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DESCRIPTION

Reference to sequence listing

[0001] This application contains a Sequence Listing in computer readable form.

FIELD OF THE INVENTION

[0002] The present invention relates to sterile filtration of a liquid lactase preparation.

BACKGROUND OF THE INVENTION

[0003] Lactase enzymes, which are also sometimes referred to as beta-galactosidase enzymes, are used commercially to break down lactose in milk to produce dairy products which are suitable for people with lactose intolerance and/or have a sweeter taste. Lactases have been isolated from a large variety of organisms, including microorganisms, such as yeast and bacteria.

[0004] WO 2009/071539 discloses use of a C-terminally truncated fragment of the extracellular lactase from *Bifidobacterium bifidum* in the production of low-lactose dairy products.

[0005] Lactase may be added to milk either before or after pasteurisation or sterilization. In general lactase will be inactivated during pasteurisation or sterilization treatment. When lactase is added before sterilization, a large amount of lactase may be required in order to reduce the storage time between addition and pasteurisation/sterilization. Alternatively, lactase may be added after pasteurisation/sterilization of the milk. In this case, lactase may be added in a lower amount, as the enzyme can digest lactose during transport and storage in the factory, shop and/or in the refrigerator of the consumer. Lactase to be added after pasteurisation/sterilization of the milk has to be sterile. One preferred way of sterilizing lactase is by sterile filtration. Sterile filtration of the lactase may be performed in-line within the production process of the lactose-free milk, for example using the Aldose® system (Tetrapak International S.A., Pully, Switzerland), or it may be performed at the production site of the lactase manufacturer. The sterile lactase solution is then dosed aseptically into the milk, for instance by means of the Flexdos® system (Tetrapak International S.A., Pully, Switzerland). Sterile filtration of the lactase solution is achieved by pumping the enzyme concentrate through a at least one filter with an absolute pore size of 0.22 µm, and that will retain particles such as fungi, bacteria, spores still present in the solution. Prior to passing through the sterile filters, the solution may be pumped through one or several prefilters with a larger pore size than 0.22 µm and that will retain large particle but will not contribute to sterility of the lactase solution.

[0006] Commercial lactase preparations used by the dairy processing industry are often aqueous solution to which one or more stabilizing agents have been added, such as glycerol. Glycerol promotes microbial as well as enzymatic stabilization. Lactase preparations comprising, e.g., 50% glycerol are somewhat viscous and may not be treated easily through sterile filters.

[0007] Improved formulations of sterile lactase are still needed, e.g., liquid formulations which can be more easily processed through sterile filters.

[0008] WO2015/132349 discloses use of e.g. sodium chloride in an aqueous liquid lactase formulation. WO 2016/071500 discloses use of e.g. sodium chloride in a spray-dried composition of a beta-galactosidase enzyme. Possible ultrafiltration of the enzyme-containing fermentation broth is suggested as a possible means for concentrating the enzyme prior to its formulation. Sterile filtration is not mentioned in any of these disclosures.

[0009] US2011/212221 and EP0145092 both disclose sterile filtration of lactase enzyme present in 50wt% glycerol and ways to improve sterile filtration by means of chromatographic purification of the enzyme.

SUMMARY OF THE INVENTION

[0010] The present inventors have surprisingly found that addition of salt to a liquid lactase preparation improves its ability to be passed through a sterile filter. In particular, it is surprising that addition of monovalent cation to a liquid lactase preparation improves its ability to be passed through a sterile filter.

[0011] The invention therefore provides a sterile-filtered liquid lactase preparation comprising at least one salt having a monovalent cation, wherein the total concentration of said at least one salt is 0.01-5% w/w, and further comprising a polyol selected among glycerol, sorbitol, monopropylene glycol, sucrose, glucose, galactose or lactose, or combinations thereof, at a concentration of 10-70% w/w, preferably 30-60% w/w, wherein the concentration of lactase in the lactase preparation is between 10 and 5000 LAU-B/g and wherein the activity of the lactase (LAU-B) is determined as set forth in the description.

[0012] The invention further provides a method of sterile filtering a liquid lactase preparation, which comprises the steps of:

1. a. providing a liquid lactase preparation comprising at least one salt having a monovalent cation, wherein the total concentration of said at least one salt is 0.01-5% w/w, and further comprising a polyol selected among glycerol, sorbitol, monopropylene glycol, sucrose, glucose, galactose or lactose, or combinations thereof, at a concentration of 10-70% w/w, preferably 30-60% w/w, wherein the concentration of lactase in the lactase preparation is between 10 and 5000 LAU-B/g; and

2. b. sterile filtering said lactase preparation;

wherein the activity of the lactase (LAU-B) is determined as set forth in the description.

DETAILED DESCRIPTION OF THE INVENTION

[0013] The present invention provides a sterile-filtered liquid lactase preparation according to claim 1 comprising at least one salt having a monovalent cation, wherein the total concentration of said at least one salt is 0.01-5% w/w.

[0014] The salt may be any salt having a monovalent cation. The lactase preparation may comprise more than one salt having a monovalent cation. In that case, the feature of 0.01-5% w/w refers to the combined concentrations of the individual salts.

[0015] The skilled person will know how to determine the individual concentrations of each salt having a monovalent cation. It may be determined, e.g., by inductive coupled plasma optical emission spectrometry (ICP-OES). The associated anions may be determined by e.g. Dionex™ ion chromatography (Dionex™-IC; Thermo Scientific™, Waltham, MA, USA).

[0016] The liquid lactase preparation of the invention as set forth in claim 1 is an aqueous liquid lactase preparation and may comprise, e.g., at least 20% w/w of water, such as at least 30% w/w, at least 40% w/w or at least 50% w/w of water. "Sterile-filtered" in the context of the present invention means that the liquid lactase preparation has been passed through a sterile filter, preferably a sterile filter with an absolute pore size of about 0.22 µm. More preferably, the sterile filter is membrane-based and is manufactured using either hydrophilic polyvinylidene fluoride (PVDF) or hydrophilic polyethersulfone (PES).

