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(71) Applicant: **DUPONT NUTRITION BIOSCIENCES APS** [DK/DK]; Langebrogade 1, DK-1411 Copenhagen K (DK).

(72) Inventors: **LARSEN, Morten Krog**; Borumtoften 4, 8471 Sabro (DK). **ZARGAHI, Masoud Rajabi**; Illerupvej 45, 2 th, 8200 Arhus (DK).

(74) Agent: **KWON, Evelyn M.**; 345 Park Avenue, New York, New York 10154 (US).

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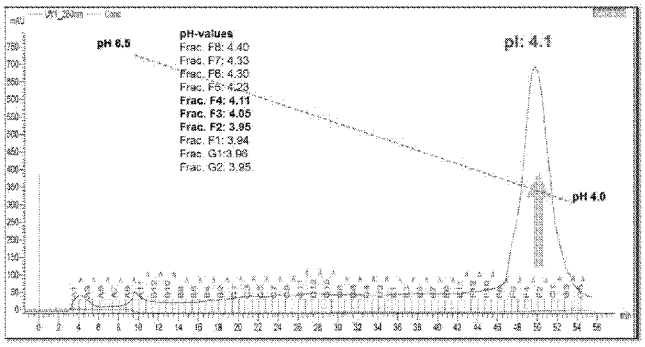
(54) Title: METHOD FOR PRODUCING A CLEAN LACTASE

Figure 1



(57) Abstract: A method for producing a clean lactase is described. The method comprises incubating a preparation comprising said lactase at a pH close to the isoelectric point of said lactase.

TITLE

METHOD FOR PRODUCING A CLEAN LACTASE

The present invention relates to a method for preparing a lactase preparation involving incubating a preparation containing a lactase enzyme at a pH close to the isoelectric point of said lactase. The invention also relates to a lactase enzyme produced by the method of the invention.

INCORPORATION BY REFERENCE OF THE SEQUENCE LISTING

The sequence listing provided in the file named “NB41025USPSP_seq_listing _ST25.txt” with a size of 8,908 bytes which was created on December 7, 2015 and which is filed herewith, is incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

The use of enzymes to improve food grade products is well known, and such enzymes are routinely produced by fermentation of microorganisms that express them. However, it is also well known in the art that when fermenting a microorganism in order to obtain a desirable enzyme, one or more undesirable enzymes are often also expressed by said microorganism. If these undesirable enzymes are not effectively inactivated they may cause stability problems during recovery of the desirable enzyme and/or during storage of the desirable enzyme. Even if these other enzymes do not cause stability problems they may indeed be unwanted as industry for many purposes requires a "pure" enzyme without side-activities.

Examples of industrial uses of enzymes are incubations with lactase, for example to render milk and milk-based products acceptable for lactose intolerant individuals. Lactase (also known as lactase-phlorizin hydrolase, or LPH), is a member of the β-galactosidase family of enzymes, and is a glycoside hydrolase involved in the hydrolysis of the disaccharide lactose into constituent galactose and glucose monomers. In humans, lactase is encoded by the LCT gene.

Lactase is an enzyme produced by many organisms and is located in the brush border of the small intestine of humans and other mammals. Lactase is essential to the complete digestion of whole milk; it breaks down lactose, a sugar which gives milk its sweetness. An individual consuming dairy products comprising lactose in the absence of lactase may experience the symptoms of lactose intolerance. Lactose is present in dairy products and more specifically in milk, skimmed milk, cream and other milk products. The breakdown of

lactose occurs in the intestinal wall of the human body (and other mammals) by the natural presence of lactase.

The nutritional and functional problems caused by lactose in most populations that lack lactase are well known and described. Members of such populations cannot hydrolyse the lactose, which in such cases passes into the large intestine, where it produces dehydration, poor calcium absorption, diarrhoea, flatulence, belching and cramps, and, in severe cases, even watery explosive diarrhoea.

Thus, an important industrial application of lactase is in the production of lactose-hydrolysed milk products for lactose intolerant individuals. Such hydrolysed milk products include pasteurized milk, UHT-milk and milk reconstituted from all or part of its original constituents with or without intermediate processing steps such as protein hydrolysis. Treatment with lactase may be done prior to and after the heat-treatment of the milk. The lactase treatment may be done by adding the enzyme to the milk. The solubility properties of lactose are such that it may lead to its crystallization, leading to a sandy or gritty texture. Such undesired texture may be found in some dairy products such as condensed milk, evaporated milk, dry milk, frozen milk, ice cream, and in confectionary products with a high content of milk. Full or partial hydrolysis of lactose by lactase eliminates this problem, providing products with a homogeneous texture and as a result a higher consumer acceptance.

