing manufacturing-scale amounts of bevacizumab, or a fragment thereof, would be particularly advantageous.

BRIEF SUMMARY OF THE INVENTION

[0006] The invention provided herein discloses, *inter alia*, methods of producing bevacizumab, or a fragment thereof, in a cell culture medium comprising at least two of copper, insulin, and cystine, and optionally comprises an animal-derived hydrolysate and/or a plant-derived hydrolysate. Also provided are methods for culturing a mammalian cell (*e.g.*, a CHO cell) comprising a nucleic acid encoding bevacizumab, or a fragment thereof, using a cell culture medium provided herein. Further disclosed herein are cell culture media compositions that enhance the amount (*e.g.*, enhance the titer) of bevacizumab, or a fragment thereof, produced from a mammalian cell in cell culture, as well as compositions comprising bevacizumab, or a fragment thereof, produced by the methods described herein.

[0007] Accordingly, in one aspect, the invention provides a method of producing bevacizumab, or a fragment thereof, comprising the step of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab or fragment thereof in a cell culture medium, wherein the cell culture medium comprises two or more components selected from the group consisting of copper, insulin, and cystine, and wherein the cell produces bevacizumab, or a fragment thereof. In a further embodiment, the cell culture medium comprises copper and insulin. In another further embodiment, the cell culture medium comprises copper and cystine. In yet another further embodiment, the cell culture medium comprises insulin and cystine. In still another further embodiment, the cell culture medium comprises copper, insulin, and cystine. In any of the embodiments herein, the cell culture medium can further comprise a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate. In some of the embodiments herein, the cell culture medium comprises copper at a concentration selected from the concentrations listed in Table 1. In some of the embodiments herein, the cell culture medium comprises insulin at a concentration selected from the concentrations listed in Table 1. In some of the embodiments herein, the cell culture medium comprises cystine at a concentration selected from the concentrations listed in Table 1. It is understood that any combination of amounts of copper, insulin and/or cystine, *e.g.*, the amounts provided in Table 1, are intended the same as if each and every combination of amounts were specifically and individually listed. In any of the embodiments herein, the cell culture medium can comprise insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L. In any of the embodiments herein, the cell culture medium can comprise copper at a concentration of from about 69.0 nM to about 400.0 nM. In any of the embodiments herein, the cell culture

medium can comprise cystine at a concentration of from about 0.8 mM to about 2.5 mM. In any of the embodiments herein, the cell culture medium can comprise an animal-derived hydrolysate at a concentration of from about 5.6 g/L to about 38.0 g/L. In any of the embodiments herein, the cell culture medium can comprise a plant-derived hydrolysate at a concentration of from about 1.4 g/L to about 6.2 g/L. In some embodiments herein, the cell culture medium is a basal cell culture medium. In some embodiments herein, the cell culture medium is a feed cell culture medium. In some embodiments herein, the cell culture medium is a basal cell culture medium comprising at least one of copper, insulin, and cystine, and where the basal cell culture medium is supplemented (*e.g.*, at a period of time following initiation of a cell culture cycle, such as any one of at least one time, two times, at least three times, at least four times, at least five times, at least six times, at least seven times, etc. of a cell culture cycle) with a feed cell culture medium comprising any one or more of insulin, an animal-derived hydrolysate and a plant-derived hydrolysate. In another variation, a feed cell culture medium comprises any one or more of insulin, an animal-derived hydrolysate, a plant-derived hydrolysate, cysteine and cystine. In another variation, a feed cell culture medium comprises insulin, an animal-derived hydrolysate, a plant-derived hydrolysate and cysteine. In another variation, a feed cell culture medium comprises any one or more of insulin, an animal-derived hydrolysate, a plant-derived hydrolysate and cystine. The feed cell culture medium may comprise the any one or more of insulin, an animal-derived hydrolysate, a plant-derived hydrolysate, cysteine and cystine in any amount provided herein. In some embodiments herein, the cell culture medium comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium (*e.g.*, such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). In some embodiments, the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration selected from the concentrations listed in Table 1. In a further embodiment, the additional amount of insulin is added to the cell culture medium at least once during the cell culture cycle. In another further embodiment, the additional amount of insulin is added to the cell culture medium at least three times during the cell culture cycle. In yet another further embodiment, the additional amount of insulin is added to the cell culture medium at least six times during the cell culture cycle. In some of the embodiments herein, the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 5.6 mg/L to about 66.0 mg/L. In some of the embodiments herein, the method further comprises the step of adding an additional amount of animal-

derived hydrolysate and/or plant-derived hydrolysate to the cell culture medium provided herein (e.g., such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). In some of the embodiments herein, the additional amount of animal-derived hydrolysate and/or plant-derived hydrolysate added to the cell culture is added in an amount to provide animal-derived hydrolysate and/or plant-derived hydrolysate in the cell culture medium at a concentration selected from the concentrations listed in Table 1. In any of the embodiments herein, the cell (e.g., a CHO cell capable of producing bevacizumab, or a fragment thereof) can be cultured at a temperature ranging from about 28 °C to about 37 °C or about 31 °C to about 37 °C. In any of the embodiments herein, bevacizumab, or a fragment thereof, can be secreted into the cell culture medium. In any of the embodiments herein, the method can further comprise the step of recovering the bevacizumab, or a fragment thereof, from the cell culture. In a particular variation, the recovered bevacizumab is purified.

[0008] Also provided herein are methods of producing bevacizumab, or a fragment thereof, in a cell culture medium comprising an animal-derived hydrolysate and a plant-derived hydrolysate and optionally further comprising copper, insulin and/or cystine. In one such aspect, the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate. In one such variation, the animal-derived hydrolysate is present in the cell culture media at a concentration of from about 5.6 g/L to about 38.0 g/L or from about 7.0 g/L to about 35.0 g/L or from about 7.0 g/L to about 25.0 g/L or from about 7.0 g/L to about 15.0 g/L or from about 8.0 g/L to about 12.0 g/L or from about 7.0 g/L to about 11.0 g/L or about any one of 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L or 50 g/L or about any one of 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, or 12 g/L or about 10 g/L. In another variation, the plant-derived hydrolysate is present in the cell culture media at a concentration of from about 1.4 g/L to about 6.2 g/L or from about 1.5 g/L to about 5.5 g/L or from about 1.5 g/L to about 4.5 g/L or from about 1.5 g/L to about 3.5 g/L or from about 1.5 g/L to about 2.5 g/L or from about 1.75 g/L to about 2.75 g/L or from about 2.0 g/L to about 3.0 g/L or from about 2.25 g/L to about 2.75 g/L or about any one of 1.75 g/L, 2.0 g/L, 2.25 g/L, 2.5 g/L, 3.0 g/L, 3.25, 3.5 g/L, 3.75 g/L, or 4.0 g/L or about any one of 2.0 g/L, 2.25 g/L, 2.5 g/L or 3.0 g/L or about 2.5 g/L. It is understood that each and every combination of amount of animal-derived hydrolysate and plant-derived hydrolysate is described the same as if each and every combination were specifically and individually listed.

[0009] In some aspects, the invention provides bevacizumab, or fragment thereof, produced by any of the methods described herein.

[0010] In other aspects, the invention provides a composition comprising: (i) bevacizumab, or a fragment thereof, produced by any of the methods described herein and (ii) a pharmaceutically acceptable carrier.

[0011] In some aspects, the invention also provides a method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the method comprising the step of contacting the mammalian cell with a cell culture medium comprising two or more components selected from the group consisting of copper, insulin and cystine. In a further embodiment, the cell culture medium comprises copper and insulin. In another further embodiment, the cell culture medium comprises copper and cystine. In yet another further embodiment, the cell culture medium comprises insulin and cystine. In still yet another further embodiment, the cell culture medium comprises copper, insulin, and cystine. In some embodiments herein, the cell culture medium further comprises a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate. In some of the embodiments herein, the cell culture medium comprises insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L. In some of the embodiments herein, the cell culture medium comprises copper at a concentration of from about 69.0 nM to about 400.0 nM. In some of the embodiments herein, the cell culture medium comprises cystine at a concentration of from about 0.8 mM to about 2.5 mM. In some of the embodiments herein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 5.6 g/L to about 38.0 g/L. In some of the embodiments herein, the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 1.4 g/L to about 6.2 g/L. In some of the embodiments herein, the cell culture medium is a basal cell culture medium. In some embodiments herein, the cell culture medium is a feed cell culture medium. In some embodiments herein, the cell culture medium is a basal cell culture medium comprising at least one of copper, insulin, and cystine, and where the basal cell culture medium is supplemented (*e.g.*, at a period of time following initiation of a cell culture cycle, such as any one of at least two times, at least three times, at least four times, at least five times, at least six times, at least seven times, etc. of a cell culture cycle) with a feed cell culture medium comprising any one or more of insulin, an animal-derived hydrolysate and a plant-derived hydrolysate. In some embodiments herein, the cell culture medium comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium. In a further embodiment, the additional amount of insulin is added to the cell culture medium at least once during the cell culture cycle. In another further embodiment, the additional amount of insulin is added to the

cell culture medium at least three times during the cell culture cycle. In yet another further embodiment, the additional amount of insulin is added to the cell culture medium at least six times during the cell culture cycle. In some embodiments, the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 5.6 mg/L to about 66.0 mg/L. In some of the embodiments herein, the method further comprises the step of adding an additional amount of animal-derived hydrolysate and/or plant-derived hydrolysate to the cell culture medium provided herein (e.g., such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). In some of the embodiments herein, the additional amount of animal-derived hydrolysate and/or plant-derived hydrolysate added to the cell culture is added in an amount to provide animal-derived hydrolysate and/or plant-derived hydrolysate in the cell culture medium at a concentration selected from the concentrations listed in Table 1. In some of the embodiments herein, the cell is cultured at a temperature ranging about 28 °C to about 37 °C or from about 31 °C to about 37 °C. In any of the embodiments herein, bevacizumab, or a fragment thereof, can be secreted into the cell culture medium. In some embodiments herein, the mammalian cell is contacted with the cell culture medium during the cell's growth phase. In some embodiments herein, the mammalian cell is contacted with the cell culture medium during the cell's production phase.

[0012] In other aspects, the invention provides a kit for supplementing a cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the kit comprising at least two of components (i)-(iii): (i) insulin in an amount to provide from about 7.0 mg/L to about 11.0 mg/L insulin in the cell culture medium; (ii) cystine in an amount to provide from about 0.8 mM to about 2.5 mM cystine in the cell culture medium; (iii) and copper in an amount to provide from about 25.0 nM to about 400.0 nM copper in the cell culture medium. In some embodiments, the kit further comprises a plant-derived hydrolysate. In a further embodiment, the kit comprises the plant-derived hydrolysate in an amount to provide from about 1.4 g/L to about 6.2 g/L plant-derived hydrolysate in the cell culture medium. In any of the embodiments herein, the kit can further comprise an animal-derived hydrolysate. In some embodiments, the kit comprises the animal-derived hydrolysate in an amount to provide from about 5.6 g/L to about 38.0 g/L animal-derived hydrolysate in the cell culture medium. The kit may additionally contain instructions for use, such as instructions for use in supplementing a cell culture medium.

[0013] In another aspect, the invention provides a cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the

cell culture medium comprising at least two of components (i)-(iii): (i) from about 7.0 mg/L to about 11.0 mg/L insulin; (ii) from about 25.0 nM to about 400.0 nM copper; and (iii) from about 0.8 mM to about 2.5 mM cystine. In some embodiments, the cell culture medium comprises from about 7.0 mg/L to about 11.0 mg/L insulin; and from about 25.0 nM to about 400.0 nM copper. In some embodiments, the cell culture medium comprises: from about 7.0 mg/L to about 11.0 mg/L insulin; and from about 0.8 mM to about 2.5 mM cystine. In some embodiments, the cell culture medium comprises from about 25.0 nM to about 400.0 nM copper; and from about 0.8 mM to about 2.5 mM cystine. In any of the embodiments herein, the cell culture medium can further comprise from about 1.4 g/L to about 6.2 g/L plant-derived hydrolysate. In any of the embodiments herein, the cell culture medium can further comprise from about 5.6 g/L to about 38.0 g/L animal-derived hydrolysate.

[0014] In yet another aspect, the invention also provides a composition comprising (a) a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof; and (b) any cell culture medium provided herein.

[0015] In another aspect, the invention provides a composition comprising: (a) bevacizumab, or a fragment thereof; and (b) any cell culture medium provided herein. In a further embodiment, bevacizumab, or a fragment thereof, is secreted into the medium by a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof.

[0016] In some aspects, also provided herein is a method of enhancing titer of bevacizumab, or a fragment thereof, from a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the method comprising the step of culturing the mammalian cell in a cell culture medium comprising at least two of insulin, copper and cystine, wherein titer is enhanced relative to culturing the mammalian cell in a cell culture medium without at least two of insulin, copper and cystine. In some embodiments, the cell culture medium comprises copper and insulin. In some embodiments, the cell culture medium comprises copper and cystine. In some embodiments, the cell culture medium comprises insulin and cystine. In some embodiments, the cell culture medium comprises copper, insulin, and cystine. In any of the embodiments herein, the cell culture medium can further comprise a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate. In any of the embodiments herein, the cell culture medium can comprise insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L. In any of the embodiments herein, the cell culture medium can comprise copper at a concentration of from about 69.0 nM to about 400.0 nM. In any of the embodiments herein, the cell culture medium can comprise cystine at a concentration of from

about 0.8 mM to about 2.5 mM. In any of the embodiments herein, the cell culture medium can comprise an animal-derived hydrolysate at a concentration of from about 5.6 g/L to about 38.0 g/L. In any of the embodiments herein, the cell culture medium can comprise a plant-derived hydrolysate at a concentration of from about 1.4 g/L to about 6.2 g/L. In some embodiments, the cell culture medium is a basal cell culture medium. In some embodiments herein, the cell culture medium is a feed cell culture medium. In some embodiments herein, the cell culture medium is a basal cell culture medium comprising at least one of copper, insulin, and cystine, and where the basal cell culture medium is supplemented (*e.g.*, at a period of time following initiation of a cell culture cycle, such as any one of at least two times, at least three times, at least four times, at least five times, at least six times, at least seven times, etc. of a cell culture cycle) with a feed cell culture medium comprising any one or more of insulin, an animal-derived hydrolysate and a plant-derived hydrolysate. In some embodiments, the cell culture medium comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium. In a further embodiment, the additional amount of insulin is added to the cell culture medium at least once during the cell culture cycle. In another further embodiment, the additional amount of insulin is added to the cell culture medium at least three times during the cell culture cycle. In yet another further embodiment, the additional amount of insulin is added to the cell culture medium at least six times during the cell culture cycle. In some embodiments herein, the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 5.6 mg/L to about 66.0 mg/L. In some of the embodiments herein, the method further comprises the step of adding an additional amount of animal-derived hydrolysate and/or plant-derived hydrolysate to the cell culture medium provided herein (*e.g.*, such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). In some of the embodiments herein, the additional amount of animal-derived hydrolysate and/or plant-derived hydrolysate added to the cell culture is added in an amount to provide animal-derived hydrolysate and/or plant-derived hydrolysate in the cell culture medium at a concentration selected from the concentrations listed in Table 1. In any of the embodiments herein, the cell may be cultured at a temperature ranging from about 28 °C to about 37 °C or from about 31 °C to about 37 °C. In any of the embodiments herein, bevacizumab, or a fragment thereof, can be secreted into the cell culture medium. In any of the embodiments herein, the method may further comprise the step of recovering the bevacizumab, or a fragment thereof, from the cell culture. In a further aspect, the recovered bevacizumab, or a fragment thereof, is purified.

[0017] In another aspect, the invention herein provides a method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, in a cell culture medium comprising at least two of insulin, copper and cystine, wherein titer of bevacizumab, or a fragment thereof, is enhanced relative to culturing the mammalian cell in a cell culture medium without at least two of insulin, copper and cystine. In some embodiments, the cell culture medium comprises copper and insulin. In some embodiments, the cell culture medium comprises copper and cystine. In some embodiments, the cell culture medium comprises insulin and cystine. In some embodiments, the cell culture medium comprises copper, insulin, and cystine. In some of the embodiments herein, the cell culture medium further comprises a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate. In some of the embodiments herein, the cell culture medium comprises insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L. In some of the embodiments herein, the cell culture medium comprises copper at a concentration of from about 69.0 nM to about 400.0 nM. In some of the embodiments herein, the cell culture medium comprises cystine at a concentration of from about 0.8 mM to about 2.5 mM. In some of the embodiments herein, the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 5.6 g/L to about 38.0 g/L. In some of the embodiments herein, the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 1.4 g/L to about 6.2 g/L. In some embodiments, the cell culture medium is a basal cell culture medium. In some embodiments herein, the cell culture medium is a feed cell culture medium. In some embodiments herein, the cell culture medium is a basal cell culture medium comprising at least one of copper, insulin, and cystine, and where the basal cell culture medium is supplemented (*e.g.*, at a period of time following initiation of a cell culture cycle, such as any one of at least two times, at least three times, at least four times, at least five times, at least six times, at least seven times, etc. of a cell culture cycle) with a feed cell culture medium comprising any one or more of insulin, an animal-derived hydrolysate and a plant-derived hydrolysate. In any of the embodiments herein, the cell culture medium can comprise insulin and the method can further comprise the step of adding an additional amount of insulin to the cell culture medium. In a further embodiment, the additional amount of insulin is added to the cell culture medium at least once during the cell culture cycle. In another further embodiment, the additional amount of insulin is added to the cell culture medium at least three times during the cell culture cycle. In yet another further embodiment, the additional amount of insulin is added to the cell culture medium at least six times during the cell culture cycle. In some of the embodiments herein, the

additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 5.6 mg/L to about 66.0 mg/L. In some of the embodiments herein, the method further comprises the step of adding an additional amount of animal-derived hydrolysate and/or plant-derived hydrolysate to the cell culture medium provided herein (e.g., such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). In some of the embodiments herein, the additional amount of animal-derived hydrolysate and/or plant-derived hydrolysate added to the cell culture is added in an amount to provide animal-derived hydrolysate and/or plant-derived hydrolysate in the cell culture medium at a concentration selected from the concentrations listed in Table 1. In some of the embodiments herein, the cell is cultured at a temperature ranging from about 28 °C to about 37 °C or from about 31 °C to about 37 °C, such as a temperature of about 31 °C, 33 °C or 35 °C. It is understood that the temperature may vary (either up or down) throughout the cell culture process, e.g., within a temperature ranging from about 28 °C to about 37 °C. In one aspect, the cell is cultured at a first temperature of about 35 °C for a first period of time (such as about 1-10 or 1-8 or 1-7 days), is cultured at a second temperature of about 33 °C for a second period of time (such as about 1-5 or 1-4 or 1-3 or 1-2 days), and is cultured at a third temperature of about 31 °C for a third period of time (such as about 1-5 or 1-4 or 1-3 or 1-2 days). In any of the embodiments herein, bevacizumab, or a fragment thereof, can be secreted into the cell culture medium. In some embodiments, the method further comprises the step of recovering the bevacizumab, or a fragment thereof, from the cell culture. In one aspect, the recovered bevacizumab, or a fragment thereof, is purified.

[0018] In another aspect, the invention provides a method of producing bevacizumab or a fragment thereof, comprising a step of culturing a mammalian cell (e.g., a CHO cell) comprising a nucleic acid encoding bevacizumab or a fragment thereof in a cell culture medium, wherein initial cell culture medium in a cell culture cycle comprises two or more components selected from the group consisting of copper at a concentration of from about 69 nM to about 1,000 nM, insulin at a concentration of from about 1.0 mg/L to about 100.0 mg/L, and cystine at a concentration of from about 0.7 mM to about 2.0 mM, and wherein the cell produces bevacizumab or the fragment. In some embodiments, the initial cell culture medium comprises (1) copper and insulin; (2) copper and cystine; (3) insulin and cystine; or (4) copper, insulin, and cystine. In some embodiments, the initial cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 50.0 mg/L. In some embodiments, the initial cell culture medium comprises insulin at a concentration of from

about 10.0 mg/L to about 20.0 mg/L. In some embodiments, the initial cell culture medium comprises insulin at a concentration of about any one of 10.0 mg/L, 15 mg/L, 20 mg/L, and 25 mg/L. In some embodiments, the initial cell culture medium comprises copper at a concentration of from about 325 nM to about 375 nM. In some embodiments, the initial cell culture medium comprises copper at a concentration of from about 325 nM to about 350 nM. In some embodiments, the initial cell culture medium comprises copper at a concentration of about any one of 330 nM, 335 nM, 339 nM, 340 nM, 345 nM and 350 nM. In some embodiments, the initial cell culture medium comprises cystine at a concentration of from about 0.7 mM to about 2.0 mM. In some embodiments, the initial cell culture medium comprises cystine at a concentration of from about 1.0 mM to about 1.6 mM. In some embodiments, the initial cell culture medium comprises cystine at a concentration of about any one of 1.0 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM and 1.6 mM. In some embodiments, the initial cell culture medium comprises an animal-derived hydrolysate and/or a plant-derived hydrolysate. In some embodiments, the initial cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 6.0 g/L to about 20.0 g/L. In some embodiments, the initial cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 8.0 g/L to about 12.0 g/L. In some embodiments, the initial cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 9.0 g/L to about 11.0 g/L. In some embodiments, the initial cell culture medium comprises an animal-derived hydrolysate at a concentration of about 13 g/L. In some embodiments, the initial cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 1.0 g/L to about 10.0 g/L. In some embodiments, the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.0 g/L to about 3.0 g/L. In some embodiments, the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.25 g/L to about 2.75 g/L. In some embodiments, the initial cell culture medium comprises a plant-derived hydrolysate at a concentration of about 2.5 g/L. In some embodiments, the cell culture medium comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate. In some embodiments, the initial cell culture medium comprises insulin and the method further comprises a step of adding an additional amount of insulin to the cell culture medium during the cell culture cycle. In some embodiments, the additional amount of insulin is added to the cell culture medium at least once, at least twice, at least three times, at least four times, at least five times, or at least six times during the cell culture cycle. In some embodiments, the

insulin added each time is from about 5 mg/L to about 25 mg/L. In some embodiments, the insulin added each time is about any one of 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, and 25 mg/L. In some embodiments, the cumulative amount of insulin added during the cell culture cycle is from about 20 mg/L to about 100 mg/L. In some embodiments, the cumulative amount of insulin added during the cell culture cycle is about any one of 20 mg/L, 25 mg/L, 30 mg/L, 35 mg/L, 40 mg/L, 45 mg/L, 50 mg/L, 55 mg/L, 60 mg/L, 65 mg/L, 70 mg/L, 75 mg/L, 80 mg/L, 85 mg/L, 90 mg/L, 95 mg/L, and 100 mg/L. In some embodiments, the initial cell culture medium comprises cystine and the method further comprises a step of adding an additional amount of cystine to the cell culture medium during the cell culture cycle. In some embodiments, cystine is added in an amount to provide from about 0.1 mM to about 1.5 mM additional cystine in the cell culture medium. In some embodiments, cystine is added in an amount to provide about 0.4 mM to about 0.7 mM (e.g., about 0.4 mM to about 0.6 mM, about 0.4 mM to about 0.5 mM) additional cystine in the cell culture medium. In some embodiments, cystine is added in a batch feed during the cell culture cycle. In some embodiments, the method further comprises at least one batch feed during the cell culture cycle. In some embodiments, the one batch feed is on day 3 (e.g., in a 14-day cell culture cycle). In some embodiments, the method comprises two, three, or four batch feeds during the cell culture cycle. In some embodiments, the method comprises a first batch feed on day 3 and a second batch feed on day 6 (e.g., in a 14 day cell culture cycle). In some embodiments, the batch feed medium comprises an animal-derived hydrolysate and/or a plant-derived hydrolysate. In some embodiments, during the cell culture cycle, the temperature of the medium is reduced by at least about 2, at least about 3, at least about 4, or at least about 5 degrees C relative to the temperature at the beginning of the culturing. In some embodiments, the temperature of the medium is reduced at least once or at least twice during the cell culture cycle. In some embodiments, the temperature is reduced on day 8 and day 10 after the beginning of the culturing. In some embodiments, the cell is cultured at a temperature ranging from about 31°C to about 35°C. In some embodiments, the cell is cultured at a first temperature of about 35°C for a first period of time, is cultured at a second temperature of about 33°C for a second period of time, and is cultured at a third temperature of about 31°C for a third period of time. In some embodiments, the cell is cultured in the medium having a pH at about 7.0 to about 7.3. In some embodiments, the method comprises (a) culturing the cell in an initial cell culture medium comprising about 10 mg/L insulin, about 325 nM to about 350 nM copper, and about 1.3 mM cystine; (b) providing a first batch feed and an insulin feed to the cell culture medium to provide additional insulin at a

concentration of about 15 mg/L on day 3 after the beginning of the culturing; and (c) providing a second batch feed comprising cystine to the cell culture medium to provide additional cystine at a concentration of about 0.4 mM to about 0.7 mM on day 6 after the beginning of the culturing; wherein the cell is cultured at an initial temperature of about 35°C, and the temperature is reduced to about 33°C on day 8 and is further reduced to about 31°C on day 10 after the beginning of the culturing. In some embodiments, the cell culture cycle is a 14-day cell culture cycle. In some embodiments, bevacizumab or a fragment thereof is secreted into the cell culture medium. In some embodiments, the method further comprises a step of recovering the bevacizumab or a fragment thereof from the cell culture. In another aspect, the invention provides bevacizumab or a fragment thereof produced by any of the methods described above. In another aspect, the invention provides a composition comprising: (i) bevacizumab or a fragment thereof produced by any of the methods described above and (ii) a pharmaceutically acceptable carrier. It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention.