[0017] The at least one salt may be a salt of Na⁺, K⁺ or NH₄⁺, preferably Na⁺ or K⁺, more preferably Na⁺. The at least one salt may be selected among NaCl, KCl, NH₄Cl, Na₂SO₄, K₂SO₄, (NH₄)₂SO₄, or a combination thereof, preferably, NaCl, KCl, (NH₄)₂SO₄, Na₂SO₄ or a combination thereof, more preferably NaCl.

[0018] The at least one salt having a monovalent cation may be a salt which is approved for use in food in at least EU.

[0019] The total final concentration of the at least one salt is at least 0.01% w/w, preferably at least 0.015% w/w, more preferably at least 0.02% w/w such as at least 0.1% w/w.

[0020] The total final concentration of the at least one salt is 0.01-5% w/w, preferably 0.015-4% w/w, more preferably 0.02-3% w/w or 0.1-2% w/w.

[0021] In further embodiments, the total final concentration of the at least one salt is in the

range from 0.01 - 5%, such as 0.01 -4.5%, for example 0.01 - 4.2%, such as 0.01 -3.5%, or for example 0.01 - 1.5 %; or is in the range from 0.01- 5%, such as 0.05- 5%, 0.1-5%, 0.5- 5%, 0.8- 5%, 1.2- 5%, 2- 5% or for example in the range from 0.5 -5%, such as 0.5 - 4%, or 0.6 - 3.8%, such as 0.7 -3.5%, for example 0.7- 3%, such as 0.7- 2.5% or for example 0.8 -2%, or 0.8 - 1.5% or for example 1.2- 3%, such as 1.3 - 3% or 1.3 to 2%, in particular 0.8 - 1.5% or 1.3 to 2%, all percentages being w/w.

[0022] The present invention further provides a sterile-filtered liquid lactase preparation as set forth in claim 1.

[0023] The salt is any salt having a monovalent cation. The lactase preparation may comprise more than one salt having a monovalent cation. Also in this case, the feature of 0.01-5% w/w refers to the combined concentrations of the individual salts.

[0024] The at least one salt having a monovalent cation may be a salt of Na⁺, K⁺ or NH₄⁺.

[0025] The at least one salt having a monovalent cation may be selected among NaCl, KCl, NH₄Cl, Na₂SO₄, K₂SO₄, (NH₄)₂SO₄, or a combination thereof, preferably, NaCl, KCl, (NH₄)₂SO₄, Na₂SO₄ or a combination thereof, more preferably NaCl.

[0026] The liquid lactase preparation according to the invention as set forth in claim 1 comprises at least one salt having a monovalent cation, and may further comprise at least one salt having a divalent cation.

[0027] A particular embodiment relates to a liquid lactase preparation according to the invention as set forth in claim 1 comprising at least one salt having a monovalent cation, and further comprising at least one salt having a divalent cation, preferably at least one salt of Mg⁺⁺.

[0028] The concentration of lactase in the lactase preparation of the invention is anywhere between 10 and 5000 LAU-B/g, such as 20-2600 LAU-B/g, 30-2000 LAU-B/g, 50-1300 LAU-B/g or 200-1300 LAU-B/g.

[0029] In the context of the present invention, the activity in LAU-B/g of a specific lactase may be determined by direct measurement of o-nitrophenyl (ONP) released from o-nitrophenyl β-D-galactopyranoside (ONPG) in a buffer containing 1.46 mg/ml substrate in 0.05 M MES, 1 mM MgSO₄ 7H₂O, 450 mg/L Brij 35 at pH6.5 and 30°C. After 600 seconds incubation, the reaction is stopped by adding 0.2 M Na₂CO₃ and the released ONP is measured at 405 nm after 126 seconds incubation. The skilled person will know how to execute this assay and determine such activity. Here, the activity is obtained by comparing to a standard curve run with a lactase of known activity, and the activity of the unknown sample calculated from this. The lactase of known activity may, e.g., be Saphera® obtained from Novozymes A/S, Denmark.

[0030] A lactase in the context of the present invention is any glycoside hydrolase having the ability to hydrolyse the disaccharide lactose into constituent galactose and glucose monomers. The group of lactases comprises but is not limited to enzymes assigned to subclass EC 3.2.1.108. Enzymes assigned to other subclasses, such as, e.g., EC 3.2.1.23, may also be lactases in the context of the present invention. A lactase in the context of the invention may have other activities than the lactose hydrolysing activity, such as for example a transgalactosylating activity. In the context of the invention, the lactose hydrolysing activity of the lactase may be referred to as its lactase activity or its beta-galactosidase activity.

[0031] The lactase may be of animal, of plant or of microbial origin. Preferred enzymes are obtained from microbial sources, in particular from a filamentous fungus or yeast, or from a bacterium.

[0032] In a preferred embodiment, the lactase is obtained from a bacterium, e.g. from the family Bifidobacteriaceae, such as from the genus *Bifidobacterium*, such as from a strain of *B. bifidum*, *B. animalis* or *B. longum*. In a more preferred embodiment, the lactase is obtained from *Bifidobacterium bifidum*.

[0033] In another preferred embodiment, the lactase is a C-terminally truncated form of a lactase obtained from the family Bifidobacteriaceae, such as from the genus *Bifidobacterium*, such as from a strain of *B. bifidum*, *B. animalis* or *B. longum*. In a more preferred embodiment, the lactase is a C-terminally truncated form of a lactase obtained from *Bifidobacterium bifidum*.

[0034] A preferred enzyme is a lactase having a sequence which is at least 50%, such as at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to SEQ ID NO: 1 or to a lactase active fragment thereof. Such lactase active fragment of SEQ ID NO: 1 may be any fragment of SEQ ID NO: 1 having lactase activity.

[0035] For purposes of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch (1970) J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al. (2000) Trends in Genetics 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labelled "longest identity" (obtained using the -no brief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

[0036] The lactase may have a C-terminal end corresponding to amino acid 1304 of SEQ ID NO: 1.