A further application of lactase in food processing is to increase the sweet taste in lactose-containing products such as milk or yoghurt. The hydrolysis of lactose in such products results in an increased sweet taste due to glucose production. Furthermore, lactase may be employed in the hydrolysis of lactose products containing dairy components, for example bread. Lactose can be added to such products to enhance flavour, retain moisture, provide browning and improve toasting properties.

Lactose hydrolysis by lactase in fermented milk products such as yoghurt will increase sweet taste. However, when the lactase is added prior to the beginning of the fermentative process, it may increase the rate of acid development and thus reduce processing times. The lactase treatment of milk or milk-derived products such as whey makes such products suitable for application in animal feed and pet food for lactose intolerant animals such as cats. The lactose hydrolysis allows the manufacture of higher concentrated whey and at the same time prevents gut problems, similar to those described earlier for lactose-deficient patients. Lactose hydrolysed whey is concentrated to produce a syrup containing 70-75% solids and is used as a food ingredient in ice cream, bakery and confectionary products.

Lactases have been described for and isolated from a large variety or organisms, including micro-organisms. Some lactases are so-called neutral lactases as they have a pH optimum between pH = 6 and pH = 8. Other lactases are classified as acidic lactases, as they have a pH optimum of between pH=3.5 and pH=5.0. The present invention encompasses both neutral and acidic lactases. In one aspect the lactase is a neutral lactase. In an alternative aspect the lactase is an acidic lactase.

The enzymatic properties of lactases like pH- and temperature optimum vary between species. In general, however, lactases that are excreted show a lower pH-optimum of pH = 3.5 to pH = 5.0; intracellular lactases usually show a higher pH optimum in the region of pH = 6.0 to pH = 7.5, but exceptions on these general rules occur. The choice for a neutral or acidic lactase depends on the pH profile in the application. In applications with neutral pH, neutral lactases are usually preferred; such applications include milk, ice cream, whey, cheese, yoghurt, milk powder etc. Acid lactases are more suited for applications in the acidic range.

Although aimed at improving the functionality and/or taste profiles of the food product, occasionally an enzyme treatment can have unexpected and undesirable side effects. An example of an undesirable side effect is the development of off-flavour as a result of the enzyme treatment, due to unwanted enzymatic activities in the enzyme preparation.

As such, it is desirable to remove unwanted enzymatic activities from enzyme preparations which are added to food-grade products. In view of its utility in this area, there is thus a need to produce isolated lactases, for example from which unwanted enzymatic activities have been removed.

Current methods used in the art to isolate and purify lactase enzymes involve column purification, which can be costly.

Thus, there is a need for new and alternative methods of producing and isolating lactases which are cost effective and relatively quick and simple to carry out.

The present invention addresses this need.

SUMMARY OF THE INVENTION

The present inventors have surprisingly found that a lactase enzyme can be produced from a preparation comprising said lactase using a simple and cost-effective method involving precipitation of the lactase at a pH close to its isoelectric point. Prior to the present invention, it had not previously been appreciated that such a relatively simple method could produce a

lactase preparation, wherein the lactase activity is retained following incubation at a pH close to its isoelectric point.

For example, acetone has been used previously in some cases to precipitate lactase enzymes, but such precipitation processes require large volumes of acetone to produce acidic lactase powders. The present invention provides an alternative, improved method for lactase precipitation in which a simple step of incubation at a pH close to the isoelectric point of said lactase is employed.

As such, the present invention provides a method for producing a lactase comprising incubating a preparation comprising said lactase at a pH close to the isoelectric point of said lactase. The method of the invention allows for production of a lactase which retains its activity following incubation at a pH close to the isoelectric point.

The invention also encompasses a lactase or lactase preparation produced by the method of the invention.

DESCRIPTION OF THE FIGURES

Figure 1 shows the chromatographic fingerprint of lactase protein obtained from a Mono P column.

Figure 2 shows the results of SDD-PAGE analysis of lactase activity. Lane 1. Crude F3031G; Lane 2. Crude after pH decrease to 4.0 and increase to 7.30; Lane 3. Supernatant pH 5.0: Lane 4. Pellet pH 5.0; Lane 5. Supernatant pH 4.5; Lane 6. Pellet pH 4.5; Lane 7. Supernatant pH 4.0; and Lane 8. Pellet pH 4.0.

BRIEF DESCRIPTION OF THE BIOLOGICAL SEQUENCES

The following sequences comply with 37 C.F.R. §§ 1.821-1.825 (“Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules”) and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (2009) and the sequence listing requirements of the European Patent Convention (EPC) and the Patent Cooperation Treaty (PCT) Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. § 1.822.

