

Fermentative production of sialylated saccharides

BACKGROUND

[0001] The present invention relates to a method for the fermentative production of sialylated saccharides, as well as to recombinant or genetically engineered microbial cells employed therein.

[0002] More than 150 structurally distinct human milk oligosaccharides (HMOs) have been identified to date. Although HMOs represent only a minor amount of total human milk nutrients, their beneficial effects on the development of breast fed infants became evident over the past decades.

[0003] Among the HMOs, sialylated HMOs (SHMOs) were observed to support the resistance to enteropathogenic bacteria and viruses. Interestingly, recent studies further demonstrated a protective effect of long-chained SHMOs against necrotizing enterocolitis, which is one of the most common and lethal diseases in preterm infants. In addition, SHMOs are believed to support an infant's brain development and its cognitive capabilities. Also, sialylated oligosaccharides have been shown to neutralize enterotoxins of various pathogenic microbes including *Escherichia coli*, *Vibrio cholerae* and *Salmonella*. Further, it was found that sialylated oligosaccharides interfere with the colonization of the gut by *Helicobacter pylori* and thereby prevent or inhibit gastric and duodenal ulcers.

[0004] Among the sialylated oligosaccharides, 3'-sialyllactose, 6'-sialyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c and disialyllacto-*N*-tetraose are the most prevalent members in human milk.

[0005] Since sialylated oligosaccharides have a complex structure, their chemical or (chemo-)enzymatic syntheses are challenging and associated with extensive difficulties, e.g. control of stereochemistry, formation of specific linkages, availability of

feedstocks, etc. As a consequence, commercially available sialylated oligosaccharides have been very expensive due to their low quantity in natural sources.

[0006] Thus, efforts in metabolic engineering of microorganisms to produce sialylated oligosaccharides have been made, since this approach is the most promising way for producing HMOs in an industrial scale. For the production of SHMOs by microbial fermentation, the microorganism is typically cultivated in the presence of exogenous sialic acid.

[0007] International Publication WO 2007/101862 A1 discloses a method for the large scale in vivo synthesis of sialylated oligosaccharides relying on the intracellular UDP-GlcNAc pool by culturing a microorganism in a culture medium, wherein said microorganism comprises heterologous genes encoding CMP-Neu5Ac synthetase, a sialic acid synthase, a GlcNAc-6-phosphate 2 epimerase and a sialyltransferase. In addition, the endogenous genes coding for sialic acid aldolase (NanA) and for ManNac kinase (NanK) were deleted.

[0008] International Publication WO 2014/153253 A1 discloses methods and compositions for engineering bacteria to produce sialylated oligosaccharides as well as a method for producing a sialylated oligosaccharide in a bacterium, said bacterium comprises an exogenous sialyltransferase, a deficient sialic acid catabolic pathway, a sialic acid synthetic capability, and a functional lactose permease gene, wherein said bacterium is cultured in the presence of lactose. The sialic acid synthetic capability comprises expressing an exogenous CMP-Neu5Ac synthetase, an exogenous sialic acid synthase, and an exogenous UDP-GlcNAc-2-epimerase.

[0009] However, it is desirable to produce sialylated oligosaccharides by microbial fermentation which does not require the presence and/or addition of exogenous sialic acid during fermentation. Also, it is desirable to produce sialylated oligosaccharides by microorganisms which does not require accessing the intracellular pool of UDP-N-acetylglucosamine (UDP-GlcNAc) as this is believed to be energetically beneficial for the cell.

SUMMARY

[0010] The object is solved, *inter alia*, by providing a method for the whole cell fermentative production of sialylated saccharides, which method does not require addition of exogenous sialic acid, and by a genetically engineered microbial cell which can synthesized sialylated saccharides in the absence of exogenous sialic acid.

[0011] According to one aspect, a method for the production of a sialylated saccharide is provided which method comprises the steps of a) providing at least one genetically engineered microbial cell which comprises (i) a sialic acid biosynthesis pathway for the intracellular biosynthesis of *N*-acetylneuraminic acid (Neu5Ac, NeuNAc), wherein said sialic acid biosynthesis pathway comprises a glucosamine-6-phosphate *N*-acetyltransferase (ii) a cytidine 5'-monophospho-(CMP)-sialic acid synthetase, and (iii) a heterologous sialyltransferase; b) cultivating the at least one genetically engineered microbial cell in a fermentation broth and under conditions permissive for the production of said sialylated saccharide; and optionally c) recovering said sialylated saccharide.

[0012] According to another aspect, a genetically engineered microbial cell for producing a sialylated saccharide is provided, wherein the microbial cell comprises (i) a sialic acid biosynthesis pathway for the intracellular biosynthesis of *N*-acetylneuraminic acid, wherein said sialic acid biosynthesis pathway comprises a glucosamine-6-phosphate *N*-acetyltransferase; (ii) a cytidine 5'-monophospho-(CMP)-*N*-acetylneuraminic acid synthetase for transferring the *N*-acetylneuraminic acid onto cytidine 5'-monophosphate to generate a CMP-activated *N*-acetylneuraminic acid; and (iii) a heterologous sialyltransferase.

[0013] According to another aspect, a sialylated saccharide is provided which is producible by a method or a genetically engineered microbial cell according to the invention.

[0014] According to another aspect, a use of a sialylated saccharide which is produced by a method or a genetically engineered microbial cell according to the invention for manufacturing a nutritional composition, preferably an infant composition, is provided.

[0015] According to yet another aspect, a nutritional composition containing at least one sialylated saccharide produced by the method or the genetically engineered microbial cell according to the invention is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 is a schematic representation of a sialic acid biosynthesis pathway that may be employed by a genetically engineered microbial cell for the fermentative production of sialylated saccharides, wherein said sialic acid biosynthesis pathway utilizes UDP-GlcNAc.

[0017] FIG. 2 is a schematic representation of a sialic acid biosynthesis pathway that may be employed by a genetically engineered microbial cell of the invention for the fermentative production of sialylated saccharides.

[0018] FIG. 3 is a schematic representation of another sialic acid biosynthesis pathway that may be employed by a genetically engineered microbial cell of the invention for the fermentative production of sialylated saccharides.

DETAILED DESCRIPTION

[0019] According to a first aspect, a method for the fermentative production of a sialylated saccharide is provided. The method comprises the steps of a) providing at least one genetically engineered microbial cell that is able to synthesize the sialylated saccharide, said at least one genetically engineered microbial cell comprises (i) a sialic acid biosynthesis pathway comprising a glucosamine-6-phosphate *N*-acetyltransferase; (ii) a cytidine 5'-monophospho-(CMP)-*N*-acetylneuraminic acid synthetase; and (iii) a

heterologous sialyltransferase; b) cultivating the at least one genetically engineered microbial cell in a fermentation broth and under conditions that are permissive for the production of said sialylated saccharide, and optionally c) recovering said sialylated saccharide.

[0020] Accordingly, in a second aspect, the invention also concerns a genetically engineered microbial cell for the fermentative production of the sialylated saccharide, wherein the microbial cell comprises (i) a sialic acid biosynthesis pathway for the intracellular biosynthesis of *N*-acetylneuraminic acid, wherein said sialic acid biosynthesis pathway comprises a glucosamine-6-phosphate *N*-acetyltransferase; (ii) a cytidine 5'-monophospho-(CMP)-sialic acid synthetase for transferring the *N*-acetylneuraminic acid onto cytidine 5'-monophosphate to generate a CMP-activated sialic acid; and (iii) a sialyltransferase for transferring the *N*-acetylneuraminic acid moiety from the CMP-activated sialic acid as donor substrate to an acceptor molecule, which acceptor molecule is a saccharide molecule, resulting in the intracellular biosynthesis of the sialylated saccharide.

[0021] The genetically engineered microbial cell comprises a sialic acid biosynthesis pathway for the intracellular biosynthesis of *N*-acetylneuraminic acid which does not utilize UDP-GlcNAc. The genetically engineered microbial cell comprises a sialic acid biosynthesis pathway for the intracellular biosynthesis of *N*-acetylneuraminic acid which a glucosamine-6-phosphate *N*-acetyltransferase. A sialic acid biosynthesis pathway using a glucosamine-6-phosphate *N*-acetyltransferase for the intracellular biosynthesis of *N*-acetylneuraminic acid does not utilize UDP-GlcNAc for the biosynthesis of sialic acid (Fig. 2 and Fig. 3).

[0022] The sialic acid biosynthesis pathway comprises the enzymatic activities of a glutamine:fructose-6-phosphate aminotransferase and an *N*-acetylneuraminic acid synthase. The sialic acid biosynthesis pathway further comprises a) the enzymatic activities of a glucosamine-6-phosphate *N*-acetyltransferase, an *N*-acetylglucosamine-6-phosphate phosphatase and an *N*-acetylglucosamine 2-epimerase (Fig. 2); and/or b) the enzymatic activities of a glucosamine-6-phosphate *N*-acetyltransferase, an *N*-acetylglucosamine-6-phosphate epimerase and an *N*-acetylmannosamine-6-phosphate phos-

phatase (Fig. 3). Therefore, it is not necessary that the genetically engineered microbial cell comprises the enzymatic activities of a phosphoglucosamine mutase, an *N*-acetylglucosamine-1-phosphate uridylyltransferase and an UDP *N*-acetylglucosamine 2-epimerase with concomitant release of UDP (Fig. 1) for intracellular sialic acid biosynthesis. Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell being capable of synthesizing sialic acid does not comprise one or more enzymatic activities selected from the group consisting of the enzymatic activities of a phosphoglucosamine mutase, an *N*-acetylglucosamine-1-phosphate uridylyltransferase and an UDP *N*-acetylglucosamine 2-epimerase with concomitant release of UDP.

[0023] The enzyme glutamine:fructose-6-phosphate aminotransferase (EC 2.6.1.16) catalyzes the conversion of fructose-6-phosphate (Frc-6P) to glucosamine-6-phosphate (GlcN-6P) using glutamine. This enzymatic reaction is typically considered to be the first step in the hexosamine biosynthesis pathway. Alternative names of the glutamine:fructose-6-phosphate aminotransferase are D-fructose-6-phosphate aminotransferase, GFAT, glucosamine-6-phosphate synthase, hexosephosphate aminotransferase, and L-glutamine–D-fructose-6-phosphate aminotransferase.

[0024] In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses a glutamine:fructose-6-phosphate aminotransferase, preferably a heterologous a glutamine:fructose-6-phosphate aminotransferase, more preferably a glutamine:fructose-6-phosphate aminotransferase which is derived from *E. coli* (*E. coli* GlmS (UniProtKB – P17169; SEQ ID NO. 67), or a functional variant of the *E. coli* GlmS. Most preferably, the functional variant is a version of the *E. coli* GlmS which shows significantly reduced sensitivity to glucosamine-6-phosphate inhibition as the wild-type enzyme does. An example of a functional variant of the *E. coli* GlmS which shows significantly reduced sensitivity to glucosamine-6-phosphate inhibition is represented by SEQ ID NO. 68).

[0025] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising a nucleotide sequence which encodes a glutamine:fructose-6-phosphate aminotransferase, preferably the *E. coli* glutamine:fructose-6-phosphate aminotransferase GlmS (SEQ ID NO. 69), or a

nucleotide sequence encoding a functional variant is a version of the *E. coli* GlmS which shows significantly reduced sensitivity to glucosamine-6-phosphate inhibition as compared to the wild-type enzyme (*glmS**54 or *glmS** (as represented by SEQ ID NO. 70)).

[0026] Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence selected from the group consisting of

- i) nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID No. 67 and SEQ ID NO. 68;
- ii) nucleotide sequences as represented by any one of SEQ ID NO. 69 and SEQ ID NO. 70;
- iii) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NO. 67 and SEQ ID NO. 68;
- iv) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences as represented by any one of SEQ ID NO. 69 and SEQ ID NO. 70;
- v) nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv.; and
- vi) fragments of any one of the nucleotide sequences of i., ii., iii., iv and v.;

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 in the genetically engineered microbial cell to provide intracellular glutamine:fructose-6-phosphate aminotransferase activity.

[0027] In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses glucosamine-6-phosphate *N*-acetyltransferase activity. Said glucosamine-6-phosphate *N*-acetyltransferase activity converts GlcN-6P to *N*-acetylglucosamine-6-phosphate (GlcNAc-6P). An example of a glucosamine-6-phosphate *N*-acetyltransferase is the *Saccharomyces cerevisiae* Gna1 (UniProtKB – P43577; SEQ ID NO. 77).

