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(54) **Title:** HIGH PH METHOD FOR PURIFICATION OF HYDROPHOBIC PROTEINS

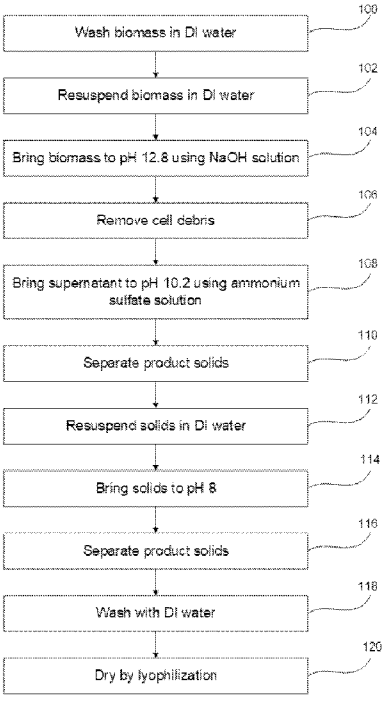

FIG. 1

(57) **Abstract:** In one aspect, the disclosure relates to a process for purification of hydrophobic proteins and/or proteins that have a tendency to aggregate when produced at high levels in cell culture. In a further aspect, the process involves subjecting intact cells to a strong base in a solution with a pH of greater than 12.5, with an optional initial pretreatment at a pH of greater than 13. The process operates efficiently with a high biomass loading of up to or greater than about 20% (w/v) and does not require the use of external flocculants. The target protein can purified by the disclosed process inexpensively and efficiently, producing a high-quality end product having few to no contaminants. This abstract is intended as a scanning tool for purposes of searching in the particular art and is not intended to be limiting of the present disclosure.

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[Continued on next page]

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

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HIGH PH METHOD FOR PURIFICATION OF HYDROPHOBIC PROTEINS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/508,018 filed on June 14, 2023, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under subaward number T-OC-A-01-0207 from Bioindustrial Manufacturing and Design Ecosystem (BioMADE). The government has certain rights in the invention.

CROSS-REFERENCE TO SEQUENCE LISTING

[0003] This application contains a sequence listing filed in ST.26 format entitled "320020_2030_Sequence_Listing.xml" created on April 29, 2024, and having a file size of 219,430 bytes. The content of the sequence listing is incorporated herein in its entirety.

BACKGROUND

[0004] Protein materials are ubiquitous in nature, playing critical protective and structural roles in forms as familiar as our own skin, hair, and fingernails, as well as providing the basis for some of our oldest technologies: fibers and textiles based on animal-derived materials like silk and wool. The development of modern biotechnology offers new possibilities for protein materials, including genetic engineering of a wide array of material properties, intrinsic biocompatibility and biodegradability, and sustainable, animal-free production in recombinant microbes. The most mature recombinant technology for protein-material production has been achieved for sequences based on various types of silk.

[0005] Recombinant and natural protein sequences have been produced at scale and manufactured into a variety of products, including blended textiles, cosmetic additives, antibodies and biologic drugs, and coatings. However, large-scale purification of proteins can be resource and materials intensive and presents difficulties when the desired proteins are insoluble, hydrophobic, or otherwise have a tendency to aggregate. Furthermore, some proteins that are naturally soluble may aggregate when produced in large quantities in a bioreactor, and many purification processes require large volumes of dilute solutions as well as the addition of flocculating agents, thereby increasing overall materials costs.

[0006] Despite advances in protein production research, there is still a scarcity of methods for

producing and purifying hydrophobic and/or aggregating proteins that are effective at removing cell debris, do not require the use of flocculants, and can be conducted using concentrated solutions and low volumes of reagents. These needs and other needs are satisfied by the present disclosure.

SUMMARY

[0007] In accordance with the purpose(s) of the present disclosure, as embodied and broadly described herein, the disclosure, in one aspect, relates to a process for purification of hydrophobic proteins and/or proteins that have a tendency to aggregate when produced at high levels in cell culture. In a further aspect, the process involves subjecting intact cells to a strong base in a solution with a pH of greater than 12.5, with an optional initial pretreatment at a pH of greater than 13. The process operates efficiently with a high biomass loading of up to or greater than about 20% (w/v) and does not require the use of external flocculants. The target protein can purified by the disclosed process inexpensively and efficiently, producing a high-quality end product having few to no contaminants.

[0008] Other systems, methods, features, and advantages of the present disclosure will be or become apparent to one with skill in the art upon examination of the following drawings and detailed description. It is intended that all such additional systems, methods, features, and advantages be included within this description, be within the scope of the present disclosure, and be protected by the accompanying claims. In addition, all optional and preferred features and modifications of the described embodiments are usable in all aspects of the disclosure taught herein. Furthermore, the individual features of the dependent claims, as well as all optional and preferred features and modifications of the described embodiments are combinable and interchangeable with one another.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] Many aspects of the present disclosure can be better understood with reference to the following drawings. The components in the drawings are not necessarily to scale, emphasis instead being placed upon clearly illustrating the principles of the present disclosure. Moreover, in the drawings, like reference numerals designate corresponding parts throughout the several views.

[0010] FIG. 1 is a flow chart showing a non-limiting, exemplary process for protein purification according to the present disclosure.

[0011] FIG. 2 is a flow chart showing an alternative non-limiting, exemplary process for protein

purification according to the present disclosure.

[0012] Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or can be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

DETAILED DESCRIPTION

[0013] Disclosed herein is a method for purifying proteins having high isoelectric points, proteins that are insoluble at low pH values, and/or proteins that are soluble under typical cellular conditions but that tend to aggregate under recombinant protein production conditions, such as, for example, inclusion bodies in proteins produced by *E. coli*.

Method for Separating a Target Protein from an Intact Cell

[0014] In one aspect, disclosed herein is a method for separating a target protein from an intact cell, the method including at least the steps of:

(a) optionally pretreating a solution including the intact cell at a pH greater than or equal to 13 for a period of time;

(b) lysing the cell at a pH value greater than 12.5 to create a first solution that includes lysed cell components;

(c) optionally, precipitating non-target molecules from the first solution;

(d) removing cell debris and precipitates from the first solution;

(e) decreasing solubility of the target protein in the first solution;

(f) separating target protein solids from the first solution;

(g) resuspending the target protein solids to create a second solution;

(h) optionally, adjusting a pH of the second solution; and

(i) separating target protein solids from the second solution.

