ferases can be distinguished due to the linkages that they form, e. g. into α-2,3-, α-2,6- and α-2,8-sialyltransferases. All these sialyltransferases transfer the sialic acid residue from cytidine 5'-monophosphate sialic acid (e. g. CMP-Neu5Ac) to a variety of acceptor molecules, usually galactose- (Gal), *N*-acetylgalactosamine- (GalNAc) or *N*-acetylglucosamine (GlcNAc) moieties or another sialic acid (Sia) moieties.

Several bacterial sialyltransferases were well characterized in the past and are already proven to be suitable for the production of 3'-SL or 6'-SL. Hardly any knowledge could have been achieved about sialyltransferases enabling the synthesis of sialylated penta- or hexasaccharides such as LST-a, LST-b or DSLNT, thereby limiting the establishment of a production process for any one of these SHMOs. As a consequence, the unavailability of highly pure amounts of these desired oligosaccharides impedes an extensive scientific evaluation of their health beneficial properties.

Therefore, there is a need for a cost-efficient process for producing one or more SHMOs, especially tetrasacharides, pentasaccharides and hexasaccharides possessing one or two sialic acid residues, in high amounts and high purity.

Summary

The object is solved, inter alia, by the identification and characterization of new sialyltransferases and their use in the production of sialylated human milk oligosaccharides by means of whole cell fermentation or biocatalysis.

According to a first example, provided is a method for producing sialylated oligo-saccharides, wherein a genetically engineered cell is used for producing said sialylated oligosaccharide. Said genetically engineered cell comprises at least one heterologous sialyltransferase which is capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

According to a second example, provided is a genetically engineered cell for use in a method for producing sialylated oligosaccharides, wherein said genetically engineered cell has been genetically engineered to express a heterologous sialyltransferase which is capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

According to a third example, provided is a recombinant nucleic acid molecule for expressing a heterologous sialyltransferase when propagated in a cell, wherein said sialyltransferase is capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

According to a fourth example, provided are sialyltransferases being capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

According to a fifth example, provided is the use of a sialyltransferase being capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides, for producing sialylated oligosaccharides.

According to a sixth example, provided is a method for producing sialylated oligosaccharides by *in vitro* biocatalysis, wherein a sialyltransferase is used, said sialyltransferase being capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule, wherein said acceptor molecule is selected from the group consisting of lactose, LNT-II and human milk oligosaccharides.

According to a seventh example, provided are sialylated oligosaccharides being produced by a method according to the first example or by a method according to the sixth example.

According to an eight example, provided is the use of sialylated oligosaccharides according to the seventh example for manufacturing a nutritional composition.

According to a ninth example, provided is a nutritional composition containing at least one sialylated oligosaccharide according to the seventh example.
According to a tenth example, provided is an infant formula containing at least one sialylated human milk oligosaccharide.

In one aspect, the present disclosure refers to a method for producing a sialylated oligosaccharide sialyllacto-N-tetraose a (LST-a), the method comprising the steps of
a) providing at least one genetically engineered cell which comprises a heterologous sialyltransferase, said heterologous sialyltransferase possessing an α-2,3-sialyltransferase activity , wherein the heterologous sialyltransferase transfers a sialic acid residue from its nucleotide-activated form as donor substrate to an acceptor molecule, wherein the acceptor molecule is the human milk oligosaccharide lacto-N-tetraose (LNT), wherein the heterologous sialyltransferase is selected from i) a polypeptide comprising an amino acid sequence of SEQ ID NO: 34; and ii) fragments of a polypeptide comprising an amino acid sequence of SEQ ID NO: 34;
b) cultivating the at least one cell in a fermentation broth and under conditions permissive for the production of said sialylated oligosaccharide; and optionally
c) recovering said sialylated oligosaccharide.

In another aspect, the present disclosure refers to a genetically engineered cell for producing a sialylated oligosaccharide sialyllacto-N-tetraose a (LST-a), wherein the genetically engineered cell comprises a heterologous sialyltransferase, said heterologous sialyltransferase possesses α-2,3-sialyltransferase activity , wherein the heterologous sialyltransferase transfers a sialic acid residue from its nucleotide-activated form as donor substrate to an acceptor molecule, wherein the acceptor molecule is the human milk oligosaccharide lacto-N-tetraose (LNT);
wherein the heterologous sialyltransferase is selected from i) a polypeptide comprising an amino acid sequence of SEQ ID NO: 34; and ii) fragments of a polypeptide comprising an amino acid sequence of SEQ ID NO: 34.

