

METHOD OF PRODUCING A COMPOSITION CONTAINING GALACTO-OLIGOSACCHARIDES

FIELD OF THE INVENTION

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The present invention relates to a galacto-oligosaccharide-containing composition as well as an efficient method of producing it.

BACKGROUND

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Human breast milk is known to contain a number of different oligosaccharides which are ascribed some of the beneficial health effects of breast feeding infants (Kunz *et al.* (2000)). For example, some oligosaccharides, such as FOS, GOS or inulin, are so-called prebiotics, which means that they promote the beneficial
15 bacteria of the gastrointestinal system and disfavour the harmful bacteria. Oligosaccharides are, due to their health promoting effects, frequently used in functional food products, such as infant formulas and clinical nutrition.

There are several approaches to the production of oligosaccharides. One approach
20 is based on isolating oligosaccharides from naturally occurring sources. Fructose-oligosaccharide (FOS) and inulin are for example found naturally in Jerusalem artichoke, burdock, chicory, leeks, onions and asparagus and may be isolated from these crops. Preparation of inulin from chicory roots is e.g. described in Frank (2002). This approach to the production of oligosaccharides is limited by the
25 availability of suitable crops and may be impossible to implement for more complex oligosaccharides.

Another approach is based on enzymatic synthesis in which enzymes catalyse the synthesis of the oligosaccharides. Yun (1996) describes the enzymatic production
30 of fructo-oligosaccharides using enzymes having fructosyltransferase activity and using sucrose as substrate for the enzyme.

Yet an example of enzymatic synthesis is described in WO 01/90,317 A2 which discloses a method of producing galacto-oligosaccharides (GOS) of the formula Gal-Gal-Glc using a special beta-galactosidase enzyme and lactose as substrate.

5 EP 2 138 586 A1 discloses process for the regio-selective manufacture of disaccharides or oligosaccharides by transglycosylation or hydrolysis of a carbohydrate donator molecule and a glycosyl acceptor molecule in the presence of a glycosidase in the presence of at least one dialkyl amide. However, EP 2 138 586 A1 does not disclose removal, during the incubation, of free leaving groups
10 released from the glycosyl donor.

WO 2009/113,030 A2 describes a process for the production of pure lactose-based galactooligosaccharides using microbial whole cells in a reactor with cross flow hollow fiber micro filtration system. However, WO 2009/113,030 A2 does not
15 contain any teaching regarding the production of hetero-galacto-oligosaccharides using a galactosyl acceptor which is not related to the donor.

WO 2012/010,597 A1 discloses a method of producing compositions containing galacto-oligosaccharides as well as galacto-oligosaccharide-containing
20 compositions as such. However, WO 2012/010,597 A1 does not disclose the removal, during the incubation, of free leaving groups released from the galactosyl donor.

SUMMARY OF THE INVENTION

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An object of the present invention is to provide improved methods of producing galacto-oligosaccharides, and particularly galacto-oligosaccharides having a different reducing end than the galactosyl donor used during the production.

30 The present inventors have found that, surprisingly, leaving groups released from the donor during synthesis of galacto-oligosaccharides act as competing galactosyl acceptors and reduces the yield of the above-mentioned galacto-oligosaccharides. This is particularly surprising as initial trials performed by the inventors have indicated that leaving groups, and particularly glucose, are poor galactosyl
35 acceptors. The present inventors have furthermore discovered that the yield of

the above-mentioned galacto-oligosaccharides (having a different reducing end than the galactosyl donor) may be increased by removing released leaving groups from the reaction mixture during the incubation of galactosyl donor, galactosyl acceptor and beta-galactosidase enzyme.

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Figures 1, 2 and 3 explain this in further detail.

Figure 1 is a schematic depiction of the main types of reactions that takes place during transgalactosylation. Reaction a) is the desired reaction and involves the transferral of a galactosyl group from the donor (lactose in this example) to the acceptor (e.g. fucose). The products of this reaction is a free leaving group (glucose in this example) and a small oligosaccharide (e.g. Gal-Fuc) having a different reducing end than the galactosyl donor.

Reaction b) is an undesired side reaction which leads to so-called self-galactosylation of the donor, e.g. further galactosylated lactose if the donor is lactose. This by-product is difficult and expensive to remove from the oligosaccharide product. The present inventors have discovered that the level of self-galactosylation can be reduced significantly by using a first enzyme having a high transgalactosylation efficiency (a low T-value) in combination with a relatively low concentration of galactosyl donor.

Reaction c) of Figure 1 is another undesired side reaction which the present inventors have recently discovered. The present inventors have observed surprisingly high levels of allo-lactose in galacto-oligosaccharide compositions and have come to the conclusion that free leaving groups, and particularly, glucose also act as acceptor if its concentration is sufficiently high.

Figure 2 is a schematic illustration of some of the molecular species present in the galacto-oligosaccharide compositions when the free leaving groups are not removed during incubation. In addition to the desired oligosaccharide products derived from galactosyl acceptor, the composition furthermore contains undesired oligosaccharides derived from galactosylation of free leaving groups, e.g. allo-lactose and further galactosylated allo-lactose.

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Figure 3 is a schematic illustration of some of the molecular species present in the galacto-oligosaccharide compositions when the free leaving groups are removed during incubation. Contrary to the galacto-oligosaccharide composition illustrated in Figure 2, the present galacto-oligosaccharide composition lacks (or has at least
5 a significantly lower concentration of) free leaving groups, allo-lactose and oligosaccharides derived from allo-lactose.

Thus, an aspect of the invention relates to a method of producing a composition comprising one or more galacto-oligosaccharide(s), the method comprising the
10 steps of:

- a) providing a mixture comprising
 - a galactosyl donor comprising a galactosyl group bound to a leaving group, which galactosyl donor has a molar weight of at most 350 g/mol,
 - 15 - a galactosyl acceptor which is different from the galactosyl donor, said galactosyl acceptor is a saccharide or a sugar-alcohol, andwherein the molar ratio between the galactosyl acceptor and the galactosyl donor is at least 1:10, and wherein the mixture comprises at least 0.05 mol/L of the galactosyl acceptor,
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- b) providing a first enzyme, said first enzyme having beta-galactosidase activity, said first enzyme contacting the mixture, and
- c) incubating the mixture and the first enzyme, thereby allowing the first enzyme
25 to release the leaving group of the galactosyl donor and transfer the galactosyl group of the galactosyl donor to the galactosyl acceptor, thus forming the galacto-oligosaccharide, step c) furthermore comprising removing from the incubating mixture, i.e. during incubation, a leaving group released from the galactosyl donor, thereby obtaining the composition comprising the one or more galacto-
30 oligosaccharide(s).

The term "removing a leaving group released from the galactosyl donor" should be understood as physical removal of free leaving groups from the incubating mixture and/or conversion of free leaving groups into one or more other chemical
35 species which may still be present in the incubating mixture. It is preferred that

such chemical species do not act as galactosyl acceptors, or at least that they are a less efficient galactosyl acceptor than the free leaving group.

5 This invention opens up for cheap and efficient production of complex galacto-oligosaccharide compositions in high yield. The present invention furthermore appears to reduce the galactosylation of free leaving groups released from of the galactosyl donor and well as the self-galactosylation of the galactosyl donor, which both result in undesired by-products which are expensive to remove from the composition.

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Preferably, the first enzyme has transgalactosylating activity in addition to beta-galactosidase activity. It may also be preferred that the first enzyme has a T-value of at most 0.9.

15 In the context of the present invention the term "transgalactosylating activity" of a beta-galactosidase enzyme relates to the ability of the enzyme to transfer a galactosyl group from a donor molecule, e.g. a lactose molecule, to a non-water molecule, e.g. another lactose molecule.

20 The T-value is a measure of the transgalactosylating efficiency of a beta-galactosidase enzyme using lactose both as galactosyl donor and acceptor. The determination of the T-value of a beta-galactosidase enzyme is performed according to the assay and the formula described in Example 3. The T-value is calculated using the formula:

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$$\text{T-value} = \frac{\text{amount of produced galactose (in mol)}}{\text{amount of used lactose (in mol)}}$$

A lactase enzyme without any transgalactosylating activity will produce one mol galactose for each used mol lactose and would have a T-value of 1. A beta-galactosidase having an extremely high transgalactosylating activity would use
30 nearly all the galactosyl groups from the lactose for transgalactosylating instead of generating galactose, and would consequently have a T-value near 0.

Yet an aspect of the invention relates to a composition comprising one or more galacto-oligosaccharide(s), which composition is obtainable by the method as described herein.

- 5 Additional objects and advantages of the invention are described below.

BRIEF DESCRIPTION OF THE FIGURES

10 Figure 1 shows a schematic illustration of the types of reactions involved in transgalactosylation.

Figure 2 shows a schematic representation of the reaction products obtained when the free leaving groups are not used.

15 Figure 3 shows a schematic representation of the reaction products obtained when the free leaving groups are not used.

20 Figure 4 shows a plot of the integrated response of di-, tri-, and tetrasaccharide of galactosylated fucose after 4 hours of incubation - with and without removal of free leaving groups during the incubation. The free leaving groups (glucose) are removed by means of enzymatic conversion. It is seen that the content of galactosylated fucose increases when the free leaving groups are removed during incubation.

25 Figure 5 shows a plot of the integrated response of di-, tri-, and tetrasaccharide of galactosylated fucose after 5 hours of incubation - with and without removal of free leaving groups during the incubation. Again, it is seen that the content of galactosylated fucose increases when the free leaving groups are removed during incubation.

30 Figure 6 show a plot of the total integrated response of galactosylated fucose compared to the total integrated response of galactosylated donor/leaving group - with and without removal of free leaving groups during the incubation. The total integrated response of galactosylated donor/leaving group is the sum of the
35 integrated responses of Gal-Glc, Gal-Gal-Glc, and Gal-Gal-Gal-Glc molecules. The

total integrated response of galactosylated fucose is the sum of the responses of Gal-Fuc, Gal-Gal-Fuc, and Gal-Gal-Gal-Fuc molecules. It is seen that the total integrated response of galactosylated fucose increases significantly when the free leaving groups are removed during incubation while the total integrated response of galactosylated donor/leaving group is almost unchanged.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned, an aspect of the invention relates to a method of producing a composition comprising one or more galacto-oligosaccharide(s), the method comprising the steps of:

- a) providing a mixture comprising
 - a galactosyl donor comprising a galactosyl group bound to a leaving group, which galactosyl donor has a molar weight of at most 350 g/mol,
 - a galactosyl acceptor which is different from the galactosyl donor, said galactosyl acceptor is a saccharide or a sugar-alcohol, andwherein the molar ratio between the galactosyl acceptor and the galactosyl donor is at least 1:10, and wherein the mixture comprises at least 0.05 mol/L of the galactosyl acceptor,
- b) providing a first enzyme, said first enzyme having beta-galactosidase activity, said first enzymes contacting the mixture, and
- c) incubating the mixture and the first enzyme, thereby allowing the first enzyme to release the leaving group of the galactosyl donor and transfer the galactosyl group of the galactosyl donor to the galactosyl acceptor, thus forming the galacto-oligosaccharide, step c) furthermore comprising removing from the incubating mixture, i.e. during incubation, a leaving group released from the galactosyl donor, thereby obtaining the composition comprising the one or more galacto-oligosaccharide(s).

In the context of the present invention, the term "glycosyl group" relates to a group obtained by removing one or two hydroxyl groups from a

monosaccharide or a lower oligosaccharide, such as a di- or tri-saccharide, or from corresponding sugar-alcohols. The term is used herein to describe various building blocks of galactosyl donors, galactosyl acceptors and oligosaccharides.

- 5 The abbreviations of the most common saccharides and their corresponding glycosyl groups are shown below.

Saccharide	Abbreviation	Name of glycosyl group
Glucose	Glc	glucosyl
Galactose	Gal	galactosyl
fucose	Fuc	fucosyl
mannose	Man	mannosyl
xylose	Xyl	xylosyl
N-acetylgalactosamine	GalNAc	N-acetylgalactosaminyl
Lactose	Lac	lactosyl

- 10 In the context of the present invention, the term "oligosaccharide" relates to a molecule comprising at least two glycosyl groups, and preferably at least three, which may be different or the same type. The at least two glycosyl groups are preferably bound via an O-glycosylic bond. An oligosaccharide may be a linear chain of glycosyl groups or it may have a branched structure. Oligosaccharides
- 15 may e.g. be represented as a stoichiometric formula, e.g. (Gal)₃Glc, or as general formulas, e.g. Gal-Gal-Gal-Glc, Gal-Gal-Glc-Gal, or Gal-(Gal-)Glc-Gal. The stoichiometric formulas provide information regarding which glycosyl groups an oligosaccharide, or a group of oligosaccharides, contains, but not the relative position of these, whereas the general formulas also contain general information
- 20 regarding the relative positions of the glycosyl groups.

