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(54) Title: CRYSTALLISATION OF HUMAN MILK OLIGOSACCHARIDES (HMO)

(57) Abstract: The present invention relates to a method of obtaining a non-acidic human milk oligosaccharide (HMO) or a precursor in crystalline form from an aqueous medium, such as a fermentation broth, by drying the aqueous medium and then treating it with a hot C_1-C_6 alcohol solvent.

CRYSTALLISATION OF HUMAN MILK OLIGOSACCHARIDES (HMO)

FIELD OF THE INVENTION

The present invention relates to a method of crystallizing non-acidic human milk oligosaccharides (HMOs) or precursors thereof from aqueous media, particularly as produced by fermentation.

BACKGROUND OF THE INVENTION

Human milk oligosaccharides (HMOs) have become of great interest in the past few years due to their important functions in human development. To date, the structures of at least 115 HMOs have been determined (see Urashima et al.: *Milk Oligosaccharides*, Nova Biomedical Books, New York, 2011, ISBN: 978-1-61122-831-1), and considerably more are probably present in human milk. The following thirteen core structures have been identified, to date, for the 115 HMOs:

No	Core name	Core structure
1	lactose (Lac)	Galβ1-4Glc
2	lacto-N-tetraose (LNT)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
3	lacto-N-neotetraose (LNnT)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
4	lacto-N-hexaose (LNH)	Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
5	lacto-N-neohexaose (LNnH)	Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
6	para-lacto-N-hexaose (para-LNH)	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
7	para-lacto-N-neohexaose (para-LNnH)	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
8	lacto-N-octaose (LNO)	Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
9	lacto-N-neooctaose (LNnO)	Galβ1-4GlcNAcβ1-3(Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
10	Iso-lacto-N-octaose (iso-LNO)	Galβ1-3GlcNAcβ1-3(Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
11	para-lacto-N-octaose (para-LNO)	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
12	lacto-N-neodecaose (LNnD)	Galβ1-3GlcNAcβ1-3[Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-6]Galβ1-4Glc
13	lacto-N-decaose (LND)	Galβ1-3GlcNAcβ1-3[Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-6]Galβ1-4Glc

Low cost ways have been sought for making industrial quantities of as many as possible of the HMOs, so that their uses in nutritional and therapeutic formulations for infants, as well as as possibly children and adults, could be discovered, developed and exploited by researchers worldwide. A few HMOs have recently been synthesized in high purity and in high yields by lengthy sequences of chemical process steps, but even lower cost ways of producing them have continued to be sought. As a result, ways have been sought for synthesizing HMOs enzymatically or by fermentation of transformed one-cell organisms, particularly *E. coli*. See Drouillard et al. *Angew. Chem. Int. Ed.* **45**,1778 (2006) and Han et al. *Biotechnol. Adv.* **30**, 1268 (2012). However, the costs of producing HMOs in such ways have remained stubbornly high because it has been difficult and expensive to purify the HMOs in high yields from the aqueous enzymatic or fermentation media, in which they were produced.

If HMOs could be isolated in crystalline form from the aqueous enzymatic or fermentation media, in which the HMOs were produced, that would provide a simple and cheap method to separate the HMOs from contaminants in such aqueous media and obtain the HMOs as pure products. Indeed, purification and isolation by crystallization generally makes any process more attractive and cost-effective industrially. See EP-A-1405856. However, there has, to date, been no commercially suitable method for purifying and isolating, by crystallization, HMOs produced enzymatically or by fermentation.

Accordingly, it is an object of the present invention to provide a method for obtaining, in crystalline form, HMOs, as well as their precursors such as fucose, N-acetyl glucosamine, galactose, N-acetyl lactosamine, lacto-N-biose and lacto-N-triose II from aqueous mixtures thereof as produced by fermentation, particularly as produced extracellularly, as well as from aqueous solutions thereof as produced enzymatically.