[0028] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a glucosamine-6-phosphate *N*-acetyltransferase, preferably a heterologous glucosamine-6-phosphate *N*-acetyltransferase, more preferably *S. cerevisiae* Gna1 (encoded by a nucleotide sequence as represented by SEQ ID NO. 78) or a functional variant thereof.

[0029] Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence selected from the group consisting of

- i) nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 77;
- ii) a nucleotide sequences as represented by SEQ ID NO. 78;
- iii) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to the nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 77;
- iv) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences as represented by SEQ ID NO. 78;
- v) nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv.; and
- vi) fragments of any one of the nucleotide sequences of i., ii., iii., iv and v.;

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 in the genetically engineered microbial cell to provide intracellular glucosamine-6-phosphate *N*-acetyltransferase activity.

[0030] In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses a *N*-acetylglucosamine-6-phosphate phosphatase activity. Said *N*-acetylglucosamine-6-phosphate phosphatase activity converts GlcNAc-6P to *N*-acetylglucosamine (GlcNAc). Examples of an *N*-acetylglucosamine-6-phosphate phosphatase are sugar phosphatases of the HAD-like superfamily which catalyze the conversion of GlcNAc6P to GlcNAc. The HAD-like superfamily of enzymes is named after the bacterial enzyme haloacid dehydrogenase and includes phosphatases. A suitable phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to

GlcNAc may be selected from the group consisting of fructose-1-phosphate phosphatase (YqaB, UniProtKB - P77475; SEQ ID NO. 79) and alpha-D-glucose 1-phosphate phosphatase (YihX, UniProtKB – P0A8Y3; SEQ ID No. 80). The *E. coli* YqaB and *E. coli* YihX enzymes are considered to also act on GlcNAc6P (Lee, S.-W. and Oh, M.-K. (2015) Metabolic Engineering 28: 143-150).

[0031] In an additional and/or alternative embodiment, the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc-6P to GlcNAc is a heterologous enzyme in the genetically engineered microbial cell. In an additional and/or alternative embodiment, the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc is selected from the group consisting of *E. coli* YqaB, *E. coli* YihX, and functional variants thereof.

[0032] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule which comprises and expresses a nucleotide sequence encoding a sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc. In an additional and/or alternative embodiment, the nucleotide sequence encoding the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc is a heterologous nucleotide sequence. In an additional and/or alternative embodiment, the nucleotide sequence encoding the sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc encodes the *E. coli* fructose-1-phosphate phosphatase or the *E. coli* alpha-D-glucose 1-phosphate phosphatase or a functional fragment of one of these two enzymes.

[0033] The *E. coli* YqaB is encoded by a nucleotide sequence as represented by SEQ ID NO. 81, whereas the *E. coli* YihX is encoded by a nucleotide sequences as represented by SEQ ID NO. 82. Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence selected from the group consisting of

- i) nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NO. 79 and SEQ ID NO. 80;

- ii) nucleotide sequences as represented by any one of SEQ ID NO. 81 and SEQ ID NO. 82;
- iii) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NO. 79 and SEQ ID NO. 80;
- iv) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences as represented by any one of SEQ ID NO. 81 and SEQ ID NO. 82;
- v) nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv.; and
- vi) fragments of any one of the nucleotide sequences of i., ii., iii., iv and v.;

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 in the genetically engineered microbial cell to provide intracellular sugar phosphatase activity which catalyze the conversion of GlcNAc6P to GlcNAc.

[0034] In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to contain a nucleic acid molecule comprising and expressing a nucleotide sequence encoding a sugar phosphatase of the HAD-like superfamily catalyzing the conversion of GlcNAc6P to GlcNAc or a functional fragment of said HAD phosphatase and/or to comprise a sugar phosphatase of the HAD-like

[0035] In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses *N*-acetylglucosamine 2-epimerase activity. *N*-acetylglucosamine 2-epimerase (EC 5.1.3.8) is an enzyme that catalyzes the conversion of *N*-acetylglucosamine (GlcNAc) to *N*-acetylmannosamine (ManNAc). The enzyme is a racemase acting on carbohydrates and their derivatives. The systematic name of this enzyme class is *N*-acyl-D-glucosamine 2-epimerase. This enzyme participates in amino-sugar metabolism and nucleotide-sugar metabolism, preferably a heterologous *N*-acetylglucosamine 2-epimerase.

[0036] In an additional and/or alternative embodiment, the genetically engineered microbial cell comprises an *N*-acetylglucosamine 2-epimerase, preferably a heterologous *N*-acetylglucosamine 2-epimerase. Examples of *N*-acetylglucosamine 2-epimerases were described from *Anabena variabilis*, *Acaryochloris* sp., *Nostoc* sp., *Nostoc punctiforme*, *Bacteroides ovatus* or *Synechocystis* sp. An example of a suitable *N*-acetylglucosamine 2-epimerase is the *N*-acetylglucosamine 2-epimerase of *B. ovatus* ATCC 8483 (UniProtKB - A7LVG6, SEQ ID NO. 83) as encoded by gene BACOVA_01816 (SEQ ID NO. 85). Another example is the *N*-acetylglucosamine 2-epimerase of *Synechocystis* sp. (strain PCC 6803) (UniProtKB - P74124; SEQ ID NO: 84) which is also known as renin-binding protein and is encoded by the *slr1975* gene (SEQ ID NO. 86).

[0037] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising a nucleotide sequence which encodes an *N*-acetylglucosamine 2-epimerase, preferably the *N*-acetylglucosamine 2-epimerase of *B. ovatus* ATCC 8483 or *Synechocystis* sp. (strain PCC 6803) or a functional variant thereof.

[0038] Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence selected from the group consisting of

- i) nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NO. 83 and SEQ ID NO. 84;
- ii) nucleotide sequences as represented by any one of SEQ ID NO. 85 and SEQ ID NO. 86;
- iii) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NO. 83 and SEQ ID NO. 84;
- iv) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences as represented by any one of SEQ ID NO. 85 and SEQ ID NO. 86;
- v) nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv.; and

vi) fragments of any one of the nucleotide sequences of i., ii., iii., iv and v.; 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 in the genetically engineered microbial cell to provide intracellular *N*-acetylglucosamine 2-epimerase activity.

[0039] In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses *N*-acetylglucosamine-6-phosphate epimerase activity and *N*-acetylmannosamine-6-phosphate phosphatase activity. *N*-acetylglucosamine-6-phosphatase epimerase converts *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) to *N*-acetylmannosamine-6-phosphate (ManNAc-6P), whereas *N*-acetylmannosamine-6-phosphate phosphatase dephosphorylates ManNAc-6P to give *N*-acetylmannosamine (ManNAc). Possessing *N*-acetylglucosamine-6-phosphate epimerase activity and *N*-acetylmannosamine-6-phosphate phosphatase activity provides an additional or alternative way for providing ManNAc for Neu5Ac production.

[0040] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains an *N*-acetylglucosamine-6-phosphate epimerase. An example of a suitable *N*-acetylglucosamine-6-phosphate epimerase is *E. coli* NanE (UniprotKB P0A761, SEQ ID NO. 87) as encoded by the *E. coli nanE* gene (SEQ ID NO. 88).

[0041] Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding an *N*-acetylglucosamine-6-phosphate epimerase, preferably a nucleotide sequence encoding *E. coli* NanE.

[0042] Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence selected from the group consisting of

- i) nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 87;
- ii) a nucleotide sequences as represented by SEQ ID NO. 88;

- iii) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to the nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 87;
 - iv) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences as represented by SEQ ID NO. 88;
 - v) nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv.; and
 - vi) fragments of any one of the nucleotide sequences of i., ii., iii., iv and v.;
- 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 in the genetically engineered microbial cell to provide intracellular *N*-acetylglucosamine-6-phosphate epimerase activity.

[0043] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains an *N*-acetylmannosamine-6-phosphate phosphatase.

[0044] Thus, in additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding an *N*-acetylmannosamine-6-phosphate phosphatase.

[0045] In an additional and/or alternative embodiment, the genetically engineered microbial cell comprises sialic acid synthase activity. The sialic acid synthase catalyzes the condensation of ManNAc and phosphoenolpyruvate (PEP) to *N*-acetylneuraminic acid (NeuNAc).

[0046] In an additional and/or alternative embodiment, the genetically engineered microbial cell comprises a sialic acid synthase or a functional variant thereof, preferably a heterologous sialic acid synthase. Examples of sialic acid synthases are known from a variety of bacterial species such as *Campylobacter jejuni*, *Streptococcus agalactiae*, *Butyrivibrio proteoclasticus*, *Methanobrevibacter ruminantium*, *Acetobacterium woodii*, *Desulfobacula toluolica*, *Escherichia coli*, *Prevotella nigescens*, *Halorhabdus*

tiamatea, *Desulfotignum phosphitoxidans*, or *Candidatus Scalindua* sp., *Idomarina loihiensis*, *Fusobacterium nucleatum* or *Neisseria meningitidis*. Preferably, the sialic acid synthase is the *N*-acetylneuraminic acid synthase NeuB of *C. jejuni* (SEQ ID NO. 89) as encoded by the *C. jejuni neuB* gene (SEQ ID NO. 90).

[0047] Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence selected from the group consisting of

- i) nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 89;
- ii) a nucleotide sequences as represented by SEQ ID NO. 90;
- iii) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to the nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 89;
- iv) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences as represented by SEQ ID NO. 90;
- v) nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv.; and
- vi) fragments of any one of the nucleotide sequences of i., ii., iii., iv and v.;

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 in the genetically engineered microbial cell to provide intracellular *N*-acetylneuraminic acid synthase activity.

[0048] The genetically engineered microbial cell possesses cytidine 5'-monophospho-(CMP)-*N*-acetylneuraminic acid synthetase activity for transferring cytidine 5'-monophosphate onto *N*-acetylneuraminic acid to generate a CMP-activated *N*-acetylneuraminic acid (CMP-NeuNAc). Several 5'-monophospho-(CMP)-sialic acid synthetases are known in the art and have been described, e.g. 5'-monophospho-(CMP)-sialic acid synthetases from *E. coli*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Streptococcus* sp., etc.

[0049] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a cytidine 5'-monophospho- (CMP)-*N*-acetylneuraminic acid synthetase, preferably a heterologous cytidine 5'-monophospho- (CMP)-*N*-acetylneuraminic acid synthetase, more preferably the *N*-acetylneuraminate cytidyltransferase NeuA from *E. coli*. *E. coli* NeuA (UnitProtKB – P13266; SEQ ID NO. 91) is encoded by the *E. coli neuA* gene (SEQ ID NO. 92).

[0050] Thus, in an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence selected from the group consisting of

- i) nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 91;
- ii) a nucleotide sequences as represented by SEQ ID NO. 92;
- iii) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to the nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 91;
- iv) nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences as represented by SEQ ID NO. 92;
- v) nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv.; and
- vi) fragments of any one of the nucleotide sequences of i., ii., iii., iv and v.;

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 in the genetically engineered microbial cell to provide *N*-acetylneuraminate cytidyltransferase activity.

[0051] The genetically engineered microbial cell possesses sialyltransferase activity, preferably a heterologous sialyltransferase activity, and more preferably a sialyltransferase activity selected from the group consisting of α -2,3-sialyltransferase activity, α -2,6-sialyltransferase activity and/or α -2,8-sialyltransferase activity. The sialyltransferase activity is capable of transferring the *N*-acetylneuraminic acid moiety from the CMP-NeuNAc to an acceptor molecule, wherein said acceptor molecule is a saccharide molecule, to provide a sialylated saccharide.

[0052] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains at least one sialyltransferase, preferably at least one heterologous sialyltransferase, wherein said sialyltransferase is capable of possessing an α -2,3-sialyltransferase activity and/or an α -2,6-sialyltransferase activity and/or an α -2,8-sialyltransferase activity for transferring the NeuNAc moiety from CMP-NeuNAc as donor substrate to the acceptor saccharide.

[0053] The term “sialyltransferase” as used herein refers to polypeptides being capable of possessing sialyltransferase activity. “Sialyltransferase activity” refers to the transfer of a sialic acid residue, preferably of an *N*-acetylneuraminic acid (Neu5Ac) residue, from a donor substrate to an acceptor molecule. The term “sialyltransferase” comprises functional fragments of the sialyltransferases described herein, functional variants of the sialyltransferases described herein, and functional fragments of the functional variants. “Functional” in this regard means that the fragments and/or variants are capable of possessing sialyltransferase activity. Functional fragments of a sialyltransferase encompass truncated versions of a sialyltransferase as encoded by it naturally occurring gene, which truncated version is capable of possessing sialyltransferase activity. Examples of truncated versions are sialyltransferases which do not comprise a so-called leader sequence which typically directs the polypeptide to a specific subcellular localization. Typically, such leader sequences are removed from the polypeptide during its subcellular transport, and are also absent in the naturally occurring mature sialyltransferase.