SEQ ID NO: 1 is the amino acid sequence of a lactase from *Lactobacillus delbrueckii* subsp. *bulgaricus*.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have surprisingly found that it is possible to produce a lactase using a quick and cost-effective method, involving incubating a preparation comprising a lactase at a pH close to the isoelectric point of the lactase.

As such, the present invention provides a method for producing a lactase comprising incubating a preparation comprising said lactase at a pH close to the isoelectric point of said lactase.

As demonstrated in Example 2 and Figure 2, when a preparation (crude extract) comprising a lactase was incubated at a pH close to the isoelectric point of the lactase, it was possible to recover the majority of the lactase activity and protein in a precipitated and resuspended pellet. This was surprising because lactases generally function as oligomeric enzymes. It was not obvious that a functioning lactase could be obtained following precipitation. The present invention represents a simplification of methods used previously in the art, and thus provides a valuable contribution to the art.

The isoelectric point (pI, pH(I), IEP), is the pH at which a particular molecule carries no net electrical charge. The isoelectric point of a lactase may be determined by methods known in the art, for example as described in present Example 1.

As used herein, the term “close to its isoelectric point” is intended to mean a pH value near to the isoelectric point of the lactase.

For example, the pH at which the lactase preparation is incubated may be within 2.0 units of the isoelectric point. For example, the pH at which the lactase preparation is incubated may be within 0.1-2.0 units, e.g. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9 or 2.0 units of the isoelectric point.

By “incubated at a pH close to the isoelectric point” is intended to mean keeping the preparation at a pH close to the isoelectric point, for example for an amount of time sufficient to allow precipitation of the lactase.

By “precipitation” is meant deposition in solid form from a solution. In the present invention the lactase may be precipitated from the preparation comprising said lactase. In one aspect 50-100% of lactase is precipitated. For example, about 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100% of lactase protein or activity is precipitated.

The lactase may be an intracellular or an extracellular produced lactase. In a preferred embodiment the lactase is extracellular.

The reference to a lactase as used herein is intended to mean any enzyme with lactase activity.

The lactase may be from a *Lactobacillus*, for example a species of *Lactobacillus* such as *Lactobacillus delbrueckii*, e.g. *Lactobacillus delbrueckii bulgaricus*. *Lactobacillus delbrueckii* subsp. *bulgaricus* was until 2014 known as *Lactobacillus bulgaricus*. The term "*Lactobacillus bulgaricus*" is used herein, but this is intended to be equivalent to *Lactobacillus delbrueckii bulgaricus*.

In a preferred embodiment the lactase is from *Lactobacillus bulgaricus*. The amino acid sequence of *L. bulgaricus* is publically available, for example as UniProtKB - P0C1Y0 (BGAL_LACDE) (www.uniprot.org/uniprot/P0C1Y0). Alternatively the lactase may be a lactase having an amino acid sequence which is at least 80, 85 or 90%, preferably at least 95% identical with the amino acid sequence of *L. bulgaricus* lactase as set out in SEQ ID NO:1:.

SEQ ID NO: 1

MSNKLVKEKRVDQADLAWLTDPEVYEVNTIPPHSDHESFQSQEELEEGKS
SLVQSLDGDWLIDYAENGQGPVNFYAEDFDDSNFKSVKVPGNLELQGFGQ
PQYVNVQYPWDGSEEIFPPQIPSKNPLASYVRYFDLDEAFWDKEVSLKFD
GAATAIYVWLNGHFVGYGEDSFTPSEFMVTKFLKKENNRLAVALYKYSSA
SWLEDQDFWRMSGLFRSVTLQAKPRLHLEDLKLTASLTDNYQKGKLEVEA
NIAYRLPNASFKLEVRDSEGDLVAEKLGPIRSEQLEFTLADLPVAAWSAE
KPNLYQVRLYLYQAGSLLEVSRQEVGFRNFELKDGIMYLNGQRIVFKGAN
RHEFDSKLGRAITEEDMIWDIKTMKRSNINAVRCSHYPNQSLFYRLCDKY
GLYVIDEANLESHGTWEKVGGHEDPSFNVPGDDQHWLGASLSRVKNMMAR
DKNHASILIWSLGNESYAGTVFAQMADYVRKADPTRVQHYEGVTHNRKFD
DATQIESRMYVPAKVIEEYLTNKPAKPFISVEYAHAMGNSVGDLAAYTAL
EKYPHYQGGFIWDWIDQGLEKDGHLLYGGDFDDRPTDYEFCGNGLVFADR
TESPKLANVKALYANLKLEVKDGQLFLKNDNLFTNSSSYYFLTSLLVDGK
LTYQSRPLTFGLEPGESGTFALPWPEVADEKGEVVYRVTAHLKEDLPWAD
EGFTVAEAEEVAQKLPEFKPEGRPDLVDSDYNLGLKGNNFQILFSKVKGW
PVSLKYAGREYLKRLPEFTFWRALTDNDRGAGYGYDLARWENAGKYARLK
DISCEVKEDSVLVKTAFTLPVALKGDLTVTYEVDGRGKIAVTADFPGAEE
AGLLPAFGLNLALPKELTDYRYYGLGPNESYPDRLEGNYLGIYQGAVKKN
FSPYLRPQETGNRSKVRWYQLFDEKGGLEFTANGADLNLSALPYSAAQIE
AADHAFELTNNYTWVRALSAQMGVGGDDSWGQKVHPEFCLDAQKARQLRL
VIQPLLLK