SUMMARY OF THE INVENTION

A first aspect of this invention relates to a method of obtaining, in crystalline form, a non-acidic human milk oligosaccharide (or HMO) or a non-acidic precursor of an HMO produced in an aqueous media, enzymatically or by fermentation, particularly

produced intracellularly in an aqueous fermentation broth, quite particularly produced by *E. coli*, said method comprising the steps of:

a) drying, preferably freeze-drying, the aqueous media, preferably after removing proteins therefrom, to produce a dry powder that contains the non-acidic HMO or HMO precursor, preferably at a concentration of at least about 15 wt%, preferably at least about 25 wt%, especially at least about 30-40%, particularly at least about 40-60%, more particularly at least 95 wt%, even more particularly at least 97 wt%, and that preferably also contains no more than about 5% water, especially no more than about 3-4% water;

b) then treating the dry powder with a hot, preferably boiling, C_1-C_6 alcohol solvent, preferably a C_1-C_4 alcohol solvent, optionally containing up to 50 v/v%, preferably up to about 35 v/v% water, especially up to about 15 v/v%, particularly up to about 5 v/v%, to produce an alcohol solution of the non-acidic HMO or HMO precursor; and

c) then crystallizing the non-acidic HMO or HMO precursor from the alcohol solution.

Advantageously, the method also comprises, between steps b) and c), one or more, preferably all, of the following intermediate steps:

i) the alcohol solution is filtered to separate insolubles;

ii) then the alcohol solution is concentrated down to at least about 60-70% of its original volume, and

iii) then, the alcohol solution is seeded with crystals of the non-acidic HMO or HMO precursor.

A second aspect of this invention relates to a method of obtaining a non-acidic HMO or HMO precursor from a powder, preferably a protein-free powder, produced by drying, preferably freeze drying, an aqueous media, in which the non-acidic HMO or HMO precursor was produced enzymatically or by fermentation, particularly produced intracellularly in an aqueous fermentation broth, quite particularly produced

by *E. coli*, comprising steps b) and c) above, and advantageously also comprising intermediate steps i) to iii).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with this invention, the term "C_1-C_6 alcohol" preferably means a hydroxy- or dihydroxy-alkanol having 1 to 6 carbon atoms, such as methanol, ethanol, *n*-propanol, *i*-propanol, ethylene glycol, 1,2-propanediol, 1,3-propanediol, butanol and an i-hexanol, especially a "C_1-C_4 alcohol", such as methanol, ethanol, 1,2-propanediol, and butanol, particularly methanol.

Also in this invention, the term "non-acidic" preferably refers to a compound containing no carboxyl group, e.g., no sialic acid group.

Also in this invention, the term "non-acidic human milk oligosaccharide" or "non-acidic HMO" preferably means a compound with one of the following core structures or desialylated analogs thereof:

Abbreviation	Chemical Structure
2'-FL	Fuc(α1-2)Gal(β1-4)Glc
3-FL	Gal(β1-4)Glc | Fuc(α1-3)
DFL	Fuc(α1-2)Gal(β1-4)Glc | Fuc(α1-3)
3'-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
FSL	Neu5Ac(α2-3)Gal(β1-4)Glc | Fuc(α1-3)
LNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
LNFP I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
LNFP II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)

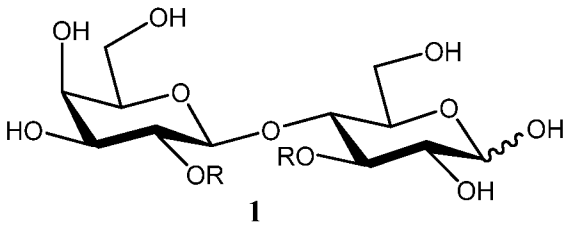
LNFP V	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
LNDFH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
LNDFH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-4) Fuc(α1-3)
LNDFH III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-3) Fuc(α1-3)
LSTa	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
LSTb	Neu5Ac(α2-6) | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
LSTc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
F-LSTa	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
F-LSTb	Neu5Ac(α2-6) | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
F-LSTc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
DS-LNT	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
FDS-LNT I	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
FDS-LNT II	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc

especially:

- LNnT and LNT,
- the monofucosyl LNTs, LNFP I, LNFP II and LNFP V,
- the difucosyl LNTs, LNDFH I and LNDFH II,
- the fucosyl lactose derivatives 2'-FL, 2',3-DFL, 3-FL, and
- the mono- and difucosyl LNnTs, LNFP III and LNDFH III.