Brief description of the drawings

FIG. 1 depicts the chemical structures of the most abundant acidic oligosaccharides in human milk: 3'-SL (A), 6'-SL (B), LST-a (C), LST-b (D), LST-c (E), and DSLNT (F).

FIG. 2 shows the maps of sialyltransferase gene overexpressing plasmids pDEST14 and pET11a.

FIG. 3 shows the *in vivo* production of sialyllacto-*N*-tetraose-a and sialyllacto-*N*-tetraose-b (marked by arrows) due to the overexpression of suitable sialyltransferases in that the separation of the intracellular fraction of *siaT9*- or *siaT19*-overexpressing *E. coli* BL21(DE3) #2130 cells by thin layer chromatography is depicted.

Detailed description

In an attempt to identify sialyltransferases which are suitable for use in a process of manufacturing a sialylated HMO, nucleic acid databases and protein databases were searched. One hundred putative sialyltransferases were identified by means of sequence similarities to known glycosyltransferases. Said putative sialyltransferases were assessed for sialyltransferase activity.
According to the first aspect, provided is a method for producing a sialylated oligosaccharide, the method comprising the steps of

a) providing at least one genetically engineered cell which comprises a heterologous sialyltransferase, said heterologous sialyltransferase being

CLAIMS

1. A method for producing a sialylated oligosaccharide sialyllacto-N-tetraose a (LST-a), the method comprising the steps of
   a) providing at least one genetically engineered cell which comprises a heterologous sialyltransferase, said heterologous sialyltransferase possessing an α-2,3-sialyltransferase activity, wherein the heterologous sialyltransferase transfers a sialic acid residue from its nucleotide-activated form as donor substrate to an acceptor molecule, wherein the acceptor molecule is the human milk oligosaccharide lacto-N-tetraose (LNT), wherein the heterologous sialyltransferase is selected from
      i) a polypeptide comprising an amino acid sequence of SEQ ID NO: 34; and
      ii) fragments of a polypeptide comprising an amino acid sequence of SEQ ID NO: 34;
   b) cultivating the at least one cell in a fermentation broth and under conditions permissive for the production of said sialylated oligosaccharide; and optionally
   c) recovering said sialylated oligosaccharide.

2. The method according to claims 1, wherein the fermentation broth contains at least one carbon source, the at least one carbon source is preferably selected from the group consisting of glucose, fructose, sucrose, glycerol, and combinations thereof.

3. The method according to claim 1 or 2, wherein the fermentation broth contains at least one compound selected from the group consisting of *N*-acetylglucosamine, galactose and sialic acid.

4. The method according to claim 1 or 2, wherein the at least one cell is cultivated in the absence of and/or without addition of one or more compounds selected from the group consisting of *N*-acetylglucosamine, galactose and sialic acid.

5. The method according to any one of claims 1 to 4, wherein the at least one cell is cultivated in the presence of at least one selected from the group consisting of lactose, lacto-*N*-triose II, LNT and LN*n*t.

6. A genetically engineered cell for producing a sialylated oligosaccharide sialyllacto-N-tetraose a (LST-a), wherein the genetically engineered cell comprises a heterologous sialyltransferase, said heterologous sialyltransferase possesses α-2,3-sialyltransferase activity, wherein the heterologous sialyltransferase transfers a sialic acid residue from

its nucleotide-activated form as donor substrate to an acceptor molecule, wherein the acceptor molecule is the human milk oligosaccharide lacto-N-tetraose (LNT);
wherein the heterologous sialyltransferase is selected from

i) a polypeptide comprising an amino acid sequence of SEQ ID NO: 34; and

ii) fragments of a polypeptide comprising an amino acid sequence of SEQ ID NO: 34.

