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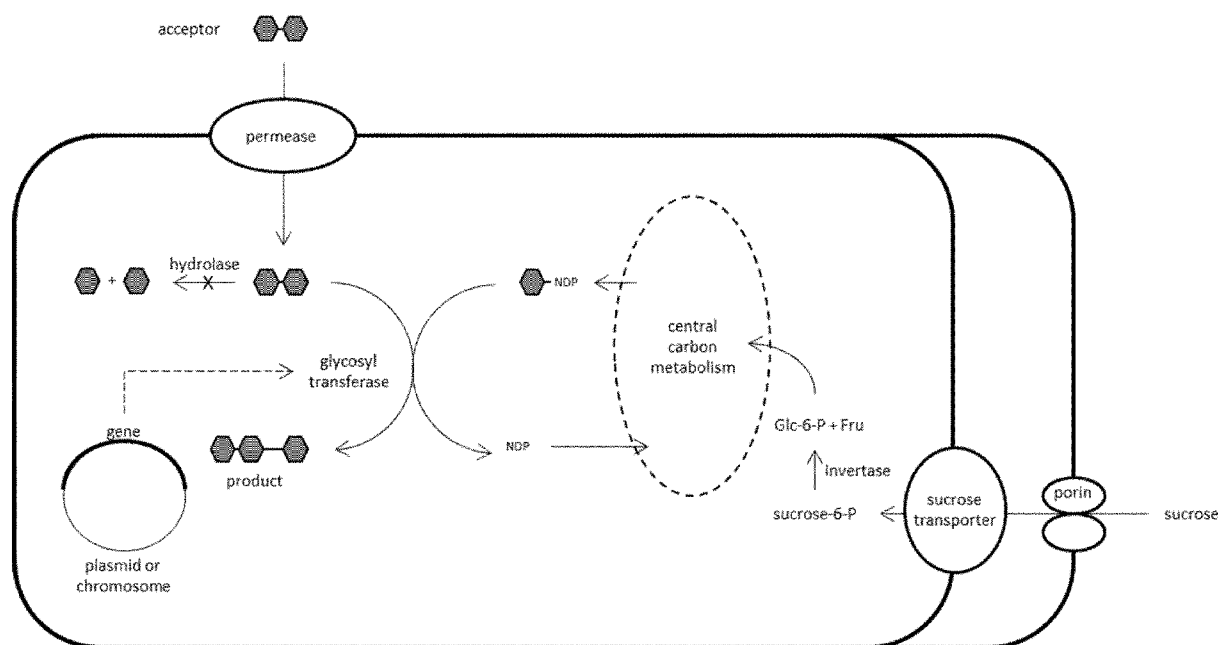


Figure 1

(57) Abstract: The invention relates to a genetically modified microorganism for making an oligosaccharide, preferably of 3-8 monosaccharide units, more preferably of 3-5 monosaccharide units, particularly a HMO, which comprises one or more genes encoding a sucrose utilization system, so the microorganism can use sucrose as a carbon and energy source.

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OLIGOSACCHARIDE PRODUCTION

FIELD OF THE INVENTION

The present invention relates to the field of biotechnology, notably to a microbial production of oligosaccharides, particularly of human milk oligosaccharides (HMOs), using a genetically modified microorganism, particularly *E. coli*, using sucrose as its exclusive carbon source.

BACKGROUND OF THE INVENTION

The fermentative syntheses of foreign or exogenous oligosaccharides using recombinant microorganisms have recently become of great commercial and industrial interest. In such syntheses, oligosaccharides of interest would be synthesized by enzymatic glycosylation of sugar acceptors mediated by one or more heterologous glycosyl transferases of the microorganisms, and the one or more activated sugar nucleotides necessary for glycosylation would be produced by the same microorganism through overexpressing one or more genes encoding endogenous activated sugar nucleotide producing enzymes. The metabolic pathways of such syntheses require a carbon source which is mainly a simple carbon building block, typically glycerol or glucose (see e.g. WO 01/04341, Priem et al. *Glycobiology* **12**, 235 (2002), Fort et al. *Chem. Comm.* 2558 (2005), Drouillard et al. *Angew. Chem. Int. Ed.* **45**, 1778 (2006), WO 2010/070104, WO 2012/112777, WO 2013/182206, WO 2014/048439). In some syntheses, lactose can be the carbon source if it also serves as an acceptor (Lee et al. *Microb. Cell Fact.* **11**:48 (2012)). As the microorganisms have been genetically manipulated, antibiotic-resistance selection marker genes have been utilized to separate the transformed microorganisms from the non-transformed ones in the inoculum and the fermentation broth. However, the use of antibiotics has been avoided by integrating the genes coding for enzymes involved in the *de novo* biosynthesis of the donor sugar in the chromosome of the microorganisms (Baumgärtner et al. *Microb. Cell Fact.* **12**:40 (2013)).

Around 50 % of wild-type *E. coli* are able to utilize sucrose as a carbon and energy source, but most of them are pathogenic. The *E. coli* strains used mainly in industry to synthesize chemical materials cannot live and grow on sucrose (Bruschi et al. *Biotechnol. Adv.* **30**, 1001 (2012)). However, in some cases, sucrose can be a cheaper carbon and energy source. For this reason, attempts have been made to create suc⁺ strains of *E. coli* that can live and grow on sucrose (e.g. GB 2155935 A, Sabri et al. *Appl. Environ. Microbiol.* **79**, 478 (2013)) and produce industrially profitable products by them such as amino acids, biofuel, carotenoids etc. (e.g. EP-A-1149911, EP-A-2239336, EP-A-2371952, EP-A-2405006, WO 2010/051849, WO 2012/078311, Kim et al. *Biores. Technol.* **130**, 288 (2013)). However, these suc⁺ transformants

have generally been less productive than suc⁻ strains (Khamduang et al. *J. Ind. Microbiol. Biotechnol.* **36**, 1267 (2009)).

WO 2012/007481 describes *E. coli* transformants that express either a sucrose phosphorylase or a sucrose invertase in combination with a fructokinase. Thereby, the microorganism is able to produce 2'-fucosyllactose, utilizing sucrose as its main carbon source. Furthermore, WO 2014/067696, WO 2015/150328, WO 2019/043029 and WO 2019/076941 describe *E.coli* transformants comprising a *csc*-gene cluster that enables them to grow on sucrose and produces fucose, 2'-FL, sialyllactose and sialic acid, respectively.

There has been, however, a continuing need for alternative processes for making oligosaccharides, particularly HMOs, using transformed microorganisms that are able to utilize more effectively sucrose as a carbon and energy source.

SUMMARY OF THE INVENTION

A first aspect of the invention relates to a process for making an oligosaccharide, preferably of 3-8 monosaccharide units, more preferably of 3-5 monosaccharide units, particularly a HMO, by glycosylating a carbohydrate acceptor which is not sucrose, preferably lactose, comprising the steps of:

- a) providing a cell, preferably an *E.coli* cell, that can internalize said acceptor into said cell and comprises
 - a recombinant gene encoding a glycosyl transferase which is able to transfer a glycosyl residue of an activated sugar nucleotide to said acceptor, internalized in said cell, or to an intermediate in the biosynthetic pathway to said oligosaccharide from said acceptor and
 - a biosynthetic pathway to make said activated sugar nucleotide in said cell,
 - b) culturing said cell in the presence of said acceptor and sucrose, and
 - c) separating said oligosaccharide from said cell, from the culture medium or from both,
- said process being characterized in that said cell also comprises one or more genes encoding a sucrose utilization system, preferably encoding a heterologous sucrose utilization system, more preferably encoding a heterologous PTS-dependent sucrose utilization transport system, still more preferably *scr* genes, so that said cell can use sucrose as a carbon source, preferably the main carbon source, more preferably the sole carbon source, for making said activated sugar nucleotide and as an energy source, preferably the main energy source, more preferably the sole energy source, for making said oligosaccharide.

A second aspect of the invention relates to a cell, preferably an *E.coli* cell, that can internalize a carbohydrate acceptor, which is not sucrose, preferably lactose, into said cell and that comprises:

- a recombinant gene encoding a glycosyl transferase which is able to transfer a glycosyl residue of an activated sugar nucleotide to said acceptor, internalized in said cell, or to an intermediate in the biosynthetic pathway to said oligosaccharide from said acceptor
- a biosynthetic pathway to make said activated sugar nucleotide in said cell, and
- one or more genes encoding a sucrose utilization system, preferably a heterologous sucrose utilization system, more preferably *scr* genes, so that said cell can use sucrose as a carbon source, preferably the main carbon source, more preferably the sole carbon source.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 is intended to illustrate the invention further. It is not intended to limit the subject matter of the invention thereto.

The engineered microorganism is a fully metabolically active cell in which the growth and the oligosaccharide synthesis may proceed simultaneously. The cell comprises a heterologous phosphotransferase (PTS)-dependent sucrose utilization transport system containing a sucrose specific porin (facilitates the sucrose diffusion through the outer membrane), a sucrose transport protein (provides intracellular sucrose-6-phosphate from extracellular sucrose) and a sucrose-6-phosphate hydrolase (provides glucose-6-phosphate and fructose). The oxidation of glucose-6-phosphate and fructose provides biological energy source by the organism's own metabolic system. Also, glucose-6-phosphate and fructose serve as carbon source for producing sugar nucleotides in the cell's natural biosynthetic pathway or in any heterologous pathway expressed in the cell. The so-produced sugar nucleotides are donors for glycosylating carbohydrate acceptors (e.g. lactose), internalized through a specific permease by the cell, and thereby manufacturing oligosaccharides of interest. The glycosylation is mediated by one or more glycosyl transferases which are directly produced by expressing heterologous genes from plasmid or the chromosome. The organism lacks any enzyme degrading either the acceptor or the oligosaccharide product in the cell.

Figure 2 Illustrate the ability to grow on sucrose with a heterologous PTS-dependent sucrose utilization transport system in the cell. The MDO strain does not contain the PTS-dependent sucrose utilization transport system, whereas MPLNnT does grow on sucrose.

DETAILED DESCRIPTION OF THE INVENTION

It has been surprisingly discovered that an exogenous mono- or disaccharide acceptor, preferably lactose, can be internalized in a suitable genetically transformed microorganism, particularly *E.coli*, by a transport mechanism of the microorganism, so that this carbohydrate acceptor can be glycosylated in the microorganism using sucrose as its carbon and energy source, and that an exogenous oligosaccharide can be produced and separated in good yield.

Thereby, an efficient, cheap and easily up-scalable process for producing oligosaccharides can be obtained. In order to make the process successful, a special oligosaccharide-producing microorganism is needed that can live on sucrose, utilize sucrose for the metabolic syntheses of the necessary nucleotide sugar donors for glycosylation, can internalize simple carbohydrate acceptors and perform glycosylation reactions on them for synthesizing more complex oligosaccharides.

The invention therefore, in a first aspect, involves a process of making an oligosaccharide by:

- a) providing a cell of a microorganism, preferably an *E.coli* cell, that can internalize sucrose and a carbohydrate acceptor, preferably lactose, into said cell and that comprises:
 - a recombinant gene encoding a glycosyl transferase which can transfer a glycosyl residue of an activated sugar nucleotide to the acceptor within the cell or to an intermediate in the biosynthetic pathway to said oligosaccharide from said acceptor, and
 - a biosynthetic pathway for making the activated sugar nucleotide from sucrose,
- b) culturing the cell in an aqueous culture medium in the presence of the acceptor and sucrose, and
- c) separating the oligosaccharide product from the cell from the culture medium or from both.

The process features the cell being transformed with one or more foreign genes encoding a sucrose utilization system that allows the cell to use sucrose as a carbon source, preferably the main carbon source, more preferably the sole carbon source, for the biosynthesis of the activated sugar nucleotide by the cell. The sucrose utilization system, with which the cell is transformed, also preferably allows the cell to use sucrose as an energy source, preferably the main energy source, more preferably the sole energy source, for the biosynthesis of the oligosaccharide.

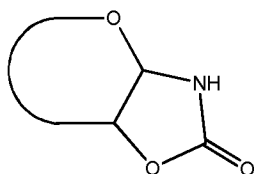
In accordance with this invention, the term "carbohydrate acceptor" or "acceptor" preferably means a mono- or disaccharide other than sucrose and its glycosides. A monosaccharide acceptor or a monosaccharide part of a disaccharide acceptor can comprise any 5-6 carbon atom sugar moiety that is an aldose (e.g. D-glucose, D-galactose, D-mannose, D-ribose, D-arabinose, L-arabinose, D-xylose, etc.), ketose (e.g. D-fructose, D-sorbose, D-tagatose, etc.), deoxysugar (e.g. L-rhamnose, L-fucose, etc.) or deoxy-aminosugar (e.g. N-acetylglucosamine, N-acetylmannosamine, N-acetylgalactosamine, etc.). In a glycoside-type carbohydrate acceptor the sugar moiety is attached to a non-sugar residue (aglycon) by either a covalent bond, which is a direct linkage between the glycosidic carbon atom of the sugar residue and any atom of the non-sugar moiety, or by a linker, which consists of one, two, three or four atoms such as -O-, -C-, -NH-, -N(OH)-, -S-, -C(=O)-, -C(=S)-, -C(=NH)-, -C(=N-OH)-, -C(=O)-O-, -O-C(=O)-, -C(=O)-

S-, -S-C(=O)-, -C(=S)-O-, -O-C(=S)-, -C(=S)-S-, -S-C(=S)-, -C(=O)-NH-, -NH-C(=O)-, -C(=NH)-O-, -O-C(=NH)-, -C(=S)-NH-, -NH-C(=S)-, -C(=NH)-S- and -S-C(=NH). Thus, the C-1 (in case of aldoses) or C-2 (in the case of ketoses) anomeric carbon atom at the reducing end of the mono- or disaccharide residue is linked to the non-sugar moiety by a covalent bond or a linker forming a O-, N-, S- or C-glycoside. Preferably, the aglycon of these glycosidic derivatives, with or without a linker, is one of the following groups:

a) $-OR_A$, wherein R_A is a linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms (such as methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, s-butyl, t-butyl, n-hexyl, etc.) or, when unsaturated, 2-24, preferably 2-6 carbon atoms (such as vinyl, allyl, propargyl, etc.), or R_A means an aryl moiety (a homoaromatic group such as phenyl or naphthyl), or R_A means a group removable by hydrogenolysis, that is a group whose bond attached to the oxygen can be cleaved by addition of hydrogen in the presence of catalytic amounts of palladium, Raney nickel or another appropriate metal catalyst known for use in hydrogenolysis, resulting in the regeneration of an OH group; such protecting groups are well known to the skilled man and are discussed in *Protective Groups in Organic Synthesis*, PGM Wuts and TW Greene, John Wiley & Sons 2007. Suitable groups include benzyl, diphenylmethyl (benzhydryl), 1-naphthylmethyl, 2-naphthylmethyl or triphenylmethyl (trityl) groups. Any of the above mentioned R_A groups can be optionally substituted by one or more groups selected from: alkyl (only for aryl and group removable by hydrogenolysis), hydroxy, alkoxy, carboxy, oxo, alkoxycarbonyl, alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylamino, arylcarbonyl, amino, mono and dialkylamino, carbamoyl, mono- and dialkyl-amino-carbonyl, alkyl-carbonylamino, cyano, alkanoyloxy, nitro, alkylthio and halogen; in case of a group removable by hydrogenolysis, such substitution, if present, is preferably on the aromatic ring(s);

b) $-X-R_B$, wherein X is N or S, and R_B means linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms (such as methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, s-butyl, t-butyl, n-hexyl, etc.) or, when unsaturated, 2-24, preferably 2-6 carbon atoms (such as vinyl, allyl, propargyl, etc.), or R_B means an aryl moiety (a homoaromatic group such as phenyl or naphthyl), or R_B means a benzyl group. Any of the above mentioned R_B groups can be optionally substituted by one or more groups selected from: alkyl (only for aryl and benzyl), hydroxy, alkoxy, carboxy, oxo, alkoxycarbonyl, alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylamino, arylcarbonyl, amino, mono and dialkylamino, carbamoyl, mono- and dialkyl-amino-carbonyl, alkyl-carbonylamino, cyano, alkanoyloxy, nitro, alkylthio and halogen;

c) a group that links the anomeric carbon atom and the adjacent carbon atom to each other by a $-NH-C(=O)-O$ -bridge, thus forming a fused 5-membered ring as depicted below in case of an aldose:



d) azide;

e) $-\text{NH}-\text{C}(\text{R}'')=\text{C}(\text{R}')_2$, wherein each R' independently is an electron withdrawing group selected from $-\text{CN}$, $-\text{COOH}$, $-\text{COO-alkyl}$, $-\text{CO-alkyl}$, $-\text{CONH}_2$, $-\text{CONH-alkyl}$ and $-\text{CON(alkyl)}_2$, or
 5 wherein the two R' -groups are linked together and form $-\text{CO}-(\text{CH}_2)_{2,4}-\text{CO}-$ and thus form with the carbon atom, to which they are attached, a 5-7 membered cycloalkan-1,3-dione, in which dione any of the methylene groups is optionally substituted with 1 or 2 alkyl groups, and wherein R'' is H or alkyl;

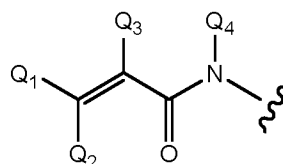
f) a residue of an amino acid, which can be any natural or non-natural amino acid, that is
 10 an alkanoic acid derivative having at least one amino group as a substituent. Preferably, the amino acid is selected from the group consisting of: α -amino acids and β -amino acids such as Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, hydroxyproline, α -methylserine, β -alanine, etc. These amino acids can be either directly or via a linker as defined above (e.g. for urea-linked glycopeptides see WO 2009/040363) bound to the
 15 carbohydrate at its C-1 (in case of aldoses) or C-2 (in the case of ketoses) anomeric carbon atom thus forming O-, N-, S- or C-glycosides. O-Glycosides (O-glycans) can be formed involving OH-containing amino acids such as serine, threonine, hydroxyproline, etc., N-glycosides (N-glycans) can be made using the α -, β -, etc. amino group of any amino acid or the additional amino group of the side chain of e.g. lysine, asparagine or glutamine, S-glycosides
 20 can be made using cysteine, while C-glycosides (C-glycans) contain a C-C bond coupling a C-atom of the amino acid to the anomeric carbon atom of the non-reducing end of the oligosaccharide part;

g) a polyethylene glycol residue. Polyethylene glycol (PEG) is a water soluble polyether of molecular formula $\text{C}_{2n}\text{H}_{4n+2}\text{O}_{n+1}$, having oxyethylene ($-\text{CH}_2-\text{O}-\text{CH}_2-$ or $\text{CH}_2-\text{CH}_2-\text{O}-$) repeating
 25 units and wherein n is 2 to 100, preferably 2 to 50, particularly 2 to 25, more particularly 2 to 10. Lower molecular weight PEGs are available in a purified form and are referred to as a "monodisperse PEG", and are also available as mixtures of PEGs and are referred to as a "polydisperse PEG". With regard to their geometry, PEGs can be in a linear, branched, star or comb configuration. Linear PEGs are preferably lower molecular weight PEGs (i.e., n is 2 to 10,
 30 preferably 3 to 6). Branched PEGs preferably have 3 to 10 linear, preferably lower molecular weight, PEG chains emanating from a central core group. Star PEGs preferably have 10 to 100 linear or branched, preferably lower molecular weight, PEG chains emanating from a central core group. Comb PEGs have multiple linear, branched and/or star, preferably lower molecular weight, PEG chains bonded to a polymer backbone. Terminal primary hydroxy group of PEGs

can be bonded by an ether bond with an alkyl group, preferably methyl. In addition, their terminal hydroxy group can be replaced by amino, alkyl amino, dialkyl amino, acylamino, thiol or alkyl thio groups or their terminal hydroxymethyl group can be oxidized to a carboxyl, which can be esterified or be present in amide form with ammonia or a primary or secondary amine. The attachment is a glycosidic-like bond;

h) a polyvinyl alcohol residue. Polyvinyl alcohol (PVA) is a water-soluble polymer of molecular formula $(C_2H_4O)_x$ having $-CH_2-CH(OH)-$ monomer units, wherein x is any desired integer. When attached to carbohydrate, any of the OH-groups can be glycosylated;

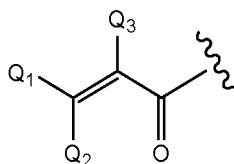
i) an α,β -unsaturated amido group of formula **A**



A

wherein Q_1 , Q_2 , Q_3 and Q_4 are, independently, H and C_1 - C_6 -alkyl, which alkyl optionally can be substituted with halogen, OH, nitro or phenyl groups. The residue of formula **A**, via its N atom, is linked to the sugar by a covalent bond, preferably to the anomeric carbon atom of the carbohydrate in the form of an N-glycoside; or

j) an α,β -unsaturated carbonyl group of formula **B**



B

wherein Q_1 , Q_2 and Q_3 are as defined at residue of formula **A**.

