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(71) **Applicant: NOVOZYMES A/S** [DK/DK]; Krogshoejvej 36, 2880 Bagsvaerd (DK).

(72) **Inventor: TAMS, Jeppe, Wegener**; Krogshoejvej 36, 2880 Bagsvaerd (DK).

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(54) **Title:** GLYCOSYLATED BETA-GALACTOSIDASE COMPOSITIONS HAVING IMPROVED TRANSGALACTOSYLATING ACTIVITY

(57) **Abstract:** The present invention relates to compositions, particularly liquid compositions, comprising polypeptides having beta-galactosidase activity, methods of making said compositions, and uses of the compositions for making e.g. dairy products. The polypeptides having beta-galactosidase activity are modified by glycation of lysine and/or arginine residues by incubating the enzyme in the presence of reducing sugars, optionally combined with a heat treatment. Thereby, transgalactosylating activity is increased.

GLYCOSYLATED BETA-GALACTOSIDASE COMPOSITIONS HAVING IMPROVED TRANSGALACTOSYLATING ACTIVITY

SEQUENCE LISTING

The present invention comprises a sequence listing, which is incorporated by reference
5 herein.

TECHNICAL FIELD

The present invention relates to compositions, particularly liquid compositions, comprising enzymes, methods of making the compositions, and uses of the same for making e.g. dairy
10 products.

BACKGROUND OF THE INVENTION

Beta-galactosidase, also known as lactase, is an enzyme known to hydrolyse the terminal non-reducing beta-D-galactose residues in beta-D-galactosidases. More particularly, under
15 normal reaction conditions, the enzyme hydrolyses its lactose substrate to the component monosaccharides D-glucose and D-galactose. Under certain conditions, certain beta-galactosidases have the ability to transfer galactose to the hydroxyl group of either glucose or galactose to form galacto-oligosaccharides (GOS) in a process called transgalactosylation.
20

A lactase from *Bifidobacterium bifidum* has been described having a high transgalactosylating activity, both in the full-length form and especially when truncated from the C-terminal end (see, e.g., Jørgensen *et al.* (2001), *Appl. Microbiol. Biotechnol.*, **57**: 647-652 or EP patent 1 283 876).
25

In WO 2009/071539, we describe a differently truncated fragment compared to Jørgensen. WO 2009/071539 discloses C-terminally truncated fragment of the extracellular lactase from *Bifidobacterium bifidum,* which was originally isolated and patented for its ability to make high amounts of galactooligosaccharides from lactose, can be used very successfully for
30 hydrolysis of lactose in milk. When tested in water + 100 g/l lactose at 37°C, the enzyme makes galactooligosaccharides with high efficiency as described in the prior art. However, when tested in milk, the ratio of hydrolytic to transgalactosylating activity has changed markedly, resulting in efficient hydrolysis and very low production of galactooligosaccharides.

35 WO 2013/182686 describes still further differently truncated fragments compared to Jørgensen, described as efficient producers of GOS when incubated with lactose even at low lactose levels such as in a milk-based product. WO 2013/182686 also describes compositions comprising a stabilizer.

WO 2015/132349 describes liquid lactase compositions comprising lactase and further comprising sodium, calcium or potassium-L-lactate or a combination thereof and optionally a sugar.

5

There remains a need to develop enzymes which are efficient producers of GOS, and industrially important formulations of the same.

SUMMARY OF THE INVENTION

10 In one embodiment, the invention provides a formulation comprising a polypeptide having beta-galactosidase activity and at least 30 wt% of a reducing sugar, preferably fructose, galactose, glucose, or lactose.

In another embodiment, the invention provides a polypeptide having beta-galactosidase 15 activity having been modified by glycation of at least one lysine and/or arginine residue.

In another embodiment, the invention provides a method of modifying a polypeptide having beta-galactosidase activity comprising contacting the polypeptide with a reducing sugar, preferably fructose, glucose, galactose, or lactose, for a time and temperature sufficient to 20 produce a polypeptide modified by glycation.

In another embodiment, the invention provides a method for producing galacto-oligosaccharides (GOS) comprising contacting a formulation of the invention or a polypeptide of the invention or a polypeptide having beta-galactosidase activity which has been modified 25 by a method of the invention with lactose.

In still another embodiment, the invention provides a method for producing galacto-oligosaccharides comprising contacting a polypeptide having a sequence comprising or consisting of amino acids 1-1304 of SEQ ID NO: 1, with lactose under conditions of high 30 temperature and high initial lactose concentration.

DETAILED DISCLOSURE OF THE INVENTION

Despite the dominant hydrolytic properties of certain beta-galactosidase or lactase enzymes, these enzymes can be forced to have transferring properties at, e.g., high lactose and high 35 temperature conditions. We have surprisingly discovered that when subjected to a pre-incubation, the previously hydrolytic-dominating enzyme can be converted to a transferring enzyme, which is also able to make GOS efficiently at lower temperatures than the unprocessed enzyme. The pre-incubation thus surprisingly results in a more robust GOS-

producing enzyme due to its heightened transferring abilities (transgalactosylase activity).

Without wishing to be bound by theory, it is believed that these incubation conditions result in glycation of the beta-galactosidase, which results in increased transferring properties. With covalent attachment of the sugar moiety, the beta-galactosidase is converted from a hydrolysing to a transferring enzyme having transgalactosylase activity.

Beta-Galactosidase

Beta-galactosidases from glycoside hydrolase family 2 (GH2) are exo-acting enzymes, which hydrolyse terminal non-reducing beta-D-galactose residues in beta-D-galactosides, e.g. lactose is hydrolysed to galactose and glucose. They belong to the enzyme class EC 3.2.1.23 with the official name beta-D-galactoside galactohydrolase. A common name used for this enzyme is lactase, as lactose is the common industrial substrate. Besides hydrolysing this enzyme class is also able to transfer galactose to other sugars and thereby make galacto-oligosaccharides (GOS). The different GH2 enzyme have various preferences for hydrolytic or beta-galactosidase activity and transgalactosylase activity and the preference can be expressed in terms of their GOS production ability, such as by the ratio of transgalactosylating activity to beta-galactosidase activity.

In the present context, the term “beta-galactosidase” means any glycoside hydrolase having the ability to hydrolyse the disaccharide lactose into its constituent galactose and glucose monomers. Enzymes assigned to subclass EC 3.2.1.108, also called lactases, are also considered a beta-galactosidase in the context of the present invention. In the context of the invention, the lactose hydrolysing activity of the beta-galactosidase may be referred to as its lactase activity or its beta-galactosidase activity.

In the context of the present invention, the polypeptide having beta-galactosidase activity preferably belongs to the enzyme class EC 3.2.1.23 or EC 3.2.1.108, preferably 3.2.1.23. The polypeptide having beta-galactosidase activity preferably belongs to glycoside hydrolase family 2 (GH2), more preferably to the glycoside hydrolase family GH2_5.

In certain applications, combinations of polypeptides having predominantly transgalactosylating activity and predominantly hydrolysing activity may be contemplated. This may be especially useful when there is a desire to reduce residual lactose after treatment with the polypeptide having beta-galactosidase activity, for example at low lactose levels.

When considering the reaction of the polypeptide in e.g. milk, carbohydrates are initially

present in the form of lactose, a disaccharide composed of galactose and glucose that is found in milk. In the formation of GOS, successive galactose molecules are added to lactose, and then after prolonged incubation a mixture of the various carbohydrates is present (glucose, galactose and ~30 different di- and polysaccharides).

5

The term “disaccharide” as used herein means two monosaccharide units bound together by a covalent bond known as a glycosidic linkage formed via a dehydration reaction, resulting in the loss of a hydrogen atom from one monosaccharide and a hydroxyl group from the other. In one aspect, the disaccharide is cellobiose, fucose, lactose, lactulose, maltose, rhamnose,
10 or sucrose, most preferably lactose.

As used herein, the term “transgalactosylase” means an enzyme that is able to transfer galactose to the hydroxyl groups of D-galactose (Gal) or D-glucose (Glc) whereby galactooligosaccharides are produced. In one embodiment, transgalactosylase activity is
15 identified by reaction of the enzyme on lactose in which the amount of galactose generated is less than the amount of glucose generated at a given time.

More particularly, the transgalactosylase activity or preference for an enzyme to hydrolyze lactose or to produce GOS can be evaluated as the amount of glucose minus galactose
20 generated at any given time during reaction or by direct quantification of GOS generated during the reaction. This measurement may be performed by one of several ways including the methods shown in the Examples herein.

When evaluating the transgalactosylating activity versus beta-galactosidase activity of an
25 enzyme, the beta-galactosidase activity is measured as concentration of galactose generated at any time point during the reaction.

In the present context, the GOS production of a polypeptide is measured as

$$\frac{(\text{Glucose} - \text{Galactose})}{\text{Galactose}},$$

30 i.e., the ratio of transgalactosylating activity to beta-galactosidase activity.

Preferably, the ratio of transgalactosylating activity to beta-galactosidase activity is at least 1, at least 2.5, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, or at least 12 as measured in high lactose conditions.

35

Polypeptides having beta-galactosidase activity useful according to the present invention

may be of animal, of plant or of microbial origin. Preferred polypeptides are obtained from microbial sources, in particular from a filamentous fungus or yeast, or from a bacterium.

The polypeptide may, e.g., be derived from a strain of *Agaricus*, e.g. *A. bisporus*; *Ascovaginospora; Aspergillus*, e.g. *A. niger, A. awamori, A. foetidus, A. japonicus, A. oryzae; Candida; Chaetomium; Chaetotomastia; Dictyostelium*, e.g. *D. discoideum; Kluveromyces*, e.g. *K. fragilis, K. lactis; Mucor*, e.g. *M. javanicus, M. mucedo, M. subtilissimus; Neurospora*, e.g. *N. crassa; Rhizomucor*, e.g. *R. pusillus; Rhizopus*, e.g. *R. arrhizus, R. japonicus, R. stolonifer; Sclerotinia*, e.g. *S. libertiana; Torula; Torulopsis; Trichophyton*, e.g. *T. rubrum; Whetzelinia*, e.g. *W. sclerotiorum*; *Bacillus*, e.g. *B. sp. B. coagulans, B. circulans, B. megaterium, B. novalis, B. subtilis, B. pumilus, B. stearothermophilus, B. thuringiensis*; *Bifidobacterium,* e.g. *B. animalis, B. bifidum, B. breve, B. infantis, B. lactis*, *B. longum; Chryseobacterium; Citrobacter*, e.g. *C. freundii; Clostridium,* e.g. *C. perfringens; Diplodia,* e.g. *D. gossypina; Enterobacter*, e.g. *E. aerogenes, E. cloacae Edwardsiella, E. tarda; Erwinia*, e.g. *E. herbicola; Escherichia*, e.g. *E. coli; Klebsiella*, e.g. *K. pneumoniae; Miriococcum; Myrothesium; Mucor; Neurospora,* e.g. *N. crassa; Proteus*, e.g. *P. vulgaris; Providencia*, e.g. *P. stuartii; Pycnoporus,* e.g. *Pycnoporus cinnabarinus, Pycnoporus sanguineus; Ruminococcus,* e.g. *R. torques; Salmonella*, e.g. *S. typhimurium*; *Serratia*, e.g. *S. liquefasciens, S. marcescens; Shigella*, e.g. *S. flexneri; Streptomyces*, e.g. *S. antibioticus, S. castaneoglobisporus, S. violeceoruber; Trametes; Trichoderma,* e.g. *T. reesei, T. viride; Yersinia*, e.g. *Y. enterocolitica*.

