

Fermentative production of carbohydrates by microbial cells utilizing a mixed feedstock

The present invention relates to the fermentative production of a saccharide of interest. Disclosed are microbial cells being capable to the saccharide of interest, wherein said microbial cells utilizes a mixed monosaccharide feedstock as main carbon- and energy source during fermentation. Also disclosed are methods for producing the saccharide of interest by utilizing said microbial cells.

Background

Human milk comprises a complex mixture of carbohydrates, fats, proteins, vitamins, minerals and trace elements. The most predominant fraction of human milk consists of carbohydrates. The carbohydrate fraction within human milk can be further divided into (i) lactose and (ii) oligosaccharides (human milk oligosaccharides, HMOs). Whereas the disaccharide lactose (galactose- β 1,4-glucose) is used as an energy source by the baby, the oligosaccharides are not metabolized by the infant.

The fraction of oligosaccharides accounts for up to one tenth of the total carbohydrate fraction and presumably consists of more than 150 structurally distinct oligosaccharides. The occurrence and concentration of these complex oligosaccharides are specific to humans and thus cannot be found in the milk of other mammals including dairy farm animals in large quantities.

The most prominent human milk oligosaccharides are 2'-fucosyllactose (2'-FL) and 3-fucosyllactose (3-FL) which together can constitute up to 1/3 of the total HMO fraction. Further prominent HMOs are lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT) and lacto-*N*-fucopentaose I (LNFP-I). Besides these neutral oligosaccharides, also acidic HMOs can be found in human milk such as 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), 3-fucosyl-3'-sialyllactose, sialyl-lacto-*N*-tetraose, and disialyl-lacto-*N*-tetraose.

Notably, the vast majority of HMOs comprise a galactose- β 1,4-glucose moiety at their reducing end, which gets extended by the addition of monosaccharide moieties

such as *N*-acetylglucosamine (GlcNAc) and/or fucose and/or galactose and/or *N*-acetylneuraminic acid (NeuNAc). The structures of HMOs are closely related to epitopes of epithelial cell surface glycoconjugates, the Lewis histoblood group antigens such as Lewis x (LeX). The structural similarity of HMOs to epithelial epitopes accounts for the protective properties of HMOs against bacterial pathogens.

The presence of oligosaccharides in human milk is known for a long time and the physiological functions of these oligosaccharides were subject to medical research for many decades. For some of the more abundant human milk oligosaccharides, specific functions have already been identified.

Besides causing local effects in the intestinal tract as indicated herein before, HMOs have also been shown to elicit systemic effects in infants by entering their systemic circulation. Also, the impact of HMOs on protein-carbohydrate interactions, e.g. selectin-leukocyte binding, can modulate immune responses and reduce inflammatory responses. In addition, it becomes more and more recognized that HMOs represent key substrates for the development of infants' microbiomes.

Due to the well-studied beneficial properties of various carbohydrates in general, and in particular of prebiotic oligosaccharides, but because of their limited availability from natural sources, an efficient and cost-effective production process for monosaccharides (e.g. of L-fucose, *N*-acetylneuraminic acid), disaccharides (e.g. lacto-*N*-biose) and oligosaccharides (e.g. 2'-FL, LNnT) is highly desirable.

Attempting for large scale production of functional carbohydrates, chemical routes to some of these carbohydrates were developed. However, such methods involve the use of several noxious chemicals, which impose the risk to contaminate the final product. At least large-scale quantities as well as qualities of functional carbohydrates being sufficient for their use in food applications cannot be provided until today through chemical synthesis.

To bypass the drawbacks that are associated with the chemical synthesis of human milk oligosaccharides, several enzymatic methods and fermentative approaches for their production were developed. Fermentative production processes have been developed for several carbohydrates such as L-fucose, *N*-acetylneuraminic acid,

2'-fucosyllactose, 3-fucosyllactose, lacto-*N*-tetraose, lacto-*N*-neotetraose, 3'-sialyllactose and 6'-sialyllactose. These production processes typically use genetically engineered bacterial cells such as recombinant *Escherichia coli*.