[0015] In some aspects, the solution including the intact cell can be contacted with one or more additives prior to step (a) or step (b). In another aspect, the first solution can be contacted with one or more additives prior to step (d). In either of these aspects, the one or more additives can

be sodium chloride, an organic solvent, calcium chloride, or any combination thereof. In an aspect, the sodium chloride can have a concentration of from about 0.37 M to about 1.23 M in the solution including the intact cell or in the first solution, or of about 0.37, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, or about 1.23 M, or a combination of any of the foregoing values, or a range encompassing any of the foregoing values. In another aspect, the calcium chloride can have a concentration of from about 0.37 M to about 0.40 M in the solution including the intact cell or in the first solution, or about 0.37, 0.38, 0.39, or about 0.40 M, or a combination of any of the foregoing values, or a range encompassing any of the foregoing values. In still another aspect, the organic solvent can be ethanol or acetone and can be present at from about 8% (v/v) to about 50% (v/v) in the solution including the intact cell or in the first solution, or about 8, 10, 15, 20, 25, 30, 35, 40, 45, or about 50% (v/v), or a combination of any of the foregoing values, or a range encompassing any of the foregoing values.

[0016] In any of these aspects, a solution containing the intact cell can be pretreated at a pH greater than or equal to 13 for a period of time prior to performing step (b). In an aspect, the pH higher than 13 can be at least 13.4. In another aspect, the period of time can be from about 5 min to about 1 hour, or about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or about 60 min, or a combination of any of the foregoing values, or a range encompassing any of the foregoing values. In a still further aspect, it was previously believed that base-catalyzed or base-assisted cell lysis carried out above pH 13 would damage target proteins, but it has been fortuitously discovered as disclosed herein that performing this step can lead to a more homogeneous lysate without damaging the target protein. In a further aspect, performing this step results in a more efficient downstream process for purification of target proteins in terms of process speed, time and materials required, and other factors.

[0017] Additional washing, resuspension, centrifugation, filtration, and drying steps can be added as needed. In some aspects, the cell is pretreated at a pH of greater than or equal to 13 in step (a), lysed in step (b), or both using a strong base such as, for example, NaOH, KOH, $Ba(OH)_2$, $Ca(OH)_2$, LiOH, RbOH, CsOH, $Sr(OH)_2$, or any combination thereof. In one aspect, the strong base is NaOH. In an aspect, and without wishing to be bound by theory, exposure to a strong base can function both to rupture cells and to dissolve the target proteins. In another aspect, endogenous host materials present can solidify under the high pH conditions of the disclosed method and can thus easily be separated from the dissolved target proteins by centrifugation and/or filtration. In a further aspect, effective separation of cell debris after NaOH treatment is important for product quality.

[0018] In one aspect, in step (c), non-target molecules can be precipitated using a first precipitation agent, a first acid, or a combination thereof.

[0019] In an aspect, significant breakdown of cell material occurs at pH levels of 12.5 or greater, which can complicate removal of cell debris and other molecules including non-target proteins. In some aspect, the pH in step (b) can be 12.5, 12.6, 12.7, 12.8, or greater.

[0020] In another aspect, step (d) can be accomplished by centrifugation, filtration, or any combination thereof. In an aspect, in smaller scale purifications, centrifugation may be sufficient for removing cell debris, while in larger scale purifications, filtration may be more effective.

[0021] In any of these aspects, in steps (c) or (e), solubility of the target protein and non-target molecules can be decreased using a first precipitation agent, a first acid, or any combination thereof. In one aspect, the first precipitation agent can be or include F^-, Cl^-, Br^-, I^-, CO_3^{2-}, HCO_3^-, SO_4^{2-}, HSO_4^-, PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, formate, acetate, Li^+, Na^+, K^+, Zn^{2+}, Al^{3+}, Fe^{3+}, Mg^{2+}, Ca^{2+}, NH_4^+, trehalose, glucose, proline, *tert*-butanol, trimethylamine N-oxide, ectoine, glycine betaine, 3-dimethylsulfoniopropionate, or any combination thereof. In a further aspect, the first acid can be sulfuric acid, phosphoric acid, carbonic acid, hydrochloric acid, or any combination thereof. In any of these aspects, in step (c), the pH of the first solution can be decreased to from about 10.9 to about 11.6, or to about 10.9, 11.0, 11.1, 11.2, 11.3, 11.4, 11.5, or about 11.6, or a combination of any of the foregoing values, or a range encompassing any of the foregoing values. In any of these aspects, in step (e), the pH of the first solution can be decreased to from about 10.2 to about 10.8, or to about 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, or about 10.8, or a combination of any of the foregoing values, or a range encompassing any of the foregoing values. In one aspect, decreasing the pH of the first solution can be accomplished using ammonium sulfate, wherein the second precipitation agent and second acid are present as one ionic compound that dissociates in water. In an aspect, various precipitation-agent/acid mixtures, including the components described herein for use in optional step (c) of the disclosed process, are contemplated for use in the disclosed process as the second precipitation agent and/or second acid instead of or in addition to the ammonium sulfate.

[0022] In a further aspect, the target protein solids can be resuspended in water in step (g). In another aspect, the pH of the second solution can be decreased to about 8 in step (h). In one aspect, the pH of the second solution in step (h) can be decreased using an aqueous solution of an acid such as, for example, 1 M HCl, although other aqueous acids at other concentrations are also contemplated and should be considered disclosed.

[0023] In another aspect, the first solution includes from about 5% to about 20%, or about 5, 10, or 20% (w/v) solids in an aqueous solution, wherein the solids include cell debris as well as the target protein. In a further aspect, a flocculant is not used during performance of the method. In an aspect, previous protein purification research has proceeded with dilute solutions, sometimes as low as 1% or less, of biomass (i.e., cell solids and the like), which required the use of flocculants to help aggregate the cell debris and separate it from solution, while with the disclosed process, it has been fortuitously discovered that when the biomass is first concentrated, higher purity and yield can be achieved in a lower working volume without the need for flocculants. In one aspect, in known protein purification processes, flocculants such as, for example, cationic polyethylenimines, chitosan, cationic poly(diallyldimethyl-ammonium chloride), cationic polyacrylamides, cationic polyamines, or any combination thereof can be used. However, the present disclosure provides effective processes for protein purification without the need for these or other flocculants.