In a preferred embodiment, the polypeptide is a beta-galactosidase from a bacterium, e.g. from the family Bifidobacteriaceae, such as from the genus *Bifidobacterium,* such as from a strain of *B. animalis, B. bifidum, B. breve, B. infantis, B. lactis*, or *B. longum*. In a more preferred embodiment, the polypeptide is a beta-galactosidase from *Bifidobacterium bifidum.*

In a preferred embodiment, the polypeptide is a beta-galactosidase from a bacterium, e.g. from the family Bacillaceae, such as from the genus *Bacillus,* such as from a strain of *B. sp. B. coagulans, B. circulans, B. megaterium, B. novalis, B. subtilis, B. pumilus, B. stearothermophilus, B. thuringiensis*; *Bifidobacterium,* e.g. *B. animalis, B. bifidum, B. breve, B. infantis, B. lactis*, *B. longum*. In a more preferred embodiment, the polypeptide is a beta-galactosidase from *Bacillus circulans* or *Bacillus infantis.*

A preferred polypeptide is a beta-galactosidase having a sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity. Such fragment of SEQ ID NO: 1 may be any fragment of SEQ ID NO:

1 having beta-galactosidase activity.

In a preferred embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention comprises an amino acid sequence which is at least 50% identical to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity. In a more preferred embodiment, the enzyme comprises an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 4, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 6, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 6.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 7, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at

least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 7.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 8, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 8.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 9, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 9.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 10, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 10.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 11, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 11.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 12, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 12.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity. Preferably, the polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at
5 least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14.

In another embodiment, a polypeptide having beta-galactosidase activity to be used in a method of the present invention has an amino acid sequence which is at least 50% identical to SEQ ID NO: 15, or a fragment thereof having beta-galactosidase activity. Preferably, the
10 polypeptide has an amino acid sequence which is at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 15.

For purposes of the present invention, the sequence identity between two amino acid sequences is determined as the output of “longest identity” using the Needleman-Wunsch
15 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 Genet.* 16: 276-277), preferably version 6.6.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. In order for the
20 Needle program to report the longest identity, the -nobrief option must be specified in the command line. The output of Needle labeled “longest identity” is calculated as follows:
(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

A beta-galactosidase may be extracellular. They may have a signal sequence at their N-
25 terminus, which is cleaved off during secretion.

A polypeptide having beta-galactosidase may be derived from any of the sources mentioned herein. The term “derived” means in this context that the polypeptide may have been isolated from an organism where it is present natively, i.e. the identity of the amino acid sequence of
30 the enzyme are identical to a native polypeptide. The term “derived” also means that the polypeptides may have been produced recombinantly in a host organism, the recombinantly produced polypeptide having either an identity identical to a native polypeptide 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 polypeptide which is a mutant
35 and/or a fragment of a native amino acid sequence. Within the meaning of a native polypeptide are included natural variants. Furthermore, the term “derived” includes polypeptides produced synthetically by, e.g., peptide synthesis. The term “derived” also encompasses enzymes which have been modified e.g. by glycosylation, phosphorylation

etc., whether *in vivo* or *in vitro*. With respect to recombinantly produced polypeptide the term “derived from” refers to the identity of the polypeptide and not the identity of the host organism in which it is produced recombinantly.

5 The polypeptide having beta-galactosidase may be obtained from a microorganism by use of any suitable technique. For instance, a beta-galactosidase polypeptide preparation may be obtained 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 polypeptide having beta-galactosidase may also be obtained by use of recombinant 10 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 beta-galactosidase in a culture medium under conditions permitting the expression of the polypeptide and recovering the polypeptide from the culture. 15 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.

A polypeptide having beta-galactosidase may be purified. The term "purified" as used herein 20 covers beta-galactosidase enzyme protein essentially free from insoluble components from the production organism. The term "purified" also covers beta-galactosidase 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 25 one or more of the unit operations: filtration, precipitation, or chromatography.

Accordingly, the polypeptide having beta-galactosidase activity 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 30 other components, particularly other proteins and most particularly other enzymes present in the cell of origin of the beta-galactosidase. The polypeptide having beta-galactosidase may be "substantially pure", i.e. free from other components from the organism in which it is produced, i.e., e.g., a host organism for recombinantly produced beta-galactosidase. Preferably, the beta-galactosidase is an at least 40% (w/w) pure enzyme protein preparation, 35 more preferably at least 50%, 60%, 70%, 80% or even at least 90% pure.

The term polypeptide having beta-galactosidase activity includes whatever auxiliary compounds may be necessary for the enzyme's catalytic activity, such as, e.g., an

appropriate acceptor or cofactor, which may or may not be naturally present in the reaction system.

The polypeptide may be in any form suited for the use in question, such as, e.g., in the form of a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a protected enzyme.

The polypeptide is added in a suitable amount to achieve the desired degree of lactose hydrolysis under the chosen reaction conditions. The polypeptide may be added at a concentration of between 100 and 15,000 LAU(C) per litre milk-based substrate, preferably between 100-10,000 LAU(C) per litre milk-based substrate. Additional preferred concentrations include e.g. 100 LAU(C)/L, 250 LAU(C)/L, 500 LAU(C)/L, 750 LAU(C)/L, 1000 LAU(C)/L, 1500 LAU(C)/L, 2000 LAU(C)/L, 5000 LAU(C)/L, 6000 LAU(C)/L, 7000 LAU(C)/L, 8000 LAU(C)/L, 9000 LAU(C)/L, 10,000 LAU(C)/L, 11,000 LAU(C)/L, 12,000 LAU(C)/L, 13,000 LAU(C)/L, 14,000 LAU(C)/L, or 15,000 LAU(C)/L.

The activity in LAU(C) of a specific beta-galactosidase may be determined by direct measurement of glucose released from lactose. The skilled person will know how to determine such activity. Alternatively, the activity may be determined by using the activity assay described in the Methods and Examples of the present application. Here, the activity is obtained by comparing to a standard curve run with a beta-galactosidase of known activity, and the activity of the unknown sample calculated from this.

The activity in LAU(B) of a specific beta-galactosidase 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 $MgSO_4$ $7H_2O$, 450 mg/L Brij 35 at pH6.5 and 30°C. After 600 seconds incubation, the reaction is stopped by adding 0.2 M Na_2CO_3 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.

The skilled person will know how to determine the lactase activity at different pH and temperature. The lactase activity at different pH and temperature is preferably determined by using a method as described in the Examples of the present application.

In one aspect, the polypeptide is a fragment having one or more (several) amino acids

deleted from the amino or carboxyl terminal of the polypeptide of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 wherein the fragment has beta-galactosidase activity. Particularly preferred are fragments which are carboxy-terminal truncations.

5 A fragment of beta-galactosidase contains at least 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, or 1300 amino acid residues.

In one aspect, the beta-galactosidase is as described in WO 2013/182686.

10 In one aspect, the beta-galactosidase is as described in WO 2015/132349.

In an aspect, the beta-galactosidase includes a polypeptide of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 and one or more fragments having beta-galactosidase activity, such as at least one, two, three, four, or five fragments.

15

Glycation

In an embodiment, the polypeptide having beta-galactosidase activity has been modified by glycation.

20 Without wishing to be bound by theory, it has been surprisingly found that glycation of the beta-galactosidase converts the polypeptide from a more hydrolysing to a more transferring enzyme having transgalactosylase activity.

"Glycation" as used herein refers to the covalent attachment of a carbohydrate to a protein.
25 Carbohydrate attachment may be via a side chain of, e.g., arginine, lysine, or N-terminal of the enzyme. Preferably, the carbohydrate attachment is via a side chain of arginine or lysine.

Glycation is sometimes referred to as (non-enzymatic) glycosylation. In the context of the present invention, glycosylation and glycation are used interchangeably and glycosylation
30 can be non-enzymatic.

In an embodiment, the polypeptide having beta-galactosidase activity has been modified by glycation of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 residues of the polypeptide.

35

In an embodiment, the polypeptide having beta-galactosidase activity has been modified by glycation of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 lysine and/or arginine residues of the polypeptide.

In an embodiment, the polypeptide having beta-galactosidase activity has been modified by glycation of at least 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50% of the lysine and/or arginine residues of the polypeptide. In one embodiment, the polypeptide having beta-galactosidase activity has been modified by glycation of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 lysine and/or arginine residues of the polypeptide.

In a preferred embodiment, the polypeptide having beta-galactosidase activity is modified by glycation of at least 1%, preferably at least 3%, more preferably at least 5%, even more preferably at least 10%, most preferably at least 20%, of the lysine and arginine residues of the polypeptide. For the avoidance of any possible doubt, this means that at least 1%, preferably at least 3%, more preferably at least 5%, even more preferably at least 10%, most preferably at least 20%, of the total number of lysine and arginine residues of the polypeptide is modified by glycation.

In another preferred embodiment, a trypsin digest of the polypeptide having beta-galactosidase activity would result in a percentage of glycated trypsin digested peptides of at least 1%, preferably at least 3%, more preferably at least 5%, at least 10% or at least 20%.

In an embodiment, incubation under suitable conditions as detailed below results in the glycation of some, substantially all, or even all of the surface residues of lysine and/or arginine. Again without wishing to be bound by theory, it is believed that some, substantially all, or even all of the glycated residues are located towards the C-terminal end of the polypeptide having beta-galactosidase activity.

Incubation Resulting in Glycation

In an embodiment, the invention provides a method of modifying a polypeptide having beta-galactosidase activity comprising contacting the polypeptide with a sugar for a time and temperature sufficient to produce a polypeptide modified by glycation.

In an embodiment, the polypeptide is contacted with a solution of 5-90 wt% sugar at pH 5-10 for a time of 3-20 hours at a temperature of 20-80°C. Preferred sugars are reducing sugars as set forth in more detail below, and particularly preferred is glucose.

Suitable conditions include contacting the polypeptide with a solution of 5-90 wt% sugar, such as 30-90 wt%, and in particular 30-70 wt%, e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90% sugar.

Suitable conditions include contacting the polypeptide at a pH in the range of 5-10, such as pH 5-8, e.g., pH 5, pH 5.5, pH 6, pH 6.5, pH 7, pH 7.5, pH 8, pH 8.5, pH 9, pH 9.5, or pH 10.

5 Suitable conditions include contacting the polypeptide for a time in the range of 3-20 hours, such as in the range of 6-16 hours, e.g., 3 hours, 3.5 hours, 4 hours, 4.5 hours, 5 hours, 5.5 hours, 6 hours, 6.5 hours, 7 hours, 7.5 hours, 8 hours, 8.5 hours, 9 hours, 9.5 hours, 10 hours, 10.5 hours, 11 hours, 11.5 hours, 12 hours, 12.5 hours, 13 hours, 13.5 hours, 14 hours, 14.5 hours, 15 hours, 15.5 hours, 16 hours, 16.5 hours, 17 hours, 17.5 hours, 18 10 hours, 18.5 hours, 19 hours, 19.5 hours, or 20 hours.