[0037] Alternatively, the lactase may be a fragment which is at least 50%, such as at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to a lactase active fragment of SEQ ID NO: 1. It may be a fragment having a C-terminal end corresponding to amino acid 887 of SEQ ID NO: 1. Or it may be a fragment having a C-terminal end corresponding to amino acid 965 of SEQ ID NO: 1. Or it may be a fragment having a C-terminal end corresponding to amino acid 1038 of SEQ ID NO: 1. Or it may be a fragment having a C-terminal end corresponding to amino acid 1142, 1211 or 1296 of SEQ ID NO: 1.

[0038] The lactase may be extracellular. It may have a signal sequence at its N-terminus, which is cleaved off during secretion.

[0039] The lactase may be obtained from any organism. The term "obtained from" means in this context that the enzyme may have been isolated from an organism where it is present natively, i.e. the identity of the amino acid sequence of the enzyme are identical to a native enzyme. The term "obtained" also means that the enzymes may have been produced recombinantly in a host organism, the recombinantly produced enzyme having either an identity identical to a native enzyme or having a modified amino acid sequence, e.g. having one or more amino acids which are deleted, inserted and/or substituted, i.e. a recombinantly produced enzyme which is a mutant and/or a fragment of a native amino acid sequence. Within the meaning of a native enzyme are included natural variants. Furthermore, the term "obtained from" includes enzymes produced synthetically by, e.g., peptide synthesis. The term "obtained from" also encompasses enzymes which have been modified e.g. by glycosylation, phosphorylation etc., whether *in vivo* or *in vitro*. With respect to recombinantly produced enzyme the term "obtained from" refers to the identity of the enzyme and not the identity of the host organism in which it is produced recombinantly.

[0040] The lactase may be produced from a microorganism by use of any suitable technique. For instance, a lactase enzyme preparation may be produced by fermentation of a suitable microorganism and subsequent isolation of a lactase preparation from the resulting fermented broth or microorganism by methods known in the art. The lactase may also be produced by use of recombinant DNA techniques. Such method normally comprises cultivation of a host cell transformed with a recombinant DNA vector comprising a DNA sequence encoding the lactase in question and the DNA sequence being operationally linked with an appropriate expression signal such that it is capable of expressing the lactase in a culture medium under conditions permitting the expression of the enzyme and recovering the enzyme from the culture. The DNA sequence may also be incorporated into the genome of the host cell. The DNA sequence may be of genomic, cDNA or synthetic origin or any combinations of these, and may be isolated or synthesized in accordance with methods known in the art.

[0041] The lactase may be purified. The term "purified" as used herein covers lactase enzyme protein essentially free from insoluble components from the production organism. The term "purified" also covers lactase enzyme protein essentially free from insoluble components from the native organism from which it is obtained. Preferably, it is also separated from some of the

soluble components of the organism and culture medium from which it is derived. More preferably, it is separated by one or more of the unit operations: filtration, precipitation, or chromatography.

[0042] Accordingly, the lactase may be purified, viz. only minor amounts of other proteins being present. The expression "other proteins" relate in particular to other enzymes. The term "purified" as used herein also refers to removal of other components, particularly other proteins and most particularly other enzymes present in the cell of origin of the lactase. The lactase may be "substantially pure", i.e. substantially free from other components from the organism in which it is produced, i.e., e.g., a host organism for recombinantly produced lactase.

[0043] The lactase in the preparation is preferably a neutral lactase. The neutral lactase preferably is active, or has its optimal activity, at a pH between pH 4 and pH 8, preferably between pH 5 and pH 7.

[0044] The lactase is preferably an extracellular produced neutral lactase.

[0045] The lactase in the preparation of the invention may be obtained as an optionally concentrated extract e.g. from a fungal, a yeast or a bacterial production strain. Preferably such extract has undergone a solid-liquid separation step, by e.g. centrifugation or filtration, to remove the solids. Such extract may contain natural components from the production cells as well as remainders of the fermentation media. Examples of such components are nucleic acid, protein, residual sugars, oligosaccharides, intrinsic enzymes, salts, minerals, intracellular components, ions, and nucleotides or other components. These components may end up, to some extent, in the lactase preparation of the invention.

[0046] The lactase in the preparation of the invention may be a concentrated non-purified lactase or it may have been purified. A concentrated non-purified lactase may have been obtained by releasing the enzyme from the host cells, removing the solids for instance by means of filtration or centrifugation and by concentrating the liquid phase comprising the lactase by means of for instance ultrafiltration. Alternatively, the lactase may have been purified, for example by using chromatography.

[0047] The pH of a lactase preparation of the invention may be in the range of pH 4-9, such as pH 5-8 or pH 5.5-7.5.

[0048] The lactase preparation of the invention is preferably enzymatically stable, more preferably microbially and enzymatically stable.

[0049] The lactase preparation of the invention further comprises a polyol at a concentration of 10-70% w/w, preferably 30-60% w/w, wherein the polyol is selected among glycerol, sorbitol, monopropylene glycol, sucrose, glucose, galactose or lactose, or combinations thereof, more preferably glycerol.

[0050] The lactase preparation to be used in a method of the invention as set forth in claim 9 may comprise more than one salt having a monovalent cation. In that case, the feature of at least 0.01-5% w/w refers to the combined concentrations of the individual salts.

[0051] A particular embodiment relates to a liquid lactase to be used in the method of the invention, said lactase comprising at least one salt having a monovalent cation, and further comprising at least one salt having a divalent cation, preferably at least one salt of Mg^{++} .

[0052] A particular embodiment relates to a liquid lactase to be used in the method of the invention as set forth in claim 9, said lactase comprising at least one salt having a monovalent cation, and at least one salt having a divalent cation, preferably at least one salt of Mg^{++} , and further comprising at least one stabilizing formulation as set forth in claim 9.