Usually, fermentative production processes as well as biocatalytic reactions to produce HMOs are based on exogenously added lactose as initial acceptor substrate for the HMO to be produced. One or more monosaccharides are added to lactose in these processes (US 7,521,212 B1; Albermann *et al.*, (2001) *Carbohydr. Res.* 334 (2) p. 97-103). The addition of monosaccharides to lactose can either be catalyzed by glycosyltransferases or by glycosidases using suitable activated monosaccharide substrates. In addition, additional monosaccharides can be added to lactose by transglycosidase reactions.

In particular the fermentative production of HMOs proved to be efficient, because the nucleotide-activated monosaccharides that are required but difficult to synthesize are provided by the metabolism of the microbial cells that are employed. The biosynthesis pathway for nucleotide-activated monosaccharides usually originates from the primary metabolism of the host cell on the level of glucose-6-phosphate or fructose-6-phosphate. The biosynthesis pathway for UDP-galactose (UDP-Gal) originates from glucose-6-phosphate while the biosynthesis of GDP-fucose, UDP-*N*-acetylglucosamine and CMP-*N*-acetylneuraminic acid originates from fructose-6-phosphate.

The efficient biosynthesis of nucleotide-activated monosaccharides in a microbial host cell, and consequently the production of desired carbohydrates by fermentative processes, clearly rely on the continuous supply of sugar phosphates such as glucose-6-phosphate and/or fructose-6-phosphate.

A major problem in said fermentative processes, usually based on the consumption of a simple and inexpensive carbon- and energy source (e.g. glycerol, glucose, sucrose) by the microbial host cell, is the limited availability of such phosphorylated/ activated saccharides in said host cell (e.g. due to competing reactions), vastly impairing the carbon flux of the product biosynthesis pathway, thus, the productivity of these processes.

To overcome aforementioned drawbacks, improved means and methods for the production of HMOs were developed. For example, WO 2012/007481 A2 discloses a engineered organism being capable to produce a saccharide, an activated saccharide, a nucleoside, a glycoside, a glycolipid and a glycoprotein, wherein said engineered organism expresses i) a gene encoding for a carbohydrate hydrolase in combination with a gene encoding for a carbohydrate kinase, ii) a gene encoding for a carbohydrate synthase, or iii) a gene encoding for a carbohydrate phosphorylase, so that said organism is capable to split a disaccharide, oligosaccharide, polysaccharide or a mixture thereof into an activated saccharide and a saccharide and wherein said organism is further genetically modified such that at least one other gene than any of the introduced genes of said organism is rendered less-functional or non-functional, and wherein said other gene encodes for an enzyme which converts said activated saccharide into biomass and/or bio-catalytic enzymes. Said engineered organism is capable of generating desired carbohydrates while utilizing a disaccharide such as sucrose, an oligosaccharide, a polysaccharide or a mixture thereof.

However, producing a desired compound by said engineered organism using sucrose as sole carbon and energy source has a major drawback in that it is difficult to sterilize sucrose. The most desirable method for sterilization is heat sterilization. However, heat sterilization of sucrose results in a considerable degree of hydrolyzation counteracting the applicability of the method described in WO 2012/007481 A2.

As an alternative to heat sterilization, sterile filtration of a sucrose solution can be employed, but sterile filtration bears a high risk of contamination leading to growth of undesired bacterial cells, in particular in industrial-scale fermentation.

Nevertheless, sucrose represents the most attractive carbon source for biotechnological applications due to its availability and low costs.

Hence, it is desired to provide microbial cells for the fermentative production of a desired carbohydrate that is capable of producing the desired carbohydrate when cultivated in the presence of an inexpensive feedstock as main carbon and energy source wherein said feedstock does not have to be hydrolyzed by the microbial cell

to be utilizable by the cell's metabolism, and a method for producing a carbohydrate of interest by means of fermentation by cultivating said microbial cell in the presence of said feedstock as main carbon and energy source.