[0024] In still another aspect, the pH value in step (b) is greater than the isoelectric point of the target protein. In yet another aspect, the target protein solids separated in step (i) can be further washed with DI water, dried by a method such as lyophilization or spray-drying, or any combination thereof.

[0025] One exemplary protein purification process is shown in **FIG. 1**. Biomass is washed in DI water **100** and resuspended in DI water **102**. The resuspended biomass is then brought to pH 12.8 using an NaOH solution **104**, under which conditions the desired protein is soluble but cell debris remains solid. The cell debris is removed **106** and the supernatant containing the protein of interest is brought to pH 10.2 using ammonium sulfate solution **108**. Desired protein solids are separated from the remaining solution **110** and the solids are resuspended in DI water **112**. The solids are then brought to pH 8 **114** and separated from the remaining solution again **116**. The solids are washed with DI water **118** and lyophilized to dry **120**. The depicted process is non-limiting; other steps and combinations of steps as described herein should also be considered disclosed.

[0026] A second exemplary protein purification process is shown in **FIG. 2**. In one aspect, this process may be especially useful for large-scale protein purifications. Harvested biomass is diluted in DI water **202**. In some experiments, the biomass is directly harvested by disc-stack centrifugation, which results in a slurry of 10-15% biomass solids in residual broth, and the dilution brings the concentration down to about 5% before proceeding. The diluted biomass is then brought to pH 12.8 using an NaOH solution **204**, under which conditions the desired protein is

soluble but cell debris remains solid. The cell debris is removed **206** and the supernatant containing the protein of interest is brought to pH 10.2 using ammonium sulfate solution **208**. Desired protein solids are separated from the remaining solution **210** and the solids are resuspended in DI water **212**. The solids are then brought to pH 8 **214** and separated from the remaining solution again **216**. The solids are washed with DI water **218** and lyophilized to dry **220**. In this second process, the biomass can be contacted with one or more additives as described herein (**203, 205**) either before raising the pH to 12.8 using an NaOH solution **204** or before removal of cell debris **206.** The depicted process is non-limiting; other steps and combinations of steps as described herein should also be considered disclosed.

Benefits of the Disclosed Process

[0027] In one aspect, the disclosed method offers numerous benefits and improvements over known protein purification processes. In an aspect, the disclosed method takes place in aqueous solution and does not require any extraction with volatile, hazardous, or expensive organic solvents, thus it is more environmentally benign and offers a cost savings over many known processes. In another aspect, due to the desirability of a high solids loading during the process, volumes of water required at various stages are lower. In a still further aspect, a lower volume of water directly results in a lower materials cost since smaller amounts of reagents are needed for pH changes and the like. In an alternative aspect, large-scale, larger-volume processes can still be carried out, but will result in much higher amounts of target protein compared to equivalent volumes of previously known processes. In some aspects, the water used in various steps of the process can be recycled, optionally after one or more purification steps. In still another aspect, the disclosed process can offer higher recovery of the target protein in a shorter amount of time and at lower cost compared to known processes.

[0028] In another aspect, a typical protein purification process can be surprisingly difficult due to particle size and solution conditions, requiring the use of a flocculant to aggregate cell debris and/or other particles. In still another aspect, the disclosed process does not require the use of any external flocculants. In a further aspect, conducting the process in the absence of external flocculants represents yet another reduction in cost and increase in efficiency when the disclosed process is compared to known processes.

[0029] In a still further aspect, both target protein yield and quality are improved over those seen with known processes, when using the disclosed process.

Process Scale-Up

[0030] In one aspect, it may be desirable to purify target proteins on an industrial scale. In some aspects, large-scale production of proteins can require modification of certain process steps in order to achieve the desired purity and yield levels. In a further aspect, large-scale production of target proteins can benefit from milder conditions. In one aspect, scaled-up separation of proteins can be challenging with 20% biomass solids and may improve with a lower solids loading such as, for example, less than 10%, about 6% or less, about 5% or less, or the like. In another aspect, scaled-up separation can be accomplished using lower centrifugation speeds and shorter centrifugation times. In an aspect, whereas bench-scale separations may take up to 1 hour of centrifugation time, the same centrifugation can be accomplished in less than 10 minutes, or in about 7 minutes, on a larger scale. In another aspect, whereas bench-scale separations may require centrifugation speeds up to 17,000 rcf, large scale separations can be accomplished at less than 10,000 rcf, or at about 6,000 rcf. In any of these aspects, optional additives such as sodium chloride, calcium chloride, and/or organic solvents as discussed above can be incorporated in order to enhance the separation process and increase product yields.

Target Proteins

[0031] Also disclosed herein are proteins purified by the disclosed method. In one aspect, the disclosed process can be particularly useful for purification of certain target proteins. In a further aspect, any protein that is found in a solid phase or as a solid aggregate after production can be subjected to the disclosed process including, but not limited to, inclusion bodies. In a still further aspect, hydrophobic proteins that are insoluble or form in aggregates in water or aqueous solutions, or inside intact cells in which they are expressed, can be purified by the disclosed process.

[0032] Also disclosed herein are target proteins produced by the disclosed method. In one aspect, the target protein produces a transparent, uniform, and freely flowing solution in DMSO. In another aspect, the target protein includes at least 5% tyrosine residues.

[0033] In one aspect, the target protein can have a sequence of Formula I:

$$A_1\text{-}E_2\text{-}P_2\text{-}(B_1\text{-}L_1\text{-}E_1\text{-}P_1)_n\text{-}B_1\text{-}L_3\text{-}E_3\text{-}P_3\text{-}G_1$$

Formula I,

wherein

A_1 is absent, is a methionine, or is an amino acid sequence 1 to 4 residues in length;

E_1 a GLY-rich amino acid sequence 8 to 58 residues in length including amino acids selected from the group consisting of glycine, leucine, tyrosine, phenylalanine, and proline, or any combination thereof;

B_1 is an ASTVH-rich sequence amino acid sequence 6 to 17 residues in length including amino acids selected from the group consisting of alanine, serine, threonine, valine, histidine, glycine, glutamine, and proline, or any combination thereof.