In the context of the present invention the term "homo-oligosaccharide" relates to an oligosaccharide containing only one type of glycosyl group. Examples of homo-oligosaccharides are Gal-Gal-Gal-Gal and Glc-Glc-Glc.

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In the context of the present invention the term "hetero-oligosaccharide" relates to an oligosaccharide which contains different glycosyl groups, e.g. Gal-Gal-Glc, or Gal-Gal-Fuc.

In the context of the present invention, the prefix "galacto-" used together with the term "oligosaccharide" indicates that the oligosaccharide contains galactosyl groups as the repeating unit. The "homo-" or "hetero-" prefix may be used together with the "galacto-" prefix. Both Gal-Gal-Glc and Gal-Gal-Gal-Gal are galacto-oligosaccharides. Gal-Gal-Glc is a hetero-galacto-oligosaccharide and Gal-Gal-Gal-Gal is a homo-galacto-oligosaccharide.

In the context of the present invention, "X" represents a galactosyl acceptor as defined herein. "-X" represents the glycosyl group corresponding to the galactosyl acceptor, and particularly the glycosyl group bound to another group. "-" symbolises the bond. The glycosyl group is preferably bound via the 3-, 4-, 5- or 6-position of the glycosyl group, and preferably via an O-glycosylic bond. In the context of the present invention, "Gal-" represents a galactosyl group bound to another group, preferably via the 1-position of the galactosyl group, and preferably via an O-glycosylic bond.

In the context of the present invention, "-Gal-" represents a galactosyl group bound to two other groups. The left bond is preferably made via the 4- or 6-position of the galactosyl group, and preferably via an O-glycosylic bond. The right bond is preferably made via the 1-position of the galactosyl group, and preferably via an O-glycosylic bond.

Bonds between two galactosyl groups are typically 1-4 or 1-6 bonds, and normally O-glycosylic bonds. A bond between a galactosyl group and a nitrogen-containing acceptor may alternatively be an N-glycosylic bond.

Method of the present invention is preferably a method of producing a composition comprising one or more galacto-oligosaccharide(s) using a galactosyl donor and a galactosyl acceptor, which one or more galacto-oligosaccharide(s) have the galactosyl acceptor as its reducing end.

In the context of the present invention the terms "method" and "process" are used interchangeably.

Step a) involves the provision of the mixture in which the oligosaccharides are to be produced.

5 The mixture is preferably a liquid mixture and may e.g. be an aqueous solution containing the galactosyl acceptor and the galactosyl donor.

10 In some embodiments of the invention the molar ratio between the galactosyl acceptor and the galactosyl donor of the mixture of step a) is at least 1:5, preferably at least 1:1, and even more preferably at least 5:1. For example, the molar ratio between the galactosyl acceptor and the galactosyl donor of the mixture of step a) may be at least 10:1, such as at least 15:1.

15 The molar ratio between the galactosyl acceptor and the galactosyl donor of the mixture of step a) may e.g. be in the range of 1:10-100:1.

20 In some embodiments of the invention the molar ratio between the galactosyl acceptor and the galactosyl donor of the mixture of step a) is in the range of 1:10-50:1, preferably in the range of 1:5-30:1, and even more preferably in the range of 1:1-20:1. For example, the molar ratio between the galactosyl acceptor and the galactosyl donor of the mixture of step a) may e.g. be in the range of 2:1-40:1, preferably in the range of 4:1-30:1, and even more preferably in the range of 10:1-25:1.

25 As mentioned, the galactosyl donors contain a galactosyl group covalently bound to a leaving group. The galactosyl group is preferably a β -D-galactopyranosyl group. Furthermore, the galactosyl group is preferably bound to the leaving group via an O-glycosidic bond from the 1-position of the galactosyl group.

30 The leaving group of the galactosyl donor may for example be a glycosyl group and/or a sugar-alcohol group. It is particularly preferred that the leaving group of the galactosyl donor is a glucosyl group, i.e. a glucose residue.

35 If the leaving group is a glycosyl group of a mono- or disaccharide or a corresponding sugar-alcohol, the galactosyl group is preferably bound to the leaving group via an O-glycosidic bond from the 1-position of the galactosyl

group, which bond attaches to the 4-position of a monosaccharide-type leaving group or to the 4'-position of a disaccharide-type leaving group.

5 In the context of the present invention, the phrase "Y and/or X" means "Y" or "X" or "Y and X". Along the same line of logic, the phrase " $X_1, X_2, \dots, X_{i-1}$, and/or X_i " means " X_1 " or " X_2 " or " X_{i-1} " or " X_i " or any combination of the components : $X_1, X_2, \dots, X_{i-1}$, and X_i .

10 In some embodiments of the invention the galactosyl donor has a molar weight of at most 1000 g/mol. For example, the galactosyl donor may have a molar weight of at most 500 g/mol. It may even be preferred that the galactosyl donor has a molar weight of at most 350 g/mol.

15 Disaccharides are a presently preferred type of galactosyl donor. Alternatively, or additionally, tri-saccharides may be used as galactosyl donors as well. Thus, it is envisioned that the mixture may contain a combination of different galactosyl donors.

20 In some preferred embodiments of the invention the galactosyl donor is lactose. Another example of a useful galactosyl donor is lactulose. Yet an example of a useful galactosyl donor is lactitol.

25 In the context of the present invention the term "lactose" relates to the disaccharide β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucose, which is also referred to as milk sugar, and which is the most predominant saccharide of bovine milk.

30 The galactosyl donor may be provided via any useful galactosyl donor source, both industrially refined sources, such as purified lactose, and/or natural sources, such as whey permeate, i.e. deproteinated whey prepared by ultrafiltration of whey.

35 The galactosyl acceptor may be any molecule capable of accepting a galactosyl group from the first enzyme and typically contains hydroxyl groups, and preferably alcoholic hydroxyl groups. The term "accepting" means that the galactosyl group of the donor should be covalently bound to the acceptor, e.g. via an O-glycosylic bond.

In some embodiments of the invention the galactosyl acceptor comprises one or more alcoholic hydroxyl group(s). For example, the galactosyl acceptor may be a polyol.

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In the context of the present invention the term "polyol" relates to a molecule comprising at least two alcoholic hydroxyl groups.

In some preferred embodiments of the invention the galactosyl acceptor is not lactose. It may furthermore be preferred that the galactosyl acceptor is not glucose.

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In some preferred embodiments of the invention the galactosyl acceptor is different from the galactosyl donor. It is particularly preferred to use a relatively cheap galactosyl donor, such as lactose, as galactosyl source, and a biologically interesting acceptor, such as fucose, as galactosyl acceptor.

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In some embodiments of the invention the galactosyl acceptor is not lactose, galactose, or glucose.

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In some embodiments of the invention the galactosyl acceptor is not glucose or oligosaccharides of the general formula $\text{Gal}-(\text{Gal})_i\text{-Glc}$, where i is a non-negative integer, i.e. for example 0, 1, 2, 3, or 4.

In some embodiments of the invention the galactosyl acceptor is not galactose or oligosaccharides of the general formula $\text{Gal}-(\text{Gal})_i\text{-Gal}$, where i is a non-negative integer.

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Galactosyl acceptors having various molar weights may be used, but galactosyl acceptors having a molar weight of at least 100 g/mol are presently preferred.

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In some embodiments of the invention the galactosyl acceptor has a molar weight of at most 1000 g/mol. For example, the galactosyl acceptor may have a molar weight of at most 500 g/mol. It may even be preferred that the galactosyl acceptor has a molar weight of at most 350 g/mol. The galactosyl acceptor may for example have a molar weight of at most 200 g/mol.

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In some preferred embodiments of the invention the galactosyl acceptor is a saccharide. The galactosyl acceptor may for example be a mono-saccharide. Alternatively, the galactosyl acceptor may be a di-saccharide.

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For example, the galactosyl acceptor may be a pentose. The galactosyl acceptor may e.g. be arabinose. Another example of a useful pentose is xylose. Yet an example of a useful pentose is ribose. The galactosyl acceptor may for example be a pentose selected from the group consisting of arabinose, xylose, and ribose.

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Hexoses are another group of useful galactosyl acceptors. The galactosyl acceptor may e.g. be mannose. Another example of a useful hexose is galactose. Yet an example of a useful hexose is tagatose. A further example of a useful hexose is fructose. The galactosyl acceptor may for example be a hexose selected from the group consisting of mannose, galactose, tagatose, and fructose.

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In some preferred embodiments of the invention the galactosyl acceptor is a deoxy-hexose. The galactosyl acceptor may for example be fucose, such as e.g. D-fucose, L-fucose, or a mixture thereof.

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Alternatively, the galactosyl acceptor may be an oligosaccharide, such as e.g. a di-saccharide or a tri-saccharide. An example of a useful di-saccharide is maltose. Another example of a useful di-saccharide is lactulose.

25 Yet a useful group of galactosyl acceptors is saccharide derivatives.

In the context of the present invention the term "saccharide derivative" pertains to a saccharide containing one or more non-hydroxyl functional group(s).

30 Examples of such functional groups are a carboxyl group, an amino group, an N-acetylamino group and/or a thiol group. Saccharides which contain an aldehyde group at the 1-position or a ketone group at the 2-position are not considered saccharide derivatives as such unless the saccharides comprise some of the non-hydroxyl functional groups mentioned above.

35 An example of a useful saccharide derivative is N-acetyl galactosamine. Another example of a useful saccharide derivative is sialic acid. Yet an example of a useful

saccharide derivative is sialyl lactose. Thus, the galactosyl acceptor may be a saccharide derivative selected from the group consisting of N-acetyl galactosamine, sialic acid, and sialyl lactose.

- 5 Another group of useful galactosyl acceptors is sugar alcohols. Thus, in some embodiments of the invention the galactosyl acceptor is a sugar alcohol. Examples of useful sugar alcohols are sorbitol, xylitol, lactitol, and/or maltitol.

Contrary to the above-mentioned galactosyl acceptors, the present inventors have
10 found that N-acetyl glucosamine and glucose are less efficient galactosyl acceptors. Thus, in some embodiments of the invention the galactosyl acceptor is not glucose or N-acetyl glucosamine.

The mixture may contain one or more further galactosyl acceptor(s) different from
15 the first type of galactosyl acceptor. The different types of galactosyl acceptors of the mixture may e.g. be selected among the galactosyl acceptor types mentioned herein.

In some preferred embodiments of the invention the produced galactosylated
20 acceptors act as a new type of galactosyl acceptor and can be galactosylated as well. In this way, galacto-oligosaccharides may be produced which have the stoichiometric formula Gal_{i+1}X , where i is a non-negative integer. Normally, the most predominant species are GalX , Gal_2X , and Gal_3X .

25 In other preferred embodiments of the invention the produced galactosylated acceptors act as a new type of galactosyl acceptor and can be galactosylated as well. In this way, galacto-oligosaccharides may be produced which have the general formula $\text{Gal}-(\text{Gal})_i\text{-X}$, where i is a non-negative integer. Normally, the most predominant species are Gal-X , Gal-Gal-X , and Gal-Gal-Gal-X .

30 In some embodiments of the invention the mixture of step a) comprises the galactosyl donor in a concentration of at most 0.7 mol/L, preferably at most 0.4 mol/L, and even more preferably at most 0.2 mol/L. The mixture may e.g. comprise the galactosyl donor in a concentration in the range of 0.001-0.7 mol/L,
35 preferably in the range of 0.01-0.5 mol/L, and even more preferred in the range of 0.02-0.2 mol/L.

Alternatively, the mixture of step a) may comprise the galactosyl donor in a concentration of at most 0.3 mol/L, preferably at most 0.1 mol/L, and even more preferably at most 0.05 mol/L. The mixture may e.g. comprise the galactosyl
5 donor in a concentration in the range of 0.001-0.2 mol/L, preferably in the range of 0.005-0.1 mol/L, and even more preferred in the range of 0.01-0.05 mol/L.

It should be noted that galactosylated galactosyl acceptor and galactosylated galactosyl donor may to a limited extent act as a galactosyl donor, but
10 galactosylated galactosyl acceptor and galactosylated galactosyl donor are not considered a galactosyl donor in the context of the present invention and do not contribute to the concentrations or ratios of galactosyl donor mentioned herein.

The galactosyl acceptor may be used in a range of difference concentrations. It is,
15 however, preferred to avoid saturating the mixture with the galactosyl acceptor since excess galactosyl acceptor normally has to be removed from the galacto-oligosaccharide-containing composition of the invention.

In some embodiments of the invention the mixture of step a) comprises the galactosyl acceptor in an amount of at least 0.05 mol/L, preferably at least 0.10 mol/L, and even more preferably at least 0.30 mol/L. Even higher concentrations of the galactosyl acceptor may be preferred, thus the mixture of step a) may e.g. comprise the galactosyl acceptor in an amount of at least 0.5 mol/L, preferably at least 0.7 mol/L, and even more preferably at least 1 mol/L.