Suitable conditions include contacting the polypeptide at a temperature in the range of 20-80 °C, such as in the range of 20-50 °C, alternatively in the range of 40-80 °C, in particular, 50-70 °C, or alternatively, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, 15 70 °C, 75 °C, or 80 °C.

In a preferred embodiment, the polypeptide having beta-galactosidase activity is contacted with a reducing sugar at pH 5-8, preferably pH 6-7, for a time of 3-100 hours, preferably 15-80 hours, at a temperature of 50-80°C, preferably 50-70°C.

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The skilled person will know how to adjust the time of the contacting with the sugar according to the amount of enzyme added and the temperature. In general, if more enzyme is added, the time of contacting can be reduced. And in general, if the reaction temperature is increased, the time of contacting can be reduced. Depending on the storage conditions of the 25 enzyme after the contacting with the sugar, the glycation process may continue on the shelf. Therefore, if the shelf temperature of the enzyme is relatively high, the time of reaction with the sugar at a specified high temperature may be reduced since the glycation process will continue during transport and storage of the enzyme before it being used by the end consumer, who may be, e.g., a dairy company or a company producing GOS as an 30 ingredient..

In another preferred embodiment, the polypeptide having beta-galactosidase activity is contacted with 30-90 wt%, preferably 40-65 wt%, of a reducing sugar.

35 Sugar

The sugar in the beta-galactosidase formulation can include monosaccharides, disaccharides, or oligosaccharides. Blends of sugars are also contemplated.

Preferably, the sugar is a reducing sugar. A reducing sugar reacts with an amino acid residue of the beta-galactosidase via the Maillard reaction.

Exemplary reducing sugars include the monosaccharides fructose, galactose, glucose, glyceraldehyde, ribose, xylose. Preferred is fructose, galactose and/or glucose and most particularly fructose and/or glucose.

Other exemplary reducing sugars include disaccharides such as cellobiose, lactose and maltose, preferably lactose and/or maltose. Also exemplary are glucose polymers e.g. maltodextrin and glycogen.

The presence of a reducing sugar can be detected by many well-known tests including the use of Benedict's reagent and/or Tollen's reagent.

Formulation

The formulation according to an embodiment of the invention may comprises a liquid composition. Liquid compositions are preferred for ease of use.

In an alternative embodiment, the formulation comprises a solid composition, e.g., a powder or a granulate.

In an embodiment, the formulation or composition according to the invention comprises a polypeptide having beta-galactosidase activity and at least 30 wt%, 31 wt%, 32 wt%, 33 wt%, 34 wt%, 35 wt%, 36 wt%, 37 wt%, 38 wt%, 39 wt%, 40 wt%, 41 wt%, 42 wt%, 43 wt%, 44 wt%, 45 wt%, 46 wt%, 47 wt%, 48 wt%, 49 wt%, 50 wt%, 51 wt%, 52 wt%, 53 wt%, 54 wt%, 55 wt%, 56 wt%, 57 wt%, 58 wt%, 59 wt%, 60 wt%, 61 wt%, 62 wt%, 63 wt%, 64 wt%, 65 wt%, 66 wt%, 67 wt%, 68 wt%, 69 wt%, 70 wt%, 71 wt%, 72 wt%, 73 wt%, 74 wt%, 75 wt%, 76 wt%, 77 wt%, 78 wt%, 79 wt%, 80 wt%, 81 wt%, 82 wt%, 83 wt%, 84 wt%, 85 wt%, 86 wt%, 87 wt%, 88 wt%, 89 wt% or 90 wt% sugar. Preferably such a beta-galactosidase composition comprises 200-20,000 LAU(C) per g.

In one suitable formulation, the composition comprises enzyme polypeptide having beta-galactosidase activity and at least 30 wt%, 31 wt%, 32 wt%, 33 wt%, 34 wt%, 35 wt%, 36 wt%, 37 wt%, 38 wt%, 39 wt%, 40 wt%, 41 wt%, 42 wt%, 43 wt%, 44 wt%, 45 wt%, 46 wt%, 47 wt%, 48 wt%, 49 wt%, 50 wt%, 51 wt%, 52 wt%, 53 wt%, 54 wt%, 55 wt%, 56 wt%, 57 wt%, 58 wt%, 59 wt%, 60 wt%, 61 wt%, 62 wt%, 63 wt%, 64 wt%, 65 wt%, 66 wt%, 67 wt%, 68 wt%, 69 wt%, 70 wt%, 71 wt%, 72 wt%, 73 wt%, 74 wt%, 75 wt%, 76 wt%, 77 wt%, 78 wt%, 79 wt%, or 80 wt% glucose. Preferably such a beta-galactosidase composition

comprises 200-20,000 LAU(C) per g.

One suitable beta-galactosidase composition comprises 200-20,000 LAU(C) per g and at least 35 wt%, 36 wt%, 37 wt%, 38 wt%, 39 wt%, 40 wt%, 41 wt%, 42 wt%, 43 wt%, 44 wt%, 45 wt%, 46 wt%, 47 wt%, 48 wt%, 49 wt%, 50 wt%, 51 wt%, 52 wt%, 53 wt%, 54 wt%, 55 wt%, 56 wt%, 57 wt%, 58 wt%, 59 wt%, 60 wt%, 61 wt%, 62 wt%, 63 wt%, 64 wt% or 65 wt% sugar, preferably in the range of 40-80 wt% sugar. A preferred beta-galactosidase composition comprises 200-20,000 LAU(C) per g and preferably 40 wt%, 60 wt%, or 80 wt% glucose.

In one embodiment, the formulation is a liquid formulation which comprises 200-15,000 LAU(C)/g, preferably 500-10,000 LAU(C)/g.

In one embodiment, the formulation is a solid formulation which comprises 1,000-20,000 LAU(C)/g, preferably 3,000-15,000 LAU(C)/g.

In an embodiment, the formulation further comprises glycerol.

However, in a preferred embodiment, the formulation is free of, or at least substantially free of, polyols or diols, such as glycerol and/or sorbitol. The amount of polyol or diol such as glycerol is preferably less than 40 wt%, less than 30 wt%, less than 25 wt%, less than 20 wt%, less than 15 wt%, less than 10 wt%, most preferably less than 5 wt%. Most preferably the formulation is free of polyol or diol such as glycerol.

In an embodiment, the formulations herein are enzymatically stable. Particularly preferred are enzymatically stable liquid enzyme formulations, and more particularly preferred are enzymatically stable liquid enzyme formulations without using glycerol. Enzymatic stability is a measure of the rate at which the activity of the enzyme decreases over time.

Also preferred are formulations, especially liquid formulations, which are microbially stable. Microbial stability is a measure of the rate at which undesired microorganisms can proliferate and grow in the composition.

In an embodiment, the formulation further comprises sodium chloride or potassium chloride, preferably in the range of 0.01-5 wt%, preferably 0.01-3 wt%, more preferably 0.01-2 wt%.

In an embodiment, the formulation further comprises a preservative. Food grade preservatives are preferred, of which benzoate, sorbate, methyl paraben, and propyl paraben

are exemplary.

In an alternative but preferred embodiment, the formulation is free of preservatives such as benzoate, sorbate, methyl paraben and/or propyl paraben.

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Uses

Production of galacto-oligosaccharides is contemplated under both *in situ* conditions from lactose already present in the milk, as well as under conditions of high initial lactose concentration (greater than 40-50% lactose (w/w)).

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In an embodiment, methods for producing galacto-oligosaccharides comprising contacting a polypeptide having beta-galactosidase activity with lactose under conditions of high temperature and high initial lactose concentration. In particular, the temperature may be, e.g., 40-80°C, such as 50°C, 60°C, 65°C, 70°C, 75°C, or 80°C. Moreover, the initial lactose

15 concentration may be above 40% (w/w), such as 40-50% (w/w), 45% (w/w), 50% (w/w), 55% (w/w), 40-60% (w/w) or even above 60% (w/w), such as 61% (w/w), 62% (w/w), 63% (w/w), 64% (w/w), 65% (w/w), 66% (w/w), 67% (w/w), 68% (w/w), 69% (w/w), 70% (w/w), 71% (w/w), 72% (w/w), 73% (w/w), 74% (w/w), 75% (w/w), or 80% (w/w) lactose.

20 In an aspect is provided a method for producing a dairy product comprising treating a milk-based substrate comprising lactose with a polypeptide having beta-galactosidase activity as described herein. Typically, under in situ conditions for applications of a polypeptide having beta-galactosidase activity in milk, initial lactose concentration is about 3-10% (w/w) lactose e.g., 3, 4, 5, 6, 7, 8, 9, or 10% (w/w), most typically about 5% (w/w).

25

The term “milk", in the context of the present invention, is to be understood as the lacteal secretion obtained by milking any mammal, such as cows, sheep, goats, buffaloes or camels.

30 “Milk-based substrate”, in the context of the present invention, may be any raw and/or processed milk material. Useful milk-based substrates include, but are not limited to solutions/suspensions of any milk or milk like products comprising lactose, such as whole or low fat milk, skim milk, buttermilk, reconstituted milk powder, condensed milk, solutions of dried milk, UHT milk, whey, whey permeate, acid whey, or cream.

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Preferably, the milk-based substrate is milk or an aqueous solution of skim milk powder. Milk powder typically has a starting lactose concentration of 36-52% (w/w/).

The milk-based substrate may be more concentrated than raw milk.

In one embodiment, the milk-based substrate has a ratio of protein to lactose of at least 0.2, preferably at least 0.3, at least 0.4, at least 0.5, at least 0.6 or, most preferably, at least 0.7.

5

The milk-based substrate may be homogenized and pasteurized according to methods known in the art.

"Homogenizing" as used herein means intensive mixing to obtain a soluble suspension or
10 emulsion. It may be performed so as to break up the milk fat into smaller sizes so that it no longer separates from the milk. This may be accomplished by forcing the milk at high pressure through small orifices.

"Pasteurizing" as used herein means reducing or eliminating the presence of live organisms,
15 such as microorganisms, in the milk-based substrate. Preferably, pasteurization is attained by maintaining a specified temperature for a specified period of time. The specified temperature is usually attained by heating. The temperature and duration may be selected in order to kill or inactivate certain bacteria, such as harmful bacteria, and/or to inactivate enzymes in the milk. A rapid cooling step may follow.' A "dairy product" in the context of the
20 present invention may be any food product wherein one of the major constituents is milk-based. Preferable, the major constituent is milk-based. More preferably, the major constituent is a milk-based substrate which has been treated with polypeptide having beta-galactosidase activity according to a method of the invention. In the context of the present invention "one of the major constituents" means a constituent having a dry matter which
25 constitutes more than 20%, preferably more than 30% or more than 40% of the total dry matter of the dairy product, whereas "the major constituent" means a constituent having a dry matter which constitutes more than 50%, preferably more than 60% or more than 70% of the total dry matter of the dairy product.