[0054] The heterologous sialyltransferase is capable of transferring a sialic acid residue from a donor substrate to an acceptor molecule. The term “capable of” with respect to the heterologous sialyltransferase refers to the sialyltransferase activity of the heterologous sialyltransferase and the provision that suitable reaction conditions are required for the heterologous sialyltransferase to possess its enzymatic activity. In the absence of suitable reaction conditions, the heterologous sialyltransferase does not possess its enzymatic activity, but retains its enzymatic activity and possesses its enzymatic activity when suitable reaction conditions are restored. Suitable reaction conditions include the presence of a suitable donor substrate, the presence of suitable acceptor molecules, the presence of essential cofactors such as – for example – monovalent or

divalent ions, a pH value in an appropriate range, a suitable temperature and the like. It is not necessary that the optimum values for each and every factor effecting the enzymatic reaction of the heterologous sialyltransferase is met, but the reaction conditions have to be such that the heterologous sialyltransferase performs its enzymatic activity. Accordingly, the term “capable of” excludes any conditions upon which the enzymatic activity of the heterologous sialyltransferase has been irreversibly impaired and also excluded exposure of the heterologous sialyltransferase to any such condition. Instead, “capable of” means that the sialyltransferase is enzymatically active, i.e. possesses its sialyltransferase activity, if permissive reactions conditions (where all requirements being necessary for the sialyltransferase to perform its enzymatic activity) are provided to the sialyltransferase.

[0055] Sialyltransferases can be distinguished on the type of sugar linkage they form. As used herein, the terms “ α -2,3-sialyltransferase” and “ α -2,3-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a sialic acid residue with an α -2,3 linkage to galactose, *N*-acetylgalactosamine or a galactose or *N*-acetylgalactosamine residue of the acceptor molecule. Likewise, the terms “ α -2,6-sialyltransferase” and “ α -2,6-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a sialic acid residue with an α -2,6 linkage to galactose, *N*-acetylgalactosamine or a galactose or *N*-acetylgalactosamine residue of the acceptor molecule. Likewise, the terms “ α -2,8-sialyltransferase” and “ α -2,8-sialyltransferase activity” refer to polypeptides and their enzymatic activity which add a sialic acid residue with an α -2,8 linkage to galactose, *N*-acetylgalactosamine or a galactose or *N*-acetylgalactosamine residue of the acceptor molecule.

[0056] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a heterologous sialyltransferase that is preferably selected from the group consisting of

- I. polypeptides comprising or consisting of an amino acid sequence as represented by any one of SEQ ID NOs: 1 to 33;
- II. polypeptides comprising or consisting of an amino acid sequence having a sequence identity of at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% to any one of the amino acid sequences as represented by any one of SEQ ID NOs: 1 to 33;

and

III. fragments of any one of the polypeptides of I. and II.

[0057] In an additional and/or alternative embodiment, the genetically engineered microbial cell has been transformed to contain a nucleic acid molecule which comprises and expresses a nucleotide sequence encoding the heterologous sialyltransferase. Preferably, a nucleotide sequence as can be inferred from Table 1. In an additional and/or alternative embodiment, the nucleotide sequence is selected from the group consisting of

- i. nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 33;
- ii. nucleotide sequences as represented by any one of SEQ ID NOs: 34 to 66;
- iii. nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 33;
- iv. nucleotide sequences having a sequence identity of at least 80 %, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% to any one of the nucleotide sequences represented by SEQ ID NOs: 34 to 66;
- v. nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv; and
- vi. fragments of any one of the nucleotide sequences of i., ii., iii., iv. and v.;

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 in the genetically engineered microbial cell to provide sialyltransferase activity.

Origin of the sialyltransferase gene	accession number of the sialyltransferase gene	Cloned as full length (FL) gene or without signal peptide (Δ)
<i>Neisseria meningitidis</i>	U60660	FL (SEQ ID NO: 5)
<i>Campylobacter jejuni</i> strain OH4384	AF130466	FL (SEQ ID NO: 10)
<i>Campylobacter jejuni</i> strain OH4384	AX934425	FL (SEQ ID NO: 12)

Origin of the sialyltransferase gene	accession number of the sialyltransferase gene	Cloned as full length (FL) gene or without signal peptide (Δ)
<i>Helicobacter acinonychis</i>	NC_008229	FL (SEQ ID NO: 11)
<i>Helicobacter acinonychis</i>	NC_008229	FL (SEQ ID NO: 33)
<i>Photobacterium</i> sp. JT-ISH-224	BAF92026	Δ 17 (SEQ ID NO: 29)
<i>Pasteurella dagmatis</i> strain DSM 22969	AFY98851	FL (SEQ ID NO: 7)
<i>Photobacterium</i> sp. JT-ISH-224	BAF92025	Δ 20 (SEQ ID NO: 3)
<i>Vibrio</i> sp. JT-FAJ-16	BAF91160	Δ 22 (SEQ ID NO: 2)
<i>Pasteurella multocida</i> PM70	AAK02272	Δ 25 (SEQ ID NO: 4)
<i>Photobacterium damsela</i> JT0160	BAA25316	FL (SEQ ID NO: 31)
<i>Streptococcus agalactiae</i>	AB050723	FL (SEQ ID NO: 17)
<i>Haemophilus somnus</i> -2336	ACA31578	FL (SEQ ID NO: 26)
<i>Haemophilus ducreyi</i> 35000HP	AF101047	FL (SEQ ID NO: 14)
<i>Haemophilus ducreyi</i> 35000HP	AAP95068	FL (SEQ ID NO: 27)
<i>Photobacterium phosphoreum</i> JT-ISH-467	BAF63530	Δ 20 (SEQ ID NO: 8)
<i>Photobacterium leiognathi</i> JT-SHIZ-119	AB500947	Δ 15 (SEQ ID NO: 28)
<i>Photobacterium leiognathi</i> JT-SHIZ-145	BAF91416	Δ 15 (SEQ ID NO: 30)
<i>Campylobacter coli</i>	YP_008473374	FL (SEQ ID NO: 1)
<i>Vibrio harveyi</i>	WP_017817635	Δ 24 (SEQ ID NO: 21)
<i>Streptococcus entericus</i>	WP_018369230	FL (SEQ ID NO: 13)
<i>Avibacterium paragallinarum</i>	WP_021724759	FL (SEQ ID NO: 9)
<i>Haemophilus parahaemolyticus</i> HK385	EIJ71207	FL (SEQ ID NO: 19)
<i>Alistipes</i> sp. CAG:268	CDC95697	Δ 17 (SEQ ID NO: 22)
<i>Alistipes</i> sp. AL-1	WP_032134786	FL (SEQ ID NO: 15)
<i>Pasteurella multocida</i> PM70	NC_002663	FL (SEQ ID NO: 6)
<i>Campylobacter jejuni</i> strain 81-176	AAL09368	FL (SEQ ID NO: 16)
<i>Alistipes shahii</i> WAL 8301	YP_007816735	Δ 21 (SEQ ID NO: 23)
<i>Actinobacillus suis</i> ATCC 33415	AIJ32009	FL (SEQ ID NO: 24)
<i>Actinobacillus capsulatus</i> DSM 19761	WP_018652686	FL (SEQ ID NO: 25)
<i>Bibersteinia trehalosi</i> USDA-ARS-USMARC-189	AHG84654	FL (SEQ ID NO: 18)
<i>Photobacterium damsela</i> subsp. <i>damsela</i> CIP 102761	EEZ40509	FL (SEQ ID NO: 32)
<i>Haemophilus somnus</i> 2336	ACA31170	FL (SEQ ID NO: 20)

[0058] Table 1: List of sialyltransferase-encoding nucleotide sequences. The sialyltransferase-encoding nucleotide sequences were either cloned as full length constructs (FL) or without a predicted signal peptide (Δ) as compared to their wild-type protein coding regions. The number behind the Δ indicates the N-terminally amino acids deleted from the corresponding sequence.

[0059] The expression "any one of SEQ ID NOs: 1 to 33" refers to any one of the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, and SEQ ID NO: 33. The same principle applies to the expression "any one of SEQ ID NOs: 34 to 66". Generally speaking, the expression "any one of SEQ ID NOs: X to Z", wherein "X" and "Z" represent a natural number, refers to all sequences (nucleotide sequences or amino acid sequences) represented by any one of the "SEQ ID NOs" comprising an identification number from X to Z.

[0060] In addition, the genetically engineered microbial cell has been genetically engineered to express the nucleotide sequence encoding the heterologous sialyltransferase. To this end, the nucleotide sequence encoding the heterologous sialyltransferase is operably linked to at least one expression control effecting transcription and/or translation of said nucleotide sequence encoding the heterologous sialyltransferase in the genetically engineered cell.

[0061] The term "operably linked" as used herein, refers to a functional linkage between the nucleotide sequence encoding the heterologous sialyltransferase and a second nucleotide sequence, the nucleic acid expression control sequence (such as promoter, operator, enhancer, regulator, array of transcription factor binding sites, transcriptional terminator, ribosome binding site), wherein the expression control sequence affects transcription and/or translation of the nucleic acid corresponding to the nucleotide sequence encoding the heterologous sialyltransferase. Accordingly, the term "promoter"

designates DNA sequences which usually "precede" a gene in a DNA polymer and provide a site for initiation of the transcription into mRNA. "Regulator" DNA sequences, also usually "upstream" of (i.e., preceding) a gene in a given DNA polymer, bind proteins that determine the frequency (or rate) of transcriptional initiation. Collectively referred to as "promoter/regulator" or "control" DNA sequence, these sequences which precede a selected gene (or series of genes) in a functional DNA polymer cooperate to determine whether the transcription (and eventual expression) of a gene will occur. DNA sequences which "follow" a gene in a DNA polymer and provide a signal for termination of the transcription into mRNA are referred to as transcription "terminator" sequences.

[0062] In an additional and/or alternative embodiment, the heterologous sialyltransferase being capable of possessing α -2,3-sialyltransferase activity is selected from the group consisting of

- I. polypeptides comprising or consisting of an amino acid sequence as represented by any one of SEQ ID NOs: 1 to 27;
- II. polypeptides comprising or consisting of an amino acid sequence having an identity of at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% to any of the amino acid sequences as represented by any one of SEQ ID NOs: 1 to 27; and
- III. fragments of any one of the polypeptides of I. and II.

[0063] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule which comprises at least one nucleotide sequence encoding said heterologous sialyltransferase being capable of possessing α -2,3-sialyltransferase activity, wherein said at least one nucleotide sequence is selected from the group consisting of

- i. nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 27;
- ii. nucleotide sequences as represented by any one of SEQ ID NOs: 34 to 60;
- iii. nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 1 to 27;

- iv. nucleotide sequences having a sequence identity of at least 80 %, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% to any one of the nucleotide sequences represented by SEQ ID NOs: 34 to 60;
 - v. nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv; and
 - vi. fragments of any one of the nucleotide sequences of i., ii., iii., iv. and v.;
- 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 in the genetically engineered cell to provide α -2,3-sialyltransferase activity.

[0064] In an additional and/or alternative embodiment, the heterologous sialyltransferase being capable of possessing α -2,3-sialyltransferase activity has a relative efficacy of at least 100-fold, at least 200-fold, at least 300-fold, at least 1000-fold, at least 10,000-fold, as compared to the relative efficacy of the sialyltransferase as represented by SEQ ID NO: 27 by means of quantitative analysis of LNT sialylation using LC-MS/MS.

[0065] In another embodiment, the heterologous sialyltransferase is capable of possessing α -2,6-sialyltransferase activity.

- [0066]** In an additional embodiment, the heterologous sialyltransferase being capable of possessing α -2,6-sialyltransferase activity is selected from the group consisting of
- I. polypeptides comprising or consisting of an amino acid sequence as represented by any one of SEQ ID NOs: 28 to 33;
 - II. polypeptides comprising or consisting of an amino acid sequence having an identity of at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% to any of the amino acid sequences as represented by any one of SEQ ID NOs: 28 to 33; and
 - III. fragments of any one of the polypeptides of I. and II.