25 The mixture may e.g. comprise the galactosyl acceptor in a concentration in the range of 0.05 mol/L – 5 mol/L, preferably in the range of 0.1 mol/L – 2 mol/L, and even more preferably in the range of 0.3 mol/L – 1 mol/L.

30 However, in some embodiments a relatively low concentration of the galactosyl acceptor is preferred in which case the mixture may e.g. comprise the galactosyl acceptor in a concentration of at most 2 mol/L, preferably at most 0.5 mol/L, and even more preferably at most 0.2 mol/L. For example, the mixture may comprise the galactosyl acceptor in a concentration in the range of 0.05 mol/L – 2 mol/L,
35 preferably in the range of 0.06 mol/L – 1 mol/L, and even more preferably in the range of 0.08 mol/L – 0.8 mol/L.

In addition to galactosyl acceptor and galactosyl donor, the mixture may furthermore contain various additives for optimizing the conditions for the enzymatic reaction.

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The mixture may for example contain one or more pH buffer(s) for adjusting the pH of the mixture to the pH optimum of the first enzyme. Alternatively, or in addition, the mixture may comprise water soluble salts containing one or more metal ions. Depending on the specific first enzyme, metal ions such as Ca^{2+} , Zn^{2+} ,
10 or Mg^{2+} may e.g. be used. Note, however, that some first enzymes are insensitive to the presence of metal ions in the mixture.

Conventional methods of synthesising oligosaccharides often employ water-activity-lowering agents, such as e.g. glycerol, ethylene glycol, propylene glycol,
15 polyethyleneglycol (PEG). The present invention advantageously makes it possible to perform efficient synthesis of galacto-oligosaccharides without the use of such water-activity-lowering agents. Thus, in some preferred embodiments of the invention the mixture contains water-activity-lowering agent in an amount of at most 5% by weight relative to the weight of the mixture, preferably at most 1%
20 by weight, and even more preferably at most 0.1% by weight. For example, the mixture may contain water-activity-lowering agent in an amount of at most 0.05% by weight relative to the weight of the mixture.

The mixture of step a) or the ingredients forming the mixture may have been heat
25 treated before the reaction with first enzyme to avoid microbial growth during the reaction. The usual heat treatment processes, such as pasteurisation (e.g. 72 degrees C for 15 seconds), high pasteurisation (e.g. 90 degrees C for 15 seconds), or UHT treatment (e.g. 140 degrees C for 4 seconds), may be used. Care should be taken when heat treating temperature labile enzymes.

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Step b) involves the provision of a first enzyme, which preferably has beta-galactosidase activity. Preferably, the first enzyme has transgalactosylating activity in addition to beta-galactosidase activity. It may also be preferred that the first enzyme has a T-value of at most 0.9. It should be noted that the method
35 may furthermore involve the use of additional enzymes, e.g. enzymes having a

different enzymatic activity than beta-galactosidase activity or transgalactosylating activity.

In the context of the present invention the term "beta-galactosidase activity" relates to enzymatic catalysis of the hydrolysis of terminal non-reducing β -D-galactose residues in β -D-galactosides, such as lactose. The first enzyme used in the invention preferably belongs to the class EC 3.2.1.23.

It is preferred that the first enzyme has a T-value of at most 0.9. In some embodiments of the invention, the T-value of the first enzyme is at most 0.8, preferably at most 0.7, and even more preferably at most 0.6. For example, the T-value of the first enzyme may be at most 0.5. Preferably the T-value of the first enzyme may be at most 0.4. It may even be more preferred that the T-value of the first enzyme is at most 0.3.

Even lower T-values may be preferred, such as at most 0.2.

Useful first enzymes may e.g. be derived from a peptide encoded by the DNA sequence of SEQ ID NO. 1. An example of such a peptide from which useful first enzymes may e.g. be derived is the peptide having the amino acid sequence of SEQ ID NO. 2.

SEQ ID NO. 1 and SEQ ID NO. 2 can be found in the PCT application WO 01/90,317 A2, where they are referred to as SEQ ID NO: 1 and SEQ ID NO: 2. Additionally, further useful first enzymes may be also be found in WO 01/90,317 A2.

In some preferred embodiments of the invention the first enzyme comprises an amino acid sequence having a sequence identity of at least 80% relative to the peptide of SEQ ID NO. 2. For example, the first enzyme may comprise an amino acid sequence having a sequence identity of at least 90% relative to the peptide of SEQ ID NO. 2, preferably at least 95%, and even more preferably at least 97.5%. In some instances the first enzyme may comprise an amino acid sequence having a sequence identity of at least 99% relative to the peptide of SEQ ID NO. 2.

In the context of the present invention the term "sequence identity" relates to a quantitative measure of the degree of identity between two amino acid sequences of equal length or between two nucleic acid sequences of equal length. If the two sequences to be compared are not of equal length, they must be
 5 aligned to the best possible fit. The sequence identity can be calculated as

$$(N_{\text{ref}} - N_{\text{dif}}) * 100 / (N_{\text{ref}}),$$

wherein N_{dif} is the total number of non-identical residues in the two sequences
 10 when aligned, and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{\text{dif}}=2$ and $N_{\text{ref}}=8$). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC ($N_{\text{dif}}=2$ and $N_{\text{ref}}=8$).
 15 Sequence identity can for example be calculated using appropriate BLAST-programs, such as the BLASTp-algorithm provided by National Center for Biotechnology Information (NCBI), USA.

In other preferred embodiments of the invention the amino acid sequence of the
 20 first enzyme has a sequence identity of at least 80% relative to the peptide of SEQ ID NO. 2. For example, the amino acid sequence of the first enzyme may have a sequence identity of at least 90% relative to the peptide of SEQ ID NO. 2, preferably at least 95%, and even more preferably at least 97.5%. In some instances the amino acid sequence of the first enzyme may have a sequence
 25 identity of at least 99% relative to the peptide of SEQ ID NO. 2.

In some preferred embodiments of the invention the first enzyme comprises an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2. For example, the first
 30 enzyme may comprise an amino acid sequence having a sequence identity of at least 90% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2, preferably at least 95%, and even more preferably at least 97.5%. In some instances the first enzyme may comprise an amino acid sequence having a sequence identity of at least 99% relative to the amino acid sequence Val (33) to
 35 Gly (950) of SEQ ID NO. 2.

In other preferred embodiments of the invention the amino acid sequence of the first enzyme has a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2. For example, the amino acid sequence of the first enzyme may have a sequence identity of at least 90%
5 relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2, preferably at least 95%, and even more preferably at least 97.5%. In some instances the amino acid sequence of the first enzyme may have a sequence identity of at least 99% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2. Thus, the first enzyme may e.g. have the amino acid sequence
10 Val (33) to Gly (950) of SEQ ID NO. 2.

In some preferred embodiments of the invention the first enzyme comprises an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2. For example, the first
15 enzyme may comprise an amino acid sequence having a sequence identity of at least 90% relative to the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2, preferably at least 95%, and even more preferably at least 97.5%. In some instances the first enzyme may comprise an amino acid sequence having a sequence identity of at least 99% relative to the amino acid sequence Val (33) to
20 Glu (917) of SEQ ID NO. 2.

In other preferred embodiments of the invention the amino acid sequence of the first enzyme has a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2. For example, the amino acid
25 sequence of the first enzyme may have a sequence identity of at least 90% relative to the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2, preferably at least 95%, and even more preferably at least 97.5%. In some instances the amino acid sequence of the first enzyme may have a sequence identity of at least 99% relative to the amino acid sequence Val (33) to Glu (917)
30 of SEQ ID NO. 2. Thus, the first enzyme may e.g. have the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2.

In some preferred embodiments of the invention the first enzyme comprises an amino acid sequence having a sequence identity of at least 80% relative to the
35 amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2. For example, the first enzyme may comprise an amino acid sequence having a sequence identity of at

least 90% relative to the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2, preferably at least 95%, and even more preferably at least 97.5%. In some instances the first enzyme may comprise an amino acid sequence having a sequence identity of at least 99% relative to the amino acid sequence Met (1) to
5 Ile (1174) of SEQ ID NO. 2.

In other preferred embodiments of the invention the amino acid sequence of the first enzyme has a sequence identity of at least 80% relative to the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2. For example, the amino acid
10 sequence of the first enzyme may have a sequence identity of at least 90% relative to the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2, preferably at least 95%, and even more preferably at least 97.5%. In some instances the amino acid sequence of the first enzyme may have a sequence identity of at least 99% relative to the amino acid sequence Met (1) to Ile (1174)
15 of SEQ ID NO. 2.

In some presently preferred embodiments of the invention the first enzyme has the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2.

20 In some embodiments of the invention the first enzyme comprises an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2. For example, the first enzyme may comprise an amino acid sequence having a sequence identity of at least 90% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2,
25 preferably at least 95%, and even more preferably at least 97.5%. In some instances the first enzyme may comprise an amino acid sequence having a sequence identity of at least 99% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2.

30 In other embodiments of the invention the amino acid sequence of the first enzyme may have a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2. For example, the amino acid sequence of the first enzyme may have a sequence identity of at least 90% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2,
35 preferably at least 95%, and even more preferably at least 97.5%. In some instances the amino acid sequence of the first enzyme may have a sequence

identity of at least 99% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2. Thus, the first enzyme may e.g. have the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2.

- 5 In some presently preferred embodiments of the invention the first enzyme has the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2.

The first enzyme may for example comprise:

- 10 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence of SEQ ID NO. 2, or
- an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2, or
- 15 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2, or
- an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2.

20

Alternatively, the first enzyme may e.g. consist of:

- an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence of SEQ ID NO. 2, or
- an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2, or
- 25 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2, or
- 30 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2.

The first enzyme may for example comprise:

- 35 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence of SEQ ID NO. 2, or

- an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2, or
- 5 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2, or
- an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2 or
- 10 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2.

Alternatively, the first enzyme may e.g. consist of:

- 15 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence of SEQ ID NO. 2, or
 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2, or
 - 20 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2, or
 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2, or
 - 25 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2.
- 30 In some embodiments of the invention, the first enzyme may e.g. comprise an amino acid sequence having a sequence identity of at least 99% relative to an amino acid sequence shown in Table 1. The first enzyme may for example comprise an amino acid sequence shown in Table 1. Alternatively, the amino acid sequence of the first enzyme may have a sequence identity of at least 99%
- 35 relative to an amino acid sequence shown in Table 1. The first enzyme may for example have an amino acid sequence shown in Table 1.

Table 1 Useful amino acid sequences (AAS).

AAS No.	Position in SEQ ID NO. 2		AAS No.	Position in SEQ ID NO. 2		AAS No.	Position in SEQ ID NO. 2	
	From	To		From	To		From	To
1	25	1122	26	27	1122	51	30	1122
2	25	1132	27	27	1132	52	30	1132
3	25	1142	28	27	1142	53	30	1142
4	25	1152	29	27	1152	54	30	1152
5	25	1162	30	27	1162	55	30	1162
6	25	1167	31	27	1167	56	30	1167
7	25	1168	32	27	1168	57	30	1168
8	25	1169	33	27	1169	58	30	1169
9	25	1170	34	27	1170	59	30	1170
10	25	1171	35	27	1171	60	30	1171
11	25	1172	36	27	1172	61	30	1172
12	25	1173	37	27	1173	62	30	1173
13	25	1174	38	27	1174	63	30	1174
14	25	1175	39	27	1175	64	30	1175
15	25	1176	40	27	1176	65	30	1176
16	25	1177	41	27	1177	66	30	1177
17	25	1178	42	27	1178	67	30	1178
18	25	1179	43	27	1179	68	30	1179
19	25	1180	44	27	1180	69	30	1180
20	25	1181	45	27	1181	70	30	1181
21	25	1186	46	27	1186	71	30	1186
22	25	1196	47	27	1196	72	30	1196
23	25	1206	48	27	1206	73	30	1206
24	25	1216	49	27	1216	74	30	1216
25	25	1226	50	27	1226	75	30	1226

5

In some embodiments of the invention, the enzyme may e.g. comprise an amino acid sequence having a sequence identity of at least 99% relative to an amino acid sequence shown in Table 2. The enzyme may for example comprise an amino acid sequence shown in Table 2. Alternatively, the amino acid sequence of the enzyme may have a sequence identity of at least 99% relative to an amino acid sequence shown in Table 2. The enzyme may for example have an amino acid sequence shown in Table 2.

10

Table 2 Useful amino acid sequences (AAS).

