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(54) **Title:** ENZYME COMPOSITION AND PREPARATION OF A DAIRY PRODUCT WITH IMPROVED PROPERTIES

(57) **Abstract:** The invention provides a dairy product obtainable by a process which comprises: a) providing a milk-based substrate comprising lactose; and b) treating said substrate with an enzyme composition comprising lactase activity, wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least about 60% sequence identity to any thereto, and wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

ENZYME COMPOSITION AND PREPARATION OF A DAIRY PRODUCT WITH IMPROVED PROPERTIES

Field of the invention

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The present invention relates to a dairy product obtainable by a process in which a milk-based substrate comprising lactose is treated with an enzyme composition comprising lactase activity. The invention also relates to the dairy product itself, to use an enzyme composition comprising lactase activity in the production of a dairy product and to the enzyme composition itself.

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Background to the invention

Lactose intolerance is perhaps the best-known food sensitivity in the United States and other parts of the world. It is estimated that about 70% of the world's population has a genetically controlled limited ability to digest lactose. Accordingly, there is a growing demand for dairy food products that contain no or only low levels of lactose.

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Lactase is used commercially to break down lactose in milk to produce dairy products which are suitable for people with lactose intolerance and/or have a sweeter taste. Because glucose and galactose are sweeter than lactose, lactase produces a more pleasant taste. Lactase is also used in the manufacture of ice cream. Lactose crystallizes at the low temperatures of ice cream, whereas glucose and galactose stay liquid and contribute to a smoother texture. Lactase is also used in the conversion of whey into syrup and for the production of condensed milk.

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It is desirable to provide new dairy products having low-amounts of lactose and methods for their production, where the method gives rise to a product which is more stable in production and/or which has with improved taste and/or shelf-life.

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Summary of the invention

In some processes for the production of dairy products, high temperatures are used. These high temperatures may lead to higher activity of contaminating side

activities present in a lactase composition such that additional low temperature steps need to be incorporated into the process for making the dairy product. The use of a lactase composition which has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity means that such a composition can be used at higher
5 temperatures without the side activities destabilising (processing of) the dairy product.

Accordingly, the invention relates to a dairy product obtainable by a process which comprises:

a) providing a milk-based substrate comprising lactose; and

b) treating said substrate at high temperature with an enzyme composition
10 comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least about 60% sequence identity to any thereto, and

15 wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

Alternatively, the invention relates to a dairy product obtainable by a process which comprises:

a) providing a milk-based substrate comprising lactose; and

b) treating said substrate at high temperature with an enzyme composition
20 comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about
25 60% sequence identity to any thereto, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

The invention also relates to a dairy product obtainable by a process which comprises:

30 a) providing a milk-based substrate comprising lactose; and

b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having lactase activity which is derived from *Bifidobacterium bifidum*, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

5 The invention also provides:

- a method for the production of a dairy product, which method comprises:
 - a) providing a milk-based substrate comprising lactose; and
 - b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

10 wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least about 60% sequence identity to any thereto, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity;

- 15
- a method for the production of a dairy product, which method comprises:
 - a) providing a milk-based substrate comprising lactose; and
 - b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

20 wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about 60% sequence identity to any thereto, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity;

- 25
- a method for the production of a dairy product, which method comprises:
 - a) providing a milk-based substrate comprising lactose; and
 - b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

30 wherein at least a part of the lactase activity is provided by a polypeptide having lactase activity which is derived from *Bifidobacterium bifidum*, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity;

- use of an enzyme having lactase activity in the production of a dairy product with improved taste and/or shelf-life, wherein dairy product obtainable by a process which comprises:

a) providing a milk-based substrate comprising lactose; and

5 b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least
10 about 60% sequence identity to any thereof, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity;

- use of an enzyme having lactase activity in the production of a dairy product with improved taste and/or shelf-life, wherein dairy product obtainable by a process
15 which comprises:

a) providing a milk-based substrate comprising lactose; and

b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having
20 an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about 60% sequence identity to any thereof, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity;

25 - use of an enzyme having lactase activity in the production of a dairy product with improved taste and/or shelf-life, wherein dairy product obtainable by a process which comprises:

a) providing a milk-based substrate comprising lactose; and

30 b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having lactase activity which is derived from *Bifidobacterium bifidum*, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity;