Preferably, the carbohydrate acceptor is a galactosyl disaccharide, particularly lactose.

Also in accordance with this invention, the term "oligosaccharide product" or "oligosaccharide" preferably means a glycosylated derivative of a carbohydrate acceptor disclosed above wherein a glycosyl residue is attached to the carbohydrate moiety of the carbohydrate acceptor by interglycosidic linkage. Preferably, an oligosaccharide product is of 3-8 monosaccharide units, particularly of 3-5 monosaccharide units, more particularly of 4 monosaccharide units or of 3 monosaccharide units. The oligosaccharide product of this invention is a recombinant product, i.e. it is made by a genetically transformed microorganism and is foreign or heterologous to the microorganism.

The term “an intermediate in the biosynthetic pathway” in relation to an oligosaccharide or more specifically an HMO, is to be understood as an intermediate oligosaccharide or HMO in the reaction steps needed to produce the desired oligosaccharide or HMO. For example, LNT-II is an intermediate in the biosynthetic pathway to LNT or LNT.

5 The genetically modified microorganism or cell used in the process of some embodiments of this invention can be selected from the group consisting of bacteria and yeasts, preferably a bacterium. Bacteria are preferably selected from the group of: *Escherichia coli*, *Bacillus* spp. (e.g. *Bacillus subtilis*), *Campylobacter pylori*, *Helicobacter pylori*, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, *Thermophilus aquaticus*, *Azorhizobium caulinodans*, *Rhizobium*
 10 *leguminosarum*, *Neisseria gonorrhoeae*, *Neisseria meningitis*, *Lactobacillus* spp., *Lactococcus* spp., *Enterococcus* spp., *Bifidobacterium* spp., *Sporolactobacillus* spp., *Micromonospora* spp., *Micrococcus* spp., *Rhodococcus* spp., *Pseudomonas*, among which *E. coli* is preferred.

Further in accordance with some embodiments of this invention, the microorganism or cell, preferably an *E.coli* cell, is genetically modified so that there is at least one alteration in its DNA
 15 sequence. The alteration can result in a change in the original characteristics of the wild type cell, e.g. the modified cell is able to perform additional chemical transformation due to the introduced new genetic material that encodes the expression of an enzymes not being in the wild type cell, or is not able to carry out transformation like degradation due to removal or deactivation of gene/genes. In this regard, a genetically modified cell contains at least one
 20 artificial alteration in its genome compared to its naturally occurring (wild type) variant. By the alteration, either a nucleic acid construct is added to the cell by way of integration into the genome or by addition via plasmid, or a nucleic acid sequence is deleted from or changed in the genome of the cell. Whatever is the case, the so-transformed cell has a genotype that is different from that before the alteration and, therefore, the modified cell shows modified
 25 feature(s). Preferably, the genetically modified cell can perform at least one additional or altered biochemical reaction, when cultured or fermented, due to the introduction of a heterologous nucleic acid sequence or the modification of a native nucleic acid sequence that encodes an enzyme that is not expressed in the wild type cell, or the genetically modified cell cannot perform a biochemical reaction due to the deletion, addition or modification of a nucleic acid
 30 sequence that encodes an enzyme found in the wild type cell. The genetically modified cell can be constructed by well-known, conventional genetic engineering techniques (e.g. Green and Sambrook: *Molecular Cloning: A laboratory Manual*, 4th ed., Cold Spring Harbor Laboratory Press (2012); *Current protocols in molecular biology* (Ausubel et al. eds.), John Wiley and Sons (2010)).

35 The terms “host cell”, “recombinant microorganism or cell” or “genetically modified microorganism or cell” are used interchangeably to designate such constructs.

The term “nucleic acid sequence encoding a polypeptide having a glycosyl transferase activity” or “nucleic acid sequence encoding glycosyl transferase” means a gene, a functional fragment thereof or a codon-optimized version thereof that express a polypeptide having a glycosyl transferase activity.

- 5 The term “gene” in the present context relates to a coding nucleic acid sequence and includes genomic DNA and cDNA.

The term “promoter” means a nucleic acid sequence involved in the binding of RNA polymerase to initiate transcription of an operably linked gene, wherein the gene includes a coding DNA sequence and other (non-coding) sequences, e.g. the 5'-untranslated region (5'-
10 UTR) located upstream of the coding sequence, which comprises a ribosomal binding site. A promoter in some embodiments of this invention is an isolated DNA sequence, i.e. not an integrated DNA fragment of the genomic DNA. The nucleotide sequence of a promoter corresponds to, or have at least 80 % identity, preferably 90% to 99.9 % identity, such as 95%, 98%, and even more preferably 100% identity with the nucleotide sequence of a fragment of
15 bacterial genomic DNA that is regarded as promoter region of a gene, e.g. a promoter region of a *glp* operon or *lac* operon of *E.coli*. A preferred promoter is the promoter of SEQ ID NO: 5.

The term “operon” means a functioning unit of genomic DNA or cDNA containing a cluster of genes under the control of a single promoter. By “*glp* operon” is meant a cluster of genes involved in the respiratory metabolism of glycerol of bacteria. Preferably, a *glp* operon promoter
20 sequence comprised in a DNA construct corresponds to or has at least 80 % identity, preferably 90-99.9 % identity, such as 95%, 98%, and even more preferably 100% identity with the nucleotide sequence of a fragment of the genomic DNA regarded as a promoter region of the corresponding *glp* operon of *E. coli*; in particular, the isolated sequence of a promoter of a *glp* operon of some embodiments of the invention corresponds to, or has said percent of identity
25 with a fragment of the genomic sequence upstream the sequence having GenBank ID: EG10396 (*glpFKX*), EG10391 (*glpABC*), EG10394 (*glpD*), EG10401 (*glpTQ*). The *E. coli* genome is referred herein to the complete genomic DNA sequence of *E coli* K-12 MG1655 (GenBank ID:U00096.3).

In some embodiments, a promoter of the *glp* operon is operably linked to the heterologous
30 glycosyl transferase genes and/or the heterologous *scr* genes. Preferably, those genes are expressed under a *glpF* promoter (WO 2019/123324, the content of which is incorporated herein by reference), in particular the *glpF* promoter of SEQ ID NO: 5. The process of some embodiments of this invention also involves transporting the exogenous carbohydrate acceptor, preferably lactose, into the genetically modified microorganism for glycosylation to produce a
35 foreign oligosaccharide of interest, preferably without adversely affecting the basic functions of the cell or destroying its integrity. In one embodiment, the transport takes place via a passive mechanism, during which the exogenous acceptor diffuses passively across the plasma

membrane of the cell. Diffusion of the acceptor into the microorganism is a function of the concentration differences between the fermentation broth and the extra- and intracellular space of the cell with respect to the acceptor, whereby the acceptor passes from the place of higher concentration to the place of lower concentration. In another embodiment, the acceptor is internalized with the aid of an active transport. In such a case, the genetically modified microorganism contains transporter proteins, called permeases, which act as enzymes and with which the microorganism is able to admit exogenous substances and to concentrate them in the cytoplasm. Specifically, lactose permease (LacY) acts specifically on galactose, simple galactosyl disaccharides such as lactose and their glycosides. The specificity towards the sugar moiety of the exogenous carbohydrate acceptor to be internalized can be altered by mutation of the microorganism by means of conventional recombinant DNA manipulation techniques. In a preferred embodiment, the internalization of exogenous lactose or its derivative takes place via an active transport mechanism mediated by a lactose permease. The genetically modified microorganism preferably lacks any enzyme activity (such as LacZ) that would degrade the acceptor. Likewise, the microorganism is not able to hydrolyze or degrade the oligosaccharide product or any oligosaccharide intermediate in the biosynthetic pathway to the oligosaccharide product.

Moreover, the genetically modified cell used in the process of the invention comprises one or more endogenous or recombinant genes encoding one or more glycosyl transferase enzymes that are able to transfer the glycosyl residue of an activated sugar nucleotide to the internalized acceptor. The gene or an equivalent DNA sequence thereof, if it is recombinant, can be introduced into the cell by conventional techniques, e.g. using an expression vector or by chromosomal integration. The origin of the heterologous nucleic acid sequence can be any animal (including human) or plant, eukaryotic cells such as those from *Saccharomyces cerevisiae*, *Saccharomyces pombe*, *Candida albicans* or from algae, prokaryotic cells such as those originated from *E. coli*, *Bacteroides fragilis*, *Photobacterium sp.*, *Bacillus subtilis*, *Campylobacter pylori*, *Helicobacter pylori*, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, *Thermophilus aquaticus*, *Azorhizobium caulinodans*, *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, or virus. The glycosyl transferase enzyme/enzymes encoded and expressed by the gene(s) or equivalent DNA sequence(s) are preferably glucosyl transferases, galactosyl transferases, N-acetylglucosaminyl transferases, N-acetylgalactosaminyl transferases, glucuronosyl transferases, xylosyl transferases, mannosyl transferases, fucosyl transferases, sialyl transferases and the like. In a preferred embodiment, the glycosyl transferases are selected from the group consisting of β -1,3-N-acetylglucosaminyl-transferase, β -1,6-N-acetylglucosaminyl-transferase, β -1,3-galactosyl-transferase, β -1,4-galactosyl-transferase, β -1,3-N-acetylgalactosaminyl-transferase, β -1,3-glucuronosyl-transferase, α -2,3-sialyl-transferase, α -2,6-sialyl-transferase, α -2,8-sialyl-transferase, α -1,2-fucosyl-transferase, α -1,3-fucosyl-transferase and α -1,4-fucosyl-transferase. More preferably,

the glycosyl transferases are selected from β -1,3-N-acetylglucosaminyl transferase, β -1,6-N-acetylglucosaminyl transferase, β -1,3-galactosyl transferase, β -1,4-galactosyl transferase, α -2,3-sialyl transferase, α -2,6-sialyl transferase, α -1,2-fucosyl transferase, α -1,3-fucosyl transferase and α -1,4 fucosyl transferase, that is from those involved in the construction of HMO core structures as well as fucosylated and/or sialylated HMOs and its glycosidic derivatives, wherein the aglycon is a moiety defined above at the group of carbohydrate acceptors. The genes encoding the above-mentioned transferases have been described in the literature.

The genetically modified microorganism or cell used in the process of the invention is capable of producing an oligosaccharide from the internalized precursor/acceptor which is preferably lactose. Said cell possesses enzymatic activity that is necessary for synthesizing an oligosaccharide, from an internalized mono- or disaccharide via consecutive glycosylation steps, wherein the internalized acceptor, e.g. lactose, is glycosylated, in a first glycosylation step, to a trisaccharide, then that trisaccharide is glycosylated, in a second glycosylating step, to a tetrasaccharide, and so forth. The glycosylation steps are mediated by respective glycosyl transferases. In this regard, the phrase "a glycosyl transferase which is able to transfer a glycosyl residue of an activated sugar nucleotide to an intermediate in the biosynthetic pathway to said oligosaccharide from said acceptor" or "a glycosyl transferase which can transfer a glycosyl residue of an activated sugar nucleotide to an intermediate in the biosynthetic pathway to said oligosaccharide from said acceptor" refers to the second, third etc. glycosylation step. For example, if lacto-N-tetraose is made from lactose, a first glycosyl transferase (a β -1,3-N-acetyl-glucosaminyl transferase) transfers GlcNAc of UDP-GlcNAc to the internalized lactose to form lacto-N-triose II, then a second glycosyl transferase (a β -1,3-galactosyl transferase) transfers Gal of UDP-Gal to the previously formed lacto-N-triose II (LNT-II) to form lacto-N-tetraose. In this regard, lacto-N-triose II is considered to be an intermediate in the biosynthetic pathway to LNT from lactose.

In the glycosyl transferase mediated glycosylation processes of this invention, activated sugar nucleotides serve as donors, and the glycosyl transferase in question transfers a monosaccharide of an appropriate donor molecule to the acceptor molecule. Each activated sugar nucleotide generally comprises a phosphorylated glycosyl residue attached to a nucleoside, and the specific glycosyl transferase enzyme accepts only the specific sugar nucleotide. Thus, preferably the following activated sugar nucleotides are involved in the glycosyl transfer: UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-glucuronic acid, UDP-Xyl, GDP-Man, GDP-Fuc and CMP-sialic acid, particularly those selected from the group consisting of UDP-Gal, UDP-GlcNAc, GDP-Fuc and CMP-sialic acid.

The genetically modified microorganism used in the process of this invention possesses a biosynthetic pathway to the above mentioned activated sugar nucleotides, that is, it has one or more sets of genes encoding one or more enzymes responsible for the synthesis of one or

more activated glycosyl nucleotides, ready for glycosylation in glycosyl transferase mediated reaction in the cell, when cultured. The sets of genes are either naturally present in the cell or introduced into the cell by means of recombinant DNA manipulation techniques. The production of the activated glycosyl nucleotides by the cell takes place under the action of enzymes
5 involved in the biosynthetic pathway of that respective sugar nucleotide stepwise reaction sequence starting from a carbon source (for a review for monosaccharide metabolism see e.g. H. H. Freeze and A. D. Elbein: *Chapter 4: Glycosylation precursors*, in: *Essentials of Glycobiology*, 2nd edition (Eds. A. Varki et al.), Cold Spring Harbour Laboratory Press (2009)).

It should be emphasized, that the production of the activated sugar nucleotides by the
10 genetically modified microorganism via its own biosynthetic pathway is advantageous compared to *in vitro* versions of transfer glycosylation, as it avoids using the very expensive sugar nucleotide type donors added exogenously, hence the donors are formed by the cell *in situ* and the phosphatidyl nucleoside leaving groups are recycled in the cell.

In addition, the microorganism used in the process of the invention comprises genes
15 encoding a sucrose utilization system, that is the cell has a capability to catabolically utilize sucrose as a carbon source, as well as an energy source. The system that enables the cell to utilize sucrose can be one normally found in the gene pool of that cell, but preferably is a heterologous system (i.e. derived from a different organism and transferred to the host cell by conventional recombinant DNA manipulation techniques). Typically, two kinds of sucrose
20 catabolism can be used: i) The phosphoenolpyruvate (PEP)-dependent phosphotransferase system ("PTS"), where sucrose is transported into the microorganism and concomitantly phosphorylated to generate intracellular sucrose-6-phosphate which is hydrolysed to glucose-6-phosphate and fructose that are then involved in the central carbon metabolism of the cell. PTS can be encoded by *scr* or *sac* genes; ii) The non-phosphotransferase-dependent system ("non-
25 PTS"), where extracellular sucrose enters the cell with the aid of a proton symport transport system (sucrose permease) and, after transport, is hydrolysed by an invertase enzyme to glucose and fructose followed by phosphorylation. In this regard, the *csc* regulon consists of genes encoding the enzymes that are responsible for the non-PTS sucrose utilization.

During fermentation in the process of this invention, the oligosaccharide-producing
30 microorganism is fed with sucrose that provides energy via glycolysis for growing, reproducing and maintaining its structure. In addition, the sucrose taken up by the cell provides, via sucrose catabolism, precursors for the synthesis of the activated sugar nucleotide(s) necessary for the glycosylation process that takes place in the cell. The internalized carbohydrate acceptor participates in the glycosyl transferase induced glycosylation reaction, in which a glycosyl
35 residue of an activated nucleotide donor produced by the cell is transferred so that the acceptor is glycosylated. Optionally, when more than one glycosyl transferase is expressed by the cell, additional glycosylation reactions can occur resulting in the formation of the target

oligosaccharide. Of course, the cell preferably lacks any enzyme activity which would degrade the oligosaccharide derivatives produced in the cell.

In a preferred realization of the process for making an oligosaccharide product, the sucrose utilization system is heterologous. This is the case when the microorganism, preferably a
5 bacterium, more preferably an *E. coli*, is a strain that is optimized for an industrially profitable transformation like oligosaccharide production, because such a strain generally has no ability to utilize sucrose. Therefore, a sucrose uptake cassette should be introduced, using an appropriate expression plasmid or via chromosome integration, in the sucrose minus cell to make it be sucrose plus. More preferably, the sucrose pathway genes comprise a PTS-
10 dependent sucrose utilization system, and especially the source regulon is *scr*. Microorganisms having *scr* genes are for example *Salmonella ssp.*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Vibrio alginolyticus*.

The *scr* genes comprise the following: *scrY*, *scrA*, *scrB*, *scrR* and *scrK*. The gene *scrA* codes for the sucrose transport protein Enzyme II^{Scr} that provides intracellular sucrose-6-phosphate
15 from extracellular sucrose via an active transport through the cell membrane and the concomitant phosphorylation. The sucrose specific ScrY porin (encoded by *scrY*) facilitate the sucrose diffusion through the outer membrane. The ScrB invertase enzyme (encoded by *scrB*) splits the accumulated sucrose-6-phosphate by hydrolysis to glucose-6-phosphate and fructose. Optionally, a fructokinase ScrK (encoded by *scrK*) phosphorylates fructose to fructose-6-
20 phosphate, however the presence of this enzyme is not crucial because the fructose can be phosphorylated by other mechanisms owned by the cell. The repressor protein ScrR (encoded by *scrR*) negatively controls the expression of the *scrYAB* genes and is induced by sucrose or fructose.

In one embodiment, the heterologous *scr* genes are introduced into the microorganism using
25 plasmids, for example by a two-plasmid system where one contains the *scrA* gene and the other does the *scrB* gene. The *scrY*, *scrR* and optionally the *scrK* gene can be carried by either plasmids. In a preferred embodiment, the heterologous *scr* genes are introduced into the genome of the microorganism, that is they are inserted in a certain site of the genome (genetic locus) of said microorganism or cell, preferably operably linked to one or more control
30 sequence(s) that is recognized by the host cell.

Also preferably, the genes coding for the glycosyl transferases are also inserted in the genome of the host cell. In this regard, the preferred oligosaccharide producing cells of the invention are plasmid-free. The advantage of having a plasmid free cell is that the production of the oligosaccharide, such as an HMO is increased and carbohydrate by-products are
35 decreased.

Also preferably, antibiotics are not added to the fermentation broth and neither during cell bank preparation and inoculum propagation in the process of some embodiments of this invention.