[0067] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule which comprises at least one nucleotide sequence encoding said heterologous sialyltransferase being capable of possessing α -2,6-sialyltransferase activity, wherein said at least one nucleotide sequence is selected from the group consisting of

- i. nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 28 to 33;
 - ii. nucleotide sequences as represented by any one of SEQ ID NOs: 61 to 66;
 - iii. nucleotide sequences having at least 80%, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% sequence identity to one of the nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 28 to 33;
 - iv. nucleotide sequences having a sequence identity of at least 80 %, 90%, 95%, 96%, 97%, 98%, 99% or more than 99% to any one of the nucleotide sequences represented by SEQ ID NOs: 61 to 66;
 - v. nucleotide sequences which are complementary to any one of the nucleotide sequences of i., ii., iii. and iv; and
 - vi. fragments of any one of the nucleotide sequences of i., ii., iii., iv. and v.;
- 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 in the genetically engineered cell to provide α -2,6-sialyltransferase activity.

[0068] In an additional and/or alternative embodiment, the heterologous sialyltransferase being capable of possessing α -2,6-sialyltransferase activity has a relative efficacy of at least 100-fold, more preferably of at least 200-fold, most preferably of at least 300-fold, as compared to the relative efficacy of the sialyltransferase as represented by SEQ ID NO: 33 by means of quantitative analysis of LNT sialylation.

[0069] In an additional and/or alternative embodiment, the heterologous sialyltransferase is capable of possessing α -2,8-sialyltransferase activity. An example of a heterologous sialyltransferase is capable of possessing α -2,8-sialyltransferase activity is the sialyltransferase CstII of *Campylobacter jejunii* OH4384.

[0070] The sialyltransferase is capable of transferring a sialic acid residue, e.g. a *N*-acetylneuraminic acid (Neu5Ac) residue, from a donor substrate, e.g. CMP-Neu5Ac, to an acceptor molecule. The acceptor molecule is a saccharide molecule, preferably a saccharide molecule set forth in Table 2.

Name	Abbreviation	Structure
<i>N</i> -acetylglucosamine	GlcNAc	GlcNAc
Galactose	Gal	Gal
<i>N</i> -acetylgalactosamine	GalNAc	GalNAc
Lactose	Lac	Gal(β1,4)Glc
<i>N</i> -acetylactosamine	LacNAc	Gal(β1,4)GlcNAc
Lacto- <i>N</i> -biose	LNB	Gal(β1,3)GlcNAc
Lactulose	LacU	Gal(β1,4)Frc
Melibiose	Mel	Gal(α1,6)Glc
Raffinose	Raf	Gal(α1,6)Glc(α1,2)Frc
2'-Fucosyllactose	2'-FL	Fuc(α1,2)Gal(β1,4)Glc
3-Fucosyllactose	3-FL	Gal(β1,4)[Fuc(α1,3)]Glc
2',3-Difucosyllactose	DFL	Fuc(α1,2)Gal(β1,4)[Fuc(α1,3)]Glc
6'-Galactosyllactose	6'-GL	Gal(β1,6)Gal(β1,4)Glc
3'-Galactosyllactose	3'-GL	Gal(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -triose II	LNT II	GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -tetraose	LNT	Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -neotetraose	LNnT	Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -fucopentaose I	LNFP I	Fuc(α1,2)Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -neofucopentaose I	LNnFP I	Fuc(α1,2)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -fucopentaose II	LNFP II	Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -fucopentaose III	LNFP III	Gal(β1,4)[Fuc(α1,3)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -fucopentaose V	LNFP V	Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto- <i>N</i> -neofucopentaose V	LNnFP V	Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto- <i>N</i> -difucohexaose I	LNDH I	Fuc(α1,2)Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)Glc
Lacto- <i>N</i> -difucohexaose II	LND	Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto- <i>N</i> -neodifucohexaose I	LNnDFH I	Gal(β1,4)[Fuc(α1,3)]GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Lacto- <i>N</i> -hexaose	LNH	Gal(β1,4)GlcNAc(β1,6)[Gal(β1,3)GlcNAc(β1,3)]Gal(β1,4)Glc
Lacto- <i>N</i> -neohexaose	LNnH	Gal(β1,4)GlcNAc(β1,6)[Gal(β1,4)GlcNAc(β1,3)]Gal(β1,4)Glc
<i>para</i> -Lacto- <i>N</i> -hexaose	<i>para</i> LNT	Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
<i>para</i> -Lacto- <i>N</i> -neohexaose	<i>para</i> LNnH	Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
3'-Sialyllactose	3'-SL	Neu5Ac(α2,3)Gal(β1,4)Glc
6'-Sialyllactose	6'-SL	Neu5Ac(α2,6)Gal(β1,4)Glc
Sialyllacto- <i>N</i> -tetraose a	LSTa	Neu5Ac(α2,3)Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)Glc
Sialyllacto- <i>N</i> -tetraose b	LSTb	Gal(β1,3)[Neu5Ac(α2,6)]GlcNAc(β1,3)Gal(β1,4)Glc
Sialyllacto- <i>N</i> -tetraose c	LSTc	Neu5Ac(α2,6)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc
Fucosyllacto- <i>N</i> -sialylpentaose a	F-LST-a	Neu5Ac(α2,3)Gal(β1,3)[Fuc(α1,4)]GlcNAc(β1,3)Gal(β1,4)Glc
Fucosyllacto- <i>N</i> -sialylpentaose b	F-LST-b	Fuc(α1,2)Gal(β1,3)[Neu5Ac(α2,6)]GlcNAc(β1,3)Gal(β1,4)Glc
Fucosyllacto- <i>N</i> -sialylpentaose c	F-LST-c	Neu5Ac(α2,3)Gal(β1,3)GlcNAc(β1,3)Gal(β1,4)[Fuc(α1,3)]Glc
Disialyllacto- <i>N</i> -tetraose	DS-LNT	Neu5Ac(α2,3)Gal(β1,3)[Neu5Ac(α2,6)]GlcNAc(β1,3)Gal(β1,4)Glc
3-Fucosyl-3'-sialyllactose	3F-3'-SL	Neu5Ac(α2,3)Gal(β1,4)[Fuc(α1,3)]Glc
3-Fucosyl-6'-sialyllactose	3F-6'-SL	Neu5Ac(α2,6)Gal(β1,4)[Fuc(α1,3)]Glc
3'-sialyl- <i>N</i> -acetylactosamine	3'-SLN	Neu5Ac(α2,3)Gal(β1,4)GlcNAc

Name	Abbreviation	Structure
6'-sialyl- <i>N</i> -acetylglucosamine	6'-SLN	Neu5Ac(α 2,6)Gal(β 1,4)GlcNAc

[0071] Table 2: List of saccharides that may be used as acceptor substrate for the production of a sialylated saccharide. The sialylated saccharide itself may also be used as acceptor substrate for the production of a further sialylated saccharide.

[0072] In an additional and/or alternative embodiment, the acceptor molecule is a monosaccharide, preferably a monosaccharide selected from the group consisting of *N*-acetylglucosamine, galactose and *N*-acetylgalactosamine.

[0073] In an additional and/or alternative embodiment, the acceptor molecule is a disaccharide, preferably a disaccharide selected from the group consisting lactose, lactulose, *N*-acetylglucosamine, lacto-*N*-biose, lactulose and melibiose.

[0074] In an additional and/or alternative embodiment, the acceptor molecule is a trisaccharide, preferably a trisaccharide selected from the group consisting of raffinose, lacto-*N*-triose II, 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-*N*-acetylglucosamine, 6'-sialyl-*N*-acetylglucosamine, 3'-galactosyllactose and 6'-galactosyllactose.

[0075] In an additional and/or alternative embodiment, the acceptor molecule is a tetrasaccharide, preferably a tetrasaccharide selected from the group consisting of lacto-*N*-tetraose, lacto-*N*-neotetraose, 2'3-difucosyllactose, 3-fucosyl-3'-sialyllactose and 3-fucosyl-6'-sialyllactose.

[0076] In an additional and/or alternative embodiment, the acceptor molecule is a pentasaccharide, preferably a pentasaccharide selected from the group consisting of sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, lacto-*N*-fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N*-neofucopentaose I and lacto-*N*-neofucopentaose V.

[0077] The term “functional variant” as used herein, with respect to an enzyme as mentioned herein, refers to polypeptide variants of the designated enzymes without loss of activity, and which share at least 70%, preferably at least 80%, at least 85%, at least 90 %, at least 95%, at least 98% or least 99% identity with the amino acid sequence of the designated enzyme. This takes into account the possibility of some variability in the genomic sequence data from which these polypeptides are derived, and also the possibility that some of the amino acids present in these polypeptides can be substituted without significantly affecting the enzyme’s catalytic activity.

[0078] The term “functional variant” also includes polypeptide variants of the designated enzymes which represent truncated variants of the enzyme without significant loss of the catalytic activity. Thus, the amino acid sequence of the truncated variants may differ from the amino acid sequences of the designated enzyme in that one, two or a stretch of more than two consecutive amino acids are absent. The truncation may be at the amino terminus (N-terminus), at the carboxyl terminus (C-terminus) and/or within the amino acid sequence of the designated enzyme.

[0079] The term “operably linked” refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, signal sequence, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence affects transcription and/or translation of the nucleic acid corresponding to the second sequence.

[0080] It is to be understood that a microbial cell already carrying one or more genes encoding said enzymes, and expressing said genes in a manner sufficient to produce NeuNAc, CMP-NeuNAc and/or the sialylated saccharide does not need to be genetically engineered to complete sialic acid biosynthesis and to transfer a sialic acid moiety to a saccharide acceptor, but may nevertheless be genetically engineered to alter the expression level of one or more of said genes to increase the intracellular level of said one or more gene products such as – for example the quantity of glutamine:fructose-6-phosphate aminotransferase, glucosamine-6-phosphate *N*-acetyltransferase, *N*-acetylglucosamine-6-phosphate phosphatase, *N*-acetylglucosamine 2-epimerase and/or *N*-acetylneuraminic acid synthase, thus increasing the rate of Neu5Ac biosynthe-

sis and, as a consequence, of the sialylated saccharide, in the genetically engineered cell.

[0081] In an additional and/or alternative embodiment, the genetically engineered microbial cell synthesizes more PEP than the wildtype of the cell. In an additional and/or alternative embodiment, the genetically engineered microbial cell has been genetically engineered to possess an enhanced PEP biosynthesis pathway. Preferably, the genetically engineered microbial cell has been genetically engineered to possess an increased phosphoenolpyruvate synthase activity, for example in that the *ppsA* gene encoding phosphoenolpyruvate synthase gene is overexpressed and/or in that the non-naturally-occurring microorganisms contains at least one additional copy of a nucleotide sequence allowing the expression of a phosphoenolpyruvate synthase or a functional variant thereof. Overexpression of *ppsA* enhances intracellular PEP synthesis such that more PEP is available for the production of sialic acid. For example, a suitable phosphoenolpyruvate synthase is PpsA of *E. coli*.

[0082] In an additional and/or alternative embodiment, the genetically engineered microbial cell contains a nucleic acid molecule comprising a nucleotide sequence encoding *E. coli* PpsA or a functional variant thereof. Said nucleotide sequence encoding *E. coli* PpsA or a functional variant thereof has a sequence identity of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to the *E. coli ppsA* gene.

[0083] In an additional and/or alternative embodiment, the genetically engineered microbial cell additionally comprises one or more genes encoding for a polypeptide being capable of possessing an enzymatic activity selected from the group consisting of sucrose permease, sucrose hydrolase, fructokinase, L-glutamine:D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate-*N*-acetyltransferase, *N*-acetylglucosamine-2-epimerase, sialic acid synthase, phosphoenolpyruvate synthase, wherein preferably at least one of these genes, preferably all, is/are overexpressed in the genetically engineered microbial cell as compared to the wild-type microbial cell.

[0084] In an additional and/or alternative embodiment, a sialic acid catabolic pathway that naturally occurs in the progenitor cell line of the genetically engineered microbial cell has been disabled in the genetically engineered microbial cell.

[0085] In an additional and/or alternative embodiment of the method and the genetically engineered microbial cell, the genetically engineered microbial cell lacks or possesses a decreased activity, as compared to the progenitor cell of the genetically engineered microbial cell, of one or more enzymatic activities selected from the group consisting of α -N-acetylgalactosaminidase (e.g. NagA), N-acetylglucosaminekinase (e.g. NagK), N-acetylneuraminate lyase (= N-acetylneuraminic acid aldolase, e.g. NanA), β -galactosidase, a glucosamine-6-phosphate deaminase, a N-acetylglucosamine-6-phosphate deacetylase, a N-acetylmannosamine kinase and/or a N-acetylmannosamine-6-phosphate epimerase.

[0086] In an additional and/or alternative embodiment of the method and the genetically engineered microbial cell, the genetically engineered microbial cell additionally comprises one or more genes encoding for a polypeptide being capable of possessing an enzymatic activity selected from the group consisting of N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyl transferase, phosphoglucosamine mutase, UDP-N-acetylglucosamine-2-epimerase, 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-guanylyltransferase.