30 A dairy product according to the invention may be, e.g., skim milk, low fat milk, whole milk, cream, UHT milk, milk having an extended shelf life, a fermented milk product, cheese, yoghurt, butter, dairy spread, butter milk, acidified milk drink, sour cream, whey based drink, ice cream, condensed milk, dulce de leche or a flavoured milk drink. A dairy product may be manufactured by any method known in the art.

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A dairy product may additionally comprise non-milk components, e.g. vegetable components such as, e.g., vegetable oil, vegetable protein, and/or vegetable carbohydrates. Dairy products may also comprise further additives such as, e.g., enzymes, flavouring agents,

microbial cultures such as probiotic cultures, salts, sweeteners, sugars, acids, fruit, fruit juices, or any other component known in the art as a component of, or additive to, a dairy product.

5 In one embodiment of the invention, one or more milk components and/or milk fractions account for at least 50% (weight/weight), such as at least 70%, e.g. at least 80%, preferably at least 90%, of the dairy product.

In one embodiment of the invention, one or more milk-based substrates having been treated 10 with lactase polypeptide having beta-galactosidase activity according to a method of the invention account for at least 50% (weight/weight), such as at least 70%, e.g. at least 80%, preferably at least 90%, of the dairy product.

In one embodiment of the invention, the dairy product is a dairy product which is not enriched 15 by addition of pre-produced galactooligosaccharides.

In one embodiment of the invention, the enzyme-treated milk-based substrate is not dried before being used as an ingredient in the dairy product.

20 In one embodiment of the invention, the dairy product is ice cream. In the present context, ice cream may be any kind of ice cream such as full fat ice cream, low fat ice cream, or ice cream based on yoghurt or other fermented milk products. Ice cream may be manufactured by any method known in the art.

25 In one embodiment of the invention, the dairy product is milk or condensed milk. Condensed milk typically has a lactose concentration of 10-20% (w/w), such as 10-16 % (w/w), and in some embodiments, 18-18.5% (w/w).

In one preferred embodiment of the invention, the dairy product is UHT milk. UHT milk in the 30 context of the present invention is milk which has been subjected to a sterilization procedure which is intended to kill all microorganisms, including the bacterial spores. UHT (ultra high temperature) treatment may be, e.g., heat treatment for 30 seconds at 130°C, or heat treatment for one second at 145°C.

35 In one preferred embodiment of the invention, the dairy product is ESL milk. ESL milk in the context of the present invention is milk which has an extended shelf life due to microfiltration and/or heat treatment and which is able to stay fresh for at least 15 days, preferably for at least 20 days, on the store shelf at 2-5°C.

In another preferred embodiment of the invention, the dairy product is a fermented dairy product, e.g., yoghurt.

A “fermented dairy product” in the context of the present invention is to be understood as any dairy product wherein any type of fermentation forms part of the production process. Examples of fermented dairy products are products like yoghurt, buttermilk, creme fraiche, quark and fromage frais. A fermented dairy product may be produced by any method known in the art.

“Fermentation” in the method of the present invention means the conversion of carbohydrates into alcohols or acids through the action of a microorganism. Preferably, fermentation in the method of the present invention comprises conversion of lactose to lactic acid.

In the context of the present invention, “microorganism” may include any bacterium or fungus being able to ferment the milk substrate.

The microorganisms used for most fermented milk products are selected from the group of bacteria generally referred to as lactic acid bacteria. As used herein, the term "lactic acid bacterium" designates a gram-positive, microaerophilic or anaerobic bacterium, which ferments sugars with the production of acids including lactic acid as the predominantly produced acid, acetic acid and propionic acid. The industrially most useful lactic acid bacteria are found within the order “Lactobacillales” which includes *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pseudoleuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Enterococcus* spp. and *Propionibacterium* spp. Additionally, lactic acid producing bacteria belonging to the group of anaerobic bacteria, bifidobacteria, i.e. *Bifidobacterium* spp., which are frequently used as food cultures alone or in combination with lactic acid bacteria, are generally included in the group of lactic acid bacteria.

Lactic acid bacteria are normally supplied to the dairy industry either as frozen or freeze-dried cultures for bulk starter propagation or as so-called "Direct Vat Set" (DVS) cultures, intended for direct inoculation into a fermentation vessel or vat for the production of a fermented dairy product. Such cultures are in general referred to as “starter cultures” or “starters”.

Commonly used starter culture strains of lactic acid bacteria are generally divided into mesophilic organisms having optimum growth temperatures at about 30°C and thermophilic

organisms having optimum growth temperatures in the range of about 40 to about 45°C. Typical organisms belonging to the mesophilic group include *Lactococcus lactis, Lactococcus lactis* subsp. *cremoris, Leuconostoc mesenteroides* subsp. *cremoris, Pseudoleuconostoc mesenteroides* subsp. *cremoris, Pediococcus pentosaceus, Lactococcus lactis* subsp. *lactis biovar. diacetylactis, Lactobacillus casei* subsp. *casei* and *Lactobacillus paracasei* subsp. *paracasei*. Thermophilic lactic acid bacterial species include as examples *Streptococcus thermophilus, Enterococcus faecium, Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus helveticus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus*.

Also the anaerobic bacteria belonging to the genus *Bifidobacterium* including *Bifidobacterium bifidum, Bifidobacterium animalis* and *Bifidobacterium longum* are commonly used as dairy starter cultures and are generally included in the group of lactic acid bacteria. Additionally, species of *Propionibacteria* are used as dairy starter cultures, in particular in the manufacture of cheese. Additionally, organisms belonging to the *Brevibacterium* genus are commonly used as food starter cultures.

Another group of microbial starter cultures are fungal cultures, including yeast cultures and cultures of filamentous fungi, which are particularly used in the manufacture of certain types of cheese and beverage. Examples of fungi include *Penicillium roqueforti, Penicillium candidum, Geotrichum candidum, Torula kefir, Saccharomyces kefir and Saccharomyces cerevisiae*.

In one embodiment of the present invention, the microorganism used for fermentation of the milk-based substrate is *Lactobacillus casei* or a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus.*

Fermentation processes to be used in a method of the present invention are well known and the person of skill in the art will know how to select suitable process conditions, such as temperature, oxygen, amount and characteristics of microorganism/s, additives such as e.g. carbohydrates, flavours, minerals, enzymes, and process time. Obviously, fermentation conditions are selected so as to support the achievement of the present invention.

As a result of fermentation, pH of the milk-based substrate will be lowered. The pH of a fermented dairy product of the invention may be, e.g., in the range 3.5-6, such as in the range 3.5-5, preferably in the range 3.8-4.8.

In a preferred embodiment, the fermented dairy product is yoghurt.

In one embodiment, is provided a method of using a polypeptide having beta-galactosidase activity as described herein, or a cell expressing such polypeptide, for producing oligosaccharides. Oligosaccharides include, without limitation, fructo-oligosaccharides, galacto-oligosaccharides, isomalto-oligosaccharides, lactosucrose, malto-oligosaccharides, mannan-oligosaccharides, and xylo-oligosaccharides. Particularly preferred are galacto-oligosaccharides (GOS).

In an embodiment, oligosaccharides are produced by contacting polypeptide as described herein with a medium that comprises a disaccharide substrate including, e.g., cellobiose, lactose, lactulose, maltose, rhamnose, sucrose, and trehalose, and incubating under conditions whereby oligosaccharides are produced. The medium comprising a polypeptide as described herein may be part of a product selected from the group consisting of cheese, yoghurt, and other fermented milk products as also described more particularly above, as well as dietary supplements and probiotic comestible products. Alternatively, the oligosaccharides can be recovered and subsequently added to the product of interest before or after its preparation.

Similarly, in an embodiment, oligosaccharides may be produced by contacting a cell expressing enzyme polypeptide as described herein in a medium that comprises a disaccharide substrate including, e.g., cellobiose, lactose, lactulose, maltose, rhamnose, sucrose, and trehalose, and incubating under conditions whereby oligosaccharides are produced. The cells may be part of a product selected from the group consisting of cheese, yoghurt, and other fermented milk products as also described more particularly above, as well as dietary supplements and probiotic comestible products. Alternatively, the oligosaccharides can be recovered and subsequently added to the product of interest before or after its preparation.

In one aspect, the use of a cell for producing a product selected from the group consisting of yoghurt, cheese, fermented milk product, dietary supplement and probiotic comestible product, is provided.

In one aspect, the polypeptides described herein may be used to prepare cheese products and in methods for making the cheese products. Cheese products may be e.g., selected from the group consisting of cream cheese, cottage cheese, and process cheese. By adding polypeptides the cheeses may contain significantly increased levels of galactooligosaccharides and reduced levels of lactose. In one aspect, the lactose levels in the final cheese product may be reduced by at least about 25 percent, preferably at least

about 50 percent, and more preferably at least about 75 percent. The polypeptides may be used to reduce lactose in cheese products to less than about 1 gram per serving, an amount that can be tolerated by most lactose-intolerant individuals.

5 The cheese products provided herein are nutritionally-enhanced cheese products having increased soluble fiber content, reduced caloric content, excellent organoleptic properties, improved texture, and flavour. Further, the polypeptides described herein may reduce the glycemic index of the cheese products because GOS are more slowly absorbed than lactose or its hydrolysis products. Finally, the polypeptides may reduce the cost of production of
10 cheese products, particularly cream cheese products, because GOS surprisingly provide improved texture to the cream cheese product, thus permitting reduced use of stabilizers, or by allowing for increased moisture content without syneresis.

In a further aspect, the use of a transgalactosylating polypeptide as disclosed herein or a cell
15 as disclosed herein, for producing galacto-oligosaccharides, is provided. In one aspect, the use of a transgalactosylating polypeptide as disclosed herein or a cell as disclosed herein, for producing galacto-oligosaccharides to be part of a product selected from the group consisting of yoghurt, cheese, fermented dairy products, dietary supplements and probiotic comestible products, is provided. In one aspect, the product is yoghurt, cheese, or fermented
20 dairy products. In one aspect, the use of a transgalactosylating polypeptide as disclosed herein or a cell as disclosed herein, for producing galacto-oligosaccharides to enhance the growth of Bifidobacterium, is provided. In one aspect, the use of a transgalactosylating polypeptide as disclosed herein or a cell as disclosed herein, for producing galacto-oligosaccharides to enhance the growth of Bifidobacterium in a mixed culture fermentation, is
25 provided.

In one aspect, a process for producing a transgalactosylating polypeptide as disclosed herein, comprising culturing a cell as disclosed herein in a suitable culture medium under conditions permitting expression of said polypeptide, and recovering the resulting polypeptide
30 from the culture, is provided. A process for producing galacto-oligosaccharides, comprising contacting of a polypeptide as disclosed herein or a cell as disclosed herein with a milk-based solution comprising lactose, is provided.