5 The carbohydrate acceptor to be glycosylated by the microorganism in the process of the invention can be a mono- or disaccharide selected from galactose, N-acetyl-glucosamine, a galactosylated monosaccharide, an N-acetyl-glucosaminylated monosaccharide, and glycosidic derivatives thereof defined above. All these carbohydrate derivatives can be easily taken up by a cell having a LacY permease by means of an active transport and accumulate in the cell before being glycosylated (WO 01/04341, Fort et al. *J. Chem. Soc., Chem. Comm.* 2558 (2005),
10 EP-A-1911850, WO 2013/182206, WO 2014/048439). This is because the cell is able to transport these carbohydrate acceptors into the cell using its LacY permease, and the cell lacks any enzymes that could degrade these acceptors, especially LacZ. Preferably the cell has a deleted or deficient *lacA* gene on the *lac* operon.

According to another preferred embodiment, the *lacI* gene for the *lac* repressor is also
15 deleted or deactivated in the microorganism. In the absence of the functioning repressor, no inducer is needed for any gene under the control of the promoter *Plac*.

According to one embodiment, the *hlyE* gene, coding for the toxin cytolysin A, is deleted or deactivated in order to eliminate the risk of unintended expression of the toxin and thus to obtain a safer manufacturing strain.

20 In one embodiment, if a *glp* promoter is used to express one or more recombinant genes in the microorganism or cell according to the invention (see above), the regulatory repressor gene *glpR* may be deleted or deactivated (see WO 2019/123324), which may result in a higher overall production of the product oligosaccharides, particularly HMOs.

According to one embodiment, the genetically modified cell used in the process of the
25 invention, particularly a LacZ⁻ *E. coli* cell, is cultured in an aqueous culture medium in the following phases:

(a) an exponential cell growth phase ensured by sucrose at least 12 hours, such as around 18 hours or 20-25 hours, and then

(b) a second (feeding) phase with sucrose which is added continuously ensuring a limited
30 cell growth that lasts until the sucrose and preferably most (e.g. at least 60 %) of the lactose have been consumed which is preferably at least 35 hours, such as at least 45 hours, 50 to 70 hours, or up to about 130 hours.

During the feeding phase, the exogenous carbohydrate acceptor, preferably lactose, to be internalized by and glycosylated in the cell, can be added to the culture medium at once,
35 sequentially or continuously. The acceptor can be added in this second phase as a pure

solid/liquid or in a form of a concentrated aqueous solution or suspension. The oligosaccharide production takes place in this second phase and can take up to 6-7 days. Preferably, this feeding phase is performed under conditions allowing the production of a culture with a high cell density.

- 5 A feature of the process of some embodiments of this invention is that there is no need to change the carbon source and/or the energy source between the growth phase and the production phase of the microorganism.

Optionally, in the case that the cells are still in possession of the lactose repressor gene *lacI*, the process further comprises the addition of an inducer to the culture medium to induce the
10 expression in the cell of enzyme(s) and/or of protein(s) involved in the transport of the acceptor and/or in the glycosylation of the internalized acceptor and/or in the biosynthesis of the activated sugar nucleotide donors. The inducer is preferably isopropyl β -D-thiogalactoside (IPTG) and is added to the culture medium in the beginning of the feeding phase. However, the use of inducer is not necessary if the cell is of *LacI⁻* genotype.

- 15 It is believed that some embodiments of the microorganisms used in the process of invention and described above are highly stable under the process conditions of this invention described above. As a result, it is believed that these microorganisms can be used to produce oligosaccharides using sucrose as their carbon and energy sources at least at the same production rate as, and in a more reliable and reproducible manner than, microorganisms using
20 glycerol and/or glucose as their carbon and/or energy sources. Beneficially, some embodiments of the genetically modified cells used in the process of the invention produce less carbohydrate by-products (e.g. 2'-FL, DFL, LNT or LNT-II, depending on what the desired product is) while the HMO titre and yield can be at least retained or even slightly increased, compared to the glucose or glycerol utilizing strains. These effects are increased even further when the
25 heterologous PTS-dependent sucrose utilization system is incorporated into the genome of the host cell. In one embodiment of the invention the amount of other HMO components compared to the desired HMO are decreased by at least 10%, preferably at least 15%, preferably at least 20%, preferably at least 25%, when the genetically modified organism of the invention is grown on sucrose as compared to a similar microorganism which does not contain the PTS-dependant
30 sucrose system and therefore is grown on glucose. In one embodiment of the invention the DFL/2'-FL ratio is decreased by at least 10%, by at least 15%, such as at least 20%, such as at least 25% when the genetically modified microorganism of the present invention is grown on sucrose, compared to a similar microorganism which does not contain the PTS-dependant sucrose system and therefore is grown on glucose. The use of the sucrose strains is therefore
35 beneficial as it facilitates the purification process. Furthermore, the HMOs made by the sucrose strains are extremely low in the sum of unspecified impurities. The reduced by-product formation provides a bigger upstream process optimisation potential, since the sucrose strain

does not waste as much energy in the production. This means that the process of making the oligosaccharide, such as an HMO, using a modified microorganism according to the invention, and growing it on sucrose as carbon source results in an improved carbon/energy utilization compared to when glucose is used as carbon source. Additionally, the sucrose is more resistant to heat sterilization than e.g. monosaccharide carbon sources like glucose, and thus the HMOs produced by the sucrose strain do not contain such degradation by-products, in particular the HMO product produced by the process of the present invention is essentially free of isomaltose, kojibiose, gentiobiose and nigerose.

At the end of the fermentation process, the oligosaccharide product has accumulated both in the intra- and the extracellular matrix of the microorganism. The product has preferably been transported out of the cell to the supernatant in a passive way, i.e. it can diffuse outside across the cell membrane. This transport can be facilitated by one or more sugar efflux transporters in the cell, i.e. proteins that promote the effluence of sugar derivatives from the cell to the supernatant. The sugar efflux transporter(s) can be homologous or heterologous and can be overexpressed under the conditions of the fermentation to enhance the export of the oligosaccharide derivative produced. The specificity towards the sugar moiety of the product to be secreted can be altered by mutation of the cell by means of conventional recombinant DNA manipulation techniques and protein engineering. Preferably, the oligosaccharide accumulates in the extracellular matrix. Alternatively, the oligosaccharide can be transported out of the cell to the supernatant by permeabilizing the cell or disrupting the cell walls in a conventional manner.

The oligosaccharide product can then be separated in a conventional manner from the aqueous culture medium, in which it was made by the cell.

A first step of separating the oligosaccharide from the culture medium preferably involves separating the oligosaccharide from the microorganism which produced it. This preferably involves clarifying the culture medium to remove suspended particulates and contaminants, particularly cells, cell components, insoluble metabolites and debris produced by culturing the genetically modified microorganism. In this step, the aqueous culture medium, which contains the oligosaccharide product, can be clarified in a conventional manner. Preferably, the culture medium is clarified by centrifugation and/or microfiltration and/or ultrafiltration.

A second step of separating the oligosaccharide from the culture medium preferably involves removing substantially all the proteins, as well as peptides, amino acids, RNA and DNA and any endotoxins and glycolipids that could interfere with the subsequent separation step, from the aqueous culture medium, preferably after it has been clarified. In this step, proteins and related impurities can be removed from the culture medium in a conventional manner. For example, proteins and related impurities are removed from the culture medium by ultrafiltration, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and/or gel filtration (i.e.,

size exclusion chromatography), particularly by chromatography, more particularly by ion exchange chromatography or hydrophobic interaction chromatography. With the exception of size exclusion chromatography, proteins and related impurities are retained by a chromatography medium or a selected membrane, while the oligosaccharide product remains in the aqueous culture medium.

If desired, the oligosaccharide product in the aqueous culture medium can then be separated from sugar-like by-product(s) and from the culture medium, after proteins and related impurities have been removed from the culture medium. This can be suitably done by subjecting the culture medium to chromatographic separation. This separation can be carried out, in case of a neutral oligosaccharide product, in a chromatographic separation column(s), filled with a conventional cationic and/or anionic ion exchange resin. The acidic cationic ion exchange resin can be in monovalent or divalent cationic form and is preferably in H^+ , K^+ , Na^+ , Mg^{2+} or Ca^{2+} form, particularly Ca^{2+} . The chromatographic separation can be carried out in a conventional manner at a pH of the solution of 2 to 9. The eluent used in the chromatographic separation is preferably water, especially demineralized water, but aqueous salt solutions can also be used. Alcohols, such as ethanol, and aqueous alcohol mixtures can also be used. Treatment with ion exchange resins removes charged particles and salts, too.

Optionally, at any phase of the separation of the oligosaccharide product from the culture medium, the aqueous solution containing said oligosaccharide can be treated with active charcoal, preferably to remove coloured bodies from the solution.

According to a preferred embodiment, the process of this invention for producing an oligosaccharide, preferably having a lactose unit at the reducing end or a glycoside thereof comprises the steps of:

(i) providing a genetically modified cell comprising

- a recombinant gene encoding a glycosyl transferase enzyme which is able to transfer the glycosyl residue of an activated sugar nucleotide to the internalized lactose or glycoside thereof,
- a biosynthetic pathway to the activated sugar nucleotide,

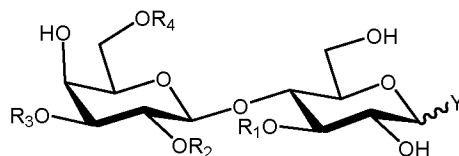
(ii) culturing the genetically modified cell in the presence of the exogenous lactose or glycoside thereof and sucrose inducing

- internalization of the exogenous lactose or glycoside thereof via an active transport mechanism by the genetically modified cell, and
- formation of the oligosaccharide having a lactose unit at the reducing end or a glycoside thereof from the internalized lactose or glycoside thereof by a glycosyl transfer mediated by the glycosyl transferase enzyme expressed by the cell,

(iii) isolating the oligosaccharide product from the cell, from the culture medium or from both, characterized in that the cell also comprises a heterologous sucrose utilization system, preferably a PTS-dependent sucrose utilization system, especially where the source regulon is *scr*, to provide sucrose as a carbon source for biosynthesis of said activated sugar nucleotide by said cell.

The genetically modified cell, used in this preferred process, can have more than one recombinant gene, encoding more than one glycosyl transferase enzyme which is able to transfer the glycosyl residue of an activated sugar nucleotide to an internalized acceptor molecule or the previously glycosylated acceptor made by the same cell, so the oligosaccharide product is formed from the internalized acceptor by multiple glycosyl transfer mediated by multiple glycosyl transferases expressed by the cell. Accordingly, the resulting oligosaccharide product can be a glycosylated lactose or a glycoside thereof. The glycosylated lactose is preferably an N-acetyl-glucosaminylated, galactosylated, fucosylated and/or sialylated lactose. In order to produce these derivatives the cell comprises one or more recombinant genes encoding an N-acetyl-glucosaminyl transferase, a galactosyl transferase, a sialyl transferase and/or a fucosyl transferase, and also comprise a biosynthetic pathway to generate the corresponding activated sugar type nucleotides, that is UDP-GlcNAc, UDP-Gal, GDP-Fuc and/or CMP-sialic acid, used as sugar donors for the transferases.

More preferably, the oligosaccharide product made by this process is characterized by formula 1



1

wherein Y is OH or a non-sugar aglycon defined above, preferably OH,

R₁ is fucosyl or H,

R₂ is fucosyl or H,

R₃ is selected from H, sialyl, N-acetyl-lactosaminyl and lacto-N-biosyl groups, wherein the N-acetyl-lactosaminyl group can carry a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; each of the N-acetyl-lactosaminyl and lacto-N-biosyl groups can be substituted with one or more sialyl and/or fucosyl residue,

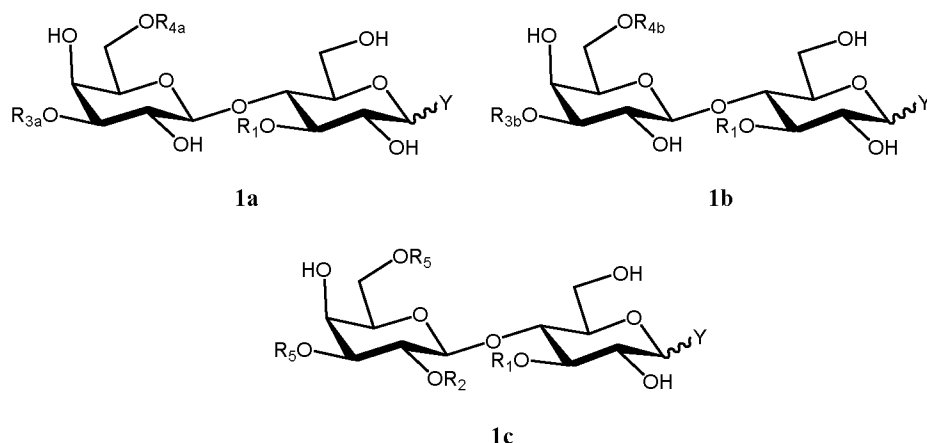
R₄ is selected from H, sialyl and N-acetyl-lactosaminyl groups optionally substituted with a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-

N-biosyl groups; each of the N-acetyl-lactosaminy and lacto-N-biosyl groups can be substituted with one or more sialyl and/or fucosyl residue,

provided that at least one of the R_1 , R_2 , R_3 and R_4 groups is different from H.

Even more preferably, the compound of formula **1** made by this process can be

5 characterized by formula **1a**, **1b** or **1c**



wherein Y, R_1 and R_2 are as defined above, preferably OH,

R_{3a} is an N-acetyl-lactosaminy group optionally substituted with a glycosyl residue comprising one N-acetyl-lactosaminy and/or one lacto-N-biosyl group; each of the N-acetyl-lactosaminy and lacto-N-biosyl groups can be substituted with one or more sialyl and/or fucosyl residue,

R_{4a} is H or an N-acetyl-lactosaminy group optionally substituted with a lacto-N-biosyl group; each of the N-acetyl-lactosaminy and lacto-N-biosyl groups can be substituted with one or more sialyl and/or fucosyl residue,

R_{3b} is a lacto-N-biosyl group optionally substituted with one or more sialyl and/or fucosyl residue(s),

R_{4b} is H or an N-acetyl-lactosaminy group optionally substituted with one or two N-acetyl-lactosaminy and/or one lacto-N-biosyl groups; each of the N-acetyl-lactosaminy and lacto-N-biosyl groups can be substituted with one or more sialyl and/or fucosyl residues,

R_5 is, independently, H or sialyl,

and wherein at least one of R_1 , R_2 or R_5 is not H.

Still more preferably, the compounds according to formulae **1a** or **1b** made by this process are characterized in that:

- the N-acetyl-lactosaminy group in the glycosyl residue of R_{3a} is attached to another N-acetyl-lactosaminy group with a 1-3 interglycosidic linkage,

- the lacto-N-biosyl group in the glycosyl residue of R_{3a} is attached to the N-acetyl-lactosaminyl group with a 1-3 interglycosidic linkage,

- the lacto-N-biosyl group in the glycosyl residue of R_{4a} is attached to the N-acetyl-lactosaminyl group with a 1-3 interglycosidic linkage,

5 - the N-acetyl-lactosaminyl group in the glycosyl residue of R_{4b} is attached to another N-acetyl-lactosaminyl group with a 1-3 or a 1-6 interglycosidic linkage,

- the lacto-N-biosyl group in the glycosyl residue of R_{4b} is attached to the N-acetyl-lactosaminyl group with a 1-3 interglycosidic linkage.

10 Yet more preferably, the compounds according to formulae **1a**, **1b** and **1c** made by the process are human milk oligosaccharides (when Y is OH) or glycosides thereof (when Y is non-sugar aglycon).

15 The preferred compounds of formula **1a** made by the process are selected from lacto-N-neotetraose (LNnT), para-lacto-N-hexaose (pLNH), para-lacto-N-neohexaose (pLNnH), lacto-N-neohexaose (LNnH), para-lacto-N-octaose (pLNO), lacto-N-neooctaose (pLNnO) and glycosides thereof, all of which can optionally be substituted with one or more sialyl and/or fucosyl residue. The preferred compounds of formula **1b** made by the process is selected from lacto-N-tetraose (LNT), lacto-N-hexaose (LNH), lacto-N-octaose (LNO), iso-lacto-N-octaose (iLNO), lacto-N-decaose (LND), lacto-N-neodecaose (LNnD) and glycosides thereof, all of them can optionally be substituted with one or more sialyl and/or fucosyl residue.

20 Particularly preferred compounds of formula **1a** or **1b** are characterized in that:

- the fucosyl residue attached to the N-acetyl-lactosaminyl and/or the lacto-N-biosyl group is linked to

25 ▪ the galactose of the lacto-N-biosyl group with 1-2 interglycosidic linkage and/or

▪ the N-acetyl-glucosamine of the lacto-N-biosyl group with 1-4 interglycosidic linkage and/or

▪ the N-acetyl-glucosamine of the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,

- the sialyl residue attached to the N-acetyl-lactosaminyl and/or the lacto-N-biosyl group is linked to

30 ▪ the galactose of the lacto-N-biosyl group with 2-3 interglycosidic linkage and/or

▪ the N-acetyl-glucosamine of the lacto-N-biosyl group with 2-6 interglycosidic linkage and/or

▪ the galactose of the N-acetyl-lactosaminyl group with 2-6 interglycosidic linkage.

According to the most preferred aspect, the compounds of subformulae **1a**, **1b** or **1c** are selected from the group of: 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 2',3-difucosyllactose (2',3-DFL), 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), 3'-sialyl-3-fucosyllactose (3'S3FL), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), lacto-N-fucopentaose I (LNFP-I), lacto-N-fucopentaose II (LNFP-II), lacto-N-fucopentaose III (LNFP-III), lacto-N-fucopentaose V (LNFP-V), sialyllacto-N-tetraose a (LST-a), sialyllacto-N-tetraose b (LST-b), sialyllacto-N-tetraose c (LST-c), Sialylfucosyllacto-N-tetraose (FLST-a), Fucosylsialyllacto-N-tetraose (FLST-b), Fucosylsialyllacto-N-neotetraose (FLST-c), lacto-N-difuco-hexaose I (LNDFH-I), Lacto-N-difucohexaose II (LNDFH-II), Lacto-N-neodifucohexaose II (LNDFH-III), Disialyllacto-N-tetraose (DS-LNT), Fucosyldisialyllacto-N-tetraose (FDS-LNT I), Disialylfucosyllacto-N-tetraose (FDS-LNT II) and their glycosides, or salts thereof. The glycosides can be alpha or beta-anomers, but preferably beta-anomers.

One preferred carbohydrate acceptor, exogenously added to the culture medium, is lactose, and the preferred oligosaccharide product is a human milk oligosaccharide (HMO). The HMOs consist of a lactose unit at the reducing end and one or more from the following monosaccharide units: N-acetyl-glucosamine, galactose, fucose and sialic acid (see Urashima et al.: *Milk Oligosaccharides*, Nova Biomedical Books, New York, 2011, ISBN: 978-1-61122-831-1; Chen *Adv. Carbohydr. Chem. Biochem.* **72**, 113 (2015)). In order to produce HMOs the cell then comprises one or more recombinant genes encoding β -1,3-N-acetyl-glucosaminyl transferase, β -1,6-N-acetyl-glucosaminyl transferase, β -1,3-galactosyl transferase, β -1,4-galactosyl transferase, α -2,3-sialyl transferase, α -2,6-sialyl transferase, α -1,2-fucosyl transferase, α -1,3-fucosyl transferase and/or α -1,4 fucosyl transferase, and also comprise a biosynthetic pathway to produce the corresponding activated sugar type nucleotides, that is UDP-GlcNAc, UDP-Gal, GDP-Fuc and/or CMP-sialic acid.