[0087] In an additional and/or alternative embodiment, the genetically engineered microbial cell comprises at least one selected from the group consisting of a functional lactose permease, a functional sialic acid transporter (exporter), wherein preferably comprises and expresses at least one nucleotide sequence encoding one selected from the group consisting of a functional lactose permease, a functional sucrose permease, a functional sialic acid transporter (exporter), wherein preferably at least one of these nucleotide sequences is overexpressed in the cell.

[0088] In an additional and/or alternative embodiment, the genetically engineered microbial cell is further modified to be capable to transfer said sole carbon source into the cell via a mechanism, that is not consuming PEP.

[0089] In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses a functional sucrose utilization system. Said functional sucrose utilization system enables cellular import of exogenously supplied sucrose and its hydrolysis such that the resulting monosaccharides glucose and fructose can be metabolically utilized by the genetically engineered cell's metabolism and for the desired sialylated oligosaccharide production.

[0090] In an additional and/or alternative embodiment, the genetically engineered microbial cell has been genetically modified to possess a functional sucrose utilization system. In an additional and/or alternative embodiment the sucrose utilization system of the non-naturally-occurring microorganism comprises a sucrose proton symport transport system, a fructokinase, an invertase and a sucrose operon repressor.

[0091] A suitable a sucrose proton symport transport system is CscB, encoded by the *cscB* gene, for example CscB of *E. coli* (UniProtKB – P30000) as encoded by the *cscB* gene of *E. coli*.

[0092] A suitable fructokinase (EC 2.7.1.4) is CscK, encoded by the *cscK* gene, for example CscK of *E. coli* (UniProtKB – P40713) as encoded by the *cscK* gene of *E. coli*.

[0093] A suitable invertase (EC 3.2.1.26) which hydrolysis terminal non-reducing β -D-fructofuranoside residues in β -D-fructofuranosides is CscA, for example CscA of *E. coli* (UniProtKB – O86076) as encoded by the *cscA* gene of *E. coli*.

[0094] A suitable sucrose operon repressor is CscR as encoded by the *cscR* gene, for example the CscR of *E. coli* (UniProtKB – P62604) as encoded by the *cscR* gene of *E. coli*.

[0095] In an additional and/or alternative embodiment, the genetically engineered cell has been genetically engineered to possess a sucrose proton symport transport system, a fructokinase, an invertase and a sucrose operon repressor or functional variants of any one of these proteins.

[0096] In an additional and/or alternative embodiment, the genetically engineered cell has been genetically engineered to possess a nucleic acid molecule comprising nucleotide sequences encoding a sucrose proton symport transport system, a fructokinase, an invertase and a sucrose operon repressor for the expression of said sucrose proton symport transport system, fructokinase, invertase and sucrose operon repressor. In an additional and/or alternative embodiment, the genetically engineered cell has been genetically engineered to express the genes *cscB*, *cscK*, *cscA*, preferably the *E. coli* genes *cscB*, *cscK*, *cscA* and *cscR*.

[0097] In an additional and/or alternative embodiment, the nucleotide sequence encoding a functional variant of *CscB*, *CscK*, *CscA* or *CscR* has a sequence identity or at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% to *E. coli cscB*, *cscK*, *cscA* or *cscR*, respectively.

[0098] In an additional and/or alternative embodiment, the non-naturally-occurring microorganism expresses a β -galactoside permease and a β -galactosidase.

[0099] In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to express a β -galactoside permease, preferably the *E. coli* lactose permease *LacY* (SEQ ID NO: 93) or a functional variant thereof and a β -galactosidase, preferably *E. coli LacZ* (SEQ ID NO: 95) or a functional variant thereof. In an additional and/or alternative embodiment, the non-naturally-occurring microorganism has been genetically engineered to carry a nucleic acid molecule comprising a nucleotide sequence encoding a β -galactoside permease, preferably a nucleotide sequence encoding the *E. coli LacY* (SEQ ID NO: 94) or a functional variant thereof, and/or a nucleotide sequence encoding a β -galactosidase, prefer-

ably a nucleotide sequence encoding *E. coli* LacZ (SEQ ID NO: 96) or a functional variant thereof.

[00100] In an additional and/or alternative embodiment, the nucleotide sequence encoding *E. coli* LacY or a functional variant thereof has a sequence identity to *E. coli lacY* of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%.

[00101] In an additional and/or alternative embodiment, the nucleotide sequence encoding *E. coli* LacZ or a functional variant thereof has a sequence identity to *E. coli lacZ* of at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%.

[00102] A non-naturally-occurring microorganism that can produce CMP-Neu5Ac, and which expresses a functional β -galactoside permease and a functional β -galactosidase allows the cultivation of said non-naturally-occurring microorganism on lactose as a sole carbon source.

[00103] The genetically engineered microbial cell that can produce sialylated saccharides may – optionally – include additional features, and may be genetically engineered to possess these additional features. These additional features are considered to improve the productivity of the non-naturally-occurring microorganism leading to higher sialylated saccharide yields.

[00104] In an additional and/or alternative embodiment the genetically engineered microbial cell has been genetically engineered to abolish UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase activity, preferably by deleting the *wcaJ* gene or a functional variant thereof, by impairing expression of the *wcaJ* gene or a functional variant thereof, or by abolishing the activity of the WcaJ enzyme by introducing mutations into the protein-coding region of the such that the polypeptide encoded by the altered nucleotide sequence does not possess enzymatic activity of WcaJ. WcaJ encodes a UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase. Said

UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase is the first enzyme in colanic acid biosynthesis.

[00105] In an additional and/or alternative embodiment, the genetically engineered microbial cell has been genetically engineered in that the β -galactosidase gene (*lacZ*) has been deleted, in that the expression of the β -galactosidase gene is impaired or in that the nucleotide sequence of the protein coding region of the β -galactosidase gene is amended such that the polypeptide being encoded by said altered nucleotide sequence(s) does not possess the enzymatic activity of the β -galactosidase.

[00106] In an additional and/or alternative embodiment, the genetically engineered microbial cell has been genetically engineered in that the gene encoding the galactose kinase (e.g. *galK* gene) has been deleted, in that the expression of the *galK* gene is impaired or in that the nucleotide sequence of the protein coding region of the *galK* gene is amended such that the polypeptide being encoded by said altered nucleotide sequence(s) does not possess the enzymatic activity of the galactose kinase. Deletion or inactivation of the *galK* gene / GalK is advantageous in that the genetically engineered microbial cell can utilize galactose as an acceptor substrate for sialylation reactions only.

[00107] In an additional and/or alternative embodiment, the genetically engineered microbial cell has been genetically engineered in that the gene encoding the *N*-acetylgalactosaminidase (*nagA*) has been deleted, that its expression has been impaired or in that the nucleotide sequence of the protein coding region is amended such that the polypeptide being encoded by said altered nucleotide sequence(s) does not possess the enzymatic activity of an *N*-acetylgalactosaminidase. Deletion or inactivation of *nagA* / NagA is advantageous in that the genetically engineered microbial cell can utilize GlcNAc or GlcNAc-6-phosphate as an acceptor for sialylation reactions only.

[00108] In an additional and/or alternative embodiment the genetically engineered microbial cell has been genetically engineered to abolish fucose isomerase activity, preferably by the deletion the *fucI* gene, by impairing expression of the *fucI*

gene, or by modifying the protein-coding region of the *fucl* gene such that the polypeptide being encoded by said altered nucleotide sequence does not possess fucose isomerase activity. For example, the *E. coli* L-fucose isomerase FucI (UniProtKB – P69922) is encoded by the *E. coli fucl* gene.

[00109] Fuculokinase catalyzes the phosphorylation of fucose. Fuculokinase is the second enzyme in the subpathway that synthesizes L-lactaldehyde and glycerone phosphate from L-fucose. The *E. coli* fuculokinase FucK (UniProtKB – P11553) is encoded by the *E. coli fucK* gene. *E. coli* fuculokinase can also phosphorylate, with lower efficiency, D-ribulose, D-xylulose and D-fructose.

[00110] In an additional and/or alternative embodiment the genetically engineered cell has been genetically engineered to abolish fucose isomerase activity, preferably by the deletion of the *fucK* gene or, by impairing expression of the *fucK* gene, or by introducing mutations into the protein-coding region of the *fucK* gene such that the polypeptide being encoded by said altered nucleotide sequence does not possess fucose isomerase activity.

[00111] *N*-acetylgalactosamine-6-phosphate deacetylase catalyzes the following reaction: *N*-acetyl-D-galactosamine 6-phosphate + H₂O → D-galactosamine 6-phosphate + acetate. *N*-acetylgalactosamine-6-phosphate deacetylase is encoded by the *agaA* gene. In *E. coli* the *N*-acetylgalactosamine-6-phosphate deacetylase AgaA (UniProtKB – P42906) is encoded by the *E. coli agaA* gene.

[00112] In an additional and/or alternative embodiment the genetically engineered microbial cell has been genetically engineered to abolish *N*-acetylgalactosamine-6-phosphate deacetylase activity, preferably by deletion of the *agaA* gene, by impairing expression of the *agaA* gene, or by introducing mutations into the protein-coding region of the *agaA* gene such that the polypeptide being encoded by said altered nucleotide sequence does not possess *N*-acetylgalactosamine-6-phosphate deacetylase activity.

[00113] In an additional and/or alternative embodiment, the at least one genetically engineered microbial cell possesses an increased production of one or more nucleotide-activated sugars selected from the group consisting of UDP-*N*-acetylglucosamine, UDP-galactose and GDP-fucose. Preferably, the at least one genetically engineered microbial cell has been further genetically engineered to possess an increased production of one or more of said nucleotide-activated sugars. The production of the at least one of said nucleotide activated sugars is increased in the further genetically engineered cell as compared to the production of the same nucleotide-activated sugar(s) in the progenitor cell of the further genetically engineered microbial cell prior to being further genetically engineered to possess an increased production of at least one of said nucleotide-activated sugars.

[00114] In an additional and/or alternative embodiment, the at least one microbial cell has been further genetically engineered to overexpress 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 uridylyltransferase, glucosamine-1-phosphate acetyl transferase, phosphoglucosamine mutase, 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 fucose kinase/L-fucose-1-phosphate-guanylyltransferase.

[00115] Presently, and as understood in the general field, and here with respect to every polynucleotide or nucleic acid discussed herein respectively, said overexpression of one or more genes or polypeptides is an overexpression as compared to the progenitor cell of the further genetically engineered microbial cell prior to being further genetically engineered to possess overexpression of said one or more genes or polypeptides.

[00116] Overexpression of one or more of said genes increases the amount of the corresponding polypeptides, i.e. enzyme(s), in the genetically engineered microbial

cell, and hence increases the corresponding enzymatic activity in the cell to enhance intracellular production of sialylated saccharides.

[00117] In an additional and/or alternative embodiment, the at least one genetically engineered cell lacks or possesses a decreased activity of one or more enzymatic activities selected from the group consisting of β -galactosidase activity, glucosamine-6-phosphate deaminase, *N*-acetylglucosamine-6-phosphate deacetylase, *N*-acetylmannosamine kinase, *N*-acetylmannosamine-6-phosphate epimerase and *N*-acetylneuraminic acid aldolase as compared to the cell prior to be genetically engineered.

[00118] In an additional and/or alternative embodiment, 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/have been deleted from the genome of the genetically engineered cell or the expression of 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/have been inactivated or at least decrease in the genetically engineered cell by further genetically engineering of cell. The expression of said genes is decreased in the further genetically engineered cell as compared to the progenitor cell of the further genetically engineered cell prior to being further genetically engineered to possess a decreased expression of said genes.

[00119] The genetically engineered microbial cell, preferably a prokaryotic cell. Appropriate microbial cells include yeast cells, bacterial cells, archaebacterial cells, algae cells, and fungal cells.

[00120] In an additional and/or alternative embodiment, the genetically engineered microbial cell is a bacterial cell, preferably a bacterial cell selected from the group consisting of *Bacillus*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Bifidobacterium*, *Sporolactobacillus* spp., *Micromonospora* spp., *Micrococcus* spp., *Rhodococcus* spp., and

Pseudomonas. Suitable bacterial species are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus thermophilus*, *Bacillus laterosporus*, *Bacillus megaterium*, *Bacillus mycoides*, *Bacillus pumilus*, *Bacillus lentus*, *Bacillus cereus*, *Bacillus circulans*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Citrobacter freundii*, *Clostridium cellulolyticum*, *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Clostridium acetobutylicum*, *Corynebacterium glutamicum*, *Enterococcus faecium*, *Enterococcus thermophilus*, *Escherichia coli*, *Erwinia herbicola* (*Pantoea agglomerans*), *Lactobacillus acidophilus*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus jensenii*, *Lactococcus lactis*, *Pantoea citrea*, *Pectobacterium carotovorum*, *Propionibacterium freudenreichii*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Streptococcus thermophilus* and *Xanthomonas campestris*.