Particularly preferred non-acidic HMOs include:

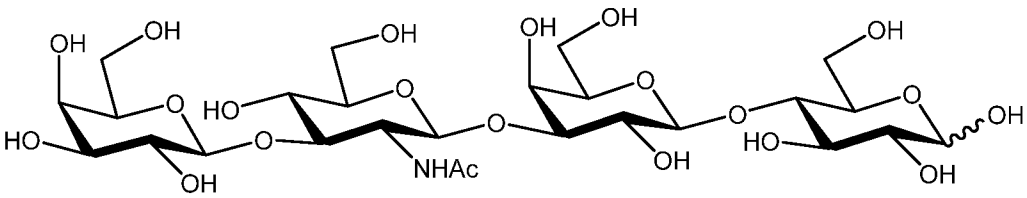
- a compound of formula **1**:



1

wherein R independently of each other is fucosyl or H, provided that at least one R is fucosyl,

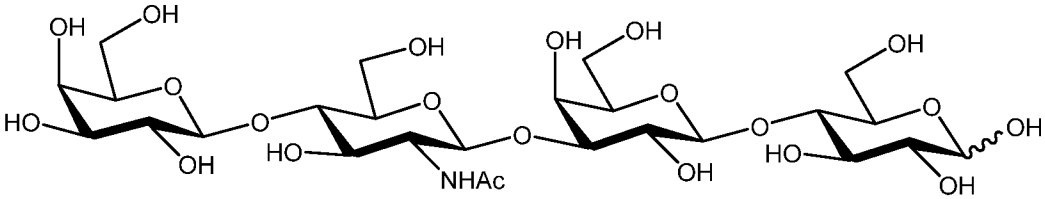
- a compound of formula **2** :



2

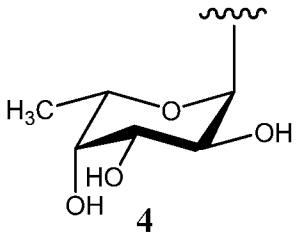
or

- a compound of formula **3**:



3

Further in this invention, the term "fucosyl" preferably means an L-fucopyranosyl group of formula **4** attached to the core compound with α-interglycosidic linkage:



4

Still further in this invention, the term "non-acidic precursor of a human milk oligosaccharide" or "non-acidic HMO precursor" preferably means fucose, N-acetyl glucosamine, galactose, N-acetyl lactosamine, lacto-N-biose or lacto-N-triose II.

Still further in this invention, the term "dry" or "dried" preferably means a water content of no more than about 10%, preferably 1-5%, particularly no more than 3-4%.

Still further in this invention, the term "hot" with regard to the alcohol solvent used in the method preferably means a temperature range from about 40-50 °C to the boiling point of that alcohol.

HMOs and their precursors can be produced in high yields, in aqueous media enzymatically of by fermentation of genetically transformed *E. coli*. See, for example, EP-A-1405856 and EP-A-1194584, Drouillard et al. *Agnew. Chem. Int. Ed.* **45**,1778 (2006) and Han et al. *Biotechnol. Adv*. **30**, 1268 (2012).

To separate a non-acidic HMO or HMO precursor in high yield as a pure crystalline material from an aqueous solution, in which it was made enzymatically, the aqueous media is preferably contacted with cationic and/or anionic ion exchange resins to remove proteins.

To separate a non-acidic HMO or HMO precursor, preferably a non-acidic HMO, in high yield as a pure crystalline material from an aqueous fermentation broth, in which it was made, the aqueous fermentation broth is preferably separated, preferably by centrifugation, from the fermented, preferably *E. coli,* cells and then proteins and salts are removed from the fermentation broth, preferably by contacting it with cationic and anionic ion exchange resins.

The resulting aqueous medium, produced enzymatically or by fermentation, is then dried to remove at least most of its water content by evaporation, azeotropic distillation, spray drying, freeze drying or a combination thereof, preferably by freeze drying. The dry powder, so-produced, contains no more than about 8-10 wt% water, preferably no more than about 5 wt% water, especially no more than 3-4 wt% water. For best results in carrying out the method of this invention, the aqueous medium, produced enzymatically or by fermentation, preferably contains at least about 10 g/l, especially at least 15-20 g/l, particularly at least about 30-40 g/l, quite particularly at least at least about 50 g/l, of the non-acidic HMO or HMO precursor, and the dry powder therefrom preferably contains the non-acidic HMO or HMO precursor at a concentration of at least about 15 wt%, preferably at least about 25 wt%, especially at least 30-40 wt%, particularly at least at least 50-60 wt%, more particularly at least 95 wt%, even more particularly at least 97 wt%.