AAS No.	Position in SEQ ID NO. 2		AAS No.	Position in SEQ ID NO. 2		AAS No.	Position in SEQ ID NO. 2	
	From	To		From	To		From	To
76	31	1122	101	32	1122	126	33	1122
77	31	1132	102	32	1132	127	33	1132
78	31	1142	103	32	1142	128	33	1142
79	31	1152	104	32	1152	129	33	1152
80	31	1162	105	32	1162	130	33	1162
81	31	1167	106	32	1167	131	33	1167
82	31	1168	107	32	1168	132	33	1168
83	31	1169	108	32	1169	133	33	1169
84	31	1170	109	32	1170	134	33	1170
85	31	1171	110	32	1171	135	33	1171
86	31	1172	111	32	1172	136	33	1172
87	31	1173	112	32	1173	137	33	1173
88	31	1174	113	32	1174	138	33	1174
89	31	1175	114	32	1175	139	33	1175
90	31	1176	115	32	1176	140	33	1176
91	31	1177	116	32	1177	141	33	1177
92	31	1178	117	32	1178	142	33	1178
93	31	1179	118	32	1179	143	33	1179
94	31	1180	119	32	1180	144	33	1180
95	31	1181	120	32	1181	145	33	1181
96	31	1186	121	32	1186	146	33	1186
97	31	1196	122	32	1196	147	33	1196
98	31	1206	123	32	1206	148	33	1206
99	31	1216	124	32	1216	149	33	1216
100	31	1226	125	32	1226	150	33	1226

- 5 In some embodiments of the invention, the enzyme may e.g. comprise an amino acid sequence having a sequence identity of at least 99% relative to an amino acid sequence shown in Table 3. The enzyme may for example comprise an amino acid sequence shown in Table 3. Alternatively, the amino acid sequence of the enzyme may have a sequence identity of at least 99% relative to an amino acid
- 10 sequence shown in Table 3. The enzyme may for example have an amino acid sequence shown in Table 3.

Table 3 Useful amino acid sequences (AAS).

AAS No.	Position in SEQ ID NO. 2		AAS No.	Position in SEQ ID NO. 2		AAS No.	Position in SEQ ID NO. 2	
	From	To		From	To		From	To
151	34	1122	176	35	1122	201	36	1122
152	34	1132	177	35	1132	202	36	1132
153	34	1142	178	35	1142	203	36	1142
154	34	1152	179	35	1152	204	36	1152
155	34	1162	180	35	1162	205	36	1162
156	34	1167	181	35	1167	206	36	1167
157	34	1168	182	35	1168	207	36	1168
158	34	1169	183	35	1169	208	36	1169
159	34	1170	184	35	1170	209	36	1170
160	34	1171	185	35	1171	210	36	1171
161	34	1172	186	35	1172	211	36	1172
162	34	1173	187	35	1173	212	36	1173
163	34	1174	188	35	1174	213	36	1174
164	34	1175	189	35	1175	214	36	1175
165	34	1176	190	35	1176	215	36	1176
166	34	1177	191	35	1177	216	36	1177
167	34	1178	192	35	1178	217	36	1178
168	34	1179	193	35	1179	218	36	1179
169	34	1180	194	35	1180	219	36	1180
170	34	1181	195	35	1181	220	36	1181
171	34	1186	196	35	1186	221	36	1186
172	34	1196	197	35	1196	222	36	1196
173	34	1206	198	35	1206	223	36	1206
174	34	1216	199	35	1216	224	36	1216
175	34	1226	200	35	1226	225	36	1226

In some embodiments of the invention, the enzyme may e.g. comprise an amino acid sequence having a sequence identity of at least 99% relative to an amino acid sequence shown in Table 4. The enzyme may for example comprise an amino acid sequence shown in Table 4. Alternatively, the amino acid sequence of the enzyme may have a sequence identity of at least 99% relative to an amino acid sequence shown in Table 4. The enzyme may for example have an amino acid sequence shown in Table 4.

Table 4 Useful amino acid sequences (AAS).

AAS No.	Position in SEQ ID NO. 2		AAS No.	Position in SEQ ID NO. 2	
	From	To		From	To
226	39	1122	251	42	1122
227	39	1132	252	42	1132
228	39	1142	253	42	1142
229	39	1152	254	42	1152
230	39	1162	255	42	1162
231	39	1167	256	42	1167
232	39	1168	257	42	1168
233	39	1169	258	42	1169
234	39	1170	259	42	1170
235	39	1171	260	42	1171
236	39	1172	261	42	1172
237	39	1173	262	42	1173
238	39	1174	263	42	1174
239	39	1175	264	42	1175
240	39	1176	265	42	1176
241	39	1177	266	42	1177
242	39	1178	267	42	1178
243	39	1179	268	42	1179
244	39	1180	269	42	1180
245	39	1181	270	42	1181
246	39	1186	271	42	1186
247	39	1196	272	42	1196
248	39	1206	273	42	1206
249	39	1216	274	42	1216
250	39	1226	275	42	1226

In some embodiments of the invention, the first enzyme may e.g. comprise an amino acid sequence having a sequence identity of at least 99% relative to an amino acid sequence shown in Table 5. The first enzyme may for example comprise an amino acid sequence shown in Table 5. Alternatively, the amino acid sequence of the first enzyme may have a sequence identity of at least 99% relative to an amino acid sequence shown in Table 5. The first enzyme may for example have an amino acid sequence shown in Table 5.

Table 5 Useful amino acid sequences (AAS).

AAS no.	Position in SEQ ID NO. 2		AAS no.	Position in SEQ ID NO. 2		AAS no.	Position in SEQ ID NO. 2	
	From	To		From	To		From	To
276	31	898	301	33	898	326	37	898
277	31	908	302	33	908	327	37	908
278	31	918	303	33	918	328	37	918
279	31	928	304	33	928	329	37	928
280	31	938	305	33	938	330	37	938
281	31	943	306	33	943	331	37	943
282	31	944	307	33	944	332	37	944
283	31	945	308	33	945	333	37	945
284	31	946	309	33	946	334	37	946
285	31	947	310	33	947	335	37	947
286	31	948	311	33	948	336	37	948
287	31	949	312	33	949	337	37	949
288	31	950	313	33	950	338	37	950
289	31	951	314	33	951	339	37	951
290	31	952	315	33	952	340	37	952
291	31	953	316	33	953	341	37	953
292	31	954	317	33	954	342	37	954
293	31	955	318	33	955	343	37	955
294	31	956	319	33	956	344	37	956
295	31	957	320	33	957	345	37	957
296	31	962	321	33	962	346	37	962
297	31	972	322	33	972	347	37	972
298	31	982	323	33	982	348	37	982
299	31	992	324	33	992	349	37	992
300	31	1002	325	33	1002	350	37	1002

In some embodiments of the invention, the first enzyme may e.g. comprise an amino acid sequence having a sequence identity of at least 99% relative to an amino acid sequence shown in Table 6. The first enzyme may for example comprise an amino acid sequence shown in Table 6. Alternatively, the amino acid sequence of the first enzyme may have a sequence identity of at least 99% relative to an amino acid sequence shown in Table 6. The first enzyme may for example have an amino acid sequence shown in Table 6.

Table 6 Useful amino acid sequences (AAS).

AAS no.	Position in SEQ ID NO. 2		AAS no.	Position in SEQ ID NO. 2		AAS no.	Position in SEQ ID NO. 2	
	From	To		From	To		From	To
351	31	865	376	33	865	401	35	865
352	31	875	377	33	875	402	35	875
353	31	885	378	33	885	403	35	885
354	31	895	379	33	895	404	35	895
355	31	905	380	33	905	405	35	905
356	31	910	381	33	910	406	35	910
357	31	911	382	33	911	407	35	911
358	31	912	383	33	912	408	35	912
359	31	913	384	33	913	409	35	913
360	31	914	385	33	914	410	35	914
361	31	915	386	33	915	411	35	915
362	31	916	387	33	916	412	35	916
363	31	917	388	33	917	413	35	917
364	31	918	389	33	918	414	35	918
365	31	919	390	33	919	415	35	919
366	31	920	391	33	920	416	35	920
367	31	921	392	33	921	417	35	921
368	31	922	393	33	922	418	35	922
369	31	923	394	33	923	419	35	923
370	31	924	395	33	924	420	35	924
371	31	929	396	33	929	421	35	929
372	31	939	397	33	939	422	35	939
373	31	949	398	33	949	423	35	949
374	31	959	399	33	959	424	35	959
375	31	969	400	33	969	425	35	969

Other examples of useful first enzymes having transgalactosylating activity can be
5 found in WO 2011/120993 A1, which is incorporated herein by reference.

In some embodiments of the invention the first enzyme may contain one or more
glycosylated amino acid(s). Alternatively, or in addition, the first enzyme may
contain one or more phosphorylated amino acid(s). Alternatively, none of the
10 amino acids of the first enzyme are glycosylated or phosphorylated.

In some preferred embodiments of the invention the first enzyme comprises at least two sub-units, each sub-unit consisting of a first enzyme as defined above.

5 The first enzyme preferably contacts the mixture and is thereby brought into contact with both the galactosyl acceptor and the galactosyl donor.

In some embodiments of the invention the mixture comprises the first enzyme and/or the second enzyme. The first enzyme and/or the second enzyme may e.g. be present in the mixture in dissolved form, e.g. as single enzyme molecules or as
10 soluble aggregate of enzyme molecules.

In other embodiments of the invention the first enzyme and/or the second enzyme is/are separate from the mixture, but brought in contact with the galactosyl acceptor and the galactosyl donor by contacting the first enzyme
15 and/or the second enzyme with the mixture. For example, first enzyme and/or second enzyme immobilised on a stationary solid phase may be used. Examples of useful stationary solid phases are e.g. a filter, a packed bed of first enzyme-containing particles, or similar structures.

20 Alternatively, the solid phase may e.g. be a free flowing, particulate solid phase, e.g. organic or inorganic beads, forming part of the mixture.

Details relating to the industrial use of enzymes including immobilisation techniques and suitable solid phase types can be found in Buchholz (2005), which
25 is incorporated herein by reference for all purposes.

The first enzyme is preferably used in a sufficient activity to obtain an acceptable yield of galacto-oligosaccharides. The optimal activity depends on the specific implementation of the process and can easily be determined by the person skilled
30 in the art.

If a high turn-over of galactosyl donor and a high yield of galacto-oligosaccharide is required, it may be preferred to use the first enzyme in a relatively high activity. For example, the activity of the first enzyme may be such that the turn-
35 over of the galactosyl donor is at least 0.02 mol/(L*h), preferably at least 0.2 mol/(L*h), and even more preferably at least 2 mol/(L*h).

The enzymatic reaction takes place during the incubation of step c). As soon as the mixture is exposed to the right conditions, which may be almost immediately when the galactosyl acceptor and the galactosyl donor are brought into contact
5 with the first enzyme, the transgalactosylation usually starts, and in some embodiments of the invention steps b) and c) occur simultaneously.

The first enzyme is capable of releasing the leaving group of the galactosyl donor and transferring the galactosyl group of the galactosyl donor to the galactosyl
10 acceptor. For example, if the galactosyl donor is lactose, glucose is released and the galactosyl group is transferred to the acceptor. The first enzyme acts as catalyst during the enzymatic reaction.

In some preferred embodiments of the invention the first enzyme furthermore
15 transfers galactosyl groups to already galactosylated galactosyl acceptors, thereby generating galactosyl acceptors containing two, three or even more galactosyl groups.

The pH of the incubating mixture is preferably near the optimum pH of the first
20 enzyme. In some embodiments of the invention the pH of the incubating mixture during step c) is in the range of pH 3-9. For example, the pH of the incubating mixture during step c) may be in the range of pH 4-8, such as in the range of pH 5-7.5.

Similar to the pH, the temperature of the incubating mixture is preferably
25 adjusted to the optimum temperature of the used first enzyme. In some embodiments of the invention the temperature of the incubating mixture of step c) is in the range of 10-80 degrees C. The temperature of the incubating mixture may e.g. be in the range of 20-70 degrees C, preferably in the range of 25-60
30 degrees C, and even more preferably in the range of 30-50 degrees C.

In the context of the present invention, the term "optimum pH of the first enzyme" relates to the pH where the first enzyme has the highest transgalactosylating activity. Along the same lines, the term "optimum
35 temperature of the first enzyme" relates to the temperature where the first enzyme has the highest transgalactosylating activity.

Step c) may furthermore involve stirring the incubating mixture.

The removal of free leaving groups takes place during the incubation of step c).

- 5 The removal may for example start immediately when step c) starts or it may be postponed until a significant amount of free leaving groups start building up in the incubating mixture.

- 10 The free leaving groups may for example be removed from the incubating mixture by microorganisms present in the incubating mixture.

- 15 Thus, in some embodiments of the invention, the method furthermore comprises providing a microorganism which is capable of converting free leaving groups released from the galactosyl donor, and allowing said microorganism, during incubation, to remove a leaving group released from the galactosyl donor.

- 20 Useful microorganisms are preferably able to selectively remove the free leaving groups from the incubating mixture and e.g. metabolise or convert these into conversion products that interfere less with the synthesis of galacto-oligosaccharides than the leaving group.

- 25 In some preferred embodiments of the invention, the removal rate of the microorganism relative to the free leaving group is at least 10 times higher than its removal rate relative to the galactosyl acceptor.