L_1 is absent or is an amino acid sequence 1 to 7 residues in length including amino acids selected from the group consisting of proline, glycine, leucine, serine, and threonine, or any combination thereof;

E_2 is absent or is E_1;

E_3 is absent or is E_1;

P_1 is absent or is proline;

P_2 is absent or is P_1;

P_3 is absent or is P_1;

L_3 is absent or is L_1;

G_1 is absent or is an amino acid sequence 1 to 4 residues in length; and

wherein n is 4 to 100.

[0034] The term ASTVH-rich sequence refers to a sequence that can comprise additional sequences and in a different order than a peptide of ASTVH. For example, in some embodiments, the ASTVH-rich sequence includes at least one alanine, at least one serine, at least one threonine, at least one valine, and at least one histidine. In some embodiments, the ASTVH-rich sequence includes two or more alanines. In some embodiments, the ASTVH-rich sequence includes two or more serines. In some embodiments, the ASTVH-rich sequence includes two or more threonines. In some embodiments, the ASTVH-rich sequence includes two or more valines. In some embodiments, the ASTVH-rich sequence includes two or more histidines. In one aspect, B_1 can be selected from one of SEQ ID NOs. 1-103.

[0035] In some embodiments, L_1 is absent or is an amino acid sequence 1 to 7 residues in length including amino acids selected from the group consisting of glycine, leucine, serine, and threonine, or any combination thereof, or L_1 can be or can be PST, PS, P, ST, or S or can have one of SEQ ID NOs. 236-242.

[0036] In still another aspect, the GLY-rich sequence can include at least one glycine, at least one leucine, and at least one tyrosine. In some embodiments, E_1 is an GLY-rich amino acid sequence 8 to 58 residues in length comprising amino acids selected from the group consisting of glycine, leucine, tyrosine, phenylalanine, and proline, or any combination thereof. In some embodiments, E_1 is a third amino sequence comprising a combination of two or more of glycine, leucine, tyrosine, phenylalanine, and proline, or can be selected from one of SEQ ID NOs. 119-235.

[0037] In some embodiments, G_1 is absent or is an amino acid sequence 1 to 4 residues in length. In some embodiments, G_1 is an amino acid sequence including serine and/or threonine. In some embodiments, G_1 is absent.

[0038] In some embodiments, n is in a range between 4-100, 4-90, 4-80, 4-70, 4-60, 4-50, 4-40, 4-30, 4-20, 4-10, 6-20, 8-20, 10-20, 10-30, 4-16, 6-16, 8-16, 10-16, 12-16, 4-12, 6-12, 8-12, or 10-12. In one aspect, n is 4. In another aspect, n is 12.

[0039] In an aspect, the target protein can be an aggregation-prone hydrophobic protein, a protein occurring in an inclusion body, or a natural or recombinant squid ring tooth protein. In an aspect, the target protein can have a sequence selected from SEQ ID NOs. 104-118, 243, and 244. In one aspect, the target protein can be or include any one of the recombinant squid ring tooth proteins identified by SEQ ID NOs. 104, 111, 112, 113, 117, or 118.

[0040] In another aspect, exemplary aggregation-prone hydrophobic proteins and/or proteins occurring in inclusion bodies can be selected from VEGF165 (examples include, but are not limited to, those disclosed in US Patent No. 9,994,612); IGF-1 (examples include, but are not limited to, those disclosed in US Patent No. 5,288,931); neublastin (examples include, but are not limited to, those disclosed in US Patent No. 8,969,042); IgG2 antibody fragment (examples include, but are not limited to, those disclosed in European Patent No. 1805320); transforming growth factor type β (TGF-β)-like (examples include, but are not limited to, those disclosed in US Patent No. 5,650,494); IL-29 (examples include, but are not limited to, those disclosed in US Patent No. 8,211,670); interleukin-2 (examples include, but are not limited to, those disclosed in US Patent No. 11,091,525); GDNF (examples include, but are not limited to, those disclosed in US Patent No. 7,226,758); memapsin (examples include, but are not limited to, those disclosed in US Patent No. 7,829,669); proNGF (examples include, but are not limited to, those disclosed in US Pre-Grant Publication No. 2018/0086805); pro-EP-B2 (examples include, but are not limited to, those disclosed in US Patent No. 8,148,105); IFN-alpha (examples include, but are not limited to, those disclosed in Japanese Patent No. 5861223); T-cell receptor (examples include, but are

not limited to, those disclosed in Japanese Patent No. 6186412); antibody Fc region (examples include, but are not limited to, those disclosed in US Patent No. 11,345,722); tenth fibronectin type III (10Fn3) domain (examples include, but are not limited to, those disclosed in US Patent No. 8,067,201); and human growth hormone (examples include, but are not limited to, those disclosed in US Patent No. 8,178,494).

[0041] Many modifications and other embodiments disclosed herein will come to mind to one skilled in the art to which the disclosed compositions and methods pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the disclosures are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. The skilled artisan will recognize many variants and adaptations of the aspects described herein. These variants and adaptations are intended to be included in the teachings of this disclosure and to be encompassed by the claims herein.

[0042] Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

[0043] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure.

[0044] Any recited method can be carried out in the order of events recited or in any other order that is logically possible. That is, unless otherwise expressly stated, it is in no way intended that any method or aspect set forth herein be construed as requiring that its steps be performed in a specific order. Accordingly, where a method claim does not specifically state in the claims or descriptions that the steps are to be limited to a specific order, it is no way intended that an order be inferred, in any respect. This holds for any possible non-express basis for interpretation, including matters of logic with respect to arrangement of steps or operational flow, plain meaning derived from grammatical organization or punctuation, or the number or type of aspects described in the specification.

[0045] All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention

is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided herein can be different from the actual publication dates, which can require independent confirmation.

[0046] While aspects of the present disclosure can be described and claimed in a particular statutory class, such as the system statutory class, this is for convenience only and one of skill in the art will understand that each aspect of the present disclosure can be described and claimed in any statutory class.

[0047] It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only and is not intended to be limiting. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosed compositions and methods belong. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the specification and relevant art and should not be interpreted in an idealized or overly formal sense unless expressly defined herein.

[0048] Prior to describing the various aspects of the present disclosure, the following definitions are provided and should be used unless otherwise indicated. Additional terms may be defined elsewhere in the present disclosure.