[0019] The specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] **FIG. 1** is a series of graphs showing the increase in antibody titer yield from bevacizumab producing CHO cells cultured in cell culture medium supplemented with insulin. **FIG.1A** and **FIG.1B** show antibody titers in experiments of six different cell culture protocols assessed during a 14 day cell culture of CHO cells producing bevacizumab. Antibody production expressed as titer (mg/L). **FIG.1A** shows the results of one batch feed on day 3. **FIG. 1B** shows the results of one batch feed on day 3 and a second batch feed on day 6.

[0021] **FIG. 2** is a series of graphs demonstrating the effect on cell biomass accumulation and antibody production by cell cultures provided with one or two batch feeds during a 14 day cell culture cycle as a relationship to cell age. **FIG. 2A** shows cell biomass accumulation in bevacizumab producing CHO cells provided with one batch feed on day 3 as compared to

bevacizumab producing CHO cells provided with one batch feed on day 3 and a second batch feed on day 6. $p < 0.004^*$ for one feed process; $p < 0.018^*$ for two feed process. **FIG. 2B** shows antibody production from bevacizumab producing CHO cells provided with one batch feed on day 3 as compared to bevacizumab producing CHO cells provided with one batch feed on day 3 and a second batch feed on day 6. $p < 0.042^*$ for one feed process; $p < 0.82$ for two feed process. * indicates significant value. “1” indicates cells fed with one batch feed on day 3; “2” indicates cells fed with one batch feed on day 3 and a second batch feed on day 6. **[0022]** **FIG. 3** is a graph demonstrating the increase in antibody yield from bevacizumab producing CHO cells grown in a new cell culture process. Protocol 1 (triangles) indicates a cell culture process that is different from the cell culture process of Protocol 2; Protocol 2 (diamonds) indicates the new cell culture process.

DETAILED DESCRIPTION

[0023] Improved and cost-effective methods of producing bevacizumab, or a fragment thereof, are provided. Cell culture media comprising components that enable a cell to consistently produce a desired amount of bevacizumab, or a fragment thereof, while maintaining acceptable product quality attributes of bevacizumab, or a fragment thereof, are described. The cell culture media provided herein may find use in producing manufacturing-scale amounts of bevacizumab, or a fragment thereof.

[0024] The methods provided herein, including: (i) a method of producing bevacizumab, or a fragment thereof; (ii) a method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof; and (iii) a method of enhancing production of bevacizumab, or a fragment thereof, (e.g., enhancing titer yields of bevacizumab, or a fragment thereof) from a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, in one aspect utilize a cell culture medium comprising two or more of copper, insulin, and cystine (such as a cell culture medium comprising copper and insulin, or a cell culture medium comprising copper and cystine, or a cell culture medium comprising insulin and cystine, or a cell culture medium comprising copper, insulin and cystine).

Bevacizumab, or fragment thereof, produced by any of the methods detailed herein is also provided, as are compositions comprising bevacizumab, or fragment thereof. In one aspect, bevacizumab, or fragment thereof, produced by any of the methods detailed herein exhibits acceptable product quality attributes of bevacizumab, or a fragment thereof, such as N-glycosylation profile, charge heterogeneity, and sequence integrity. In a particular variation, the product quality attributes of bevacizumab, or a fragment thereof, are acceptable if they are

substantially similar to bevacizumab, or a fragment thereof produced by a method that does not use a cell culture medium comprising at least two of copper, insulin, and cystine. A cell culture medium comprising two or more of copper, insulin, and cystine (e.g., a cell culture medium comprising: (i) copper and insulin; (ii) copper and cystine; (iii) insulin and cystine; or (iv) copper, insulin and cystine) is also provided. In one variation, the cell culture medium comprising two or more of copper, insulin, and cystine enhances production of bevacizumab, or a fragment thereof, (e.g., enhances titer yields of bevacizumab, or a fragment thereof) by a mammalian cell cultured in the medium relative to culturing the mammalian cell in a cell culture medium without at least two of insulin, copper and cystine. Also provided herein is a composition comprising a cell culture medium comprising at least two of copper, insulin, and cystine and (i) a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof and/or (ii) bevacizumab, or a fragment thereof. A culturing vessel comprising any of the cell culture media provided herein are provided. In one aspect, the culturing vessel is a manufacturing scale culturing vessel, such as a culturing vessel capable of containing at least 2 liters, at least 10 liters, at least 100 liters, at least 500 liters, at least 1,000 liters, at least 2,500 liters, at least 5,000 liters, at least 7,500 liters, at least 10,000 liters, at least 12,000 liters or more of a cell culture medium provided herein as is required for producing manufacturing scale amounts of bevacizumab from cell culture. Thus, the methods provided herein may find use in a manufacturing-scale production of bevacizumab, or a fragment thereof.

I. Definitions

[0025] The term “bevacizumab” refers to a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. (1997) *Cancer Res.* 57:4593-4599, also known as “rhuMAb VEGF” or “AVASTIN®”. It comprises mutated human IgG1 framework regions and antigen-binding complementarity-determining regions from the murine anti-human VEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF to its receptors. Approximately 93% of the amino acid sequence of bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the sequence is derived from the murine antibody A4.6.1. bevacizumab binds to the same epitope as the monoclonal anti-VEGF antibody A4.6.1 produced by hybridoma ATCC HB 10709. Bevacizumab and other humanized anti-VEGF antibodies are further described in U.S. Patent No. 6,884,879.

[0026] The terms “medium” and “cell culture medium” refer to a nutrient source used for growing or maintaining cells. As is understood by a person of skill in the art, the nutrient

source may contain components required by the cell for growth and/or survival or may contain components that aid in cell growth and/or survival. Vitamins, essential or non-essential amino acids (e.g., cysteine and cystine), and trace elements (e.g., copper) are examples of medium components. Any media provided herein may also be supplemented with any one or more of insulin, plant hydrolysates and animal hydrolysates.

[0027] “Culturing” a cell refers to contacting a cell with a cell culture medium under conditions suitable to the survival and/or growth and/or proliferation of the cell.

[0028] “Batch culture” refers to a culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process.

[0029] “Fed batch cell culture,” as used herein refers to a batch culture wherein the cells and culture medium are supplied to the culturing vessel initially, and additional culture nutrients are fed, continuously or in discrete increments, to the culture during the culturing process, with or without periodic cell and/or product harvest before termination of culture.

[0030] “Perfusion culture” is a culture by which the cells are restrained in the culture by, *e.g.*, filtration, encapsulation, anchoring to microcarriers, etc., and the culture medium is continuously or intermittently introduced and removed from the culturing vessel.

[0031] “Culturing vessel” refers to a container used for culturing a cell. The culturing vessel can be of any size so long as it is useful for the culturing of cells.

[0032] The term “titer” as used herein refers to the total amount of recombinantly expressed antibody produced by a cell culture divided by a given amount of medium volume. Titer is typically expressed in units of milligrams of antibody per milliliter of medium. Titer can be expressed or assessed in terms of a relative measurement, such as a percentage increase in titer as compared obtaining the protein product under different culture conditions.

[0033] A “nucleic acid” refers to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer.

[0034] An “isolated nucleic acid” means and encompasses a non-naturally occurring, recombinant or a naturally occurring sequence outside of or separated from its usual context. An isolated nucleic acid molecule is other than in the form or setting in which it is found in

nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the protein where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

[0035] An “isolated” protein (*e.g.*, an isolated antibody) is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. Isolated protein includes the protein *in situ* within recombinant cells since at least one component of the protein's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

[0036] A “purified” protein (*e.g.*, antibody) means that the protein has been increased in purity, such that it exists in a form that is more pure than it exists in its natural environment and/or when initially produced and/or synthesized and/or amplified under laboratory conditions. Purity is a relative term and does not necessarily mean absolute purity.

[0037] “Contaminants” refer to materials that are different from the desired protein product (*e.g.*, different from an antibody product). A contaminant may include, without limitation: host cell materials, such as CHOP; nucleic acid; a variant, fragment, aggregate or derivative of the desired protein; another polypeptide; endotoxin; viral contaminant; cell culture media components, etc.

[0038] The term “antibody” herein is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity. An antibody can be human, humanized and/or affinity matured.

[0039] The terms “full length antibody,” “intact antibody” and “whole antibody” are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain an Fc region.

[0040] “Antibody fragments” comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab',

F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[0041] Papain digestion of antibodies produces two identical antigen-binding fragments, called “Fab” fragments, each with a single antigen-binding site, and a residual “Fc” fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')₂ fragment that has two antigen-combining sites and is still capable of cross-linking antigen. The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0042] “Fv” is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a “dimeric” structure analogous to that in a two-chain Fv species. It is in this configuration that the three HVRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0043] “Single-chain Fv” or “scFv” antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see, *e.g.*, Pluckthün, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315, 1994.

[0044] The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *e.g.*, the individual antibodies comprising the population are identical except for possible mutations, *e.g.*, naturally

occurring mutations, that may be present in minor amounts. Thus, the modifier “monoclonal” indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity *in vivo*, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

[0045] The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler and Milstein, *Nature*, 256:495-97 (1975); Hongo *et al.*, *Hybridoma*, 14 (3): 253-260 (1995), Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage-display technologies (see, e.g., Clackson *et al.*, *Nature*, 352: 624-628 (1991); Marks *et al.*, *J. Mol. Biol.* 222: 581-597 (1992); Sidhu *et al.*, *J. Mol. Biol.* 338(2): 299-310 (2004); Lee *et al.*, *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee *et al.*, *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits *et al.*, *Nature* 362: 255-258

(1993); Bruggemann *et al.*, *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks *et al.*, *Bio/Technology* 10: 779-783 (1992); Lonberg *et al.*, *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild *et al.*, *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

[0046] A “human antibody” is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries. Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581 (1991). Also available for the preparation of human monoclonal antibodies are methods described in Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner *et al.*, *J. Immunol.*, 147(1):86-95 (1991). See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.*, 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, *e.g.*, immunized xenomice (see, *e.g.*, U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSETM technology). See also, for example, Li *et al.*, *Proc. Natl. Acad. Sci. USA*, 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

[0047] The term “hypervariable region,” “HVR,” or “HV,” when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, *e.g.*, Xu *et al.*, *Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, N.J., 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain. See, *e.g.*, Hamers-Casterman *et al.*, *Nature* 363:446-448 (1993); Sheriff *et al.*, *Nature Struct. Biol.* 3:733-736 (1996).

[0048] A number of HVR delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the

most commonly used (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). The AbM HVRs represent a compromise between the Kabat HVRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" HVRs are based on an analysis of the available complex crystal structures. The residues from each of these HVRs are noted below.

Loop	Kabat	AbM	Chothia	Contact
L1	L24-L34	L24-L34	L26-L32	L30-L36
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B (Kabat Numbering)
H1	H31-H35	H26-H35	H26-H32	H30-H35 (Chothia Numbering)
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

[0049] HVRs may comprise "extended HVRs" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat *et al.*, *supra*, for each of these definitions.

[0050] "Framework" or "FR" residues are those variable domain residues other than the HVR residues as herein defined.

[0051] The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat *et al.*, *supra*. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (*e.g.* residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence

[0052] The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (e.g., Kabat *et al.*, *Sequences of Immunological Interest*. 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The “EU numbering system” or “EU index” is generally used when referring to a residue in an immunoglobulin heavy chain constant region (e.g., the EU index reported in Kabat *et al.*, *supra*). The “EU index as in Kabat” refers to the residue numbering of the human IgG1 EU antibody.

[0053] The term “pharmaceutical formulation” refers to a preparation which is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. Such formulations are sterile.

[0054] “Pharmaceutically acceptable” carriers, excipients, or stabilizers are ones which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed (*Remington's Pharmaceutical Sciences* (20th edition), ed. A. Gennaro, 2000, Lippincott, Williams & Wilkins, Philadelphia, PA). Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TweenTM, polyethylene glycol (PEG), and PluronicsTM.

[0055] A “sterile” formulation is aseptic or free or essentially free from all living microorganisms and their spores.

[0056] As used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to “a compound” optionally includes a combination of two or more such compounds, and the like.

[0057] It is understood that aspects and embodiments of the invention described herein include “comprising,” “consisting,” and “consisting essentially of” aspects and embodiments.

[0058] Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description

referring to “about X” includes description of “X.” Numeric ranges are inclusive of the numbers defining the range.

[0059] Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the present invention encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, but also the main group absent one or more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the claimed invention.

II. Cell Culture Media

[0060] Cell culture media provided herein may find use in methods (*e.g.*, a method of producing bevacizumab, or a fragment thereof; a method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof; and/or a method of enhancing production of bevacizumab, or a fragment thereof, such as by enhancing titer yields of bevacizumab, from a mammalian cell comprising a nucleic acid encoding bevacizumab) and in compositions (*e.g.*, a composition comprising a cell culture medium comprising at least two of copper, insulin, and cystine and (i) a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof and/or (ii) bevacizumab, or a fragment thereof) as detailed herein.

[0061] In some aspects, the cell culture medium provided herein comprises components (*e.g.*, at least two of copper, insulin, and cystine) that may be used in culturing a cell that produces bevacizumab, or a fragment thereof, wherein the cell, when cultured in the presence of the media components (*e.g.*, at least two of copper, insulin, and cystine), produces bevacizumab, or a fragment thereof, in a desired amount, which may be in an amount that is greater than the amount of bevacizumab produced by a cell cultured in a cell culture medium that does not contain the media components (*e.g.*, a cell culture medium that does not contain at least two of copper, insulin, and cystine). In one aspect, the cell culture media provided herein is used in culturing a cell that produces bevacizumab, or a fragment thereof, wherein the cell, when cultured in the presence of the media components (*e.g.*, at least two of copper, insulin, and cystine), produces bevacizumab, or a fragment thereof, in a desired amount and with an acceptable quality attribute, such as an acceptable molecular weight. As used herein, “an acceptable quality attribute” of bevacizumab can refer to a chemical and/or physical attribute required for regulatory approval or marketing of bevacizumab and may be the chemical

and/or physical attribute used in assessing lot-to-lot consistency of batches of bevacizumab, or a fragment thereof, produced by a cell.

[0062] In other aspects of the invention, cell culture media components (*e.g.*, at least two of copper, insulin, and cystine) have been identified as capable of providing antibody-producing cells with improved or acceptable quality attributes that contribute to higher production of bevacizumab (*e.g.*, results in higher titer of bevacizumab) as compared to cells that produce bevacizumab and are cultured in a cell culture medium that does not contain these components (a cell culture medium that does not contain at least two of copper, insulin and cystine). Certain identified media components (*e.g.*, at least two of copper, insulin, and cystine) can be used to provide an antibody-producing cell (*e.g.*, a CHO cell) with the capability of producing bevacizumab, or a fragment thereof, with an acceptable titer, which in one aspect is a titer greater than the titer obtained when the cells produce bevacizumab, or a fragment thereof, in a cell culture medium that does not comprise at least two of copper, insulin, and cystine. As used herein, “an acceptable titer” of an antibody produced from a cultured cell (*e.g.*, bevacizumab produced from a CHO cell) can as a non-limiting example refer to the amount of antibody required to meet manufacturing-scale production of the antibody or to the amount of antibody required to assess consistency in lot-to-lot batches of the antibody product. The cell culture media provided herein may improve the amount of bevacizumab that is produced from a cell comprising a nucleic acid encoding bevacizumab as compared to the amount of bevacizumab produced from the cell cultured in a different media.

[0063] A cell culture medium for use in culturing a cell for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, is provided, wherein the cell culture medium comprises any one or more of: (a) copper; (b) insulin; (c) cystine; (d) an animal-derived hydrolysate; and (e) a plant-derived hydrolysate. In some embodiments, the cell culture medium comprises 2 or 3 or 4 or 5 of components (a), (b), (c), (d) and (e). It is understood that the cell culture medium provided herein may contain any combination of components (a), (b), (c), (d) and (e) the same as if each and every combination were specifically and individually listed. For example, it is understood that a cell culture medium comprising three of components (a), (b), (c), (d) and (e) may comprise any combination of the components so long as at least three of the components are present (*e.g.*, a cell culture medium comprising components (a), (b) and (c) or comprising components (a), (d) and (e) or comprising components (c), (d) and (e) are contemplated). In some embodiments, a cell culture medium provided herein comprises components (a), (b), (c), (d) and (e). In some embodiments, a cell culture medium provided herein comprises (a) and (b). In some

embodiments, a cell culture medium provided herein comprises (a) and (c). In some embodiments, a cell culture medium provided herein comprises (b) and (c).

[0064] In some aspects, a cell culture medium as provided herein contains one or more media components selected from the group consisting of copper, insulin, cystine in amounts as described in Table 1. In some embodiments, the cell culture medium further comprises an animal-derived hydrolysate in amounts as described in Table 1. In other embodiments, the cell culture medium further comprises a plant-derived hydrolysate in amounts as described in Table 1. In some embodiments, the cell culture medium further comprises both an animal-derived hydrolysate and a plant-derived hydrolysate in amounts as described in Table 1.

[0065] It is also understood that a cell culture medium provided herein may comprise any one or more of the cell culture medium components of Table 1 (any one or more of copper, insulin, cystine, an animal-derived hydrolysate and a plant-derived hydrolysate) in any of the amounts listed in Table 1, the same as if each and every combination of components and amounts were specifically and individually listed. In one variation, the cell culture medium provided herein comprises two or three or four or each of copper, insulin, cystine, an animal-derived hydrolysate and a plant-derived hydrolysate in any of the amounts listed in Table 1, the same as if each and every combination of components and amounts were specifically and individually listed. In one aspect, the cell culture medium comprises at least two of copper, insulin and cystine in any of the amounts listed in Table 1, and an in further variation further comprises an animal-derived hydrolysate and/or a plant-derived hydrolysate in any of the amounts listed in Table 1.

Table 1. Exemplary Amounts of Media Components

Component	Amount of Component in Medium
(a) Insulin	from about 1.0 mg/L to about 100.0 mg/L; from about 5.0 mg/L to about 80.0 mg/L; from about 5.0 mg/L to about 60.0 mg/L; from about 5.0 mg/L to about 50.0 mg/L; from about 5.0 mg/L to about 40.0 mg/L; from about 5.0 mg/L to about 30.0 mg/L; from about 5.0 mg/L to about 25.0 mg/L; from about 10.0 mg/L to about 25.0 mg/L; from about 10.0 mg/L to about 30.0 mg/L; from about 15.0 mg/L to about 20.0 mg/L; from about 5.0 mg/L to about 15.0 mg/L; from about 6.0 mg/L to about 12.0 mg/L; from about 7.0 mg/L to about 11.0 mg/L; from about 8.0 mg/L to about 10.0 mg/L; from about 10

	mg/L to about 100 mg/L; from about 10 mg/L to about 50 mg/L; from about 10 mg/L to about 35 mg/L; from about 10 mg/L to about 250 mg/L; from about 1.0 mg/L to about 66 mg/L; from about 1.0 mg/L to about 60 mg/L; from about 1.0 mg/L to about 50 mg/L; from about 1.0 mg/L to about 40 mg/L; from about 1.0 mg/L to about 30 mg/L; from about 1.0 mg/L to about 20 mg/L; from about 1.0 mg/L to about 10 mg/L; from about 10 mg/L to about 66 mg/L; from about 20 mg/L to about 66 mg/L; from about 30 mg/L to about 66 mg/L; from about 40 mg/L to about 66 mg/L; from about 50 mg/L to about 66 mg/L; from about 60 mg/L to about 66 mg/L; from about 5.6 mg/L to about 66 mg/L; from about 10 mg/L to about 60 mg/L; from about 20 mg/L to about 50 mg/L; from about 30 mg/L to about 40 mg/L; from about 1 mg/L to about 14 mg/L; from about 1.3 mg/L to about 13 mg/L; from about 1.6 mg/L to about 12 mg/L; from about 1.4 mg/L to about 11 mg/L; from about 5.6 mg/L to about 14 mg/L; from about 5.9 mg/L to about 13 mg/L; from about 6.2 mg/L to about 12 mg/L; from about 7 mg/L to about 11 mg/L; about any of 1.0 or 2.0 or 3.0 or 4.0 or 5.0 or 6.0 or 7.0 or 8.0 or 9.0 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 34 or 35 or 36 or 37 or 38 or 39 or 40 or 41 or 42 or 43 or 45 mg/L; any of 5.6 mg/L, 6 mg/L, 6.2 mg/L, 7 mg/L, 8 mg/L, 9 mg/L, 10 mg/L, 11 mg/L, 12 mg/L, 13 mg/L, 14 mg/L or 15 mg/L or 16 mg/L or 17 mg/L or 18 mg/L or 19 mg/L or 20 mg/L or 21 mg/L or 22 mg/L or 23 mg/L or 24 mg/L or 25 mg/L; at least about any of 1.0 or 3.0 or 5.0 or 7.0 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 mg/L and no more than about 44 or 24 or 14 or 11 mg/L.
(b) Cystine	from about 0.5 mM to about 2.5 mM; from about 0.5 mM to about 2.0 mM; from about 0.5 mM to about 1.75 mM; from about 0.5 mM to about 2.5 mM; from about 0.8 mM to about 2.5 mM; from about 0.8 mM to about 2.25 mM; from about 0.8 mM to about 2.0 mM; from about 0.8 mM to about 1.75 mM; from about 0.8 mM to about 1.6 mM; from about 0.8 mM to about 1.25 mM; from about 0.8 mM to

	about 1.0 mM; from about 1.0 mM to about 1.6 mM; from about 1.0 mM to about 2.5 mM; from about 1.25 mM to about 2.5 mM; from about 1.5 mM to about 2.5 mM; from about 1.75 mM to about 2.5 mM; from about 2.0 mM to about 2.5 mM; from about 2.25 mM to about 2.5 mM; from about 0.9 mM to about 2.0 mM; from about 0.8 mM to about 1.75 mM; from about 0.9 mM to about 1.5 mM; from about 1.0 mM to about 1.25 mM; from about 1.0 mM to about 2.0 mM; from about 1.0 mM to about 1.5 mM; from about 1.2 mM to about 1.4 mM; about any of 0.8 or 0.9 or 1.0 or 1.1 or 1.2 or 1.3 or 1.4 or 1.5 or 1.6 mM; any of 0.8 mM, 0.85 mM, 0.9 mM, 0.95 mM, 1.0 mM, 1.05 mM, 1.1 mM, 1.15 mM, 1.2 mM, 1.25 mM, 1.3 mM, 1.35 mM, 1.4 mM, 1.5 mM, 1.55 mM, 1.6 mM, 1.65 mM, 1.7 mM, or 1.75 mM; at least about any of 0.8 or 0.9 or 1.0 or 1.1 mM and no more than about 1.75 or 1.6 or 1.5 or 1.4 mM.
(c) Copper	from about 69 nM to about 1,000.0 nM; from about 20 nM to about 480.0 nM; from about 20 nM to about 400 nM; from about 20 nM to about 350 nM; from about 20 nM to about 300 nM; from about 20 nM to about 250 nM; from about 20 nM to about 200 nM; from about 20 nM to about 150 nM; from about 20 nM to about 100 nM; from about 20 nM to about 50 nM; from about 50 nM to about 480 nM; from about 100 nM to about 480 nM; from about 150 nM to about 480 nM; from about 200 nM to about 480 nM; from about 250 nM to about 480 nM; from about 300 nM to about 480 nM; from about 325 nM to about 375 nM; from about 350 nM to about 480 nM; from about 400 nM to about 480 nM; from about 50 nM to about 450 nM; from about 100 nM to about 400 nM; from about 150 nM to about 350 nM; from about 200 nM to about 300 nM; from about 22 nM to about 440 nM; from about 26 nM to about 400 nM; from about 30 nM to about 360 nM; from about 54 nM to about 480 nM; from about 62 nM to about 440 nM; from about 69 nM to about 400 nM; from about 80 nM to about 400 nM; from about 100 nM to about 400 nM; from about 125 nM to about 400 nM; from about 150 nM to about 400 nM; from about 200 nM to about 400 nM; from about 250 nM to

	about 400 nM; from about 300 nM to about 400 nM; from about 325 nM to about 375 nM; from about 325 nM to about 350 nM; any of about 25 or 26 or 27 or 28 or 29 or 30 or 40 or 50 or 60 or 69 or 100 or 110 or 120 or 125 or 130 or 140 or 150 or 160 or 170 or 175 or 180 or 190 or 200 or 210 or 220 or 225 or 230 or 240 or 250 or 260 or 270 or 275 or 280 or 290 or 300 or 310 or 320 or 325 or 330 or 335 or 336 or 337 or 338 or 339 or 340 or 345 or 350 or 360 or 370 or 375 or 380 or 390 or 400 nM; any of 54 nM, 56 nM, 58 nM, 60 nM, 62 nM, 64 nM, 66 nM, 68 nM, 69 nM, 70 nM, 71 nM, 72 nM, 73 nM, 74 nM, 75 nM, 100 nM, 125 nM, 150 nM, 175 nM, 200 nM, 225 nM, 250 nM, 275 nM, 300 nM, 325 nM, 350 nM, 375 nM, or 400 nM; at least any of about 20 or 25 or 30 or 35 or 40 or 45 or 50 or 55 or 60 or 65 or 70 or 80 nM and no more than about 420 or 400 or 380 or 360 nM.
(d) Animal-derived hydrolysate	from about 6.0 g/L to about 20 g/L; from about 5.6 g/L to about 38 g/L; from about 5.6 g/L to about 30 g/L; from about 5.6 g/L to about 25 g/L; from about 5.6 g/L to about 20 g/L; from about 7.0 g/L to about 20 g/L; from about 9.0 to about 11.0 g/L; from about 5.6 g/L to about 10 g/L; from about 5.6 to about 38 g/L; from about 10 g/L to about 38 g/L; from about 15 g/L to about 38 g/L; from about 20 g/L to about 38 g/L; from about 25 g/L to about 38 g/L; from about 30 g/L to about 38 g/L; from about 35 g/L to about 38 g/L; from about 10 g/L to about 30 g/L; from about 15 g/L to about 25 g/L; from about 5.6 g/L to about 14 g/L; from about 5.9 g/L to about 13 g/L; from about 6.2 g/L to about 12 g/L; from about 7.0 g/L to about 11.0 g/L; from about 7.0 g/L to about 35.0 g/L; from about 7.0 g/L to about 25.0 g/L; from about 7.0 g/L to about 15.0 g/L; from about 8.0 g/L to about 12.0 g/L; any of about 2.8 or 3.0 or 3.2 or 3.4 or 3.6 or 3.8 or 4.0 or 4.2 or 4.4 or 4.6 or 4.8 or 5.0 or 5.2 or 5.4 or 5.6 or 6 or 6.2 or 7 or 7.4 or 7.8 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 20 or 25 or 30 or 35 or 40 or 45 or 50 g/L; any of 2.8 g/L, 3.0 g/L, 3.2 g/L, 3.4 g/L, 3.6 g/L, 3.8 g/L, 4.0 g/L, 4.2 g/L, 4.4 g/L, 4.6 g/L, 4.8 g/L, 5.0 g/L or 6 g/L, 6.2 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, or 14 g/L; at least any of about 2.8 or 3.2 or 3.4 or 3.6 or