The object is solved in that genetically engineered microbial cells are provided which are capable of producing a desired carbohydrate when cultivated on a mixed monosaccharide feedstock as main carbon and energy source, wherein said mixed monosaccharide feedstock consists of glucose and at least one additional monosaccharide selected from the group consisting of fructose and galactose, and wherein the microbial cells possess an increased intracellular availability of at least one sugar phosphate, and by providing a method for the fermentative production of a carbohydrate of interest which method comprises cultivating said genetically engineered microbial cells in the presence of said mixed monosaccharide feedstock as main carbon and energy source.

Summary

According to a first aspect, provided is a genetically engineered microbial cell for the production of a carbohydrate of interest, wherein said microbial cell possesses an increased intracellular availability of at least one sugar phosphate, and is capable of producing a carbohydrate of interest when cultivated in the presence of a mixed monosaccharide feedstock as main carbon and energy source, wherein said mixed monosaccharide feedstock consists of glucose and at least one additional monosaccharide selected from the group consisting of fructose and galactose.

According to a second aspect, provided is the use of a genetically engineered microbial cell as described herein for the production of a carbohydrate of interest, wherein said microbial cell is cultivated in the presence of a monosaccharide mixture consisting of glucose and at least one additional monosaccharide from the group of fructose and galactose.

According to a third aspect, provided is a method for the fermentative production of a carbohydrate of interest, the method comprising the steps of:

- a) providing a genetically engineered microbial cell that is capable of producing the carbohydrate of interest, wherein said microbial cell possesses an increased intracellular availability of at least one sugar phosphate;
- b) cultivating said genetically engineered microbial cell in a culture medium that is permissive for the production of said carbohydrate of interest, wherein the culture medium comprises a mixed monosaccharide feedstock as main carbon and energy source, wherein said mixed monosaccharide feedstock consist of glucose and at least one additional monosaccharide selected from the group consisting of fructose and galactose; and
- c) recovering said carbohydrate of interest.

In a fourth aspect, provided is the use of the carbohydrate of interest that has been produced by the genetically engineered microbial cell and/or by the method described herein for the manufacturing a pharmaceutical and/or nutritional composition.

Brief description of the drawings

Fig. 1 shows a schematic representation of an exemplary wild-type microbial cell (e.g. *E. coli*) illustrating naturally occurring metabolic pathways leading to and originating from the sugar phosphates fructose-1-phosphate and glucose-6-phosphate.

Fig. 2 shows a schematic representation of an exemplary genetically modified microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular glucose-6-phosphate availability, are indicated.

Fig. 3 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular glucose-6-phosphate availability, are indicated.

Fig. 4 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic

modifications, leading to an increased intracellular glucose-6-phosphate availability, are indicated.

Fig. 5 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular glucose-6-phosphate availability and an increased intracellular fructose-6-phosphate availability, are indicated.

Fig. 6 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular glucose-6-phosphate availability and an increased intracellular fructose-6-phosphate availability, are indicated.

Fig. 7 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular glucose-6-phosphate availability and an increased intracellular fructose-6-phosphate availability, are indicated.

Fig. 8 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular glucose-6-phosphate availability and an increased intracellular fructose-6-phosphate availability, are indicated.

Fig. 9 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular glucose-6-phosphate availability and an increased intracellular fructose-6-phosphate availability, are indicated.

Fig. 10 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular availability of glucose, glucose-6-phosphate and fructose-6-phosphate, are indicated.

Fig. 11 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular availability of mannose and/or mannose-6-phosphate, are indicated.

Fig. 12 shows a schematic drawing of another exemplary embodiment of a genetically engineered microbial cell of the invention, wherein genetic modifications, leading to an increased intracellular availability of galactose-1-phosphate and fructose-6-phosphate, are indicated.

Fig. 13 displays growth characteristics of *E. coli* strains during cultivation on glucose (A) or on a mixed-monosaccharide feedstock consisting of glucose and fructose (B) as sole carbon- and energy source.

Detailed description

According to the first aspect, provided is a genetically engineered microbial cell that is capable to produce a carbohydrate of interest. In an additional embodiment, the carbohydrate of interest is a carbohydrate that does not naturally occur in the wild type progenitor of the genetically engineered microbial cell.