- an enzyme composition comprising lactase activity, wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least about 60% sequence identity to any thereto, which has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity;

- an enzyme composition comprising lactase activity, wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about 60% sequence identity to any thereto, which has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity; and

- an enzyme composition comprising lactase activity, wherein at least a part of the lactase activity is provided by a polypeptide having lactase activity which is derived from *Bifidobacterium bifidum* which has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

Description of the sequence listing

SEQ ID NOs: 1 and 2 set out the polypeptide sequences of lactase enzymes from *Bifidobacterium bifidum*. SEQ ID NO: 1 may also be defined with reference to SEQ ID NO: 2 in WO01/90317. SEQ ID NO: 2 may also be defined with reference to the Bbg3 sequence described in Goulas T.K., Goulas A.K., Tzortzis G., Gibson G.R., Appl. Microbiol. Biotechnol. 76(6), 1365 and 1372 (2007).

SEQ ID NO: 3, a C-terminal truncation variant, may also be defined with reference to SEQ ID NO: 2 as described in WO2009/071539.

SEQ ID NO: 4, another C-terminal truncation variant, may also be defined with reference to SEQ ID NO: 2 as described in WO2014/184189.

Detailed description of the invention

Throughout the present specification and the accompanying claims, the words "comprise", "include" and "having" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where
5 the context allows.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to one or at least one) of the grammatical object of the article. By way of example, "an element" may mean one element or more than one element.

The embodiments as described in the summary of the invention are now described
10 in more detail.

The treatment of the substrate may involve any process wherein a substrate is contacted with the enzyme preparation. The treatment may involve any process wherein the substrate is incubated in the presence of the enzyme preparation.

Typically, the process described herein is a high temperature process. A high
15 temperature process herein may be a process which is carried out at at least about 45°C, for example at at least about 45°C or at a higher temperature such as at least 50°C, at least 55°C, at least 60°C or at least above 60°C such as at least 61, 62, 63, 64 or 65°C.

The enzyme preparation may be added to the substrate in any suitable manner.
20 The process may be any process wherein a product is produced, for instance a nutritive product, preferably a dairy product.

Milk-based substrate in this 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
25 whole milk, low fat milk, skim milk, buttermilk, reconstituted milk from milk powder, condensed milk, solutions of dried milk, UHT milk or cream.

As used herein, a dairy product encompasses any composition that contains milk protein, for instance casein and/or whey protein. Examples are milk, milk-derived products, fermented milk products (e.g. yoghurt, for example stirred, set or greek),
30 condensed milk, evaporated milk, dry milk, frozen milk, ice cream, whey; and/or cheese, for example fresh or hard cheese.

The dairy product may also be a milk powder and/or a hydrolysate.

A dairy product may be plain or flavoured.

Preferred are dairy products which are to be made using high temperature conditions, for example a starch containing product such as a custard. Yet another preferred dairy product is ice cream. Preferred dairy products are custard and ice cream.

5 The milk protein may be derived from a mammal or a plant sources or mixtures thereof. Preferably, the milk protein is from a mammal source. Mammals sources of milk protein include, but are not limited to cow, sheep, goat, buffalo, camel, llama, mare and deer. In an embodiment, the milk protein is from a mammal selected from the group consisting of cow, sheep, goat, buffalo, camel, llama, mare and deer, and combinations
10 thereof. Plant sources of milk protein include, but are not limited to, milk extracted from soy bean, pea, peanut, barley, rice, oat, quinoa, almond, cashew, coconut, hazelnut, hemp, sesame seed and sunflower seed. Soy bean milk protein is preferred. In addition, the term "milk" refers to not only whole milk, but also skim milk or any liquid component derived thereof.

15 The invention also relates to the use of the enzyme preparation according to the invention to improve processability, especially in higher temperature process and/or prevent or reduce the development of off-flavor and/or to improve texture.

As used herein, an enzyme preparation has a reduced activity of a given enzyme if the activity of the given enzyme in the preparation has been reduced as
20 compared with an identical preparation in which no steps are taken to reduce activity of the enzyme.