Definitions

[0049] As used herein, “comprising” is to be interpreted as specifying the presence of the stated features, integers, steps, or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps, or components, or groups thereof. Moreover, each of the terms “by”, “comprising,” “comprises”, “comprised of,” “including,” “includes,” “included,” “involving,” “involves,” “involved,” and “such as” are used in their open, non-limiting sense and may be used interchangeably. Further, the term “comprising” is intended to include examples and aspects encompassed by the terms “consisting essentially of” and “consisting of.” Similarly, the term “consisting essentially of” is intended to include examples encompassed by the term “consisting of.

[0050] As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a strong base,” “a target protein,” or “a precipitation agent,” include, but are not limited to, mixtures or combinations of two or more such strong bases, target proteins, or

precipitation agents, and the like.

[0051] It should be noted that ratios, concentrations, amounts, and other numerical data can be expressed herein in a range format. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms a further aspect. For example, if the value "about 10" is disclosed, then "10" is also disclosed.

[0052] When a range is expressed, a further aspect includes from the one particular value and/or to the other particular value. For example, where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the disclosure, e.g. the phrase "x to y" includes the range from 'x' to 'y' as well as the range greater than 'x' and less than 'y'. The range can also be expressed as an upper limit, e.g. 'about x, y, z, or less' and should be interpreted to include the specific ranges of 'about x', 'about y', and 'about z' as well as the ranges of 'less than x', less than y', and 'less than z'. Likewise, the phrase 'about x, y, z, or greater' should be interpreted to include the specific ranges of 'about x', 'about y', and 'about z' as well as the ranges of 'greater than x', greater than y', and 'greater than z'. In addition, the phrase "about 'x' to 'y'", where 'x' and 'y' are numerical values, includes "about 'x' to about 'y'".

[0053] It is to be understood that such a range format is used for convenience and brevity, and thus, should be interpreted in a flexible manner to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited. To illustrate, a numerical range of "about 0.1% to 5%" should be interpreted to include not only the explicitly recited values of about 0.1% to about 5%, but also include individual values (e.g., about 1%, about 2%, about 3%, and about 4%) and the sub-ranges (e.g., about 0.5% to about 1.1%; about 5% to about 2.4%; about 0.5% to about 3.2%, and about 0.5% to about 4.4%, and other possible sub-ranges) within the indicated range.

[0054] As used herein, the terms "about," "approximate," "at or about," and "substantially" mean that the amount or value in question can be the exact value or a value that provides equivalent results or effects as recited in the claims or taught herein. That is, it is understood that amounts,

sizes, formulations, parameters, and other quantities and characteristics are not and need not be exact, but may be approximate and/or larger or smaller, as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art such that equivalent results or effects are obtained. In some circumstances, the value that provides equivalent results or effects cannot be reasonably determined. In such cases, it is generally understood, as used herein, that "about" and "at or about" mean the nominal value indicated ±10% variation unless otherwise indicated or inferred. In general, an amount, size, formulation, parameter or other quantity or characteristic is "about," "approximate," or "at or about" whether or not expressly stated to be such. It is understood that where "about," "approximate," or "at or about" is used before a quantitative value, the parameter also includes the specific quantitative value itself, unless specifically stated otherwise.

[0055] As used herein, the term "effective amount" refers to an amount that is sufficient to achieve the desired modification of a physical property of the composition or material. For example, an "effective amount" of a strong base refers to an amount that is sufficient to achieve the desired improvement in the method step modulated by the strong base, e.g. achieving the desired level of cell lysis accompanied by increased solubility for the target protein. The specific level in terms of wt% in a composition required as an effective amount will depend upon a variety of factors including the amount and type of protein component, amount and type of cells being lysed, total biomass in solution, and scale of the purification reaction.

[0056] As used herein, the terms "optional" or "optionally" means that the subsequently described event or circumstance can or cannot occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

[0057] Unless otherwise specified, temperatures referred to herein are based on atmospheric pressure (i.e. one atmosphere).

[0058] Now having described the aspects of the present disclosure, in general, the following Examples describe some additional aspects of the present disclosure. While aspects of the present disclosure are described in connection with the following examples and the corresponding text and figures, there is no intent to limit aspects of the present disclosure to this description. On the contrary, the intent is to cover all alternatives, modifications, and equivalents included within the spirit and scope of the present disclosure.

EXAMPLES

[0059] The following examples are put forth so as to provide those of ordinary skill in the art with

a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the disclosure and are not intended to limit the scope of what the inventors regard as their disclosure. Efforts have been made to ensure accuracy with respect to numbers (*e.g.*, amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

Example 1: Process Optimization

[0060] Process parameters were varied to determine optimum and preferred conditions for protein purification.

Solids loading

[0061] Solids loading is defined as the ratio of biomass solids on a dry basis to aqueous alkaline solution. Solids loading percentages (w/v) of 5%, 10%, and 20% were tested.

pH 13.3 Step

[0062] In some experiments, the solution was adjusted to pH 13.3 for 1 hour at the beginning of the extraction, prior to adjustment to pH 12.8.

Mass Yield

[0063] Mass yield is defined as the dry-basis yield of product based on the input dry biomass.

Quality Test

[0064] 75 mg of dry product was mixed with 500 µL of neat DMSO and inverted slowly overnight in a capped tube at room temperature. The sample passes the quality test if the resulting solution is transparent, uniform, and freely flowing, while the sample fails the test if the resulting solution is opaque or exhibits any visible gelling.

Conclusions

[0065] Results of solids loading, pH 13.3 step experiments, and quality testing are presented in Table 1:

Table 1: Dependence of Product Yield and Quality on Solids Loading and pH Treatment During Alkaline Extraction

Solids Loading	pH 13.3 Step	Mass Yield	Quality Test
5%	**No**	**7.9%**	**Fail**
10%	**No**	**3.3%**	**Fail**

10%	**Yes**	**1.9%**	**Pass**
20%	**No**	**5.8%**	**Pass**

[0066] Solids loading of 10% or less results in samples failing quality tests associated with unacceptable purity for downstream processing, while solids loading of 20% or greater and/or performance of an additional extraction step at pH 13.3 result in samples passing quality tests.