[00121] In an alternative embodiment, the genetically engineered cell is a yeast cell, preferably selected from the group consisting of *Saccharomyces* sp., in particular *Saccharomyces cerevisiae*, *Saccharomycopsis* sp., *Pichia* sp., in particular *Pichia pastoris*, *Hansenula* sp., *Kluyveromyces* sp., *Yarrowia* sp., *Rhodotorula* sp., and *Schizosaccharomyces* sp.

[00122] The genetically engineered cell has been genetically engineered to comprise a NeuNAc biosynthesis pathway, a cytidine 5'-monophospho- (CMP)-sialic acid synthetase activity, and a sialyltransferase activity.

[00123] The term "genetically engineered" as used herein refers to the modification of the microbial cell's genetic make-up using molecular biological methods. The modification of the microbial cell's genetic make-up may include the transfer of genes within and/or across species boundaries, inserting, deleting, replacing and/or modifying nucleotides, triplets, genes, open reading frames, promoters, enhancers, terminators and other nucleotide sequences mediating and/or controlling gene expression. The modification of the microbial cell's genetic make-up aims to generate a genetically modified organism possessing particular, desired properties. Genetically engineered microbial cells can contain one or more genes that are not present in the native (not genetically

engineered) form of the cell. Techniques for introducing exogenous nucleic acid molecules and/or inserting exogenous nucleic acid molecules (recombinant, heterologous) into a cell's hereditary information for inserting, deleting or altering the nucleotide sequence of a cell's genetic information are known to the skilled artisan. Genetically engineered microbial cells can contain one or more genes that are present in the native form of the cell, wherein said genes are modified and re-introduced into the microbial cell by artificial means. The term "genetically engineered" also encompasses microbial cells that contain a nucleic acid molecule being endogenous to the cell, and that has been modified without removing the nucleic acid molecule from the cell. Such modifications include those obtained by gene replacement, site-specific mutations, and related techniques.

[00124] The term "heterologous" as used herein refers to a polypeptide, amino acid sequence, nucleic acid molecule or nucleotide sequence that is foreign to a cell or organism, i.e. to a polypeptide, amino acid sequence, nucleic acid molecule or nucleotide sequence that does not naturally occurs in said cell or organism. A "heterologous sequence" or a "heterologous nucleic acid" or "heterologous polypeptide", as used herein, is one that originates from a source foreign to the particular host cell (e.g. from a different species), or, if from the same source, is modified from its original form. Thus, a heterologous nucleic acid operably linked to a promoter is from a source different from that from which the promoter was derived, or, if from the same source, is modified from its original form. The heterologous sequence may be stably introduced, e.g. by transfection, transformation, conjugation or transduction, into the genome of the host microbial host cell, thus representing a genetically modified host cell. Techniques may be applied which will depend on the host cell the sequence is to be introduced. Various techniques are known to a person skilled in the art and are, e.g., disclosed in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Accordingly, a "heterologous polypeptide" is a polypeptide that does not naturally occur in the cell, and a "heterologous sialyltransferase" is a sialyltransferase that does not naturally occur in the microbial cell.

[00125] In an aspect, provided is a method by means of which a sialylated saccharide can be produced by fermentation, i.e. by means of whole cell biocatalysis, using a genetically engineered microbial cell as set forth herein before. The production of said

sialylated saccharide does not require addition of *N*-acetylglucosamine, *N*-acetylmannosamine and/or *N*-acetylneuraminic acid to the fermentation broth and/or cultivating the genetically engineered microbial cell in the presence of *N*-acetylglucosamine, *N*-acetylmannosamine and/or *N*-acetylneuraminic acid for the intracellular biosynthesis of the sialylated saccharide.

[00126] In the method, the at least one genetically engineered microbial cell is cultivated in a fermentation broth and under conditions which are permissive for the production of the saccharide comprising at least one *N*-acetylneuraminic acid moiety.

[00127] In an additional and/or alternative embodiment, 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.

[00128] While the process and the genetically modified/engineered microbial cell employs a carbon source in the fermentation broth, it is not necessary to add glucosamine and/or *N*-acetylneuraminic acid and/or *N*-acetylglucosamine and/or *N*-acetylmannosamine to the fermentation broth, since the *N*-acetylneuraminic acid is produced intracellularly by the genetically engineered microbial cell. Thus, in an additional and/or alternative embodiment, the at least one genetically engineered microbial cell is cultivated in the absence of and/or without addition of one or more selected from the group consisting of glucosamine, *N*-acetylglucosamine, *N*-acetylmannosamine and *N*-acetylneuraminic acid. The genetically engineered microbial cell may be cultivated in the absence and/or without addition of galactose, as far as galactose is not supplied as an acceptor substrate for the sialyltransferase reaction. In an additional and/or alternative embodiment, the at least one genetically engineered microbial cell is cultivated in the presence of one or more monosaccharides (e.g. galactose), disaccharides (e.g. lactose), trisaccharides (e.g. lacto-*N*-triose II), tetrasaccharides (e.g. lacto-*N*-tetraose) and/or pentasaccharides (e.g. sialyllacto-*N*-tetraose a).

[00129] According to an additional and/or alternative embodiment, the at least one genetically engineered microbial cell is cultivated in the presence of at least one acceptor substrate selected from the group consisting of galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, lactose, lactulose, *N*-acetyllactosamine, lacto-*N*-biose, lacto-*N*-triose, 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-*N*-acetyllactosamine, 6'-sialyl-*N*-acetyllactosamine, 3'-galactosyllactose, 6'-galactosyllactose, lacto-*N*-triose II, lacto-*N*-tetraose, lacto-*N*-neotetraose, 2'3-difucosyllactose, 3-fucosyl-3'-sialyllactose and 3-fucosyl-6'-sialyllactose. These substrates are imported into the cell and used as acceptor molecules in the cell.

[00130] The genetically engineered cell requires a carbon source for growth, proliferation and production of sialylated oligosaccharides. In an additional and/or alternative embodiment, the genetically engineered cell may grow on an inexpensive sole carbon source, such as – for example – glycerol, glucose or sucrose. Said sole carbon source provides an educt for CMP-sialic acid biosynthesis in the genetically engineered cell. Hence, for the production of sialylated oligosaccharides, it is not necessary to cultivate the genetically engineered cell in the presence of Neu5Ac, ManNAc, GlcNAc or glucosamine (GlcN).

[00131] The method comprises the optional step of recovering the sialylated saccharide that has been produced by the at least one genetically engineered microbial cell during its cultivation in the fermentation broth. The sialylated saccharide can be recovered from the fermentation broth after the genetically engineered microbial cells have been removed, for example by centrifugation, and/or can be recovered from the cells, for example in that the cells are harvested from the fermentation broth by centrifugation and are subjected to a cell lysis step. Subsequently, the sialylated saccharides can be further purified from the fermentation broth and/or cell lysates by suitable techniques known to the skilled artisan. Suitable techniques include microfiltration, ultrafiltration, diafiltration, simulated moving bed type chromatography, electrodialysis, reverse osmosis, gel filtration, anion exchange chromatography, cation exchange chromatography, and the like.

[00132] The method and the genetically engineered microbial cell that is employed in the method are used for the production of a sialylated saccharide. The term “sialylated saccharide” refers to a saccharide molecule comprising at least one *N*-acetylneuraminic acid moiety.

[00133] In an additional and/or alternative embodiment, the sialylated saccharide is an oligosaccharide. The term “oligosaccharide” as used herein refers to polymers of monosaccharide residues, wherein said polymers comprise at least two monosaccharide residues, but no more than 10 monosaccharide residues, preferably no more than 7 monosaccharide residues. The oligosaccharides are either a linear chain of monosaccharides or are branched. In addition, the monosaccharide residues of the oligosaccharides may feature a number of chemical modifications. Accordingly, the oligosaccharides may comprise one or more non-saccharide moieties. The term “sialylated oligosaccharide” as used herein refers to oligosaccharides comprising one or more *N*-acetylneuraminic acid moieties.

[00134] According to additional and/or alternative embodiment, the sialylated oligosaccharide is selected from the group consisting of 3'-sialyllactose, 6'-sialyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, fucosyl-sialyllacto-*N*-tetraose a, fucosyl-sialyllacto-*N*-tetraose b, fucosyl-sialyllacto-*N*-tetraose c, disialyllacto-*N*-tetraose, fucosyldisialyllacto-*N*-tetraose I, fucosyldisialyllacto-*N*-tetraose II, 3'-sialylgalactose, 6'-sialylgalactose, 3'-sialyl-*N*-acetyllactosamine and 6'-sialyl-*N*-acetyllactosamine.

[00135] In another aspect of the invention, the use of a genetically engineered microbial cell as described herein before for the production of a sialylated saccharide in a whole cell fermentation process is provided, i.e. the sialylated saccharide is synthesized by the genetically engineered microbial cell.

[00136] In another aspect of the invention, a sialylated saccharide is provided that has been produced by the method and/or by using the genetically engineered microbial cell as described herein before. In an additional and/or alternative embodiment,

the sialylated saccharide is a sialylated oligosaccharide, preferably a sialylated oligosaccharide selected from the group consisting of 3'-sialyllactose, 6'-sialyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, fucosyl-sialyllacto-*N*-tetraose a, fucosyl-sialyllacto-*N*-tetraose b, fucosyl-sialyllacto-*N*-tetraose c, disialyllacto-*N*-tetraose, fucosyldisialyllacto-*N*-tetraose I, fucosyldisialyllacto-*N*-tetraose II, 3'-sialylgalactose, 6'-sialylgalactose, 3'-sialyl-*N*-acetyllactosamine and 6'-sialyl-*N*-acetyllactosamine.

[00137] In another aspect of the invention, provided is the use of a sialylated saccharide that has been produced by a method as described herein before and/or by using the genetically engineered microbial cell as described herein before for the manufacture of a nutritional composition.

[00138] Thus, according to another aspect of the invention, provided is a nutritional composition containing at least one sialylated saccharide, preferably at least one sialylated oligosaccharide, which has been produced by the method and/or by the genetically engineered microbial cell as described herein before. In an additional and/or alternative embodiment, the sialylated oligosaccharide is selected from the group consisting of 3'-sialyllactose, 6'-sialyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, fucosyl-sialyllacto-*N*-tetraose a, fucosyl-sialyllacto-*N*-tetraose b, fucosyl-sialyllacto-*N*-tetraose c, disialyllacto-*N*-tetraose, fucosyldisialyllacto-*N*-tetraose I, fucosyldisialyllacto-*N*-tetraose II.

[00139] In an additional and/or alternative embodiment, the nutritional composition further contains at least one neutral HMO, preferably 2'-FL.

[00140] In an additional and/or alternative embodiment, the nutritional composition contains 3-SL, 6-SL and 2'-FL.

[00141] In an additional embodiment, the nutritional composition is selected from the group consisting of medicinal, pharmaceutical, formulations, infant formula and dietary supplements.

[00142] The nutritional composition may be present in liquid form or in solid form including, but not limited to, powders, granules, flakes and pellets.

[00143] The present invention will be further described with respect to particular embodiments, but the invention is not limited thereto but only by the claims. Furthermore, the terms first, second and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequence, either temporally, spatially, in ranking or in any other manner. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

[00144] It is to be noticed that the term “comprising”, used in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps. It is thus to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more other features, integers, steps or components, or groups thereof. Thus, the scope of the expression “a device comprising means A and B” should not be limited to devices consisting only of components A and B. It means that with respect to the present invention, the only relevant components of the device are A and B.

[00145] Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to one of ordinary skill in the art from this disclosure, in one or more embodiments.

[00146] Similarly, it should be appreciated that in the description of exemplary embodiments of the invention, various features of the invention are sometimes grouped together in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure and aiding in the understanding of one or more of the various inventive aspects. This method of disclosure, however, is not to be interpreted as reflecting an intention that the claimed invention requires more features than are expressly recited in each claim. Rather, as the following claims reflect, inventive aspects lie in less than all features of a single foregoing disclosed embodiment. Thus, the claims following the detailed description are hereby expressly incorporated into this detailed description, with each claim standing on its own as a separate embodiment of this invention.