The microbial cell possesses an increased intracellular availability of at least one sugar phosphate as compared to the intracellular availability of said at least one sugar phosphate in the corresponding wild-type cell. The genetically engineered microbial cell is capable to produce said carbohydrate of interest, and produces said carbohydrate of interest when being cultivated in a culture medium comprising a mixed monosaccharide feedstock as main carbon and energy source for the microbial cell, wherein the mixed monosaccharide feedstock consists of glucose and at least one additional monosaccharide selected from the group consisting of fructose and galactose.

The genetically engineered microbial cell is able to produce the carbohydrate of interest due to being genetically engineered. Hence, the genetically engineered microbial cell expresses one or more heterologous gene(s), wherein the activity of the polypeptide(s) encoded by said heterologous gene(s) enable(s) the microbial cell to synthesize the carbohydrate of interest.

The term "functional gene" as used herein, refers to a nucleic acid molecule comprising a nucleotide sequence which encodes a protein or polypeptide, and which also contains regulatory sequences operably linked to said protein-coding nucleotide sequence such that the nucleotide sequence which encodes the protein or polypeptide can be expressed in/by the microbial cell bearing said functional gene. Thus, when cultivated at conditions that are permissive for the expression of the functional gene, said functional gene is expressed, and the microbial cell expressing said functional gene typically comprises the protein or polypeptide that is encoded by the protein coding region of the functional gene. As used herein, the terms "nucleic acid" and "polynucleotide" refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence includes the complementary sequence thereof.

The term "operably linked" as used herein, shall mean a functional linkage between a nucleic acid expression control sequence (such as a promoter, signal sequence, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence affects transcription and/or translation of the nucleic acid corresponding to the second sequence. Accordingly, the term "Promoter" designates DNA sequences which usually "precede" a gene in a DNA polymer and provide a site for initiation of the transcription into mRNA. "Regulator" DNA sequences, also usually "upstream" of (i.e., preceding) a gene in a given DNA polymer, bind proteins that determine the frequency (or rate) of transcriptional initiation. Collectively referred to as "promoter/regulator" or "control" DNA sequence, these sequences which precede a selected gene (or series of genes) in a functional DNA polymer cooperate to determine whether the transcription (and eventual expression) of a gene will occur. DNA sequences which "follow" a gene in a DNA polymer and provide a signal for termination of the transcription into mRNA are referred to as transcription "terminator" sequences.

The term "recombinant", as used herein with reference to a bacterial host cell indicates that the bacterial cell replicates a heterologous nucleic acid, or expresses

a peptide or protein encoded by a heterologous nucleic acid (i.e., a sequence that is "foreign to said cell"). Recombinant cells can contain genes that are not found within the native (non-recombinant) form of the cell. Recombinant cells can also contain genes found in the native form of the cell wherein the genes are modified and re-introduced into the cell by artificial means. The term also encompasses cells that contain a nucleic acid endogenous to the cell that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques. Accordingly, a "recombinant polypeptide" is one which has been produced by a recombinant cell. A "heterologous sequence" or a "heterologous nucleic acid", as used herein, is one that originates from a source foreign to the particular host cell (e.g. from a different species), or, if from the same source, is modified from its original form. Thus, a heterologous nucleic acid operably linked to a promoter is from a source different from that from which the promoter was derived, or, if from the same source, is modified from its original form. The heterologous sequence may be stably introduced, e.g. by transfection, transformation, conjugation or transduction, into the genome of the host microorganism cell, wherein techniques may be applied which will depend on the host cell the sequence is to be introduced. Various techniques are known to a person skilled in the art and are, e.g., disclosed in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Accordingly, a "genetically engineered host cell" or "genetically engineered microbial cell" is understood as a bacterial cell or a yeast cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous polynucleotide sequence.

Thus, the nucleic acid sequences as used in the present invention, may, e.g., be comprised in a vector which is to be stably transformed/transfected or otherwise introduced into host microorganism cells.

A great variety of expression systems can be used to produce the polypeptides of the invention. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast

chromosomal elements, from viruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression system constructs may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides and to synthesize a polypeptide in a host may be used for expression in this regard. The appropriate DNA sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, *supra*.