The treatment of milk products with a polypeptide that converts lactose into
35 monosaccharides or GOS has several advantages. The products may be consumed by people with lactose intolerance that would otherwise exhibit symptoms such as flatulence and diarrhea. Dairy products treated with lactase will also have a higher sweetness than similar untreated products due to the higher perceived sweetness of glucose and galactose

compared to lactose. This effect is particularly interesting for applications such as yoghurt and ice-cream where high sweetness of the end product is desired and this allows for a net reduction of carbohydrates in the consumed product. In ice-cream production, a phenomenon termed sandiness is often seen, where the lactose molecules crystallizes due to the relative low solubility of the lactose. When lactose is converted into monosaccharides or GOS the mouth feeling of the ice-cream is much improved over the non-treated products. The presence of a sandy feeling due to lactose crystallization can be eliminated and the raw material costs can be decreased by replacement of skimmed milk powder by when powder. The main effects of the enzymatic treatment are increased sweetness.

Another interesting use of the polypeptides having beta-galactosidase activity is in infant, follow-on or toddler formula. Infant formula is a manufactured food designed and marketed for feeding to babies and infants under 12 months of age, usually prepared for bottle-feeding or cup-feeding from a powder (mixed with water) or a liquid (with or without additional water). The most commonly used infant formulae contain purified cow's milk whey and casein as a protein source, a blend of vegetable oils as a fat source, lactose as a carbohydrate source, a vitamin-mineral mix, and other ingredients.

In many countries, the addition or carry-over of glycerol to infant, follow-on or toddler formula is prohibited by law, therefore in applications for infant, follow-on or toddler formula, formulations of polypeptides having beta-galactosidase activity must be free of glycerol.

In one embodiment, the polypeptides having transgalactosylating activity may be used together with other enzymes such as proteases, including chymosin or rennin, lipases such as phospholipases, amylases, and transferases.

PREFERRED EMBODIMENTS

1. A formulation comprising a polypeptide having beta-galactosidase activity and at least 30 wt% of a reducing sugar, preferably fructose, galactose, glucose, or lactose.

2. The formulation of embodiment 1, wherein the polypeptide having beta-galactosidase activity has been modified by glycation of at least one lysine and/or arginine residue.

3. The formulation of any of the preceding embodiments, wherein the polypeptide having beta-galactosidase activity has been modified by glycation of at least 1%, preferably at least 3%, more preferably at least 5%, even more preferably at least

10%, most preferably at least 20%, of the lysine and arginine residues of the polypeptide.

4. The formulation of any of the preceding embodiments, which is an enzyme formulation.

5. The formulation of any of the preceding embodiments having an activity of 200-20,000 LAU(C)/g, preferably 500-15,000 LAU(C)/g.

6. The formulation of any of the preceding embodiments which is a liquid formulation, preferably having an activity of 200-15,000 LAU(C)/g, more preferably 500-10,000 LAU(C)/g.

7. The formulation of any of the preceding embodiments which is a solid formulation, preferably having an activity of 1,000-20,000 LAU(C)/g, more preferably 3,000-15,000 LAU(C)/g.

8. The formulation of any of the preceding embodiments, comprising 40-65 wt% sugar.

9. The formulation of any of the preceding embodiments, wherein the sugar is glucose.

10. The formulation of any of the preceding embodiments, which is substantially free of glycerol.

11. The formulation of any of the preceding embodiments, which further comprises sodium chloride or potassium chloride, preferably in the range of 0.01-5 wt%, preferably 0.01-3 wt%, more preferably 0.01-2 wt%.

12. The formulation of any of the preceding embodiments, wherein the polypeptide having beta-galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4, or a fragment thereof having

beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 6, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 7, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 8, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 9, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 10, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 11, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 12, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 15, or a fragment thereof having beta-galactosidase activity.

13. The formulation of any of the preceding embodiments, wherein the polypeptide having beta-galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2 to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least

98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity.

14. The formulation of any of the preceding embodiments, wherein the polypeptide having beta-galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or 100% identical to amino acids 1-1304 of SEQ ID NO: 1 and has a length of 900-1350 amino acids, preferably 1300-1305 amino acids, more preferably 1302 or 1304 amino acids.

15. A polypeptide having beta-galactosidase activity having been modified by glycation of at least one lysine and/or arginine residue.

16. The polypeptide of embodiment 15, which has been modified by glycation of at least 1%, preferably at least 3%, more preferably at least 5%, even more preferably at least 10%, most preferably at least 20%, of the lysine and arginine residues of the polypeptide.

17. The polypeptide of any of embodiments 15-16 which has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4, or a fragment thereof

having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 6, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 7, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 8, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 9, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 10, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 11, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 12, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 15, or a fragment thereof having beta-galactosidase activity.

18. The polypeptide of any of embodiments 15-17 which has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2 to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a

fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity.

19. The polypeptide of any of embodiments 15-18 which has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or 100% identical to amino acids 1-1304 of SEQ ID NO: 1 and has a length of 900-1350 amino acids, preferably 1300-1305 amino acids, more preferably 1302 or 1304 amino acids.

20. A method of modifying a polypeptide having beta-galactosidase activity comprising contacting the polypeptide with a reducing sugar, preferably fructose, glucose, galactose, or lactose for a time and temperature sufficient to produce a polypeptide modified by glycation.

21. The method of embodiment 20 which is a method of modifying by glycation a polypeptide having beta-galactosidase activity.

22. The method of any of embodiments 20-21, wherein the polypeptide having beta-galactosidase activity modified by glycation has improved transgalactosylating activity as compared to the polypeptide having beta-galactosidase activity which has not been modified by glycation.

23. The method of any of embodiments 20-22, wherein the polypeptide having beta-galactosidase activity is modified by glycation of at least 1%, preferably at least 3%, more preferably at least 5%, even more preferably at least 10%, most preferably at least 20%, of the lysine and arginine residues of the polypeptide.

24. The method of any of embodiments 20-23, comprising contacting the polypeptide having beta-galactosidase activity with 30-90 wt% of a reducing sugar, preferably fructose, glucose, or galactose, at pH 5-8 for a time of 3-100 hours at a temperature

of 20-80°C.

25. The method of any of embodiments 20-24, comprising contacting the polypeptide having beta-galactosidase activity at pH 5-8, preferably pH 6-7, for a time of 3-100 hours, preferably 15-80 hours, at a temperature of 50-80°C, preferably 50-70°C.

26. The method of any of embodiments 20-25, comprising contacting the polypeptide having beta-galactosidase activity with 30-90 wt%, preferably 40 wt%, 60 wt%, or 80 wt% of a reducing sugar, preferably glucose.

27. The method of any of embodiments 20-25, comprising contacting the polypeptide having beta-galactosidase activity with 30-90 wt%, preferably 40-65 wt%, of a reducing sugar.

28. The method of any of embodiments 20-27, wherein the reducing sugar is fructose, glucose or galactose, preferably glucose.

29. The method of any of embodiments 20-28, wherein the polypeptide having beta-galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 6, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 7, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 8, or a fragment thereof having beta-galactosidase

activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 9, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 10, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 11, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 12, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 15, or a fragment thereof having beta-galactosidase activity.

30. The method of any of embodiments 20-29, wherein the polypeptide having beta-galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2 to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95%

or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity.

31. The method of any of embodiments 20-30, wherein the polypeptide having beta-galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or 100% identical to amino acids 1-1304 of SEQ ID NO: 1 and has a length of 900-1350 amino acids, preferably 1300-1305 amino acids, more preferably 1302 or 1304 amino acids.

32. A method for producing galacto-oligosaccharides (GOS) comprising contacting the formulation of any of embodiments 1-14 or the polypeptide of any of embodiments 15-19 or a polypeptide having beta-galactosidase activity which has been modified by the method of any of claims 20-31 with lactose.

33. A method for producing galacto-oligosaccharides (GOS) comprising contacting a polypeptide having a sequence comprising or consisting of amino acids 1-1304 of SEQ ID NO: 1, with lactose under conditions of high temperature and high initial lactose concentration.

34. The method of embodiment 33, wherein the temperature is 40-80°C, such as 50°C, 60°C, 65°C, 70°C, 75°C, or 80°C and wherein the initial lactose concentration is above 40% (w/w), such as 40-50% (w/w), 45% (w/w), 50% (w/w), 55% (w/w), 40-60% (w/w) or even above 60% (w/w), such as 61% (w/w), 62% (w/w), 63% (w/w), 64% (w/w), 65% (w/w), 66% (w/w), 67% (w/w), 68% (w/w), 69% (w/w), 70% (w/w), 71% (w/w), 72% (w/w), 73% (w/w), 74% (w/w), 75% (w/w), or 80% (w/w) lactose.

EXAMPLES

MATERIALS AND METHODS

Activity Assay (LAU(C))

Principle:

Lactase hydrolyzes lactose to form α-D-glucose. The α-D-glucose is phosphorylated by ATP, in a reaction catalyzed by hexokinase. The glucose-6-phosphate formed is oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. Concomitant with this reaction an equimolar amount of NAD+ is reduced to NADH with a resulting increase in absorbance at 340 nm.

Reagents:

15% (w/v) Brij L23: Weigh out 508.0 ± 0.4g of Brij® L23 (Sigma B4184) into a beaker. Add approx. 300mL ultrapure water and stir. Transfer the Brij® L23 quantitatively to a 1 L volumetric flask. Fill to the mark with ultrapure water. Stir until homogenous. Storability: 2 months in refrigerator.

Colour reagent: (Glucose reagent kit (GHK) (0.1 M Tris, 2.1 mM ATP, 2.1 mM NAD, 4 mM Mg2+, <0.1% NaN3, 4 mMMg2+, >7.5 kU/L hexokinase, > 7.5 kU/L G-6-P-DH, pH 7.8)): Open a vial of Glucose (HK) Reagent A, Thermo Fisher Scientific (Art. no.: 981304 or 981779) and a vial of Glucose (HK) Reagent B, Thermo Fisher Scientific (Art. no.: 981304 or 981779). Pour 1 vial of reagent B into 1 vial of reagent A. Put on the cap. Mix well by slowly and gently turning up and down the vial 10-15 times. Use the whole mixture in reagent A vial, or pour needed amount into an appropriate container. Storability: 1 month in refrigerator.
Dissolution buffer/dilution buffer (0.01 M Citric acid monohydrate, 0.0225% (w/v) Brij® L23, 1 mM MgSO4, 7H2O, pH 4.5): Weigh out 21.0 ± 0.1 g of Citric acid monohydrate (Cas. No. 5949-29-1) and transfer quantitative to a 10 L volumetric flask. Weigh out 2.46 ± 0.01 g of MgSO4, 7H2O (Cas. no. 10034-99-8) and transfer quantitative to the volumetric flask. Add approximately 9 L of demineralized water and stir until completely dissolved. Add 15 mL of 15% (w/v) Brij L23 to the volumetric flask and stir. Add approximately 35 mL of 4 M NaOH (Cas. No. 1310-73-2) and stir. Adjust pH to 4.50 ± 0.05 using e.g. 4 M NaOH or e.g. HCl as appropriate. Fill to the mark with demineralized water and stir. Storability: 13 days at room temperature.

Substrate (31.6 % w/w lactose monohydrate, 0.01M citric monohydrate, 0.0225 (w/v) Brij L23, 1 mM MgSO4, 7H2O): Weigh out 7.9 ± 0.2 g of Lactose monohydrate (Cas. No. 10039-26-6) directly into a beaker. Dissolve to a total volume of 25.0 ± 0.1 g of dissolution buffer. Heat up and stir until fully dissolved with no boiling of the substrate. Storability: 6 hours at room temperature.