[0053] The concentration of lactase in the lactase preparation to be used in a method of the invention is anywhere between 10 and 5000 LAU-B/g, such as 20-2600 LAU-B/g, 30-2000 LAU-B/g, 50-1300 LAU-B/g or 200-1300 LAU-B/g.

[0054] In a preferred embodiment, the lactase to be used in a method of the invention is obtained from a bacterium, e.g. from the family Bifidobacteriaceae, such as from the genus *Bifidobacterium*, such as from a strain of *B. bifidum*, *B. animalis* or *B. longum*. In a more preferred embodiment, the lactase is obtained from *Bifidobacterium bifidum*.

[0055] In another preferred embodiment, the lactase is a C-terminally truncated form of a lactase obtained from the family Bifidobacteriaceae, such as from the genus *Bifidobacterium*, such as from a strain of *B. bifidum*, *B. animalis* or *B. longum*. In a more preferred embodiment, the lactase is a C-terminally truncated form of a lactase obtained from *Bifidobacterium bifidum*.

[0056] A preferred enzyme is a lactase having a sequence which is at least 50%, such as at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to SEQ ID NO: 1 or to a lactase active fragment thereof. Such lactase active fragment of SEQ ID NO: 1 may be any fragment of SEQ ID NO: 1 having lactase activity.

[0057] The lactase may have a C-terminal end corresponding to amino acid 1304 of SEQ ID NO: 1. The lactase may be extracellular. It may have a signal sequence at its N-terminus, which is cleaved off during secretion.

[0058] The lactase in the preparation to be used in a method of the invention is preferably a neutral lactase. The neutral lactase preferably is active, or has its optimal activity, at a pH between pH 4 and pH 8, preferably between pH 5 and pH 7.

[0059] The lactase to be used in a method of the invention is preferably an extracellular produced neutral lactase.

[0060] The lactase preparation to be used in a method of the invention comprises 10-70% w/w, preferably 30-60% w/w polyol selected among glycerol, sorbitol, monopropylene glycol, sucrose, glucose, galactose or lactose, more preferably glycerol.

[0061] Sterile filtering of the lactase preparation may be performed according to standard operational procedures known to the skilled person either by keeping the feed flow of the lactase solution to the sterile filter constant, or by keeping the transmembrane pressure (TMP) in the filter system constant; the filtration regime with a constant TMP should be preferred, following general recommendations from filter manufacturers. When performing a sterile filtration under constant TMP regime and as the filter accumulates unwanted particles larger than the filter cut-off, the feed flow of enzyme solution to the filter will have to be gradually decreased according to procedures known to the skilled person.

[0062] Sterile filtration may be performed in-line within a production process of a dairy product. Alternatively, sterile filtration may be performed prior to addition of the lactase preparation to the dairy product, in which case the sterile lactase solution is dosed aseptically into the milk.

[0063] The lactase preparation obtained from the enzyme supplier may be diluted with, e.g., water prior to its addition to the dairy product. If sterile filtration is performed in-line within a production process of a dairy product, the lactase preparation obtained from the enzyme supplier may be diluted prior to the sterile filtration. If the salt concentration in the lactase preparation obtained from the enzyme supplier is sufficiently high, even after dilution enough salt will be present to improve the sterile filterability.

[0064] A lactase preparation to be used in a method of the invention has an improved filterability compared to a second liquid lactase preparation having the same composition except that it does not comprise the at least one salt.

[0065] Improved filterability may be determined as described in Example 1 of the present application. In one embodiment, "improved filterability" means that the amount of sterile lactase obtained per a defined unit of time, under constant TMP filtration regime or constant feed flow filtration regime, is relatively larger than for a standard liquid lactase preparation.

[0066] In one embodiment, the lactase preparation to be used in a method of the invention has an improved filterability compared to a second liquid lactase preparation having the same composition except that it does not comprise the at least one salt, where improved filterability is defined as a larger amount of filtrate obtained per a defined unit of time, e.g. 600 seconds, and under either constant TMP or constant feed flow filtration regime compared to the second liquid lactase preparation.

[0067] In another embodiment, the lactase preparation to be used in a method of the invention has an improved filterability compared to a second liquid lactase preparation having the same composition except that it does not comprise the at least one salt, where improved filterability is defined as a larger amount of filtrate obtained per a defined unit of time, e.g. 600 seconds, and

under constant TMP filtration regime compared to the second liquid lactase preparation.

[0068] In another embodiment, the lactase preparation to be used in a method of the invention has an improved filterability compared to a second liquid lactase preparation having the same composition except that it does not comprise the at least one salt, where improved filterability is defined as a larger amount of filtrate obtained per a defined unit of time, e.g. 600 seconds, and under constant feed flow filtration regime compared to the second liquid lactase preparation.

[0069] Also disclosed is a method (not according to the claims, present for illustrative purposes only) of sterile filtering a liquid lactase preparation, which comprises the steps of:

1. a. providing a liquid lactase preparation,
2. b. adding to said liquid lactase preparation at least one salt having a monovalent or divalent cation, wherein the at least one salt is added at a concentration of at least 0.01% w/w of the lactase preparation which is to be sterile filtered, and
3. c. sterile filtering the lactase preparation obtained in step b.

[0070] In said step b. at least one salt is added to the lactase preparation at a concentration of at least 0.01% w/w of the lactase preparation which is to be sterile filtered, the at least 0.01% w/w refers to the amount of added salt and is not including any possible carry-over from production of the lactase.

[0071] Also disclosed is a method (not according to the claims, present for illustrative purposes only) of improving the filterability of a liquid lactase preparation, which comprises addition to the lactase preparation of at least one salt selected among NaCl, KCl, NH_4Cl , MgCl_2 , Na_2SO_4 , K_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 or a combination thereof, preferably, NaCl, KCl, MgCl_2 , $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 or a combination thereof, wherein the salt is added at a concentration of at least 0.01% w/w of the lactase preparation.