The art is rich in patent and literature publications relating to "recombinant DNA" methodologies for the isolation, synthesis, purification and amplification of genetic materials for use in the transformation of selected host organisms. Thus, it is common knowledge to transform host organisms with "hybrid" viral or circular plasmid DNA which includes selected exogenous (i.e. foreign or "heterologous") DNA sequences. The procedures known in the art first involve generation of a transformation vector by enzymatically cleaving circular viral or plasmid DNA to form linear DNA strands. Selected foreign DNA strands usually including sequences coding for desired protein product are prepared in linear form through use of the same/similar enzymes. The linear viral or plasmid DNA is incubated with the foreign DNA in the presence of ligating enzymes capable of effecting a restoration process and "hybrid" vectors are formed which include the selected exogenous DNA segment "spliced" into the viral or circular DNA plasmid.

The term "nucleotide sequence encoding..." generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA, and generally represents the portion of a gene which encodes a certain polypeptide or protein. The term includes, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded regions, or a mixture of single- and double-stranded regions. The term also encompasses polynucleotides that include a

single continuous region or discontinuous regions encoding the polypeptide (for example, interrupted by integrated phage or an insertion sequence or editing) together with additional regions that also may contain coding and/or non-coding sequences.

The term "variant(s)" as used herein, refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains the essential (enzymatic) properties of the reference polynucleotide or polypeptide. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to the persons skilled in the art.

Within the scope of the present invention, also nucleic acid/polynucleotide and polypeptide polymorphic variants, alleles, mutants, and interspecies homologs are comprised by those terms, that have an amino acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to a polypeptide encoded by a wildtype protein.

Accordingly, a “functional variant” of any of the genes/proteins disclosed therein, is meant to designate sequence variants of the genes/proteins still retaining the same or somewhat lesser activity of the gene or protein the respective fragment is derived from.

The genetically engineered microbial cell possesses at least one monosaccharide transporter for translocating at least one monosaccharide from the culture medium said microbial cell is cultivated in into its cytoplasm. Said at least one monosaccharide transporter translocates a monosaccharide selected from the group consisting of glucose, fructose and galactose.

The monosaccharide that has been translocated across the cell membrane has to be phosphorylated in order to become accessible for the cell's metabolism. Depending on the monosaccharide transporter that has translocated the monosaccharide across the cell membrane, the monosaccharide(s) are either directly phosphorylated, i.e. while being transferred into the cell, or are subsequently phosphorylated by a suitable kinase. Translocation of the monosaccharide by a phosphoenolpyruvate:sugar phosphotransferase dependent transport (PEP-PTS) leads to direct phosphorylation, whereas translocation of a monosaccharide by means of a phosphoenolpyruvate:sugar phosphotransferase independent transport (non-PEP-PTS) requires subsequent phosphorylation of the monosaccharide by an intracellular kinase.

In an additional and/or alternative embodiment, the microbial cell comprises a glucose-translocating phosphotransferase system (PtsG). The glucose-translocating phosphotransferase system catalyzes the phosphorylation of incoming glucose concomitantly with its translocation across the cell membrane.

The general mechanism of the Pts system is the following: a phosphoryl group from phosphoenolpyruvate (PEP) is transferred via a signal transduction pathway, to enzyme I (EI) which in turn transfers it to a phosphoryl carrier, the histidine protein (HPr). Phospho-HPr then transfers the phosphoryl group to a sugar-specific permease, a membrane-bound complex known as enzyme 2 (EII), which transports the sugar to the cell. EII consists of at least three structurally distinct domains IIA,

IIB and IIC. These can either be fused together in a single polypeptide chain or exist as two or three interactive chains, formerly called enzymes II (EII) and III (EIIE).

The first domain (IIA or EIIEA) carries the first permease-specific phosphorylation site, a histidine which is phosphorylated by phospho-HPr. The second domain (IIB or EIIEB) is phosphorylated by phospho-IIA on a cysteinyl or histidyl residue, depending on the sugar transported. Finally, the phosphoryl group is transferred from the IIB domain to the sugar substrate concomitantly with the sugar uptake processed by the IIC domain. This third domain (IIC or EIIEC) forms the translocation channel and the specific substrate-binding site.