Standard: Enzyme standard with identified LAU(C)/g(available from Novozymes A/S, Denmark) is used as standard, diluted in dissolution buffer in the range from 0.197-0.7880 LAU(C)/mL.

Procedure:

1. 50 uL of substrate is incubated for 540 seconds at 50°C. Blank (50 uL of dissolution buffer) is subtracted out.
2. 25 uL sample in dissolution buffer is added.

3. The reaction is incubated for 1800 seconds followed by addition of 160 uL colour reagent.
4. After 300 seconds, the absorbance is measured at 340 nm.

Calculation of Enzyme Activity:

5 The enzyme activity of the diluted sample is read from the standard curve. Calculation of activity of a sample in LAU(C)/g is as stated in the formula:

$$Activity\ Unit/g = \frac{S \cdot V \cdot F}{W}$$

S = Reading from the standard curve in LAU(C)/mL
V = Volume of the measuring flask used in mL
10 F = Dilution factor for second dilution
W = Weight of sample in g

Application in Yoghurt

Commercial homogenized milk with 1.5% fat is pasteurized at 90°C for 20 min. 200 ml of the 15 milk is transferred into baby bottles and tempered to 43°C. The milk is inoculated with a frozen probiotic yoghurt culture e.g. Chr. Hansen, Denmark, (F-DVS ABY-3) using an inoculation level of 0.02%. At the same time, enzyme is added to the milk. The milk samples are fermented at 43°C until pH reached 4.55 within approximately five hours. The yoghurts are then stirred, cooled to 25°C and placed at 8°C for storage. Samples are collected 2 hours 20 after addition of culture and enzyme, at end pH (pH 4.55) and after 20-24 hours (Day 1) of storage at 8°C. The biological activity is stopped by addition of sulphuric acid. Proteins are precipitated adding perchloric acid and MQW containing standards are then added. Lactose hydrolysis is measured using a Dionex ICS-3000 system equipped with a Carbopac20 connected with an electrochemical detector (ED). Peaks are identified and quantified by 25 comparing with known standards of lactose, glucose and galactose. Content of DP2 saccharides, particularly lactose, and GOS in the form of DP3+ are identified and quantified. Vivinal GOS (Friesland Campina) is a useful standard for GOS quantification.

Application in Yoghurt

30 Commercial homogenized milk with 1.5% fat is pasteurized at 90°C for 20 min. 200 ml of the milk is transferred into baby bottles and tempered to 43°C. The milk is inoculated with a frozen probiotic yoghurt culture e.g. Chr. Hansen, Denmark, (F-DVS ABY-3) using an inoculation level if 0.02%. At the same time enzyme is added to the milk. The milk samples are fermented at 43°C until pH reached 4.55 within approximately five hours. The yoghurts 35 are then stirred, cooled to 25°C and placed at 8°C for storage. Samples are collected 2 hours after addition of culture and enzyme, at end pH (pH 4.55) and after 1, 2, 3 and 7 days of storage at 8°C. The biological activity is stopped by addition of sulphuric acid. Proteins are

precipitated adding perchloric acid and MQW containing standards are then added. Lactose hydrolysis is measured using a Dionex ICS-3000 system equipped with a Carbopac20 connected with an electrochemical detector (ED). Peaks are identified and quantified by comparing with known standards of lactose, glucose and galactose. Content of DP2 saccharides, particularly lactose, and GOS in the form of DP3+ are identified and quantified. Vivinal GOS (Friesland Campina) is a useful standard for GOS quantification.

Application in 1.5% Milk

Commercial homogenized milk with 1.5% fat is transferred to tubes (10 ml) and heated in water baths to 40°C, 50°C and 55°C, respectively. Enzyme is then added to the milk samples. Samples are collected 2 hours and 4 hours after addition of the enzyme. The biological activity is stopped by addition of sulphuric acid. Proteins are precipitated adding perchloric acid and MQW containing standards is then added. Lactose hydrolysis is measured using a Dionex ICS-3000 system equipped with a Carbopac20 connected with an electrochemical detector (ED). Peaks are identified and quantified by comparing with known standards of lactose, glucose and galactose. Content of DP2 saccharides, particularly lactose, and GOS in the form of DP3+ are identified and quantified. Vivinal GOS (Friesland Campina) is a useful standard for GOS quantification.

Application in Skimmed Milk Solution

100 ml 9% skimmed milk solution having approximately 5% lactose is made by mixing 9 g skimmed milk powder (Kerry) in 91 ml ionic water. 10 ml of the solution is transferred to a test tube containing a magnetic stirring bar and placed in a water bath at 37°C. After 15 min enzyme is added. Milk samples are taken at regular intervals up till 4 hrs. and the enzyme inactivated by heating to 99°C for 10 min in a thermomixer. Samples are diluted appropriately and filtered through a 0.20 um filter. Lactose hydrolysis is measured using a Dionex BioLC equipped with a Dionex PA1 column and a Pulsed Amperiometrisk Detektor (PAD). Peaks are identified and quantified by comparing with known standards of lactose, glucose and galactose. Content of DP2 saccharides, particularly lactose, and GOS in the form of DP3+ are identified and quantified. Vivinal GOS (Friesland Campina) is a useful standard for GOS quantification.

Application in 1.5% Milk – High Temperature

Commercial homogenized milk with 1.5% fat is transferred to tubes (10 ml) and tempered to 63°C. Enzyme is added to the milk samples. At 63°C samples are collected 15 minutes, 30 minutes, 2 hours and 4 hours after addition of the enzyme. The enzymatic activity in the samples is stopped by addition of sulphuric acid and proteins precipitated by addition of perchloric acid before HPLC analysis. Lactose hydrolysis is measured using a Dionex ICS-

3000 system equipped with a Carbopac20 connected with an electrochemical detector (ED). Peaks are identified and quantified by comparing with known standards of lactose, glucose and galactose. Content of DP2 saccharides, particularly lactose, and GOS in the form of DP3+ are identified and quantified. Vivinal GOS (Friesland Campina) is a useful standard for GOS quantification.

Application in Whey Permeate Solution

100 ml 15 or 30%(w/w) whey permeate containing primarily lactose and ions is made by mixing 15 or 30 g spray-dried whey permeate powder (Variolac, Arla) in 85 or 70 ml ionic water respectively. The solution is poured in a flask containing a magnetic stirring bar and placed in a water bath at 37°C. After 15 min, enzyme is added. Milk samples are taken at regular intervals up till 5.5 hrs. and the enzyme inactivated by heating to 99°C for 10 min in a thermomixer. Samples are diluted appropriately and filtered through a 0.20 um filter. Lactose hydrolysis is measured using a Dionex BioLC equipped with a Dionex PA1 column and a Pulsed Amperiometrisk Detektor (PAD). Peaks are identified and quantified by comparing with known standards of lactose, glucose and galactose. Content of DP2 saccharides, particularly lactose, and GOS in the form of DP3+ are identified and quantified. Vivinal GOS (Friesland Campina) is a useful standard for GOS quantification.

EXAMPLE 1

Production of Polypeptide

Bifidobacterium bifidum β-galactosidase (BBB) having the sequence shown as SEQ ID NO: 1 is expressed in *Bacillus licheniformis*

EXAMPLE 2

Glycation

BBB-un_1: Untreated *Bifidobacterium bifidum* β-galactosidase (BBB-un_1) is expressed in *Bacillus licheniformis* according to Example 1 and concentrated using ultra filtration (cut-off 10 kDa) and finally formulated with glycerol 50% (w/w). Activity of this sample is 7210 LAU(C)/g.

To a 100 ml Distec vessel, 100 grams of 66% (w/w) sugar (glucose (Glc), galactose (Gal) or lactose (Lac)) solution with 20 mM succinic acid buffer pH 6.5 is added and preheated to 60°C for 15 min. Then 10 ml BBB-un_1 without glycerol is added and incubated at 60°C with mixing for 16 hr. The solution is cooled to room temperature and dialyzed (cut-off 12 kDa) against 5 mM succinic acid buffer pH 6.5 for 16 hr at 5°C and then concentrated to ~5 ml using Amicon cell cut-off 10 kDa and finally added the same volume of glycerol to give a conc. of 50% glycerol (v/v). The three samples generated from this procedure are termed

BBB-Glc, BBB-Gal and BBB-Lac referring to the sugar used for the incubation.

Filter-aided sample preparation (FASP) MS data from tryptic digests was made and glycated peptides were identified as Lys and Arg + 1 Hexose, causing 1 missed cleavage site. The % of glycated trypsin digested peptides were estimated to be 0.66%, 31%, 36% and 27% for untreated, lactose treated, glucose treated and galactose treated respectively. Thus, mass spectrometery of peptides made from trypsin digest confirms glycation on lysine and arginine residues of BBB-Glc, BBB-Gal and BBB-Lac but these glycations are not present in BBB-un_1.

GOS Production at 25°C

To evaluate GOS produced at 25°C, 50ul 1280 LAU(C)/g enzyme (BBB-un_1, BBB-Glc, BBB-Gal or BBB-Lac) is mixed with 950 ul preheated 66.5 % lactose*H_2O (w/w), 20 mM succinate pH 6.5 in an Eppendorf tube which gives a final concentration of 60% lactose. This mixture is then incubated at 25°C with 1000 rpm for 22 hr and applied on ice. Inactivation of the enzyme is then performed by diluting the 1ml GOS product with 49 ml 0.04 M NaOH, 1mM EDTA and incubated for 5 min at room temp. Then an additional 40x dilution with milli Q water (i.e. 2000x dilution in total) is made and applied to a PA1 column (High-Performance Anion-Exchange Chromatography) with Pulsed Amperometric Detection (HPAEC-PAD).

GOS Production at 65°C

In order to evaluate GOS produced at 65°C, 50ul 192 LAU(C)/g enzyme (BBB-un_1, BBB-Glc, BBB-Gal or BBB-Lac) is mixed with 950 ul preheated 66.5 % lactose*H20 (w/w), 20 mM succinate pH 6.5 in an Eppendorf tube which gives a final concentration of 60 % lactose. This mixture is then incubated at 65°C with 1000 rpm for 22 hr and applied on ice. Inactivation of the enzyme is then performed by diluting the 1ml GOS product with 49 ml 0.04 M NaOH, 1mM EDTA and incubated for 5 min at room temp. Then an additional 40x dilution with milli Q water (i.e. 2000x dilution in total) is made and applied to a PA1 column (High-Performance Anion-Exchange Chromatography) with Pulsed Amperometric Detection (HPAEC-PAD).

Table 1.