[0072] Also disclosed is a use (not according to the claims, present for illustrative purposes only) of at least one salt selected among NaCl, KCl, NH_4Cl , MgCl_2 , Na_2SO_4 , K_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$, MgSO_4 or a combination thereof, preferably, NaCl, KCl, MgCl_2 , $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 or a combination thereof, for improving the filterability of a liquid lactase preparation.

[0073] Also disclosed is a method (not according to the claims, present for illustrative purposes only) of sterile filtering a liquid lactase preparation, which comprises the steps of:

1. a. providing a liquid lactase preparation,
2. b. adding to said liquid lactase preparation at least one salt having a monovalent cation, wherein the at least one salt is added at a concentration of at least 0.01% w/w of the lactase preparation which is to be sterile filtered, and
3. c. sterile filtering the lactase preparation obtained in step b.

[0074] In said step b. at least one salt is added to the lactase preparation at a concentration of at least 0.01% w/w of the lactase preparation which is to be sterile filtered, the at least 0.01% w/w refers to the amount of added salt and is not including any possible carry-over from production of the lactase.

EXAMPLE 1

Improved filterability of *Bifidobacterium bifidum* lactase through sterile filter as a function of salt content (only compositions with added monovalent cations are according to the invention):

[0075] An unstandardized enzyme concentrate of *B. bifidum* lactase (SEQ ID NO: 1) expressed in *Bacillus licheniformis* and that had been produced in large scale was chosen as start material. The unstandardized concentrate had been originally formulated in large scale with 66% w/w glycerol at pH 5.7. The total sodium, potassium, magnesium, chloride and sulphate ions were measured in the unstandardized concentrate by means of ICP-OES and Dionex™ IC, respectively.

Method	Total ion concentration in the unstandardized concentrate [mg/kg]
Dionex™ IC for total chloride	123
Dionex™ IC for total sulphate	Below detection limit of 100
ICP-OES for total magnesium	17
ICP-OES for total potassium	50
ICP-OES for total sodium	42

[0076] The concentrate was then diluted with glycerol and water in absence or presence of different salts down to an enzyme concentration equivalent to 900 LAU-B/g lactase units: the unstandardized lactase concentrate was first pre-diluted with pure glycerol prior to adding salt as powder; a control sample did not contain added salt. Water was eventually added to a final volume of 1L, as described in the table here below. The final glycerol concentration was 50% w/w in all samples. Upon dilution of the unstandardized lactase concentrate the cations and anions present in the concentrate were diluted so extensively that the total amount of ions leftover in the diluted samples was very low to negligible compared to the different salt added

voluntarily in this experiment.

Samples	Unstandardized lactase concentrate + glycerol [g]	Salt [g]	Cold tap water [g]
900 LAU-B/g (control)	575	0	425
900 LAU-B/g + 0.1 % w/w NaCl	575	1	424
900 LAU-B/g + 1 % w/w NaCl	575	10	415
900 LAU-B/g + 2 % w/w NaCl	575	20	405
900 LAU-B/g + 1 % w/w Na ₂ SO ₄	575	10	415
900 LAU-B/g + 1 % w/w (NH ₄) ₂ SO ₄	575	10	415
900 LAU-B/g + 1 % w/w MgCl ₂ , 6 H ₂ O	575	10	415
900 LAU-B/g + 1 % w/w KCl	575	10	415

[0077] The unstandardized lactase concentrate and the pure glycerol solution were both kept at temperatures between 5°C and 10°C prior to start. All salts tested here were food grade quality and supplied by Sigma Aldrich Corp. (St. Louis, MO, USA). Thorough mixing was achieved with a magnetic stir bar under cold conditions (5-10°C).

[0078] All diluted enzyme solutions were then sterile filtered on 47 mm Fluorodyne® II DFL membrane filter discs (Pall, Port Washington, NY, USA) with a cutoff of 0.2 µm. The filter discs were maintained in a 47 mm inline stainless steel filter holder (Sartorius, Göttingen, Germany) and the diluted enzyme solutions were pumped through the filters by means of a FilterTec normal flow filtration system (Parker Hannifin Corp., Oxnard, CA, USA) under constant flow regime of 10 ml/min and until the system pressure reached a maximum of 1.8 bar. The filtrations were carried out at room temperature, within 30-60 min after diluting the unstandardized enzyme concentrate.

[0079] The table here below lists the volume $V_{\max}$ of the different solutions that were needed to reach an arbitrary, maximum pressure set point of 1.8 bar in the filtration system. The volume $V_{\max}$ of the "control" sample was set to 100%; the volumes for the other solutions are therefore proportional to the base 100.

Samples	$V_{\max}$ of filtrate to reach a system pressure of 1.8 bar [%]
900 LAU-B/g (control)	100
900 LAU-B/g + 0.1 % w/w NaCl	127

Samples	$V_{\max}$ of filtrate to reach a system pressure of 1.8 bar [%]
900 LAU-B/g + 1 % w/w NaCl	122
900 LAU-B/g + 2 % w/w NaCl	116
900 LAU-B/g + 1% w/w Na ₂ SO ₄	115
900 LAU-B/g + 1 % w/w (NH ₄) ₂ SO ₄	132
900 LAU-B/g + 1% w/w MgCl ₂ , 6 H ₂ O	135
900 LAU-B/g + 1 % w/w KCl	148

[0080] All 900 LAU-B/g lactase solutions containing salt tested here showed a measurable improvement in filterability through a sterile filter compared to the control sample: the volume $V_{\max}$ of lactase solution needed to reach the maximum pressure set point was higher in all accounts.

EXAMPLE 2

Improved filterability of *Bifidobacterium bifidum* lactase through sterile filter under very low salt conditions.

[0081] An unstandardized enzyme concentrate of *B. bifidum* lactase (SEQ ID NO: 1) expressed in *Bacillus licheniformis* and that had been produced in large scale was chosen as start material. The unstandardized concentrate had been originally formulated in large scale with 66 % w/w glycerol at pH 5.7.