	3.8 or 4.0 or 4.2 or 4.6 or 5.0 or 5.6 or 6 or 6.2 or 7 or 8 or 9 g/L and no more than about 14 or 13 or 12 or 11 g/L.
(e) Plant-derived hydrolysate	from about 1.0 g/L to about 10.0 g/L; from about 1.4 g/L to about 6.2 g/L; from about 1.4 g/L to about 6.0 g/L; from about 1.4 g/L to about 5.5 g/L; from about 1.4 g/L to about 5.0 g/L; from about 1.4 g/L to about 4.5 g/L; from about 1.4 g/L to about 4.0 g/L; from about 1.4 g/L to about 3.5 g/L; from about 1.4 g/L to about 3.0 g/L; from about 1.4 g/L to about 2.5 g/L; from about 1.4 g/L to about 2.0 g/L; from about 2.0 g/L to about 6.2 g/L; from about 2.5 g/L to about 6.2 g/L; from about 3.0 g/L to about 6.2 g/L; from about 3.5 g/L to about 6.2 g/L; from about 4.0 g/L to about 6.2 g/L; from about 4.5 g/L to about 6.2 g/L; from about 5.0 g/L to about 6.2 g/L; from about 5.5 g/L to about 6.2 g/L; from about 1.5 g/L to about 6.0 g/L; from about 2.0 g/L to about 5.5 g/L; from about 2.5 g/L to about 5.0 g/L; from about 3.0 g/L to about 4.5 g/L; from about 3.0 g/L to about 3.2 g/L; from about 3.5 g/L to about 4.0 g/L; from about 1.4 g/L to about 3.4 g/L; from about 1.5 g/L to about 3.0 g/L; from about 1.75 g/L to about 2.8 g/L; from about 1.5 g/L to about 5.5 g/L; from about 1.5 g/L to about 4.5 g/L; from about 1.5 g/L to about 3.5 g/L; from about 1.5 g/L to about 2.5 g/L from about 1.75 g/L to about 2.75 g/L; from about 2.0 g/L to about 3.0 g/L; from about 2.25 g/L to about 2.75 g/L; any of about 1.4 or 1.5 or 1.75 or 2.0 or 2.25 or 2.5 or 2.8 or 3.0 or 3.1 or 3.25 or 3.4 or 3.5 or 3.75 or 4.0 g/L; any of 1.4 g/L, 1.5 g/L, 1.75 g/L, 2.0 g/L, 2.25 g/L, 2.5 g/L, 2.8 g/L, 3.0 g/L, 3.2 g/L, 3.25 g/L, 3.4, 3.75, or 4.0 g/L; at least any of about 1.4 or 1.5 or 1.75 g/L and no more than about 3.4 or 3 or 2.8 g/L.

[0066] In one variation, insulin is present in the cell culture media at a concentration of from about 1.0 mg/L to about 100.0 mg/L or from about 5.0 mg/L to about 80.0 mg/L or from about 5.0 mg/L to about 60.0 mg/L or from about 5.0 mg/L to about 50.0 mg/L or from about 5.0 mg/L to about 40.0 mg/L or from about 5.0 mg/L to about 30.0 mg/L or from about 5.0 mg/L to about 25.0 mg/L or from about 10.0 mg/L to about 25.0 mg/L or from about 10.0

mg/L to about 30.0 mg/L or from about 15.0 mg/L to about 20.0 mg/L or from about 5.0 mg/L to about 15.0 mg/L or from about 6.0 mg/L to about 12.0 mg/L or from about 7.0 mg/L to about 11.0 mg/L or from about 8.0 mg/L to about 10.0 mg/L or at a concentration of about any one of 5.0 mg/L, 6.0 mg/L, 7.0 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L, 11.0 mg/L, 12.0 mg/L, 13.0 mg/L, 14.0 mg/L, 15.0 mg/L, 16.0 mg/L, 17.0 mg/L, 18.0 mg/L, 19.0 mg/L, 20.0 mg/L, 21.0 mg/L, 22.0 mg/L, 23.0 mg/L, 24.0 mg/L, 25.0 mg/L, 26.0 mg/L, 27.0 mg/L, 28.0 mg/L, 29.0 mg/L or 30.0 mg/L or at a concentration of about any one of 7 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L or 11.0 mg/L.

[0067] In one variation, copper is present in the cell culture media at a concentration of from about 69.0 nM to about 400.0 nM or from about 80 nM to about 400 nM or from about 100 nM to about 400 nM or from about 125 nM to about 400 nM or from about 150 nM to about 400 nM or from about 200 nM to about 400 nM or from about 250 nM to about 400 nM or from about 300 nM to about 400 nM or from about 325 nM to about 375 nM or from about 325 nM to about 350 nM or at a concentration of about any one of 100 nM, 125 nM, 150 nM, 175 nM, 200 nM, 225 nM, 250 nM, 275 nM, 300 nM, 325 nM, 350 nM, 375 nM or 400 nM or at a concentration of about any one of 330 nM, 335 nM, 340 nM, 345 nM or 350 nM or at a concentration of about 335 nM, 336 nM, 337 nM, 338 nM, 339 nM or 400 nM or at a concentration of about 339 nM.

[0068] In one variation cystine is present in the cell culture medium at a concentration of from about 0.8 mM to about 2.5 mM or from about 0.8 mM to about 2.0 mM or from about 0.8 mM to about 1.75 mM or from about 0.8 mM to about 1.5 mM or from about 1.0 mM to about 2.0 mM or from about 1.0 mM to about 1.5 mM or from about 1.2 mM to about 1.4 mM or at a concentration of about any one of 0.8 mM or 0.9 mM or 1.0 mM or 1.1 mM or 1.2 mM or 1.3 mM or 1.4 mM or 1.5 mM or at a concentration of about any one of 1.1 mM, 1.3 mM or 1.5 mM or at a concentration of about 1.3 mM.

[0069] In one variation, an animal-derived hydrolysate is present in the cell culture media at a concentration of from about 5.6 g/L to about 38.0 g/L or from about 7.0 g/L to about 35.0 g/L or from about 7.0 g/L to about 25.0 g/L or from about 7.0 g/L to about 15.0 g/L or from about 8.0 g/L to about 12.0 g/L or from about 7.0 g/L to about 11.0 g/L or about any one of 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L or 50 g/L or about any one of 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, or 12 g/L or about 10 g/L.

[0070] In one variation, an plant-derived hydrolysate is present in the cell culture media at a concentration of from about 1.4 g/L to about 6.2 g/L or from about 1.5 g/L to about 5.5 g/L or from about 1.5 g/L to about 4.5 g/L or from about 1.5 g/L to about 3.5 g/L or from about 1.5

g/L to about 2.5 g/L or from about 1.75 g/L to about 2.75 g/L or from about 2.0 g/L to about 3.0 g/L or from about 2.25 g/L to about 2.75 g/L or about any one of 1.75 g/L, 2.0 g/L, 2.25 g/L, 2.5 g/L, 3.0 g/L, 3.25, 3.5 g/L, 3.75 g/L, or 4.0 g/L or about any one of 2.0 g/L, 2.25 g/L, 2.5 g/L or 3.0 g/L or about 2.5 g/L.

[0071] In a further variation, cysteine is present in the cell culture medium. Cysteine may in one aspect be added to a basal cell culture medium (e.g., by supplementing the basal cell culture medium with a feed medium comprising cysteine). In one variation, a cell culture medium comprises cysteine (which may be added to a basal cell culture medium that does not comprise cysteine via a feed medium comprising cysteine) in a concentration of from about 0.5 mM to about 5.0 mM or from about 1.0 mM to about 12.0 mM or from about 2.0 mM to about 10.0 mM or from about 2.0 mM to about 8.0 mM or about 1.0 mM to about 10.0 mM or from about 1.0 mM to about 8.0 mM or from about 2.0 mM to about 12.0 mM or from about 3.0 mM to about 12.0 mM or from about 4.0 mM to about 12.0 mM or from about 5.0 mM to about 12.0 mM or from about 6.0 mM to about 12.0 mM or from about 6.0 mM to about 10.0 mM or from about 6.0 mM to about 8.0 mM or about any one of 0.5 mM, 0.8 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, 5.0 mM, 5.5 mM, 6.0 mM, 6.5 mM, 7.0 mM, 7.5 mM, 8.0 mM, 8.5 mM or 9.0 mM. In one aspect, cysteine is added to a basal cell culture media (which addition may be at any point in time of the cell culture cycle and may be in one or more amounts, which may be the same or different), in an amount such that cysteine is present in the cell culture media at a concentration of about 7.5 mM.

[0072] In a further variation, cystine is present in the cell culture medium. Cystine may in one aspect be added to a basal cell culture medium (e.g., by supplementing the basal cell culture medium which may or may not already comprise cystine with a feed medium comprising cystine). In one variation, a cell culture medium comprises cystine (which may be added to a basal cell culture medium via a feed medium comprising cystine) in a concentration of from about 0.5 mM to about 5.0 mM, such as in an amount to provide about 0.8 mM cysteine in the cell culture medium.

[0073] In certain embodiments, the cell culture medium comprises cystine but is free of cysteine.

[0074] The cell culture medium (e.g., a basal cell culture medium) may further be supplemented with an additional cell culture medium components (e.g., such as via a feed cell culture medium). In one aspect, the additional cell culture medium component comprises insulin. In another aspect, the additional cell culture medium component comprises insulin and cysteine. A cell culture media provided herein may be supplemented with any amount of

insulin and/or cysteine that is suitable for culturing a cell. In one aspect, insulin is added to a cell culture medium (e.g., added to a basal cell culture medium at one or more point in time of the cell culture cycle) in an amount to provide a concentration of insulin in the cell culture of about 15 mg/L or about 25 mg/L. In one aspect, insulin is added to a cell culture medium (e.g., added to a basal cell culture medium at one or more point in time of the cell culture cycle) in an amount to provide a concentration of insulin in the cell culture selected from the group consisting of: from about 1.0 mg/L to about 100.0 mg/L; from about 10.0 mg/L to about 100.0 mg/L; from about 10.0 mg/L to about 50.0 mg/L; from about 10.0 mg/L to about 35.0 mg/L; from about 10.0 mg/L to about 25.0 mg/L; from about 5.0 mg/L to about 80.0 mg/L; from about 5.0 mg/L to about 60.0 mg/L; from about 5.0 mg/L to about 50.0 mg/L; from about 5.0 mg/L to about 30.0 mg/L; from about 5.0 mg/L to about 25.0 mg/L; from about 10.0 mg/L to about 25.0 mg/L; from about 10.0 mg/L to about 30.0 mg/L; from about 15.0 mg/L to about 20.0 mg/L; from about 5.0 mg/L to about 15.0 mg/L; from about 6.0 mg/L to about 12.0 mg/L; from about 7.0 mg/L to about 11.0 mg/L; from about 8.0 mg/L to about 10.0 mg/L; about any one of 5.0 mg/L, 6.0 mg/L, 7.0 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L, 11.0 mg/L, 12.0 mg/L, 13.0 mg/L, 14.0 mg/L, 15.0 mg/L, 16.0 mg/L, 17.0 mg/L, 18.0 mg/L, 19.0 mg/L, 20.0 mg/L, 21.0 mg/L, 22.0 mg/L, 23.0 mg/L, 24.0 mg/L, 25.0 mg/L, 26.0 mg/L, 27.0 mg/L, 28.0 mg/L, 29.0 mg/L or 30.0 mg/L; about any one of 7 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L and 11.0 mg/L. In another aspect, cysteine is added to a cell culture medium (e.g., added to a basal cell culture medium at one or more point in time of the cell culture cycle) in an amount to provide a concentration of cysteine in the cell culture selected from the group consisting of: from about 1.0 mM to about 12.0 mM or from about 2.0 mM to about 10.0 mM or from about 2.0 mM to about 8.0 mM or about 1.0 mM to about 10.0 mM or from about 1.0 mM to about 8.0 mM or from about 2.0 mM to about 12.0 mM or from about 3.0 mM to about 12.0 mM or from about 4.0 mM to about 12.0 mM or from about 5.0 mM to about 12.0 mM or from about 6.0 mM to about 12.0 mM or from about 6.0 mM to about 10.0 mM or from about 6.0 mM to about 8.0 mM or about any one of 5.0 mM, 5.5 mM, 6.0 mM, 6.5 mM, 7.0 mM, 7.5 mM, 8.0 mM, 8.5 mM or 9.0 mM. In another aspect, cystine is added to a cell culture medium (e.g., added to a basal cell culture medium at one or more point in time of the cell culture cycle) in an amount to provide a concentration of cystine in the cell culture of from about 0.1 mM to about 1.5 mM, such as a concentration of about 0.2 mM.

[0075] In some aspects, a cell culture medium provided herein comprises from about 5.0 mg/L to about 14.0 mg/L, from about 5.5 mg/L to about 13.0 mg/L, from about 6.0 mg/L to

about 12.0 mg/L, from about 7.0 mg/L to about 11.0 mg/L, from about 8.0 mg/L to about 10.0 mg/L, or from about 8.5 mg/L to about 14.0 mg/L insulin. It is understood that the cell culture medium comprising insulin may further comprise any one or more of copper and cystine in any amount provided herein. For example, it is understood that a cell culture medium comprising from about 6.0 mg/L to about 12.0 mg/L insulin may further comprise from about 70 nM to about 400 nM of copper and/or from about 0.5 mM to about 2.5 mM cystine and may further comprise an animal-derived hydrolysate and/or plant-derived hydrolysate, for example an animal-derived hydrolysate from about 5.5 g/L to about 40.0 g/L and/or a plant-derived hydrolysate from about 1.5 g/L to about 6.5 g/L.

[0076] In other aspects, a cell culture medium provided herein comprises from about 65 nM to about 400 nM, from about 70 nM to about 375 nM, from about 75 nM to about 350 nM, from about 80 nM to about 325 nM, from about 85 nM to about 300 nM, or from about 90 nM to about 275 nM copper. It is understood that the cell culture medium comprising copper may further comprise any one or more of insulin and cystine in any amount provided herein. For example, it is understood that a cell culture medium comprising from about 85 nM to about 300 nM copper may further comprise from about 0.8 mM to about 1.75 mM of cystine and/or from about 8.0 mg/L to about 12.0 mg/L insulin and may further comprise an animal-derived hydrolysate and/or plant-derived hydrolysate, for example an animal-derived hydrolysate from about 5.5 g/L to about 40.0 g/L and/or a plant-derived hydrolysate from about 1.5 g/L to about 6.5 g/L.

[0077] In some aspects, a cell culture medium provided herein comprises from about 0.8 mM to about 1.75 mM, from about 0.9 mM to about 1.50 mM, from about 1.0 mM to about 1.40 mM, or from about 1.0 mM to about 1.30 mM cystine. It is understood that the cell culture medium comprising cystine may further comprise any one or more of insulin and copper in any amount provided herein. For example, it is understood that a cell culture medium comprising from about 0.8 mM to about 1.75 mM cystine may further comprise from about 70 nM to about 375 nM of copper and/or from about 8.0 mg/L to about 12.0 mg/L insulin and may further comprise an animal-derived hydrolysate and/or plant-derived hydrolysate, for example an animal-derived hydrolysate from about 5.5 g/L to about 40.0 g/L and/or a plant-derived hydrolysate from about 1.5 g/L to about 6.5 g/L.

[0078] In some embodiments, the cell culture medium further comprises an animal-derived hydrolysate in amounts as described in Table 1. In other embodiments, the cell culture medium further comprises a plant-derived hydrolysate in amounts as described in Table 1. In

some embodiments, the cell culture medium further comprises both an animal-derived hydrolysate and a plant-derived hydrolysate in amounts as described in Table 1.

[0079] In some aspects, a cell culture medium provided herein comprises from about 1.5 g/L to about 6.0 g/L, from about 2.0 g/L to about 5.5 g/L, from about 2.5 g/L to about 5.0 g/L, from about 3.0 g/L to about 4.5 g/L, or from about 3.5 g/L to about 4.0 g/L plant-derived hydrolysate. It is understood that the cell culture medium comprising plant-derived hydrolysate may further comprise any one or more of cystine, insulin and copper in any amount provided herein. For example, it is understood that a cell culture medium comprising from about 0.8 mM to about 1.75 mM cystine may further comprise from about 70 nM to about 375 nM of copper and/or from about 8.0 mg/L to about 12.0 mg/L insulin and may further comprise an animal-derived hydrolysate, for example an animal-derived hydrolysate from about 6.0 g/L to about 20.0 g/L.

[0080] In some aspects, a cell culture medium provided herein comprises from about 6.0 g/L to about 35.0 g/L, from about 7.0 g/L to about 30.0 g/L, from about 8.0 g/L to about 25.0 g/L, from about 9.0 g/L to about 20 g/L, or from about 10.0 g/L to about 15.0 g/L animal-derived hydrolysate. It is understood that the cell culture medium comprising animal-derived hydrolysate may further comprise any one or more of cystine, insulin and copper in any amount provided herein. For example, it is understood that a cell culture medium comprising from about 0.8 mM to about 1.75 mM cystine may further comprise from about 70 nM to about 375 nM of copper and/or from about 8.0 mg/L to about 12.0 mg/L insulin and may further comprise an plant-derived hydrolysate, for example an plant-derived hydrolysate from about 1.5 g/L to about 3.0 g/L.

[0081] In some of the embodiments herein, the cell culture medium comprises from about 0.9 mM to about 1.5 mM cystine. In some of the embodiments herein, the cell culture medium comprises from about 1.4 mg/L to about 11.0 mg/L insulin or from about 1.44 mg/L to about 66.0 mg/L insulin. In some of the embodiments herein, the cell culture medium comprises from about 26.0 nM to about 400.0 nM copper. In some aspects, a cell culture medium comprises two or more components selected from: (a) about 69.0 nM to about 400.0 nM copper, (b) from about 7.0 mg/L to about 11.0 mg/L insulin or from about 1.44 mg/L to about 66 mg/L insulin, and (c) from about 0.8 mM to about 2.5 mM cystine.

[0082] Cell culture media components described herein (e.g., a cell culture media comprising any one or more of copper, insulin, cystine, an animal-derived hydrolysate and a plant-derived hydrolysate) may be added to a cell culture medium in a form that is known in the art, such as a salt, a hydrate or combination thereof. The cell culture media components can

also be provided to the cell culture media as a solution, an extract, or in solid form. In some embodiments, the copper is provided to the cell culture medium as CuSO₄. As a non-limiting example, cystine may be provided to the cell culture medium as the disodium salt monohydrate powder. Protein hydrolysates, also known as peptones, are typically manufactured by enzymatic digestion of a variety of biologically based starting materials such as animal tissues, milk-derived products, microorganisms or plants. The hydrolysate used in the cell culture medium provided herein can be derived from a plant or an animal (e.g., plant-derived hydrolysate and/or animal-derived hydrolysate). A plant hydrolysate as described herein can be derived from, but not limited to, wheat gluten, maize, cereal, soy, or cottonseed. An animal hydrolysate as described herein can be derived from, but not limited to, bovine, chicken, caprine, equine, human, ovine, porcine, or rabbit or other animals.

[0083] A method of preparing a cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, is also provided, wherein the method comprises combining any two or more of copper, insulin and cystine in a composition suitable for cell culture. In one aspect, the method comprises adding any two or more of copper, insulin and cystine to a composition suitable for cell culture, wherein the two or more of copper, insulin and cystine may be added to the composition sequentially or simultaneously. In a further variation, a method of preparing a cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, is provided, wherein the method comprises combining any two or more of copper, insulin and cystine in a composition suitable for cell culture at a first period of time and wherein the method further comprises adding an amount of insulin at a second period of time, such as at least once, at least twice, at least three times, at least four times, at least five times, at least six times, at least seven times, etc. of a cell growth cycle. In some embodiments, a cell growth cycle is at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, or any amount of days wherein the cells may remain in cell culture while still remaining viable. In one variation of a method of preparing a cell culture medium, cystine is added in an amount to provide about 0.9 mM to about 1.5 mM cystine in the cell culture medium. In another variation of a method of preparing a cell culture medium, insulin is added in an amount to provide from about 1.4 mg/L to about 11.0 mg/L insulin or from about 1.44 mg/L to about 66.0 mg/L insulin in the cell culture medium. In another variation of a method of preparing a cell culture medium, copper is added in an amount to provide from about 26.0 nM to about 400.0 nM copper in the cell culture medium. In some aspects, a cell

culture medium is prepared by combining two or more components selected from: (a) copper in an amount to provide about 69.0 nM to about 400.0 nM copper in the cell culture medium, (b) insulin in an amount to provide from about 7.0 mg/L to about 11.0 mg/L or from about 1.44 mg/L to about 66 mg/L insulin in the cell culture medium, and (c) cystine in an amount to provide from about 0.8 mM to about 2.5 mM cystine in the cell culture medium.

[0084] In some embodiments herein, the cell culture medium is a basal cell culture medium. In other embodiments herein, the cell culture medium is a feed cell culture medium. In some embodiments herein, the cell culture medium is a basal cell culture medium comprising at least one of copper, insulin, and cystine, and where the basal cell culture medium is supplemented (*e.g.*, at a period of time following initiation of a cell culture cycle, such as any one of at least two times, at least three times, at least four times, at least five times, at least six times, at least seven times, etc. of a cell culture cycle) with a feed cell culture medium comprising any one or more of insulin, an animal-derived hydrolysate and a plant-derived hydrolysate.

