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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/081673 A1

(54) Title: PURIFIED LACTASE

(57) Abstract: The present invention relates to a lactase solution comprising a lactase solution comprising less than 10 g/kg of poly and oligosaccharides, a process for the production of such a lactase solution from an untreated lactase solution, a sterilized lactase solution and to a process for the production of milk containing sterilized lactase, whereby such lactose is filter sterilized in-line with the milk production process.

PURIFIED LACTASE

Field of the invention

The present invention relates to purified lactase and the production thereof.

Background of the invention

Lactase or beta-galactosidase (EC: 3.2.1.23) is an enzyme, which can convert lactose (disaccharides) into the monosaccharides glucose and galactose. Lactose is present in dairy products and more particularly in milk, skimmed milk, cream and other milk products. The breakdown of lactose occurs in the intestinal wall of the human body (and in other mammals) by the natural presence of lactase.

Many humans (and other mammals) suffer from lactose-intolerance, wherein lactase is absent or partially absent in their digestive system. In case where lactose is part of the food or feed, decreased digestion of lactose may lead to intestinal trouble.

Nowadays lactase is added to milk to breakdown the lactose present. 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. Although lactase is an active enzyme one has to keep in mind that milk is processed and stored generally at temperatures between 0 and 8°C.

The other possibility is the addition of the enzyme after pasteurisation or sterilization of the milk and before packing. In this case lactase may be added in a lower amount, as it may be at least 10 to 24 hours before the milk is consumed. The enzyme can digest lactose, which may be present during transport and storage in the factory, shop and in the refrigerator of the consumer.

There are several ways to sterilize lactase, for example by chemical and/or heat treatment. However, because of its application in food or feed, sterile filtration is a preferred option.

In the journal Voedingsmiddelentechnologie 13 (1980), 23, a method, which is also described in British patent specification 1477087, is further illustrated. Lactase, usually used by the dairy processing industry as an aqueous solution to which one or more stabilizing agents, such as glycerol, can be added, is filtered before use. The filtered enzyme solution is pumped through a sterile filter then injected via a dosing device into a production line of previously sterilized or pasteurised milk and then mixed with the milk which is subsequently packed under aseptic conditions in uniform packs.

However, in practice, the sterile filter often blocks due to degraded protein, poly- and oligosaccharides remaining in the enzyme solution despite filtering. According to EP 145092, such degradation generally increases the longer the enzyme is stored prior use and may be promoted by the considerable period of time between the production of the enzyme and its use in the dairy processing industry. The repeated cleaning or replacement of the sterile filters is not an option since stopping the whole process requires sterilisation before starting again.

EP 145092 describes a process for the sterile filtration of lactase within 14 days of it being produced. EP 145092 describes that lactase should be sterile filtered after recovery and purification of lactase produced by fermentation, but before the formation of degradation products which are sufficient to clog the sterile filter. However the approach of sterile filtering freshly produced lactase solution does not fulfil the need of lactase solution which can be filtered in-line and which can be added to the sterilized/pasteurised milk. The lactase described in EP 145092 is derived from the yeast *Kluyveromyces* that is used widely in the dairy industry. The polysaccharides are probably parts of host cell walls, which are formed during the recovery process.

Summary of the invention

The present invention provides a lactase solution comprising less than 10 g/kg of poly and oligosaccharides.

The present invention also provides a process for the production of a lactase solution, whereby the poly and oligosaccharides present in an untreated solution are separated from the lactase solution.

The present invention also provides a process for producing lactase containing milk whereby the lactase is sterilized before the lactase is added to the milk.

The present invention also provides a sterilized lactase solution and also dairy products comprising the sterilized lactase solution.

Detailed description of the invention

The present invention relates to purified lactase and in particular to novel processes for the production of lactase free from poly and oligosaccharides. The removal of poly and oligosaccharides allows easier filter sterilization of the lactase solution since the filter does not become blocked with poly and oligosaccharides. As a result, purified lactase can be filter sterilized "in line" with a milk production process, thereby negating the need for such a filter to be repeatedly unblocked.