- For example, the removal rate of the microorganism relative to the free leaving group may be at least 10^2 times higher than its removal rate relative to the galactosyl acceptor, preferably at least 10^3 times higher, and even more preferred at least 10^4 times higher than its removal rate relative to the galactosyl acceptor.
- 30 The removal rate of the microorganism relative to the free leaving group may e.g. be at least 10^5 times higher than its removal rate relative to the galactosyl acceptor. It may even be preferred that the removal rate of the microorganism relative to the free leaving group is at least 10^6 times higher, and even more preferred at least 10^7 times higher than its removal rate relative to the galactosyl acceptor.
- 35

In the context of the present invention, the term "removal rate" pertains to the number of moles of free leaving group, donor, acceptor or mono-galactosylated acceptor that the microorganism is capable of removing from the incubating mixture per minute.

5

In some preferred embodiments of the invention, the removal rate of the microorganism relative to the free leaving group is at least 10 times higher than its removal rate relative to the galactosyl donor.

10 For example, the removal rate of the microorganism relative to the free leaving group may be at least 10^2 times higher than its removal rate relative to the galactosyl donor, preferably at least 10^3 times higher, and even more preferred at least 10^4 times higher than its removal rate relative to the galactosyl donor. The removal rate of the microorganism relative to the free leaving group may e.g. be
15 at least 10^5 times higher than its removal rate relative to the galactosyl donor. It may even be preferred that the removal rate of the microorganism relative to the free leaving group is at least 10^6 times higher, and even more preferred at least 10^7 times higher than its removal rate relative to the galactosyl donor.

20 In some preferred embodiments of the invention, the removal rate of the microorganism relative to the free leaving group is at least 10 times higher than its removal rate relative to the mono-galactosylated galactosyl acceptor.

For example, the removal rate of the microorganism relative to the free leaving
25 group may be at least 10^2 times higher than its removal rate relative to the mono-galactosylated galactosyl acceptor, preferably at least 10^3 times higher, and even more preferred at least 10^4 times higher than its removal rate relative to the mono-galactosylated galactosyl acceptor. The removal rate of the microorganism relative to the free leaving group may e.g. be at least 10^5 times higher than its
30 removal rate relative to the mono-galactosylated galactosyl acceptor. It may even be preferred that the removal rate of the microorganism relative to the free leaving group is at least 10^6 times higher, and even more preferred at least 10^7 times higher than its removal rate relative to the mono-galactosylated galactosyl acceptor.

35

The microorganism is preferably a yeast or a bacterium.

Saccharomyces cerevisiae is an example of a useful yeast, and useful bacteria could for example be Lac Z-negative E.coli strains or other bacteria which can metabolise glucose but not lactose.

5

Alternatively, or additionally, free leaving groups may be removed from the incubating mixture by means of specific enzymes that convert the leaving groups into other chemical species.

10 Thus, in some preferred embodiments of the invention, the method furthermore comprises providing a second enzyme which is capable of converting free leaving groups released from the galactosyl donor and allowing said second enzyme, during incubation, to convert a leaving group released from the galactosyl donor.

15 The term "converting free leaving groups" pertains to the process of converting the free leaving groups into chemical species which preferably are poorer galactosyl acceptors than the free leaving groups as such. The conversion may involve degradation of the free leaving group into several smaller chemical species or it may simply involve a transformation of the free leaving group into a different
20 chemical species.

The present invention may for example pertain to a method of producing a composition comprising one or more galacto-oligosaccharide(s), the method comprising the steps of:

25

a) providing a mixture comprising

- a galactosyl donor comprising a galactosyl group bound to a leaving group, which galactosyl donor has a molar weight of at most 350 g/mol,
- a galactosyl acceptor which is different from the galactosyl donor,

30

said galactosyl acceptor is a saccharide or a sugar-alcohol, and

wherein the molar ratio between the galactosyl acceptor and the galactosyl donor is at least 1:10, and wherein the mixture comprises at least 0.05 mol/L of the galactosyl acceptor,

35

b) providing a first enzyme and a second enzyme, said first enzyme having beta-galactosidase activity and a T-value of at most 0.9, said second enzyme being

capable of converting free leaving groups released from the galactosyl donor, and said first and second enzymes contacting the mixture, and

- 5 c) allowing the first enzyme to release the leaving group of the galactosyl donor and transferring the galactosyl group of the galactosyl donor to the galactosyl acceptor, thus forming the galacto-oligosaccharide, and allowing the second enzyme to convert a leaving group released from the galactosyl donor, thereby obtaining the composition comprising the galacto-oligosaccharide.
- 10 The second enzyme may for example have hexose oxidase activity, i.e. belonging to the enzyme class EC 1.1.3.5 and having the ability to catalyze the oxidation of beta-D-glucose with O₂ to form D-glucono-1,5-lactone and hydrogen peroxide.

- 15 It is even more preferred that the second enzyme has glucose oxidase activity, i.e. belonging to the enzyme class EC 1.1.3.4 and having the ability to catalyze the oxidation of beta-D-glucose with O₂ to form D-glucono-1,5-lactone and hydrogen peroxide without significant oxidation of glucose-containing disaccharides such as lactose.

- 20 In an aqueous, acidic environment, D-glucono-1,5-lactone typically hydrolyses to form gluconic acid.

- Alternatively, the second enzymes may have hexokinase activity (EC 2.7.1.1) or glucokinase activity (EC 2.7.1.2).

- 25 Other useful second enzymes may e.g. be found in handbooks which are available to the person skilled in the art.

- 30 It is preferred that the second enzyme is selective in its conversion of the free leaving groups and only to a limited extent, and preferably not at all, converts the galactosyl donor, the galactosyl acceptor and/or the galacto-oligosaccharides.

- Thus, in some preferred embodiments of the invention, the specificity constant of the second enzyme relative to the free leaving group is at least 10 times higher
- 35 than its specificity constant relative to the galactosyl acceptor.

For example, the specificity constant of the second enzyme relative to the free leaving group may be at least 10^2 times higher than its specificity constant relative to the galactosyl acceptor, preferably at least 10^3 times higher, and even more preferred at least 10^4 times higher than its specificity constant relative to the galactosyl acceptor. The specificity constant of the second enzyme relative to the free leaving group may e.g. be at least 10^5 times higher than its specificity constant relative to the galactosyl acceptor. It may even be preferred that the specificity constant of the second enzyme relative to the free leaving group is at least 10^6 times higher, and even more preferred at least 10^7 times higher than its specificity constant relative to the galactosyl acceptor.

The term "specificity constant" is determined as k_{cat}/K_m and incorporates the rate constants for all steps in the reaction. Because the specificity constant reflects both affinity and catalytic ability, it is useful for comparing different enzymes against each other, or the same enzyme with different substrates. The theoretical maximum for the specificity constant is called the diffusion limit and is about 10^8 to 10^9 ($M^{-1} s^{-1}$). At this point every collision of the enzyme with its substrate will result in catalysis, and the rate of product formation is not limited by the reaction rate but by the diffusion rate.

k_{cat} and K_m of the second enzyme is determined at 37 degrees using 50 mM Na_3PO_4 buffer adjusted to pH 6.5. The buffer furthermore contains the salts and co-factors which are required for optimal performance of the enzyme. In some preferred embodiments of the invention, the specificity constant of the second enzyme relative to the free leaving group is at least 10 times higher than its specificity constant relative to the galactosyl donor.

For example, the specificity constant of the second enzyme relative to the free leaving group may be at least 10^2 times higher than its specificity constant relative to the galactosyl donor, preferably at least 10^3 times higher, and even more preferred at least 10^4 times higher than its specificity constant relative to the galactosyl donor. The specificity constant of the second enzyme relative to the free leaving group may e.g. be at least 10^5 times higher than its specificity constant relative to the galactosyl donor. It may even be preferred that the specificity constant of the second enzyme relative to the free leaving group is at

least 10^6 times higher, and even more preferred at least 10^7 times higher than its specificity constant relative to the galactosyl donor.

5 In some preferred embodiments of the invention, the specificity constant of the second enzyme relative to the free leaving group is at least 10 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor (Gal-X).

10 For example, the specificity constant of the second enzyme relative to the free leaving group may be at least 10^2 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor, preferably at least 10^3 times higher, and even more preferred at least 10^4 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor. The specificity constant of the second enzyme relative to the free leaving group may
15 e.g. be at least 10^5 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor. It may even be preferred that the specificity constant of the second enzyme relative to the free leaving group is at least 10^6 times higher, and even more preferred at least 10^7 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.

20 In some preferred embodiments of the invention, the specificity constant of the second enzyme relative to the free leaving group is:

- at least 10 times higher than its specificity constant relative to the galactosyl donor,
- 25 - at least 10 times higher than its specificity constant relative to the galactosyl acceptor, and
- at least 10 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.

30 For example, the specificity constant of the second enzyme relative to the free leaving group may be:

- at least 10^2 times higher than its specificity constant relative to the galactosyl donor,
- at least 10^2 times higher than its specificity constant relative to the galactosyl
35 acceptor, and

- at least 10^2 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.

- Preferably, the specificity constant of the second enzyme relative to the free leaving group is:
- at least 10^3 times higher than its specificity constant relative to the galactosyl donor,
 - at least 10^3 times higher than its specificity constant relative to the galactosyl acceptor, and
 - at least 10^3 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.

- Alternatively, the specificity constant of the second enzyme relative to the free leaving group may be:
- at least 10^4 times higher than its specificity constant relative to the galactosyl donor,
 - at least 10^4 times higher than its specificity constant relative to the galactosyl acceptor, and
 - at least 10^4 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.

- The specificity constant of the second enzyme relative to the free leaving group may be:
- at least 10^5 times higher than its specificity constant relative to the galactosyl donor,
 - at least 10^5 times higher than its specificity constant relative to the galactosyl acceptor, and
 - at least 10^5 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.

- It is even more preferred that the specificity constant of the second enzyme relative to the free leaving group is:
- at least 10^6 times higher than its specificity constant relative to the galactosyl donor,
 - at least 10^6 times higher than its specificity constant relative to the galactosyl acceptor, and

- at least 10^6 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.

The specificity constant of the second enzyme relative to the free leaving group
5 may for example be:

- at least 10^7 times higher than its specificity constant relative to the galactosyl donor,
- at least 10^7 times higher than its specificity constant relative to the galactosyl acceptor, and
- 10 - at least 10^7 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.

It is particularly preferred that the second enzyme has glucose oxidase activity and the leaving group of the galactosyl donor is a glucosyl group in which case the
15 free leaving group is glucose. If the second enzyme has glucose oxidase activity lactose is a preferred galactosyl donor.

Glucose oxidase requires O_2 as oxidant and it may be necessary to add extra O_2 (g) to the incubating mixture if the normal amount of dissolved O_2 in water is
20 insufficient. It is also possible to add co-factors such as FAD (flavin adenine dinucleotide) or NAD (nicotinamide adenine dinucleotide) to the incubating mixture if required.

As mentioned above, H_2O_2 is formed when glucose oxidase catalyses the oxidation
25 of glucose to D-glucono-1,5-lactone, and high levels of H_2O_2 may be problematic for the process, e.g. due to undesired oxidation of the used enzymes. In some embodiments of the invention, the method furthermore involves providing a peroxidase or a catalase which contacts the incubating mixture and which catalyses the degradation of H_2O_2 , e.g. to H_2O and O_2 . Both glucose oxidases and
30 catalases are well-known in the prior art and commercially available. An example of a useful glucose oxidase is Glyzyme BG (Novozymes, Denmark). An example of a useful catalase enzyme is Catazyme 25L (Novozymes, Denmark). Another example is catalase enzyme from *Micrococcus lysodeikticus* (Sigma-Aldrich, Denmark).

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The method may furthermore involve contacting the incubating mixture with a lactonase, and preferably a gluconolactonase (EC 3.1.1.17), i.e. an enzyme capable of hydrolysing D-glucono-1,5-lactone into gluconic acid or its corresponding base gluconate.

5

Thus, in some preferred embodiments of the invention, the incubating mixture is brought in contact with an enzyme having glucose oxidase activity, an enzyme having glucose oxidase activity catalase activity and an enzyme having gluconolactonase activity.

10

In some preferred embodiments of the invention, the method furthermore comprises providing a removal agent capable of removing at least some of the conversion product obtained by converting the leaving group with the second enzyme, and allowing the removal agent, during the incubation, to remove at least some of the conversion product.

15

In the context of the present invention the term "removal agent" pertains to a particulate or molecular agent capable of capturing conversion product and removing it from the incubating mixture, e.g. by precipitation.

20

The removal agent may for example comprise, or even consist of, a salt of a divalent or trivalent metal ion. Examples of useful metal ions are Ca^{2+} , Fe^{2+} , Al^{3+} , or Zn^{2+} .