It is defined here that in order to determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino

acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions (i.e. overlapping positions) x 100). Preferably, the two sequences are the same length.

The skilled person will be aware of the fact that several different computer programs are available to determine the homology between two sequences. For instance, a comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm.

In one embodiment the lactase is not from a *Kluyveromyces* species, for example *K. lactis*.

The method of the invention may be performed on any lactase preparation which contains a lactase, and, in a preferred embodiment , at least one further enzyme. The method is particularly useful when the preparation is a culture or fermentation broth. The method of the invention may be applied to an untreated culture broth (whole culture broth) , to a homogenized culture broth, or to a whole or homogenized culture broth.

According to the method of the invention, during the incubation the temperature should be held in the range of from 2-75°C, in particular in the range of from 10-70°C, preferably in the range of from 5-25°C.

The incubation time may be anything from 1 second to a couple of weeks, typically the holding time will be in the range of from 1 second to 24 hours, , preferably in the range of from 1 second to 4 hours.

In one aspect of the invention no further or additional purification steps are performed. In a preferred embodiment no column purification of the lactase is performed, for example, no ion exchange, affinity or size exclusion column purification is performed, nor column purification based on hydrophobic interaction.

The invention also encompasses a lactase enzyme produced by any aspect of the method of the invention.

Also encompassed are food and beverage products comprising the lactase according to the invention.

A lactase according to the invention may be used in the production of a food or beverage product, preferably a dairy product.

The dairy product may be selected from milk, ice cream, whey, cheese, yoghurt, and milk powder etc. In one embodiment the product is in the form of a milk drink.

The lactase according to the present invention or produced by a method according to the invention may be substantially free of unwanted enzymatic activities, and hence the method of the invention can also be used to purify lactase from a preparation comprising said lactase and other enzymes.

As used herein, a lactase "substantially free" from unwanted enzymatic activities may encompass any lactase, in which the unwanted enzymatic activity or activities is not present or present at a sufficiently low level that, upon effective dosage of the lactase in the relevant production process, no observable activity of the unwanted enzyme activities is observed.

Unwanted enzymatic activities can be any enzymatic activities that are not the desired lactase activity. For example, the unwanted enzyme activity may be an enzyme selected from the group consisting of lipases, amylases, cellulases, oxidoreductases, xylanases, isomerases, peptidases and proteases. In one aspect the unwanted enzymatic activity is aryl sulfatase.

In a further aspect of the invention, the invention provides a lactase which comprises less than e.g. 20-1000 units of arylsulfatase activity per NLU, for example, less than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 units activity per NLU.

The lactase according to the invention may advantageously be used in food and feed products to hydrolyse lactose without the formation of off-flavour compounds.

By way of example, the preparation of intracellular lactases requires the disruption of the cells to release the lactase enzyme. At the same time, other cytoplasmic enzymes are released. The quality of an industrial preparation of the lactase may be determined by the ratio of unwanted enzyme activities to lactase activity. Proteases are critical unwanted enzymes since they are known to lead to unwanted side effects in application, such as milk clotting or off-flavour formation in milk.

"Off-flavour" compounds include p-cresol (produced by aryl sulfatase), and bitter peptides.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

EXAMPLE 1

DETERMINATION OF THE ISOELECTRIC POINT OF L. BULGARICUS LACTASE

Background

Chromatofocusing is a chromatography technique that separates proteins according to differences in their isoelectric point (pI). The pI of each protein is the pH at which the protein has zero surface charge. Proteins with different pI can be separated by being passed through a chromatofocusing column while a pH gradient is generated on the column by specifically designed and matched amphoteric buffers. Proteins elute in order of their isoelectric points.

Materials

ÄKTA explorer system was used for protein purification and according to the method described by the manufacturer, GE, USA – Cat. No. 18-1112-41.

The MonoP column was obtained from GE Life sciences, USA - Cat. No. 17-0611-01.

PD-10 column was obtained from GE Life sciences, USA - Cat. No. 17-0851-01.

Na- dihydrogen phosphate was obtained from Merck, Germany - Cat. No. 1.06346.