[0085] Individual media components provided herein may be present in amounts that result in one or more advantageous properties for culturing cells comprising a nucleic acid encoding bevacizumab, or a fragment thereof, and/or for bevacizumab production from cell culture. Advantageous properties include, but are not limited to, increased cell viability, increase in the amount of bevacizumab produced from the cell (*e.g.*, enhanced bevacizumab titer) and/or reduced oxidation of bevacizumab in cell culture. Advantageous properties of the cell culture media provided herein may also include maintaining or enhancing the amount of bevacizumab produced by the cells (*e.g.*, antibody titer) while maintaining the N-glycosylation profile, the charge heterogeneity and/or the amino acid sequence integrity of bevacizumab, or a fragment thereof. These advantageous properties are applicable to methods of culturing a cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof and to methods of producing bevacizumab, or a fragment thereof in cell culture as described herein.

[0086] A cell culture medium provided herein in one aspect results in one or more favorable product quality attribute or advantageous property when used in a method of producing bevacizumab, or a fragment thereof. In one variation, use of the cell culture medium provided herein increases the amount of bevacizumab produced by the cells (*e.g.*, enhances antibody titer) as compared to the amount of bevacizumab, or a fragment thereof, produced by culturing the cell producing bevacizumab in a different cell culture medium.

[0087] As would be understood by the skilled artisan, the cell culture media detailed herein may comprise other components (e.g., besides the one or more of copper, insulin, and cystine, and optionally peptone hydrolysate) that are useful for cell culture. For example, it is understood that the cell culture media may comprise additional components such as amino acids (e.g., glutamine, arginine, or asparagine), vitamins (including but not limited to B vitamins such as any one or more of vitamin B1, vitamin B2, vitamin B3, vitamin B6, vitamin B7, vitamin B9, or vitamin B12), trace elements, transition metals (including but not limited to nickel, iron (e.g., ferric iron or ferrous iron), or zinc), and other media components. Any media provided herein may also be supplemented with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), ions (such as sodium, chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), trace elements and glucose or an equivalent energy source. Additional cell culture media components, such as those listed herein, may be included in the cell culture medium at appropriate concentrations that would be known to those skilled in the art.

III. Methods and Uses of the Invention

[0088] Provided herein are methods of culturing cells used in the production bevacizumab, or a fragment thereof, and use of cell culture media that comprise one or more of copper, insulin and cystine. In some aspects, a method is provided for culturing a mammalian cell comprising a nucleic acid encoding bevacizumab or fragment thereof, wherein the method comprises the step of contacting the mammalian cell with a cell culture medium comprising at least two of copper, insulin and cystine, wherein the cell culture medium may additionally comprise a plant-derived hydrolysate, an animal-plant-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate. In some embodiments, the cell culture media comprises insulin. In some of the embodiments herein, the cell culture media comprises copper. In some of the embodiments herein, the cell culture media comprises cystine. In some of the embodiments herein, the cell culture media comprises copper and cystine. In some of the embodiments herein, the cell culture media comprises copper and insulin. In some of the embodiments herein, the cell culture media comprises insulin and cystine. In some of the embodiments herein, the amount of the components in the cell culture medium (e.g., the amount of copper, insulin, cystine, plant-derived hydrolysate and/or animal-derived hydrolysate) is in an amount selected from a value provided in Table 1. In some embodiments, the method further comprises the step of adding an additional amount of

insulin to the medium. The additional amount of insulin can be added to the cell culture medium at least once, at least three times, at least 6 times or at least 12 times during the cell culture cycle. In some of the embodiments herein, the additional amount of insulin added to the cell culture is added in an amount to provide insulin in the cell culture medium at a concentration selected from Table 1 such as from about 1 mg/L to about 44 mg/L. In some aspects, the cell culture medium further comprises an animal-derived hydrolysate, a plant-derived hydrolysate, or both an animal-derived hydrolysate and a plant-derived hydrolysate. [0089] In some other aspects, a method is provided for culturing a cell comprising a nucleic acid encoding bevacizumab or fragment thereof, wherein the method comprises the step of contacting the cell with a cell culture medium comprising two or more components selected from the group consisting of copper, insulin, and cystine. In some embodiments herein, the cell culture medium comprises insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L. In some embodiments herein, the cell culture medium comprises copper at a concentration of from about 69.0 nM to about 400.0 nM. In some embodiments herein, the cell culture medium comprises cystine at a concentration of from about 0.8 mM to about 2.5 mM. In some embodiments herein the cell culture medium comprises insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L and copper at a concentration of from about 69.0 nM to about 400.0 nM. In some embodiments herein the cell culture medium comprises insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L and cystine at a concentration of from about 0.8 mM to about 2.5 mM. In some embodiments herein the cell culture medium comprises copper at a concentration of from about 69.0 nM to about 400.0 nM and cystine at a concentration of from about 0.8 mM to about 2.5 mM. In some embodiments herein the cell culture medium comprises insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L, copper at a concentration of from about 69.0 nM to about 400.0 nM and cystine at a concentration of from about 0.8 mM to about 2.5 mM. In any of the embodiments herein, the cell culture medium may comprise cystine, insulin and/or copper in an amount selected from Table 1. In some of the embodiments herein, the method further comprises the step of adding an additional amount of insulin to the cell culture medium provided herein (e.g., such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). The additional amount of insulin can be added to the cell culture medium at least once, at least two times, at three times, at least six times, at least nine times, at least twelve times, or at least fourteen times during the cell culture cycle. In some of the embodiments herein, the additional amount of insulin added to the cell culture is added in an amount to provide insulin in the cell culture

medium at a concentration selected from Table 1 such as 5.6 mg/L to about 66 mg/L. In some aspects, the cell culture medium further comprises an animal-derived hydrolysate, a plant-derived hydrolysate, or both an animal-derived hydrolysate and a plant-derived hydrolysate. In some of the embodiments herein, the method further comprises the step of adding an additional amount of animal-derived hydrolysate and plant-derived hydrolysate to the cell culture medium provided herein (e.g., such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). In some of the embodiments herein, the additional amount of animal-derived hydrolysate and plant-derived hydrolysate added to the cell culture is added in an amount to provide animal-derived hydrolysate and plant-derived hydrolysate in the cell culture medium at a concentration selected from the concentrations listed in Table 1.

[0090] In another aspect, provided herein are methods of producing bevacizumab or a fragment thereof, wherein the method comprises the step of contacting a cell capable of producing bevacizumab or a fragment thereof with a cell culture medium comprising two or more components selected from the group consisting of copper, insulin, and cystine. In some embodiments herein, the cell culture medium comprises insulin at a concentration of from about 7.0 mg/L to about 11.0 mg/L. In some embodiments herein, the cell culture medium comprises copper at a concentration of from about 69.0 nM to about 400.0 nM. In some embodiments herein, the cell culture medium comprises cystine at a concentration of from about 0.8 mM to about 2.5 mM. In any of the embodiments herein, the cell culture medium may comprise cystine, insulin or copper in an amount selected from Table 1. In some of the embodiments herein, the method further comprises the step of adding an additional amount of insulin to the cell culture medium provided herein. Insulin may be added to the cell culture medium in any amount that is suitable for cell culture. In one aspect, insulin is added to the cell culture medium in an amount to provide insulin in the cell culture medium at a concentration selected from the concentrations listed in Table 1. In a particular aspect, insulin is added to the cell culture medium in an amount to provide insulin in the cell culture medium at a concentration selected from the group consisting of: from about 1.0 mg/L to about 100.0 mg/L; from about 5.0 mg/L to about 80.0 mg/L; from about 5.0 mg/L to about 60.0 mg/L; from about 5.0 mg/L to about 50.0 mg/L; from about 5.0 mg/L to about 40.0 mg/L; from about 5.0 mg/L to about 30.0 mg/L; from about 5.0 mg/L to about 25.0 mg/L; from about 10.0 mg/L to about 25.0 mg/L; from about 10.0 mg/L to about 30.0 mg/L; from about 15.0 mg/L to about 20.0 mg/L; from about 5.0 mg/L to about 15.0 mg/L; from about 6.0 mg/L to about 12.0 mg/L; from about 7.0 mg/L to about 11.0 mg/L and from about 8.0

mg/L to about 10.0 mg/L. In another aspect, insulin is added to the cell culture medium in an amount to provide insulin in the cell culture medium at a concentration of about any one of 5.0 mg/L, 6.0 mg/L, 7.0 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L, 11.0 mg/L, 12.0 mg/L, 13.0 mg/L, 14.0 mg/L, 15.0 mg/L, 16.0 mg/L, 17.0 mg/L, 18.0 mg/L, 19.0 mg/L, 20.0 mg/L, 21.0 mg/L, 22.0 mg/L, 23.0 mg/L, 24.0 mg/L, 25.0 mg/L, 26.0 mg/L, 27.0 mg/L, 28.0 mg/L, 29.0 mg/L or 30.0 mg/L. In a further aspect, insulin is added to the cell culture medium in an amount to provide insulin in the cell culture medium at a concentration of about any one of 7 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L or 11.0 mg/L. The additional amount of insulin can be added to the cell culture medium at any time during the cell culture cycle. For example, insulin may be added at any one or more of days 1-20 for a 20 day cell culture cycle (e.g., at any one or more of days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20). When an additional amount of insulin is added, it may be added in any amount, which amount may be the same or different when insulin is added more than once during a cell culture cycle. It is therefore appreciated that for a 14 day cell culture cycle, insulin may be added at any one or more of days 1-14 (e.g., at any one or more of days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14) in any amount, which amount may be the same or different when insulin is added more than one during a cell culture cycle. The additional amount of insulin can be added to the cell culture medium at least once, at least two times, at three times, at least six times, at least nine times, at least twelve times, or at least fourteen times during the cell culture cycle. In some of the embodiments herein, the additional amount of insulin added to the cell culture is added in an amount to provide insulin in the cell culture medium at a concentration selected from Table 1 such as 5.6 mg/L to about 66 mg/L. In some aspects, the cell culture medium further comprises an animal-derived hydrolysate, a plant-derived hydrolysate, or both an animal-derived hydrolysate and a plant-derived hydrolysate. In some of the embodiments herein, the method further comprises the step of adding an additional amount of animal-derived hydrolysate and plant-derived hydrolysate to the cell culture medium provided herein (e.g., such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). In some of the embodiments herein, the additional amount of animal-derived hydrolysate and plant-derived hydrolysate added to the cell culture is added in an amount to provide animal-derived hydrolysate and plant-derived hydrolysate in the cell culture medium at a concentration selected from the concentrations listed in Table 1.

[0091] In another aspect, provided herein are methods of producing bevacizumab or a fragment thereof, comprising a step of culturing a mammalian cell comprising a nucleic acid

encoding bevacizumab or a fragment thereof in a cell culture medium, wherein initial cell culture medium in a cell culture cycle comprises two or more components selected from the group consisting of copper at a concentration of from about 69 nM to about 1,000 nM, insulin at a concentration of from about 1.0 mg/L to about 100.0 mg/L, and cystine at a concentration of from about 0.7 mM to about 2.0 mM, and wherein the cell produces bevacizumab or the fragment. In some embodiments, the initial cell culture medium comprising (1) copper and insulin; (2) copper and cystine; (3) insulin and cystine; or (4) copper, insulin, and cystine. In some embodiments, the initial cell culture medium comprises copper, cystine, and/or insulin in a concentration selected from the concentrations as described in Table 1. In some embodiments, the initial cell culture medium comprises an animal-derived hydrolysate and/or a plant-derived hydrolysate in a concentration selected from the concentrations as described in Table 1. In some embodiments, the initial cell culture medium comprises insulin and the method further comprises a step of adding an additional amount of insulin to the cell culture medium during the cell culture cycle. In some embodiments, the additional amount of insulin is added to the cell culture medium at least once, at least twice, at least three times, at least four times, at least five times, or at least six times during the cell culture cycle. In some embodiments, the insulin added each time is from about 5 mg/L to about 25 mg/L (e.g., about any one of 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L and 25 mg/L). In some embodiments, the cumulative amount of insulin added during the cell culture cycle is from about 20 mg/L to about 100 mg/L (e.g., about any one of 20 mg/L, 25 mg/L, 30 mg/L, 35 mg/L, 40 mg/L, 45 mg/L, 50 mg/L, 55 mg/L, 60 mg/L, 65 mg/L, 70 mg/L, 75 mg/L, 80 mg/L, 85 mg/L, 90 mg/L, 95 mg/L, and 100 mg/L). In some embodiments, the initial cell culture medium comprises cystine and the method further comprises a step of adding an additional amount of cystine to the cell culture medium during the cell culture cycle. For example, cystine is added in an amount to provide from about 0.1 to about 1.5 mM additional cystine in the cell culture medium (e.g., about 0.1 mM, about 0.2 mM, about 0.3 mM, about 0.4 mM, about 0.5 mM, about 0.6 mM, about 0.7 mM, about 1 mM, or about 1.5 mM additional cystine in the cell culture medium). In some embodiments, cystine is added in a batch feed during the cell culture cycle. In some embodiments, the method further comprises at least one batch feed during the cell culture cycle (e.g., two, three, or four batch feeds during the cell culture cycle). In some embodiments, the batch feed medium further comprises an animal-derived hydrolysate and/or a plant-derived hydrolysate (e.g., in a concentration selected from the concentrations as described in Table 1). In some embodiments, during the cell culture cycle, the temperature of the medium is reduced by at least about 2, at least about 3, at least about 4,

or at least about 5 degrees C relative to the temperature at the beginning of the culturing. In some embodiments, the temperature of the medium is reduced at least once or at least twice during the cell culture cycle. In some embodiments, the temperature is reduced on day 8 and day 10 after the beginning of the culturing. In some embodiments, the cell is cultured at a temperature ranging from about 31°C to about 35°C. In some embodiments, the cell is cultured at a first temperature of about 35°C for a first period of time, is cultured at a second temperature of about 33°C for a second period of time, and is cultured at a third temperature of about 31°C for a third period of time. In some embodiments, the cell is cultured in the medium having a pH at about 7.0 to about 7.3.

[0092] As used herein, the term “initial cell culture medium” refers to the cell culture medium at the beginning of a cell culture cycle. In some embodiments, the initial cell culture medium is the cell culture medium after cells are inoculated in a basal medium.

[0093] As used herein, the term “cumulative” refers to the total amount of a particular component or components added over the cell culture cycle, including components added at the beginning of the cell culture cycle and subsequently added components. In some embodiments, the cumulative amount is the total concentration of a component or components added into the cell culture medium as measured or calculated in the cell culture medium after addition. In some embodiments, the component or components are added into the cell culture medium by a feed solution containing concentrated component or components. For example, if the initial culture medium has 10 mg/L insulin, and additional amount of insulin at 15 mg/L is added during the cell culture cycle to achieve an increase of 15 mg/L in the cell culture medium, the cumulative amount of the insulin added during the cell culture cycle is 25 mg/L.

[0094] In some embodiments, a cell culture cycle refers to a period of time from inoculating cells into a basal cell culture medium for producing a polypeptide expressed by the cells to the end of the production period. In some embodiments, the cell culture cycle is at least 10 days, at least 11 days, at least 12 days, at least 13 days, at least 14 days, at least 15 days, at least 16 days, at least 17 days, at least 18 days, at least 19 days, at least 20 days, at least 21 days, at least 22 days, at least 23 days, or any amount of days wherein the cells may remain viable for producing the polypeptide expressed by the cells.

[0095] In some of the embodiments herein, the method increases the amount of bevacizumab or fragment thereof produced by the mammalian cell as compared to the amount of bevacizumab or fragment thereof the mammalian cell produces when cultured in a cell culture medium that does not comprise one or more of components listed in Table 1. In some

embodiments, the amount of bevacizumab or fragment thereof produced by a cell cultured in a cell culture medium comprising at least two of copper, insulin and cystine is increased by at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% as compared to the amount of bevacizumab or fragment thereof produced by the cell when cultured in a cell culture medium that does not comprise at least two of copper, insulin and cystine.

[0096] The cell culture medium provided herein can be used a basal cell culture medium and/or as a feed cell culture medium. In some embodiments, a cell culture medium provided herein is used in a method for culturing the cell during the cell's growth phase. In some embodiments, a cell culture medium provided herein is used in a method for culturing the cell during the cell's production phase.

[0097] It is understood that any of the methods detailed herein including: (i) a method of producing bevacizumab, or a fragment thereof; (ii) a method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof; and (iii) a method of enhancing production of bevacizumab, or a fragment thereof, (*e.g.*, enhancing titer yields of bevacizumab, or a fragment thereof) from a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, may be carried out in any suitable scale (*e.g.*, any scale that produces bevacizumab, or a fragment thereof). In one aspect, any of the methods detailed herein are performed on a scale that is commensurate in scope with commercial production of bevacizumab, or a fragment thereof. For example, in one variation, a cell capable of producing bevacizumab may be cultured in a cell culture medium provided herein wherein the culturing occurs in a culturing vessel that is capable of holding a commercial batch of bevacizumab, such as in a culturing vessel capable of holding at least 10,000 L of cell culture (*e.g.*, the methods in one aspect are carried out on at least a 10,000 L cell culture scale, such as a 12,000 L cell culture scale).

[0098] In further embodiments of the methods provided herein, the bevacizumab or a fragment thereof is recovered from the cell culture. A composition comprising the recovered bevacizumab or a fragment thereof can be subjected to at least one purification step before assessment of, *e.g.*, a quality attribute. In a further embodiment, the composition is a pharmaceutical composition comprising bevacizumab or a fragment thereof and a pharmaceutically acceptable carrier.

[0099] Other methods and cell culture media are provided throughout, such as in the Brief Summary of the Invention and elsewhere.

Polypeptide Production

[0100] The cell culture media detailed herein can be used in a method of culturing cells to produce bevacizumab or a fragment thereof. The medium may be used in a method of culturing cells capable of producing bevacizumab or a fragment thereof, whether by batch culture, fed batch culture or perfusion culture. In one embodiment, bevacizumab or a fragment thereof is directly secreted into the medium by the host cell. In another embodiment, bevacizumab or a fragment thereof is released into the medium by lysis of a cell comprising a nucleic acid encoding the antibody or fragment thereof.

[0101] Bevacizumab or a fragment thereof that is expressible in a host cell may be produced in accordance with the present disclosure and may be present in the compositions provided.

[0102] Methods for producing antibodies and fragments thereof, in cell culture are well known in the art. Provided herein are non-limiting exemplary methods for producing an antibody (e.g., full length antibodies, antibody fragments and multispecific antibodies) in cell culture. See *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F.M. Ausubel, et al. eds., 2003); *Short Protocols in Molecular Biology* (Ausubel et al., eds., J. Wiley and Sons, 2002); *Current Protocols in Protein Science*, (Horswill et al., 2006); *Antibodies, A Laboratory Manual* (Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R.I. Freshney, 6th ed., J. Wiley and Sons, 2010) for generally well understood and commonly employed techniques and procedures for the production of antibodies (e.g., bevacizumab), which are all incorporated herein by reference in their entirety.

Cell Culture and Antibody Production

[0103] Generally the cells are combined (contacted) with any of the cell culture media described herein under one or more conditions that promote any of cell growth, maintenance and/or antibody production. Methods of culturing a cell and producing an antibody employ a culturing vessel (bioreactor) to contain the cell and cell culture medium. The culturing vessel can be composed of any material that is suitable for culturing cells, including glass, plastic or metal. Typically, the culturing vessel will be at least 1 liter and may be 10, 100, 250, 500, 1000, 2500, 5000, 8000, 10,000 liters or more (e.g., a 12,000 liter vessel). In one aspect the culturing vessel is capable of containing at least 2 liters, at least 10 liters, at least 100 liters, at least 500 liters, at least 1,000 liters, at least 2,500 liters, at least 5,000 liters, at least 7,500

liters, at least 10,000 liters, at least 12,000 liters or more of a cell culture medium provided herein as is required for producing manufacturing scale amounts of bevacizumab from cell culture. Thus, the compositions and methods provided herein may find use in a manufacturing-scale production of bevacizumab, or a fragment thereof. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. Culturing conditions that may be adjusted during the culturing process include but are not limited to pH and temperature. In some of the embodiments herein, the pH is at least 7.0, 7.15, 7.2, 7.25, 7.30, 7.35, 7.4, 7.45, or 7.50 but no more than 8.0. The number of cells comprising a nucleic acid encoding bevacizumab, or a fragment thereof, that can be inoculated into a cell culture medium provided herein will be apparent to one of skill in the art. For example, about 1.0×10^6 to about 2.0×10^6 cells (including any of about 1.1×10^6 , about 1.2×10^6 about 1.3×10^6 , about 1.4×10^6 , about 1.5×10^6 , about 1.6×10^6 , about 1.7×10^6 , about 1.8×10^6 or about 1.9×10^6) comprising a nucleic acid encoding bevacizumab, or a fragment thereof, can be inoculated in a medium provided herein for initiation of a cell culture cycle. In one aspect, the number of cells comprising a nucleic acid encoding bevacizumab, or a fragment thereof, that can be inoculated into a cell culture medium provided herein is from about 1.2×10^6 to about 1.8×10^6 cells or from about 1.3×10^6 to about 1.7×10^6 cells or from about 1.5×10^6 to about 1.7×10^6 cells.

[0104] A cell culture is generally maintained in the initial growth phase under conditions conducive to the survival, growth and viability (maintenance) of the cell culture. The precise conditions will vary depending on the cell type, the organism from which the cell was derived, and the nature and character of the expressed antibody or fragment thereof.

[0105] The temperature of the cell culture in the initial growth phase will be selected based primarily on the range of temperatures at which the cell culture remains viable. For example, during the initial growth phase, CHO cells grow well at 37 °C. In general, most mammalian cells grow well within a range of about 25 °C. to 42 °C. Preferably, mammalian cells grow well within the range of about 35 °C. to 40 °C. Those of ordinary skill in the art will be able to select appropriate temperature or temperatures in which to grow cells, depending on the needs of the cells and the production requirements.

[0106] In one embodiment of the present invention, the temperature of the initial growth phase is maintained at a single, constant temperature. In another embodiment, the temperature of the initial growth phase is maintained within a range of temperatures. For example, the temperature may be steadily increased or decreased during the initial growth

phase. Alternatively, the temperature may be increased or decreased by discrete amounts at various times during the initial growth phase. One of ordinary skill in the art will be able to determine whether a single or multiple temperatures should be used, and whether the temperature should be adjusted steadily or by discrete amounts.

[0107] The cells may be cultured during the initial growth phase for a greater or lesser amount of time. In one variation, the cells are cultured for a period of time sufficient to achieve a viable cell density that is a given percentage of the maximal viable cell density that the cells would eventually reach if allowed to grow undisturbed. For example, the cells may be cultured for a period of time sufficient to achieve a desired viable cell density of 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 percent of maximal viable cell density.

[0108] In another embodiment the cells are allowed to grow for a defined period of time. For example, depending on the starting concentration of the cell culture, the temperature at which the cells are cultured, and the intrinsic growth rate of the cells, the cells may be cultured for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more days. In some cases, the cells may be allowed to grow for a month or more.

[0109] The cell culture may be agitated or shaken during the initial culture phase in order to increase oxygenation and dispersion of nutrients to the cells. In accordance with the present invention, one of ordinary skill in the art will understand that it can be beneficial to control or regulate certain internal conditions of the bioreactor during the initial growth phase, including but not limited to pH, temperature, oxygenation, etc. For example, pH can be controlled by supplying an appropriate amount of acid or base and oxygenation can be controlled with sparging devices that are well known in the art.

[0110] An initial culturing step is a growth phase, wherein batch cell culture conditions are modified to enhance growth of recombinant cells, to produce a seed train. The growth phase generally refers to the period of exponential growth where cells are generally rapidly dividing, *e.g.* growing. During this phase, cells are cultured for a period of time, usually 1 to 4 days, *e.g.* 1, 2, 3, or 4 days, and under such conditions that cell growth is optimal. The determination of the growth cycle for the host cell can be determined for the particular host cell by methods known to those skilled in the art.

[0111] In the growth phase, a basal culture medium provided herein and cells may be supplied to the culturing vessel in batch. The culture medium in one aspect contains less than about 5% or less than 1% or less than 0.1% serum and other proteins derived from plants or animals (*e.g.*, animal-derived hydrolysates and/or plant-derived hydrolysates). In some

embodiments, the basal medium does not comprise an animal-derived or plant-derived hydrolysate. However, serum and animal-derived proteins can be used if desired. At a particular point in their growth, the cells may form an inoculum to inoculate a culture medium at the start of culturing in the production phase. Alternatively, the production phase may be continuous with the growth phase. The cell growth phase is generally followed by a polypeptide production phase (*e.g.*, antibody production phase).