[00147] Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

[00148] Furthermore, some of the embodiments are described herein as a method or combination of elements of a method that can be implemented by a processor of a computer system or by other means of carrying out the function. Thus, a processor with the necessary instructions for carrying out such a method or element of a method forms a means for carrying out the method or element of a method. Furthermore, an element described herein of an apparatus embodiment is an example of a means for carrying out the function performed by the element for the purpose of carrying out the invention.

[00149] In the description and drawings provided herein, numerous specific details are set forth. However, it is understood that embodiments of the invention may be practiced without these specific details. In other instances, well-known methods, structures and techniques have not been shown in detail in order not to obscure an understanding of this description.

[00150] The invention will now be described by a detailed description of several embodiments of the invention. It is clear that other embodiments of the invention can be configured according to the knowledge of persons skilled in the art without departing from the true spirit or technical teaching of the invention, the invention being limited only by the terms of the appended claims.

[00151] EXAMPLES

[00152] Figs. 1 to 3 show schemes displaying alternative pathways for intracellular biosynthesis of NeuNAc, CMP-NeuNAc and sialylated saccharides.

[00153] With cells genetically modified as described herein, the fermentative production of sialylated saccharides can be achieved. A provided sole carbon source (e.g. sucrose) gets imported into the microbial cell and is metabolized yielding fructose-6-phosphate (Fig. 1 to Fig. 3). Next, L-glutamine:D-fructose-6-phosphate aminotransferase (GlmS) effects conversion of fructose-6-phosphate to glucosamine-6-phosphate (Fig. 1 to Fig. 3), which in turn is metabolized by glucosamine-6-phosphate *N*-acetyltransferase (Gna1) to *N*-acetylglucosamine-6-phosphate (Fig. 2 and Fig. 3). *N*-acetylglucosamine-6-phosphate can be converted to i) *N*-acetylmannosamine-6-phosphate by an *N*-acetylglucosamine-6-phosphate epimerase (NanE) and further to *N*-acetylmannosamine by an *N*-acetylmannosamine-6-phosphate phosphatase (Fig. 3) or ii) to *N*-acetylglucosamine by an *N*-acetylglucosamine-6-phosphate phosphatase (YihX/YqaB) and further metabolized to *N*-acetylmannosamine by an *N*-acetylglucosamine 2-epimerase (Slr1975) (Fig. 2). Sialic acid synthase (NanA) converts *N*-acetylmannosamine into *N*-acetyl neuraminic acid, which gets converted into CMP-*N*-acetylneuraminic acid by CMP-sialic acid synthetase (Fig. 1 to Fig. 3). An acceptor substrate may be supplied to the culture broth and imported into the cell and modified or *de novo* synthesized by the recombinant host cell. The acceptor substrate is ligated with *N*-acetylneuraminic acid in a reaction catalyzed by a sialyltransferase (SiaT) yielding the sialylated saccharide, which may be exported into the culture broth.

[00154] Example 1: Production of various sialylated oligosaccharides

[00155] Gene sequences of characterized or putative sialyltransferases were received from the literature and public databases. Since sialyltransferases are often described to exhibit higher activity when their signal peptide is deleted, we analyzed the corresponding protein sequences by the on-line prediction tool SignalP (Petersen et al., Nature Methods, 2011 Sep 29;8(10):785-6). Genes were synthetically synthesized by GenScript cooperation either, as annotated, in a full-length form or, when a signal peptide is predicted, as a truncated variant lacking the N-terminal signal peptide.

[00156] The sialyltransferases 1 to 26 were each subcloned as an operon with *neuA* into pDEST14 by SLIC using gene specific primers, yielding plasmids of the general kind: pDEST14-siaT-neuA. The remaining sialyltransferases 27 to 100 were directly subcloned by GenScript cooperation into plasmid pET11a using restriction sites NdeI and BamHI. Both expression systems allow the IPTG-inducible gene expression. For in vitro activity screenings, the plasmids were transformed to an *E. coli* BL21(DE3) strain lacking LacZ activity.

[00157] The *E. coli* strains harboring the plasmids for *siaT9* (α -2,3-sialyltransferase) and *siaT18* (α -2,6-sialyltransferase) expression were grown at 30°C in 100 ml shake flasks filled with 20 ml of 2YT medium supplemented with ampicillin 100 μ g ml⁻¹. When the cultures reached an OD₆₀₀ of 0.1 to 0.3, gene expression was induced by addition of 0.3 mM IPTG and the incubation was continued for 12 to 16 hours. Cells were harvested by centrifugation and mechanically disrupted in a defined volume of 50 mM Tris-HCl pH7.5 using glass beads. The protein extract was kept on ice until the assay started. The *in vitro* assay was carried out in a total volume of 25 μ l including 50 mM Tris-HCl pH7.5, 5 mM MgCl₂, 10 mM CMP-Neu5Ac and 5 to 20 mM of the appropriate acceptor substrates. The assay started with the addition of 3 μ l protein extract and continued for 16 hours. Formation of sialylated oligosaccharides resulting from the activity of the sialyltransferases was determined by thin layer chromatography.

[00158] Therefore, samples were applied on Silica Gel 60 F₂₅₄ (Merck KGaA, Darmstadt, Germany)-plates. A mixture of butanol:acetone:acetic acid:H₂O (35/35/7/23 (v/v/v/v)) was used as mobile phase. For detection of the separated substances, the TLC plate was soaked with thymol reagent (0.5 g thymol solved in 95 ml ethanol, 5 ml sulfuric acid added) and heated. Sialylated reaction products run slower than their acceptor substrates.

Acceptor substrate	SiaT9	SiaT18
Galactose	+	+
Lactose	+	+
Lacto- <i>N</i> -tetraose	+	+
Sucrose	-	-

[00159] Table 3: *In vitro* analyses determining sialyltransferase activities of two exemplary sialyltransferases depending on the supplied acceptor substrate. The formation of sialylated saccharides was determined by thin layer chromatography. (+) A sialylated reaction product was detectable. (-) A sialylated reaction product was not detectable.

[00160] Both sialyltransferases were capable to sialylate galactose or diverse oligosaccharides containing at least one galactose residue. No sialylated oligosaccharide was detectable when sucrose was applied to the reaction (Table 3).

[00161] Example 2: Metabolic engineering of an *E. coli* BL21(DE3) strain for the production of *N*-acetylneuraminic acid

[00162] Metabolic engineering was achieved by the mutagenesis and deletions of specific endogenous genes and the genomic integration of heterologous genes. The genes *lacZ* and *araA* were inactivated by mutagenesis using mismatch-oligonucleotides as described by Ellis *et al.*, (Proc. Natl. Acad. Sci. USA 98: 6742-6746 (2001)).

[00163] Genomic deletions were generated according to the method of Datsenko and Wanner (Proc. Natl. Acad. Sci. USA 97:6640-6645 (2000)). To prevent the degradation of *N*-acetylglucosamine the following genes were deleted from the genome of *E. coli* strain BL21 (DE3): *N*-acetylglucosamine specific PTS enzyme II (*nagE*), *N*-acetylglucosamine-6-phosphate deacetylase (*nagA*), and glucosamine-6-phosphate deaminase (*nagB*). The whole *N*-acetylneuraminic acid catabolic gene cluster encoding *N*-acetylmannosamine kinase (*nanK*), *N*-acetylmannosamine-6-phosphate epimerase (*nanE*), *N*-acetylneuraminic acid aldolase (*nanA*) and the sialic acid permease (*nanT*) was also deleted. The genes *manX*, *manY* and *manZ*, encoding a phosphoenolpyruvate-dependent phosphotransferase system facilitating the import of glucosamine, were also deleted. The *wzxC-wcaJ* genes were also deleted. The *wcaJ* gene encodes an UDP-glucose:undecaprenyl phosphate glucose-1-phosphate transferase catalyzing the first step in colanic acid synthesis (Stevenson *et al.*, J. Bacteriol. 1996, 178:4885-4893). In addition, the genes *fucI* and *fucK* and *agaA* were deleted, encoding L-fucose isomerase, L-fuculose kinase, and *N*-acetylgalactosamine-6-phosphate deacetylase, respectively.

[00164] The genomic integration of heterologous genes was achieved by transposition, using either the EZ-Tn5TM transposase (Epicentre, USA) or the hyperactive C9-mutant of the mariner transposase *Himar1* (Proc. Natl. Acad. Sci. 1999, USA 96:11428-11433). To produce EZ-Tn5 transposomes the gene of interest together with a FRT-site flanked antibiotic resistance marker (alternatively the resistance marker gene was flanked by *lox66-lox71* sites) was amplified. The resulting PCR-product carried at both termini the 19-bp Mosaic End recognition sites for the EZ-Tn5 transposase. For integration using *Himar1* transposase expression constructs (operons) of interest were similarly cloned together with a FRT-site/*lox66-lox71*-site flanked by antibiotic resistance markers and transferred into the pEcomar vector, which encodes the hyperactive C9-mutant of the mariner transposase *Himar1* under the control of the arabinose-inducible promoter P_{araB}. All genes were codon-optimized for expression in *E. coli* and prepared synthetically by GenScript Corp.

[00165] The expression fragment <P_{let}-*lacY*-FRT-*aadA*-FRT> was integrated by using the EZ-Tn5 transposase. After successful integration of the gene for the lactose importer LacY from *E. coli* K12 TG1 (GenBank: ABN72583) the resistance gene was

eliminated from streptomycin resistant clones by the FLP recombinase encoded on plasmid pCP20 (Proc. Natl. Acad. Sci. 2000, USA 97:6640-6645). The *csc*-gene cluster from *E. coli* W (GenBank: CP002185.1), comprising the genes for sucrose permease, fructokinase, sucrose hydrolase, and a transcriptional repressor (genes *cscB*, *cscK*, *cscA*, and *cscR*, respectively), enabling the strain to grow on sucrose as a sole carbon source, was also inserted in the genome. This cluster was integrated into the genome of the *E. coli* BL21(DE3) strain by transposition using plasmid pEcomar-*cscABKR*.

[00166] The resulting strain was further modified for the production of NeuNAc by the genomic integration of the following expression cassettes: <P_{tet}-*slr1975-gna1-lox66-aacC1-lox71*> (SEQ ID NO. 97), <P_{tet}-*neuB-lox66-kanR-lox71*> (SEQ ID NO. 98), <P_{tet}-*slr1975-P_{I5}-neuB-FRT-dhfr-FRT*> (SEQ ID NO. 99), <P_{tet}-*glmS*-gna1-lox66-aacC1-lox71*> (SEQ ID NO. 100) and <P_{tet}-*ppsA-lox66-aacC1-lox71*> (SEQ ID NO. 101). Except for the *dhfr* expression cassette, all resistance marker genes were removed in a stepwise manner from the genome (before the next round of gene integration) by introducing plasmid pKD-Cre (SEQ ID NO. 102) followed by selection on 2YT agar plates containing 100 µg·mL⁻¹ ampicillin and 100 mM L-arabinose at 30°C. Resistant clones were subsequently transferred to 2YT agar plates lacking ampicillin as well as the selective antibiotic used for genomic integration. The plates were incubated at 42°C to cure the cells of the plasmid. Clones that were sensitive to ampicillin and the selective antibiotic were used for further experiments and modifications.

[00167] The gene *slr1975* (GenBank: BAL35720) encodes *Synechocystis* sp. PCC6803 *N*-acetylglucosamine 2-epimerase. The gene *gna1* (GenBank: NP_116637) encodes a glucosamine-6-phosphate acetyltransferase from *Saccharomyces cerevisiae*. The gene *neuB* (GenBank: AF305571) encodes a sialic acid synthase from *Campylobacter jejuni*. The gene *glmS** is a mutated version of the *E. coli* L-glutamine:D-fructose-6-phosphate aminotransferase gene (Metab Eng. 2005 May;7(3):201-14). The gene *ppsA* (GenBank: ACT43527) encodes the phosphoenolpyruvate synthase of *E. coli* BL21(DE3).

[00168] For the generation of <P_{tet}-*slr1975-gna1-lox66-aacC1-lox71*>, the genes *slr1975* and *gna1* were subcloned as an operon behind the constitutive promotor P_{tet} and

fused to the gentamycin resistance gene (flanked by lox66/lox71 sites) and inserted into the pEcomar vector by blunt-end ligation. The resulting expression cassette was integrated into the genome using vector pEcomar-*slr195-gna1-aacC1* and the hyperactive C9-mutant of the mariner transposase *Himar1* under the control of the arabinose-inducible promoter P_{araB} .