25 In preferred embodiments of the invention, the removal agent comprises, or even consists of, CaCO_3 .

Negatively charged conversion products such as e.g. gluconate, the deprotonated form of gluconic acid, may be removed using anion exchange chromatography.

30

Thus, the removal agent may comprise, or even consist of, an anion exchange material.

In some embodiments of the invention the anion exchange material comprises a solid phase and one or more cationic group(s).

35

Preferably, at least some of the cationic groups are attached to the outer surface of the solid phase and/or to the surface of pores which are accessible through the surface of the solid phase.

- 5 In some embodiments of the invention the solid phase of the anion exchange material comprises one or more components selected from the group consisting of a plurality of particles, a filter, and a membrane.

The solid phase may for example comprise, or even consists of,
10 polysaccharide. Cross-linked polysaccharides are particularly preferred. Examples of useful polysaccharides are cellulose, agarose, and/or dextran. Alternatively, the solid phase may comprise, or even consists of, a non-carbohydrate polymer. Examples of useful non-carbohydrate polymers are methacrylate, polystyrene, and/or styrene-divinylbenzene.

15 In some preferred embodiments of the invention the cationic groups comprise, or even consists of, amino groups. Tertiary amino groups are particularly preferred and result in quaternary ammonium groups under appropriate pH conditions. Quaternary ammonium groups provide strong anion exchange
20 characteristics to the anion exchange material.

Alternatively, or additionally, the cationic groups may comprise one or more primary or secondary amino groups. A substantial amount of primary or secondary amino groups typically provides the anion exchange material with
25 weak anion exchange characteristics. More details regarding anion exchange chromatography and its industrial implementation can be found in *Scopes*, which is incorporated herein by reference for all purposes.

30 When an anion exchange material is used for capturing the conversion product, the total binding capacity of the anion exchange material may e.g. be at least 30% (mol/mol) relative to the total amount of conversion product produced during the incubation. For example, the total binding capacity of the anion exchange material may be at least 50% (mol/mol) relative to the total amount of
35 conversion product produced during the incubation. Alternatively, the total binding

capacity of the anion exchange material may be at least 80% (mol/mol) relative to the total amount of conversion product produced during the incubation.

- When employed, the removal agent is preferably present in an amount sufficient
- 5 to precipitate or capture the theoretical amount of converted leaving group formed during the synthesis process.

- Thus, the total binding capacity of the anion exchange material may e.g. be at least 100% (mol/mol) relative to the total amount of conversion product produced
- 10 during the incubation. For example, the total binding capacity of the anion exchange material may be at least 150% (mol/mol) relative to the total amount of conversion product produced during the incubation. Alternatively, the total binding capacity of the anion exchange material may be at least 200% (mol/mol) relative to the total amount of conversion product produced during the incubation.

- 15 The total amount of conversion product produced during the incubation may for example be estimated from the planned process conditions and kinetic data relating to the used enzyme(s).

- 20 Alternatively, the expected amount of released leaving groups may be used as guidance for dosing the anion exchange material. Thus, the total binding capacity of the anion exchange material may e.g. be at least 30% (mol/mol) relative to the total amount of leaving groups released during the incubation. For example, the total binding capacity of the anion exchange material may be at least 50%
- 25 (mol/mol) relative to the total amount of leaving groups released during the incubation. Alternatively, the total binding capacity of the anion exchange material may be at least 80% (mol/mol) relative to the total amount of leaving groups released during the incubation.

- 30 The total binding capacity of the anion exchange material may e.g. be at least 100% (mol/mol) relative to the total amount of leaving groups released during the incubation. For example, the total binding capacity of the anion exchange material may be at least 150% (mol/mol) relative to the total amount of leaving groups released during the incubation. Alternatively, the total binding capacity of
- 35 the anion exchange material may be at least 200% (mol/mol) relative to the total amount of leaving groups released during the incubation.

The inventors have discovered that the present method surprisingly provides a high yield of galacto-oligosaccharides even though a relatively low concentration of the galactosyl donor is used. The relatively low concentration of galactosyl donor additionally reduces the degree of self-galactosylation of the donor, i.e. when the galactosyl group of a first galactosyl donor is transferred to a second galactosyl donor instead of to a galactosyl acceptor.

In some preferred embodiments of the invention, step c) comprises addition of further galactosyl donor to the mixture. This is particularly preferred when a relatively low concentration of the galactosyl donor is used. By adding more galactosyl donor one avoids the galactosyl donor being depleted in the mixture and the concentration of galactosyl donor may be controlled during the enzymatic reaction.

The addition of further galactosyl donor may involve discrete addition(s) of galactosyl donor, e.g. at least once during the enzymatic reaction. Alternatively, or additionally, the addition of further galactosyl donor may be a continuous addition during the enzymatic reaction. The further galactosyl donor is preferably of the same type as used in step a).

Step c) may for example involve measuring the concentration of galactosyl donor during incubation and adding extra galactosyl donor if the concentration is too low.

In some preferred embodiments of the invention, the concentration of galactosyl donor of the mixture during step c) is maintained at a concentration in the range of 0.01-1 mol/L, preferably in the range of 0.01-0.5 mol/L, and preferably in the range of 0.03-0.3 mol/L.

For example, the concentration of galactosyl donor of the mixture during step c) may be maintained at a concentration in the range of 0.02-0.1 mol/L.

Step c) may furthermore comprise addition of further galactosyl acceptor. This makes it possible to control the concentration of galactosyl acceptor of the

mixture during step c) and e.g. to keep the galactosyl acceptor concentration substantially constant if this is desired.

5 In order to produce significant amounts of galacto-oligosaccharides, which contain two or three transferred galactosyl groups, the process should consume more galactosyl donor than galactosyl acceptor. Thereby more of the galactosyl acceptors will become galactosylated two or three times. Thus, in some preferred embodiments of the invention the molar ratio between the consumed galactosyl donor and the consumed galactosyl acceptor is at least 1:1, and preferably at
10 least 5:1, and even more preferably at least 10:1.

Step c) typically ends by inactivating the enzymes e.g. by denaturing the first enzyme by heat treatment or by breaking the contact between the first enzyme and the carbohydrates of the incubated mixture. If the first enzyme an enzyme
15 immobilised to a solid phase, the contact can e.g. be broken by separating the solid phase and the incubated mixture. If the first enzyme has been dissolved in the mixture, the first enzyme can be separated from the incubated mixture by ultrafiltration using a filter which retains the first enzyme but allows for the passage of oligo-saccharides.

20

Often it is required to enrich and/or purify the galacto-oligosaccharides of the composition and reduce the concentration of the galactosyl acceptor, the galactosyl donor and the released leaving group.

25 Thus, in some preferred embodiments of the invention the method furthermore comprises the step:

d) enriching the galacto-oligosaccharides of the composition of step c).

30 In the context of the present invention, the term "enriching the galacto-oligosaccharides" relates to increasing the relative amount of the galacto-oligosaccharides of the composition on a dry weight basis. This is typically done by removing some of the other solids of the composition, e.g. the lower saccharides, and optionally also the first enzyme, if required.

35

The enrichment of step d) may for example involve chromatographic separation and/or nanofiltration. Details regarding such processes are described in Walstra *et al.* (2006) which is incorporated herein by reference for all purposes.

5 In some embodiments of the invention the enrichment involves that at least 50% (w/w on dry weight basis) of the molecules having a molar weight of at most 200 g/mol are removed from the composition of step c). For example, the enrichment may involve that at least 80% (w/w on dry weight basis) of the molecules having
10 c).

In other embodiments of the invention the enrichment involves that at least 50% (w/w on dry weight basis) of the molecules having a molar weight of at most 350 g/mol are removed from the composition of step c). For example, the enrichment
15 may involve that at least 80% (w/w on dry weight basis) of the molecules having a molar weight of at most 350 g/mol are removed from the composition of step c).

As an alternative, or in addition, to the enrichment it may be preferred that step
20 d) comprises one or more processes which increase the concentration of the galacto-oligosaccharides in the composition. Examples of useful concentration steps are e.g. reverse osmosis, evaporation, and/or spray-drying.

Step d) may furthermore involve removing removal agent and/or converted
25 leaving groups bound to the removal agent from the composition of step c). Such removal may e.g. be performed by filtration, sedimentation, or centrifugation.

The galacto-oligosaccharide-containing composition provided by the method may for example be in the form of a dry powder or in the form of a syrup.
30

The production of a dry powder typically requires one or more process steps, such as concentrating, evaporating, and/or spray-drying. Thus, in some preferred embodiments of the invention step d) furthermore involves concentrating, evaporating, and/or spray-drying the composition in liquid form to obtain the
35 composition in powder form. It is particularly preferred to spray-dry the liquid composition of step d) to obtain a powdered composition. Step d) may for

example comprise the enrichment step followed by concentration step, e.g. nanofiltration, reverse osmosis, or evaporation, followed by a spray-drying step. Alternatively, step d) may comprise the concentration step followed by an enrichment step, followed by a spray-drying step. Concentrating the galacto-
5 oligosaccharides of the composition prior to the enrichment may make the subsequent enrichment process more cost-efficient.

Efficient spray-drying may require addition of one or more auxiliary agent(s), such as maltodextrin, milk protein, caseinate, whey protein concentrate, and/or
10 skimmed-milk powder.

The present process may e.g. be implemented as a batch process. The present process may alternatively be implemented as a fed-batch process. The present process may alternatively be implemented as a continuous process.

15 The present process may furthermore involve recirculation of first enzyme and/or unused galactosyl acceptor back to the mixture. The recirculation may e.g. form part of step d). For example, step d) may involve separating galactosyl acceptor and/or the first enzyme from the galacto-oligosaccharide-containing composition and recirculating galactosyl acceptor and/or first enzyme to step a) or c). In the
20 case of a batch process or a fed-batch process, the galactosyl acceptor and/or the first enzyme may be recirculated to the mixture of the next batch.

In the case of a continuous process, the galactosyl acceptor may be recirculated
25 back to part of the process line corresponding to step a) or step c). The first enzyme may be recirculated back to part of the process line corresponding to step b) or step c).

It should be noted that the details and features related to steps a) and b) need
30 not relate to the actual start of a production process but should at least occur sometime during the process. However, in some embodiments of the invention the concentration of the galactosyl donor is kept within the range described in step a) during the entire duration of step c).

35 If the method is implemented as a batch or feed batch process, step a) preferably pertains to the composition of the mixture when the synthesis starts. If the

method is a continuous process, step a) preferably pertains to the composition of the mixture during the synthesis under steady-state operation.

It may be perceived as desirable that the level of galactosylated galactosyl donor is kept as low as possible, as galactosylated galactosyl donor may be perceived as an undesired impurity, which is tricky to separate from the galactosylated galactosyl acceptor. In some preferred embodiments of the invention, the incubating mixture of step c) contains at most 0.5 mol/L galactosylated galactosyl donor. The incubating mixture of step c) may for example contain at most 0.1 mol/L galactosylated galactosyl donor. Even more preferably the incubating mixture of step c) contains at most 0.01 mol/L galactosylated galactosyl donor, and preferably substantially no galactosylated galactosyl donor.

In some preferred embodiments of the invention where the donor is lactose, the total concentration of allo-lactose and galactosylated allo-lactose is kept as low as possible, as these compounds also are perceived as an undesired impurities, which is difficult to separate from the galactosylated galactosyl acceptor.

In some preferred embodiments of the invention, the incubating mixture of step c) contains a total amount of allo-lactose and galactosylated allo-lactose of at most 0.5 mol/L. The incubating mixture of step c) may for example contain a total amount of allo-lactose and galactosylated allo-lactose of at most 0.1 mol/L. Even more preferably the incubating mixture of step c) contains a total amount of allo-lactose and galactosylated allo-lactose of at most 0.01 mol/L, and preferably substantially no allo-lactose and galactosylated allo-lactose at all.

Yet an aspect of the invention relates to a composition comprising galacto-oligosaccharides, which composition is obtainable by the method as defined herein.

A further aspect of the invention is a galacto-oligosaccharide-containing composition, e.g. the above-mentioned composition, said galacto-oligosaccharide-containing composition comprising:

- a first galacto-oligosaccharide having the general formula Gal-X,

- a second galacto-oligosaccharide having the stoichiometric formula $(\text{Gal})_2\text{X}$, such as e.g. the general formula Gal-Gal-X,
 - a third galacto-oligosaccharide having the stoichiometric formula $(\text{Gal})_3\text{X}$, such as e.g. the general formula Gal-Gal-Gal-X, and
- 5 wherein X is a glycosyl group, which is not lactosyl or glucosyl.

The galacto-oligosaccharide-containing composition described herein may for example be a food ingredient.

10

As described above, "X" or "-X" is preferably a glycosyl group of one of the galactosyl acceptors mentioned herein.

15

In some embodiments of the invention "X" or "-X" is a glycosyl group of a monosaccharide, which is not glucose. In other embodiments of the invention "-X" is a glycosyl group of a disaccharide, which is not lactose.