Tris (hydroxymethyl) aminomethane was obtained from Sigma-Aldrich, Germany – Cat. No. 154563.

Polybuffer 74 was obtained from GE Life sciences, USA - Cat. No. 17-0713-01.

Nu-PAGE gel was obtained from Invitrogen, USA – Cat. No. NP0321.

Mes running buffer was obtained from Invitrogen, USA – Cat. No. NP0002.

SeeBlue standard marker was obtained from Invitrogen, USA – Cat. No. LC5925.

SDS-PAGE analyses was carried out using Xcell Mini-cell and according to the method described by the manufacturer, Invitrogen, USA – Cat. No. EI0001.

Sample:

Lactobacillus bulgaricus lactase (fermentation number F3031G)

Methods

Desalting, PD-10 desalting column

The PD-10 column was prepared as described by the manufacturer and equilibrated with 20 mM Tris/HCl, pH 8.5 (buffer A). The Sample was diluted 2x with desalting buffer and desalted on the column.

Chromatofocusing, Mono P HR10/8

The column was prepared as described by the manufacturer and equilibrated with 20 mM Tris/HCl, pH 8.5 (buffer A). The sample (2 ml desalted sample) was applied to the column at a flow rate of 1.5 ml/min and the column was washed with buffer A. The bound proteins were eluted according to differences in their isoelectric point with a linier pH gradient of 8.5–4.0 using 10% Polybuffer 74, pH 4.0/HCl (buffer B). During the entire run, fractions of approx. 1.0 ml were collected and subjected to pH-measurement and lactase enzyme assay.

Lactase assay

Lactase (Neutral) Beta-Galactosidase activity - FCC IV was determined using the following parameters:

Substrate: ONPG, pH 6.50;

10 µL sample/blank;

90 µL substrate;

Incubation for 5 min at 32ºC;

Read at 420 nm using a ELISA reader.

Results and discussion

The chromatographic fingerprint of lactase protein obtained from the Mono P column is shown in Figure 1. As shown, the lactase protein elutes at the end of pH gradient having the pI of approx. 4.1 (see Table 1).

Table 1

	1	2	3	4	5	6	7	8	9	10
A	A1 0.002	A9 0.001	0.001	0.002	0.003	0.002	0.000	0.004	0.012	G1 0.083
B	0.001	0.003	0.001	0.002	0.000	0.002	0.002	0.002	0.015	0.044
C	0.000	0.002	0.001	0.000	0.002	0.002	0.001	0.004	0.053	0.025
D	0.000	-0.001	-0.001	-0.000	0.004	-0.001	0.001	0.001	0.080	0.013
E	0.000	0.001	-0.000	-0.000	0.001	0.000	0.001	0.005	F4 0.185	G5 0.010
F	0.001	0.003	0.002	0.002	0.003	0.002	0.003	0.004	F3 0.234	crude 3.872
G	0.000	0.000	0.000	-0.001	0.000	-0.001	0.000	0.002	F2 0.132	0.000
H	A8 0.001	-0.000	0.000	-0.000	0.001	-0.000	-0.000	0.001	F1 0.145	0.001

EXAMPLE 2

EVALUATION OF THE EFFICIENCY OF ISOELECTRIC POINT PRECIPITATION AND RECOVERY OF *L. BULGARICUS* LACTASE PROTEIN.

The isoelectric point of the *L. bulgaricus* lactase protein was determined to be 4.1 as described in Example 1.

Sample: Crude F3031G - 496 NLU/ml - pH 7.31 (30 mL)

Method

The pH was stepwise adjusted down to 5.0, 4.5 and 4.0 by the addition of ice cold H_2SO_4 solution (2 M).

Samples were taken (2 mL) at each pH point and pellets were separated from supernatants by 5 min centrifugation at 10000*g*. Pellets were re-solubilized by the addition of 2.0 mL of PEM buffer. The Supernatants were added 200 µL of a 1.0 M Na-P buffer (pH 8.0). Both supernatants and pellets were subjected to lactase assay and SDS-PAGE analysis.

The remaining sample was added NaOH (1.0 M) to bring the pH to its original value (7.3) - without removal of pellet, precipitation was completely reversible.

SDD-PAGE analysis was carried out using the following parameters: 10 µL sample, 30 µL 100mM Na-P, pH 6.5, 17 µL 5x sample buffer +DTT, 5 min heat, and 10 µL load. The results are shown in Figure 2.