[0112] During the polypeptide production phase, the cell culture may be maintained under a second set of culture conditions (as compared to the growth phase) conducive to the survival and viability of the cell culture and appropriate for expression of the desired polypeptide (*e.g.*, bevacizumab or fragment thereof). For example, during the subsequent production phase, CHO cells express recombinant polypeptides well within a range of 25°C to 35°C. Multiple discrete temperature shifts may be employed to increase cell density or viability or to increase expression of the recombinant polypeptide. In one embodiment, a method of increasing polypeptide production (*e.g.*, increasing production of bevacizumab or fragment thereof) comprises a one or more temperature shift step during the polypeptide production phase. In a further embodiment, a one or more temperature shift step comprises a shift of the temperature from 37°C to 35°C, from 35°C to 33°C, or from 33°C to 31°C. In some embodiments herein, a one or more temperature shift step comprises a shift of temperature from about 37°C on day 0 to 35°C on day 1 to 33°C on day 8 and to 31°C on day 10. In some embodiments herein, a one or more temperature shift step comprises a shift of temperature from about 37°C on day 0 to 35°C on day 3 to 33°C on day 8 and to 31°C on day 10. In some embodiments herein, a one or more temperature shift step comprises a shift of temperature from about 37°C on day 0 to 34°C on day 2.5.

[0113] The cells may be maintained in the subsequent production phase until a desired cell density or production titer is reached. In one embodiment, the cells are maintained in the subsequent production phase until the titer of the recombinant polypeptide (*e.g.*, bevacizumab or fragment thereof) reaches a maximum. In other embodiments, the culture may be harvested prior to this point. For example, the cells may be maintained for a period of time sufficient to achieve a viable cell density of 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 percent of maximal viable cell density. In some cases, it may be desirable to allow the viable cell density to reach a maximum, and then allow the viable cell density to decline to some level before harvesting the culture.

[0114] In certain cases, it may be beneficial or necessary to supplement the cell culture during the subsequent production phase with nutrients or other medium components that have

been depleted or metabolized by the cells. For example, it might be advantageous to supplement the cell culture with nutrients or other medium components observed to have been depleted during monitoring of the cell culture. Alternatively or additionally, it may be beneficial or necessary to supplement the cell culture prior to the subsequent production phase. As non-limiting examples, it may be beneficial or necessary to supplement the cell culture with hormones and/or other growth factors, particular ions (such as sodium, chloride, calcium, magnesium, and phosphate), buffers, vitamins, nucleosides or nucleotides, trace elements (inorganic compounds usually present at very low final concentrations), amino acids, lipids, or glucose or other energy source. In one aspect, a basal cell culture is supplemented with insulin and/or plant-derived hydrolysate and/or an animal-derived hydrolysate as detailed herein.

[0115] In some embodiments herein, the methods of the invention comprise the supplementation of an additional amount of insulin into the cell culture during the cell production phase. For example, an additional 15 mg/L of insulin may be added to the cell culture on day of the production phase of the cell culture cycle. In another example, an additional 5 mg/L of insulin may be added to the cell culture at least three times during the production phase of the cell culture cycle. In still another example, an additional 5 mg/L of insulin may be added to the cell culture at least six times during the production phase of the cell culture cycle. A cell culture cycle may be at least 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days long. In some embodiments, the cell culture cycle is up to 20 days long. In some embodiments, the cell culture is at least 20 days long. In some embodiments, a cell may be cultured for more than one cell culture cycle. In some of the embodiments herein, the method further comprises the step of adding an additional amount of animal-derived hydrolysate and plant-derived hydrolysate to the cell culture medium provided herein (*e.g.*, such as via a feed medium introduced to the basal cell culture medium at a period of time following initiation of the cell culture cycle). In some of the embodiments herein, the additional amount of animal-derived hydrolysate and plant-derived hydrolysate added to the cell culture is added in an amount to provide animal-derived hydrolysate and plant-derived hydrolysate in the cell culture medium at a concentration selected from the concentrations listed in Table 1.

Antibody Purification

[0116] Bevacizumab or a fragment thereof preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

[0117] The culture medium or lysate may be centrifuged to remove particulate cell debris. Bevacizumab or a fragment thereof thereafter may be purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification. One skilled in the art will appreciate that purification methods suitable for the antibody or fragment thereof of interest may require modification to account for changes in the character of the antibody or fragment thereof upon expression in recombinant cell culture. An antibody or fragment thereof can be generally purified using chromatographic techniques (*e.g.*, affinity chromatography with a low pH elution step and ion exchange chromatography to remove process impurities). Purified bevacizumab or a fragment thereof may be concentrated to provide a concentrated protein composition, *e.g.*, one with an antibody concentration of at least 100 mg/mL or 125 mg/mL or 150 mg/mL or a concentration of about 100 mg/mL or 125 mg/mL or 150 mg/mL. It is understood that concentrated polypeptide products may be concentrated up to levels that are permissible under the concentration conditions, *e.g.*, up to a concentration at which the polypeptide is no longer soluble in solution. Non-limiting examples of methods for producing and purifying antibodies for drug formulations are described in Kelley, B. *MAbs.*, 2009, 1(5):443-452, which is incorporated herein in its entirety by reference.

IV. Pharmaceutical Formulations

[0118] Compositions comprising the cell culture medium provided herein and one or more other component, such as a cell or a desired antibody or fragment thereof (*i.e.*, bevacizumab or fragment thereof), are also provided. A mammalian cell comprising a nucleic acid encoding bevacizumab or fragment thereof can secrete the antibody or fragment thereof into a cell culture medium of the invention during cell culture. Accordingly,

compositions of the invention may comprise a mammalian cell that produces bevacizumab or fragment thereof and a cell culture medium provided herein into which the bevacizumab or fragment thereof is secreted. Compositions comprising bevacizumab or fragment thereof and a cell culture medium provided herein are also contemplated. In some aspects of the invention, a composition comprises (a) a mammalian cell comprising a nucleic acid encoding bevacizumab or fragment thereof; and (b) a cell culture medium as provided herein. In some aspects, the composition comprises (a) bevacizumab or fragment thereof; and (b) a cell culture medium as provided herein, wherein the antibody or fragment thereof is secreted into the medium by a mammalian cell comprising an isolated nucleic acid encoding bevacizumab or fragment thereof. In other aspects, the composition comprises: (a) bevacizumab or fragment thereof; and (b) a cell culture medium as provided herein, wherein the bevacizumab or fragment thereof is released into the medium by lysis of a mammalian cell comprising an isolated nucleic acid encoding the bevacizumab or fragment thereof. The mammalian cell of the composition may be any mammalian cell detailed herein (e.g., a CHO cell) and the medium of the composition may be any medium detailed herein, such as a medium comprising one or more compounds as detailed in Table 1.

[0119] Compositions (e.g., pharmaceutical formulations) of bevacizumab or a fragment thereof produced by any of the methods described herein are prepared by mixing bevacizumab or fragment thereof having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), which may be in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers, antioxidants, preservatives, low molecular weight (less than about 10 residues) polypeptides, proteins; hydrophilic polymers; amino acids; monosaccharides, disaccharides, and other carbohydrates, chelating agents, sugars, salt-forming counter-ions, metal complexes (e.g. Zn-protein complexes), and/or non-ionic surfactants. In some embodiments, the pharmaceutical formulation is administered to a mammal such as a human. Pharmaceutical formulations of bevacizumab or fragment thereof can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Accordingly, antibody-containing formulations as provided

herein may be suitable for injection, such as subcutaneous injection into an individual (e.g., subcutaneous injection into a human). The pharmaceutical formulations to be used for *in vivo* administration are generally sterile. Sterility may be readily accomplished, for example by filtration through sterile filtration membranes.

[0120] In some aspects, a composition (e.g., pharmaceutical formulation) as provided herein comprises bevacizumab or fragment thereof at a concentration of at least 100 mg/mL, 125 mg/mL, 150 mg/mL, 200 mg/mL, or 250 mg/mL, or at a concentration of about 100 mg/mL, about 125 mg/mL, about 150 mg/mL, about 175 mg/mL, or about 200 mg/mL. In other aspects, a composition (e.g., pharmaceutical formulation) as provided herein comprises bevacizumab or fragment thereof at a concentration of at least 1 mg/mL, 10 mg/mL, 25 mg/mL, 50 mg/mL, or 75 mg/mL, or at a concentration of about 1 mg/mL, about 10 mg/mL, about 25 mg/mL, about 50 mg/mL, or about 75 mg/mL.

V. Articles of Manufacture and Kits

[0121] A kit for supplementing a cell culture medium with at least two of copper, insulin and cystine are provided. The at least two of copper, insulin and cystine may be present in an amount to provide a concentration of the components at provided in Table 1. The kit may contain dried constituents to be reconstituted, and may also contain instructions for use (e.g., for use in supplementing a medium with the kit constituents). The kit may contain the constituents provided herein in amounts suitable to supplement a cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab or fragment thereof. In one aspect, a kit comprises cystine in an amount to provide from about 0.9 mM to about 1.5 mM cystine in the cell culture medium. In some embodiments herein, the kit further comprises insulin in an amount to provide from about 1.4 mg/L to about 11 mg/L insulin in the cell culture medium. In some embodiments herein, the kit further comprises copper in an amount to provide from about 26 nM to about 400 nM copper in the cell culture medium. In some embodiments, a kit comprises two or more constituents selected from the group consisting of insulin in an amount to provide from about 7.0 mg/L to about 11.0 mg/L insulin in the cell culture medium, cystine in an amount to provide from about 0.8 mM to about 2.5 mM cystine in the cell culture medium, and copper in an amount to provide from about 25.0 nM to about 400.0 nM copper in the cell culture medium.

[0122] In any of the aspects herein, the kit may further comprise an animal-derived hydrolysate or a plant-derived hydrolysate or both an animal-derived hydrolysate and a plant-derived hydrolysate. In some of the embodiments herein, the kit further comprises a plant-

derived hydrolysate in an amount to provide from about 1.4 g/L to about 6.2 g/L plant-derived hydrolysate in the cell culture medium. In some of the embodiments herein, the kit further comprises an animal-derived hydrolysate in an amount to provide from about 5.6 g/L to about 38.0 g/L animal-derived hydrolysate in the cell culture medium.

[0123] In another aspect of the invention, an article of manufacture is provided comprising a container which holds the cell culture medium of the invention and optionally provides instructions for its use. Suitable containers include, for example, bottles and bags. The container may be formed from a variety of materials such as glass or plastic. The container holds the cell culture medium and the label on, or associated with, the container may indicate directions for use (e.g., for use in culturing cells). The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents and package inserts with instructions for use.

[0124] An article of manufacture comprising a container which holds bevacizumab, or a fragment thereof produced by a method detailed herein and optionally provides instructions for its use is also provided.

EMBODIMENTS

[0125] Various embodiments and aspects of the invention are detailed herein and throughout. Embodiments include, without limitation, the following:

[0126] Method 1: A method of producing bevacizumab, or a fragment thereof, comprising the step of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab or fragment thereof in a cell culture medium, wherein the cell culture medium comprises two or more components selected from the group consisting of copper, insulin, and cystine, and wherein the cell produces bevacizumab, or a fragment thereof.

[0127] Method 2: A method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the method comprising the step of contacting the mammalian cell with a cell culture medium comprising two or more components selected from the group consisting of copper, insulin and cystine.

[0128] Method 3: A method of enhancing the amount of bevacizumab, or a fragment thereof, produced from a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the method comprising the step of culturing the mammalian cell in a cell culture medium comprising at least two of insulin, copper and cystine, wherein the amount of bevacizumab, or a fragment thereof, produced from the mammalian cell is

enhanced relative to culturing the mammalian cell in a cell culture medium without at least two of insulin, copper and cystine.

[0129] Method 4: A method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, in a cell culture medium comprising at least two of insulin, copper and cystine, wherein the amount of bevacizumab, or a fragment thereof, produced from the mammalian cell is enhanced relative to culturing the mammalian cell in a cell culture medium without at least two of insulin, copper and cystine.

[0130] For any of methods 1-4, the method may comprise any one or more of the following features (i)-(xviii) or sub-feature thereof or any combination of feature or sub feature:

- (i) the cell culture medium comprises copper and insulin
- (ii) the cell culture medium comprises copper and cystine
- (iii) the cell culture medium comprises insulin and cystine
- (iv) the cell culture medium comprises copper, insulin, and cystine
- (v) the cell culture medium (including a medium containing any of features (i)-(iv)) further comprises a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate.
- (vi) the cell culture medium (including a medium containing any one or more or all of features (i)-(v)) comprises insulin at a concentration of any one of:
 - a. from about 1.0 mg/L to about 100.0 mg/L
 - b. from about 10.0 mg/L to about 100.0 mg/L
 - c. from about 10.0 mg/L to about 50.0 mg/L
 - d. from about 10.0 mg/L to about 35.0 mg/L
 - e. from about 10.0 mg/L to about 25.0 mg/L
 - f. from about 5.0 mg/L to about 80.0 mg/L
 - g. from about 5.0 mg/L to about 60.0 mg/L
 - h. from about 5.0 mg/L to about 50.0 mg/L
 - i. from about 5.0 mg/L to about 40.0 mg/L
 - j. from about 5.0 mg/L to about 25.0 mg/L
 - k. from about 10.0 mg/L to about 25.0 mg/L

- l. from about 10.0 mg/L to about 40.0 mg/L
- m. from about 15.0 mg/L to about 20.0 mg/L
- n. from about 5.0 mg/L to about 15.0 mg/L
- o. from about 6.0 mg/L to about 12.0 mg/L
- p. from about 7.0 mg/L to about 11.0 mg/L
- q. from about 8.0 mg/L to about 10.0 mg/L
- r. about any one of 5.0 mg/L, 6.0 mg/L, 7.0 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L, 11.0 mg/L, 12.0 mg/L, 13.0 mg/L, 14.0 mg/L, 15.0 mg/L, 16.0 mg/L, 17.0 mg/L, 18.0 mg/L, 19.0 mg/L, 20.0 mg/L, 21.0 mg/L, 22.0 mg/L, 23.0 mg/L, 24.0 mg/L, 25.0 mg/L, 26.0 mg/L, 27.0 mg/L, 28.0 mg/L, 29.0 mg/L or 30.0 mg/L or 31.0 mg/L or 32 mg/L or 33 mg/L or 34 mg/L or 35 mg/L or 36 mg/L or 37 mg/L or 38 mg/L or 39 mg/L or 40 mg/L
- s. about any one of 7 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L or 11.0 mg/L
- t. about 25 mg/L

(vii) the cell culture medium (including a medium containing any one or more or all of features (i)-(vi)) comprises copper at a concentration of any one of:

- a. from about 69 nM to about 1,000 nM
- b. from about 69.0 nM to about 400.0 nM
- c. from about 80 nM to about 400 nM.
- d. from about 100 nM to about 400 nM
- e. from about 125 nM to about 400 nM
- f. from about 150 nM to about 400 nM
- g. from about 200 nM to about 400 nM
- h. from about 250 nM to about 400 nM
- i. from about 300 nM to about 400 nM
- j. from about 325 nM to about 375 nM
- k. from about 325 nM to about 350 nM
- l. about any one of 100 nM, 125 nM, 150 nM, 175 nM, 200 nM, 225 nM, 250 nM, 275 nM, 300 nM, 325 nM, 350 nM, 375 nM or 400 nM.
- m. about any one of 330 nM, 335 nM, 340 nM, 345 nM or 350 nM
- n. about 335 nM, 336 nM, 337 nM, 338 nM, 339 nM or 400 nM
- o. about 339 nM

- (viii) the cell culture medium (including a medium containing any one or more or all of features (i)-(vii)) comprises cystine at a concentration of any one of:
 - a. from about 0.7 mM to about 2.0 mM
 - b. from about 0.8 mM to about 2.5 mM
 - c. from about 0.8 mM to about 2.0 mM
 - d. from about 0.8 mM to about 1.75 mM
 - e. from about 0.8 mM to about 1.6 mM
 - f. from about 1.0 mM to about 2.0 mM
 - g. from about 1.0 mM to about 1.6 mM
 - h. from about 1.2 mM to about 1.4 mM
 - i. about any one of 0.8 mM or 0.9 mM or 1.0 mM or 1.1 mM or 1.2 mM or 1.3 mM or 1.4 mM or 1.5 mM
 - j. about any one of 1.1 mM, 1.3 mM or 1.5 mM
- (ix) the cell culture medium (including a medium containing any one or more or all of features (i)-(viii)) comprises an animal-derived hydrolysate at a concentration of any one of:
 - a. from about 6.0 g/L to about 20.0 g/L
 - b. from about 5.6 g/L to about 38.0 g/L
 - c. from about 7.0 g/L to about 25.0 g/L
 - d. from about 7.0 g/L to about 20.0 g/L
 - e. from about 7.0 g/L to about 15.0 g/L
 - f. from about 8.0 g/L to about 12.0 g/L
 - g. from about 9.0 g/L to about 11.0 g/L
 - h. from about 7.0 g/L to about 11.0 g/L
 - i. about any one of 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L or 50 g/L
 - j. about any one of 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, or 12 g/L
 - k. about 10 g/L
 - l. about 13 g/L
- (x) the cell culture medium (including a medium containing any one or more or all of features (i)-(ix)) comprises a plant-derived hydrolysate at a concentration of any one of:
 - a. from about 1.0 g/L to about 10.0 g/L

- b. from about 1.4 g/L to about 11.0 g/L
- c. from about 1.4 g/L to about 6.2 g/L
- d. from about 1.5 g/L to about 5.5 g/L
- e. from about 1.5 g/L to about 4.5 g/L
- f. from about 1.5 g/L to about 3.5 g/L
- g. from about 2.0 g/L to about 3.0 g/L
- h. from about 1.5 g/L to about 2.5 g/L
- i. from about 1.75 g/L to about 2.75 g/L
- j. from about 2.25 g/L to about 2.75 g/L
- k. about any one of 1.75 g/L, 2.0 g/L, 2.25 g/L, 2.5 g/L, 3.0 g/L, 3.25, 3.5 g/L, 3.75 g/L, or 4.0 g/L
- l. about any one of 2.0 g/L, 2.25 g/L, 2.5 g/L or 3.0 g/L
- m. about 2.5 g/L
- n. about 3.1 g/L

(xi) the cell culture medium (including a medium containing any one or more or all of features (i)-(x)) comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate

(xii) the cell culture medium (including a medium containing any one or more or all of features (i)-(xi)) comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium, wherein the additional amount of insulin may: (a) be added to the cell culture medium once or at least three times or at least six times during the cell culture cycle and (b) may be added in an amount to provide insulin in the cell culture medium at a concentration of any one of:

- a. from about 1.0 mg/L to about 100.0 mg/L
- b. from about 10.0 mg/L to about 100.0 mg/L
- c. from about 10.0 mg/L to about 50.0 mg/L
- d. from about 10.0 mg/L to about 35.0 mg/L
- e. from about 10.0 mg/L to about 25.0 mg/L
- f. from about 5.0 mg/L to about 80.0 mg/L
- g. from about 5.0 mg/L to about 60.0 mg/L

- h. from about 5.0 mg/L to about 50.0 mg/L
- i. from about 5.0 mg/L to about 40.0 mg/L
- j. from about 5.0 mg/L to about 25.0 mg/L
- k. from about 10.0 mg/L to about 25.0 mg/L
- l. from about 10.0 mg/L to about 40.0 mg/L
- m. from about 15.0 mg/L to about 20.0 mg/L
- n. from about 5.0 mg/L to about 15.0 mg/L
- o. from about 6.0 mg/L to about 12.0 mg/L
- p. from about 7.0 mg/L to about 11.0 mg/L
- q. from about 8.0 mg/L to about 10.0 mg/L
- r. about any one of 5.0 mg/L, 6.0 mg/L, 7.0 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L, 11.0 mg/L, 12.0 mg/L, 13.0 mg/L, 14.0 mg/L, 15.0 mg/L, 16.0 mg/L, 17.0 mg/L, 18.0 mg/L, 19.0 mg/L, 20.0 mg/L, 21.0 mg/L, 22.0 mg/L, 23.0 mg/L, 24.0 mg/L, 25.0 mg/L, 26.0 mg/L, 27.0 mg/L, 28.0 mg/L, 29.0 mg/L or 30.0 mg/L or 31.0 mg/L or 32 mg/L or 33 mg/L or 34 mg/L or 35 mg/L or 36 mg/L or 37 mg/L or 38 mg/L or 39 mg/L or 40 mg/L
- s. about any one of 7 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L or 11.0 mg/L
- t. about 25 mg/L
- u. about 15 mg/L

(xiii) the method further comprises the step of adding cysteine to the cell culture medium (including a medium containing any one or more or all of features (i)-(xii)), which cysteine may be added to the cell culture medium (a) as a component of a batch feed that is added to a basal medium that does not comprise cysteine and/or (b) may be added in an amount to provide cysteine in the cell culture medium at a concentration of from about 0.5 to about 5.0 mM or from about 0.5 to about 2.0 mM or from about 0.5 to about 2.0 mM (such as at a concentration of 0.8 mM) or from about 7.0 to about 8.0 mM (such as at a concentration of about 7.5 mM)

(xiv) the method further comprises the step of adding cystine to the cell culture medium (including a medium containing any one or more or all of features (i)-(xii)), which cystine may be added to the cell culture medium as a component of a batch feed that is added to a basal medium and which may be added in an

amount to provide cystine in the cell culture medium at a concentration of from about 0.1 to about 1.5 mM (such as at a concentration of 0.2 mM)

- (xv) the cell is cultured (e.g., in any cell culture media including those having any one or more or all of features (i)-(xiv)) at a temperature ranging from about 28 °C to about 37 °C or from about 31 °C to about 35 °C
- (xvi) the cell is cultured (e.g., in any cell culture media including those having any one or more or all of features (i)-(xiv)) at a first temperature of about 35 °C for a first period of time, is cultured at a second temperature of about 33 °C for a second period of time, and is cultured at a third temperature of about 31 °C for a third period of time
- (xvii) bevacizumab, or a fragment thereof, is secreted into the cell culture medium (including a medium containing any one or more or all of features (i)-(xvi))
- (xviii) the method further comprises the step of recovering the bevacizumab, or a fragment thereof, from the cell culture (including a medium containing any one or more or all of features (i)-(xvi))

[0131] Also provided herein is bevacizumab, or fragment thereof, produced by any method provided herein, including without limitation any of methods 1-4, which method may further comprise any one or more or all of the features (i)-(xviii) or sub-feature thereof or any combination of the foregoing.

[0132] Also provided is a composition comprising: (i) bevacizumab, or a fragment thereof, produced by any method provided herein, including without limitation any of methods 1-4, which method may further comprise any one or more or all of the features (i)-(xviii) or sub-feature thereof or any combination of the foregoing and (ii) a pharmaceutically acceptable carrier.