20

In some preferred embodiments of the invention "X" or "-X" is a fucosyl group. In other preferred embodiments of the invention "X" or "-X" is a galactosyl group.

In some preferred embodiments of the invention the galacto-oligosaccharide-containing composition has a molar ratio between:

25

- the total amount of the galacto-oligosaccharides Gal-X, $(\text{Gal})_2\text{X}$, and $(\text{Gal})_3\text{X}$, and
- the total amount of the galacto-oligosaccharides Gal-Glc, Gal_2Glc , Gal_3Glc

30

of at least 5:95. For example, the above-mentioned molar ratio may be at least 1:4, preferably at least 1:1, and even more preferably at least 2:1. It may even be preferred that the above-mentioned molar ratio is at least 5:1, preferably at least 10:1, and even more preferably at least 20:1.

In other preferred embodiments of the invention the galacto-oligosaccharide-containing composition has a molar ratio between:

35

- the total amount of the galacto-oligosaccharides Gal-X, Gal-Gal-X, and Gal-Gal-Gal-X, and

- the total amount of the galacto-oligosaccharides Gal-Glc, Gal-Gal-Glc, Gal-Gal-Gal-Glc
of at least 5:95. For example, the above-mentioned molar ratio may be at least
1:4, preferably at least 1:1, and even more preferably at least 2:1. It may even
5 be preferred that the above-mentioned molar ratio is at least 5:1, preferably at
least 10:1, and even more preferably at least 20:1.

It is even possible that the galacto-oligosaccharide-containing composition does
not contain any galacto-oligosaccharides of the formula Gal-Glc, Gal-Gal-Glc, and
10 Gal-Gal-Gal-Glc at all.

In some embodiments of the invention the galacto-oligosaccharide-containing
composition has a molar ratio between the first galacto-oligosaccharide, the
second galacto-oligosaccharide, and the third galacto-oligosaccharide in the range
15 of 50-99 : 1-45 : 0.5-25.

In other embodiments of the invention the galacto-oligosaccharide-containing
composition has a molar ratio between the first galacto-oligosaccharide, the
second galacto-oligosaccharide, and the third galacto-oligosaccharide in the range
20 of 20-45 : 20-45: 20-45.

In further embodiments of the invention the galacto-oligosaccharide-containing
composition has a molar ratio between the first galacto-oligosaccharide, the
second galacto-oligosaccharide, and the third galacto-oligosaccharide in the range
25 of 0.5-25 : 1-45 : 50-98.

In some preferred embodiments of the invention the galacto-oligosaccharide-
containing composition comprises a total amount of the first galacto-
oligosaccharide, second galacto-oligosaccharide, and third galacto-oligosaccharide
30 of at least 10% by weight relative to the total weight of the galacto-
oligosaccharide-containing composition. For example, the galacto-oligosaccharide-
containing composition may comprise a total amount of the first galacto-
oligosaccharide, second galacto-oligosaccharide, and third galacto-oligosaccharide
of at least 20% by weight relative to the total weight of the galacto-
35 oligosaccharide-containing composition, preferably at least 30% by weight, even

more preferably at least 40% relative to the total weight of the galacto-oligosaccharide-containing composition.

Even higher levels of the first, second, and third galacto-oligosaccharides may be preferred. Thus, in some preferred embodiments of the invention the galacto-oligosaccharide-containing composition comprises a total amount of the first galacto-oligosaccharide, second galacto-oligosaccharide, and third galacto-oligosaccharide of at least 50% by weight relative to the total weight of the galacto-oligosaccharide-containing composition. For example, the galacto-oligosaccharide-containing composition may comprise a total amount of the first galacto-oligosaccharide, second galacto-oligosaccharide, and third galacto-oligosaccharide of at least 60% by weight relative to the total weight of the galacto-oligosaccharide-containing composition, preferably least 70% by weight, even more preferably at least 80% relative to the total weight of the galacto-oligosaccharide-containing composition.

Yet an aspect of the invention relates to a food product comprising the galacto-oligosaccharide-containing composition described herein.

In some embodiments of the invention the food product is a functional food product such as infant formula or a product for clinical nutrition.

In other embodiments of the invention the food product is a baked product, e.g. comprising baked dough, such as bread or similar products.

In further embodiments of the invention the food product is a dairy product, e.g. a fresh dairy product such as milk, or a fermented dairy product such as yoghurt.

In still further embodiments of the invention the food product is a pet food product.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

The invention will now be described in further details in the following non-limiting

examples.

EXAMPLES

Example 1: Preparation of a beta-galactosidase having transgalactosylating activity

A working volume of 750 mL fermentation medium was inoculated with a 2 mL starter-culture of Lysogeny broth (LB) medium with 100 mg/L ampicillin with an OD₆₀₀ of 3.0 grown for 12 hours. The fermentation was performed in EC medium containing 2 % (w/v) yeast extract, 2 % (w/v) soy peptone, 1 % (w/v) glucose and 100 mg/L ampicillin. The *E. coli* strain expressing OLGA347 β -galactosidase (having the sequence Val (33) – Ile (1174) of SEQ ID NO 2) was prepared as described earlier (Jørgensen *et al.*, US patent No. 6,555,348 B2, Examples 1 and 2). The fermentor was from Applikon with glass dished bottom vessels with a total volume of 2 L and equipped with two Rushton impellers. During the fermentation, pH was maintained at pH 6.5 by appropriate addition of 2 M NaOH and 2 M H₃PO₄ and temperature was controlled at 37 degrees C. Oxygen was supplied by bubbling with air at a rate of 1-2 L/min, and pO₂ was maintained at 30% by increasing the agitation rate. Growth was followed by off-line OD₆₀₀ readings. The culture was harvested by centrifugation after approximately 10 h of growth at an OD₆₀₀ value of 29.7. The 650 mL culture supernatant was stored at -20 degrees C. The periplasmic proteins were isolated from the cell pellet by osmotic shock by resuspending the cell pellet in 200 mL sucrose buffer (30 mM Tris-HCl, 40% sucrose, 2 mM EDTA, pH 7.5) and incubating for 10 min at room temperature. After centrifugation, the supernatant was discarded and the pellet resuspended in 200 mL of cold water. 83 μ L of a saturated MgCl₂ solution was added, and the supernatant containing the periplasmic proteins were collected by a centrifugation step. The periplasmic fraction was filter sterilized through a 0.2 μ m Millipak 40 filter and stored at -20 degrees C.

The β -galactosidase activity of the 200 mL periplasmic fraction and the 650 mL culture supernatant was determined using *o*-nitrophenyl- β -D-galactopyranoside (OPNG) as a substrate according to protocol (J. Sambrook and D.W. Russell, Molecular Cloning – A laboratory manual, 3rd edition (2001), pp. 17.48-17.51).

The majority of the activity was found in the periplasmic fraction (525 units, corresponding to 98%).

Example 2: Preparation of a second beta-galactosidase having transgalactosylating activity

A second beta-galactosidase (OLGA917) was prepared along the lines described in Example 1 but based on the expression of the amino acid sequence Val (33) – Glu (917) of SEQ ID NO. 2.

Example 3: Determination of the T-value of a beta-galactosidase enzyme

The T-value of a beta-galactosidase enzyme is determined according to the assay and formula given below.

Assay:

Prepare 3.3 mL enzyme solution consisting of the beta-galactosidase enzyme to be tested, 10 mM sodium citrate, 1 mM magnesium citrate, 1 mM calcium-citrate, Milli-Q water (Millipore, USA), and having a pH of 6.5. The enzyme solution should contain the beta-galactosidase enzyme in an amount sufficient to use 33% (w/w) of the added lactose in 1 hour under the present assay condition. The temperature of the enzyme solution should be 37 degrees C.

At time = T_0 82.5 mg lactose monohydrate (for biochemistry, Merck Germany) is added to and mixed with the enzyme solution, and the mixture is subsequently incubated at 37 degrees C for 4 hours. Precisely 1 hour after T_0 a 100 μ L sample is collected and is diluted 1:5 with Milli-Q water and inactivated by heating to 85 °C for 10 min. The inactivated mixture is kept at -20 degrees C until the characterization.

Characterisation:

The determination of the amount (in mol) of produced galactose and the amount of used lactose (in mol) may be performed using any suitable analysis technique. For example, the diluted mixture may be analyzed by HPLC according to the

method described by Richmond *et al.* (1982) and Simms *et al.* (1994). Other useful analysis techniques are described in El Razzi (2002).

- Another example of a suitable analysis technique is ISO 5765-2:2002 (IDF 79-2: 2002) "Dried milk, dried ice-mixes and processed cheese – Determination of lactose content – Part 2: Enzymatic method utilizing the galactose moiety of the lactose".

Calculation of the T-value:

- 10 The T-value is calculated according to the following formula using the data obtained from the characterization of the diluted mixture of the assay:

$$\text{T-value} = \frac{\text{amount of produced galactose (in mol)}}{\text{amount of used lactose (in mol)}}$$

Example - T-value of the OLGA347 enzyme:

- 15 The above-mentioned assay was performed using the OLGA347 enzyme of Example 1.

- The diluted mixture obtained from the assay was analyzed with respect to converted (i.e. used) lactose and generated galactose via analytical HPLC. The HPLC apparatus was from Waters and equipped with a differential refractometer (RI-detector) and a BioRad Aminex HPX-87C column (300x7.8 mm, 125-0055). Elution of saccharides was performed isocratically with 0.05 g/L CaAcetate, a flow rate of 0.3 mL/min. and an injection volume of 20 µL.

- 25 The obtained data was appropriately baseline corrected by automated software, peaks were individually identified and integrated. Quantification was performed by using external standards of lactose monohydrate (for biochemistry, Merck, Germany), D-(+)-glucose monohydrate (for biochemistry, Merck Eurolab, France), and D-(+)-galactose (≥99%, Sigma-Aldrich, Italy).

30

The conversion of lactose and the formation of galactose to each time T was calculated from the quantified data. At time T=1h 29% of the lactose in the collected 100 µL sample had been converted, which corresponds to 2.3 µmol

lactose. At time $T=1$ 0.5 μmol galactose had been formed in the collected 100 μL sample. The T-value can therefore be calculated to $0.5 \mu\text{mol} / 2.3 \mu\text{mol} = 0.2$.

The diluted mixture obtained from the assay was also analyzed with respect to converted (i.e. used) lactose and generated galactose via the enzymatic method ISO 5765-2. A Boehringer Mannheim Lactose/D-Galactose test-kit from R-Biopharm (Cat. No. 10 176 303 035) was used and the test performed according to protocol. The enzymatic method confirmed a T-value of the OLGA347-enzyme of 0.2.

Example - T-value of the OLGA917 enzyme:

The above-mentioned T-value assay was performed using the OLGA917 enzyme produced in Example 2 and the T-value was determined following the procedure explained above.

The T-value of the OLGA917 enzyme was determined to 0.3.

Example - T-value of conventional lactase enzyme:

The above-mentioned assay was performed using the commercially available conventional lactase enzyme Lactozym Pure 2600L (Novozymes, Denmark). The diluted mixture obtained from the assay was analyzed as described for the OLGA347 enzyme. Tri- and tetra-saccharides were not present in detectable amounts and equal amounts of glucose and galactose were seen. The corresponding T-value is 1.

The T-values of commercially available beta-galactosidase from *Escherichia coli* (Product number: G6008, Sigma-Aldrich, Germany) and *Aspergillus oryzae* (Product number: G5160, Sigma-Aldrich, Germany) have also been determined, and both enzymes have a T-value of approx. 1.0.

Example 4: Synthesis of L-fucosyl-containing hetero-galacto-oligosaccharides by sequential addition of donor molecules and conversion of glucose leaving groups

L-(-)-Fucose and lactose monohydrate in amounts as found in Table 7 were dissolved in 100 mL MilliQ water. Each sample was maintained at a temperature of 37 degrees C and pH is maintained at 6.5 by continuous addition of NaOH. Air

- was continuously pumped through the mixture at a rate of 100 L/min. Glucose Oxidase enzyme, GOX, DuPont, Denmark, was added to sample 2. Glucose Oxidase enzyme, Gluzyme, Novozymes, Denmark, was added to sample 3. Catalase enzyme, from *Micrococcus lysodeikticus*, Sigma-Aldrich, Denmark, was added to sample 2 and 3. OLGA917 enzyme of Example 2 was added to each sample. MilliQ water was added to a total process volume of 250 mL. This time was defined as T=0. Lactose monohydrate was added to the samples over a 5 hour period in 30 min. intervals.

10 Table 7:

Sample	1 (Control)	2	3
Start concentration of L-Fucose	10 %	10 %	10 %
Start concentration of lactose monohydrate	5 %	5%	5%
Addition of lactose / 30 min.	2.63 g	2.63 g	2.63 g
Amount of Glucose oxidase	-	100.45 mg	110.50 mg
Specific activity of Glucose oxidase	-	11,000 U/g	10,000 U/g
Amount of catalase	-	20 µL	20 µL
Specific activity of catalase	-	170,000 U/mL	170,000 U/mL
Amount of OLGA enzyme	5 mL	5 mL	5 mL

Test samples from T=4 h and T= 5h were analysed by HPLC according to Example 7 and were found to contain significant amounts of galactosylated fucose.