The present invention provides a solution of lactase, which can be stored and which, after storage, is still free of clogging compounds such as poly and or oligo saccharides. Preferably this solution can be stored after recovery and purification from the fermentation process for at least 15 days, preferably more than 30 days and more preferably more than 120 days.

Free of poly- and oligosaccharides means that less than 10 g/kg of poly- and oligosaccharides are present in the lactase solution. Preferably less than 5 g/kg, more preferably less than 2 g/kg and most preferably less than 1g/kg of poly- and oligosaccharides are present in the lactase solution.

The lactase solution of the present invention is very suitably of sterile filtration, since the low concentration of compounds such as poly- and oligosaccharides, allows the lactase solution to be sterile filtered with less chance of clogging the filter. The filter can be used for a long time preferably at least four times longer than in case of use of lactase solution, which is not substantially free of polysaccharides.

The lactase solution is preferably an aqueous solution of lactase. The lactase solution in general will contain from 10 to 100000 NLU/g, preferably from 100 to 10000 NLU/g.

The lactase solution may comprise one or more solvents or other additives, which bring the enzyme activity to the desired level and may further stabilize the enzyme. Suitable solvents are, for example, sorbitol and glycerol. These solvents may be added to a concentration of from 10 to 70 w/w%, or more preferably 30 to 70% w/w, of the lactase solution. Suitable additives, which stabilize the enzyme, are for example, hydrolysed lactose, glucose, mannitol and salt buffers. According to one embodiment of

the present invention the lactase solution, which is generally free of clogging compounds such as polysaccharides, can be obtained by purifying an untreated lactase solution in a chromatographic process whereby all the compounds responsible for clogging a filter are separated from the lactase solution.

Even in cases where the untreated lactase solution is stored for at least 15 days or even more than 30 days before the chromatographic process, the purified lactase solution can still be easily sterile filtered without the filter becoming clogged.

Unexpectedly, the use of chromatography makes it possible to remove all (poly- and oligosaccharides, proteins, peptides etc.) compounds, which might clog the sterile filter. Surprisingly, it was found that only one chromatographic step was required to remove all polysaccharides and all other unknown non-protein compounds, which are responsible for clogging the sterile filter. One has to keep in mind that the lactase solution although being recovered and purified from the fermentation broth, will contain at least polysaccharides, which are at least partly converted into degradation products. Typical concentration of polysaccharides in untreated lactase solutions are from 10 to 100 g/kg.

Commercial lactase preparations may contain from 40 to 60% w/w of glycerol. The viscosity of the lactase preparation is therefore expected to be related to the amount of glycerol present. However we have found the viscosity of a lactase preparation can be reduced significantly by the removal of polysaccharides from the lactase preparation. This reduction of viscosity makes it possible to pass the lactase preparation through the sterile filter at a reduced pressure difference or at similar pressure difference on the filter, to allow more lactase solution to pass through the filter compared to a lactase solution not purified according to the present process. The invention provides lactase solutions comprising from 10 to 70 w/w% of glycerol, preferably 30 to 70 w/w% and more preferably 40 to 70 w/w% of glycerol having a viscosity of less than 100 mPa, preferably less than 80 mPa and even more preferably less than 60 mPa.

Separation of the clogging compounds from the lactase solution can be achieved by binding of the lactase to an appropriate chromatographic resin. Suitable resins which can be used to separate clogging compounds from a lactase solution are for instance anion and cation exchange resins. Anion exchangers, for instance Q-sepharose, can be used when a lactase solution at a pH above its isoelectric point is applied to an anion exchanger equilibrated in the same pH. Lactase, but not clogging components, is bound

to the resin. The bound lactase can be eluted (desorbed), free of clogging compounds, from the resin by increasing the ionic strength and/or changing the pH of the anion exchange resin. The change in ionic strength and/or pH during desorption or elution can take place under a stepwise or continuous gradient.