According to a preferred embodiment, the process of the invention relates to the production of neutral (non-acidic) HMOs. The neutral HMOs do not contain sialic acid residue in their structure and therefore can be non-fucosylated neutral HMOs (referred to as N-acetylated HMOs or core HMOs) and fucosylated HMOs. The N-acetylated HMOs has only three types of monosaccharide in their structure: glucose, galactose and N-acetyl-glucosamine, and the N-acetylated HMOs are preferably selected from the group consisting of lacto-N-neotetraose, para-lacto-N-hexaose, para-lacto-N-neohexaose, lacto-N-neohexaose, para-lacto-N-octaose, lacto-N-neooctaose, lacto-N-tetraose, lacto-N-hexaose, lacto-N-octaose, iso-lacto-N-octaose, lacto-N-decaose and lacto-N-neodecaose, more preferably lacto-N-neotetraose, lacto-N-tetraose, lacto-N-hexaose, para-lacto-N-hexaose, para-lacto-N-neohexaose and lacto-N-neohexaose. The group of fucosylated neutral HMOs encompasses N-acetylated HMOs disclosed above that are fucosylated and difucosylated lactoses (2'-FL, 3-FL, DFL).

According, the process, in a preferred embodiment, relates to the production of neutral HMOs comprising:

(i) providing a genetically modified *E. coli* cell comprising:

(ia) - a recombinant gene encoding a N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to internalized lactose or to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-GlcNAc; and/or

- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to the internalized lactose or to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to GDP-Fuc,

(ib) - optionally a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-Gal,

(ii) culturing the genetically modified *E. coli* cell in the presence of exogenous lactose and sucrose, thereby inducing:

- internalization of the exogenous lactose, and
- formation, within the cell, of neutral HMO, and then

(iii) separating the neutral HMO from the cell, from the culture medium or from both, characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of UDP-GlcNAc and/or GDP-Fuc, and optionally UDP-Gal by the cell, and as energy source for making said neutral HMO.

The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, and particularly does not contain *scrK*.

According to another preferred embodiment, the process of this invention for producing an N-acetylated HMO, preferably of 3-6 monosaccharide units, comprises the steps of:

(i) providing a genetically modified *E. coli* cell comprising:

- a recombinant gene encoding a N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to internalized lactose or to an intermediate in the biosynthetic pathway to said N-acetylated HMO from lactose,

- optionally a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to the N-acetyl-glucosaminylated lactose to an intermediate in the biosynthetic pathway to said N-acetylated HMO from lactose, and
- one or more genes encoding a biosynthetic pathway to UDP-GlcNAc and optionally to UDP-Gal,

(ii) culturing the genetically modified *E. coli* cell in the presence of exogenous lactose and sucrose, thereby inducing:

- internalization of the exogenous lactose, and
- formation, within the cell, of an N-acetylated HMO, and then

(iii) separating the N-acetylated HMO from the cell, from the culture medium or from both, characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of UDP-GlcNAc and optionally UDP-Gal by the cell, and as energy source for making said N-acetylated HMO.

The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, and particularly does not contain *scrK*.

If the N-acetyl-glucosaminyl transferase is a β -1,3-N-acetyl-glucosaminyl transferase and no recombinant gene encoding a galactosyl transferase is present in the cell, the product is preferably lacto-N-triose II, and if a β -1,3- or a β -1,4-galactosyl transferase is also present in the cell, the product is preferably LNT or LNnT, respectively.

According to another preferred embodiment, the process of this invention for producing a fucosylated N-acetylated HMO, preferably LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II or LNDFH-III, comprises the steps of:

(i) providing a genetically modified *E. coli* cell comprising:

- a recombinant gene encoding a N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-GlcNAc;
- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to GDP-Fuc, and
- a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to an intermediate in the biosynthetic pathway to said

fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-Gal,

(ii) culturing the genetically modified *E. coli* cell in the presence of exogenous lactose and sucrose, thereby inducing:

- 5 - internalization of the exogenous lactose, and
- formation, within the cell, of the fucosylated N-acetylated HMO, and then

(iii) separating the fucosylated N-acetylated HMO from the cell, from the culture medium or from both,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose
10 utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of UDP-GlcNAc, GDP-Fuc and optionally UDP-Gal by the cell, and as energy source for making said fucosylated N-acetylated HMO.

The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, and particularly does not contain *scrK*.

15 According to yet another preferred embodiment, the process of this invention for producing a fucosylated lactose, preferably 2'-FL, 3-FL or DFL, comprises the steps of:

(i) providing a genetically modified *E. coli* cell comprising:

- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to internalized lactose, and
- 20 - one or more genes encoding a biosynthetic pathway to GDP-Fuc,

(ii) culturing the cell in the presence of exogenous lactose and sucrose, thereby inducing:

- internalization of the exogenous lactose, and
- formation of the fucosylated lactose, and then

(iii) separating the fucosylated lactose product from the cell, from the culture medium or from
25 both,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of GDP-Fuc by the cell, and as energy source for making said fucosylated lactose.

The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes,
30 more preferably *scrY*, *scrA*, *scrB* and *scrR*, which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%, such as 95%, such as 98% identity to the respective sequence and particularly does not contain *scrK*.

If the fucosyl transferase is an α -1,2-fucosyl transferase, the product is preferably 2'-fucosyllactose (2'-FL), if the fucosyl transferase is an α -1,3-fucosyl transferase, the product is preferably 3-fucosyllactose (3-FL), and if both α -1,2- and α -1,3-fucosyl transferases are expressed in the cell, the product is preferably difucosyllactose (DFL).

5 According to still another preferred embodiment, the process of this invention for producing a sialylated HMO, preferably of 3-5 monosaccharide units, comprises the steps of:

(i) providing a genetically modified *E. coli* cell comprising:

- a recombinant gene encoding a sialyl transferase enzyme which is able to transfer the sialyl residue of CMP-sialic acid to internalized lactose or to an intermediate in the biosynthetic pathway to said sialylated HMO from lactose, and
- one or more genes encoding a biosynthetic pathway to CMP-sialic acid,

(ii) culturing the genetically modified *E. coli* cell in the presence of exogenous lactose and sucrose, thereby inducing:

- internalization of the exogenous lactose, and
- formation of a sialylated HMO, and then

(iii) separating the sialylated HMO from the cell, from the culture medium or from both,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of CMP-sialic acid by the cell, and as energy source for making said sialylated HMO.

The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR* which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%, such as 95%, such as 98% identity to the respective sequence, and particularly does not contain *scrK*.

25 If the sialyl transferase is an α -2,3-sialyl transferase, the product is preferably 3'-sialyllactose, and if the sialyl transferase is an α -2,6-sialyl transferase, the product is preferably 6'-sialyllactose.

According to another preferred embodiment, the process of this invention for producing a fucosylated and sialylated HMO, preferably of 3-5 monosaccharide units, comprises the steps of:

(i) providing a genetically modified *E. coli* cell comprising:

- a recombinant gene encoding a sialyl transferase enzyme which is able to transfer the sialyl residue of CMP-sialic acid to internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated and sialylated HMO from lactose,

- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated and sialylated HMO from lactose, and

- one or more genes encoding a biosynthetic pathway to CMP-sialic acid and GDP-Fuc,

5 (ii) culturing the genetically modified *E. coli* cell in the presence of exogenous lactose and sucrose, thereby inducing:

- internalization of the exogenous lactose, and

- formation of a sialyl-fucosyl-lactose, and then

10 (iii) separating the fucosylated and sialylated HMO from the cell, from the culture medium or from both,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of CMP-sialic acid and GDP-Fuc by the cell, and as energy source for making said fucosylated and sialylated HMO.

15 The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR* which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%, such as 95%, such as 98% identity to the respective sequence, and particularly does not contain *scrK*.

20 If the sialyl transferase is an α -2,3-sialyl transferase and the fucosyl transferase is an α -1,3-fucosyl transferase, the product is preferably 3'-sialyl-3-fucosyllactose.

According to another preferred embodiment, the process of this invention for producing a fucosylated LNT or LNnT, comprises the steps of:

(i) providing a genetically modified *E. coli* cell comprising:

25 - a recombinant gene encoding a N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to internalized lactose to make lacto-N-triose II,

- a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to the lacto-N-triose II to make LNT or LNnT,

- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to the LNT or LNnT,

30 - one or more genes encoding a biosynthetic pathway to GDP-Fuc

- one or more genes encoding a biosynthetic pathway to UDP-GlcNAc and to UDP-Gal,

(ii) culturing the genetically modified *E. coli* cell in the presence of exogenous lactose and sucrose, thereby inducing:

- internalization of the exogenous lactose, and
- formation of a fucosylated LNT or LNnT, and then

(iii) separating the fucosylated LNT or LNnT from the cell, from the culture medium or from both,

5 characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of UDP-GlcNAc, UDP-Gal and GDP-Fuc by the cell, and as energy source for making said fucosylated LNT or LNnT.

10 The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%, such as 95%, such as 98% identity to the respective sequence, and particularly does not contain *scrK*.

15 A second aspect of the invention relates to providing a genetically modified microorganism that can internalize sucrose and a carbohydrate acceptor, which is not sucrose, preferably lactose, or a glycoside thereof, into said microorganism and that comprises:

- a recombinant gene encoding a glycosyl transferase which can transfer a glycosyl residue of an activated sugar nucleotide to the acceptor within the microorganism,
 - a biosynthetic pathway for making the activated sugar nucleotide from sucrose, and
 - one or more genes encoding a heterologous PTS-dependent sucrose utilization system,
- 20 preferably *scr* genes, so that said cell can use sucrose as a carbon source, preferably the main carbon source, more preferably the sole carbon source, for making said activated sugar nucleotide and as an energy source, preferably the main energy source, more preferably the sole energy source, thereby making an oligosaccharide or a glycoside thereof comprising the internalized carbohydrate acceptor in its structure.

25 The aglycon moieties of the glycosides of the internalized carbohydrate acceptors or the oligosaccharide products are disclosed in the first aspect.

The genetically modified microorganism or cell of this invention can be selected from the group consisting of bacteria and yeasts, preferably a bacterium. Bacteria are preferably selected from the group of: *Escherichia coli*, *Bacillus* spp. (e.g. *Bacillus subtilis*), *Campylobacter pylori*, *Helicobacter pylori*, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, *Thermophilus aquaticus*, *Azorhizobium caulinodans*, *Rhizobium leguminosarum*, *Neisseria gonorrhoeae*, *Neisseria meningitis*, *Lactobacillus* spp., *Lactococcus* spp., *Enterococcus* spp., *Bifidobacterium* spp., *Sporolactobacillus* spp., *Micromonospora* spp., *Micrococcus* spp., *Rhodococcus* spp., *Pseudomonas*, among which *E. coli* is preferred, especially *E. coli* K-12 or *E. coli* BL-21 .

Further in accordance with some embodiments of this invention, the microorganism or cell, preferably an *E.coli* cell, is genetically modified so that there is at least one alteration in its DNA sequence. The alteration can result in a change in the original characteristics of the wild type cell, e.g. the modified cell is able to perform additional chemical transformation due to the introduced new genetic material that encodes the expression of an enzymes not being in the wild type cell, or is not able to carry out transformation like degradation due to removal or deactivation of gene/genes. In this regard, a genetically modified cell contains at least one artificial alteration in its genome compared to its naturally occurring (wild type) variant. By the alteration, either a nucleic acid construct is added to the cell by way of integration into the genome or by addition via plasmid, or a nucleic acid sequence is deleted from or changed in the genome of the cell. Whatever is the case, the so-transformed cell has a genotype that is different from that before the alteration and, therefore, the modified cell shows modified feature(s). Preferably, the genetically modified cell can perform at least one additional or altered biochemical reaction, when cultured or fermented, due to the introduction of a heterologous nucleic acid sequence or the modification of a native nucleic acid sequence that encodes an enzyme that is not expressed in the wild type cell, or the genetically modified cell cannot perform a biochemical reaction due to the deletion, addition or modification of a nucleic acid sequence that encodes an enzyme found in the wild type cell. The genetically modified cell can be constructed by well-known, conventional genetic engineering techniques (e.g. Green and Sambrook: *Molecular Cloning: A laboratory Manual*, 4th ed., Cold Spring Harbor Laboratory Press (2012); *Current protocols in molecular biology* (Ausubel et al. eds.), John Wiley and Sons (2010)). The terms "host cell", "recombinant microorganism or cell" or "genetically modified microorganism or cell" are used interchangeably to designate such constructs.

The term "promoter" means a nucleic acid sequence involved in the binding of RNA polymerase to initiate transcription of an operably linked gene, wherein the gene includes a coding DNA sequence and other (non-coding) sequences, e.g. the 5'-untranslated region (5'-UTR) located upstream of the coding sequence, which comprises a ribosomal binding site. A promoter in some embodiments of this invention is an isolated DNA sequence, i.e. not an integrated DNA fragment of the genomic DNA. The nucleotide sequence of a promoter corresponds to, or have at least 80 % identity, preferably 90-99.9 % identity with the nucleotide sequence of a fragment of bacterial genomic DNA that is regarded as promoter region of a gene, e.g. a promoter region of a *glp* operon or *lac* operon of *E.coli*. By "operon" is meant a functioning unit of genomic DNA containing a cluster of genes under the control of a single promoter. By "*glp* operon" is meant a cluster of genes involved in the respiratory metabolism of glycerol of bacteria. Preferably, a *glp* operon promoter sequence comprised in a DNA construct corresponds to or has at least 80 % identity, preferably 90-99.9 % identity with the nucleotide sequence of a fragment of the genomic DNA regarded as a promoter region of the corresponding *glp* operon of *E. coli*; in particular, the isolated sequence of a promoter of a *glp*

operon of some embodiments of the invention corresponds to, or has said percent of identity with a fragment of the genomic sequence upstream the sequence having GenBank ID: EG10396 (*glpFKX*), EG10391 (*glpABC*), EG10394 (*glpD*), EG10401 (*glpTQ*). The *E. coli* genome is referred herein to the complete genomic DNA sequence of *E. coli* K-12 MG1655 (GenBank ID:U00096.3).

In some embodiments, a promoter of the *glp* operon is operably linked to the heterologous glycosyl transferase genes and/or the heterologous *scr* genes. Preferably, those genes are expressed under a *glpF* promoter described in WO 2019/123324, the content of which is incorporated herein by reference, even more preferably by the *glpF* promoter of SEQ ID NO: 5.

The genetically modified microorganism or cell of this invention is able to transport the exogenous carbohydrate acceptor, preferably lactose, into the cell for glycosylation to produce a foreign oligosaccharide of interest, preferably without adversely affecting the basic functions of the cell or destroying its integrity. In one embodiment, the transport takes place via a passive mechanism, during which the exogenous acceptor diffuses passively across the plasma membrane of the cell. Diffusion of the acceptor into the microorganism is a function of the concentration differences between the fermentation broth and the extra- and intracellular space of the cell with respect to the acceptor, whereby the acceptor passes from the place of higher concentration to the place of lower concentration. In another embodiment, the acceptor is internalized with the aid of an active transport. In such a case, the genetically modified microorganism contains transporter proteins, called permeases, which act as enzymes and with which the microorganism is able to admit exogenous substances and to concentrate them in the cytoplasm. Specifically, lactose permease (LacY) acts specifically on galactose, simple galactosyl disaccharides such as lactose and their glycosides. The specificity towards the sugar moiety of the exogenous carbohydrate acceptor to be internalized can be altered by mutation of the microorganism by means of conventional recombinant DNA manipulation techniques. In a preferred embodiment, the internalization of exogenous lactose or its derivative takes place via an active transport mechanism mediated by a lactose permease. The genetically modified microorganism preferably lacks any enzyme activity (such as LacZ) that would degrade the acceptor. Likewise, the microorganism is not able to hydrolyze or degrade the oligosaccharide product or any oligosaccharide intermediate in the biosynthetic pathway to the oligosaccharide product.

Moreover, the genetically modified cell of the invention comprises one or more endogenous or recombinant genes encoding one or more glycosyl transferase enzymes that are able to transfer the glycosyl residue of an activated sugar nucleotide to the internalized acceptor. The gene or an equivalent DNA sequence thereof, if it is recombinant, can be introduced into the cell by conventional techniques, e.g. using an expression vector or by chromosomal integration. The origin of the heterologous nucleic acid sequence can be any animal (including human) or plant,

eukaryotic cells such as those from *Saccharomyces cerevisiae*, *Saccharomyces pombe*, *Candida albicans* or from algae, prokaryotic cells such as those originated from *E. coli*, *Bacteroides fragilis*, *Photobacterium sp.*, *Bacillus subtilis*, *Campylobacter pylori*, *Helicobacter pylori*, *Agrobacterium tumefaciens*, *Staphylococcus aureus*, *Thermophilus aquaticus*,

5 *Azorhizobium caulinodans*, *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, or virus. The glycosyl transferase enzyme/enzymes encoded and expressed by the gene(s) or equivalent DNA sequence(s) are preferably glucosyl transferases, galactosyl transferases, N-acetylglucosaminyl transferases, N-acetylgalactosaminyl transferases, glucuronosyl transferases, xylosyl transferases, mannosyl
10 transferases, fucosyl transferases, sialyl transferases and the like. In a preferred embodiment, the glycosyl transferases are selected from the group consisting of β -1,3-N-acetylglucosaminyl-transferase, β -1,6-N-acetylglucosaminyl-transferase, β -1,3-galactosyl-transferase, β -1,4-galactosyl-transferase, β -1,3-N-acetylglactosaminyl-transferase, β -1,3-glucuronosyl-transferase, α -2,3-sialyl-transferase, α -2,6-sialyl-transferase, α -2,8-sialyl-transferase, α -1,2-
15 fucosyl-transferase, α -1,3-fucosyl-transferase and α -1,4-fucosyl-transferase. More preferably, the glycosyl transferases are selected from β -1,3-N-acetylglucosaminyl transferase, β -1,6-N-acetylglucosaminyl transferase, β -1,3-galactosyl transferase, β -1,4-galactosyl transferase, α -2,3-sialyl transferase, α -2,6-sialyl transferase, α -1,2-fucosyl transferase, α -1,3-fucosyl
20 transferase and α -1,4 fucosyl transferase, that is from those involved in the construction of HMO core structures as well as fucosylated and/or sialylated HMOs and its glycosidic derivatives, wherein the aglycon is a moiety defined above at the group of carbohydrate acceptors. The genes encoding the above-mentioned transferases have been described in the literature.

The genetically modified microorganism or cell of the invention is capable of producing an oligosaccharide from an internalized precursor/acceptor which is preferably lactose and not
25 sucrose. Said cell possesses enzymatic activity that is necessary for synthesizing an oligosaccharide, from an internalized mono- or disaccharide, or a glycoside thereof, via consecutive glycosylation steps, wherein the internalized acceptor, e.g. lactose, is glycosylated, in a first glycosylation step, to a trisaccharide, then that trisaccharide is glycosylated, in a second glycosylating step, to a tetrasaccharide, and so forth. The glycosylation steps are
30 mediated by respective glycosyl transferases.

In the glycosyl transferase mediated glycosylation processes, activated sugar nucleotides serve as donors, and the glycosyl transferase in question transfers a monosaccharide of an appropriate donor molecule to the acceptor molecule. Each activated sugar nucleotide generally comprises a phosphorylated glycosyl residue attached to a nucleoside, and the specific glycosyl
35 transferase enzyme accepts only the specific sugar nucleotide. Thus, preferably the following activated sugar nucleotides are involved in the glycosyl transfer: UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, UDP-glucuronic acid, UDP-Xyl, GDP-Man, GDP-Fuc and CMP-sialic

acid, particularly those selected from the group consisting of UDP-Gal, UDP-GlcNAc, GDP-Fuc and CMP-sialic acid.