Thus, an enzyme preparation having lactase activity and having reduced activity of a given enzyme, such as arylsulfatase activity, invertase activity, lipase activity and/or amylase activity, may be one which is subject to a biochemical step intended to
25 reduce arylsulfatase activity, invertase activity, lipase activity and/or amylase activity (as compared to process in which such a step is not used). An enzyme preparation with reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity may also thus encompass an enzyme preparation obtained by purifying a crude enzyme preparation which lactase activity, wherein at least some of the arylsulfatase
30 activity, invertase activity, lipase activity and/or amylase activity is separated from the lactase.

Alternatively, a host microorganism used to express a lactase may be modified so that the activity of one or more given enzymes, such as arylsulfatase, invertase,

lipase and/or amylase (expressed by the host), is reduced partly or completely, for example by modification or part or complete deletion of a nucleic acid sequence encoding an arylsulfatase, invertase, lipase and/or amylase or by modification or part or complete deletion of a nucleic acid sequence encoding a polypeptide involved in the regulation of an arylsulfatase, invertase, lipase and/or amylase. That is to say, a genetic approach may be used to produce an enzyme composition comprising lactase activity having reduced activity of a given enzyme, such as arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

Accordingly, the invention provides a process in which a modified host is used to express a lactase having reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity and then that lactase composition is used as described herein.

Preferably, the enzyme composition is reduced in/substantially free from arylsulfatase activity. Alternatively, the enzyme composition is reduced in/substantially free from lipase activity. Alternatively, the enzyme composition is reduced in/substantially free from lipase activity. Alternatively, the enzyme composition is reduced in/substantially free from amylase activity. Combinations are reduced activity/substantially free from: arylsulfatase and invertase activity; arylsulfatase and lipase activity; arylsulfatase and amylase activity; invertase and lipase activity; invertase and amylase activity; lipase and amylase activity; arylsulfatase, invertase and lipase activity; arylsulfatase, invertase and amylase activity; invertase, lipase and amylase activity or arylsulfatase, invertase, lipase and amylase activity. Any such combination may be combined with a reduction in protease activity.

All four of these activities may be reduced in a composition of the invention or a composition of the invention may be substantially free of all four activities. Combinations of such reductions may be used to define a composition of the invention. Thus, a composition of the invention may have reduced activity of or may be substantially free of two or three such activities.

The activity of a given enzyme, such as arylsulfatase activity, invertase activity, lipase activity and/or amylase activity, in an enzyme composition comprising lactase activity may be reduced by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or more. The percentage decrease represents the reduction as compared with an identical composition where no steps are

taken to reduce arylsulfatase activity, invertase activity, lipase activity and/or amylase activity. Thus, one or more genetic modifications may be made to reduce arylsulfatase activity, invertase activity, lipase activity and/or amylase activity by the desired amount (as compared to a non-modified host).

5 An enzyme composition of the invention may be substantially free from arylsulfatase activity, invertase activity, lipase activity and/or amylase activity. This typically implies a reduction in activity of at least about 50%, but more typically of at least about 80% or higher, such as a reduction of at least about 95% or a greater reduction.

10 A purification step that has the effect that the activity of protease relative to the activity of lactase is reduced may be used. Preferably, the purifying results in a reduction of arylsulfatase activity, invertase activity, lipase activity and/or amylase activity of at least 50%, preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 99%. The skilled person will appreciate that this is understood to mean that preferably $(a_{\text{enzyme,pur}}/a_{\text{lactase,pur}})/(a_{\text{enzyme,crude}}/a_{\text{lactase,crude}}) \leq 0.5$, preferably ≤ 0.2 , preferably ≤ 0.1 , preferably ≤ 0.05 , preferably ≤ 0.01 ., wherein

$a_{\text{enzyme,pur}}$ = arylsulfatase activity, invertase activity, lipase activity and/or amylase activity in purified enzyme preparation (unit/ml)

20 $a_{\text{lactase,pur}}$ = activity of lactase in purified enzyme preparation (unit/ml)

$a_{\text{enzyme,crude}}$ = arylsulfatase activity, invertase activity, lipase activity and/or amylase activity in crude enzyme preparation (unit/ml)

$a_{\text{lactase,crude}}$ = activity of lactase in crude enzyme preparation (unit/ml)