The same separation can be achieved with a cation exchanger, for instance SP-sepharose, wherein the lactase preparation is applied to the resin below its isoelectric point. We have found that HAP (hydroxyapatite) chromatography does not give separation of the poly and oligosaccharides from the lactase. A possible explanation might be that poly and oligosaccharides are also bound to the HAP matrix.

Preferred resins are hydrophobic interaction media (HIC). On a HIC media, separation can be obtained based on the differences in hydrophobicity. Different HIC resins are available which contain different ligands, for instance ethyl, propyl, butyl, phenyl and octyl. By applying an aqueous lactase solution under conditions which, permit binding of lactase to the resin, it is possible to separate lactase from the clogging compounds.

A typical protocol for promoting binding or adsorption to a HIC resin is applying an aqueous lactase solution under non-denaturing pH and a relatively high ionic strength to the resin. The high ionic strength can be obtained by adding salts to the lactase solution. Appropriate salts are for instance ammonium sulphate, sodium chloride and sodium sulphate.

After the binding or adsorption step, lactase can be eluted or desorbed by decreasing the ionic strength. The change in ionic strength and/or pH during elution or desorption can take place under a stepwise or continuous gradient. Other suitable resins are for instance gelfiltration media and hydrophobic charge induction media (combination of HIC and ion exchange chromatography).

The lactase provided in the present invention produced by a yeast, preferably a *Kluyveromyces* strain more preferably *K. lactis* or *K. fragilis.* Such lactase is purified according to the invention resulting in a lactase solution which is easy to sterilize. The removal of all compounds responsible for clogging the sterile filter is very surprising because after the fermentation process, the lactase, which is being recovered and purified, may contain polysaccharides, which are at least partly converted into degradation products. Therefore it is believed that protease and other contaminating proteins also present in lactase solution are removed in the chromatography step. The

sterile filter preferably used to sterilize the lactase solution is in general present in the milk in-line production process. The sterile filtering treatment is preferably carried out in-line with respect to the milk production process, whereby one or more membrane filters are used. A suitable sterile filter is for example a membrane filter having a pore size of 0.22 µm.

The lactase solution according to the invention is advantageously used in the preparation of pasteurised milk.

Example 1

The example describes a typical chromatographic purification procedure of lactase using phenyl sepharose LS as chromatographic resin.

The commercial product Maxilact® 5000 LX (obtainable from DSM, The Netherlands) containing 5000 NLU/g was diluted 5 times with demineralised water, ammonium sulphate is added to a final concentration of 1 M after which the pH was corrected to 7.5.

A 20 ml sample was applied to a 20 ml HiPrep phenyl 16/10 column having a diameter of 16 mm and length of 10 cm, at a linear flow rate of 150 cm/h. The column was equilibrated with 1 M ammonium sulphate in 100 mM Tris pH 7.5. After loading the column was washed with equilibration buffer at a flow rate of 150 cm/h until the baseline was reached. Elution of lactase was done under a step gradient at 150 cm/h (100 mM Tris pH 7.5). After elution of the lactase the lactase solution was desalted and concentrated to a final activity of approximately 10.000 NLU/g. After concentration the lactase solution was formulated with glycerol at a final concentration of 50 % w/w, the final lactase activity after formulation is approximately 5.000 NLU/g.

Lactase activity was determined by the hydrolysis of the substrate o-nitrophenyl-ß-galactopyranoside (ONPG) into o-nitrophenyl and galactose. The reaction was terminated by the addition of sodium carbonate. The absorbance of the o-Nitrophenyl formed, being yellow in alkaline medium, was used to measure the activity of the enzyme (expresses as NLU/g). The procedure is published in the. *FCC, fourth edition, July 1, 1996, page 801 to 802 /Lactase (neutral) (ß-galactosidase) activity*

The results are shown in the Table 1

Table 1

Sample	Activity NLU/g	g/kg sugars		Poly- and oligosaccharides g/kg
		before inversion	after inversion	
Maxilact LX 5000	5000	1	57.6	56.6
Purified Lactase	5000	< 1	2.21	< 2

Poly- and oligosaccharide content was determined by measuring the amount of free sugar and the amount of sugar present after the acid inversion of the polysaccharides.