In accordance with this invention, the dry, preferably protein-free powder is then treated with a hot, preferably boiling, C_1-C_6 alcohol solvent, preferably a C_1-C_4 alcohol solvent, especially methanol, to produce an alcohol solution of the non-acidic HMO or HMO precursor. In this regard, the alcohol solvent can, if desired, be heated, preferably to boiling, only after it is used to dissolve the non-acidic HMO or HMO precursor to produce the alcohol solution. The alcohol solvent preferably contains no water, but where water enhances the solubility differential between the desired product and fermentation by-products, the alcohol solvent can suitably contain up to about 50 v/v% water, preferably up to 30 v/v% water, especially no more than 15 v/v%, particularly no more than 5 v/v%. In this regard, the dry powder can be dissolved in water or aqueous alcohol before treating it with the alcohol solvent. Optionally, the alcohol solvent contains a filtration aid, such as activated carbon or diatomaceous earth, to be removed in a subsequent filtration step. The amount of alcohol solvent used is not critical, but preferably at least about 4-10 ml, especially about 5-7 ml, of alcohol are used per gram of the dry powder. Depending on the choice of solvent and its water content, if any, at least about 33 wt%, preferably at least 40-60 wt%, of the solids content of the dry powder become dissolved in the alcohol solution.

Preferably for certain non-acidic HMO or HMO precursors, the alcohol solution is then filtered, preferably while still hot, especially boiling hot, to remove insoluble fermentation by-products. Depending on the choice of solvent and its water content, if any, at least about 30 wt%, preferably about 40-60 wt%, especially about about 45-55 wt%, of the solids content of the dry powder are filtered from the alcohol solution. The hot alcohol solution, preferably after filtration, is then concentrated by partial evaporation down to at least about 60-70 % of its original volume, preferably down to about 40-60 %, especially down to about 45-55 %, of its original volume. The concentrated alcohol solution is then allowed to cool somewhat, preferably down to about 30-50 °C, especially about 35-45 °C. Seed crystals of the desired non-acidic HMO or HMO precursor can be added to the concentrated alcohol solution while it is still warm, preferably at about 30-50 °C, especially at about 35-45 °C. The concentrated alcohol solution is then allowed to cool, for example to room temperature, and precipitated crystals of the desired non-acidic HMO or HMO precursor can then be filtered from the solution. If desired, the precipitated crystals can then be recrystallized using a hot C_1-C_6 alcohol solvent, preferably the same alcohol solvent as was used for the original crystallization, optionally containing up to about 50 v/v% water.

In a preferred embodiment, the non-acidic HMO to be obtained by crystallization is a fucosylated HMO and is preferably a compound of formula **1**, above (2'-FL, 3-FL or 2',3-DFL). Its aqueous medium, produced enzymatically or by fermentation (see e.g. Drouillard et al. *Angew. Chem. Int. Ed.* **45**, 1778 (2006), WO 2010/070104, WO 2010/142305, WO 2012/112777) is dried, by removing at least most of its water content by evaporation, azeotropic distillation, spray drying, freeze drying or a combination thereof, preferably by freeze drying, to form a dry powder that contains no more than about 8-10 wt% water, preferably no more than about 5 wt% water, especially no more than 3-4 wt% water. For best results, the aqueous medium, preferably contains at least about 15-20 g/l, particularly at least about 30-40 g/l, quite particularly at least at least about 50 g/l, of a compound of formula **1**, and the dry powder therefrom preferably contains the compound of formula **1** at a concentration of at least about 30-40 wt%, preferably at least at least 50-60 wt%, more particularly at least 95 wt%, even more particularly at least 97 wt%. The dry, preferably protein-