25 The purification can be effected in any suitable manner. In a preferred embodiment, the purifying is by chromatography. Processes for purifying enzyme preparations using chromatography are known per se. Selecting the most appropriate chromatographic separation methods depend on molecular characteristics of both the relevant enzyme and of the relevant arylsulfatase activity present. Relevant molecular characteristics are the isoelectric point, hydrophobicity, molecular surface charge distribution, molecular weight of the relevant enzyme and the side activity as well as
30 several other protein chemical properties. A practical background on the use of these characteristics in selecting the appropriate chromatographic separation process, can be found in a.o. the Protein Purification Handbook (issued by Amersham Pharmacia

Biotech, nowadays GE Healthcare Bio-Sciences, Diegem, Belgium). Suitable chromatographic separation methods comprise ion exchange chromatography, affinity chromatography, size exclusion chromatography, hydrophobic interaction chromatography and others. For the present invention ion exchange chromatography or hydrophobic interaction chromatography are preferred.

An enzyme preparation of the invention may encompass an enzyme preparation wherein the ratio of the arylsulfatase activity, invertase activity, lipase activity and/or amylase activity divided by the activity of the lactase is below a specified value.

Preferred ratio's may vary depending on the enzyme and application used.

By arylsulfatase activity is meant the sulphuric ester hydrolase activity able to cleave a phenol sulfate into the phenol and sulfate moiety as described for EC 3.1.6.1.

By invertase activity is meant the activity that catalyzes the hydrolysis (breakdown) of sucrose as described in EC 3.2.1.26.

By lipase activity is meant the activity that catalyzes the hydrolysis of lipids.

By amylase activity is meant the activity that catalyzes the hydrolysis of starch into sugars.

By protease activity is meant the activity that catalyzes the hydrolysis of proteins.

An enzyme composition of the invention having lactase activity and having reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity may also have one or more of reduced protease activity. An enzyme composition of the invention may have reduced activity of all of these enzyme activities. An enzyme composition of the invention may be substantially free from all of these enzyme activities. Additionally, the enzyme preparation may also be free from protease.

At least a part of the lactase activity in the enzyme composition may be provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least about 60% sequence identity to any thereto. Reference to a truncated version refers to a C-terminal truncated version.

Alternatively, at least a part of the lactase activity in the enzyme composition may be provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about 60% sequence identity to any thereto.

In addition or alternatively, at least a part of the lactase activity is provided by a polypeptide having lactase activity which is derived from *Bifidobacterium bifidum*. The term "derived from" also includes the terms "originated from," "obtained from," "obtainable from," "isolated from," and "created from," and generally indicates that one specified material find its origin in another specified material or has features that can be described with reference to the another specified material. As used herein, a substance (e.g., a nucleic acid molecule or polypeptide) "derived from" a microorganism preferably means that the substance is native to that microorganism or is a variant based on a substance native to the microorganism (for example a truncated version of a polypeptide derived from the microorganism and/or a version which contains one or more substitutions as compared to a polypeptide derived from the microorganism).

The polypeptide set out in SEQ ID NO: 1 or SEQ ID NO: 2 of the invention is the full-length wild-type sequence of a lactase from *Bifidobacterium bifidum*.

A polypeptide having lactase activity suitable for use in the invention may have comprise an amino acid sequence having at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about, 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 2.

SEQ ID NO: 1 and 2 both comprise a signal sequence which is positioned at amino acids 1 to 32 for both of them. As the signal sequence will most likely be cleaved off during production in a host cell, a polypeptide having lactase activity for use in the invention preferably comprises:

- an amino acid sequence having at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about, 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to amino acids 33 to 1752 of SEQ ID NO: 1.
- an amino acid sequence having at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about,

86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to amino acids 33 to 1935 of SEQ ID NO: 2.

5 A truncated version of the polypeptide of SEQ ID NO: 1 or SEQ ID NO: 2, or more preferably a truncated version of amino acids 33 to 1752 of SEQ ID NO: 1 or a truncated version of amino acids 33 to 1935 of SEQ ID NO:2, may be used or a polypeptide comprising an amino acid sequence having at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about, 10 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to such a truncated version of SEQ ID NO: 1 OR SEQ ID NO: 2 or more preferred to a truncated version of amino acids 33 to 1752 of SEQ ID NO: 1 or amino acids 33 to 1935 of SEQ ID NO:2.