Example 2: Process Scale-Up

[0067] Attempts to scale-up the disclosed process revealed tradeoffs related to the efficiency with which cell debris can be separated at various solids-loading levels. Scaled-up separation was challenging with 20% solids and improved separation under milder conditions such as lower centrifugation speeds and shorter separation times was needed. Several chemical additives were identified that could be applied before alkaline extraction or before cell-debris separation. These additives enable better product yields and quality in conjunction with lower biomass loading and milder separation conditions.

[0068] Additives tested were as follows, either alone or in combination:

1. NaCl, applied at final concentrations in the range of 0.6-1.3 M prior to alkaline extraction.
2. Solvents, including acetone and ethanol, at concentrations in the range of 8-50% v/v just prior to cell-debris separation
3. $CaCl_2$ up to a concentration of 0.4 M just prior to cell-debris separation

[0069] Various combinations of the above additives can be used to enable performance improvements including good product quality from biomass solids loading as low as 5-6% and higher product yields by a factor of 1.5-3× compared to the process described in Example 1. Centrifugation time was reduced from 1 hour (as in Example 1) to 7 minutes and centrifugation speed was reduced from 17,000 rcf (as in Example 1) to 6,000 rcf.

[0070] Exemplary combinations of additives are shown in Table 2:

Table 2: Additives for Scale-Up of Alkaline Extraction

Solids Loading %	Na Molarity	Ca Molarity	Solvent	Solvent % (v/v)	Mass Yield %	Quality Test[a]
6	1.23	0.00	Acetone	16.7	9.0	Pass
6	0.37	0.00	Acetone	50.0	18.0	Pass
5	0.66	0.37	Acetone	8.3	7.3	Pass
5	0.66	0.37	Ethanol	8.3	9.8	Pass
6	0.80	0.40	Acetone	8.0	11.2	Pass

[a] See Example 1 for a description of the Quality Test.

[0071] It should be emphasized that the above-described embodiments of the present disclosure are merely possible examples of implementations set forth for a clear understanding of the principles of the disclosure. Many variations and modifications may be made to the above-described embodiment(s) without departing substantially from the spirit and principles of the disclosure. All such modifications and variations are intended to be included herein within the scope of this disclosure and protected by the following claims.

CLAIMS

What is claimed is:

1. A method for separating a target protein from an intact cell, the method comprising:
    (a) optionally pretreating a solution comprising the intact cell at a pH greater than or equal to 13 for a period of time;
    (b) lysing the cell at a pH value greater than 12.5 to create a first solution that includes lysed cell components;
    (c) optionally, precipitating non-target molecules from the first solution;
    (d) removing cell debris and precipitates from the first solution;
    (e) decreasing solubility of the target protein in the first solution;
    (f) separating target protein solids from the first solution;
    (g) resuspending the target protein solids to create a second solution;
    (h) optionally, adjusting a pH of the second solution; and
    (i) separating target protein solids from the second solution.
2. The method of claim 1, further comprising contacting the solution comprising the intact cell before step (a) or (b) with one or more additives, or contacting the first solution before step (d) with one or more additives, or both
3. The method of claim 2, wherein the one or more additives comprise sodium chloride, an organic solvent, calcium chloride, or any combination thereof.
4. The method of claim 3, wherein the sodium chloride has a concentration of from about 0.37 M to about 1.23 M in the solution comprising the intact cell or in the first solution.
5. The method of claim 3, wherein the calcium chloride has a concentration of from about 0.37 M to about 0.40 M in the solution comprising the intact cell or in the first solution.
6. The method of claim 3, wherein the organic solvent comprises ethanol or acetone.
7. The method of claim 6, wherein the organic solvent is present at from about 8% (v/v) to about 50% (v/v) in the solution comprising the intact cell or in the first solution.
8. The method of any one of claims 1-7, wherein, in step (a), the pH higher than 13 is at least 13.4.

9. The method of any one of claims 1-7, wherein the period of time is from about 5 min to about 1 hour.

10. The method of any one of claims 1-7, wherein the cell is pretreated in step (a), lysed in step (b), or both using a strong base.

11. The method of claim 10, wherein the strong base comprises NaOH, KOH, $Ba(OH)_2$, $Ca(OH)_2$, LiOH, RbOH, CsOH, $Sr(OH)_2$, or any combination thereof.

12. The method of claim 10, wherein step (c) is carried out using a first precipitation agent, a first acid, or a combination thereof.

13. The method of claim 12, wherein the first precipitation agent comprises F^-, Cl^-, Br^-, I^-, CO_3^{2-}, HCO_3^-, SO_4^{2-}, HSO_4^-, PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, formate, acetate, Li^+, Na^+, K^+, Zn^{2+}, Al^{3+}, Fe^{3+}, Mg^{2+}, Ca^{2+}, NH_4^+, trehalose, glucose, proline, *tert*-butanol, trimethylamine N-oxide, ectoine, glycine betaine, 3-dimethylsulfoniopropionate, or any combination thereof.

14. The method of claim 12, wherein the first acid comprises sulfuric acid, phosphoric acid, carbonic acid, hydrochloric acid, or any combination thereof.

15. The method of any one of claims 1-7, wherein step (d) is accomplished by centrifugation, filtration, or any combination thereof.

16. The method of any one of claims 1-7, wherein step (e) is carried out using a second precipitation agent, a second acid, or a combination thereof.

17. The method of claim 16, wherein the second precipitation agent comprises F^-, Cl^-, Br^-, I^-, CO_3^{2-}, HCO_3^-, SO_4^{2-}, HSO_4^-, PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, formate, acetate, Li^+, Na^+, K^+, Zn^{2+}, Al^{3+}, Fe^{3+}, Mg^{2+}, Ca^{2+}, NH_4^+, trehalose, glucose, proline, *tert*-butanol, trimethylamine N-oxide, ectoine, glycine betaine, 3-dimethylsulfoniopropionate, or any combination thereof.

18. The method of claim 16, wherein the second acid comprises sulfuric acid, phosphoric acid, carbonic acid, hydrochloric acid, or any combination thereof.

19. The method of claim 16, wherein the second precipitation agent comprises ammonium sulfate.

20. The method of any one of claims 1-7, where pH is decreased to 10.2 in step (e).

21. The method of any one of claims 1-7, wherein the target protein solids are resuspended in water in step (g).

22. The method of any one of claims 1-7, wherein a pH of the second solution is decreased to about 8 in step (h).

23. The method of claim 22, wherein the pH of the solution is accomplished using 1 M HCl.

24. The method of any one of claims 1-7, wherein the first solution comprises from about 5% to about 20% (w/v) solids in an aqueous solution, wherein the solids comprise cell debris and the target protein.