The genetically modified microorganism of this invention possesses a biosynthetic pathway to the above mentioned activated sugar nucleotides, that is, it has one or more sets of genes encoding one or more enzymes capable of the synthesis of one or more activated glycosyl nucleotides, ready for glycosylation in glycosyl transferase mediated reaction in the cell, when cultured. The sets of genes are either naturally present in the cell or introduced into the cell by means of recombinant DNA manipulation techniques. The production of the activated glycosyl nucleotides by the cell takes place under the action of enzymes involved in the biosynthetic pathway of that respective sugar nucleotide stepwise reaction sequence starting from a carbon source (for a review for monosaccharide metabolism see e.g. H. H. Freeze and A. D. Elbein: *Chapter 4: Glycosylation precursors*, in: *Essentials of Glycobiology*, 2nd edition (Eds. A. Varki et al.), Cold Spring Harbour Laboratory Press (2009)).

It is emphasized that the production of the activated sugar nucleotides by the genetically modified microorganism via its own biosynthetic pathway is advantageous compared to *in vitro* versions of transfer glycosylation, as it avoids using the very expensive sugar nucleotide type donors added exogenously, hence the donors are formed by the cell *in situ* and the phosphatidyl nucleoside leaving groups are recycled in the cell.

In addition, the microorganism of the invention comprises genes encoding a sucrose utilization system, that is the cell has a capability to catabolically utilize sucrose as a carbon source, as well as an energy source. The system that enables the cell to utilize sucrose can be one normally found in the gene pool of that cell but preferably is a heterologous system (i.e. derived from a different organism and transferred to the host cell by conventional recombinant DNA manipulation techniques). Typically, two kinds of sucrose catabolism can be used. According to the phosphoenolpyruvate (PEP)-dependent phosphotransferase system ("PTS"), sucrose is transported into the microorganism and concomitantly phosphorylated to generate intracellular sucrose-6-phosphate which is hydrolysed to glucose-6-phosphate and fructose that are then involved in the central carbon metabolism of the cell. PTS can be encoded by *scr* or *sac* genes. According to non-phosphotransferase-dependent system ("non-PTS"), extracellular sucrose enters the cell with the aid of a proton symport transport system (sucrose permease) and, after transport, is hydrolysed by an invertase enzyme to glucose and fructose followed by phosphorylation. In this regard, the *csc* regulon consists of genes encoding the enzymes that are responsible for the non-PTS sucrose utilization.

In a preferred embodiment, the sucrose utilization system is heterologous. This is the case when the microorganism, preferably a bacterium, more preferably an *E. coli*, such as *K-12* or *BL21*, is a strain that is optimized for an industrially profitable transformation like oligosaccharide production, because such a strain generally has no ability to utilize sucrose.

Therefore, a sucrose uptake cassette should be introduced, using an appropriate expression plasmid or via chromosome integration, in the sucrose minus cell to make it be sucrose plus. More preferably, the sucrose pathway genes comprise a PTS-dependent sucrose utilization system, and especially the source regulon is *scr*. Microorganisms having *scr* genes are for example *Salmonella* *ssp.*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Vibrio alginolyticus*.

The *scr* genes comprise the following: *scrY*, *scrA*, *scrB*, *scrR* and *scrK*. The gene *scrA* codes for the sucrose transport protein Enzyme II^{Scr} that provides intracellular sucrose-6-phosphate from extracellular sucrose via an active transport through the cell membrane and the concomitant phosphorylation. The sucrose specific ScrY porin (encoded by *scrY*) facilitate the sucrose diffusion through the outer membrane. The ScrB invertase enzyme (encoded by *scrB*) splits the accumulated sucrose-6-phosphate by hydrolysis to glucose-6-phosphate and fructose. Optionally, a fructokinase ScrK (encoded by *scrK*) phosphorylates fructose to fructose-6-phosphate, however the presence of this enzyme is not crucial because the fructose can be phosphorylated by other mechanisms owned by the cell. The repressor protein ScrR (encoded by *scrR*) negatively controls the expression of the *scrYAB* genes and is induced by sucrose or fructose.

Table 1: List of Scr proteins encoded by the *scr* genes

Organism	<i>scrA</i>	<i>scrB</i>	<i>scrR</i>	ScrY
<i>Salmonella</i> <i>ssp</i>	ALE63092.1	CAA47974.1	CAA47975.1	CAA40656.1
<i>Klebsiella pneumoniae</i>	AHM80898.1	WP_004191473.1	CDQ53602.1	WP_004147437.1
<i>Vibrio alginolyticus</i>	SQA41727.1	ARP45128.1	KLI71527.1	ARP45132.1

In one embodiment, the heterologous *scr* genes are introduced into the microorganism using plasmids, for example by a two-plasmid system where one contains the *scrA* gene and the other plasmid contains the *scrB* gene. The *scrY*, *scrR* and optionally the *scrK* gene can be carried by either plasmids.

In one embodiment the *scr* genes are selected from the sequences listed in table 1.

In one embodiment the *scrY* and *scrA* is on one plasmid and encodes the polypeptides of WP_004147437.1 (SEQ ID NO: 1) and AHM80898.1 (SEQ ID NO: 2), respectively, and the *scrB* and *scrR* is on the second plasmid encodes the polypeptides of CAA47974.1 (SEQ ID NO: 3) and CAA47975.1 (SEQ ID NO: 4), respectively.

In one embodiment the genetically modified microorganism comprises 4 genes which encode PTS-dependent sucrose utilization system, where the genes encode

- i) sucrose porin *scrY* of SEQ ID NO: 1 or a homologue thereof which is at least 85% identical to SEQ ID NO: 1, such as 90%, 95% or 98% identical to SEQ ID NO: 1; and

- ii) PTS permease *scrA* of SEQ ID NO: 2 or a homologue thereof which is at least 85% identical to SEQ ID NO: 2, such as 90%, 95% or 98% identical to SEQ ID NO: 2; and
- iii) sucrose-6-phosphate hydrolase *scrB* of SEQ ID NO: 3 or a homologue thereof which is at least 85% identical to SEQ ID NO: 3, such as 90%, 95% or 98% identical to SEQ ID NO: 3; and
- iv) repressor protein *scrR* of SEQ ID NO: 4 or a homologue thereof which is at least 85% identical to SEQ ID NO: 4 such as 90%, 95% or 98% identical to SEQ ID NO: 4

In a preferred embodiment, the heterologous *scr* genes are introduced into the genome of the microorganism, that is they are chromosomally integrated by insertion in a certain site of the genome (genetic locus) of said microorganism or cell, preferably operably linked to one or more control sequence(s) that is recognized by the host cell. Preferably, *scrY* and *scrA* are operably linked to one promoter element (an operon) and *scrB* and *scrR* is operably linked to another promoter element. The sequence of the two promoter elements may be identical for the two operons. In a referred embodiment the promoter element is *pglpF*, in particular the *pglpF* promoter of SEQ ID NO: 5. Other *pglpF* promoters are described in WO2019/123324, a preferred *pglpF* promoter is the one of SEQ ID NO:5.

In one embodiment the *scr* genes are introduced into the mixed acid fermentation pathway. Mixed acid fermentation results in a mixture of end products including lactate, acetate, succinate, formate and ethanol. The formation of these end products is driven by known pathways into which the *scr* genes can be introduced.

Also preferably, the genes coding for the glycosyl transferases are also inserted in the genome of the host cell (that is, chromosomally integrated).

Also preferably, if one or more recombinant genes involved in the biosynthetic pathway to the activated sugar type nucleotides, preferably to UDP-GlcNAc, UDP-Gal, GDP-Fuc and/or CMP-sialic acid, necessary for the production of the oligosaccharide, preferably the HMO, are present in the genetically modified cell of the invention, it is (they are) also inserted in the genome of the host cell.

In this regard, the most preferred oligosaccharide producing cells of the invention are plasmid-free.

The control sequences or promoters operably linked to heterologous DNA sequences encoding the glycosyl transferases necessary for making the oligosaccharide product or the proteins necessary for the sucrose uptake can be any suitable promoter. In some embodiments, a promoter of the *glp* operon is used, preferably *glpF* or its variant (WO 2019/123324, the content of which is incorporated herein by reference).

The carbohydrate acceptor to be glycosylated by the microorganism of the invention can be a mono- or disaccharide selected from galactose, N-acetyl-glucosamine, a galactosylated

monosaccharide, an N-acetyl-glucosaminylated monosaccharide, a lactose, and glycosidic derivatives thereof defined above. All these carbohydrate derivatives can be easily taken up by a cell having a LacY permease by means of an active transport and accumulate in the cell before being glycosylated (WO 01/04341, Fort et al. *J. Chem. Soc., Chem. Comm.* 2558 (2005), EP-A-1911850, WO 2013/182206, WO 2014/048439). This is because the cell is able to transport these carbohydrate acceptors into the cell using its LacY permease, and the cell lacks any enzymes that could degrade these acceptors, especially LacZ, this can be achieved by deleting or mutating the *lacZ* gene to render it incapable of expressing functional LacZ. Preferably the cell has a deleted or deficient *lacA* gene on the *lac* operon.

According to another preferred embodiment, the *lacI* gene for the *lac* repressor is also deleted or deactivated in the microorganism. In the absence of the functioning repressor, no inducer is needed for expressing any gene under the control of the promoter Plac.

According to one embodiment, the *hlyE* gene, coding for the toxin cytolysin A, is deleted or deactivated in order to eliminate the risk of unintended expression of the toxin and thus to obtain a safer manufacturing strain.

In one embodiment, if a *glp* promoter is used to express one or more recombinant genes in the microorganism or cell according to the invention (see above), the regulatory repressor gene *glpR* may be deleted or deactivated (WO 2019/123324), which may result in a higher overall production of the product oligosaccharides, particularly HMOs.

It is believed that the microorganisms of invention described above are highly stable under the process conditions of this invention described above. As a result, it is believed that these microorganisms can be used to produce oligosaccharides using sucrose as their carbon and energy sources at least at the same production rate as, and in a more reliable and reproducible manner than microorganisms using glycerol and/or glucose as their carbon and/or energy sources. Beneficially, the genetically modified cells used in the process of the invention produce less carbohydrate by-products while the HMO titre and yield can be at least retained or even slightly increased, compared to the glucose or glycerol utilizing strains. The use of the sucrose strains is therefore beneficial as it facilitates the purification process. Furthermore, the HMOs made by the sucrose strains are extremely low in the sum of unspecified impurities. The reduced by-product formation provides a bigger upstream process optimisation potential, since the sucrose strain does not waste as much energy in the production. Additionally, the sucrose is more resistant to heat sterilization than e.g. monosaccharide carbon sources like glucose, and thus the HMOs produced by the sucrose strain do not contain such degradation by-products. In particular the HMO product is free of isomaltose, kojibiose, gentiobiose and nigerose.

The genetically modified cell of the invention can have more than one recombinant gene, encoding more than one glycosyl transferase enzyme which is able to transfer the glycosyl

residue of an activated sugar nucleotide to an internalized acceptor molecule or the previously glycosylated acceptor made by the same cell, so the oligosaccharide product is formed from the internalized acceptor by multiple glycosyl transfer mediated by multiple glycosyl transferases expressed by the cell. Accordingly, the resulting oligosaccharide product can be a glycosylated lactose or a glycoside thereof. The glycosylated lactose is preferably an N-acetyl-glucosaminylated, galactosylated, fucosylated and/or sialylated lactose. In order to produce these derivatives the cell comprises one or more recombinant genes encoding an N-acetyl-glucosaminyl transferase, a galactosyl transferase, a sialyl transferase and/or a fucosyl transferase, and also comprise a biosynthetic pathway to the corresponding activated sugar type nucleotides, that is UDP-GlcNAc, UDP-Gal, GDP-Fuc and/or CMP-sialic acid.

According to a preferred embodiment, the genetically modified *E. coli* cell is suitable for producing a neutral HMO, preferably of 3-6 monosaccharide units, and comprises

- (ia) - a recombinant gene encoding a N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to internalized lactose or to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-GlcNAc; and/or
- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to internalized lactose or to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to GDP-Fuc,
- (ib) - optionally a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-Gal,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of UDP-GlcNAc and/or GDP-Fuc, and optionally UDP-Gal by the cell, and as energy source for making said neutral HMO. The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%, such as 95%, such as 98% identity to the respective sequence and particularly does not contain *scrK*.

According to a preferred embodiment, the genetically modified *E. coli* cell suitable for producing an N-acetylated HMO, preferably of 3-6 monosaccharide units, comprises:

- a recombinant gene encoding a N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to internalized lactose or to an intermediate in the biosynthetic pathway to said N-acetylated HMO from lactose,

5 - optionally a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to the N-acetyl-glucosaminylated lactose or to an intermediate in the biosynthetic pathway to said N-acetylated HMO from lactose, and

- one or more genes encoding a biosynthetic pathway to UDP-GlcNAc and optionally to UDP-Gal,

10 characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of UDP-GlcNAc and optionally UDP-Gal by the cell, and as energy source for making said N-acetylated HMO. The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%,
15 such as 95%, such as 98% identity to the respective sequence, and particularly does not contain *scrK*.

If the N-acetyl-glucosaminyl transferase is a β -1,3-N-acetyl-glucosaminyl transferase and no recombinant gene encoding a galactosyl transferase is present in the cell, the product is preferably lacto-N-triose II, and if a β -1,3- or a β -1,4-galactosyl transferase is also present in the
20 cell, the product is preferably LNT or LNnT, respectively.

According to another preferred embodiment, the genetically modified *E. coli* cell is suitable for producing a fucosylated N-acetylated HMO, preferably LNFP-I, LNFP-II, LNFP-III, LNFP-V, LNFP-VI, LNDFH-I, LNDFH-II or LNDFH-III, and comprises:

25 - a recombinant gene encoding an N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-GlcNAc;

30 - a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to GDP-Fuc, and

35 - a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-Gal,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of UDP-GlcNAc, GDP-Fuc and UDP-Gal by the cell, and as energy source for making said fucosylated N-acetylated HMO. The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%, such as 95%, such as 98% identity to the respective sequence and particularly does not contain *scrK*.

According to yet another preferred embodiment, the genetically modified *E. coli* cell is suitable for producing a fucosylated HMO, preferably 2'-FL, 3-FL or DFL, and comprises:

- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to internalized lactose, and
- one or more genes encoding a biosynthetic pathway to GDP-Fuc,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of GDP-Fuc by the cell, and as energy source for making said fucosylated HMO. The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%, such as 95%, such as 98% identity to the respective sequence and particularly does not contain *scrK*.

If the fucosyl transferase is an α -1,2-fucosyl transferase, the product is preferably 2'-fucosyllactose, if the fucosyl transferase is an α -1,3-fucosyl transferase, the product is preferably 3-fucosyllactose, and if both α -1,2- and α -1,3-fucosyl transferases are expressed in the cell, the product is preferably difucosyllactose.

According to still another preferred embodiment, the genetically modified *E. coli* cell suitable for producing a sialylated HMO, preferably of 3-5 monosaccharide units, more preferably a sialylated lactose, comprises:

- a recombinant gene encoding a sialyl transferase enzyme which is able to transfer the sialyl residue of CMP-sialic acid to internalized lactose or to an intermediate in the biosynthetic pathway to said sialylated HMO from lactose, and
- one or more genes encoding a biosynthetic pathway to CMP-sialic acid,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of CMP-sialic acid by the cell, and as energy source for making said sialylated HMO. The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more

preferably *scrY*, *scrA*, *scrB* and *scrR*, which encode the proteins of SEQ ID NO: 1, 2, 3 and 4, respectively or homologues thereof with at least 85%, such as 90%, such as 95%, such as 98% identity to the respective sequence, and particularly does not contain *scrK*.

If the sialyl transferase is an α -2,3-sialyl transferase, the product is preferably 3'-sialyllactose, and if the sialyl transferase is an α -2,6-sialyl transferase, the product is preferably 6'-sialyllactose.

According to another preferred embodiment, the genetically modified *E. coli* cell suitable for producing a fucosylated and sialylated HMO, preferably of 3-5 monosaccharide units, more preferably a sialyl-fucosyl-lactose, comprises:

- a recombinant gene encoding a sialyl transferase enzyme which is able to transfer the sialyl residue of CMP-sialic acid to internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated and sialylated HMO from lactose,
- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated and sialylated HMO from lactose, and
- one or more genes encoding a biosynthetic pathway to CMP-sialic acid and GDP-Fuc, characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of CMP-sialic acid and GDP-Fuc by the cell, and as energy source for making said fucosylated and sialylated HMO. The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, and particularly does not contain *scrK*.

If the sialyl transferase is an α -2,3-sialyl transferase and the fucosyl transferase is an α -1,3-fucosyl transferase, the product is preferably 3'-sialyl-3-fucosyllactose.

According to another preferred embodiment, the genetically modified *E. coli* cell is suitable for producing a fucosylated LNT or LNnT and comprises:

- a recombinant gene encoding a N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to the internalized lactose to make lacto-N-triose II,
- a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to the lacto-N-triose II to make LNT or LNnT,
- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to the LNT or LNnT,
- one or more genes encoding a biosynthetic pathway to GDP-Fuc
- one or more genes encoding a biosynthetic pathway to UDP-GlcNAc and to UDP-Gal,

characterized in that the cell also comprises a heterologous PTS-dependent sucrose utilization system to provide sucrose as a carbon source, as well as an energy source, for biosynthesis of UDP-GlcNAc, UDP-Gal and GDP-Fuc by the cell, and as energy source for making said fucosylated LNT or LNnT. The heterologous PTS-dependent sucrose utilization system preferably comprises *scr* genes, more preferably *scrY*, *scrA*, *scrB* and *scrR*, and particularly does not contain *scrK*.

EMBODIMENTS

The present invention specifically discloses the following embodiments:

1. A genetically modified microorganism for making an oligosaccharide or a glycoside of said oligosaccharide, comprising:

- a. a recombinant gene encoding a glycosyl transferase which can transfer a glycosyl residue of an activated sugar nucleotide to a carbohydrate acceptor or a glycoside of said acceptor,
- b. a biosynthetic pathway for making said activated sugar nucleotide from sucrose, and
- c. nucleic acid sequences which encode PTS-dependent sucrose utilization system, where the nucleic acid sequences encode:

- i) sucrose porin *scrY* of SEQ ID NO: 1 or a homologue thereof which is at least 85% identical to SEQ ID NO: 1, such as 90%, 95% or 98% identical to SEQ ID NO: 1; and

- ii) PTS permease *scrA* of SEQ ID NO: 2 or a homologue thereof which is at least 85% identical to SEQ ID NO: 2, such as 90%, 95% or 98% identical to SEQ ID NO: 2; and

- iii) sucrose-6-phosphate hydrolase *scrB* of SEQ ID NO: 3 or a homologue thereof which is at least 85% identical to SEQ ID NO: 3, such as 90%, 95% or 98% identical to SEQ ID NO: 3; and

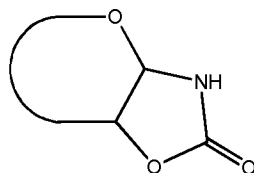
- iv) repressor protein *scrR* of SEQ ID NO: 4 or a homologue thereof which is at least 85% identical to SEQ ID NO: 4 such as 90%, 95% or 98% identical to SEQ ID NO: 4.