free powder is then treated with a hot, C_1-C_4 alcohol solvent, preferably boiling methanol, to produce an alcohol solution of a compound of formula **1**. In this regard, the alcohol solvent can, if desired, be heated, preferably to boiling, only after it is used to dissolve a compound of formula **1** to produce the alcohol solution. The alcohol solvent preferably contains no water. Preferably, the alcohol solution is then filtered, preferably while still hot, especially boiling hot, to remove insoluble fermentation by-products. The hot alcohol solution, preferably after filtration, is then concentrated by partial evaporation down to at least about 60-70 % of its original volume, preferably down to about 40-60 %, especially down to about 45-55 %, of its original volume. The concentrated alcohol solution is then allowed to cool somewhat, preferably down to about 30-50 °C, especially about35-45 °C, seed crystals are then preferably added to the concentrated alcohol solution while it is still warm, preferably at about 30-50 °C, especially at about 35-45 °C. The concentrated alcohol solution is then allowed to cool, for example to room temperature, and precipitated crystals of a compound of formula **1** can then be filtered from the solution. If desired, the precipitated crystals can then be recrystallized using a hot C_1-C_4 alcohol solvent, preferably the same alcohol solvent as was used for the original crystallization, which can contain water.

In another preferred embodiment, the non-acidic HMO to be crystallized has no fucosyl residue and is preferably LNT or LNnT. Its aqueous medium, produced enzymatically or by fermentation (see e.g. WO 01/04341, Priem et al. *Glycobiology* **12**, 235 (2002)) is dried, by removing at least most of its water content, by means of evaporation, azeotropic distillation, spray drying, freeze drying or combination thereof, preferably by freeze drying, to form a dry powder that contains no more than about 8-10 wt% water, preferably no more than about 5 wt% water, especially no more than 3-4 wt% water and preferably contains LNT or LNnT at a concentration of at least about 30-40 wt%, preferably at least at least 50-60 wt%, more particularly at least 95 wt%, even more particularly at least 97 wt%. The dry, preferably protein-free powder is then treated with a hot C_1-C_4 alcohol solvent, preferably boiling methanol, which can contain up to 30-40 % of water, to produce an alcohol solution of LNT or LNnT. In this regard, the alcohol solvent, water or a mixture thereof can, if desired, be heated, preferably to around 60 °C, only after it is used to dissolve LNT or LNnT

to produce the alcohol solution. Optionally, the alcohol solution can be filtered, preferably while still hot, to remove insoluble fermentation by-products. The hot alcohol solution is then allowed to cool somewhat, preferably down to room temperature or 0-4 °C, and precipitated crystals of LNT or LNnT can then be filtered from the solution. It is possible to assist crystallization by adding seed crystals. If desired, the precipitated crystals can then be recrystallized using a hot C_1-C_4 alcohol solvent, preferably the same alcohol solvent as was used for the original crystallization.

EXAMPLES

Example 1

2'-*O*-fucosyllactose (α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucose ["2'-FL"]) was expressed by an *E. coli* strain K12 that had been transformed with a glycosyl transferase gene and that had been cultivated in an aqueous medium containing glycerol (as a carbon and energy source) and lactose (as an acceptor for glycosylation with fucose, produced internally in the *E. coli*). The fermentation was carried out as described in Drouillard et al. *Angew. Chem. Int. Ed.* **45**, 1778 (2006).

When fermentation ended, the aqueous fermentation broth, containing about 30 g/l of 2'-FL, was centrifuged to remove the *E. coli* cells from the broth. The remaining liquid was treated with an anionic ion exchange resin and with a cationic ion exchange resin to remove the cell proteins from the liquid. The cell- and protein-free broth was then freeze dried, so that it contained about 50-55 wt% of 2'-FL and no more than about 4 wt% water.

The dried powder (1 g) was then treated with boiling anhydrous methanol (6 ml). About 50 wt% of the solids content of the dry powder dissolved in the methanol. The methanol solution was then filtered while still boiling hot to remove insoluble fermentation by-products (amounting to about 50 wt% of the solids content of the dried powder).The filtered methanol solution was then kept hot to partially evaporate it to about 50 % of its original volume and then allowed to cool somewhat. Seed crystals of 2'-FL were then added to the still warm (40 °C), concentrated methanol solution.

The concentrated methanol solution was then allowed to cool to room temperature (22 °C), and precipitated crystals (0.45 g) of 2'-FL were filtered from the solution with high purity.

Example 2

9.17 g of LNT (approximately 80%, obtained after fermentation with genetically modified *E. coli* according to the teaching of WO 01/04341, treatment with resins and freeze-drying) was dissolved in water (30 ml) and MeOH (60 ml) by heating to 60 °C, and the solution was stirred at room temperature for 2 h followed by 4 °C over night. Filtration, washing with MeOH-H_2O 3:1 and drying (50 °C and vacuum overnight) gave lacto-N-tetraose (5.89 g). Purity 95% (HPLC).