Lane 1. Crude F3031G

Lane 2. Crude after pH down to 4.0 and up to 7.30

Lane 3. Supernatant pH 5.0

Lane 4. Pellet pH 5.0

Lane 5. Supernatant pH 4.5

Lane 6. Pellet pH 4.5

Lane 7. Supernatant pH 4.0

Lane 8. Pellet pH 4.0

Lactase activity was assayed using the following parameters: Lactase assay

Substrate: FCC IV NLU, 10 µl of 100x diluted sample/blank (diluted in PEM buffer), 190 µl substrate, 5 min/32°C/420nm.

Results

The results are shown in Table 2 below:

Sample	Relative lactase activity
Crude	598
Crude pH 4.0&7.30	412
Supnat pH 5.0	431
Pellet pH 5.0	73
Supnat pH 4.5	43
Pellet pH 4.5	522
Supnat pH 4.0	0
Pellet pH 4.0	427

The addition of H_2SO_4 caused rapid and smooth precipitation of lactase protein started at pH 5.0 and completed at pH 4.0. The resolubilization of the lactase protein pellet was fast and 100%, and the resulting solution was crystal clear. The highest lactase recovery (activity) was achieved at pH 4.5. However, pH 4.0 generated the highest lactase protein pellet as judged by SDS-PAGE analysis (the decrease in enzyme activity at this pH was most likely due to protein instability/aggregation).

This condition of lactase protein precipitation/recovery might be improved by the pH fine-tuning (depending on the nature of the crude).

The recovered lactase solution had a decrease in odour.

All documents referred to herein are hereby incorporated by reference in their entirety, with special attention to the subject matter for which they are referred Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, cellular immunology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method for producing a lactase comprising incubating a preparation comprising said lactase at a pH close to the isoelectric point of said lactase.

2. A method for reducing unwanted enzymatic activities in a preparation comprising lactase, comprising incubating said preparation at a pH close to the isoelectric point of said lactase.

3. The method of claim 1 or claim 2 wherein the lactase is precipitated from said preparation.

4. The method of claim 3 wherein about, or at least about, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100% of lactase protein or activity is precipitated from said preparation.

5. The method of any one of claims 1 to 4 wherein said pH is within 0.8 units of the isoelectric point of said lactase.

6. The method of any one of claims 1 to 5, wherein said lactase is from a *Lactobacillus*.

7. The method of any one of claims 1 to 6 wherein said lactase is from *Lactobacillus bulgaricus*.

8. The method of any one of claims 1 to 7 wherein said lactase is from *Lactobacillus bulgaricus*, and said isoelectric point is 4.1.

9. The method of any one of claims 1 to 8 wherein the lactase is not from a *Kluyveromyces* species, preferably not from *K. lactis.*

10. The method of any one of claims 1 to 9 comprising the step of forming a pellet of said lactase, and subsequently dissolving said pellet in a medium.

11. The method of any one of claims 1 to 10 wherein a further purification step is not performed.

12. The method of any one of claims 1 to 11 wherein a column purification step is not performed.

13. The method of any one of claims 1 to 12 wherein said preparation comprising a lactase is a fermentation broth.

14. The method of claim 2, wherein said unwanted enzymatic activities are selected from lipases, amylases, cellulases, oxidoreductases, xylanases, isomerases, peptidases and proteases.

15. The method of claim 2 wherein the unwanted enzymatic activity is aryl sulfatase.

16. A lactase produced by the method of any one of claims 1 to 15.

17. A lactase produced by the method of any one of claims 1 to 15, which is substantially free of unwanted enzymatic activities.

18. A food or beverage product comprising the lactase of claim 16 or claim 17.

19. The food or beverage product of claim 18 which is a dairy product, preferably selected from milk, ice cream, whey, cheese, yoghurt, and milk powder.

20. Use of a lactase produced by the method of any one of claims 1 to 15 for use in the production of a food or beverage product according to claim 18 or claim 19.