25. The method of claim 24, wherein the first solution comprises 20% (w/v) solids in the aqueous solution.

26. The method of any one of claims 1-7, wherein a flocculant is not used during performance of the method.

27. The method of any one of claims 1-7, wherein the pH value in step (b) is greater than the isoelectric point of the target protein.

28. The method of any one of claims 1-7, wherein the target protein solids separated in step (i) are further washed with DI water, dried by lyophilization, dried by spray-drying, or any combination thereof.

29. A target protein produced by the method of any one of claims 1-7.

30. The target protein of claim 31, wherein the target protein produces a transparent, uniform, and freely flowing solution in DMSO.

31. The target protein of claim 29, wherein the target protein comprises at least 5% tyrosine residues.

32. The target protein of claim 29, wherein the target protein forms a solid aggregate in the intact cell.

33. The target protein of claim 29, wherein the target protein occurs in an inclusion body.

34. The target protein of claim 29, wherein the target protein comprises VEGF165; IGF-1; neublastin; IgG2 antibody fragment; transforming growth factor type β (TGF-β)-like; IL-29; interleukin-2; GDNF; memapsin; proNGF; pro-EP-B2; IFN-alpha; T-cell receptor; antibody Fc region; tenth fibronectin type III (10Fn3) domain; human growth hormone, or any combination thereof.

35. The target protein of claim 29, wherein the target protein has a sequence selected from one of SEQ ID NOs. 104-118, 243, or 244.

36. The target protein of claim 29, wherein the target protein comprises a natural or recombinant squid ring tooth protein.

37. The target protein of claim 36, wherein the natural or recombinant squid ring tooth protein has a sequence selected from one of SEQ ID NOs. 104, 111, 112, 113, 117, or 118.

38. The target protein of claim 29, wherein the target protein has a sequence of Formula I:

$$A_1\text{-}E_2\text{-}P_2\text{-}(B_1\text{-}L_1\text{-}E_1\text{-}P_1)_n\text{-}B_1\text{-}L_3\text{-}E_3\text{-}P_3\text{-}G_1$$

Formula I,

wherein

A_1 is absent, is a methionine, or is an amino acid sequence 1 to 4 residues in length;

E_1 a GLY-rich amino acid sequence 8 to 58 residues in length including amino acids selected from the group consisting of glycine, leucine, tyrosine, phenylalanine, and proline, or any combination thereof;

B_1 is an ASTVH-rich sequence amino acid sequence 6 to 17 residues in length including amino acids selected from the group consisting of alanine, serine, threonine, valine, histidine, glycine, glutamine, and proline, or any combination thereof.

L_1 is absent or is an amino acid sequence 1 to 7 residues in length including amino acids selected from the group consisting of proline, glycine, leucine, serine, and threonine, or any combination thereof;

E_2 is absent or is E_1;

E_3 is absent or is E_1;

P_1 is absent or is proline;

P_2 is absent or is P_1;

P_3 is absent or is P_1;

L_3 is absent or is L_1;

G_1 is absent or is an amino acid sequence 1 to 4 residues in length; and

wherein n is 4 to 100.

39. The target protein of claim 38, wherein L_1 is selected from PST, PS, P, ST, or S or SEQ ID NOs. 236-242.

40. The target protein of claim 38, wherein B_1 is selected from SEQ ID NOs. 1-103.
41. The target protein of claim 38, wherein E_1 comprises a combination of two or more of glycine, leucine, tyrosine, phenylalanine, and proline.
42. The target protein of claim 38, wherein E_1 is selected from SEQ ID NOs. 119-235.
43. The target protein of claim 38, wherein G_1 comprises serine, threonine, or a combination thereof.
44. The target protein of claim 38, wherein n is from 4 to 12.

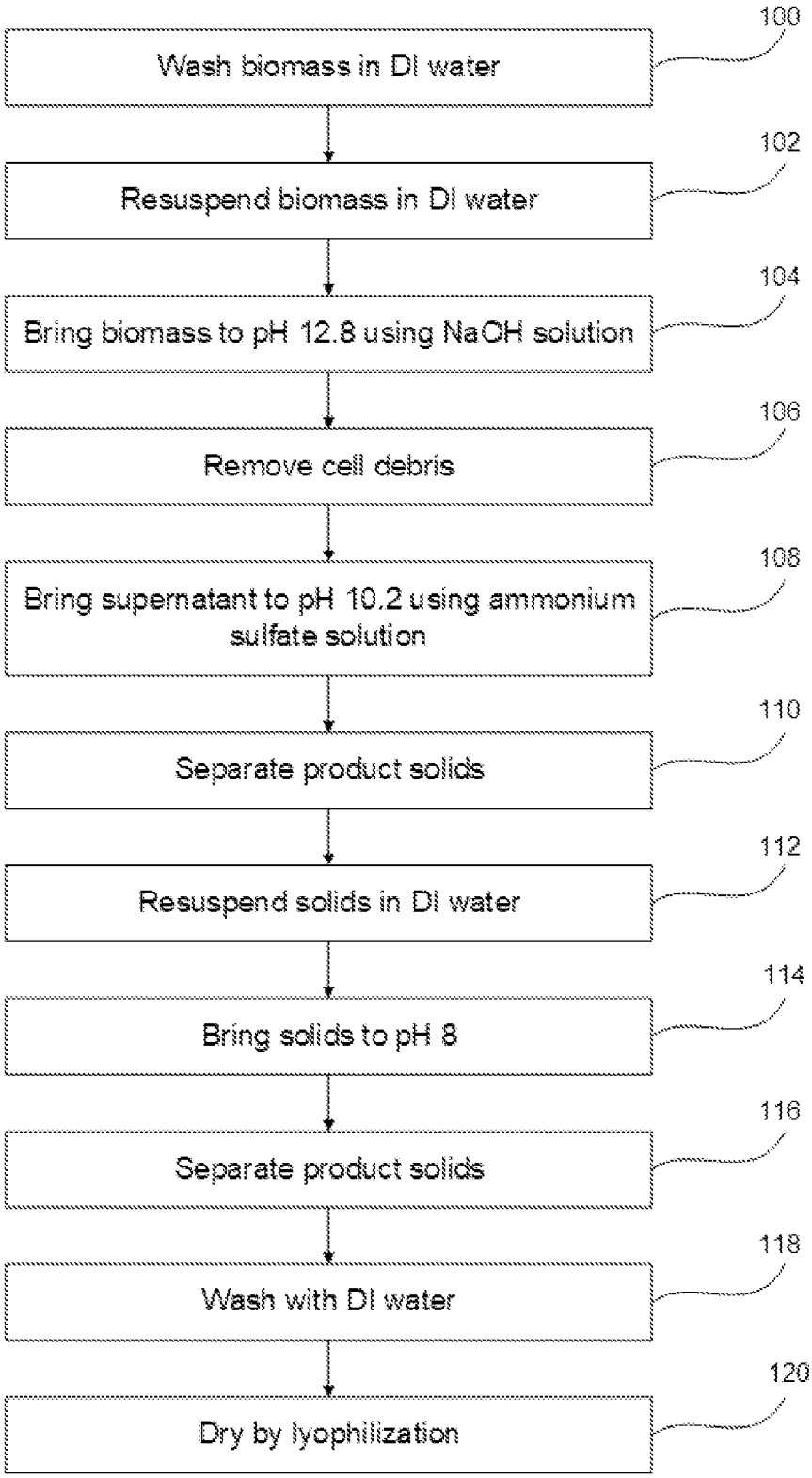
Wash biomass in DI water
100
Resuspend biomass in DI water
102
Bring biomass to pH 12.8 using NaOH solution
104
Remove cell debris
106
Bring supernatant to pH 10.2 using ammonium sulfate solution
108
Separate product solids
110
Resuspend solids in DI water
112
Bring solids to pH 8
114
Separate product solids
116
Wash with DI water
118
Dry by lyophilization
120