[0082] The concentrate was then diluted with glycerol and water in absence or presence of sodium chloride down to an enzyme concentration equivalent to 900 LAU-B/g lactase units per gram: the unstandardized lactase concentrate was first pre-diluted with pure glycerol prior to adding salt as powder; a control sample did not contain added salt. Water was eventually added to a final volume of 1L, as described in the table here below. The final glycerol concentration was 50% w/w in all samples. Upon dilution of the unstandardized lactase concentrate the cations and anions present were diluted so extensively that the total amount of ions leftover in the samples were very low to negligible compared to the different salt added voluntarily during this experiment.

Samples	Unstandardized lactase concentrate + glycerol [g]	Salt [g]	Cold tap water [g]
900 LAU-B/g (control)	575	0	425
900 LAU-B/g + 0.01 % w/w NaCl	575	0.1	424.9
900 LAU-B/g + 0.1 % w/w NaCl	575	1	424

[0083] The unstandardized lactase concentrate and the pure glycerol solution were both kept at temperatures around 10 °C to 15 °C prior to start. All salts tested here were food grade quality and supplied by Sigma Aldrich Corp. (St. Louis, MO, USA). Thorough mixing was achieved with a magnetic stir bar under mildly cold conditions (15 °C) in a water bath.

[0084] All diluted enzyme solutions were then sterile filtered on 47 mm Fluorodyne® II DFL membrane filter discs (Pall, Port Washington, NY, USA) with a cutoff of 0.2 µm. The filter discs were maintained in a 47 mm inline stainless steel filter holder (Sartorius, Göttingen, Germany) and the diluted enzyme solutions were pumped through the filters by means of a FilterTec normal flow filtration system (Parker Hannifin Corp., Oxnard, CA, USA) under constant flow regime of 10 ml/min and until the system pressure reached a maximum of 1.8 bar. The filtrations were carried out at room temperature, within 30-60 min after diluting the unstandardized enzyme concentrate.

[0085] The table here below lists the volume $V_{\max}$ of the different solutions that was needed to reach an arbitrary, maximum pressure set point of 1.8 bar in the filtration system. The volume $V_{\max}$ of the "control" sample was set to 100%; the volumes for the other solutions are therefore proportional to the base 100.

Samples	$V_{\max}$ of filtrate to reach a system pressure of 1.8 bar [%]
900 LAU-B/g (control)	100
900 LAU-B/g + 0.01 % w/w NaCl	105
900 LAU-B/g + 0.1 % w/w NaCl	120

[0086] The addition of a very low sodium chloride amount (0.01% w/w) to the lactase concentrate showed already a measurable improvement in filterability through a sterile filter compared to the control sample. The $V_{\max}$ % for the sample containing 0.1% w/w NaCl is very close to the one observed in Example 1.

SEQUENCE LISTING

[0087]

<110> Novozymes A/S

<120> Lactase preparation

<130> 14400-WO-PCT

<160> 1

<170> PatentIn version 3.5

<210> 1

<211> 1304

<212> PRT

<213> Bifidobacterium bifidum

<400> 1

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Pro	Glu	Val	Val	Tyr	Ser	Ser	Ala	Val	Asp	Ser	Lys	Gln	Asn	Arg	Thr
		20						25					30		

Ser	Asp	Phe	Asp	Ala	Asn	Trp	Lys	Phe	Met	Leu	Ser	Asp	Ser	Val	Gln
		35					40					45			

Ala	Gln	Asp	Pro	Ala	Phe	Asp	Asp	Ser	Ala	Trp	Gln	Gln	Val	Asp	Leu
	50					55					60				

Pro	His	Asp	Tyr	Ser	Ile	Thr	Gln	Lys	Tyr	Ser	Gln	Ser	Asn	Glu	Ala
65					70					75					80

Glu	Ser	Ala	Tyr	Leu	Pro	Gly	Gly	Thr	Gly	Trp	Tyr	Arg	Lys	Ser	Phe
				85					90					95	

Thr	Ile	Asp	Arg	Asp	Leu	Ala	Gly	Lys	Arg	Ile	Ala	Ile	Asn	Phe	Asp
			100					105					110		

Gly	Val	Tyr	Met	Asn	Ala	Thr	Val	Trp	Phe	Asn	Gly	Val	Lys	Leu	Gly
		115					120					125			

Thr	His	Pro	Tyr	Gly	Tyr	Ser	Pro	Phe	Ser	Phe	Asp	Leu	Thr	Gly	Asn
	130						135				140				

Ala	Lys	Phe	Gly	Gly	Glu	Asn	Thr	Ile	Val	Val	Lys	Val	Glu	Asn	Arg
145					150					155					160

Leu	Pro	Ser	Ser	Arg	Trp	Tyr	Ser	Gly	Ser	Gly	Ile	Tyr	Arg	Asp	Val
				165					170					175	