21. A method, or food, or use substantially as described herein.

Figure 1

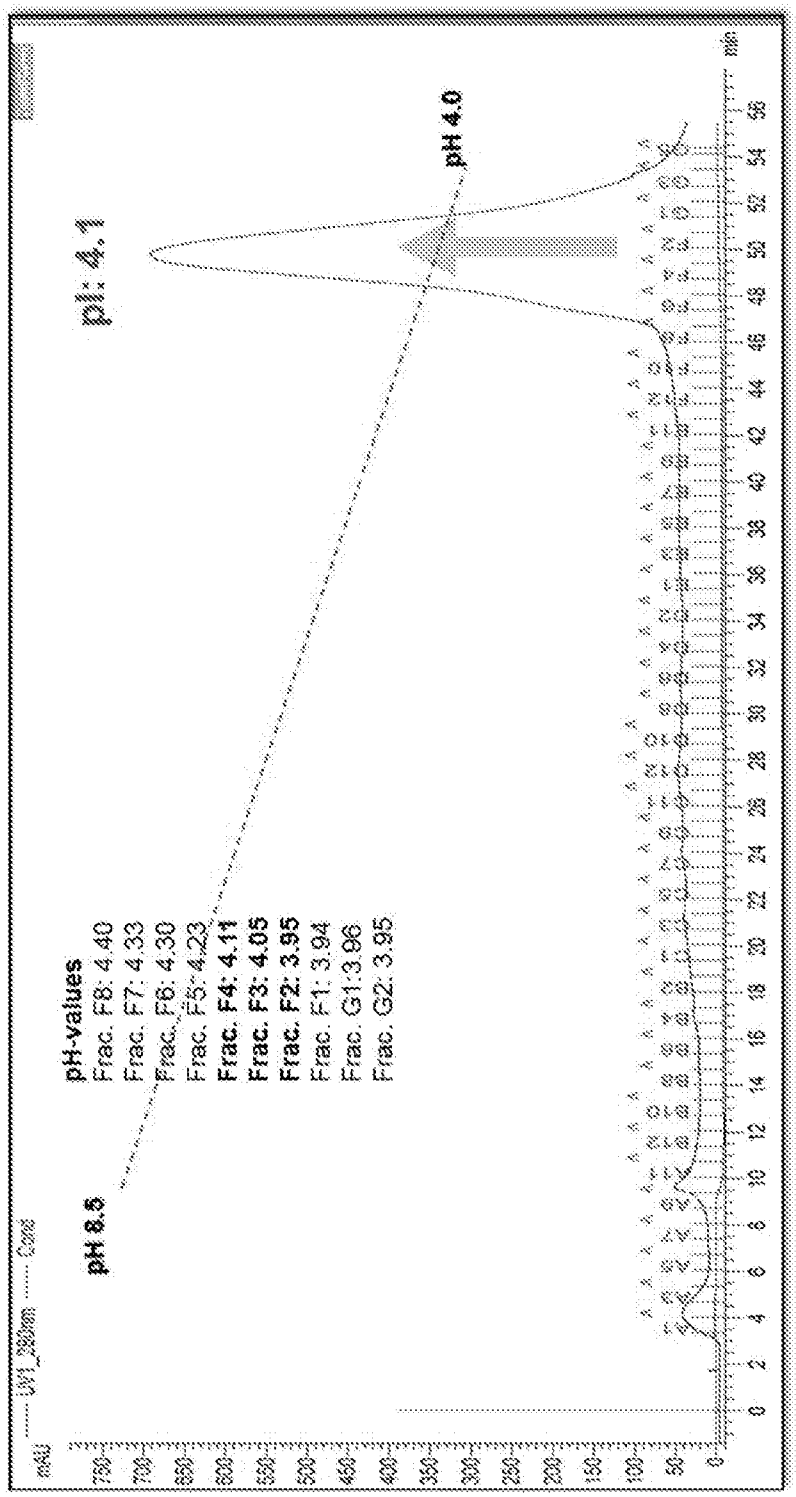
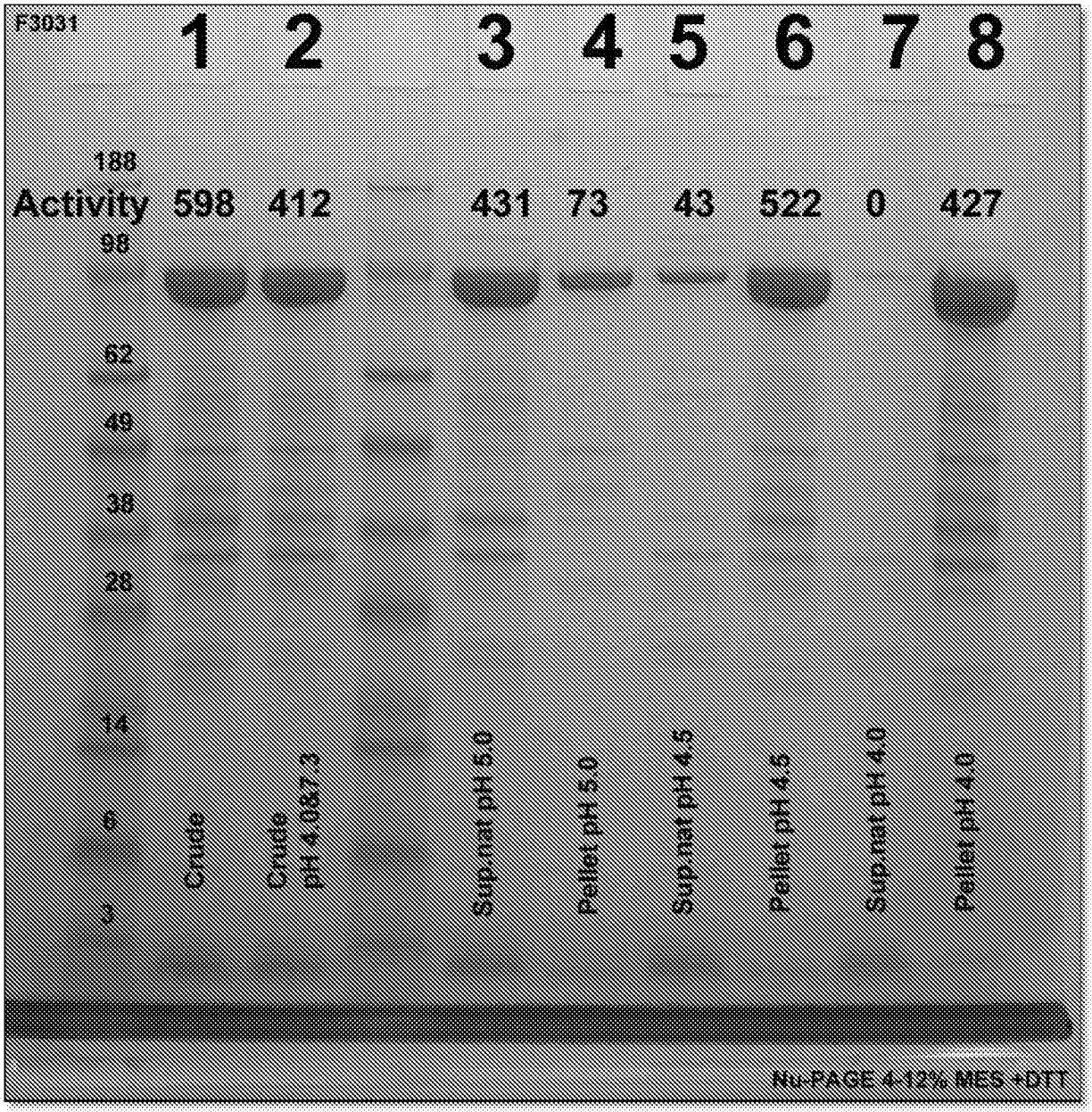