2. A genetically modified microorganism for making an oligosaccharide or a glycoside of said oligosaccharide, comprising:

- a recombinant gene encoding a glycosyl transferase which can transfer a glycosyl residue of an activated sugar nucleotide to a carbohydrate acceptor or a glycoside of said acceptor,
- a biosynthetic pathway for making said activated sugar nucleotide from sucrose, and
- one or more genes encoding a heterologous PTS-dependent sucrose utilization system, so that said cell is capable to use sucrose as a carbon source for making said activated sugar nucleotide and as energy source for making said oligosaccharide,

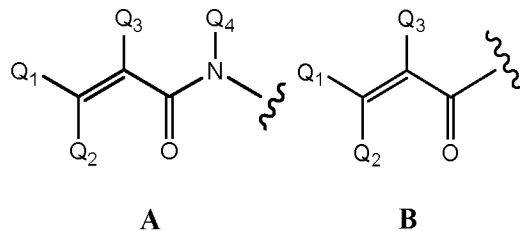
wherein the carbohydrate acceptor is not sucrose and said heterologous PTS-dependent sucrose utilization system comprises proteins encoded by *scr* genes *scrY* for a sucrose porin, *scrA* for a PTS permease, *scrB* for a sucrose-6-phosphate hydrolase and *scrR* for a repressor protein.

- 5 3. The genetically modified microorganism of embodiment 1 or 2, wherein the glycosyl transferase can transfer a glycosyl residue to a carbohydrate acceptor internalized by the microorganism.
4. The genetically modified microorganism of any one of embodiment 1 to 3, wherein the glycosyl transferase can transfer a glycosyl residue of an activated sugar nucleotide to an
- 10 intermediate in the biosynthetic pathway to said oligosaccharide from said acceptor.
5. The genetically modified microorganism of any one of embodiment 1 to 4, wherein an aglycon in the glycoside of a recombinant oligosaccharide or a carbohydrate acceptor is selected from the group consisting of:
 - -OR_A , wherein R_A is a linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms or, when unsaturated, 2-24, preferably 2-6 carbon atoms, or R_A means an aryl moiety, or R_A means a group removable by hydrogenolysis,
 - -X-R_B , wherein X is N or S, and R_B means linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms or, when unsaturated, 2-24, preferably 2-6 carbon atoms, or R_B means an aryl moiety, or R_B means a benzyl group,
 - 20 - a group that links an anomeric carbon atom and an adjacent carbon atom to each other by a
 - -NH-C(=O)-O- bridge, thus forming a fused 5-membered ring, as depicted in case of an aldose:



- 25 - azide,
- $\text{-NH-C(R'')=C(R')}_2$, wherein each R' independently is an electron withdrawing group selected from -CN, -COOH, -COO-alkyl, -CO-alkyl, -CONH₂, -CONH-alkyl and -CON(alkyl)₂, or wherein the two R' -groups are linked together and form -CO-(CH₂)₂₋₄-CO- and thus form with the carbon atom, to which they are attached, a 5-7 membered
- 30 cycloalkan-1,3-dione, in which dione any of its methylene groups is optionally substituted with 1 or 2 alkyl groups, and wherein R'' is H or alkyl,
- a residue of an amino acid,

- a polyethylene glycol residue,
- a polyvinyl alcohol residue, and
- an α,β -unsaturated amido group of formula A or an α,β -unsaturated carbonyl group of formula B



5

wherein Q2, Q3 and Q4 are, independently, H and C₁-C₆-alkyl, which alkyl is optionally substituted with halogen, OH, nitro or phenyl groups.

6. The genetically modified microorganism of any one of embodiment 1 to 5, which is *E. coli*.

7. The genetically modified microorganism of any one of embodiment 1 to 6, wherein one or
10 more of the following genes are deleted or deactivated: glpR, lacZ, lacI, lacA and hlyE.

8. The genetically modified microorganism according to embodiment 7, wherein lacZ is deleted or deactivated, and optionally one or more of the following genes are deleted or deactivated: glpR, lacI, lacA and hlyE.

9. The genetically modified microorganism of any one of embodiment 1 to 7, wherein
15 recombinant gene encoding a glycosyl transferase is chromosomally integrated in the microorganism.

10. The genetically modified microorganism according to embodiment 9, which is plasmid-free.

11. The genetically modified microorganism of any one of embodiment 1 to 10, wherein the glycosyl transferase(s) is operably linked to a glp, such as a glpF promoter, such as the glpF
20 promoter of SEQ ID NO: 5.

12. The genetically modified microorganism of any one of embodiment 1 to 11, wherein the scr genes are chromosomally integrated in the genome of the microorganism.

13. The genetically modified microorganism of embodiment 12, wherein scrY and scrA are operably linked and scrB and scrR are operably linked.

25 14. The genetically modified microorganism of any one of embodiments 2 to 13, wherein the scrYA genes are present in SEQ ID NO: 6, or a homologous sequence encoding identical proteins.

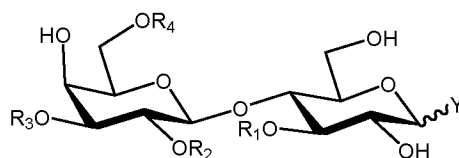
15. The genetically modified microorganism of any one of embodiments 2 to 14, wherein the the scrBR genes present in SEQ ID NO: 7 or a codon homologous sequence encoding identical
30 proteins.

16. The genetically modified microorganism of any one of embodiments 1 to 15, wherein the *scr* genes are operably linked to a *glp*, such as a *glpF* promoter, such as the *glpF* promoter of SEQ ID NO: 5.

17. The genetically modified microorganism of any one of embodiments 1 to 16, wherein the the *scrYA* and *scrBR* genes are integrated at two sites in the mixed-acid fermentation pathway.

18. The genetically modified microorganism of any one of embodiment 1 to 17, which is plasmid-free.

19. The genetically modified microorganism of any one of embodiment 1 to 18 for producing an oligosaccharide of formula 1

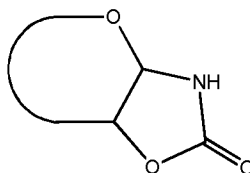


1

wherein:

Y is

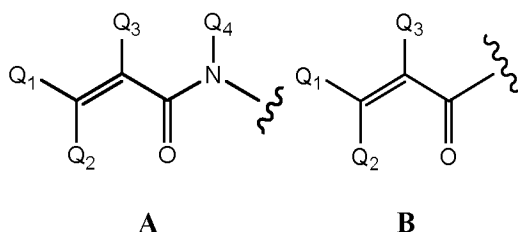
- -OH,
- -OR_A, wherein R_A is a linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms or, when unsaturated, 2-24, preferably 2-6 carbon atoms, or R_A means an aryl moiety, or R_A means a group removable by hydrogenolysis,
- -X-R_B, wherein X is N or S, and R_B means linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms or, when unsaturated, 2-24, preferably 2-6 carbon atoms, or R_B means an aryl moiety, or R_B means a benzyl group,
- a group that links an anomeric carbon atom and an adjacent carbon atom to each other by a -NH-C(=O)-O-bridge, thus forming a fused 5-membered ring, as depicted in case of an aldose:



- azide,
- -NH-C(R'')=C(R')₂, wherein each R' independently is an electron withdrawing group selected from -CN, -COOH, -COO-alkyl, -CO-alkyl, -CONH₂, -CONH-alkyl and -

CON(alkyl)₂, or wherein the two R'-groups are linked together and form -CO-(CH₂)₂₋₄-CO- and thus form with the carbon atom, to which they are attached, a 5-7 membered cycloalkan-1,3-dione, in which dione any of its methylene groups is optionally substituted with 1 or 2 alkyl groups, and wherein R'' is H or alkyl,

- 5 - a residue of an amino acid,
 - a polyethylene glycol residue,
 - a polyvinyl alcohol residue, or
 - an α,β-unsaturated amido group of formula A or an α,β-unsaturated carbonyl group of formula B



10 wherein Q2, Q3 and Q4 are, independently, H and C₁-C₆-alkyl, which alkyl is optionally substituted with halogen, OH, nitro or phenyl groups,

R₁ is fucosyl or H,

R₂ is fucosyl or H,

- 15 R₃ is selected from H, sialyl, N-acetyl-lactosaminy and lacto-N-biosyl groups, wherein the N-acetyl-lactosaminy group optionally carries a glycosyl residue comprising one or more N-acetyl-lactosaminy and/or one or more lacto-N-biosyl groups; each of the N-acetyl-lactosaminy and lacto-N-biosyl groups is optionally substituted with one or more sialyl and/or fucosyl residue,

- 20 R₄ is selected from H, sialyl and N-acetyl-lactosaminy groups optionally substituted with a glycosyl residue comprising one or more N-acetyl-lactosaminy and/or one or more lacto-N-biosyl groups; each of the N-acetyl-lactosaminy and lacto-N-biosyl groups is optionally substituted with one or more sialyl and/or fucosyl residue,

provided that at least one of the R₁, R₂, R₃ and R₄ groups is different from H.

- 25 20. The genetically modified microorganism of embodiment 19, wherein Y is -OH.

21. The genetically modified microorganism of embodiment 19 or 20, wherein the oligosaccharide of formula 1 represents a human milk oligosaccharide (HMO).

22. The genetically modified microorganism of embodiment 21, wherein the microorganism is *E. coli* and the HMO is a neutral HMO, and the *E. coli* comprises:

- (i) - a recombinant gene encoding an N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to an internalized lactose or to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-GlcNAc; and/or
- 5 - a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to an internalized lactose or to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to GDP-Fuc,
- (ii) - optionally a recombinant gene encoding a galactosyl transferase enzyme which is able
- 10 to transfer a galactosyl residue of UDP-Gal to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-Gal.

23. The genetically modified microorganism of embodiment 21, wherein the microorganism is *E. coli* and the HMO is an N-acetylated HMO, and the *E. coli* comprises:

- 15 - a recombinant gene encoding an N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to an internalized lactose or to an intermediate in the biosynthetic pathway to said N-acetylated HMO from lactose,
- optionally a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer a galactosyl residue of UDP-Gal to N-acetyl-glucosaminylated lactose or to an
- 20 intermediate in the biosynthetic pathway to said N-acetylated HMO from lactose, and
- one or more genes encoding a biosynthetic pathway to UDP-GlcNAc and optionally to UDP-Gal.

24. The genetically modified microorganism of embodiment 22 or 23, wherein:

- the N-acetyl-glucosaminyl transferase is a β -1,3-N-acetyl-glucosaminyl transferase, no
- 25 recombinant gene encoding a galactosyl transferase is present in the cell, and the N-acetylated HMO is lacto-N-triose II,
- the N-acetyl-glucosaminyl transferase is a β -1,3-N-acetyl-glucosaminyl transferase, the galactosyl transferase is a β -1,3-galactosyl transferase, and the N-acetylated HMO is lacto-N-tetraose, or
- 30 - the N-acetyl-glucosaminyl transferase is a β -1,3-N-acetyl-glucosaminyl transferase, the galactosyl transferase is a β -1,4-galactosyl transferase, and the N-acetylated HMO is lacto-N-neotetraose.

25. The genetically modified microorganism of embodiment 21, wherein the microorganism is *E. coli* and the HMO is an N-acetylated HMO, and the *E. coli* comprises:

- 35 - a recombinant gene encoding an N-acetyl-glucosaminyl transferase enzyme which is able to transfer a GlcNAc of UDP-GlcNAc to an internalized lactose or to an intermediate in the

biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-GlcNAc; and

- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer a fucosyl residue of GDP-Fuc to an internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to GDP-Fuc, and
- a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer a galactosyl residue of UDP-Gal to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-Gal.

26. The genetically modified microorganism of embodiment 21, wherein the microorganism is *E. coli* and the HMO is 2'-FL, 3-FL or DFL, and the *E. coli* comprises:

- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer a fucosyl residue of GDP-Fuc to an internalized lactose, and
- one or more genes encoding a biosynthetic pathway to GDP-Fuc.

27. The genetically modified microorganism according to embodiment 26, wherein the DFL/2'-FL ratio is decreased by at least 15%, preferably at least 20%, when the genetically modified organism is grown on sucrose as compared to an organism grown on glucose.

28. The genetically modified microorganism of embodiment 21, wherein the microorganism is *E. coli* and the HMO is a sialylated HMO, and the *E. coli* comprises:

- a recombinant gene encoding a sialyl transferase enzyme which is able to transfer a sialyl residue of CMP-sialic acid to an internalized lactose or to an intermediate in the biosynthetic pathway to said sialylated HMO from lactose, and
- one or more genes encoding a biosynthetic pathway to CMP-sialic acid.

29. The genetically modified microorganism of embodiment 21, wherein the microorganism is *E. coli* and the HMO is a fucosylated and sialylated HMO, and the *E. coli* comprises:

- a recombinant gene encoding a sialyl transferase enzyme which is able to transfer a sialyl residue of CMP-sialic acid to an internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated and sialylated HMO from lactose, and
- a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer a fucosyl residue of GDP-Fuc to an internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated and sialylated HMO from lactose, and
- one or more genes encoding a biosynthetic pathway to CMP-sialic acid and GDP-Fuc.

30. The genetically modified microorganism of any one of embodiments 21 to 29, wherein the *E. coli* is selected from K12 or B21.

31. A process for making an oligosaccharide or a glycoside of said oligosaccharide by glycosylating a carbohydrate acceptor, or a glycoside of said acceptor, wherein the carbohydrate acceptor is not sucrose, comprising the steps of:

- a) providing of a genetically modified microorganism of any one of embodiments 1 to 30,
- b) culturing said cell in the presence of said acceptor and sucrose, and
- c) separating said oligosaccharide from said cell, from the culture medium or from both.

32. . The process of embodiment 31, wherein the genetically modified microorganism is *E. coli*, such as K12 or B21.

33. The process of embodiment 31 or 32, wherein the oligosaccharide is a human milk oligosaccharide (HMO).

34. The process of embodiment 31, wherein the HMO is a neutral HMO.

35. The process of embodiment 34, wherein the neutral HMO is an N-acetylated HMO selected from the group consisting of lacto-N-neotetraose, para-lacto-N-hexaose, para-lacto-N-neohexaose, lacto-N-neohexaose, para-lacto-N-octaose, lacto-N-neooctaose, lacto-N-tetraose, lacto-N-hexaose, lacto-N-octaose, iso-lacto-N-octaose, lacto-N-decaose and lacto-N-neodecaose, more preferably lacto-N-neotetraose, lacto-N-tetraose, lacto-N-hexaose, para-lacto-N-hexaose, para-lacto-N-neohexaose and lacto-N-neohexaose.

36. The process of embodiment 34 or 35, where the HMO is selected from LNT or LNnT.

37. The process of embodiment 34, wherein the neutral HMO is a fucosylated HMO.

38. The process of embodiment 34 or 37, wherein the neutral HMO is a fucosylated lactose selected from 2'-fucosyllactose, 3- fucosyllactose or difucosyllactose.

39. The process of embodiment 34 or 38, wherein the neutral HMO is 2'-fucosyllactose.

40. The process of embodiment 36 or 39, wherein the amount of other HMO components are decreased by at least 10%, preferably at least 15%, preferably at least 20%, preferably at least 25%, when the genetically modified organism is grown on sucrose as compared to an organism grown on glucose.

41. The process of any one of embodiments 31 to 40, wherein the oligosaccharide separated from the broth and the cell is substantially free of isomaltose, kojibiose, gentiobiose and nigerose.

DESCRIPTION OF SEQUENCES

SEQ ID NO	Description	Public ref.
1	Amino acid sequence of ScrY	WP_004147437.1
2	Amino acid sequence of ScrA	AHM80898.1
3	Amino acid sequence of ScrB	WP_000056853.1
4	Amino acid sequence of ScrR	WP_000851062.1

5	DNA sequence of pglpF promoter	WO 2019/123324
6	Promoter-operon DNA sequence: Contains promoter Pscr, genes <i>scrY</i> and <i>scrA</i> (originating from <i>Klebsiella pneumoniae</i>) SEQ ID NO: 6 contains 2 mismatches compared to the public sequence	X57401.1
7	DNA sequence encoding scrBR operon: originating from <i>Salmonella typhimurium</i> . There are 5 mismatches compared to the public sequence	X67750.1

EXAMPLES

Example 1: Comparative test for making LNnT by a glycerol or sucrose utilizing *E. coli*

Bacterial strains:

- 5 Both strains were constructed from *Escherichia coli* K12 strain DH1 which was obtained from the Deutsche Sammlung von Mikroorganismen (reference DSM 5346) by deleting the genes: *lacZ nanKETA lacA melA wcaJ mdoH*, by inserting a Plac promoter, and maintaining genes involved in the UDP-GlcNAc and UDP-Gal biosynthesis. The glycerol utilizing strain (strain I) contains a pBBR3 -lgtA-tet plasmid carrying *N. meningitidis lgtA* gene for β -1,3-N-
- 10 acetylglucosaminyl transfease and the tetracycline resistant gene, and a pBS-galT-amp plasmid carrying *Helicobacter pylori galT* gene for β -1-4-galactosyl transferase and the ampicillin resistant gene. The sucrose utilizing strain (strain II) contains the two following plasmids:
- pBS-scrBR-galT-amp which is a pUC derivative carrying the *galT* gene encoding an β -1,4-
 - 15 galactosyl transferase, the *scrR* gene encoding a sucrose repressor, the *scrB* gene encoding a sucrose-6-phosphate hydrolase and the ampicillin resistance gene;
 - pBBR3-scrYA-lgtA-tet which is a pBBR1-MCS3 derivative carrying the *lgtA* gene encoding a β -1,3-N-acetylglucosaminyl transferase, the *scrA* gene encoding a PTS system sucrose-specific EIIBC component, the *scrY* gene encoding a sucrose porin and the tetracycline
 - 20 resistance gene.

Fermentation procedure:

Glucose, glycerol, sucrose and lactose were each sterilized at 120 °C. Isopropyl thio- β -D-galactopyranoside (IPTG) was filter sterilized.

- 25 The culture was carried out in a 3 l fermenter containing ≈ 0.9 l of mineral culture medium (Samain et al. *J. Biotechnol.* **72**, 33 (1999); does not contain antibiotics for the sucrose system). The temperature was kept at 33 °C and the pH regulated at 6.8 with 28% NH₄OH. The inoculum of the producing strain consisted in a LB medium (20 ml) supplemented with ampicillin and tetracycline for strain I or M9 medium (20 ml) supplemented with sucrose for strain II was added

to the fermenter. The exponential growth phase started with the inoculation and stopped until exhaustion of the carbon source (glucose for strain I or sucrose for strain II, ≈ 17.5 g/l) initially added to the medium. A lactose solution (70 g of lactose/500 ml of water) was then added before starting the feeding with the carbon source (500 g/l solution, 4.5 g/h of glycerol for strain I and 3 g/h of sucrose for strain II). The inducer (isopropyl thio- β -D-galactopyranoside, IPTG, 1-2 ml of a 50 mg/ml solution) was also added. The glycerol-fed fermentation (strain I) lasted for 90 hours after which the cells died (LNnT titre: 45 g/l). The sucrose-fed fermentation (strain II) produced an LNnT concentration of 56 g/l after 116 hours.

Example 2: production of 2'-FL by a sucrose utilizing *E. coli*

Bacterial strain:

The strain was constructed from *Escherichia coli* K12 strain DH1 which was obtained from the Deutsche Sammlung von Mikroorganismen (reference DSM 5346) by deleting the genes: *lacZ nanKETA lacA melA wcaJ mdoH* and by inserting a Plac promoter to upstream the *gmd* gene. In addition the strain contains the following plasmids:

- pBS-futC-scrBR-amp which is a pUC derivative carrying the *futC* gene encoding an α -1,2-fucosyl transferase, the *scrR* gene encoding a sucrose repressor, the *scrB* gene encoding a sucrose-6-phosphate hydrolase and the ampicillin resistance gene;

- pBBR3-GMAB-scrYA-tet which is a pBBR1-MCS3 derivative carrying the *manB*, *manC*, *gmd* and *wcaG* genes involved in the GDP-Fuc biosynthesis, the *scrA* gene encoding a PTS system sucrose-specific EIIBC component, the *scrY* gene encoding a sucrose porin and the tetracycline resistance gene.