7. The method according to any one of claims 1 to 5, or the genetically engineered cell according to claim 6, wherein

the genetically engineered cell comprises a nucleic acid molecule comprising a nucleotide sequence which encodes the heterologous sialyltransferase, said nucleotide sequence being selected from the group consisting of

i. nucleotide sequences encoding a polypeptide as represented by SEQ ID NO: 34;
ii. nucleotide sequences encoding fragments of a polypeptide comprising an amino acid sequence of SEQ ID NO: 34 ; and
iii. nucleotide sequences which are complementary to any one of the nucleotide sequences of i. and ii.;

wherein said nucleotide sequence is operably linked to at least one nucleic acid expression control sequence effecting transcription and/or translation of said nucleotide sequence encoding the heterologous sialyltransferase in the genetically engineered cell.

8. The method according to any one of claims 1 to 5 or 7, or the genetically engineered cell according to claim 6 or 7, wherein the genetically engineered cell possesses an increased production of one or more nucleotide-activated sugars selected from the group consisting of CMP-*N*-acetylneuraminic acid, UDP-*N*-acetylglucosamine, UDP-galactose and GDP-fucose as compared to cell before being genetically engineered.

9. The method according to any one of claims 1 to 5 or 7 to 8, or the genetically engineered cell according to any one of claims 6 to 8, wherein the genetically engineered cell possesses an overexpression, as compared to the cell prior to be genetically engineered, of one or more genes encoding for a polypeptide being capable of possessing an enzymatic activity selected from the group consisting of L-glutamine:D-fructose-6-phosphate aminotransferase, *N*-acetylglucosamine-1-phosphate uridyltransferase, glucosamine-1-phosphate acetyl transferase, phosphoglucosamine mutase, glucosamine-6-phosphate-*N*-acetyltransferase, *N*-acetylglucosamine-2-epimerase, UDP-*N*-acetylglucosamine-2-epimerase, sialic acid synthase, phosphoenolpyruvate synthase, CMP-sialic acid synthetase, UDP-galactose-

4-epimerase, galactose-1-phosphate uridylyltransferase, phosphoglucomutase, glucose-1-phosphate uridylyltransferase, phosphomannomutase, mannose-1-phosphate guanosyltransferase, GDP-mannose-4,6-dehydratase, GDP-L-fucose synthase and fucosekinase/L-fucose-1-phosphate-guanyltransferase.

10. The method according to any one of claims 1 to 5 or 7 to 9, or the genetically engineered cell according to any one of claims 7 to 11, wherein the cell lacks or possesses a decreased activity, as compared to the prior to be genetically engineered, of one or more enzymatic activities selected from the group consisting of β-galactosidase, a glucosamine-6-phosphate deaminase, a *N*-acetylglucosamine-6-phosphate deacetylase, a *N*-acetylmannosamine kinase, a *N*-acetylmannosamine-6-phosphate epimerase and a *N*-acetylneuraminic acid aldolase.

11. The method according to any one of claims 1 to 5 or 7 to 10, or the genetically engineered cell according to any one of claims 6 to 10, wherein one or more of the genes encoding a β-galactosidase, a glucosamine-6-phosphate deaminase, a *N*-acetylglucosamine-6-phosphate deacetylase, a *N*-acetylmannosamine kinase, a *N*-acetylmannosamine-6-phosphate epimerase and a *N*-acetylneuraminic acid aldolase has been deleted or its expression has been inactivated in the genetically engineered cell.

12. The method according to any one of claims 1 to 5 or 7 to 77, or the genetically engineered cell according to any one of claims 6 to 11, wherein the at least one cell comprises at least one selected from the group consisting of a functional lactose permease, a functional fucose permease and a functional sialic acid transporter (importer), preferably comprises and expresses at least one nucleotide sequence encoding one selected from the group consisting of a functional lactose permease, a functional fucose permease and a functional sialic acid transporter (importer).

13. The method according to any one of claims 1 to 5 or 7 to 121, or the genetically engineered cell according to any one of claims 6to 12, wherein the cell possesses activity of at least one glycosyltransferase selected from the group consisting of a β-1,3-N-acetylglucosaminyltransferase, a β-1,3-galactosyltransferase, a β-1,4-galactosyltransferase, a α-2,3-sialyltransferase and a α-2,6-sialyltransferase.