The following non-acidic HMOs and precursors are crystallized in essentially the same manner after fermentation thereof:

lacto-N-triose II (US 7521212, Priem et al. *Glycobiology* **12**, 235 [2002]),
LNnT(US 7521212, or Priem et al. *Glycobiology* **12**, 235 [2002]),
LNDFH III (Dumon et al. *Biotechnol. Prog.* **20**, 412 [2004]),
LNFP V Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]Glc (Dumon et al. *Glycoconj. J.* **18**, 465 [2001]).

CLAIMS

1. A method of obtaining, in crystalline form, a non-acidic HMO or a non-acidic precursor of an HMO produced in an aqueous media, enzymatically or by fermentation, particularly produced intracellularly in an aqueous fermentation broth, quite particularly produced by *E. coli*, said method comprising the steps of:

a) drying, preferably freeze-drying, the aqueous media, preferably after removing proteins therefrom, to produce a dry powder that contains the non-acidic HMO or HMO precursor;

b) then treating the dry powder with a hot C_1-C_6 alcohol solvent, preferably a C_1-C_4 alcohol solvent, especially methanol, optionally containing up to 50 v/v%, preferably up to 35 v/v%, especially no more than 15 v/v%, particularly no more than 5 v/v%, water, to produce an alcohol solution of the non-acidic HMO or HMO precursor; and

c) then crystallizing the non-acidic HMO or HMO precursor from the alcohol solution.

2. The method of claim 1, wherein the dry powder is at least about 15 wt%, preferably at least about 25 wt%, especially at least 30-40 wt%, particularly at least 50-60 wt%, more particularly at least 95 wt%, even more particularly at least 97 wt% non-acidic HMO or HMO precursor.

3. The method of any one of the claims 1-2, wherein the dry powder contains no more than 8-10 wt% water, preferably no more than about 5 wt% water, especially no more than 3-4 wt% water.

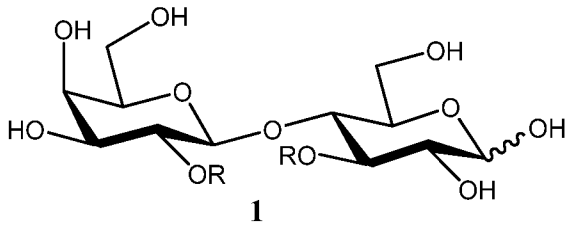
4. The method of any one of the claims 1-3, which also comprises, between steps b) and c), one or more, preferably all, of the following intermediate steps:

i) the alcohol solution is filtered to separate insolubles;

ii) then the alcohol solution is concentrated down to at least about 60-70 %, preferably down to about 40-60 %, especially down to about 45-55 %, of its original volume, and

iii) then, the alcohol solution is seeded with crystals of the non-acidic HMO or HMO precursor.

5. The method of any one of the claims 1-4, wherein the non-acidic HMO is a fucosylated HMO, preferably a compound of formula **1**



1

wherein R independently of each other is fucosyl or H, provided that at least one R is fucosyl.

6. The method of claim 5, comprising the steps of:

a) drying, preferably freeze-drying, the aqueous media, preferably after removing proteins therefrom, to produce a dry powder that contains a compound of formula **1**;

b) then treating the dry powder with a hot C_1-C_4 alcohol solvent, preferably methanol, that preferably contains no water, to produce an alcohol solution of compound of formula **1**; and

c) then crystallizing the compound of formula **1** from the alcohol solution.

7. The method of claim 6, which also comprises, between steps b) and c), an intermediate step wherein the alcohol solution is concentrated down to 40-60 %, preferably down to 45-55 %, of its original volume, preferably by keeping it hot to partially evaporate it.

8. The method of claim 7, wherein the hot alcohol solution is cooled to 30-50 °C, preferably 35-45 °C, after being concentrated in the intermediate step above.

9. The method of any one of the claims 5-8, wherein the compound of formula **1** is 2'-FL, and the alcohol is methanol.

10. The method of any one of the claims 1-4, wherein the non-acidic HMO is a HMO having no fucosyl residue, preferably LNT or LNnT.