Fermentation procedure:

Sucrose and lactose were each sterilized at 120 °C. Isopropyl thio- β -D-galactopyranoside (IPTG) was filter sterilized.

The culture was carried out in a 2 l fermenter containing ≈ 0.9 l of mineral culture medium (Samain et al. *J. Biotechnol.* **72**, 33 (1999); does not contain antibiotics). The temperature was kept at 33 °C and the pH regulated at 6.8 with 28% NH_4OH . The inoculum of the producing strain consisted in a M9 medium supplemented with sucrose (20 ml) was added to the fermenter. The exponential growth phase started with the inoculation and stopped until exhaustion of sucrose (≈ 22 g/l) initially added to the medium. The inducer (isopropyl thio- β -D-galactopyranoside, IPTG, 1-2 ml of a 50 mg/ml solution) was then added. A feeding with a lactose + sucrose solution (160 g of lactose + 500 g of sucrose/l in water) started with a rate of 9 ml/h for 6 hours, then was continued for 115 hours at a rate of 6 ml/h. The concentration of 2'-FL was around 100 g/l in the supernatant at the end of the fermentation.

Example 3: Comparative test for making 2'-FL by a glucose or sucrose utilizing *E. coli*

Gene targeting in the chromosomal DNA was done using standard DNA manipulation techniques, e.g. as disclosed in Warming et al. *Nucleic Acids Res.* **33**, e36 (2005). Insertion of genetic cassettes in the chromosomal DNA was done by gene Gorging as described by Herring et al. *Gene* **311**, 153 (2003).

The strains were derived from an *E. coli* platform strain that was constructed from *E. coli* K12 DH1 (genotype: F^- , λ^- , *gyrA96*, *recA1*, *relA1*, *endA1*, *thi-1*, *hsdR17*, *supE44*, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), www.dsmz.de, reference DSM 4235) by disrupting (deletions of) the genes *lacZ*, *nanKETA*, *lacA*, *mela*, *wcaJ*, *mdoH* and by inserting a *P_{lac}* promoter upstream the *gmd* gene. In addition, two copies of codon optimized *futC* encoding an α 1,2-fucosyl transferase under the control of the *glpF* promoter were integrated into the genome (chromosome) of the *E. coli* platform strain in loci related to sugar metabolism and expressed under the control of the *glpF* promoter (see WO 2019/123324); a single copy of the colanic acid cluster (*gmd-wcaG-wcaH-wcaI-manC-manB*) was integrated in a locus involved in the utilization of alternative carbon sources and expressed under the control of the *glpF* promoter; *lacI* was deleted from the lac operon (Δ *lacI*); *hlyE* gene, coding for the toxin cytolysin A, was deleted (in order to obtain a safer manufacturing strain, eliminating the risk of unintended expression of the toxin); and the functional regulatory gene *glpR*, which is a repressor of the *glpF* promoter, were deleted (see WO 2019/123324). The resulting strain was able to produce 2'-FL when grown on glucose, however, was not able to grow on sucrose (strain 1).

To make it able to grow on sucrose, strain 1 was further modified by inserting a) *scrYA* from *Klebsiella pneumoniae* (GeneBank accession ID: X57401.1 or SEQ ID NO: 6, herein) in another locus involved in sugar metabolism and expressed under the control of a *glpF* promoter with a modified SD sequence (*P_{glpF}_SD1*, see WO 2019/123324 and SEQ ID NO: 5 herein), b) *scrBR* from *Salmonella typhimurium* (GeneBank accession ID: X67750.1, or SEQ ID NO: 7 herein) in one of the loci that enables sugar metabolism under the control of the *P_{scr}* promoter (strain 2). The strains are plasmid-free.

The fermentations were carried out in 250 ml fermenters (DASBOX Mini Bioreactor system, Eppendorf) starting with 100 ml of defined mineral culture medium, consisting of 25 g/kg carbon source (glucose or sucrose), $(\text{NH}_4)_2\text{HPO}_4$, KH_2PO_4 , $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, KOH, NaOH, trace element solution, citric acid, antifoam and thiamine. The trace element solution contained Mn, Cu, Fe, Zn as sulfate salts and citric acid. Lactose was added pre-inoculation at 80 g/kg. The pH throughout fermentation was controlled at 6.8 by titration with 14 % NH_4OH solution. Aeration was at 1 vvm using air and dissolved oxygen was controlled above 20 % of air saturation. Fermentations were started by inoculation with 2 % (v/v) of pre-cultures grown in a similar medium. After depletion of the carbon source contained in the batch medium, a sterile feed

solution containing glucose or sucrose, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, trace metal solution and lactose was fed continuously at a constant feed rate in a carbon-limited manner. The lactose concentration in the broth was maintained above 15 g/kg throughout and until the end of the fermentations. End-of-fermentation was at approximately 120 hours.

Throughout the fermentation, samples were taken in order to determine the concentration of 2'-FL, DFL, lactose and other minor by-products using HPLC. Total broth samples were diluted three-fold in deionized water and boiled for 20 minutes. This was followed by centrifugation at 17000g for 3 minutes, where after the resulting supernatant was analysed by HPLC. The above measurements were used to accurately calculate the 2'-FL titre, the ratio of DFL/2'-FL and the yield of 2'-FL on the carbon source.

While the only change between the two tested strains was the introduction of the sucrose operon genes on the chromosome, the fermentation data in table 2 below show clear evidence that by-product formation in the form of DFL was significantly reduced by approximately 25 % at the end-of-fermentation, while 2'-FL titre and yield can be at least retained or even slightly increased, compared to the glucose reference strain. The use of the sucrose technology is therefore beneficial as it facilitates the purification process. The reduced DFL formation will furthermore provide a bigger upstream process optimisation potential, since the sucrose strain does not waste as much energy on the production of the GDP-fucose precursor.

Table 2

strain	carbon source	number of replicate runs	DFL/2'-FL ratio EoF*	2'-FL titre EoF*	2'-FL accumulated yield index EoF*
strain 1 (no scr)	glucose	3	9.9 % (0.6% SD**)	124.4 g/l (1.1 g/l SD**)	100 % (6.9 % SD**)
strain 2 (scrABRY)	sucrose	2	7.3 %	125.9 g/l	111.5 %

*EoF = end-of-fermentation 119.3 h (± 1.4 h)

**SD = standard deviation

Example 4: Comparative test for making 2'-FL by a glucose or sucrose utilizing *E. coli*

This test comprised strain 1, disclosed in Example 3 above, and a modified strain 2, referred to as strain 2', which differs from strain 2 disclosed in Example 3 above in that the regulatory gene *glpR* was not deleted and thus remained functional.

The strains were fermented separately, under equivalent industrialized conditions based on the conditions disclosed in Example 3, with glucose for strain 1 and sucrose for strain 2'. The product 2'-FL was purified and isolated from the broths using identical unit step operations, without crystallization.

Analysis of the purified samples showed substantial difference in the presence of specific carbohydrate impurities. Product of strain 1 (fermented with glucose) had more than 2 w/w% of glucose degradation products, containing (amongst other products) isomaltose, kojibiose, gentiobiose and nigerose, while the product of strain 2' (fermented with sucrose) was totally free of these impurities. Furthermore, the sucrose-manufactured product is extremely low in the sum of unspecified impurities compared to the glucose-manufactured product, i.e. 0.05 w/w% vs 0.26 w/w%. The overall advantage of the sucrose technology is thereby two-fold, firstly to obtain a product with higher purity (thereby alleviating the need for costly crystallisation) and secondly, a 2 % higher carbon efficiency, since the carbon source upon heat sterilisation was not converted into non-metabolisable carbohydrate impurities.

Example 5: comparative test for making LNT

A strain was derived from an *E. coli* platform strain that was constructed from *E. coli* K12 DH1 (genotype: *F*⁻, *λ*⁻, *gyrA96*, *recA1*, *relA1*, *endA1*, *thi-1*, *hsdR17*, *supE44*, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), www.dsmz.de, reference DSM 4235) by disrupting (deletions of) the genes *lacZ*, *nanKETA*, *lacA*, *mela*, *wcaJ*, *mdoH* and by inserting a *Plac* promoter upstream the *gmd* gene. In addition, three copies of codon optimized *lgtA* coding sequence for β1,3-N-acetyl glucosaminyl transferase were integrated into the genome (chromosome) of the *E. coli* platform strain in loci related to sugar metabolism and expressed under the control of the *glpF* promoter; two copies of codon optimized *galTK* encoding β1,3-galactosyl transferase (WO 2020/115671) was integrated into the genome of the *E. coli* platform strain in another loci involved in sugar metabolism and expressed under the control of the *glpF* promoter. The integration of the genes of the sucrose operon *scrYABR* (while split into *scrYA* and *scrBR*) was done at two sites in the mixed-acid fermentation pathway, thereby deleting genes that cause formation of undesired fermentative metabolites under anaerobic conditions, but not under normal aerobic fermentation conditions. In addition, an expression cassette containing the *glpF* promoter linked to a codon optimized DNA encoding a putative MFS transporter protein from *Rosenbergiella nectarea* (GenBank accession ID: WP_092672081.1), which facilitates the export of LNT from the cell, was inserted in a locus involved in the utilization of alternative carbon sources. Moreover, *lacI* and *hlyE* were deleted. The so-constructed strain is plasmid-free and able to grow on glucose and sucrose.

The fermentations were carried out in 250 ml fermenters (DASBOX Mini Bioreactor system, Eppendorf) starting with 100 ml of defined mineral culture medium, consisting of 30 g/kg carbon source (glucose or sucrose), (NH₄)₂HPO₄, KH₂PO₄, MgSO₄ x 7H₂O, KOH, NaOH, trace element solution, citric acid, antifoam and thiamine. The trace element solution contained Mn, Cu, Fe, Zn as sulfate salts and citric acid. Lactose was provided by bolus addition of 80 g/kg approximately 5 hours after the end of the batch phase, i.e. at approximately 18-19 h elapsed fermentation time (ELT). The pH throughout fermentation was controlled at 6.8 by titration with 14 % NH₄OH-

solution. Aeration was at 1 vvm using air and dissolved oxygen was controlled above 23 % of air saturation. Fermentations were started by inoculation with 2 % (v/v) of pre-cultures grown in a similar medium. After depletion of the carbon source contained in the batch medium, a sterile feed solution containing glucose or sucrose, $(\text{NH}_4)_2\text{SO}_4$, trace metal solution and anti-foam was fed continuously at a constant feed rate in a carbon-limited manner. Lactose was almost depleted at 72.8 h ELT in the sucrose fermentations, while it was still at around 8-10 g/l in the glucose fermentation, indicating a higher lactose utilisation rate and hence product formation. End-of-fermentation samples were taken and analysed at 72.8 h.

Throughout the fermentation, samples were taken in order to determine the concentration of LNT and lactose. Total broth samples were diluted three-fold in deionized water and boiled for 20 minutes. This was followed by centrifugation at 17000g for 3 minutes, where after the resulting supernatant was analysed by HPLC. The HPLC measurements were used to accurately calculate the LNT titre, by-product ratios (not shown) and the accumulated yield of LNT on the carbon source. The latter takes also smaller variations in feed rates and dilutions into account and is therefore an important parameter for direct comparison.

This study describes a direct comparison of a sucrose-utilising strain in fermentations with sucrose versus glucose as carbon source. The processes were identical except for the type of carbon source used. The data demonstrate clearly superiority of sucrose in terms of LNT productivity and carbon yield. The table 3 below shows that the average carbon yield was increased by 17% when using sucrose instead of glucose. This is also reflected by the 19 % higher final LNT titre at end-of-fermentation and the increased lactose consumption rate (lactose was only added by bolus addition and not in the feed solution). Overall, the use of the sucrose technology is therefore beneficial as it not only facilitates the purification process due to the higher final titre, but also leads to a more efficient carbon source conversion to product.

Table 3: LNT production from strains with and without heterologous PTS-dependent sucrose utilization system.

carbon source	number of replicate runs	lactose concentration EoF*	LNT titre EoF*	LNT accumulated yield index EoF*
Sucrose (scrABRY)	2	1.6 g/l	78.1 g/l	117.5 %
Glucose (no scr)	2	9.2 g/l	65.4 g/l	100 %

*EoF = end-of-fermentation 72.8 h

Example 6 Strain without *scr* genes cannot grow on sucrose

This example illustrates that the background strain used for constructing the above strains are not capable of growing on sucrose.

The background strain, MDO, was constructed from *Escherichia coli* K12 DH1. The *E. coli* K12 DH1 genotype is: F⁻, λ⁻, gyrA96, recA1, relA1, endA1, thi-1, hsdR17, supE44. In addition to the *E. coli* K12 DH1 genotype MDO has the following modifications: *lacZ* deletion of 1,5kbp, *lacA*: deletion of 0,5kbp, *nanKETA*: deletion of 3,3kbp, *melA*: deletion of 0,9kbp, *wcaJ*: deletion of 0,5kbp, *mdoH*: deletion of 0,5kbp, and insertion of Plac promoter upstream of the *gmd* gene.

The strain MPLNnT can produce the tetrasaccharide HMO, LNnT, while the MDO strain does not form a product of biotechnological interest. The strain MPLNnT bears the genes that are responsible for the biosynthesis of LNnT, namely *lgtA* (encoding a beta-1,3-N-acetyloglucosamine transferase) from *N. meningitidis* and *galT* (encoding a beta-1,4-galactosyltransferase) from *H. pylori*, as well as four additional genes conferring the ability to grow on sucrose as the sole carbon and energy source. These four genes are organized in 2 operons, *scrBR* and *scrYA*, which are controlled by a wild-type sucrose promoter (Pscr) and a synthetic dual promoter (PglpF_SD1-Pscr), respectively and integrated into the genome.

The strains were grown in 96-well microtiter plates using a 4-day protocol. During the first 24 hours, cells were grown to high densities while in the next 72 hours cells were transferred to a medium containing sucrose as the sole carbon and energy source. Specifically, during day 1, fresh inoculums were prepared using a Luria-Bertani broth containing 20% glucose. After 24 hours of incubation of the prepared cultures at 34 °C, cells were transferred to a basal minimal medium (200 uL) supplemented with magnesium sulphate and thiamine to which an initial bolus of 20 % glucose solution and 15 g/L sucrose solution as carbon source was provided to the cells. After inoculation of the new medium, cells were shaken at 1200 rpm at 28 °C for 72 hours. The cells were grown in a batch mode of cultivation in microtiter plates that were compatible with the Varioskan LUX Multimode Microplate Reader from ThermoFisher Scientific.

In the present Example, it is demonstrated that the background strain, MDO, is not able to grow on sucrose whereas a strain with the chromosomally integrated *scrBR* and *scrYA* genes grows very efficiently on sucrose (Figure 2).

The MPLNnT stain has also been subjected to fermentation and has shown to be capable of producing between 83 and 85% LNnT relative to the total amount of HMO produced when grown on sucrose.

Example 7 Genomic integration vs plasmid expression of *scrYA* and *scrBR*

The benefit of integrating the genes necessary for HMO production into the genome as compared to expressing them from high-copy-number expression plasmids was analysed.

The plasmid containing strain of example 2 and the chromosomal strain 2' of example 4 were compared in a head-to-head analysis. The chromosomal strain 2' of example 4 has two copies of *futC*, which is necessary to achieve levels of futC which is similar to those obtained from the high expression plasmid pBS in example 2. Furthermore, the expression plasmids in the strain

of example 2 have antibiotic markers (amp and tet) to secure that the plasmids are maintained within the cell when propagated, such markers are not needed in chromosomal strains, which is an advantage in production since antibiotics can be avoided in cell banking and during propagation.

- 5 The strains were fermented separately, under equivalent industrialized conditions based on the conditions disclosed in Example 3, with sucrose for both strains. The product 2'-FL was purified and isolated from the broths using identical unit step operations, without crystallization. The results are shown in table 4 below.

Table 4. 2'-FL expression from plasmid strain and chromosomal strain

	accumulated yield index		Number of runs	DFL/2FL	
	Mean [%]	St dev [%]		Mean [g/g]	St dev [g/g]
Plasmid strain (Ex 2)	96.0	5.1	26	0.149	0.022
Chromosomal strain 2' (ex 4)	100.0	4.9	7	0.059	0.013

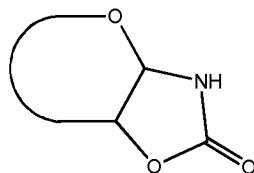
10

From this it can be seen that the 2'FL yield is slightly higher (but statistically significant) for the chromosomal strain. In addition the amount of DFL by-product is reduced for the chromosomal strain in that the DFL/2'FL ratio is lower, this is a benefit in the down-stream processing since less by-product needs to be removed to obtain pure 2'FL.

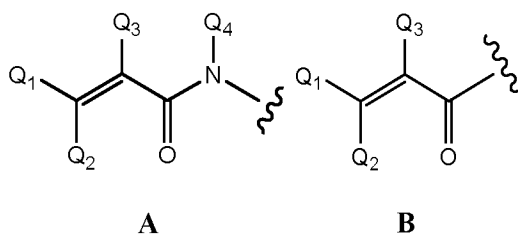
CLAIMS

1. A genetically modified microorganism for making an oligosaccharide or a glycoside of said oligosaccharide, comprising:
 - a. a recombinant gene encoding a glycosyl transferase which can transfer a glycosyl residue of an activated sugar nucleotide
 - i. to a carbohydrate acceptor or a glycoside of said acceptor, where said acceptor is internalized by the microorganism, and/or
 - ii. to an intermediate in the biosynthetic pathway to said oligosaccharide from said acceptor,
 - b. a biosynthetic pathway for making said activated sugar nucleotide from sucrose, and
 - c. one or more genes encoding a heterologous PTS-dependent sucrose utilization system, so that said cell is capable to use sucrose as a carbon source for making said activated sugar nucleotide and as energy source for making said oligosaccharide, wherein the carbohydrate acceptor is not sucrose, or a glycoside of said acceptor, and said heterologous PTS-dependent sucrose utilization system comprises proteins encoded by *scr* genes *scrY* for a sucrose porin, *scrA* for a PTS permease, *scrB* for a sucrose-6-phosphate hydrolase and *scrR* for a repressor protein, and wherein the recombinant gene encoding a glycosyl transferase is inserted in the genome of the microorganism and/or the *scr* genes are inserted in the genome of the microorganism.
2. The genetically modified microorganism according to claim 1, which is plasmid-free.
3. The genetically modified microorganism according to claim 1 or 2, wherein the *scr* genes are operably linked to a *glp promoter*, such as a *glpF* promoter, such as the *glpF* promoter of SEQ ID NO: 5.
4. The genetically modified microorganism according to claim 1 to 3, wherein an aglycon in the glycoside of an oligosaccharide or a carbohydrate acceptor is selected from the group consisting of:
 - OR_A, wherein R_A is a linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms or, when unsaturated, 2-24, preferably 2-6 carbon atoms, or R_A means an aryl moiety, or R_A means a group removable by hydrogenolysis,
 - X-R_B, wherein X is N or S, and R_B means linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms or, when unsaturated, 2-24, preferably 2-6 carbon atoms, or R_B means an aryl moiety, or R_B means a benzyl group,
 - a group that links an anomeric carbon atom and an adjacent carbon atom to each other by

-NH-C(=O)-O-bridge, thus forming a fused 5-membered ring₁ as depicted in case of an aldose:

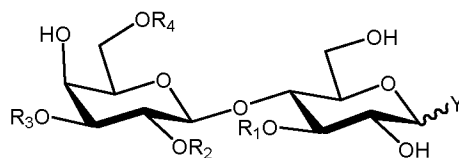


- azide,
- -NH-C(R'')=C(R')₂, wherein each R' independently is an electron withdrawing group selected from -CN, -COOH, -COO-alkyl, -CO-alkyl, -CONH₂, -CONH-alkyl and -CON(alkyl)₂, or wherein the two R'-groups are linked together and form -CO-(CH₂)₂₋₄-CO- and thus form with the carbon atom, to which they are attached, a 5-7 membered cycloalkan-1,3-dione, in which dione any of its methylene groups is optionally substituted with 1 or 2 alkyl groups, and wherein R'' is H or alkyl,
- a residue of an amino acid,
- a polyethylene glycol residue,
- a polyvinyl alcohol residue, and
- an α,β -unsaturated amido group of formula A or an α,β -unsaturated carbonyl group of formula B



wherein Q₂, Q₃ and Q₄ are, independently, H and C₁-C₆-alkyl, which alkyl is optionally substituted with halogen, OH, nitro or phenyl groups.