15 An example of such a polypeptide, i.e. a polypeptide having lactase activity suitable for use in the invention, is the Bbg3 polypeptide described in Goulas T.K., Goulas A.K., Tzortzis G., Gibson G.R., Appl. Microbiol. Biotechnol. 76(6), 1365 and 1372 (2007).

20 Suitable truncated versions of the polypeptide of SEQ ID NO: 1 or SEQ ID NO: 2 include SEQ ID NO: 2 in WO2009/071539 (herein SEQ ID NO: 3) and SEQ ID NOs: 1 or 2 in WO2014/184189 (SEQ ID NO: 2 of WO2014/184189 is SEQ ID NO: 4 in this patent application).

25 In a preferred embodiment, the invention provides a dairy product or a method according to the invention in which SEQ ID NO: 3 or 4 is used or a polypeptide comprising an amino acid sequence having at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about, 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, 30 at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to SEQ ID NO: 3 or SEQ ID NO: 4.

SEQ ID NO: 3 and 4 both comprise a signal sequence which is positioned at amino acids 1 to 27 for both of them. As the signal sequence will most likely be cleaved off during production in a host cell, a polypeptide having lactase activity for use in the invention preferably comprises:

- 5 - an amino acid sequence having at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about, 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 10 97%, at least about 98% or at least about 99% sequence identity to amino acids 28 to 1341 of SEQ ID NO: 3, more preferably amino acids 28-1331 of SEQ ID NO: 3 (as amino acids 1332-1341 comprise a purification tag which is preferably removed)
- an amino acid sequence having at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about, 15 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98% or at least about 99% sequence identity to amino 20 acids 28 to 1331 of SEQ ID NO: 4.

Preferably, the invention concerns a dairy product obtainable by a process which comprises:

- a) providing a milk-based substrate comprising lactose; and
- b) treating said substrate at high temperature with an enzyme composition 25 comprising lactase activity

wherein at least part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1, 2, 3 or 4 or a sequence having at least 60% sequence identity to SEQ ID NO: 1, 2, 3 or 4

- wherein the enzyme composition has reduced arylsulfatase activity, invertase 30 activity, lipase activity and/or amylase activity.

More preferably, at least part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in amino acids 33 to 1752 of SEQ ID NO: 1, amino acids 33 to 1935 of SEQ ID NO: 2, amino acids 28

to 1331 of SEQ ID NO:3 or amino acids 28 to 1331 of SEQ ID NO: 4 or a sequence having at least 60% sequence identity to any thereto.

The invention also concerns a method for the production of a dairy product, which method comprises:

- 5 a) providing a milk-based substrate comprising lactose; and
b) treating said substrate at high temperature with an enzyme composition comprising lactase activity

wherein at least part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1, 2, 3
10 or 4 or a sequence having at least 60% sequence identity to SEQ ID NO: 1, 2, 3 or 4

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

More preferably, at least part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in amino acids
15 33 to 1752 of SEQ ID NO: 1, amino acids 33 to 1935 of SEQ ID NO: 2, amino acids 28 to 1331 of SEQ ID NO:3 or amino acids 28 to 1331 of SEQ ID NO: 4 or a sequence having at least 60% sequence identity to any thereto.

The terms “sequence homology” or “sequence identity” or “homology” or
20 “identity” are used interchangeably herein. For the purpose of this invention, it is defined here that in order to determine the percentage of sequence homology or sequence identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes. In order to optimize the alignment between the two sequences gaps may be introduced in any of the two
25 sequences that are compared. Such alignment can be carried out over the full length of the sequences being compared. Alternatively, the alignment may be carried out over a shorter length, for example over about 20, about 50, about 100 or more nucleic acids/based or amino acids. The sequence identity is the percentage of identical matches between the two sequences over the reported aligned region.