The polysaccharide contents were determined by means of High Performance Liquid Chromatography (HPLC). The detection was performed using a RI (refraction index)-detector. The column used was a BioRad Aminex HPX 87N, length 30 cm, inner diameter 7.8 mm, thermostated at 85 °C. The mobile phase was a solution of 0.71 g sodium sulphate in 1 liter water at a flow rate of 0.68 ml per minute.

Two different samples pre-treatment were performed, both with and without acid inversion. The sample pre-treatment without inversion was done by weighing 5 g sample in a volumetric flask of 50 ml, dissolving in mobile phase and injecting 5 µl onto the column.

The sample pre-treatment with inversion was done by weighing 2 g sample in a centrifuge tube, adding 3.00 ml water and 2.50 ml hydrochloric acid 2.58 mol/l, heating for 75 minutes at 100 °C and adding 2.50 ml sodium hydroxide 2.58 mol/l. 5 µl of the resulting solution was injected onto the column.

The glucose content was calculated using a standard solution with a concentration of 400 mg glucose in 50 ml mobile phase. The concentrations of trisaccharide, disaccharide and fructose were calculated using a response factor, relative to glucose.

Example 2

The example describes the sterile filtration of lactase solutions.

A syringe was filled with 1 ml Maxilact LX 5000, and a sterile filter, Millex GV 0.22 µm from Millipore with a surface of 4.91 cm^2, was placed on top of the syringe. After applying hand pressure it was not possible to filter the product through the filter, increasing the pressure caused the filter to break.

Another syringe was filled with 1 ml of purified lactase formulated with 50 % w/w glycerol (end concentration), prepared as described in Example 1, and a sterile filter, Millex GV 0.22 µm from Millipore with a surface of 4.91 cm^2, was placed on top of the syringe. After applying hand pressure it was surprisingly easy to sterile filter at least 1 ml of the lactase solution through the filter.

Therefore, the use of chromatography allowed all compounds which may have clogged the sterile filter to be removed, and as a result sterile filtration was not problematic.

Example 3

The example describes viscosity measurements of formulated lactase solutions.

The viscosity of both the commercially available Maxilact LX 5000 and of the purified lactase formulated with 50 % w/w glycerol (end concentration), prepared as described in Example 1 was measured. Commercially available Maxilact also contains 50% w/w glycerol.

The viscosity was measured with a Physica UDS 200 at 25 °C, using a MK 21 cone probe.

Table 2 shows the viscosity of the purified lactase formulated at 5.000 NLU/g with 50 % glycerol (end concentration) significantly drops as a result of the purification in which all clogging compounds are removed.

Table 2: Results of viscosity measurements

Product	Viscosity mPa shear rate = 100 (s-1)
Commercial Maxilact LX 5000	170
Purified lactase formulated with 50 % glycerol	40