[0133] A kit for supplementing a cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, is also provided, the kit comprising at least two of components (i)-(iii):

- (i) insulin in an amount to provide a concentration of any one of:
 - a. from about 1.0 mg/L to about 100.0 mg/L
 - b. from about 10.0 mg/L to about 100.0 mg/L
 - c. from about 10.0 mg/L to about 50.0 mg/L
 - d. from about 10.0 mg/L to about 35.0 mg/L

- e. from about 10.0 mg/L to about 25.0 mg/L
- f. from about 5.0 mg/L to about 80.0 mg/L
- g. from about 5.0 mg/L to about 60.0 mg/L
- h. from about 5.0 mg/L to about 50.0 mg/L
- i. from about 5.0 mg/L to about 40.0 mg/L
- j. from about 5.0 mg/L to about 25.0 mg/L
- k. from about 10.0 mg/L to about 25.0 mg/L
- l. from about 10.0 mg/L to about 40.0 mg/L
- m. from about 15.0 mg/L to about 20.0 mg/L
- n. from about 5.0 mg/L to about 15.0 mg/L
- o. from about 6.0 mg/L to about 12.0 mg/L
- p. from about 7.0 mg/L to about 11.0 mg/L
- q. from about 8.0 mg/L to about 10.0 mg/L
- r. about any one of 5.0 mg/L, 6.0 mg/L, 7.0 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L, 11.0 mg/L, 12.0 mg/L, 13.0 mg/L, 14.0 mg/L, 15.0 mg/L, 16.0 mg/L, 17.0 mg/L, 18.0 mg/L, 19.0 mg/L, 20.0 mg/L, 21.0 mg/L, 22.0 mg/L, 23.0 mg/L, 24.0 mg/L, 25.0 mg/L, 26.0 mg/L, 27.0 mg/L, 28.0 mg/L, 29.0 mg/L or 30.0 mg/L or 31.0 mg/L or 32 mg/L or 33 mg/L or 34 mg/L or 35 mg/L or 36 mg/L or 37 mg/L or 38 mg/L or 39 mg/L or 40 mg/L
- s. about any one of 7 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L or 11.0 mg/L.
- t. about 25 mg/L

(ii) cystine in an amount to provide a concentration of any one of:

- a. from about 0.7 mM to about 2.0 mM
- b. from about 0.8 mM to about 2.5 mM
- c. from about 0.8 mM to about 2.0 mM
- d. from about 0.8 mM to about 1.75 mM
- e. from about 0.8 mM to about 1.6 mM
- f. from about 1.0 mM to about 2.0 mM
- g. from about 1.0 mM to about 1.6 mM
- h. from about 1.2 mM to about 1.4 mM

- i. about any one of 0.8 mM or 0.9 mM or 1.0 mM or 1.1 mM or 1.2 mM or 1.3 mM or 1.4 mM or 1.5 mM
- j. about any one of 1.1 mM, 1.3 mM or 1.5 mM

(iii) copper at a concentration of any one of:

- a. from about 69.0 nM to about 1,000.0 nM
- b. from about 69.0 nM to about 400.0 nM
- c. from about 80 nM to about 400 nM.
- d. from about 100 nM to about 400 nM
- e. from about 125 nM to about 400 nM
- f. from about 150 nM to about 400 nM
- g. from about 200 nM to about 400 nM
- h. from about 250 nM to about 400 nM
- i. from about 300 nM to about 400 nM
- j. from about 325 nM to about 375 nM
- k. from about 325 nM to about 350 nM
- l. about any one of 100 nM, 125 nM, 150 nM, 175 nM, 200 nM, 225 nM, 250 nM, 275 nM, 300 nM, 325 nM, 350 nM, 375 nM or 400 nM.
- m. about any one of 330 nM, 335 nM, 340 nM, 345 nM or 350 nM
- n. about 335 nM, 336 nM, 337 nM, 338 nM, 339 nM or 400 nM
- o. about 339 nM

[0134] The kit may further comprise other components, including any one or more of:

- i. an animal-derived hydrolysate, such as in an amount to provide concentration of any one of:
 - a. from about 6.0 g/L to about 20.0 g/L
 - b. from about 5.6 g/L to about 38.0 g/L
 - c. from about 7.0 g/L to about 25.0 g/L
 - d. from about 7.0 g/L to about 20.0 g/L
 - e. from about 7.0 g/L to about 15.0 g/L
 - f. from about 8.0 g/L to about 12.0 g/L
 - g. from about 9.0 g/L to about 11.0 g/L
 - h. from about 7.0 g/L to about 11.0 g/L
 - i. about any one of 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L or 50 g/L
 - j. about any one of 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, or 12 g/L
 - k. about 10 g/L
 - l. about 13 g/L
- ii. plant-derived hydrolysate, such as in an amount to provide a concentration of any one of:
 - a. from about 1.0 g/L to about 10.0 g/L
 - b. from about 1.4 g/L to about 11.0 g/L

- c. from about 1.4 g/L to about 6.2 g/L
- d. from about 1.5 g/L to about 5.5 g/L
- e. from about 1.5 g/L to about 4.5 g/L
- f. from about 1.5 g/L to about 3.5 g/L
- g. from about 2.0 g/L to about 3.0 g/L
- h. from about 1.5 g/L to about 2.5 g/L
- i. from about 1.75 g/L to about 2.75 g/L
- j. from about 2.25 g/L to about 2.75 g/L
- k. about any one of 1.75 g/L, 2.0 g/L, 2.25 g/L, 2.5 g/L, 3.0 g/L, 3.25, 3.5 g/L, 3.75 g/L, or 4.0 g/L
- l. about any one of 2.0 g/L, 2.25 g/L, 2.5 g/L or 3.0 g/L
- m. about 2.5 g/L
- n. about 3.1 g/L

[0135] A cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, is also provided, the cell culture medium comprising at least two of components (i)-(iii):

- (i) insulin in an amount to provide a concentration of any one of:
 - a. from about 1.0 mg/L to about 100.0 mg/L
 - b. from about 10.0 mg/L to about 100.0 mg/L
 - c. from about 10.0 mg/L to about 50.0 mg/L
 - d. from about 10.0 mg/L to about 35.0 mg/L
 - e. from about 10.0 mg/L to about 25.0 mg/L
 - f. from about 5.0 mg/L to about 80.0 mg/L
 - g. from about 5.0 mg/L to about 60.0 mg/L
 - h. from about 5.0 mg/L to about 50.0 mg/L
 - i. from about 5.0 mg/L to about 40.0 mg/L
 - j. from about 5.0 mg/L to about 25.0 mg/L
 - k. from about 10.0 mg/L to about 25.0 mg/L
 - l. from about 10.0 mg/L to about 40.0 mg/L
 - m. from about 15.0 mg/L to about 20.0 mg/L
 - n. from about 5.0 mg/L to about 15.0 mg/L
 - o. from about 6.0 mg/L to about 12.0 mg/L
 - p. from about 7.0 mg/L to about 11.0 mg/L
 - q. from about 8.0 mg/L to about 10.0 mg/L

- r. about any one of 5.0 mg/L, 6.0 mg/L, 7.0 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L, 11.0 mg/L, 12.0 mg/L, 13.0 mg/L, 14.0 mg/L, 15.0 mg/L, 16.0 mg/L, 17.0 mg/L, 18.0 mg/L, 19.0 mg/L, 20.0 mg/L, 21.0 mg/L, 22.0 mg/L, 23.0 mg/L, 24.0 mg/L, 25.0 mg/L, 26.0 mg/L, 27.0 mg/L, 28.0 mg/L, 29.0 mg/L or 30.0 mg/L or 31.0 mg/L or 32 mg/L or 33 mg/L or 34 mg/L or 35 mg/L or 36 mg/L or 37 mg/L or 38 mg/L or 39 mg/L or 40 mg/L
- s. about any one of 7 mg/L, 8.0 mg/L, 9.0 mg/L, 10.0 mg/L or 11.0 mg/L
- t. about 25 mg/L

(ii) cystine in an amount to provide a concentration of any one of:

- a. from about 0.7 mM to about 2.0 mM
- b. from about 0.8 mM to about 2.5 mM
- c. from about 0.8 mM to about 2.0 mM
- d. from about 0.8 mM to about 1.75 mM
- e. from about 0.8 mM to about 1.6 mM
- f. from about 1.0 mM to about 2.0 mM
- g. from about 1.0 mM to about 1.6 mM
- h. from about 1.2 mM to about 1.4 mM
- i. about any one of 0.8 mM or 0.9 mM or 1.0 mM or 1.1 mM or 1.2 mM or 1.3 mM or 1.4 mM or 1.5 mM
- j. about any one of 1.1 mM, 1.3 mM or 1.5 mM

(iii) copper at a concentration of any one of:

- a. from about 69.0 nM to about 1,000.0 nM
- b. from about 69.0 nM to about 400.0 nM
- c. from about 80 nM to about 400 nM.
- d. from about 100 nM to about 400 nM
- e. from about 125 nM to about 400 nM
- f. from about 150 nM to about 400 nM
- g. from about 200 nM to about 400 nM
- h. from about 250 nM to about 400 nM
- i. from about 300 nM to about 400 nM
- j. from about 325 nM to about 375 nM
- k. from about 325 nM to about 350 nM

1. about any one of 100 nM, 125 nM, 150 nM, 175 nM, 200 nM, 225 nM, 250 nM, 275 nM, 300 nM, 325 nM, 350 nM, 375 nM or 400 nM.
- m. about any one of 330 nM, 335 nM, 340 nM, 345 nM or 350 nM
- n. about 335 nM, 336 nM, 337 nM, 338 nM, 339 nM or 400 nM
- o. about 339 nM

[0136] The cell culture medium may comprise other components, including any one or more of:

- 1) an animal-derived hydrolysate, such as in an amount to provide concentration of any one of:
 - a. from about 6.0 g/L to about 20.0 g/L
 - b. from about 5.6 g/L to about 25.0 g/L
 - c. from about 7.0 g/L to about 25.0 g/L
 - d. from about 7.0 g/L to about 20.0 g/L
 - e. from about 7.0 g/L to about 15.0 g/L
 - f. from about 8.0 g/L to about 12.0 g/L
 - g. from about 9.0 g/L to about 11.0 g/L
 - h. from about 7.0 g/L to about 11.0 g/L
 - i. about any one of 5 g/L, 10 g/L, 15 g/L, 20 g/L or 25 g/L
 - j. about any one of 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, or 12 g/L
 - k. about 10 g/L
 - l. about 13 g/L
- 2) plant-derived hydrolysate, such as in an amount to provide a concentration of any one of:
 - a. from about 1.0 g/L to about 10.0 g/L
 - b. from about 1.4 g/L to about 11.0 g/L
 - c. from about 1.4 g/L to about 6.2 g/L
 - d. from about 1.5 g/L to about 5.5 g/L
 - e. from about 1.5 g/L to about 4.5 g/L
 - f. from about 1.5 g/L to about 3.5 g/L
 - g. from about 2.0 g/L to about 3.0 g/L
 - h. from about 1.5 g/L to about 2.5 g/L
 - i. from about 1.75 g/L to about 2.75 g/L
 - j. from about 2.25 g/L to about 2.75 g/L

- k. about any one of 1.75 g/L, 2.0 g/L, 2.25 g/L, 2.5 g/L, 3.0 g/L, 3.25, 3.5 g/L, 3.75 g/L, or 4.0 g/L
- l. about any one of 2.0 g/L, 2.25 g/L, 2.5 g/L or 3.0 g/L
- m. about 2.5 g/L
- n. about 3.1 g/L

[0137] The cell culture medium may further be supplemented with additional cell culture medium components, where the additional cell culture medium components may comprise, e.g., insulin and/or cysteine, such as insulin in an amount to provide any concentration of insulin provided herein, including the concentrations listed in the present embodiments (such as 15 mg/L) and/or cysteine in an amount to provide any concentration of cysteine provided herein (such as 0.8 mM), including the concentrations listed in the present embodiments.

[0138] The cell culture medium may further be supplemented with additional cell culture medium components, where the additional cell culture medium components may comprise, e.g., insulin and/or cysteine and/or cystine, such as insulin in an amount to provide any concentration of insulin provided herein, including the concentrations listed in the present embodiments (such as 15 mg/L) and/or cysteine in an amount to provide any concentration of cysteine provided herein (such as 0.8 mM), including the concentrations listed in the present embodiments and/or cystine in an amount to provide any concentration of cystine provided herein (such as 0.2 mM), including the concentrations listed in the present embodiments.

[0139] Also provided herein is a composition comprising (a) a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof; and (b) a cell culture medium detailed herein, including without limitation a cell culture medium provided in the present embodiments. Further provided is a composition comprising: (a) bevacizumab, or a fragment thereof; and (b) a cell culture medium detailed herein, including without limitation a cell culture medium provided in the present embodiments.

[0140] Also provided herein is a method of producing bevacizumab or a fragment thereof, comprising a step of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab or a fragment thereof in a cell culture medium, wherein initial cell culture medium in a cell culture cycle comprises two or more components selected from the group consisting of copper at a concentration of from about 69 nM to about 1,000 nM, insulin at a concentration of from about 1.0 mg/L to about 100.0 mg/L, and cystine at a concentration of from about 0.7 mM to about 2.0 mM, and wherein the cell produces bevacizumab or the fragment.

[0141] In some embodiments, without limitation, the method may comprise any one or more of the following features (i)-(xxiii) or sub-feature thereof or any combination of feature or sub feature:

- (i) the initial cell culture medium comprises copper and insulin
- (ii) the initial cell culture medium comprises copper and cystine
- (iii) the initial cell culture medium comprises insulin and cystine
- (iv) the initial cell culture medium comprises copper, insulin, and cystine
- (v) the initial cell culture medium (including a medium containing any one or more or all of features (i)-(iv)) comprises insulin at a concentration of any one of:
 - a. from about 10.0 mg/L to about 50.0 mg/L
 - b. from about 10.0 mg/L to about 20.0 mg/L
 - c. about any one of 10.0 mg/L, 15.0 mg/L, 20.0 mg/L, and 25.0 mg/L
- (vi) the initial cell culture medium (including a medium containing any one or more or all of features (i)-(v)) comprises copper at a concentration of any one of:
 - a. from about 325 nM to about 375 nM
 - b. from about 325 nM to about 350 nM
 - c. about any one of 330 nM, 335 nM, 339 nM, 340 nM, 345 nM and 350 nM
- (vii) the initial cell culture medium (including a medium containing any one or more or all of features (i)-(vi)) comprises cystine at a concentration of any one of:
 - a. from about 0.7 mM to about 2.0 mM
 - b. from about 1.0 mM to about 1.6 mM
 - c. about any one of 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM 1.4 mM, 1.5 mM and 1.6 mM
- (viii) the initial cell culture medium (including a medium containing any of features (i)-(vii)) further comprises a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate

- (ix) the initial cell culture medium (including a medium containing any one or more or all of features (i)-(viii)) comprises an animal-derived hydrolysate at a concentration of any one of:
 - a. from about 6.0 g/L to about 20.0 g/L
 - b. from about 8.0 g/L to about 12.0 g/L
 - c. from about 9.0 g/L to about 11.0 g/L
 - d. about 13 g/L
- (x) the initial cell culture medium (including a medium containing any one or more or all of features (i)-(ix)) comprises a plant-derived hydrolysate at a concentration of any one of:
 - a. from about 1.0 g/L to about 10.0 g/L
 - b. from about 2.0 g/L to about 3.0 g/L
 - c. from about 2.25 g/L to about 2.75 g/L
 - d. about 2.5 g/L
- (xi) the initial cell culture medium (including a medium containing any one or more or all of features (i)-(x)) comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate
- (xii) the initial cell culture medium (including a medium containing any one or more or all of features (i)-(xi)) comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium during the cell culture cycle, and optionally wherein a) the additional amount of insulin may be added to the cell culture medium at least once, at least twice, at least three times, at least four times, at least five times, or at least six times during the cell culture cycle; and/or b) the insulin added each time is any one of: from about 5.0 mg/L to about 25.0 mg/L or about any one of 5.0 mg/L, 10.0 mg/L, 15.0 mg/L, 20.0 mg/L, and 25.0 mg/L; and/or c) the cumulative amount of insulin added during the cell culture cycle is any one of: from about 20.0 mg/L to about 100.0 mg/L or about any one of 20.0 mg/L, 25.0 mg/L, 30 mg/L, 35 mg/L, 40 mg/L, 45 mg/L, 50 mg/L, 55 mg/L, 60 mg/L, 65 mg/L, 70 mg/L, 75 mg/L, 80 mg/L and 85 mg/L
- (xiii) the initial cell culture medium (including a medium containing any one or more or all of features (i)-(xii)) comprises cystine and the method further comprises a step of adding an additional amount of cystine to the cell culture

medium during the cell culture cycle, and optionally wherein a) cystine is added in an amount to provide any one of: from about 0.1 mM to about 1.5 mM additional cystine in the cell culture medium or from about 0.4 mM to about 0.7 mM (e.g., about 0.4 mM to about 0.6 mM, about 0.4 mM to about 0.5 mM) additional cystine in the cell culture medium; and/or b) the cystine is added in a batch feed during the cell culture cycle

(xiv) the method further comprises the step of adding any one of: at least one batch feed during the cell culture cycle, or two, three, or four batch feeds during the cell culture cycle; and optionally wherein the batch feed medium may comprise a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate

(xv) during the cell culture cycle, the temperature of the medium (including a medium containing any one or more or all of features (i)-(xiv)) is reduced by at least 2, at least about 3, at least about 4, or at least about 5 degrees C relative to the temperature at the beginning of the culturing

(xvi) during the cell culture cycle, the temperature of the medium (including a medium containing any one or more or all of features (i)-(xv)) is a) reduced at least once or at least twice during the cell culture cycle; and/or b) wherein the temperature is reduced on day 8 and day 10 after the beginning of the culturing

(xvii) the cell is cultured (e.g., in any cell culture media including those having any one or more or all of features (i)-(xvi)) at a temperature ranging from about 31°C to about 35°C

(xviii) the cell is cultured (e.g., in any cell culture media including those having any one or more or all of features (i)-(xvii)) at a first temperature of about 35 °C for a first period of time, is cultured at a second temperature of about 33 °C for a second period of time, and is cultured at a third temperature of about 31 °C for a third period of time

(xix) the cell is cultured in the medium (e.g., in any cell culture media including those having any one or more or all of features (i)-(xviii)) having a pH at about 7.0 to about 7.3

(xx) the method comprises (a) culturing the cell in an initial cell culture medium comprising about 10 mg/L insulin, about 325 nM to about 350 nM copper, and about 1.3 mM cystine; (b) providing a first batch feed and an insulin feed to

the cell culture medium to provide additional insulin at a concentration of about 15 mg/L on day 3 after the beginning of the culturing; and (c) providing a second batch feed comprising cystine to the cell culture medium to provide additional cystine at a concentration of about 0.4 mM to about 0.7 mM on day 6 after the beginning of the culturing; wherein the cell is cultured at an initial temperature of about 35°C, and the temperature is reduced to about 33°C on day 8 and is further reduced to about 31°C on day 10 after the beginning of the culturing

- (xxi) bevacizumab, or a fragment thereof, is secreted into the cell culture medium (including a medium containing any one or more or all of features (i)-(xx))
- (xxii) the method further comprises the step of recovering the bevacizumab, or a fragment thereof, from the cell culture (including a medium containing any one or more or all of features (i)-(xxi))
- (xxiii) the mammalian cell is a Chinese hamster ovary cell

[0142] Also provided herein is bevacizumab, or fragment thereof, produced by any method provided herein, which method may further comprise any one or more or all of the features (i)-(xxiii) or sub-feature thereof or any combination of the foregoing.

[0143] Also provided is a composition comprising: (i) bevacizumab, or a fragment thereof, produced by any method provided herein, which method may further comprise any one or more or all of the features (i)-(xxiii) or sub-feature thereof or any combination of the foregoing and (ii) a pharmaceutically acceptable carrier.

[0144] The following Examples are provided to illustrate but not to limit the invention.

EXAMPLES

Example 1: Impact of Cell Culture Medium Components on Amount of Bevacizumab Produced by a Mammalian Cell Line.

[0145] Chinese hamster ovary (CHO) cells producing bevacizumab were cultured in cell culture media containing various amounts of insulin, and the impact of insulin on the amount of bevacizumab produced was assessed. Production of bevacizumab was initiated in cell culture by inoculating cells in basal medium containing 339 nM copper, 1% animal hydrolysate and 0.25% plant hydrolysate and a batch feed medium was added on day 3 over a 14 day cell culture cycle in a bioreactor. The basal cell culture medium of a control cell culture was supplemented with less than 10 mg/L of insulin and no additional insulin was given during the cell culture cycle. In two representative experimental cases (case 1 and case

2), the insulin level in the basal cell culture media was 10 mg/L. Additional insulin was added three to six times during the cell culture cycle so as to provide cell culture case 1 and cell culture case 2 with a final amount of 25 mg/L and 40 mg/L insulin at the end of the 14 day cell culture cycle, respectively (Table A). The cells were cultured at 37°C on day 1 and the temperature was subsequently shifted down to 35°C on day 1, 33°C on day 8, and 31°C on day 10. Titer improvement was quantified by percent increase over the control. Total addition of insulin during the cell culture cycle from less than 10 mg/L to 25 mg/L or 40 mg/L led to a titer improvement of about 16% or about 18% as compared with the control, respectively (Table A).

Table A. Exemplary insulin addition protocol and results

	Insulin addition case 1	Insulin addition case 2
Total insulin concentration added during a 14 day duration	25 mg/L	40 mg/L
Titer improvement percent increase	16 %	18 %

[0146] CHO cells producing bevacizumab were cultured in cell culture media containing different amounts of hydrolysate from different sources and the impact of the specific hydrolysate on the amount of bevacizumab produced was assessed. In two representative protocols, production of bevacizumab was initiated in cell culture by inoculating cells in basal cell culture medium containing 339 nM copper and a batch feed medium was added on day 3 over a 14 day cell culture cycle in a bioreactor. The basal cell culture medium for Protocol 1 had 1% animal-derived hydrolysate without plant-derived hydrolysate while Protocol 2 had basal cell culture medium supplemented with 0.75% animal-derived hydrolysate in combination with 0.25% plant-derived hydrolysate (Table B). Analysis of the antibody titer produced by the two protocols demonstrated that Protocol 2 provided a 27% increase in the amount of bevacizumab produced from the cells as compared to the amount of bevacizumab produced by cells cultured using Protocol 1 (Table B).

Table B. Exemplary experimental protocols

	Protocol 1	Protocol 2
Porcine peptone	1%	0.75%
Plant peptone	None	0.25%
Titer improvement percent increase	Not Applicable	27%

Example 2: Impact of Cell Culture Medium Components on Amount of Bevacizumab Produced by a Mammalian Cell Line.

[0147] CHO cells producing bevacizumab were cultured in basal media with either the amino acid cysteine in the monomer form (Cys, cysteine) or in the dimer form (Cys-Cys, cystine) (Table C). Production of bevacizumab was initiated in cell culture by inoculating cells in basal medium containing 339 nM copper, 1% animal hydrolysate and 0.25% plant hydrolysate and a batch feed was added on day 3 over a 14 day cell culture cycle in a bioreactor. Insulin levels in the basal media were at a concentration of either less than 10 mg/L or 10 mg/L. Two of the experimental cell cultures (Cysteine + Insulin and Cystine + Insulin) had additional insulin added three times or six times during the cell culture cycle so as to provide a final amount of 25 mg/L or 40 mg/L (Table C). The cells were cultured at 37°C on day 1 the temperature was subsequently shifted down to 35°C on day 1, 33°C on day 8, and 31°C on day 10. Titer improvement was quantified by percent increase over the control. Replacement of cysteine (Cys) by cystine (Cys-Cys) improved titer by 11% over the control. Impact of insulin addition was also observed in basal media made using cystine (Cys-Cys).

Table C. Summary of protocols and results

	Cysteine (Control)	Cystine	Cysteine+Insulin	Cystine+Insulin
Cysteine (Cys) concentration in basal media	2.6 mM	0 mM	2.6 mM	0 mM
Cystine (Cys-Cys) concentration in basal media	0 mM	1.3 mM	0 mM	1.3 mM
Total insulin concentration added during a 14 day duration	Less than 10 mg/L	Less than 10 mg/L	25 mg/L	40 mg/L
Titer improvement percent increase	Not Applicable	11%	11%	14%

Example 3: Impact of Insulin Addition on Amount of Bevacizumab Produced by a Mammalian Cell Line.

[0148] Chinese hamster ovary (CHO) cells producing bevacizumab were cultured in cell culture media containing various amounts of insulin, and the impact of insulin addition on the amount of bevacizumab produced was assessed. Production of bevacizumab was initiated in cell culture by inoculating cells in basal medium. After inoculation, the medium (*i.e.*, initial cell culture medium) contained 300 nM copper sulfate (CuSO₄), 1% animal hydrolysate and 0.25% plant hydrolysate, and 1.3 mM cystine. The medium also contained 10 mg/L or 20 mg/L of insulin after inoculation (Table D). A batch feed medium was added on day 3 or a two batch feed mediums were added (one batch feed on day 3 and a second batch feed on day 6) over a 14 day cell culture cycle in a bioreactor. Some cell cultures received additional insulin one to six times during the cell culture cycle so as to provide the cell culture with a cumulative amount of 25 mg/L to 85 mg/L insulin at the end of the 14 day cell culture cycle (Table D). The cells were cultured at 37°C on day 1 and the temperature was subsequently shifted down to 35°C on day 1, 33°C on day 8, and 31°C on day 10. Titer improvement was compared across the different cultures in two representative experiments and it was determined that 10 mg/L insulin in the initial cell culture medium with addition of insulin on

day 3 for a cumulative amount of 25 mg/L insulin produced the highest titer yield (FIG. 1A (one batch feed) and 1B (two batch feeds)). Cell cultures containing 20 mg/mL insulin in the initial cell culture medium with no additional insulin supplementation demonstrated comparable titer yields to cultures that had 10 mg/mL insulin in the initial cell culture medium and additional insulin added during the cell culture cycle indicating that insulin in the initial cell culture medium was an important factor for increasing titer yield (Table D, FIG. 1A and 1B).