	(Glc-Gal)/ Gal	**(Glc-Gal)/ Gal**
	25°C	65°C
BBB-un_1	0.79	10

BBB-Lac	6.9	12
BBB-Glc	7.8	13
BBB-Gal	6.0	12

As seen in Table 1, untreated *Bifidobacterium bifidum* beta-galactosidase (BBB-un_1) has low transgalactosylating activity at 25°C with a (Glc-Gal)/Gal ratio of 0.79 compared with the glycated BBB forms (BBB-Glc, BBB-Gal and BBB-Lac) which have 7-10 fold higher (Glc-
5 Gal)/Gal ratio when incubated at the same process conditions. At 65°C, the difference is less pronounced between untreated and glycated BBB and only a 1.2-1.3 fold increase in (Glc-Gal)/Gal ratio is seen. However, it is surprising that all enzyme BBB-un_1, BBB-Glc, BBB-Gal and BBB-Lac have a pronounced increase in (Glc-Gal)/Gal ratio at elevated temperature, 65°C compared to 25°C, especially for BBB-un which has a 13-fold increase in (Glc-Gal)/Gal
10 ratio.

EXAMPLE 3

Sample:

BBB-un_2: Untreated *Bifidobacterium bifidum* β-galactosidase (BBB-un_2) is expressed in
15 *Bacillus licheniformis* according to Example 1 and concentrated using ultrafiltration (cut-off 10 kDa) and finally formulated with glucose 40% (w/w), 60% (w/w) or 80% (w/w), with an enzyme concentration of 7575 LAU(C)/g, 9200 LAU(C)/g and 4600 LAU(C)/g, respectively.

Glycation of enzyme samples:

20 Enzyme solution formulated with glucose are incubated for 16 h and 40 h at three different temperatures 50°C, 55°C and 60°C, see Table 2.

GOS production at 25°C

To evaluate GOS produced at 25°C, 50 μl enzyme sample as shown in Table 2 is mixed with
25 950 μl preheated 66.5 % lactose*H20 (w/w), 20 mM succinate pH 6.5 in an Eppendorf tube which gives a final concentration of 60 % lactose. This mixture is then incubated at 25°C with 1000 rpm for 22 hr and applied on ice. Inactivation of the enzyme is then performed by diluting the 1ml GOS product with 49 ml 0.04 M NaOH, 1mM EDTA and incubated for 5 min at room temp. Then an additional 40x dilution with milli Q water (i.e. 2000x dilution in total) is
30 made and applied to a PA1 column (which is High-Performance Anion-Exchange Chromatography, HPAEC) and carbohydrates were detected with Pulsed Amperometric Detection (PAD).

Results & Discussion

Table 2

	Temperature	Time	Glucose conc.	(Glc-Gal)/Gal
LAU(C)/g	°C	h	%	Ratio
7575	No heat treatment		40	0.5
7575	50	16	40	0.9
7575	55	16	40	0.9
7575	60	16	40	1.4
7575	50	40	40	1.6
7575	55	40	40	2.1
7575	60	40	40	2.5
9200	No heat treatment		60	0.5
9200	50	16	60	0.9
9200	55	16	60	1.4
9200	60	16	60	2.0
9200	50	40	60	2.2
9200	55	40	60	2.9
9200	60	40	60	3.3
4600	50	16	80	1.9
4600	55	16	80	4.3
4600	60	16	80	5.0
4600	50	40	80	5.3
4600	55	40	80	6.1
4600	60	40	80	6.1

Table 2 shows that there is an increase in the (Glc-Gal)/Gal ratio when the temperature is increased from 50 to 60°C and the (Glc-Gal)/Gal ratios are higher than control where no heat
5 treatment has been made. Prolonged incubation times also increases the (Glc-Gal)/Gal ratio, i.e. higher values are obtained after 40 h compared to 16 h. The effect of the temperature and time is the same for enzymes formulated with 40%, 60% and 80% glucose.

10 **EXAMPLE 4**

Glycation of enzyme samples

Untreated *Bifidobacterium bifidum* β-galactosidase (BBB-un) is expressed in *Bacillus licheniformis* according to Example 1 and concentrated using ultrafiltration (cut-off 10 kDa) and formulated with glucose at levels as indicated in the table by incubating for 44 h at 55°C
15 then stored at 4°C.

GOS production in milk at 5°C

One ml semi-skim milk is applied in 2 ml Eppendorf tube and heated to 90°C for 5 min and cooled in ice-bath for at least 30 min. Then 10 µl diluted enzyme sample is added and incubated for 24 h at 5°C. The reaction is stopped by adding 5 µl HAc and heated to 90°C for 5 min and centrifuged at 20,000 *g* for 5min. Then 50 µl supernatant is added to 500 µl Milli Q water + 10 µl Carrez I solution in a 5 ml Eppendorf tube and mixed followed by adding 10 µl Carrez II solution and mixed. Then 4.43 ml milli Q water is added and centrifuged at 20,000 *g* for 5 min at room temperature. Then 1 ml of supernatant is added to 4 ml water and filtered through a 0.20 µm filter into a HPLC vial and applied to a PA1 HPAEC column and carbohydrates are detected with PAD.

GOS production in milk at 42°C

One ml semi-skim milk is applied in 2 ml Eppendorf tube and heated to 90°C for 5 min and cooled in ice-bath for at least 30 min. Then 10 µl diluted enzyme sample is added and incubated for 6 h at 42°C. The reaction is stopped by adding 5 µl HAc and heated to 90°C for 5 min and centrifuged at 20,000 *g* for 5min. Then 50 µl supernatant is added to 500 µl Milli Q water + 10 µl Carrez I solution in a 5 ml Eppendorf tube and mixed followed by adding 10 µl Carrez II solution and mixed. Then 4.43 ml milli Q water is added and centrifuged at 20,000 *g* for 5 min at room temperature. Then 1 ml of supernatant is added to 4 ml water and filtered through a 0.20 µm filter into a HPLC vial and applied to a PA1 HPAEC column and carbohydrates are detected with PAD.

GOS production in 35% reconstituted skim milk powder at 42°C

One ml 35% (w/w) reconstituted skim milk powder is applied in 2 ml eppendorf tube and heated to 90°C for 5 min and cooled in ice-bath for at least 30 min. Then 10 µl diluted enzyme sample is added and incubated for 6 h at 42°C. The reaction is stopped by adding 5 µl HAc and heated to 90°C for 5 min and centrifuged at 20,000 g for 5min. Then 50 µl supernatant is added to 500 µl Milli Q water + 10 µl Carrez I solution in a 5 ml Eppendorf tube and mixed followed by adding 10 µl Carrez II solution and mixed. Then 4.43 ml milli Q water is added and centrifuged at 20,000 g for 5 min at room temperature. Then 0.35 ml of supernatant is added to 4.65 ml water and filtered through a 0.20 µm filter into a HPLC vial and applied to a PA1 column (which is High-Performance Anion-Exchange Chromatography, HPAEC) and carbohydrates are detected with Pulsed Amperometric Detection (PAD).

Results & Discussion

Table 3 Data from GOS production in milk at 5°C

Enzyme amount	Temperature	Time	Glucose conc.	(Glc-Gal)/ Gal	mean dev.
LAU(C)/mL	°C	h	%	ratio	

640	55	44	40	0.34	0.01
640	55	44	60	1.06	0.03
640	No heat treatment		40	0.07	0.002

Table 4 Data from GOS production in milk at 42°C

Enzyme amount	Temperature	Time	Glucose conc.	(Glc-Gal)/ Gal	mean dev.
LAU(C)/mL	°C	h	%	ratio	
290	55	44	40	0.31	0.02
290	55	44	60	0.51	0.02
290	No heat treatment		40	0.05	0.004

Table 5 Data from GOS production in 35% (w/w) reconstituted skim milk powder at 42°C

Enzyme amount	Temperature	Time	Glucose conc.	(Glc-Gal)/ Gal	mean dev.
LAU(C)/mL	°C	h	%	ratio	
840	55	44	40	0.68	0.005
840	55	44	60	1.8	0.2
840	No heat treatment		40	0.07	0.003

5

Table 3, 4 and 5 shows that (Glc-Gal)/Gal ratio is increased for both 40% and 60% glucose formulations when incubated at 55°C for 44 h compared to 40% glucose control (No heat treatment). These results show that GOS can be generated in-situ (in milk) at 5°C which is the common storage temperature of milk but also at 42°C which is useful for yoghurt
10 application as 42°C is a common fermentation temperature. An even higher (Glc-Gal)/Gal ratio can be achieved in 35% (w/w) reconstituted skim milk powder (table 5), i.e. increasing the dry matter content and lactose concentration therefore increasing transferase efficiency.

EXAMPLE 5

15 *Glycosylation of enzyme samples*

Untreated *Bifidobacterium bifidum* β-galactosidase (BBB) having the sequence shown as SEQ ID NO: 1 is expressed in *Bacillus licheniformis* and concentrated using ultrafiltration (cut-off 10 kDa) to 23000 LAU(B)/g and formulated with either 60% (w/w) glucose (3 gram glucose + 2 gram BBB-un) or 60% (w/w) glycerol (3 gram glycerol + 2 gram BBB-un) and
20 incubated for 66 h at 50°C or unformulated diluted with water with same dilution i.e. 3 gram water + 2 gram BBB-un and incubated for 30 min at 50°C

GOS Production at 25°C

To evaluate GOS produced at 25°C, 50ul 770 LAU(B)/g enzyme is mixed with 950 ul
25 preheated 66.5 % lactose*H20 (w/w), 20 mM succinate pH 6.5 in an Eppendorf tube which

gives a final concentration of 60 % lactose. This mixture is then incubated at 25°C with 1000 rpm for 22 hr and applied on ice. Inactivation of the enzyme is then performed by diluting the 1ml GOS product with 9 ml 0.04 M NaOH and incubated for 5 min at room temp. Then an additional 200x dilution with milli Q water (i.e. 2000x dilution in total) is made and applied to a PA1 column (High-Performance Anion-Exchange Chromatography) with Pulsed Amperometric Detection (HPAEC-PAD).

Table 6

	Enzyme amount	Temperature	Time	(Glc-Gal)/Gal	mean dev.
Formulation	LAU(B)/g	°C	h	ratio	
BBB treated in 60% glucose	770	50	66	5,5	0,1
BBB treated in 60% glycerol	770	50	66	0,70	0,05
BBB treated in water	770	50	0,5	0,68	0,01

The incubation in 60% glucose is made at 50°C for 66 h to ensure glycation of the *Bifidobacterium bifidum* β-galactosidase (BBB). Incubation in 60% glycerol (which is not a reducing sugar) is included as a control. The sample without formulating agent (BBB treated in water) is included as another control. Due to instability of the enzyme when no stabilizer is added (e.g. glucose or glycerol), the enzyme would not be stable for 66 h at 50°C and therefore the "BBB treated in water" sample was incubated only for 0.5 h at 50°C.

Table 6 shows that only high (Glc-Gal)/Gal ratio (5.5) is obtained by incubating *Bifidobacterium bifidum* β-galactosidase (BBB) with glucose and not with controls formulated in glycerol or without formulation agents (water). Thus, these results show that it is not heating of the enzyme sample as such that transforms the enzyme to get a high (Glc-Gal)/Gal ratio. It is the incubation with glucose at conditions that enable glycation of the enzyme that ensures the transformation from low to high (Glc-Gal)/Gal ratio.