Thr	Leu	Thr	Val	Thr	Asp	Gly	Val	His	Val	Gly	Asn	Asn	Gly	Val	Ala
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180	185	190
Ile Lys Thr Pro Ser Leu Ala Thr Gln Asn Gly Gly Asn Val Thr Met 195 200 205		
Asn Leu Thr Thr Lys Val Ala Asn Asp Thr Lys Ala Ala Ala Asn Ile 210 215 220		
Thr Leu Lys Gln Thr Val Phe Pro Lys Gly Gly Lys Thr Asp Ala Ala 225 230 235 240		
Ile Gly Thr Val Thr Thr Ala Ser Lys Ser Ile Ala Ala Gly Ala Ser 245 250 255		
Ala Asp Val Thr Ser Thr Ile Thr Ala Ala Ser Pro Lys Leu Trp Ser 260 265 270		
Ile Lys Asn Pro Asn Leu Tyr Thr Val Arg Thr Glu Val Leu Asn Gly 275 280 285		
Gly Lys Val Leu Asp Thr Tyr Asp Thr Glu Tyr Gly Phe Arg Trp Thr 290 295 300		
Gly Phe Asp Ala Thr Ser Gly Phe Ser Leu Asn Gly Glu Lys Val Lys 305 310 315 320		
Leu Lys Gly Val Ser Met His His Asp Gln Gly Ser Leu Gly Ala Val 325 330 335		
Ala Asn Arg Arg Ala Ile Glu Arg Gln Val Glu Ile Leu Gln Lys Met 340 345 350		
Gly Val Asn Ser Ile Arg Thr Thr His Asn Pro Ala Ala Lys Ala Leu 355 360 365		
Ile Asp Val Cys Asn Glu Lys Gly Val Leu Val Val Glu Glu Val Phe 370 375 380		
Asp Met Trp Asn Arg Ser Lys Asn Gly Asn Thr Glu Asp Tyr Gly Lys 385 390 395 400		
Trp Phe Gly Gln Ala Ile Ala Gly Asp Asn Ala Val Leu Gly Gly Asp 405 410 415		
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Asp Arg Asn Ala Pro Ser Val Ile Met Trp Ser Leu Gly Asn Glu Met 435 440 445		
Met Glu Gly Ile Ser Gly Ser Val Ser Gly Phe Pro Ala Thr Ser Ala 450 455 460		
Lys Leu Val Ala Trp Thr Lys Ala Ala Asp Ser Thr Arg Pro Met Thr 465 470 475 480		
Tyr Gly Asp Asn Lys Ile Lys Ala Asn Trp Asn Glu Ser Asn Thr Met 485 490 495		

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480              490              495
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500              505              510

Asp Gly Ala Asn Tyr Asp Lys Ile Arg Thr Thr His Pro Ser Trp Ala
515              520              525

Ile Tyr Gly Ser Glu Thr Ala Ser Ala Ile Asn Ser Arg Gly Ile Tyr
530              535              540

Asn Arg Thr Thr Gly Gly Ala Gln Ser Ser Asp Lys Gln Leu Thr Ser
545              550              555

Tyr Asp Asn Ser Ala Val Gly Trp Gly Ala Val Ala Ser Ser Ala Trp
565              570              575

Tyr Asp Val Val Gln Arg Asp Phe Val Ala Gly Thr Tyr Val Trp Thr
580              585              590

Gly Phe Asp Tyr Leu Gly Glu Pro Thr Pro Trp Asn Gly Thr Gly Ser
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Gly Ala Val Gly Ser Trp Pro Ser Pro Lys Asn Ser Tyr Phe Gly Ile
610              615              620

Val Asp Thr Ala Gly Phe Pro Lys Asp Thr Tyr Tyr Phe Tyr Gln Ser
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Gln Trp Asn Asp Asp Val His Thr Leu His Ile Leu Pro Ala Trp Asn
645              650              655

Glu Asn Val Val Ala Lys Gly Ser Gly Asn Asn Val Pro Val Val Val
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Tyr Thr Asp Ala Ala Lys Val Lys Leu Tyr Phe Thr Pro Lys Gly Ser
675              680              685

Thr Glu Lys Arg Leu Ile Gly Glu Lys Ser Phe Thr Lys Lys Thr Thr
690              695              700

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705              710              715

Thr Ala His Lys Asn Met Tyr Leu Thr Trp Asn Val Pro Trp Ala Glu
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740              745              750

Glu Gly Ser Thr Glu Gly Asn Ala Ser Val Thr Thr Thr Gly Lys Ala
755              760              765

Ala Lys Leu Lys Ala Asp Ala Asp Arg Lys Thr Ile Thr Ala Asp Gly
770              775              780

Lys Asp Leu Ser Tyr Ile Glu Val Asp Val Thr Asp Ala Asn Gly His
785              790              795

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Gly Lys Leu Val Gly Val Asp Asn Gly Ser Ser Pro Asp His Asp Ser
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Tyr Gln Ala Asp Asn Arg Lys Ala Phe Ser Gly Lys Val Leu Ala Ile
      835                      840                      845

Val Gln Ser Thr Lys Glu Ala Gly Glu Ile Thr Val Thr Ala Lys Ala
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Asp Gly Leu Gln Ser Ser Thr Val Lys Ile Ala Thr Thr Ala Val Pro
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Gly Thr Ser Thr Glu Lys Thr Val Arg Ser Phe Tyr Tyr Ser Arg Asn
      885                      890                      895

Tyr Tyr Val Lys Thr Gly Asn Lys Pro Ile Leu Pro Ser Asp Val Glu
      900                      905                      910

Val Arg Tyr Ser Asp Gly Thr Ser Asp Arg Gln Asn Val Thr Trp Asp
      915                      920                      925

Ala Val Ser Asp Asp Gln Ile Ala Lys Ala Gly Ser Phe Ser Val Ala
      930                      935                      940

Gly Thr Val Ala Gly Gln Lys Ile Ser Val Arg Val Thr Met Ile Asp
      945                      950                      955                      960

Glu Ile Gly Ala Leu Leu Asn Tyr Ser Ala Ser Thr Pro Val Gly Thr
      965                      970                      975

Pro Ala Val Leu Pro Gly Ser Arg Pro Ala Val Leu Pro Asp Gly Thr
      980                      985                      990

Val Thr Ser Ala Asn Phe Ala Val Asp Trp Thr Lys Pro Ala Asp Thr
      995                      1000                      1005

Val Tyr Asn Thr Ala Gly Thr Val Lys Val Pro Gly Thr Ala Thr
      1010                      1015                      1020

Val Phe Gly Lys Glu Phe Lys Val Thr Ala Thr Ile Arg Val Gln
      1025                      1030                      1035

Arg Ser Gln Val Thr Ile Gly Ser Ser Val Ser Gly Asn Ala Leu
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Arg Leu Thr Gln Asn Ile Pro Ala Asp Lys Gln Ser Asp Thr Leu
      1055                      1060                      1065

Asp Ala Ile Lys Asp Gly Ser Thr Thr Val Asp Ala Asn Thr Gly
      1070                      1075                      1080