CLAIMS

1. A lactase solution comprising less than 10 g/kg of poly and oligosaccharides.
2. A lactase solution according to claim 1 which after storage for at least 15 days, preferably 30 days comprises less than 10 g/kg of poly and oligosaccharides.
3. A lactase solution according to claim 1 or 2, which is an aqueous solution.
4. A lactase solution according to any one of claims 1-3, further comprising stabilizing solvent.
5. A lactase solution according to claim 4, wherein the stabilizing solvent is sorbitol or glycerol.
6. A lactase solution according to any one of the preceding claims further comprising at least one stabilizing additive.
7. A lactase solution according to claim 6, wherein the additive is selected from hydrolysed lactose, glucose, mannitol and salt buffers.
8. A lactase solution according to any one of the preceding claims, wherein lactose is contained in an amount of from 10 to 100,000 NLU/g.
9. A lactase solution comprising from 10 to 70 w/w% glycerol and having a viscosity of less than 100 mPa, preferably less than 80mPa.
10. A lactase solution according to any one of claims 1-9, wherein the lactase is produced in a *Kluyveromyces* strain.
11. A process for the production of a lactase solution according to any one of claims 1 to 10, wherein the poly and oligosaccharides present in an untreated solution of lactase are separated from the lactase solution.
12. A process according to claim 11, wherein the poly and oligosaccharides are removed from an untreated solution of lactase by a chromatographic process.
13. A sterilized lactase solution wherein a lactase solution of any one of claims 1-10 is sterilized on a sterile filter.
14. A dairy product comprising a sterilized lactase solution according to claim 13.

15. A process for producing lactase containing milk whereby a lactase solution according to any one of claims 1-10 is sterilized before addition to the milk.

16. The process according to claim 15, wherein sterilization is carried out on at least one filter positioned in line with the milk production process.

17. A process according to claim 15 or 16 whereby the milk is pasteurised or sterilized milk.

18. A process for producing sterilized lactase whereby a lactase solution according to any one of claims 1-10 is sterilized preferably with a sterile filter.

19. Use of chromatography to separate poly and oligosaccharides from a lactase solution.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/03680

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/38 A23J1/18 A23J3/20 A23C9/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23J C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 01 04276 A (UNIV LIEGE) 18 January 2001 (2001-01-18) Table I page 12	1-3,8, 11-14,19 4-7,9, 10,15-18
X Y	US 4 237 230 A (IIDA TAKAO ET AL) 2 December 1980 (1980-12-02) column 4, line 1-13; example 3	1-3,8, 11-14,19 4-7,9, 10,15-18
X	US 4 007 283 A (CRISAN ELI V ET AL) 8 February 1977 (1977-02-08) column 4, line 17-22	1-3,11, 12
X	GB 1 306 751 A (BAXTER LABORATORIES INC) 14 February 1973 (1973-02-14) example 4	1-3,11, 12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2002	24/06/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Stolz, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/03680

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 145 092 A (GIST BROCADES NV) 19 June 1985-(1985-06-19) cited in the application page 4, line 33-39	4-7,9, 10,15-18
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; PREV199191123138, 1991 RAHIM AND LEE: "Production and characterization of beta galactosidase from psychrotrophic Bacillus subtilis KL88" XP002174412 abstract	1-3,11, 12
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; PREV198987081285, 1989 HUSSEIN L. ET AL.: "Reduction of Lactose in Milk by purified Lactase produced by Kluyveromyces lactis" XP002174413 abstract	1-3, 10-12
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; PREV197968040855, 1979 PARK ET AL.: "Production and characterization of beta galactosidase EC 3.2.1.23 from Aspergillus orizae" XP002174414 abstract	1-3,12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/03680

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 1-12 partially
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

International Application No. PCT/EP 02 /03680

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. Claims: 1-8, 10 partially, 11-19

   A solution comprising lactase and a certain amount of poly- or oligosaccharides

2. Claims: 9,10

   a lactase solution containing glycerol and having a viscosity of less than 100 mPa.

International Application No. PCT/EP 02 /03680

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-12 partially

The claimed subject matter is defined in terms which have not been used in the prior art. Many lactase solutions and preparations have been described, however without assessing the degree of contamination by oligo- or polysaccharides. A comparison of the claimed subject matter with that of the prior art is therefore not possible.
According to the instant description and claims, the claimed subject matter can be obtained by including whatever chromatographic step in the preparation of the enzyme. Consequently, the search has been limited to processes for the preparation of lactase solutions involving a chromatographic step and the resulting products.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/03680

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