EXAMPLE 6

Sample:

BBB-1: *Bifidobacterium bifidum* β-galactosidase having the sequence shown as SEQ ID NO: 1 has been expressed in *Bacillus licheniformis* and column purified and then finally formulated with 60% glucose (BBB-1-G) and incubated for 66 h at 50°C as shown in Table 6 and then stored at -20°C. Control sample (BBB-1-C) were not formulated with glucose and just stored at -20°C.

Kluyveromyces lactis β-galactosidase (Lactozym® Pure) has been expressed in *Kluyveromyces lactis* and concentrated using UF (cut-off 10 kDa) and finally formulated with 60% glucose with same enzyme protein conc. ([ep]) as BBB-1-G and incubated for 66 h at 50°C as shown in Table 6 and then stored at -20°C. Control sample was not formulated with glucose and just stored at -20°C and has the same enzyme protein conc. ([ep]) as BBB-1-C.

Bacillus circulans β-galactosidase having the sequence shown as amino acids 28-1737 of SEQ ID NO: 14 has been expressed in *Bacillus subtilis* and column purified and then finally formulated with 60% glucose with same enzyme protein conc. ([ep]) as BBB-1-G and incubated for 66 h at 50°C as shown in Table 6 and then stored at -20°C. Control sample was not formulated with glucose and just stored at -20°C and has the same enzyme protein conc. ([ep]) as BBB-1-C.

BBB-2: *Bifidobacterium bifidum* β-galactosidase having the sequence shown as SEQ ID NO: 1 has been expressed in *Bacillus licheniformis* and concentrated using UF (cut-off 10 kDa) and finally formulated with 50% glycerol and incubated for 4 weeks (672 h) at 40 °C as shown in Table 6 and then stored at -20°C. Control sample was not incubated at 40°C but just stored at -20°C during the 4 weeks

BBB-3: *Bifidobacterium bifidum* β-galactosidase having the sequence shown as SEQ ID NO: 1 has been expressed in *Bacillus licheniformis* and concentrated using UF (cut-off 10 kDa) and finally formulated with 40% glucose and incubated for 4 weeks (672 h) at 40 °C as shown in Table 6 and then stored at -20°C. Control sample was not incubated at 40°C but just stored at -20°C during the 4 weeks

GOS production in regular milk

5°C for 24 h (Results are shown in Table 6).

Two ml semi-skim milk (Arla, purchased in a local Danish supermarket, 4.7 g lactose and 3.5 g protein per 100 g) was transferred into a 5 ml Eppendorf tube (double determinations for each dose including control). Then 20 µl of enzyme dilution was added (see Table 6) and mixed followed by an incubation at 5°C for 24 h. After incubation, 10 µl concentrated acetic acid was added to each sample and the solutions were heated to 90°C for 5 min. After inactivation, the samples were centrifuged at 14,000 rpm for 5 min at room temperature. One ml supernatant was transferred to another tube and kept frozen until analysed by HPLC.

Determination of ratio of (Glc-Gal)/Gal

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a PA1 column for quantitative determination of galactose (Gal) and glucose (Glc) is performed as follows.

5 50 µl sample is mixed together with 500 µl Milli Q water + 10 µl Carrez I solution in a 5 ml Eppendorf tube, and then mixed with 10 µl Carrez II solution. Then 4.43 ml milli Q water is added and centrifugated at 14,000 rpm for 5 min at room temperature. One ml supernatant is mixed with 4 ml Milli-Q water and filtered through a 0.2 µm filter into a HPLC vial and applied on a PA1 column. Quantitative determination of Glc and Gal was made using a known
10 standard of Glc and Gal, respectively.

Table 6

	Enzyme amount	Temperature	Time	(Glc-Gal)/Gal	mean dev.
	LAU(B)/g	°C	h	ratio	
BBB-1-G (60% glucose)	260	50	66	3.39	0.03
K.lactis (60% glucose)	"same [ep] as BBB-1-G"	50	66	0.40	0.02
B.circulans (60% glucose)	"same [ep] as BBB-1-G"	50	66	4.09	0.07
BBB-1-C - control	640	No heat treatment		-0.04	0.005
K.lactis - control	"same [ep] as BBB-1-C"	No heat treatment		0.00	0.02
B.circulans - control	"same [ep] as BBB-1-C"	No heat treatment		0.00	0.001
BBB-2 (50% glycerol)	640	40	672	-0.02	0.02
BBB-3 (40% glucose)	640	40	672	0.75	0.14
BBB-2 (50% glycerol) - control	640	-20	672	-0.02	0.02
BBB-3 (40% glucose) - control	640	-20	672	-0.01	0.01

A pronounced increase in (Glc-Gal)/Gal ratio is seen when *Bifidobacterium bifidum* β-
15 galactosidase and *Bacillus circulans* β-galactosidase (both GH2_5) is incubated with 60%

glucose at 50°C for 66 h with a (Glc-Gal)/Gal ratio of 3.39 and 4.09, respectively. Whereas a smaller increase in (Glc-Gal)/Gal ratio is seen for *Kluyveromyces lactis* β-galactosidase (GH2_6). This suggests that for subfamily 5 of the glycosyl hydrolase family 2 (GH2_5), glycation has a more pronounced affect to shift the enzyme molecule from having a hydrolytic to a transferase activity, than for subfamily 6 of GH2 (GH2_6).

All control samples BBB-1-C, *K. lactis* and *B. circulans* have a (Glc-Gal)/Gal ratio close to zero. When compared to the small but positive values for the controls of Example 5, this is as expected as there is only ~5% lactose in milk whereas the controls in Table 5 were incubated at 60% lactose (a high lactose conc. favours transferase activity).

Glycation in lower glucose conc. and lower temperatures could also be achieved at prolonged incubation times as seen in Table 6, where BBB-3 incubated in 40% glucose at 40°C for 672 h resulted in a (Glc-Gal)/Gal ratio of 0.75 compared to control which has a value of zero. An additional control was made incubating *Bifidobacterium bifidum* β-galactosidase in 40% glycerol at 40°C for 672 h which has no detectable effect on the (Glc-Gal)/Gal ratio as a value of zero was obtained just as for the control. This additional experiment confirms that glycation and not heat-treatment as such is responsible for transforming the enzyme from a hydrolytic enzyme to a more transferring enzyme.

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

CLAIMS

1. A formulation comprising a polypeptide having beta-galactosidase activity and at least 30 wt% of a reducing sugar, preferably fructose, galactose, glucose, or lactose;
5 wherein the polypeptide having beta-galactosidase activity has been modified by glycation of at least one lysine and/or arginine residue.

2. The formulation of claim 1, wherein the polypeptide having beta-galactosidase activity has been modified by glycation of at least 1%, preferably at least 3%, more preferably
10 at least 5%, of the lysine and arginine residues of the polypeptide.

3. The formulation of any of claims 1 or 2 having an activity of 200-20,000 LAU(C)/g,.

4. The formulation of any of claims 1-3 which is a liquid formulation and which preferably
15 has an activity of 200-15,000 LAU(C)/g, more preferably 500-10,000 LAU(C)/g.

5. The formulation of any of claims 1-4, comprising 40-65 wt% sugar, wherein the sugar is preferably glucose.

20 6. The formulation of any of claims 1-5, which is substantially free of glycerol, and which optionally further comprises sodium chloride or potassium chloride, preferably in the range of 0.01-5 wt%, preferably 0.01-3 wt%, more preferably 0.01-2 wt%.

7. The formulation of any of claims 1-6, wherein the polypeptide having beta-
25 galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-
30 1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at
35 least 98% identical to SEQ ID NO: 4, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least

98% identical to SEQ ID NO: 6, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 7, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 8, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 9, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 10, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 11, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 12, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 15, or a fragment thereof having beta-galactosidase activity.

8. The formulation of any of claims 1-7, wherein the polypeptide having beta-galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2 to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4, or a fragment thereof having beta-galactosidase activity; at least 50% identical,

such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity.

9. The formulation of any of claims 1-8, wherein the polypeptide having beta-galactosidase activity has an amino acid sequence which is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or 100% identical to amino acids 1-1304 of SEQ ID NO: 1 and has a length of 900-1350 amino acids, preferably 1300-1305 amino acids, more preferably 1302 or 1304 amino acids.

10. A method of modifying by glycation a polypeptide having beta-galactosidase activity comprising contacting the polypeptide with 30-90 wt% of a reducing sugar, preferably fructose, glucose, galactose, or lactose, for a time and temperature sufficient to produce a polypeptide modified by glycation.

11. The method of claim 10, wherein the polypeptide having beta-galactosidase activity modified by glycation has improved transgalactosylating activity as compared to the polypeptide having beta-galactosidase activity which has not been modified by glycation.

12. The method of any of claims 10-11, wherein the polypeptide having beta-galactosidase activity is modified by glycation of at least 1%, preferably at least 3%, more preferably at least 5%, of the lysine and arginine residues of the polypeptide.

13. The method of any of claims 10-12, comprising contacting the polypeptide having beta-galactosidase activity at pH 5-8, preferably pH 6-7, for a time of 3-100 hours, preferably 15-80 hours, at a temperature of 50-80°C, preferably 50-70°C.

14. The method of any of claims 10-13, wherein the polypeptide having beta-galactosidase activity is at least 50%, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 1-1304 of SEQ ID NO: 1 or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, at least 70%, at least 80%, at least 90%, at least 95%

or at least 98% identical to amino acids 28-1931 of SEQ ID NO: 2, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to amino acids 28-1331 of SEQ ID NO: 3, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 4, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 5, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 6, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 7, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 8, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 9, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 10, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 11, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 12, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 13, or a fragment thereof having beta-galactosidase activity; at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 14, or a fragment thereof having beta-galactosidase activity; or at least 50% identical, such as at least 60%, such as at least 70%, at least 80%, at least 90%, at least 95% or at least 98% identical to SEQ ID NO: 15, or a fragment thereof having beta-galactosidase activity.

15. A method for producing galacto-oligosaccharides (GOS) comprising contacting the formulation of any of claims 1-9 or a polypeptide having beta-galactosidase activity

which has been modified by the method of any of claims 10-14 with lactose.

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/062539

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23C9/12 A23C9/13 C12N9/38 C12N9/26
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)
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 practicable, search terms used)

EPO-Internal, WPI Data, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CARLOS VERA ET AL: "Determination of the transgalactosylation activity of Aspergillus oryzae [beta]-galactosidase: effect of pH, temperature, and galactose and glucose concentrations", CARBOHYDRATE RESEARCH, vol. 346, no. 6, 4 February 2011 (2011-02-04), pages 745-752, XP055242416, GB ISSN: 0008-6215, DOI: 10.1016/j.carres.2011.01.030 abstract page 747, column 2, paragraph 3 - page 751, column 2, paragraph 4 -----	1-15

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

[X] See patent family annex.

* Special categories of cited documents :

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"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

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"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

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Date of the actual completion of the international search	Date of mailing of the international search report
20 July 2018	03/08/2018
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Schlegel, Birgit

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 2010/098561 A2 (GENOFOCUS CO LTD [KR]; CHOI JAE YOUL [KR]; PAN JAE GU [KR]; PARK SEUNG) 2 September 2010 (2010-09-02) paragraph [0052]; claims 1-9; example 5; sequence 1 -----	1-15
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/062539

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