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Gly Gly Ala Asn Pro Ser Ala Trp Thr Asn Trp Ala Tyr Ser Lys
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 Gln Gln Leu Gly Gln Ile Val Met Tyr Phe Phe Arg Asp Ser Asn
 1115 1120 1125
 Ala Val Arg Phe Pro Asp Ala Gly Lys Thr Lys Ile Gln Ile Ser
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 1145 1150 1155
 Ala Ala Gln Glu Ser Ser Glu Arg Val Lys Pro Tyr Thr Tyr Asp
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 1175 1180 1185
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 Glu Ile Glu Leu Lys Thr Ala Thr Ser Lys Phe Val Thr Asn Thr
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 Asp Ser Val Leu Ala Ala Gly Ser Tyr Asn Thr Pro Ala Ile Ile
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 Asp His Val Thr Arg Lys Thr Phe Thr Ile Asn Leu Gly Thr Glu
 1280 1285 1290
 Gln Glu Phe Pro Ala Asp Ser Asp Glu Arg Asp
 1295 1300

REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patent documents cited in the description

- [WO2009071539A](#) [0004]
- [WO2015132349A](#) [0008]
- [WO2016071500A](#) [0008]
- [US2011212221A](#) [0009]
- [EP0145092A](#) [0009]

Non-patent literature cited in the description

- NEEDLEMANWUNSCHJ. Mol. Biol., 1970, vol. 48, 443-453 [0035]
- RICE et al.EMBOSS: The European Molecular Biology Open Software SuiteTrends in Genetics, 2000, vol. 16, 276-277 [0035]

PATENTKRAV

1. Sterilfiltreret flydende lactasepræparat, som omfatter mindst ét salt med en monovalent kation, hvor den samlede koncentration af det mindst ene salt er 0,01-5% vægt/vægt, og som endvidere omfatter en polyol valgt blandt glycerol, sorbitol, monopropylenglycol, saccharose, glucose, galactose eller lactose eller kombinationer deraf i en koncentration på 10-70% vægt/vægt, fortrinsvis 30-60% vægt/vægt, hvor koncentrationen af lactase i lactasepræparatet er på mellem 10 og 5000 LAU-B/g, og hvor aktiviteten af lactasen (LAU-B) bestemmes som anført i beskrivelsen.
2. Flydende lactasepræparat ifølge krav 1, hvor saltet er et salt af Na^+ , K^+ eller NH_4^+ .
3. Flydende lactasepræparat ifølge krav 1, hvor saltet er valgt blandt NaCl , KCl , NH_4Cl , Na_2SO_4 , K_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$ eller en kombination deraf, fortrinsvis NaCl , KCl , $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 eller en kombination deraf, mere fortrinsvis NaCl .
4. Flydende lactasepræparat ifølge et hvilket som helst af de foregående krav, hvor den samlede koncentration af det mindst ene salt er 0,015-4% vægt/vægt, fortrinsvis 0,02-3% vægt/vægt eller 0,1-2% vægt/vægt.
5. Flydende lactasepræparat ifølge et hvilket som helst af de foregående krav, hvor polyolen er glycerol.
6. Flydende lactasepræparat ifølge et hvilket som helst af de foregående krav, hvor lactasen har en aminosyresekvens, som er mindst 50% identisk med SEQ ID NO: 1.
7. Flydende lactasepræparat ifølge et hvilket som helst af de foregående krav, hvor lactasen har en C-terminal ende svarende til aminosyren 1304 ifølge SEQ ID NO: 1.
8. Flydende lactasepræparat ifølge et hvilket som helst af kravene 1-7, som endvidere omfatter mindst ét salt med en divalent kation, fortrinsvis mindst ét salt af Mg^{++} .
9. Fremgangsmåde til sterilfiltrering af et flydende lactasepræparat, hvilken fremgangsmåde omfatter følgende trin:
 - a. tilvejebringelse af et flydende lactasepræparat, som omfatter mindst ét salt med en monovalent kation, hvor den samlede koncentration af det mindst ene salt er 0,01-5% vægt/vægt, og som endvidere omfatter en polyol valgt blandt glycerol, sorbitol, monopropylenglycol, saccharose, glucose, galactose eller lactose eller kombinationer deraf i en koncentration på 10-70% vægt/vægt, fortrinsvis 30-60% vægt/vægt, hvor koncentrationen af lactase i lactasepræparatet er på mellem 10 og 5000 LAU-B/g, og
 - b. sterilfiltrering af lactasepræparatet;
 og hvor aktiviteten af lactasen (LAU-B) bestemmes som anført i beskrivelsen.

10. Fremgangsmåde ifølge krav 9, hvor det mindst ene salt er valgt blandt et salt af Na^+ , K^+ eller NH_4^+ .
11. Fremgangsmåde ifølge et hvilket som helst af kravene 9-10, hvor det mindst ene salt er valgt blandt NaCl , KCl , NH_4Cl , Na_2SO_4 , K_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$ eller en kombination deraf, fortrinsvis
- 5 NaCl , KCl , $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 eller en kombination deraf, mere fortrinsvis NaCl .
12. Fremgangsmåde ifølge et hvilket som helst af kravene 9-11, hvor den samlede koncentration af det mindst ene salt er 0,015-4% vægt/vægt, fortrinsvis 0,02-3% vægt/vægt eller 0,1-2% vægt/vægt.
13. Fremgangsmåde ifølge et hvilket som helst af kravene 9-12, hvor lactasen har en
- 10 aminosyresekvens, som er mindst 50% identisk med SEQ ID NO: 1.
14. Fremgangsmåde ifølge et hvilket som helst af kravene 9-13, hvor lactasepræparatet har en forbedret filtrerbarhed i sammenligning med et andet flydende lactasepræparat, som har samme sammensætning bortset fra, at det ikke omfatter det mindst ene salt med en monovalent kation.
15. Fremgangsmåde ifølge et hvilket som helst af kravene 9-14, hvor sterilfiltreringen udføres
- 15 inline i en fremstillingsproces for et mejeriprodukt.