FIG. 1

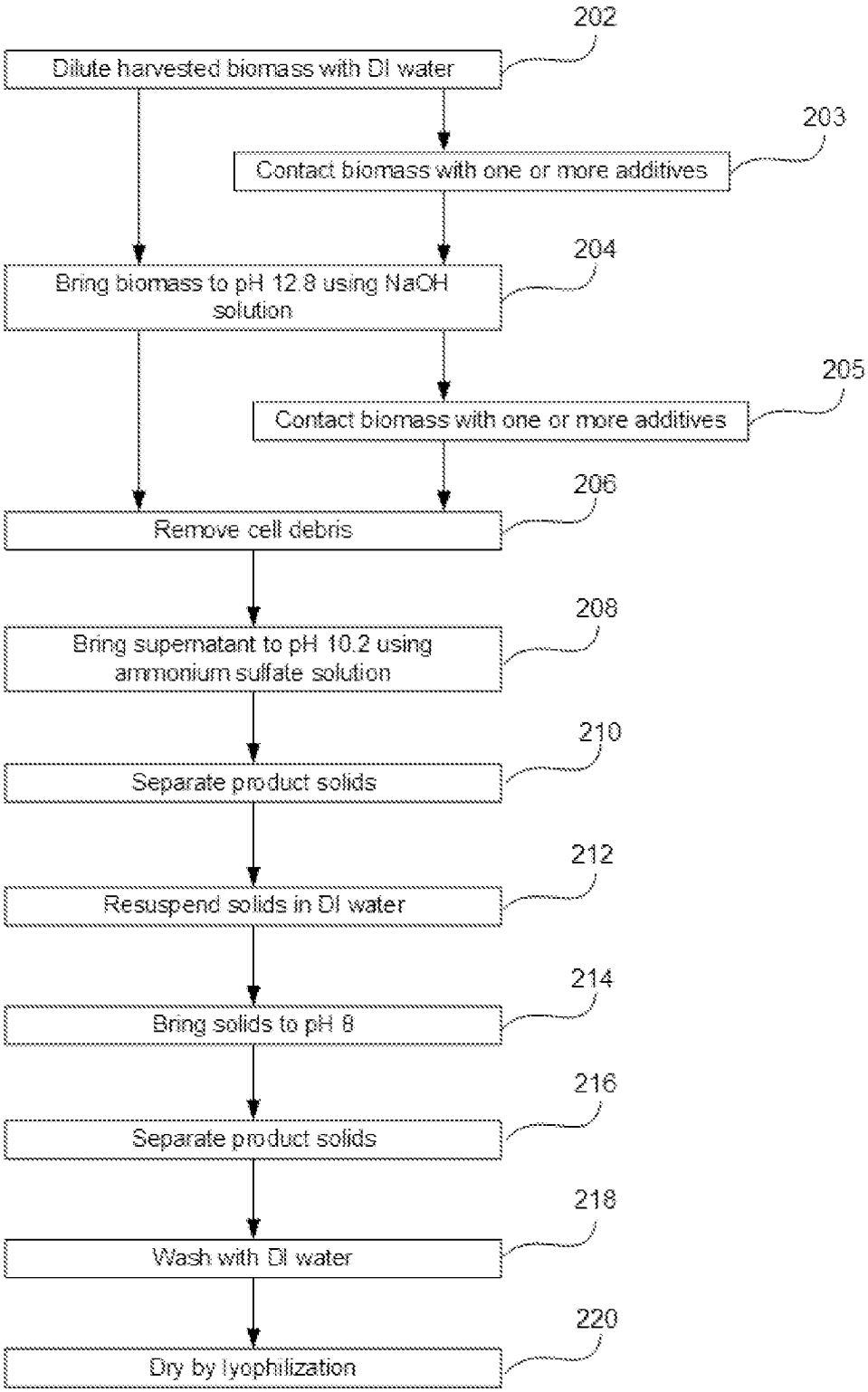
202
Dilute harvested biomass with DI water
203
Contact biomass with one or more additives
204
Bring biomass to pH 12.8 using NaOH solution
205
Contact biomass with one or more additives
206
Remove cell debris
208
Bring supernatant to pH 10.2 using ammonium sulfate solution
210
Separate product solids
212
Resuspend solids in DI water
214
Bring solids to pH 8
216
Separate product solids
218
Wash with DI water
220
Dry by lyophilization

FIG. 2