11. The method of claim 10, comprising the steps of:

a) drying, preferably freeze-drying, the aqueous media, preferably after removing proteins therefrom, to produce a dry powder that contains LNT or LNnT;

b) then treating the dry powder with a hot C_1-C_4 alcohol solvent, preferably methanol, that preferably contains up to 30-40 % of water, to produce an alcohol solution LNT or LNnT; and

c) then crystallizing LNT or LNnT from the alcohol solution.

12. The method of any one of the claims 10-11, wherein the non-acidic HMO is LNT, and the alcohol is methanol.

13. A method of obtaining a non-acidic HMO or HMO precursor from a powder, preferably a protein-free powder, produced by drying, preferably freeze drying, an aqueous media, in which the non-acidic HMO or HMO precursor was produced enzymatically or by fermentation, particularly produced intracellularly in an aqueous fermentation broth, quite particularly produced by *E. coli*, comprising carrying out the method of any one of the claims 1-12.

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2013/050416

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H3/06 A61K31/702 C07H13/04 C07H1/08 C12P19/18
C12P19/26 C12N9/00 C12N9/10 C12N9/38
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07H A61K C12P C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BEILSTEIN Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RICHARD KUHN ET AL: "KRISTALLISIERTE FUCOSIDO-LACTOSE", CHEMISCHE BERICHTE, VCH, DE, vol. 89, no. 11, 1 November 1956 (1956-11-01), page 2513, XP009074773, ISSN: 0009-2940	1-9,13
Y	page 2513, paragraph 1 - paragraph 2	1-9,13
X	WO 2011/150939 A1 (GLYCOM AS [DK]; AGOSTON KAROLY [HU]; BAJZA ISTVAN [HU]; DEKANY GYULA [) 8 December 2011 (2011-12-08)	1-9,13
Y	page 2, paragraph 2 - paragraph 4 page 17, paragraph crystallization procedures - page 19	1-9,13

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 2014	25/04/2014

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

2

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NAM SOO HAN ET AL: "Biotechnological production of human milk oligosaccharides", BIOTECHNOLOGY ADVANCES, vol. 30, no. 6, 1 November 2012 (2012-11-01), pages 1268-1278, XP055074947, ISSN: 0734-9750, DOI: 10.1016/j.biotechadv.2011.11.003 cited in the application page 1272, paragraph 3 - page 1274	1-9,13
X	Richard Kuhn ET AL: "Die Konstitution der Lacto-N-fucopentaose II. Ein Beitrag zur Spezifität der Blutgruppensubstanz Lea", Chemische Berichte, 1 February 1958 (1958-02-01), pages 364-374, XP055113657, DOI: 10.1002/cber.19580910221 Retrieved from the Internet: URL:http://onlinelibrary.wiley.com/store/10.1002/cber.19580910221/asset/19580910221_ftp.pdf?v=1&t=htv66hdf&s=a346c0e574efb3f1dc49129665d53ba15d3be5db	1-4,10, 11,13
Y	page 369, last paragraph	1-4, 10-13
X	WO 2011/100980 A1 (GLYCOM AS [DK]; BAJZA ISTVAN [HU]; DEKANY GYULA [AU]; AGOSTON KAROLY [) 25 August 2011 (2011-08-25)	1-4,10, 11,13
Y	page 22, line 15 - line 27 page 46 - page 47; examples 33-34	1-4, 10-13
Y	EP 1 405 856 A1 (KYOWA HAKKO KOGYO KK [JP]) 7 April 2004 (2004-04-07) cited in the application page 6 - page 8; examples 1-3 claim 15	1-4, 10-13

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2013/050416

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 5-9(completely); 1-4, 13(partially)

A method for obtaining crystals of compound of formula 1

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2. claims: 10-12(completely); 1-4, 13(partially)

A method for obtaining crystals of compounds of claim 10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK2013/050416

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011150939 A1	08-12-2011	CA 2798675 A1	08-12-2011
		CN 103025749 A	03-04-2013
		EP 2576578 A1	10-04-2013
		US 2013165406 A1	27-06-2013
		WO 2011150939 A1	08-12-2011
WO 2011100980 A1	25-08-2011	CN 102803282 A	28-11-2012
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		JP 4347042 B2	21-10-2009
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		US 2004149200 A1	05-08-2004
		WO 02100875 A1	19-12-2002