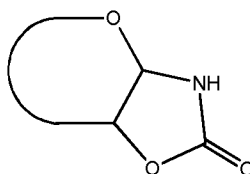
5. The genetically modified microorganism according to any of the claims 1 to 4, which is *E. coli*.
6. The genetically modified microorganism according to any of the claims 1 to 5, wherein one or more of the following genes are deleted or deactivated: lacZ, lacI, lacA and hlyE.
7. The genetically modified microorganism according to claim 6, wherein lacZ is deleted or deactivated, and optionally one or more of the following genes are deleted or deactivated: lacI, lacA and hlyE.
8. The genetically modified microorganism according to any of the claims 1 to 7 for producing an oligosaccharide of formula 1:

**1**

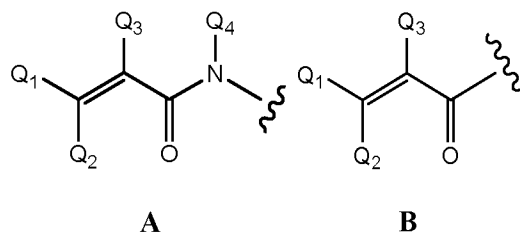
wherein:

Y is

- -OH,
- -OR_A, wherein R_A is a linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms or, when unsaturated, 2-24, preferably 2-6 carbon atoms, or R_A means an aryl moiety, or R_A means a group removable by hydrogenolysis,
- -X-R_B, wherein X is N or S, and R_B means linear or branched hydrocarbon chain having, when saturated, 1-24, preferably 1-6 carbon atoms or, when unsaturated, 2-24, preferably 2-6 carbon atoms, or R_B means an aryl moiety, or R_B means a benzyl group,
- a group that links an anomeric carbon atom and an adjacent carbon atom to each other by a
 - NH-C(=O)-O-bridge, thus forming a fused 5-membered ring₁ as depicted in case of an aldose:



- azide,
- -NH-C(R'')=C(R')₂, wherein each R' independently is an electron withdrawing group selected from -CN, -COOH, -COO-alkyl, -CO-alkyl, -CONH₂, -CONH-alkyl and -CON(alkyl)₂, or wherein the two R'-groups are linked together and form -CO-(CH₂)₂₋₄-CO- and thus form with the carbon atom, to which they are attached, a 5-7 membered cycloalkan-1,3-dione, in which dione any of its methylene groups is optionally substituted with 1 or 2 alkyl groups, and wherein R'' is H or alkyl,
- a residue of an amino acid,
- a polyethylene glycol residue,
- a polyvinyl alcohol residue, or
- an α,β-unsaturated amido group of formula A or an α,β-unsaturated carbonyl group of formula B



wherein Q2, Q3 and Q4 are, independently, H and C₁-C₆-alkyl, which alkyl is optionally substituted with halogen, OH, nitro or phenyl groups,

R₁ is fucosyl or H,

R₂ is fucosyl or H,

R₃ is selected from H, sialyl, N-acetyl-lactosaminy and lacto-N-biosyl groups, wherein the N-acetyl-lactosaminy group optionally carries a glycosyl residue comprising one or more N-acetyl-lactosaminy and/or one or more lacto-N-biosyl groups; each of the N-acetyl-lactosaminy and lacto-N-biosyl groups is optionally substituted with one or more sialyl and/or fucosyl residue,

R₄ is selected from H, sialyl and N-acetyl-lactosaminy groups optionally substituted with a glycosyl residue comprising one or more N-acetyl-lactosaminy and/or one or more lacto-N-biosyl groups; each of the N-acetyl-lactosaminy and lacto-N-biosyl groups is optionally substituted with one or more sialyl and/or fucosyl residue,

provided that at least one of the R₁, R₂, R₃ and R₄ groups is different from H.

9. The genetically modified microorganism according to claim 8, wherein Y is -OH.
10. The genetically modified microorganism according to claim 9, wherein the oligosaccharide of formula 1 represents a human milk oligosaccharide (HMO).
11. The genetically modified microorganism according to claim 10, wherein the microorganism is *E. coli* and the HMO is a neutral HMO, and the *E. coli* comprises:
 - i. a recombinant gene encoding an N-acetyl-glucosaminy transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to the internalized lactose or to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-GlcNAc; and/or
 - ii. a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to the internalized lactose or to an intermediate in the biosynthetic pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to GDP-Fuc, and
 - iii. optionally a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to an intermediate in the biosynthetic

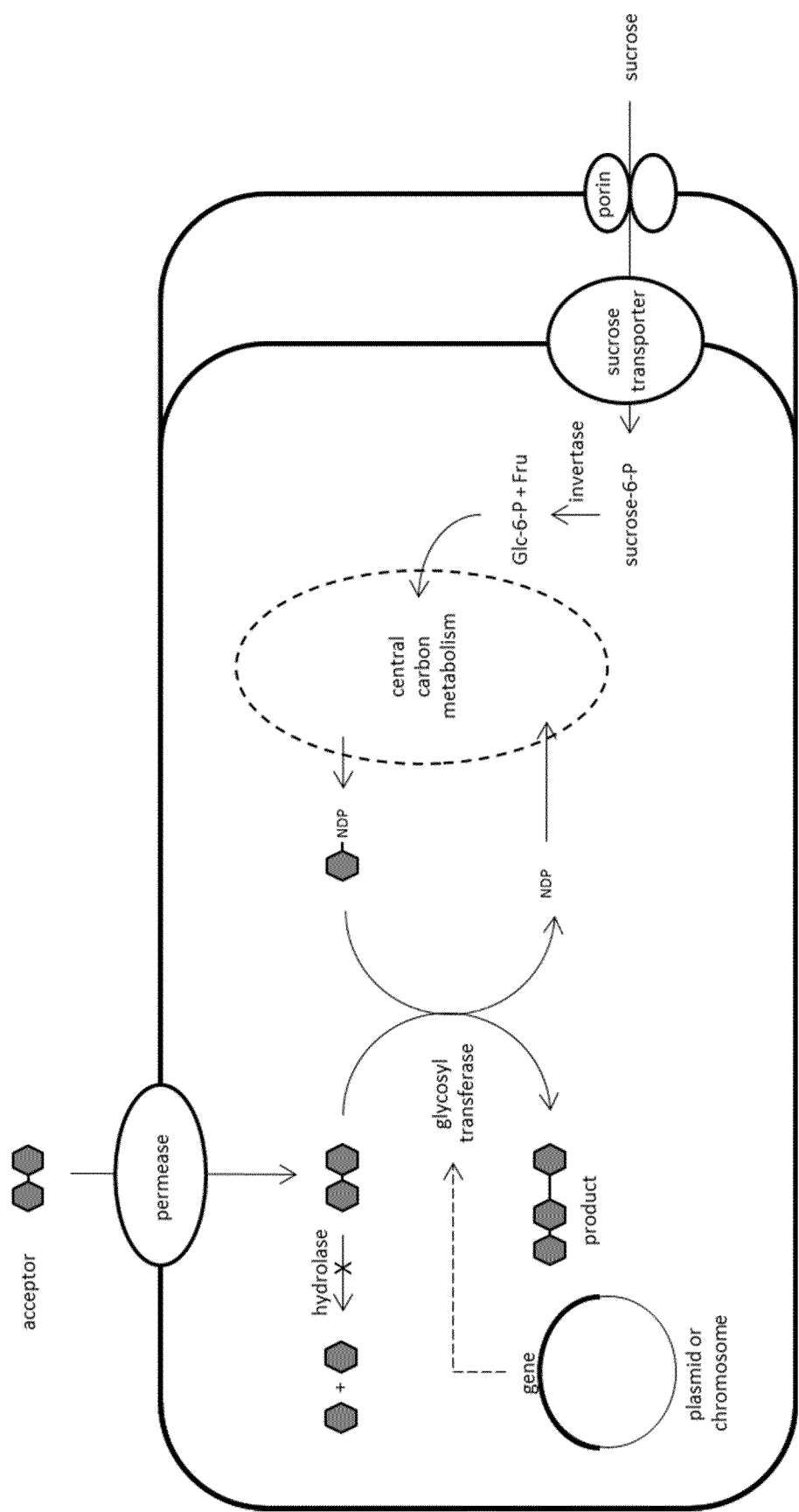
pathway to said neutral HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-Gal.

12. The genetically modified microorganism according to claim 10, wherein the microorganism is *E. coli* and the HMO is an N-acetylated HMO, and the *E. coli* comprises:
 - i. a recombinant gene encoding an N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to the internalized lactose or to an intermediate in the biosynthetic pathway to said N-acetylated HMO from lactose, and
 - ii. optionally a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to the N-acetyl-glucosaminylated lactose or to an intermediate in the biosynthetic pathway to said N-acetylated HMO from lactose; and
 - iii. one or more genes encoding a biosynthetic pathway to UDP-GlcNAc and optionally to UDP-Gal.
13. The genetically modified microorganism according to claim 12, wherein:
 - i. the N-acetyl-glucosaminyl transferase is a β -1,3-N-acetyl-glucosaminyl transferase, no recombinant gene encoding a galactosyl transferase is present in the cell, and the N-acetylated HMO is lacto-N-triose II, or
 - ii. the N-acetyl-glucosaminyl transferase is a β -1,3-N-acetyl-glucosaminyl transferase, the galactosyl transferase is a β -1,3-galactosyl transferase, and the N-acetylated HMO is lacto-N-tetraose, or
 - iii. - the N-acetyl-glucosaminyl transferase is a β -1,3-N-acetyl-glucosaminyl transferase, the galactosyl transferase is a β -1,4-galactosyl transferase, and the N-acetylated HMO is lacto-N-neotetraose.
14. The genetically modified microorganism according to claim 10, wherein the microorganism is *E. coli* and the HMO is an N-acetylated HMO, and the *E. coli* comprises:
 - i. a recombinant gene encoding an N-acetyl-glucosaminyl transferase enzyme which is able to transfer the GlcNAc of UDP-GlcNAc to the internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-GlcNAc; and
 - ii. a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to the internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to GDP-Fuc, and
 - iii. a recombinant gene encoding a galactosyl transferase enzyme which is able to transfer the galactosyl residue of UDP-Gal to an intermediate in the biosynthetic pathway to said fucosylated N-acetylated HMO from lactose, and one or more genes encoding a biosynthetic pathway to UDP-Gal.

15. The genetically modified microorganism according to claim 10, wherein the microorganism is *E. coli* and the HMO is 2'-FL, 3-FL or DFL, and the *E. coli* comprises:
 - i. a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to the internalized lactose, and
 - ii. one or more genes encoding a biosynthetic pathway to GDP-Fuc.
16. The genetically modified microorganism according to claim 15, wherein the DFL/2'-FL ratio is decreased by at least 15%, preferably at least 20%, when the genetically modified organism is grown on sucrose compared to a similar microorganism which does not contain the PTS-dependant sucrose system and is grown on glucose.
17. The genetically modified microorganism according to claim 10, wherein the microorganism is *E. coli* and the HMO is a sialylated HMO, and the *E. coli* comprises:
 - i. a recombinant gene encoding a sialyl transferase enzyme which is able to transfer the sialyl residue of CMP-sialic acid to the internalized lactose or to an intermediate in the biosynthetic pathway to said sialylated HMO from lactose, and
 - ii. one or more genes encoding a biosynthetic pathway to CMP-sialic acid.
18. The genetically modified microorganism according to claim 10, wherein the microorganism is *E. coli* and the HMO is a fucosylated and sialylated HMO, and the *E. coli* comprises:
 - i. a recombinant gene encoding a sialyl transferase enzyme which is able to transfer the sialyl residue of CMP-sialic acid to the internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated and sialylated HMO from lactose,
 - ii. a recombinant gene encoding a fucosyl transferase enzyme which is able to transfer the fucosyl residue of GDP-Fuc to the internalized lactose or to an intermediate in the biosynthetic pathway to said fucosylated and sialylated HMO from lactose, and
 - iii. one or more genes encoding a biosynthetic pathway to CMP-sialic acid and GDP-Fuc.
19. A process for making an oligosaccharide or a glycoside of said oligosaccharide by glycosylating a carbohydrate acceptor, or a glycoside of said acceptor, wherein the carbohydrate acceptor is not sucrose, comprising the steps of:
 - a. providing of a genetically modified microorganism according to any one of claims 1 to 18,
 - b. culturing said cell in the presence of said acceptor and sucrose, and
 - c. separating said oligosaccharide from said cell, from the culture medium or from both.
20. The process according to claim 19, wherein the genetically modified microorganism is *E. coli*.
21. The process according to claim 19 or 20, wherein the utilization of the sucrose carbon source is improved compared to when glucose is used as carbon source.

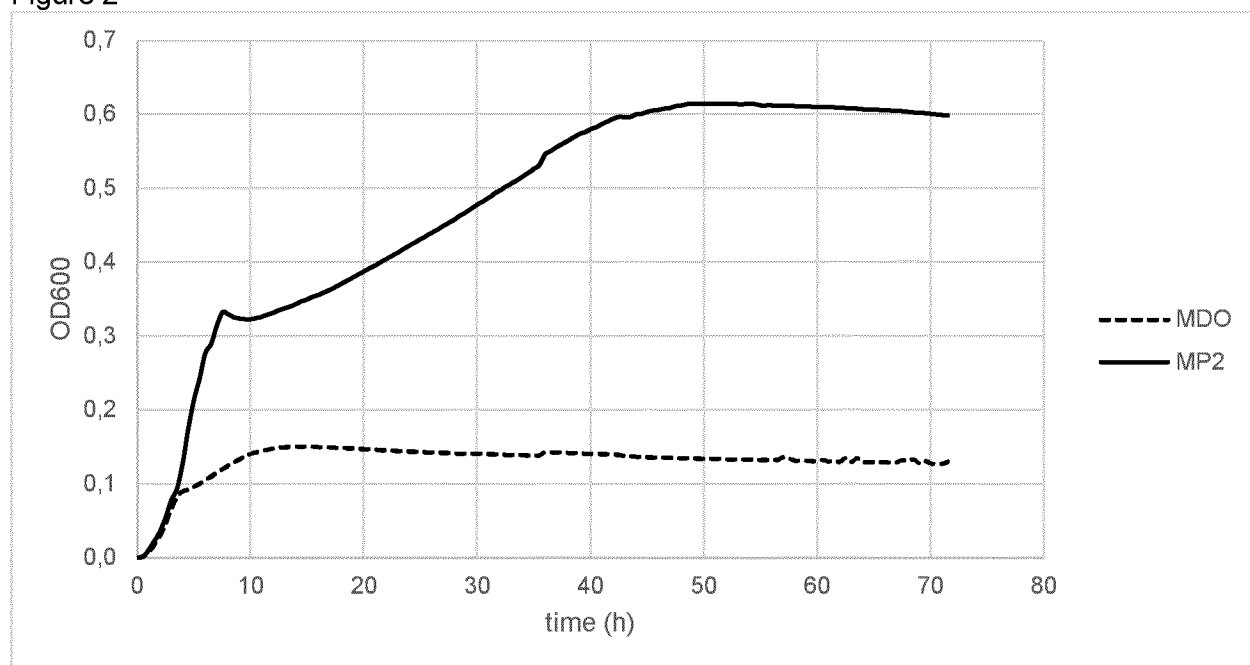
22. The process according to claim 19 to 21, wherein the oligosaccharide is a human milk oligosaccharide (HMO).
23. The process of any one of claims 19 to 22, wherein the oligosaccharide separated from the said cell, from the culture medium or from both is substantially free of isomaltose, kojibiose, gentiobiose and nigerose.

Figure 1



2/2

Figure 2



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069310

A. CLASSIFICATION OF SUBJECT MATTER					
INV.	C12P19/00	C12P19/04	C12P19/12	C12P19/18	C12P19/26
	C07K14/255	C07K14/26	C12N9/12	C12N9/24	C12N15/52
ADD.					
According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED					
Minimum documentation searched (classification system followed by classification symbols)					
C12P C07K C12N C12R					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)					
EPO-Internal, BIOSIS, Sequence Search, WPI Data					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category*	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
Y	WO 2019/076941 A1 (JENNEWEIN BIOTECHNOLOGIE GMBH [DE]) 25 April 2019 (2019-04-25) cited in the application the whole document examples 1-5				1-23
Y	WO 2019/043029 A1 (JENNEWEIN BIOTECHNOLOGIE GMBH [DE]) 7 March 2019 (2019-03-07) cited in the application the whole document examples 1,2				1-23
	----- -/--				
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.					
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family					
Date of the actual completion of the international search			Date of mailing of the international search report		
19 October 2021			27/10/2021		
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016			Authorized officer van de Kamp, Mart		

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069310

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2015/150328 A1 (JENNEWEIN BIOTECHNOLOGIE GMBH [DE]) 8 October 2015 (2015-10-08) cited in the application the whole document example 1 table 2 figures 1,2	1-23
Y	----- WO 2014/067696 A1 (JENNEWEIN BIOTECHNOLOGIE GMBH [DE]) 8 May 2014 (2014-05-08) cited in the application the whole document example 1	1-23
Y	----- WO 2015/197082 A1 (GLYCOM AS [DK]) 30 December 2015 (2015-12-30) the whole document page 11, line 16 - page 12, line 6 examples 1,2 figure 1	1,2,4-23
Y	----- WO 2019/123324 A1 (GLYCOM AS [DK]) 27 June 2019 (2019-06-27) cited in the application the whole document -----	3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2021/069310

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2021/069310

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2019076941 A1	25-04-2019	AU 2018350754 A1 BR 112020007532 A2 CN 111556873 A EP 3473644 A1 EP 3697805 A1 JP 2020537530 A KR 20200067176 A PH 12020550441 A1 SG 11202003360Q A US 2020332331 A1 WO 2019076941 A1	07-05-2020 06-10-2020 18-08-2020 24-04-2019 26-08-2020 24-12-2020 11-06-2020 26-04-2021 28-05-2020 22-10-2020 25-04-2019
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WO 2015150328 A1	08-10-2015	DK 2927316 T3 EP 2927316 A1 EP 3461890 A1 ES 2715010 T3 JP 6360908 B2 JP 2017509346 A KR 20170003546 A PL 2927316 T3 WO 2015150328 A1	04-03-2019 07-10-2015 03-04-2019 31-05-2019 18-07-2018 06-04-2017 09-01-2017 31-05-2019 08-10-2015
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WO 2019123324 A1	27-06-2019	BR 112020012207 A2 CN 111727253 A EP 3728596 A1 US 2021102216 A1 WO 2019123324 A1	24-11-2020 29-09-2020 28-10-2020 08-04-2021 27-06-2019