[00169] For the generation of $\langle P_{tet}\text{-}neuB\text{-}lox66\text{-}kanR\text{-}lox71 \rangle$, *neuB* was cloned behind the constitutive promotor P_{tet} and fused to the kanamycin resistance gene (flanked by lox66/lox71 sites). The resulting expression cassette was integrated into the genome using the EZ-Tn5 transposase. For the generation of $\langle P_{tet}\text{-}slr1975\text{-}P_{t5}\text{-}neuB\text{-}FRT\text{-}dhfr\text{-}FRT \rangle$, the genes *slr1975* and *neuB* were separately subcloned behind the constitutive promoters P_{tet} and P_{t5} , respectively, and fused to the trimethoprim resistance gene (flanked by FRT sites). The resulting expression cassette was integrated into the genome by using the EZ-Tn5 transposase.

[00170] Expression cassette $\langle P_{tet}\text{-}glmS^*\text{-}gna1\text{-}lox66\text{-}aacC1\text{-}lox71 \rangle$ was generated by cloning *glmS*^{*} and *gna1* as an operon behind the constitutive promotor P_{tet} . This construct was further fused to the gentamycin resistance gene (flanked by lox66/lox71 sites). The resulting expression cassette was integrated into the genome by using the EZ-Tn5 transposase.

[00171] For the generation of $\langle P_{tet}\text{-}ppsA\text{-}lox66\text{-}aacC1\text{-}lox71 \rangle$, the *ppsA* gene was cloned behind the constitutive promoter P_{tet} and fused to the gentamycin resistance gene (flanked by lox66/lox71 sites). The resulting expression cassette was integrated into the genome by using the EZ-Tn5 transposase.

[00172] Altogether, the cumulative genome modifications gave rise to the Neu5Ac-producing strain *E. coli* #NANA1.

[00173] Example 3: Generation and cultivation of a microbial cell line for the production of 3'-sialyllactose

[00174] Strain *E. coli* #NANA1 was further modified by the genomic integration of <P_{tet}-*siaT9*-P_{t5}-*neuA*-lox66-*aacC1*-lox71> (SEQ ID NO: 103) by using the EZ-Tn5 transposase yielding a 3'-SL production strain. The gene *siaT9* (GenBank: BAF91160), co-don-optimized for expression in *E. coli* and prepared synthetically by GenScript, encodes an α -2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16. The gene *neuA* (GenBank: AF305571) encodes a CMP-sialic acid synthetase from *Campylobacter jejuni*.

[00175] Cultivation of the strain was conducted in 96-well plates. Therefore, single colonies of the strain were transferred from agar plates into microtiter plates containing 200 μ L of the minimal medium containing 7 g l⁻¹ NH₄H₂PO₄, 7 g l⁻¹ K₂HPO₄, 2 g l⁻¹ KOH, 0.3g l⁻¹ citric acid, 5 g l⁻¹ NH₄Cl, 1 ml l⁻¹ antifoam, 0.1 mM CaCl₂, 8 mM MgSO₄, trace-elements and 2% sucrose as carbon source. Trace elements consisted of 0.101 g l⁻¹ nitrilotriacetic acid, pH 6.5, 0.056 g l⁻¹ ammonium ferric citrate, 0.01 g l⁻¹ MnCl₂ x 4 H₂O, 0.002 g l⁻¹ CoCl₂ x 6 H₂O, 0.001g l⁻¹ CuCl₂ x 2 H₂O, 0.002 g l⁻¹ boric acid, 0.009 g l⁻¹ ZnSO₄ x 7 H₂O, 0.001 g l⁻¹ Na₂MoO₄ x 2 H₂O, 0.002 g l⁻¹ Na₂SeO₃, 0.002 g l⁻¹ NiSO₄ x 6 H₂O. Cultivation was conducted for approximately 20 hours at 30°C under vigorous shaking. Subsequently, 50 μ L of the culture broth was transferred to deepwell 96 well plates (2.0 mL) containing 400 μ L of minimal medium per well.

[00176] After an incubation of another 48 hours, cultivation was stopped and the 3'-sialyllactose level in the supernatant was determined by mass spectrometry. Mass spectrometry analysis was performed by MRM (multiple reaction monitoring) using a LC Triple-Quadrupole MS detection system. Precursor ions are selected and analyzed in quadrupole 1, fragmentation takes place in the collision cell using argon as CID gas, selection of fragment ions is performed in quadrupole 3. Chromatographic separation of lactose, 3'-sialyllactose and 6'-sialyllactose after dilution of culture supernatant 1:100 with H₂O (LC/MS Grade), was performed on a XBridge Amide HPLC column (3.5 μ m, 2.1 $\times$ 50 mm (Waters, USA) with a XBridge Amide guard cartridge (3.5 μ m, 2.1 $\times$ 10 mm) (Waters, USA). Column oven temperature of the HPLC system was 50°C. The mobile phase was composed of acetonitrile:H₂O with 10 mM ammonium acetate. A 1 μ l

sample was injected into the instrument; the run was performed for 3.60 min with a flow rate of 400 $\mu\text{l}/\text{min}$. 3'-sialyllactose and 6'-sialyllactose were analyzed by MRM in ESI positive ionization mode. The mass spectrometer was operated at unit resolution. Sialyllactose forms an ion of m/z 656.2 $[\text{M}+\text{Na}]$. The precursor ion of sialyllactose was further fragmented in the collision cell into the fragment ions m/z 612.15, m/z 365.15 and m/z 314.15. Collision energy, Q1 and Q3 Pre Bias were optimized for each analyte individually. Quantification methods were established using commercially available standards (Carbosynth, Compton, UK). At the end of the cultivation a 3'-SL titer in the culture supernatant of approx. 0.6 g L^{-1} was reached.

[00177] Example 4: Generation and cultivation of a microbial cell line for the production of 6'-sialyllactose

[00178] Strain *E. coli* #NANA1 was further modified by the genomic integration of $\langle P_{\text{tet}}\text{-}siaT18\text{-}P_{\text{t5}}\text{-}neuA\text{-}lox66\text{-}aacC1\text{-}lox71 \rangle$ (SEQ ID NO: 104) by using the EZ-Tn5 transposase yielding a 6'-SL production strain. The gene *siaT18* (GenBank: AB500947), codon-optimized for expression in *E. coli* and prepared synthetically by GenScript, encodes an α -2,6-sialyltransferase from *Photobacterium leiognathi* JT-SHIZ-119. The gene *neuA* (GenBank: AF305571) encodes a CMP-sialic acid synthetase from *Campylobacter jejuni*.

[00179] As described in example 2, a cultivation in a 96-well plate was conducted using this 6'-SL production strain. At the end of the cultivation a 6'-SL titer in the culture supernatant of approx. 0.9 g L^{-1} was reached.

[00180] Example 5: Composition of an infant formula containing sialyllactose

Infant formula: Skimmed milk
Vegetable oils (palm oil, rapeseed oil, sunflower oil)
Human milk oligosaccharides
L-Fucose
6'-sialyllactose
Skimmed milk powder
Oil of *Mortierella alpine*
Fish oil
Calcium carbonate
Potassium chloride
Vitamin C
Sodium chloride
Vitamin E
Iron acetate
Zinc sulphate
Niacin
Calcium-D-pantothenate
Copper sulphate
Vitamin A
Vitamin B1
Vitamin B6
Magnesium sulphate
Potassium iodate
Folic acid
Vitamin K
Sodium selenite
Vitamin D

Claims

1. A method for the fermentative production of a saccharide comprising at least one *N*-acetylneuraminic acid moiety, the method comprising the steps of
 - a) providing at least one genetically engineered microbial cell which comprises
 - (i) a sialic acid biosynthesis pathway comprising a glucosamine-6-phosphate *N*-acetyltransferase, an *N*-acetylglucosamine-6-phosphate phosphatase and an *N*-acetylglucosamine 2-epimerase;
 - (ii) a cytidine 5'-monophospho-(CMP)-*N*-acetylneuraminic acid synthetase, wherein the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding the cytidine 5'-monophospho-(CMP)-*N*-acetylneuraminic acid synthetase, the nucleotide sequence being selected from the group consisting of
 - i) nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 91; and
 - ii) a nucleotide sequence as represented by SEQ ID NO. 92; and
 - (iii) a heterologous sialyltransferase;
 - b) cultivating the at least one genetically-engineered microbial cell in a fermentation broth and under conditions permissive for the production of said saccharide comprising at least one *N*-acetylneuraminic acid moiety; and optionally
 - c) recovering said saccharide comprising at least one *N*-acetylneuraminic acid moiety.

2. A genetically-engineered microbial cell for the fermentative production of a saccharide comprising at least one *N*-acetylneuraminic acid moiety, wherein the genetically-engineered microbial cell comprises
 - (i) a synthetic sialic acid biosynthesis pathway comprising a glucosamine-6-phosphate *N*-acetyltransferase, an *N*-acetylglucosamine-6-phosphate phosphatase and an *N*-acetylglucosamine 2-epimerase;
 - (ii) a cytidine 5'-monophospho-(CMP)-*N*-acetylneuraminic acid synthetase, wherein the genetically engineered microbial cell contains a nucleic acid molecule comprising and expressing a nucleotide sequence encoding the cytidine 5'-monophospho-(CMP)-*N*-acetylneuraminic acid synthetase, the nucleotide se-

- quence being selected from the group consisting of nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 91
- i) nucleotide sequences encoding a polypeptide as represented by SEQ ID NO. 91;
 - ii) a nucleotide sequence as represented by SEQ ID NO. 92; and
 - (iii) a heterologous sialyltransferase.
3. The method according to claim 1 or the genetically engineered microbial cell according to claim 2, wherein the genetically engineered microbial cell contains a heterologous sialyltransferase that is preferably selected from the group consisting of
- I. polypeptides comprising or consisting of an amino acid sequence encoded by a nucleotide sequence of any one of SEQ ID NOs: 1 to 33;
 - II. polypeptides comprising or consisting of an amino acid sequence having a sequence similarity of at least 80% to any one of the amino acid sequences as encoded by a nucleotide sequence of any one of SEQ ID NOs: 1 to 33; and
 - III. fragments of any one of the polypeptides of I. and II.
4. The method according to claim 1 or 3 or the genetically engineered microbial cell according to claim 2 or 3, wherein the genetically engineered microbial cell contains a nucleic acid molecule which comprises and expresses a nucleotide sequence encoding the heterologous sialyltransferase possessing α 2,3-sialyltransferase activity or α 2,6-sialyltransferase activity, the nucleotide sequence being selected from the group consisting of
- i. nucleotide sequences as represented by any one of SEQ ID NOs: 1 to 33;
 - ii. nucleotide sequences encoding a polypeptide as represented by any one of SEQ ID NOs: 34 to 66.
5. The method according to any one of claims 1 and 3 to 4 or the genetically engineered microbial cell according to any one of claims 2 to 4, wherein the sialyltransferase transfers a sialic acid residue from a donor substrate to an acceptor molecule, wherein the acceptor molecule is selected from the group consisting of *N*-acetylglucosamine, galactose, *N*-acetylgalactosamine, lactose, lactulose, *N*-

acetyllactosamine, lacto-*N*-biose, lactulose, melibiose, raffinose, lacto-*N*-triose II, 2'-fucosyllactose, 3-fucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-*N*-acetyllactosamine, 6'-sialyl-*N*-acetyllactosamine, 3'-galactosyllactose, 6'-galactosyllactose, lacto-*N*-tetraose, lacto-*N*-neotetraose, 2'3-difucosyllactose, 3-fucosyl-3'-sialyllactose, 3-fucosyl-6'-sialyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, lacto-*N*-fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N*-neofucopentaose I and lacto-*N*-neofucopentaose V.

6. The method according to any one of claims 1 and 3 to 5, 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.
7. The method according to any one of claims 1 and 3 to 6, wherein the at least one genetically engineered microbial cell is cultivated in the absence of and/or without addition of one or more selected from the group consisting of glucosamine, *N*-acetylglucosamine, *N*-acetylmannosamine and *N*-acetylneuraminic acid.
8. The method according to any one of claims 1 and 3 to 7, wherein the saccharide comprising at least one *N*-acetylneuraminic acid moiety is selected from the group consisting of 3'-sialylgalactose, 6'-sialylgalactose, 3'-sialyl-*N*-acetyllactosamine, 6'-sialyl-*N*-acetyllactosamine, 3'-sialyllactose, 6'-sialyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, fucosyl-sialyllacto-*N*-tetraose a, fucosyl-sialyllacto-*N*-tetraose b, fucosyl-sialyllacto-*N*-tetraose c, disialyllacto-*N*-tetraose, fucosyldisialyllacto-*N*-tetraose I, fucosyldisialyllacto-*N*-tetraose II.
9. Use of a genetically engineered microbial cell according to any one of claims 2 to 5 for the production of a sialylated saccharide in a whole cell fermentation process.