pI: 4.1
pH 4.0
pH 8.6
pH-values
Frac. F8: 4.40
Frac. F7: 4.33
Frac. F6: 4.30
Frac. F5: 4.23
Frac. F4: 4.11
Frac. F3: 4.05
Frac. F2: 3.95
Frac. F1: 3.94
Frac. G1:3.96
Frac. G2: 3.95
mAU
UV1_280nm
Conc
min

Figure 2


F3031
1 2 3 4 5 6 7 8
188
Activity 598 412 431 73 43 522 0 427
98
62
49
38
28
14
6
3
Crude
Crude pH 4.0&7.3
Sup.nat pH 5.0
Pellet pH 5.0
Sup.nat pH 4.5
Pellet pH 4.5
Sup.nat pH 4.0
Pellet pH 4.0
Nu-PAGE 4-12% MES +DTT

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/066519

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23C9/12 C12N9/38
ADD.

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)
A23C C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, 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	GB 1 306 751 A (BAXTER LABORATORIES INC) 14 February 1973 (1973-02-14) abstract page 4, column 2, line 93 ff.; example 4 -----	1-10, 12-15
X	WO 2007/060247 A2 (DSM IP ASSETS BV [NL]; SWAAF DE MAXIMILIAAN PETER MAR [NL]; DIJK VAN A) 31 May 2007 (2007-05-31) abstract pages 1, 2, 10 ----- -/--	16-21

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

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 February 2017	08/03/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brero, Alessandro

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JIAN CHEN ET AL: "Large-scale purification of human granulocyte-macrophage colony-stimulating factor expressed in Bombyx mori pupae", APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY ; PART A: ENZYME ENGINEERING AND BIOTECHNOLOGY, vol. 141, no. 1, 1 April 2007 (2007-04-01) , pages 149-159, XP055347141, New York ISSN: 0273-2289, DOI: 10.1007/s12010-007-9217-1 abstract ----- C Bramaud ET AL: "Whey protein fractionation: isoelectric precipitation of alpha-lactalbumin under gentle heat treatment", Biotechnology and bioengineering, 20 November 1997 (1997-11-20), pages 391-397, XP055347142, United States DOI: 10.1002/(SICI)1097-0290(19971120)56:4<391: :AID-BIT5>3.0.CO;2-J Retrieved from the Internet: URL:http://onlinelibrary.wiley.com/doi/10.1002/%28SICI%291097-0290%2819971120%2956:4%3C391::AID-BIT5%3E3.0.CO;2-J/pdf [retrieved on 2017-02-16] abstract -----	1-21 1-21

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International application No
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