30 A comparison of sequences and determination of percentage of sequence identity between two sequences can be accomplished using a mathematical algorithm. The skilled person will be aware of the fact that several different computer programs are available to align two sequences and determine the identity between two

sequences (Kruskal, J. B. (1983) An overview of sequence comparison In D. Sankoff and J. B. Kruskal, (ed.), Time warps, string edits and macromolecules: the theory and practice of sequence comparison, pp. 1-44 Addison Wesley). The percent sequence identity between two amino acid sequences or between two nucleotide sequences may be determined using the Needleman and Wunsch algorithm for the alignment of two sequences. (Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453). Both amino acid sequences and nucleotide sequences can be aligned by the algorithm. The Needleman-Wunsch algorithm has been implemented in the computer program NEEDLE. For the purpose of this invention the NEEDLE program from the EMBOSS package was used (version 2.8.0 or higher, EMBOSS: The European Molecular Biology Open Software Suite (2000) Rice, P. Longden, I. and Bleasby, A. Trends in Genetics 16, (6) pp276—277, <http://emboss.bioinformatics.nl/>). For protein sequences EBLOSUM62 is used for the substitution matrix. For nucleotide sequence, EDNAFULL is used. The optional parameters used are a gap-open penalty of 10 and a gap extension penalty of 0.5. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.

After alignment by the program NEEDLE as described above the percentage of sequence identity between a query sequence and a sequence of the invention is calculated as follows: Number of corresponding positions in the alignment showing an identical amino acid or identical nucleotide in both sequences divided by the total length of the alignment after subtraction of the total number of gaps in the alignment. The identity defined as herein can be obtained from NEEDLE by using the NOBRIEF option and is labeled in the output of the program as “longest-identity”.

The nucleic acid and protein sequences of the present invention can further be used as a “query sequence” to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403—10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules as described herein. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules as described herein. To obtain

gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17): 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See the homepage of the
5 National Center for Biotechnology Information at <http://www.ncbi.nlm.nih.gov/>.

A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

10 The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

The present invention is further illustrated by the following Examples:

EXAMPLES

15 **Example 1: Preparation of a lactase composition comprising substantially no arylsulfatase activity, invertase activity, lipase activity and/or amylase activity and its use in the preparation of an ice-cream**

20 A lactase composition is prepared by expressing lactases in *Bacillus subtilis*. Lac16 is described in WO2009/071538 (SEQ ID NO: 2; herein SEQ ID NO: 3) and lac19 (herein SEQ ID NO: 2) is described as Bbg3 in Goulas T.K., Goulas A.K., Tzortzis G., Gibson G.R., Appl. Microbiol. Biotechnol. 76(6), 1365 and 1372 (2007). Alternatively, a lactase having SEQ ID NO: 3 or 4, or more preferably amino acids 28-
25 1331 of SEQ ID NO: 3 or 4, is used.

Standard genetic techniques, such as overexpression of enzymes in the host cells, genetic modification of host cells, or hybridisation techniques, are known methods in the art, such as described in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, *Cold Spring Harbor Laboratory Press*, or F. Ausubel et al, eds., "Current protocols in molecular biology",
30 Green Publishing and Wiley Interscience, New York (1987). Methods for transformation, genetic modification etc of fungal host cells are known from e.g. EP-A-0

635 574, WO 98/46772, WO 99/60102 and WO 00/37671, WO90/14423, EP-A-0481008, EP-A-0635 574 and US 6,265,186.

Enzymes were isolated from the fermentation broth of *Bacillus subtilis* strains and purified using standard centrifugation and filtration techniques.

5 The compositions are prepared so that they comprise substantially no arylsulfatase activity, invertase activity, lipase activity and/or amylase activity. This can be carried out using a biochemical method to reduce the amount of protease in the composition. Alternatively, it can be carried out by modifying the host organism in which the lactase is prepared such that the amount of protease produced by the host
10 organism is reduced.

The compositions are then used in the manufacture of an ice-cream. This comprises the steps of:

- Reception raw materials (liquid and dry)
- Blending, hydration and lactose hydrolyzation at $>60^{\circ}\text{C}$
- 15 • Pasteurization
- Homogenization
- Cooling
- Fat crystallization
- Freezing
- 20 • Packaging
- Wrapping
- Storage and transport

The raw materials can be split into 2 groups liquid and dry raw materials. The liquid raw materials are usually: condensed milk; cream; corn sugar; cane sugar and water.
25 The dry ingredients are usually flavors; fruit; nuts; stabilizers; milk powder; whey powder; emulsifiers; egg-powder; and sugar.

The dry ingredients are blended with the liquid ingredients prior to pasteurization of the total ice-cream mix together with the enzyme composition comprising lactase. The dry ingredients are given enough time to completely hydrate in order to prevent
30 lumps formation and ensure proper solubilization at the moment of pasteurization. Alternatively, the lactase composition may be added with the wet ingredients.