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Example 5: Synthesis of L-fucosyl-containing hetero-galacto-oligosaccharides and conversion and removal of glucose leaving groups but without sequential addition of donor molecules

- 10 L-(-)-Fucose and lactose monohydrate in amounts as found in Table 8 were dissolved in 100 mL MilliQ water. Each sample was maintained at a temperature of 37 degrees C and the pH was maintained at 6.5 by continuous addition of NaOH. Air was continuously pumped through the mixture at a rate of 100 L/min. Anion exchange resin, Dowex 66, Sigma-Aldrich, USA was prepared and added to
- 15 sample 3. Glucose Oxidase enzyme, GOX, DuPont, Denmark, was added to sample 2 and 3. Catalase enzyme, from *Micrococcus lysodeikticus*, Sigma-Aldrich, Denmark, was added to sample 2 and 3. OLGA917 enzyme of Example 2 was added to each sample. MilliQ water was added to a total process volume as found in Table 8. This time was defined as T=0.

20

Table 8:

Sample	1 (Control)	2	3
Start concentration of L-Fucose	10 %	10 %	10 %
Start concentration of lactose monohydrate	15 %	15%	15%
Amount of Glucose oxidase	-	100.45 mg	100.45 mg
Specific activity of Glucose	-	11,000 U/g	11,000 U/g

oxidase			
Amount of catalase	-	20 µL	20 µL
Specific activity of catalase	-	170,000 U/mL	170,000 U/mL
Volume of ion exchange resin	-	-	40 mL
Weak base capacity	-	-	1.35 meq/mL
Amount of OLGA enzyme	5 mL	5 mL	5 mL
Total process volume	250 mL	250 mL	250 mL

Test samples from T=4 h and T= 5h were analysed by HPLC according to Example 7 and were also found to contain significant amounts of galactosylated fucose.

5 Example 6: Synthesis of L-fucosyl-containing hetero-galacto-oligosaccharides by sequential addition of donor molecules, conversion of glucose leaving groups, and removal of converted leaving groups

10 L-(-)-Fucose and lactose monohydrate in amounts as found in Table 9 were dissolved in 100 mL MilliQ water. Each sample was maintained at a temperature of 37 degrees C and the pH was maintained at 6.5 by continuous addition of NaOH. Air was continuously pumped through the mixture at a rate of 100 L/min. Anion exchange resin, Dowex 66, Sigma-Aldrich, USA was prepared and added to each sample. Glucose Oxidase enzyme, GOX, Danisco/DuPont, Denmark, was
15 added to sample 2. Glucose Oxidase enzyme, Gluzyme, Novozymes, Denmark, was added to sample 3. Catalase enzyme, from *Micrococcus lysodeikticus*, Sigma-Aldrich, Denmark, was added to sample 2 and 3. OLGA917 enzyme of Example 2 was added to each sample. MilliQ water was added to a total process volume of 250 mL. This time was defined as T=0. Lactose monohydrate was added to the
20 samples over a 5 hour period in 30 min. intervals.

Table 9:

Sample	1 (Control)	2	3
Start concentration of L-Fucose	10 %	10 %	10 %
Start concentration of lactose monohydrate	5 %	5%	5%
Addition of lactose / 30 min.	1,06 g	1,06 g	1,06 g
Amount of Glucose oxidase	-	40 mg	45 mg
Specific activity of Glucose oxidase	-	11,000 U/g	10,000 U/g
Amount of catalase	-	10 µL	10 µL
Specific activity of catalase	-	170,000 U/mL	170,000 U/mL
Volume of ion exchange resin	40 mL	40 mL	40 mL
Weak base capacity	1.35 meq/mL	1.35 meq/mL	1.35 meq/mL
Amount of OLGA enzyme	2 mL	2 mL	2 mL

At times T=4 and 5 h 1500 µL test samples were taken and analysed by HPLC and
5 Mass Spectroscopy according to Example 7. The samples were found to contain

significant amounts of galactosylated fucose. The results of the Mass Spectroscopy analysis is illustrated in Figures 4-6.

Figure 4 shows a plot of the integrated response of di-, tri-, and tetrasaccharide of galactosylated fucose after 4 hours of incubation - with and without removal of free leaving groups during the incubation. The free leaving groups (glucose) are removed by means of enzymatic conversion. It is seen that the content of galactosylated fucose increases when the free leaving groups are removed during incubation.

10

Figure 5 shows a plot of the integrated response of di-, tri-, and tetrasaccharide of galactosylated fucose after 5 hours of incubation - with and without removal of free leaving groups during the incubation. Again, it is seen that the content of galactosylated fucose increases when the free leaving groups are removed during incubation.

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Figure 6 show a plot of the total integrated response of galactosylated fucose (total HOS) compared to the total integrated response of galactosylated donor/leaving group (total GOS) - with and without removal of free leaving groups during the incubation. The total integrated response of galactosylated donor/leaving group is the sum of the integrated responses of Gal-Glc, Gal-Gal-Glc, and Gal-Gal-Gal-Glc molecules. The total integrated response of galactosylated fucose is the sum of the responses of Gal-Fuc, Gal-Gal-Fuc, and Gal-Gal-Gal-Fuc molecules. It is seen that the total integrated response of galactosylated fucose increases significantly when the free leaving groups are removed during incubation while the total integrated response of galactosylated donor/leaving group is almost unchanged.

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Conclusion:

This example demonstrates that the ratio between the galactosylated acceptor (the intended product) and galactosylated donor/leaving groups (by-product) is increased significantly when the free leaving groups are removed during incubation.

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Example 7: Characterisation of samples containing L-fucosyl-containing hetero-galacto-oligosaccharides

Collected samples were diluted 1:10 with Milli-Q water and inactivated by heating
5 to 85 °C for 15 min. The inactivated mixture was kept at –20 degrees C until the characterization.

Samples were characterized by use of analytical HPLC. The samples were filtered
using a 0.22 µm filter. The HPLC apparatus was from Waters and equipped with a
10 differential refractometer (RI-detector) and a BioRad Aminex HPX-87C column
(300x7.8 mm, 125-0055). Elution of saccharides was performed isocratically with
0.05 g/L CaAcetate, a flow rate of 0.3 mL/min. and an injection volume of 20 µL.

Mass spectrometry analysis was performed with an Agilent 1200 API-ES LC/MSD
15 Quadropole 6410 scanning masses between 100 and 1000 amu (gas temperature:
350 °C, drying gas flow: 13.0 L/min, nebulizer pressure: 40 psig). The column
used for LC separation is a HyperCarb 2.1x150 mm from Thermo Scientific,
Denmark, running at 25 degrees Celcius. Elution was performed with 5 mM AcNH4
aqueous solution and acetonitrile.

20 Ion chromatograms were extracted based on the common ionization patterns from
the electrospray interfase. The peaks in the extracted chromatograms were
evaluated based on the mass spectra and subsequently integrated, thus providing
integrated responses for various carbohydrate species of the test sample.

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CLAIMS

1. A method of producing a composition comprising one or more galacto-
5 oligosaccharide(s), the method comprising the steps of:
- a) providing a mixture comprising
- a galactosyl donor comprising a galactosyl group bound to a leaving
group, which galactosyl donor has a molar weight of at most 350 g/mol,
 - 10 - a galactosyl acceptor which is different from the galactosyl donor,
said galactosyl acceptor is a saccharide or a sugar-alcohol, and
- wherein the molar ratio between the galactosyl acceptor and the galactosyl donor
is at least 1:10, and wherein the mixture comprises at least 0.05 mol/L of the
galactosyl acceptor,
- 15 b) providing a first enzyme, said first enzyme having beta-galactosidase
activity and transgalactosylating activity, said first enzyme contacting the mixture,
and
- 20 c) incubating the mixture and the first enzyme, thereby allowing the first enzyme
to release the leaving group of the galactosyl donor and transfer the galactosyl
group of the galactosyl donor to the galactosyl acceptor, thus forming the galacto-
oligosaccharide, step c) furthermore comprising removing from the incubating
mixture a leaving group released from the galactosyl donor, thereby obtaining the
25 composition comprising the one or more galacto-oligosaccharide(s).
2. The method according to claim 1, wherein the leaving group of the galactosyl
donor is a glycosyl group.
- 30 3. The method according to claim 1 or 2, wherein the galactosyl donor is lactose
or lactitol.
4. The method according to any of the preceding claims, wherein the first enzyme
35 has a T-value of at most 0.9.

5. The method according to any of the preceding claims, wherein the first enzyme comprises:

- 5 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence of SEQ ID NO. 2, or
- an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Met (1) to Ile (1174) of SEQ ID NO. 2, or
- 10 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Gly (950) of SEQ ID NO. 2, or
- an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2, or
- 15 - an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2.

6. The method according to any of the preceding claims, wherein the first enzyme
20 comprises an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Ile (1174) of SEQ ID NO. 2.

7. The method according to any of the preceding claims, wherein the first enzyme
25 comprises an amino acid sequence having a sequence identity of at least 80% relative to the amino acid sequence Val (33) to Glu (917) of SEQ ID NO. 2.

8. The method according to any of the preceding claims, wherein the first enzyme has:

- 30 - a sequence identity of at least 99% relative to an amino acid sequence shown in Table 1, or
- a sequence identity of at least 99% relative to an amino acid sequence shown in Table 2, or
- a sequence identity of at least 99% relative to an amino acid sequence
35 shown in Table 3, or

- a sequence identity of at least 99% relative to an amino acid sequence shown in Table 4, or
- a sequence identity of at least 99% relative to an amino acid sequence shown in Table 5, or
- 5 - a sequence identity of at least 99% relative to an amino acid sequence shown in Table 6.

9. The method according to any of the preceding claims, furthermore comprising
10 providing a microorganism which is capable of converting free leaving groups released from the galactosyl donor, and allowing said microorganism, during incubation, to remove a leaving group released from the galactosyl donor.

10. The method according to claim 9, wherein
- 15 a) the removal rate of the microorganism relative to the free leaving group is at least 10 times higher than its removal rate relative to the galactosyl acceptor,
 - b) the removal rate of the microorganism relative to the free leaving
20 group is at least 10 times higher than its removal rate relative to the galactosyl donor, or
 - c) the removal rate of the microorganism relative to the free leaving
25 group is at least 10 times higher than its removal rate relative to mono-galactosylated galactosyl acceptor.

11. The method according to any of the preceding claims, furthermore comprising
providing a second enzyme which is capable of converting free leaving groups
released from the galactosyl donor, and allowing said second enzyme, during
30 incubation, to convert a leaving group released from the galactosyl donor.

12. The method according to claim 11, wherein the second enzyme has glucose
oxidase activity.
35

13. The method according to any of the claims 11-12, wherein
- a) the specificity constant of the second enzyme relative to the free leaving group is at least 10 times higher than its specificity constant relative to the galactosyl acceptor,
 - 5 b) the specificity constant of the second enzyme relative to the free leaving group is at least 10 times higher than its specificity constant relative to the galactosyl donor, or
 - 10 c) the specificity constant of the second enzyme relative to the free leaving group is at least 10 times higher than its specificity constant relative to the mono-galactosylated galactosyl acceptor.
- 15 14. The method according to any of the claims 11-13, wherein the second enzyme has glucose oxidase activity and the leaving group of the galactosyl donor is glucose.
- 20 15. The method according to any of the claims 12-14, wherein the method furthermore comprises providing an enzyme having catalase activity which enzyme contacts the incubating mixture.
- 25 16. The method according to any of the claims 11-15, wherein the method furthermore comprises providing a removal agent capable of removing at least some of the conversion product obtained by converting the leaving group with the second enzyme, and allowing the removal agent, during the incubation, to remove at least some of the conversion product.
- 30 17. The method according to claim 16, wherein the removal agent comprises a salt of a divalent or trivalent metal ion.
18. The method according to claim 13, wherein the removal agent comprises, or even consists of, an anion exchange material.

19. The method according to claim 18, wherein the total binding capacity of the anion exchange material is at least 30% (mol/mol) relative to the total amount of conversion product produced during the incubation.

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20. The method according to any of the preceding claims, wherein step c) comprises addition of further galactosyl donor.

21. The method according to any of the preceding claims, wherein the
10 concentration of galactosyl donor of the mixture during step c) is maintained at a concentration in the range of 0.01-1 mol/L.

22. The method according to any of the preceding claims furthermore comprising the step:

15

d) enriching the galacto-oligosaccharide of the composition of step c).

23. The method according to claim 22, wherein the step d) furthermore involves
20 removing removal agent and/or converted leaving groups bound to the removal agent from the composition of step c).