Table D. Exemplary insulin addition protocol

Protocol	Insulin in initial cell culture medium	Insulin feed	Day of insulin feed	Cumulative insulin in cell culture at 14 days
1	20 mg/L	None	None	20 mg/L
2	10 mg/L	15 mg/L	Day 3	25 mg/L
3	10 mg/L	5 mg/L	Days 3, 6, and 9	25 mg/L
4	10 mg/L	5 mg/L	Days 3, 5, 7, 9, 11, and 13	40 mg/L
5	10 mg/L	15 mg/L	Days 3, 6, and 9	55 mg/L
6	10 mg/L	25 mg/L	Days 3, 6, and 9	85 mg/L

Example 4: Impact of Additional Batch Media Feed on Amount of Bevacizumab Produced by a Mammalian Cell Line.

[0149] Four different samples of CHO cells producing bevacizumab were cultured in cell culture media and fed with one or two batch media feeds to determine the impact on the amount of bevacizumab produced. Production of bevacizumab was initiated in cell culture by inoculating cells in basal medium. After inoculation, the medium (i.e., initial cell culture medium) contained 300 nM copper sulfate (CuSO₄), 1% animal hydrolysate and 0.25% plant hydrolysate, and 1.3 mM cystine. In one protocol, a batch feed medium was added on day 3 over a 14 day cell culture cycle in a bioreactor. In a second protocol, a batch feed medium was added on day 3 and day 6 over a 14 day cell culture cycle in a bioreactor (Table E). The batch feed medium delivered on day 3 contained 2.5% animal hydrolysate and 0.625% plant hydrolysate and was added into the cell culture in a volume of 125 ml/L. The batch feed

medium delivered on day 6 contained 7.5 mM cysteine and 7.5 mM cystine and was added into the cell culture in a volume of 90 mL/L. Accordingly, the cell culture had an increase of 0.62 mM in cystine concentration after the feed. The cells were cultured at 37°C on day 1 and the temperature was subsequently shifted down to 35°C on day 1, 33°C on day 8, and 31°C on day 10. Titer improvement was observed in cell cultures receiving a second feed on day 6 with cultures receiving only one feed demonstrating an average titer, and the two-feed process led to a titer increase of up to 0.3 g/L (about 20% titer increase). Measurement of biomass accumulation (FIG. 2A) and titer (FIG. 2B) at Day 14 indicated that a second feed on day 6 improved yield and that this second feed was more significant for cultures with higher cell ages where higher growth and an earlier decline in viability is observed.

Table E. Exemplary batch feed addition protocol

Media	Protocol 1	Protocol 2
Initial cell culture medium	1% Animal hydrolysate 0.25% Plant hydrolysate 1.3 mM Cystine 10 mg/L Insulin 300 nM CuSO ₄	1% Animal hydrolysate 0.25% Plant hydrolysate 1.3 mM Cystine 10 mg/L Insulin 300 nM CuSO ₄
Batch Feed on Day 3	2.5% Animal hydrolysate 0.625% Plant hydrolysate (provided as 125 mL/L)	2.5% Animal hydrolysate 0.625% Plant hydrolysate (provided as 125 mL/L)
Batch Feed on Day 6	None	7.5 mM Cysteine 7.5 mM Cystine (provided as 90 mL/L)

[0150] Analysis of the above described results provided for the development of a new process for culturing CHO cells producing bevacizumab. The new process incorporated the use of an initial cell culture medium containing 10 mg/L insulin as well as cystine instead of cysteine, a batch feed on day 3 and day 6 during cell culture and the addition of insulin on day 3 of cell culture (Table F, Protocol 2). Addition of these components to the cell culture process resulted in a titer increase up to about 60%. See FIG. 3 and Table F, Protocol 2 as compared Protocol 1.

Table F. New cell culture process

Media	Protocol 1	Protocol 2
Initial cell culture medium	1% Animal hydrolysate 2.6 mM Cysteine 2 mg/L Insulin	1% Animal hydrolysate 0.25% Plant hydrolysate 1.3 mM Cystine 10 mg/L Insulin 300 nM CuSO ₄
Batch Feed on Day 3	2.5% Animal hydrolysate (provided as 125 mL/L)	2.5% Animal hydrolysate 0.625% Plant hydrolysate (provided as 125 mL/L)
Insulin Feed on Day 3	None	15 mg/L Insulin
Batch Feed on Day 6	None	7.5 mM Cysteine 7.5 mM Cystine (provided as 90 mL/L)
Temperature	33°C	35°C on day 1, 33°C on day 8, and 31°C on day 10

CLAIMS

What is claimed is:

1. A method of producing bevacizumab, or a fragment thereof, comprising the step of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab or fragment thereof in a cell culture medium, wherein the cell culture medium comprises two or more components selected from the group consisting of copper, insulin, and cystine, and wherein the cell produces bevacizumab, or a fragment thereof.
2. The method of claim 1, wherein the cell culture medium comprises copper and insulin.
3. The method of claim 1, wherein the cell culture medium comprises copper and cystine.
4. The method of claim 1, wherein the cell culture medium comprises insulin and cystine.
5. The method of claim 1, wherein the cell culture medium comprises copper, insulin, and cystine.
6. The method of any one of claims 1-5, wherein the cell culture medium further comprises a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate.
7. The method of any one of claims 1-6, wherein the cell culture medium comprises insulin at a concentration of from about 1.0 mg/L to about 100.0 mg/L.
8. The method of any one of claims 1-6, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 100.0 mg/L.
9. The method of any one of claims 1-6, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 50.0 mg/L.
10. The method of any one of claims 1-6, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 35.0 mg/L.
11. The method of any one of claims 1-6, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 25.0 mg/L.
12. The method of any one of claims 1-6, wherein the cell culture medium comprises insulin at a concentration of about 25 mg/L.
13. The method of any one of claims 1-12, wherein the cell culture medium comprises copper at a concentration of from about 69 nM to about 1,000 nM.

14. The method of any one of claims 1-12, wherein the cell culture medium comprises copper at a concentration of from about 325 nM to about 375 nM.
15. The method of any one of claims 1-12, wherein the cell culture medium comprises copper at a concentration of from about 325 nM to about 350 nM.
16. The method of any one of claims 1-12, wherein the cell culture medium comprises copper at a concentration of about any one of 330 nM, 335 nM, 339 nM, 340 nM, 345 nM or 350 nM.
17. The method of any one of claims 1-12, wherein the cell culture medium comprises copper at a concentration of about 339 nM.
18. The method of any one of claims 1-17, wherein the cell culture medium comprises cystine at a concentration of from about 0.7 mM to about 2.0 mM.
19. The method of any one of claims 1-17, wherein the cell culture medium comprises cystine at a concentration of from about 1.0 mM to about 1.6 mM.
20. The method of any one of claims 1-17, wherein the cell culture medium comprises cystine at a concentration of from about 1.2 mM to about 1.4 mM
21. The method of any one of claims 1-17, wherein the cell culture medium comprises cystine at a concentration of about 1.3 mM.
22. The method of any one of claims 1-21, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 6.0 g/L to about 20.0 g/L.
23. The method of any one of claims 1-21, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 8.0 g/L to about 12.0 g/L.
24. The method of any one of claims 1-21, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 9.0 g/L to about 11.0 g/L.
25. The method of any one of claims 1-21, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of about 13 g/L.
26. The method of any one of claims 1-25, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 1.0 g/L to about 10.0 g/L.
27. The method of any one of claims 1-25, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.0 g/L to about 3.0 g/L.

28. The method of any one of claims 1-25, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.25 g/L to about 2.75 g/L
29. The method of any one of claims 1-25, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of about 3.1 g/L.
30. The method of any one of claims 1-29, wherein the cell culture medium comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate.
31. The method of any one of claims 1-30, wherein the cell culture medium comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium.
32. The method of claim 31, wherein the additional amount of insulin is added to the cell culture medium once during the cell culture cycle.
33. The method of claim 31, wherein the additional amount of insulin is added to the cell culture medium at least three times during the cell culture cycle.
34. The method of claim 31, wherein the additional amount of insulin is added to the cell culture medium at least six times during the cell culture cycle.
35. The method of any one of claims 31-34, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 1.0 mg/L to about 100.0 mg/L.
36. The method of any one of claims 31-34, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 100.0 mg/L.
37. The method of any one of claims 31-34, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 50.0 mg/L.

38. The method of any one of claims 31-34, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 35.0 mg/L.
39. The method of any one of claims 31-34, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 25.0 mg/L.
40. The method of any one of claims 31-34, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of about 15 mg/L.
41. The method of any one of claims 1-40, wherein the method further comprises the step of adding cysteine to the cell culture medium.
42. The method of claim 41, wherein cysteine is added in an amount to provide from about 0.5 to about 2.0 mM cysteine in the cell culture medium.
43. The method of claim 41, wherein cysteine is added in an amount to provide about 0.8 mM cysteine in the cell culture medium.
44. The method of any one of claims 1-43, wherein the method further comprises the step of adding cystine to the cell culture medium.
45. The method of claim 44, wherein cystine is added in an amount to provide from about 0.1 to about 1.5 mM cystine in the cell culture medium.
46. The method of claim 44, wherein cystine is added in an amount to provide about 0.2 mM cystine in the cell culture medium.
47. The method of any one of claims 1-46, wherein the cell is cultured at a temperature ranging from about 28 °C to about 37 °C.
48. The method of claim 47, wherein the cell is cultured at a temperature ranging from about 31 °C to about 35 °C.
49. The method of any one of claims 1-46, wherein the cell is cultured at a first temperature of about 35 °C for a first period of time, is cultured at a second temperature of about 33 °C for a second period of time, and is cultured at a third temperature of about 31 °C for a third period of time.
50. The method of any one of claims 1-49, wherein bevacizumab, or a fragment thereof, is secreted into the cell culture medium.
51. The method of any one of claims 1-50, further comprising the step of recovering the bevacizumab, or a fragment thereof, from the cell culture.

52. Bevacizumab, or fragment thereof, produced by the method of any one of claims 1-51.
53. A composition comprising: (i) bevacizumab, or a fragment thereof, produced by the method of any one of claims 1-51 and (ii) a pharmaceutically acceptable carrier.
54. A method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the method comprising the step of contacting the mammalian cell with a cell culture medium comprising two or more components selected from the group consisting of copper, insulin and cystine.
55. The method of claim 54, wherein the cell culture medium comprises copper and insulin.
56. The method of claim 54, wherein the cell culture medium comprises copper and cystine.
57. The method of claim 54, wherein the cell culture medium comprises insulin and cystine.
58. The method of claim 54, wherein the cell culture medium comprises copper, insulin, and cystine.
59. The method of any one of claims 54-58, wherein the cell culture medium further comprises a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate.
60. The method of any one of claims 54-59, wherein the cell culture medium comprises insulin at a concentration of from about 1.0 mg/L to about 100.0 mg/L.
61. The method of any one of claims 54-59, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 100.0 mg/L.
62. The method of any one of claims 54-59, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 50.0 mg/L.
63. The method of any one of claims 54-59, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 35.0 mg/L.
64. The method of any one of claims 54-59, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 25.0 mg/L.
65. The method of any one of claims 54-59, wherein the cell culture medium comprises insulin at a concentration of about 25 mg/L.
66. The method of any one of claims 54-65, wherein the cell culture medium comprises copper at a concentration of from about 69 nM to about 1,000 nM.

67. The method of any one of claims 54-65, wherein the cell culture medium comprises copper at a concentration of from about 325 nM to about 375 nM.
68. The method of any one of claims 54-65, wherein the cell culture medium comprises copper at a concentration of from about 325 nM to about 350 nM.
69. The method of any one of claims 54-65, wherein the cell culture medium comprises copper at a concentration of about any one of 330 nM, 335 nM, 339 nM, 340 nM, 345 nM or 350 nM.
70. The method of any one of claims 54-65, wherein the cell culture medium comprises copper at a concentration of about 339 nM.
71. The method of any one of claims 54-70, wherein the cell culture medium comprises cystine at a concentration of from about 0.7 mM to about 2.0 mM.
72. The method of any one of claims 54-70, wherein the cell culture medium comprises cystine at a concentration of from about 1.0 mM to about 1.6 mM.
73. The method of any one of claims 54-70, wherein the cell culture medium comprises cystine at a concentration of from about 1.2 mM to about 1.4 mM
74. The method of any one of claims 54-70, wherein the cell culture medium comprises cystine at a concentration of about 1.3 mM.
75. The method of any one of claims 54-74, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 6.0 g/L to about 20.0 g/L.
76. The method of any one of claims 54-74, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 8.0 g/L to about 12.0 g/L.
77. The method of any one of claims 54-74, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 9.0 g/L to about 11.0 g/L.
78. The method of any one of claims 54-74, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of about 13 g/L.
79. The method of any one of claims 54-78, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 1.0 g/L to about 10.0 g/L.
80. The method of any one of claims 54-78, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.0 g/L to about 3.0 g/L.

81. The method of any one of claims 54-78, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.25 g/L to about 2.75 g/L
82. The method of any one of claims 54-78, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of about 3.1 g/L.
83. The method of any one of claims 54-82, wherein the cell culture medium comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate.
84. The method of any one of claims 54-83, wherein the cell culture medium comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium.
85. The method of claim 84, wherein the additional amount of insulin is added to the cell culture medium once during the cell culture cycle.
86. The method of claim 84, wherein the additional amount of insulin is added to the cell culture medium at least three times during the cell culture cycle.
87. The method of claim 84, wherein the additional amount of insulin is added to the cell culture medium at least six times during the cell culture cycle.
88. The method of any one of claims 84-87, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 1.0 mg/L to about 100.0 mg/L.
89. The method of any one of claims 84-87, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 100.0 mg/L.
90. The method of any one of claims 84-87, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 50.0 mg/L.
91. The method of any one of claims 84-87, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 35.0 mg/L.
92. The method of any one of claims 84-87, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 25.0 mg/L.

93. The method of any one of claims 84-87, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of about 15 mg/L.
94. The method of any one of claims 54-93, wherein the method further comprises the step of adding cysteine to the cell culture medium.
95. The method of claim 94, wherein cysteine is added in an amount to provide from about 0.5 to about 2.0 mM cysteine in the cell culture medium.
96. The method of claim 94, wherein cysteine is added in an amount to provide about 0.8 mM cysteine in the cell culture medium.
97. The method of any one of claims 54-96, wherein the method further comprises the step of adding cystine to the cell culture medium.
98. The method of claim 97, wherein cystine is added in an amount to provide from about 0.1 to about 1.5 mM cystine in the cell culture medium.
99. The method of claim 97, wherein cystine is added in an amount to provide about 0.2 mM cystine in the cell culture medium.
100. The method of any one of claims 54-99, wherein the cell culture medium comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate.
101. The method of any one of claims 54-100, wherein the cell is cultured at a temperature ranging from about 28 °C to about 37 °C.
102. The method of claim 101, wherein the cell is cultured at a temperature ranging from about 28 °C to about 35 °C.
103. The method of any one of claims 54-100, wherein the cell is cultured at a first temperature of about 35 °C for a first period of time, is cultured at a second temperature of about 33 °C for a second period of time, and is cultured at a third temperature of about 31 °C for a third period of time.
104. The method of any one of claims 54-103, wherein bevacizumab, or a fragment thereof, is secreted into the cell culture medium.
105. The method of any one of claims 54-104, wherein the mammalian cell is contacted with the cell culture medium during the cell's growth phase.
106. The method of any one of claims 54-105, wherein the mammalian cell is contacted with the cell culture medium during the cell's production phase.

107. A kit for supplementing a cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the kit comprising at least two of components (i)-(iii): (i) insulin in an amount to provide from about 1.0 mg/L to about 100.0 mg/L insulin in the cell culture medium; (ii) cystine in an amount to provide from about 0.7 mM to about 2.0 mM cystine in the cell culture medium; (iii) and copper in an amount to provide from about 69.0 nM to about 1,000.0 nM copper in the cell culture medium.
108. The kit of claim 107, wherein the kit further comprises a plant-derived hydrolysate.
109. The kit of claim 108, wherein the kit comprises the plant-derived hydrolysate in an amount to provide from about 1.0 g/L to about 10.0 g/L plant-derived hydrolysate in the cell culture medium.
110. The kit of any one of claims 107-109, wherein the kit further comprises an animal-derived hydrolysate.
111. The kit of claim 110, wherein the kit comprises the animal-derived hydrolysate in an amount to provide from about 6.0 g/L to about 20.0 g/L animal-derived hydrolysate in the cell culture medium.
112. A cell culture medium for use in culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the cell culture medium comprising at least two of components (i)-(iii): (i) from about 1.0 mg/L to about 100.0 mg/L insulin; (ii) from about 69.0 nM to about 1,000.0 nM copper; and (iii) from about 0.7 mM to about 2.0 mM cystine.
113. The cell culture medium of claim 112, wherein the cell culture medium comprises: (i) from about 1.0 mg/L to about 100.0 mg/L insulin; and (ii) from about 69.0 nM to about 1,000.0 nM copper.
114. The cell culture medium of claim 112, wherein the cell culture medium comprises: (i) from about 1.0 mg/L to about 100.0 mg/L insulin; and (iii) from about 0.7 mM to about 2.0 mM cystine.
115. The cell culture medium of claim 112, wherein the cell culture medium comprises: (ii) from about 69.0 nM to about 1,000.0 nM copper; and (iii) from about 0.7 mM to about 2.0 mM cystine.
116. The cell culture medium of any one of claims 112-115, wherein the cell culture medium further comprises from about 1.0 g/L to about 10.0 g/L plant-derived hydrolysate.

117. The cell culture medium of any one of claims 112-116, wherein the cell culture medium further comprises from about 6.0 g/L to about 20.0 g/L animal-derived hydrolysate.
118. The cell culture medium of any one of claims 112-117, wherein the cell culture medium comprises insulin in a concentration selected from the group consisting of: from about 1.0 mg/L to about 100.0 mg/L; from about 10.0 mg/L to about 100.0 mg/L; from about 10.0 mg/L to about 50.0 mg/L; from about 10.0 mg/L to about 35.0 mg/L; from about 10.0 mg/L to about 25.0 mg/L; and about 10.0 mg/L.
119. The cell culture medium of any one of claims 112-118, wherein the cell culture medium comprises copper in a concentration selected from the group consisting of: from about 69 nM to about 1,000 nM; from about 300 nM to about 400 nM; from about 325 nM to about 375 nM; from about 325 nM to about 350 nM; about any one of 330 nM, 335 nM, 339 nM, 340 nM, 345 nM or 350 nM; and about 339 nM.
120. The cell culture medium of any one of claims 112-119, wherein the cell culture medium comprises cystine in a concentration selected from the group consisting of: from about 0.7 mM to about 2.0 mM; from about 1.0 mM to about 2.0 mM; from about 1.0 mM to about 1.6 mM; from about 1.2 mM to about 1.4 mM; and about 1.3 mM.
121. A composition comprising (a) a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof; and (b) a cell culture medium according to any one of claims 112-120.
122. A composition comprising: (a) bevacizumab, or a fragment thereof; and (b) a cell culture medium according to any one of claims 112-120.

123. The composition of claim 122, where the bevacizumab, or a fragment thereof, is secreted into the medium by a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof.
124. A method of enhancing the amount of bevacizumab, or a fragment thereof, produced from a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, the method comprising the step of culturing the mammalian cell in a cell culture medium comprising at least two of insulin, copper and cystine, wherein the amount of bevacizumab, or a fragment thereof, produced from the mammalian cell is enhanced relative to culturing the mammalian cell in a cell culture medium without at least two of insulin, copper and cystine.
125. The method of claim 124, wherein the cell culture medium comprises copper and insulin.
126. The method of claim 124, wherein the cell culture medium comprises copper and cystine.
127. The method of claim 124, wherein the cell culture medium comprises insulin and cystine.
128. The method of claim 124, wherein the cell culture medium comprises copper, insulin, and cystine.
129. The method of any one of claims 124-128, wherein the cell culture medium further comprises a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate.
130. The method of any one of claims 124-129, wherein the cell culture medium comprises insulin at a concentration of from about 1.0 mg/L to about 100.0 mg/L.
131. The method of any one of claims 124-129, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 100.0 mg/L.
132. The method of any one of claims 124-129, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 50.0 mg/L.

133. The method of any one of claims 124-129, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 35.0 mg/L.
134. The method of any one of claims 124-129, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 25.0 mg/L.
135. The method of any one of claims 124-129, wherein the cell culture medium comprises insulin at a concentration of about 25.0 mg/L.
136. The method of any one of claims 124-135, wherein the cell culture medium comprises copper at a concentration of from about 69 nM to about 1,000 nM.
137. The method of any one of claims 124-135, wherein the cell culture medium comprises copper at a concentration of from about 325 nM to about 375 nM.
138. The method of any one of claims 124-135, wherein the cell culture medium comprises copper at a concentration of from about 325 nM to about 350 nM.
139. The method of any one of claims 124-135, wherein the cell culture medium comprises copper at a concentration of about any one of 330 nM, 335 nM, 339 nM, 340 nM, 345 nM or 350 nM.
140. The method of any one of claims 124-135, wherein the cell culture medium comprises copper at a concentration of about 339 nM.
141. The method of any one of claims 124-140, wherein the cell culture medium comprises cystine at a concentration of from about 0.7 mM to about 2.0 mM.
142. The method of any one of claims 124-140, wherein the cell culture medium comprises cystine at a concentration of from about 1.0 mM to about 1.6 mM.
143. The method of any one of claims 124-140, wherein the cell culture medium comprises cystine at a concentration of from about 1.2 mM to about 1.4 mM.
144. The method of any one of claims 124-140, wherein the cell culture medium comprises cystine at a concentration of about 1.3 mM.
145. The method of any one of claims 124-144, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 6.0 g/L to about 20.0 g/L.
146. The method of any one of claims 124-144, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 8.0 g/L to about 12.0 g/L.

147. The method of any one of claims 124-144 wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 9.0 g/L to about 11.0 g/L.
148. The method of any one of claims 124-144, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of about 13 g/L.
149. The method of any one of claims 124-148, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 1.0 g/L to about 10.0 g/L.
150. The method of any one of claims 124-148, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.0 g/L to about 3.0 g/L.
151. The method of any one of claims 124-148, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.25 g/L to about 2.75 g/L
152. The method of any one of claims 124-148, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of about 3.1 g/L.
153. The method of any one of claims 124-152, wherein the cell culture medium comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate.
154. The method of any one of claims 124-153, wherein the cell culture medium comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium.
155. The method of claim 154, wherein the additional amount of insulin is added to the cell culture medium once during the cell culture cycle.
156. The method of claim 154, wherein the additional amount of insulin is added to the cell culture medium at least three times during the cell culture cycle.
157. The method of claim 154, wherein the additional amount of insulin is added to the cell culture medium at least six times during the cell culture cycle.

158. The method of any one of claims 154-157, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 1.0 mg/L to about 100.0 mg/L.
159. The method of any one of claims 154-157, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 100.0 mg/L.
160. The method of any one of claims 154-157, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 50.0 mg/L.
161. The method of any one of claims 154-157, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 35.0 mg/L.
162. The method of any one of claims 154-157, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 25.0 mg/L.
163. The method of any one of claims 154-157, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of about 15.0 mg/L.
164. The method of any one of claims 124-163, wherein the method further comprises the step of adding cysteine to the cell culture medium.
165. The method of claim 164, wherein cysteine is added in an amount to provide from about 0.5 to about 2.0 mM cysteine in the cell culture medium.
166. The method of claim 164, wherein cysteine is added in an amount to provide about 0.8 mM cysteine in the cell culture medium.
167. The method of any one of claims 124-166, wherein the method further comprises the step of adding cystine to the cell culture medium.
168. The method of claim 167, wherein cystine is added in an amount to provide from about 0.1 to about 1.5 mM cystine in the cell culture medium.
169. The method of claim 167, wherein cystine is added in an amount to provide about 0.2 mM cystine in the cell culture medium.
170. The method of any one of claims 124-169, wherein the cell is cultured at a temperature ranging from about 28 °C to about 37 °C.
171. The method of claim 170, wherein the cell is cultured at a temperature ranging from about 31 °C to about 35 °C.