The temperature of hydration of the dry ingredients is typically above 60°C and no hydrolysis of lactose-containing ingredients is carried out prior to blending with the other ice-cream mix ingredients.

The ice-cream is evaluated by a sensory panel for taste and texture characteristics.

5

CLAIMS

1. A dairy product obtainable by a process which comprises:
a) providing a milk-based substrate comprising lactose; and
5 b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,
wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence
10 having at least about 60% sequence identity to any thereto, and
wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.
2. A dairy product obtainable by a process which comprises:
15 a) providing a milk-based substrate comprising lactose; and
b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,
wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ
20 ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about 60% sequence identity to any thereto, and
wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.
- 25 3. A dairy product obtainable by a process which comprises:
a) providing a milk-based substrate comprising lactose; and
b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,
wherein at least a part of the lactase activity is provided by a polypeptide
30 having lactase activity which is derived from *Bifidobacterium bifidum*, and
wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

4. A dairy product according to any one of claims 1 to 3, wherein said high temperature is a temperature of at least about 45°C.

5. A dairy product according to any one of claims 1 to 4, which is ice cream or custard.

6. A method for the production of a dairy product, which method comprises:

a) providing a milk-based substrate comprising lactose; and

b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least about 60% sequence identity to any thereto, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

7. A method for the production of a dairy product, which method comprises:

a) providing a milk-based substrate comprising lactose; and

b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about 60% sequence identity to any thereto, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

8. A method for the production of a dairy product, which method comprises:

a) providing a milk-based substrate comprising lactose; and

b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having lactase activity which is derived from *Bifidobacterium bifidum*, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

5

9. A method according to any one of claims 6 to 8, wherein said high temperature is a temperature of at least about 45°C.

10. A method according to any one of claims 6 to 9, wherein said dairy product is ice cream or custard.

10

11. A method according to claim 10, wherein said dairy product is ice cream and wherein blending and hydration of raw material and lactose hydrolysis are performed at a temperature of at least about 60°C, preferably above 60°C.

15

12. Use of an enzyme having lactase activity in the production of a dairy product with improved taste and/or shelf-life, wherein dairy product obtainable by a process which comprises:

a) providing a milk-based substrate comprising lactose; and

20

b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least about 60% sequence identity to any thereto, and

25

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

13. Use of an enzyme having lactase activity in the production of a dairy product with improved taste and/or shelf-life, wherein dairy product obtainable by a process which comprises:

30

a) providing a milk-based substrate comprising lactose; and

b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about 60% sequence identity to any thereto, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

10 14. Use of an enzyme having lactase activity in the production of a dairy product with improved taste and/or shelf-life, wherein dairy product obtainable by a process which comprises:

a) providing a milk-based substrate comprising lactose; and

15 b) treating said substrate at high temperature with an enzyme composition comprising lactase activity,

wherein at least a part of the lactase activity is provided by a polypeptide having lactase activity which is derived from *Bifidobacterium bifidum*, and

wherein the enzyme composition has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

20

15. An enzyme composition comprising lactase activity, wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, a truncated version of either thereof or a sequence having at least about 60% sequence identity to any thereto, which has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

25

16. An enzyme composition comprising lactase activity, wherein at least a part of the lactase activity is provided by a polypeptide having an amino acid sequence which comprises the sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2 or SEQ ID NO: 3 or SEQ ID NO: 4 or a sequence having at least about 60% sequence identity to any thereto, which has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

30

17. An enzyme composition comprising lactase activity, wherein at least a part of the lactase activity is provided by a polypeptide having lactase activity which is derived from *Bifidobacterium bifidum* which has reduced arylsulfatase activity, invertase activity, lipase activity and/or amylase activity.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/057490

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23C9/00 A23C9/20 A23G9/40
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23C A23G

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

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| X | WO 2013/182686 A1 (DUPONT NUTRITION BIOSCI APS [DK]) 12 December 2013 (2013-12-12) page 2, line 16 - page 63, line 2 claims; examples; sequences ----- | 1-17 |
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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

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"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 24 May 2017 | Date of mailing of the international search report 02/06/2017 |
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/057490

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