172. The method of any one of claims 124-169, wherein the cell is cultured at a first temperature of about 35 °C for a first period of time, is cultured at a second temperature of about 33 °C for a second period of time, and is cultured at a third temperature of about 31 °C for a third period of time.
173. The method of any one of claims 124-172, wherein bevacizumab, or a fragment thereof, is secreted into the cell culture medium.
174. The method of any one of claims 124-173, further comprising the step of recovering the bevacizumab, or a fragment thereof, from the cell culture.
175. A method of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab, or a fragment thereof, in a cell culture medium comprising at least two of insulin, copper and cystine, wherein the amount of bevacizumab, or a fragment thereof, produced from the mammalian cell is enhanced relative to culturing the mammalian cell in a cell culture medium without at least two of insulin, copper and cystine.
176. The method of claim 175, wherein the cell culture medium comprises copper and insulin.
177. The method of claim 175, wherein the cell culture medium comprises copper and cystine.
178. The method of claim 175, wherein the cell culture medium comprises insulin and cystine.
179. The method of claim 175, wherein the cell culture medium comprises copper, insulin, and cystine.
180. The method of any one of claims 175-179, wherein the cell culture medium further comprises a plant-derived hydrolysate, an animal-derived hydrolysate or both a plant-derived hydrolysate and an animal-derived hydrolysate.
181. The method of any one of claims 175-180, wherein the cell culture medium comprises insulin at a concentration of from about 1.0 mg/L to about 100.0 mg/L.
182. The method of any one of claims 175-180, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 100.0 mg/L.
183. The method of any one of claims 175-180, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 50.0 mg/L.
184. The method of any one of claims 175-180, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 35.0 mg/L.

185. The method of any one of claims 175-180, wherein the cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 25.0 mg/L.
186. The method of any one of claims 175-180, wherein the cell culture medium comprises insulin at a concentration of about 25.0 mg/L.
187. The method of any one of claims 175-186, wherein the cell culture medium comprises copper at a concentration of from about 69 nM to about 1,000 nM.
188. The method of any one of claims 175-186, wherein the cell culture medium comprises copper at a concentration of from about 325 nM to about 375 nM.
189. The method of any one of claims 175-186, wherein the cell culture medium comprises copper at a concentration of from about 325 nM to about 350 nM.
190. The method of any one of claims 175-186, wherein the cell culture medium comprises copper at a concentration of about any one of 330 nM, 335 nM, 339 nM, 340 nM, 345 nM or 350 nM.
191. The method of any one of claims 175-186, wherein the cell culture medium comprises copper at a concentration of about 339 nM.
192. The method of any one of claims 175-191, wherein the cell culture medium comprises cystine at a concentration of from about 0.7 mM to about 2.0 mM.
193. The method of any one of claims 175-191, wherein the cell culture medium comprises cystine at a concentration of from about 1.0 mM to about 1.6 mM.
194. The method of any one of claims 175-191, wherein the cell culture medium comprises cystine at a concentration of from about 1.2 mM to about 1.4 mM
195. The method of any one of claims 175-191, wherein the cell culture medium comprises cystine at a concentration of about 1.3 mM.
196. The method of any one of claims 175-195, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 6.0 g/L to about 20.0 g/L.
197. The method of any one of claims 175-195, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 8.0 g/L to about 12.0 g/L.
198. The method of any one of claims 175-195, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 9.0 g/L to about 11.0 g/L.

199. The method of any one of claims 175-195, wherein the cell culture medium comprises an animal-derived hydrolysate at a concentration of about 13 g/L.
200. The method of any one of claims 175-199, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 1.0 g/L to about 10.0 g/L.
201. The method of any one of claims 175-199, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.0 g/L to about 3.0 g/L.
202. The method of any one of claims 175-199, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.25 g/L to about 2.75 g/L
203. The method of any one of claims 175-199, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of about 3.1 g/L.
204. The method of any one of claims 175-203, wherein the cell culture medium comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate.
205. The method of any one of claims 175-204, wherein the cell culture medium comprises insulin and the method further comprises the step of adding an additional amount of insulin to the cell culture medium.
206. The method of claim 205, wherein the additional amount of insulin is added to the cell culture medium once during the cell culture cycle.
207. The method of claim 205, wherein the additional amount of insulin is added to the cell culture medium at least three times during the cell culture cycle.
208. The method of claim 205, wherein the additional amount of insulin is added to the cell culture medium at least six times during the cell culture cycle.
209. The method of any one of claims 205-208, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 1.0 mg/L to about 100.0 mg/L.
210. The method of any one of claims 205-208, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 100.0 mg/L.

211. The method of any one of claims 205-208, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 50.0 mg/L.
212. The method of any one of claims 205-208, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 35.0 mg/L.
213. The method of any one of claims 205-208, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of from about 10.0 mg/L to about 25.0 mg/L.
214. The method of any one of claims 205-208, wherein the additional amount of insulin is added in an amount to provide insulin in the cell culture medium at a concentration of about 15.0 mg/L.
215. The method of any one of claims 175-214, wherein the method further comprises the step of adding cysteine to the cell culture medium.
216. The method of claim 215, wherein cysteine is added in an amount to provide from about 0.5 to about 2.0 mM cysteine in the cell culture medium.
217. The method of claim 215, wherein cysteine is added in an amount to provide about 0.8 mM cysteine in the cell culture medium.
218. The method of any one of claims 175-217, wherein the method further comprises the step of adding cystine to the cell culture medium.
219. The method of claim 218, wherein cystine is added in an amount to provide from about 0.1 to about 1.5 mM cystine in the cell culture medium.
220. The method of claim 218, wherein cystine is added in an amount to provide about 0.2 mM cystine in the cell culture medium.
221. The method of any one of claims 175-220, wherein the cell is cultured at a temperature ranging from about 28 °C to about 37 °C.
222. The method of claim 221, wherein the cell is cultured at a temperature ranging from about 31 °C to about 35 °C.
223. The method of any one of claims 175-220, wherein the cell is cultured at a first temperature of about 35 °C for a first period of time, is cultured at a second temperature of about 33 °C for a second period of time, and is cultured at a third temperature of about 31 °C for a third period of time.
224. The method of any one of claims 175-223, wherein bevacizumab, or a fragment thereof, is secreted into the cell culture medium.

225. The method of any one of claims 175-223, further comprising the step of recovering the bevacizumab, or a fragment thereof, from the cell culture.
226. A method of producing bevacizumab or a fragment thereof, comprising a step of culturing a mammalian cell comprising a nucleic acid encoding bevacizumab or a fragment thereof in a cell culture medium, wherein initial cell culture medium in a cell culture cycle comprises two or more components selected from the group consisting of copper at a concentration of from about 69 nM to about 1,000 nM, insulin at a concentration of from about 1.0 mg/L to about 100.0 mg/L, and cystine at a concentration of from about 0.7 mM to about 2.0 mM, and wherein the cell produces bevacizumab or the fragment.
227. The method of claim 226, wherein the initial cell culture medium comprises (1) copper and insulin; (2) copper and cystine; (3) insulin and cystine; or (4) copper, insulin, and cystine.
228. The method of claim 226 or 227, wherein the initial cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 50.0 mg/L.
229. The method of claim 228, wherein the initial cell culture medium comprises insulin at a concentration of from about 10.0 mg/L to about 20.0 mg/L.
230. The method of claim 228, wherein the initial cell culture medium comprises insulin at a concentration of about any one of 10.0 mg/L, 15 mg/L, 20 mg/L, and 25 mg/L.
231. The method of any one of claims 226-230, wherein the initial cell culture medium comprises copper at a concentration of from about 325 nM to about 375 nM.
232. The method of any one of claims 226-230, wherein the initial cell culture medium comprises copper at a concentration of from about 325 nM to about 350 nM.
233. The method of any one of claims 226-230, wherein the initial cell culture medium comprises copper at a concentration of about any one of 330 nM, 335 nM, 339 nM, 340 nM, 345 nM and 350 nM.
234. The method of any one of claims 226-233, wherein the initial cell culture medium comprises cystine at a concentration of from about 0.7 mM to about 2.0 mM.
235. The method of any one of claims 226-233, wherein the initial cell culture medium comprises cystine at a concentration of from about 1.0 mM to about 1.6 mM.
236. The method of any one of claims 226-233, wherein the initial cell culture medium comprises cystine at a concentration of about any one of 1.0 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM and 1.6 mM.

237. The method of any one of claims 226-236, wherein the initial cell culture medium comprises an animal-derived hydrolysate and/or a plant-derived hydrolysate.
238. The method of any one of claims 226-236, wherein the initial cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 6.0 g/L to about 20.0 g/L.
239. The method of any one of claims 226-236, wherein the initial cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 8.0 g/L to about 12.0 g/L.
240. The method of any one of claims 226-236, wherein the initial cell culture medium comprises an animal-derived hydrolysate at a concentration of from about 9.0 g/L to about 11.0 g/L.
241. The method of any one of claims 226-236, wherein the initial cell culture medium comprises an animal-derived hydrolysate at a concentration of about 13 g/L.
242. The method of any one of claims 226-241, wherein the initial cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 1.0 g/L to about 10.0 g/L.
243. The method of any one of claims 226-242, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.0 g/L to about 3.0 g/L.
244. The method of any one of claims 226-243, wherein the cell culture medium comprises a plant-derived hydrolysate at a concentration of from about 2.25 g/L to about 2.75 g/L.
245. The method of any one of claims 226-244, wherein the initial cell culture medium comprises a plant-derived hydrolysate at a concentration of about 2.5 g/L.
246. The method of any one of claims 226-245, wherein the cell culture medium comprises both an animal-derived hydrolysate and a plant-derived hydrolysate, and wherein the animal-derived hydrolysate is present in a greater amount than the plant-derived hydrolysate.
247. The method of any one of claims 226-246, wherein the initial cell culture medium comprises insulin and the method further comprises a step of adding an additional amount of insulin to the cell culture medium during the cell culture cycle.
248. The method of claim 247, wherein the additional amount of insulin is added to the cell culture medium at least once, at least twice, at least three times, at least four times, at least five times, or at least six times during the cell culture cycle.

249. The method of claim 248, wherein the insulin added each time is from about 5 mg/L to about 25 mg/L.
250. The method of claim 248, wherein the insulin added each time is about any one of 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, and 25 mg/L.
251. The method of any one of claims 248-250, wherein the cumulative amount of insulin added during the cell culture cycle is from about 20 mg/L to about 100 mg/L.
252. The method of claim 251, wherein the cumulative amount of insulin added during the cell culture cycle is about any one of 20 mg/L, 25 mg/L, 30 mg/L, 35 mg/L, 40 mg/L, 45 mg/L, 50 mg/L, 55 mg/L, 60 mg/L, 65 mg/L, 70 mg/L, 75 mg/L, 80 mg/L and 85 mg/L.
253. The method of any one of claims 226-252, wherein the initial cell culture medium comprises cystine and the method further comprises a step of adding an additional amount of cystine to the cell culture medium during the cell culture cycle.
254. The method of claim 253, wherein cystine is added in an amount to provide from about 0.1 to about 1.5 mM additional cystine in the cell culture medium.
255. The method of claim 254, wherein cystine is added in an amount to provide about 0.4 to about 0.7 mM additional cystine in the cell culture medium.
256. The method of any one of claims 253-255, wherein cystine is added in a batch feed during the cell culture cycle.
257. The method of any one of claims 226-256, wherein the method further comprises at least one batch feed during the cell culture cycle.
258. The method of claim 257, wherein the method comprises two, three, or four batch feeds during the cell culture cycle.
259. The method of claim 257 or 259, wherein the batch feed medium comprises an animal-derived hydrolysate and/or a plant-derived hydrolysate.
260. The method of any one of claims 226-259, wherein during the cell culture cycle, the temperature of the medium is reduced by at least about 2, at least about 3, at least about 4, or at least about 5 degrees C relative to the temperature at the beginning of the culturing.
261. The method of claim 260, wherein the temperature of the medium is reduced at least once or at least twice during the cell culture cycle.
262. The method of claim 261, wherein the temperature is reduced on day 8 and day 10 after the beginning of the culturing.

263. The method of any one of claims 226-262, wherein the cell is cultured at a temperature ranging from about 31°C to about 35°C.
264. The method of claim 263, wherein the cell is cultured at a first temperature of about 35°C for a first period of time, is cultured at a second temperature of about 33°C for a second period of time, and is cultured at a third temperature of about 31°C for a third period of time.
265. The method of any one of claims 226-264, wherein the cell is cultured in the medium having a pH at about 7.0 to about 7.3.
266. The method of any one of claims 226-265, wherein the method comprises (a) culturing the cell in an initial cell culture medium comprising about 10 mg/L insulin, about 325 nM to about 350 nM copper, and about 1.3 mM cystine; (b) providing a first batch feed and an insulin feed to the cell culture medium to provide additional insulin at a concentration of about 15 mg/L on day 3 after the beginning of the culturing; and (c) providing a second batch feed comprising cystine to the cell culture medium to provide additional cystine at a concentration of about 0.4 to about 0.7 mM on day 6 after the beginning of the culturing; wherein the cell is cultured at an initial temperature of about 35°C, and the temperature is reduced to about 33°C on day 8 and is further reduced to about 31°C on day 10 after the beginning of the culturing.
267. The method of any one of claims 226-266, wherein bevacizumab or a fragment thereof is secreted into the cell culture medium.
268. The method of any one of claims 226-267, further comprising a step of recovering the bevacizumab or a fragment thereof from the cell culture.
269. The method of any one of claims 226-268, wherein the mammalian cell is a Chinese hamster ovary cell.
270. Bevacizumab or a fragment thereof produced by the method of any one of claims 226-269.
271. A composition comprising: (i) bevacizumab or a fragment thereof produced by the method of any one of claims 226-269 and (ii) a pharmaceutically acceptable carrier.

FIG. 1

FIG. 1A

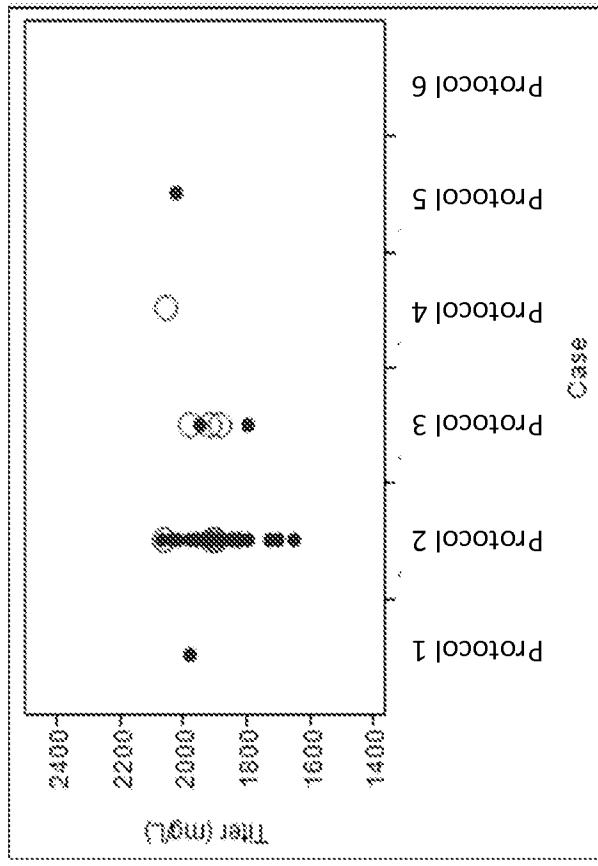


FIG. 1B

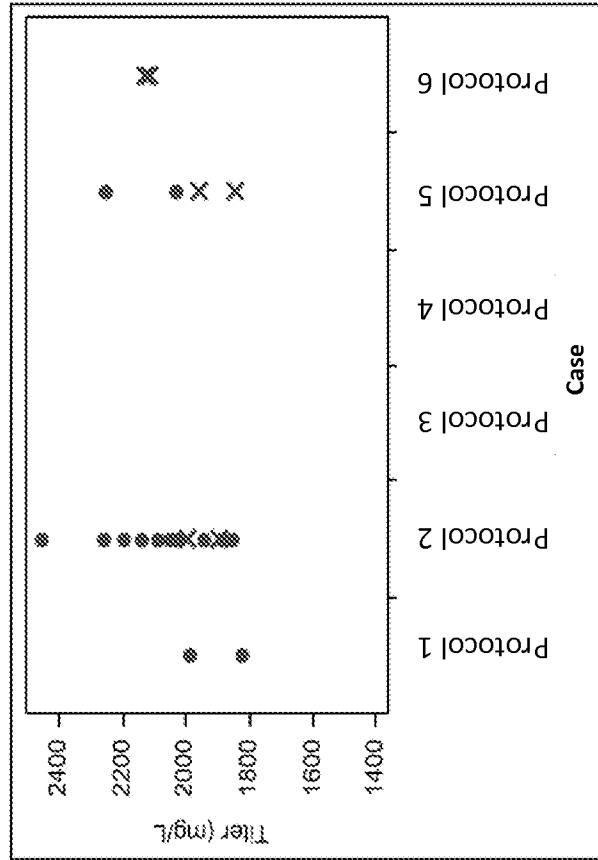
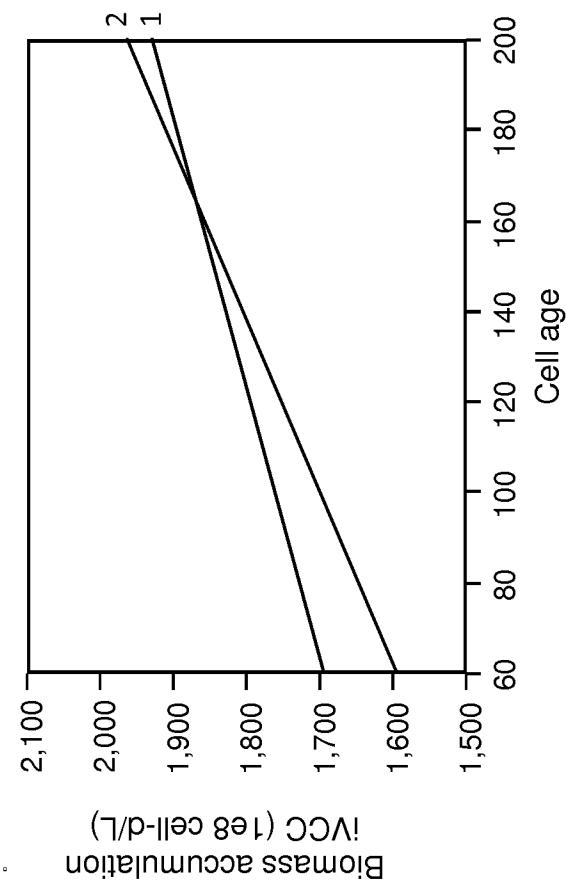


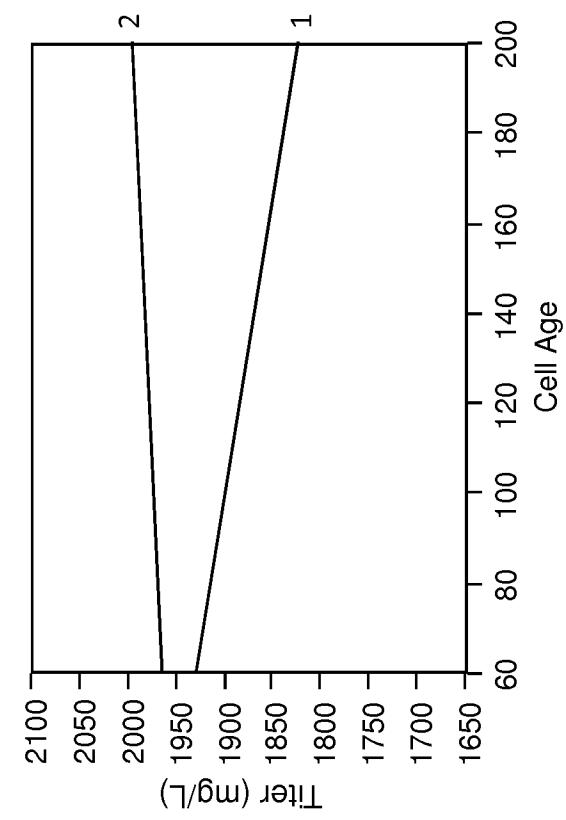
FIG. 2

FIG. 2A



1 = One Feed process
 2 = Two Feed process

FIG. 2B



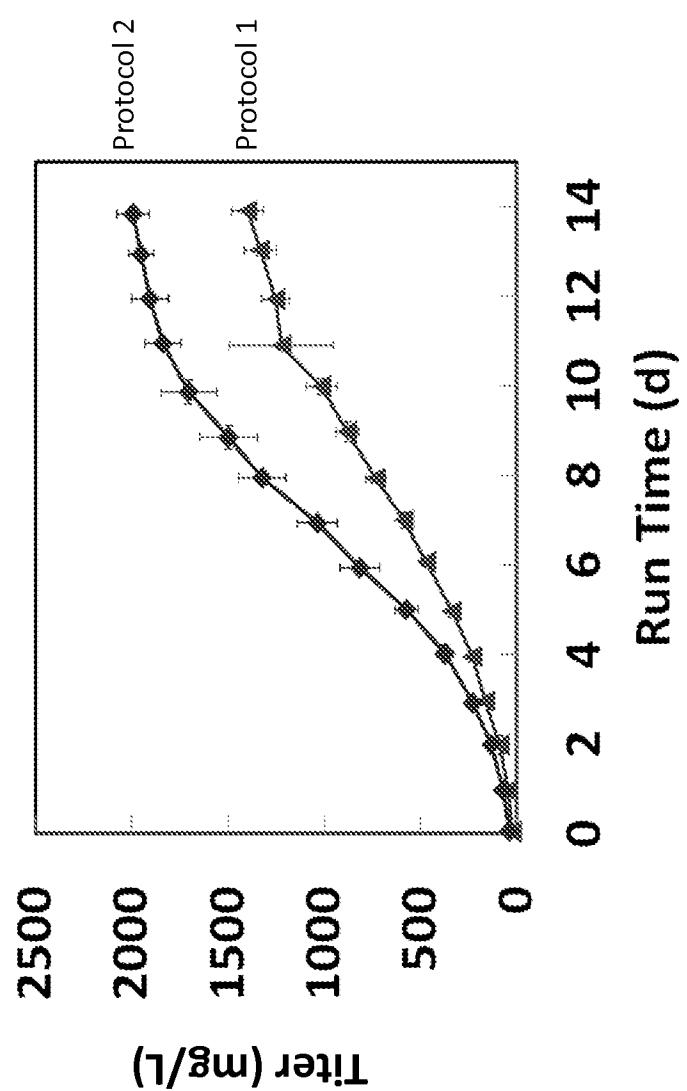


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/29758

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/395; C07K 16/00; C12N 5/00 (2014.01)

USPC - 530/287.1, 386, 380, 350; 424/130.1; 435/404, 325, 41

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)

IPC(8): A61K 39/395; C07K 16/00; C12N 5/00 (2014.01)

USPC: 530/287.1, 386, 380, 350; 424/130.1; 435/404, 325, 41

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)

MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; Dialog ProQuest; Entrez Pubmed; bevacizumab, 'Avastin,' 'cell culture medium,' copper, insulin, cystine, kit, 'plant-derived hydrolysate,' 'animal-derived hydrolysate,' 'culture yield'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0177640 A1 (BURG, J et al.) July 12, 2012; abstract; paragraphs [0016], [0021], [0026], [0027], [0040], [0103]-[0108], [0130], [0131], [0134]-[0136], [0192], [0249]	1-5, 6/1-6/5, 54-58, 59/34-59/58, 112-115, 116/112-116/115, 124-128, 129/124-129/128, 175-179, 180/175-180/179, 226, 227, 228/226-228/227, 229/228/226-229/228/227, 230/228/226-230/228/227
Y	WO 2012/145682 A1 (JERUMS, MI et al.) October 26, 2012; page 2, lines 21-36; page 3, lines 2-3; page 5, lines 10-19; page 13, lines 9-23	1-5, 6/1-6/5, 54-58, 59/34-59/58, 107-109, 110/107-110/109, 111/110/107-111/110/109 112-115, 116/112-116/115, 124-128, 129/124-129/128, 175-179, 180/175-180/179, 226, 227, 228/226, 228/227, 229/228/226-229/228/227, 230/228/226-230/228/227

Further documents are listed in the continuation of Box C.

* 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

28 July 2014 (28.07.2014)

Date of mailing of the international search report

19 AUG 2014

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/29758

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0129926 A1 (FIKE, RM et al.) June 2, 2011; abstract; paragraphs [0003], [0014], [0032], [0034], [0056], [0093]-[0095], [[0185], [0186], [0188], [0526]	107-109, 110/107-110/109, 111/110/107- 111/110/109
Y	US 2010/0098725 A1 (LIU, J et al.) April 22, 2010; paragraphs [0070], [0073], [0482]	107-109, 110/107-110/109, 111/110/107- 111/110/109, 112-115, 116/112-116/115, 226, 227, 228/226, 228/227, 229/228/226, 229/228/227, 230/228/226, 230/228/227
Y	WO 2003/046162 A2 (KATINGER, H et al.) June 5, 2003; page 7, line 4-10; page 19, line 1-4	113, 115, 116/113, 116/115

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/29758

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.: 7-53, 60-106, 117-123, 130-174, 181-225, 231-271
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:

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 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.:

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.