Thus, the PtsG system acquires exogenous glucose and provides glucose-6-phosphate in the microbial cell. Glucose-6-phosphate can be utilized in the UDP-galactose biosynthesis pathway and/or converted to fructose-6-phosphate which in turn may be used for generating energy-rich triphosphates in the central metabolism and/or, for example, in the biosynthesis of nucleotide activated saccharides such as GDP-fucose.

In an additional and/or alternative embodiment, the genetically engineered microbial cell comprises a fructose transporter for translocating fructose (Frc) from the culture medium into the microbial cell's cytoplasm. A suitable fructose transporter for uptake of free fructose is an isoform (PtsG-F) as described by Kornberg *et al.* PNAS 97: 1808-1812 (2000)).

The internalized fructose may then be phosphorylated by a fructokinase (FrK) to provide fructose-6-phosphate (Fru-6-P). Fructose-6-phosphate may be utilized in the UDP-galactose biosynthesis pathway and/or in other metabolic pathways such as generating energy-rich triphosphates in the central metabolism and/or, for example, in the biosynthesis of nucleotide activated saccharides such as GDP-fucose.

In an additional and/or alternative embodiment, the genetically engineered microbial cell comprises a fructose-translocating phosphotransferase system (PtsF). The fructose-translocating phosphotransferase system catalyzes the phosphorylation of incoming fructose concomitantly with its translocation across the cell membrane.

Thus, the PtsF system acquires exogenous fructose and provides fructose-1-phosphate in the microbial cell. The PtsF system comprises a membrane-spanning protein FruA, a 1-phosphofructose kinase (FruK) and a diphosphoryl transfer protein FruB. Fructose is translocated by means of FruA and FruB to provide fructose-1-phosphate in the cytoplasm. Fructose-1-phosphate can be further phosphorylated by a phosphofructokinase (FruK) to yield fructose-1,6-bisphosphate which in turn may be used by the microbial cell for generating energy-rich triphosphates in the central metabolism.

Another suitable PtsF System comprises LevD, LevE, LevF and LevG. LevD is the fructose-specific phosphotransferase enzyme IIA component. LevE is the fructose-specific phosphotransferase enzyme IIB component. LevF is the fructose permease IIC component, LevG is the fructose permease IID component. Corresponding genes *levD*, *levE*, *levF* and *levG* are – for example known from *Bacillus subtilis* (strain 168). Said PtsF system provides fructose-1-phosphate in the cell.

In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses an UDP-galactose biosynthesis pathway for intracellular formation of UDP-galactose (UDP-Gal). UDP-galactose is required as a substrate for galactosyltransferases wherein the activity of said galactosyltransferases may lead to the formation of galactosylated disaccharides or galactosylated oligosaccharides.

UDP-galactose may be provided by the microbial cells' naturally occurring metabolism, i.e. by the activity of a phosphoglucomutase catalyzing the interconversion of glucose-1-phosphate and glucose 6-phosphate, an UTP-glucose-1-phosphate-uridylyltransferase which catalyzes the formation of UDP-glucose from glucose-1-phosphate and UTP, and an UDP-glucose-4-epimerase catalyzing the reversible conversion of UDP-glucose to UDP-galactose.

The intracellular supply of UDP-galactose can be improved by genetic engineering in that one or more of the genes encoding the phosphoglucomutase, the UDP-glucose-1-phosphate-uridylyltransferase, the UDP-glucose-4-epimerase, and functional variants thereof are overexpressed and/or in that one or more additional copies of one or more of the genes encoding one or more of the genes encoding the phosphoglucomutase, the UDP-glucose-1-phosphate-uridylyltransferase, the UDP-

glucose-4-epimerase, and functional variants thereof are expressed in the microbial cell. An example of a gene encoding a phosphoglucomutase is the *pgm* gene (acc. no. NP_415214) found in *E. coli* K-12, an example of a gene encoding an UDP-glucose-1-phosphate-uridylyltransferase is the *E. coli galU* gene (acc. no. NP_415752) found in *E. coli* K-12, and an example of a gene encoding a UDP-glucose-4-epimerase is the *E. coli galE* gene (NP_415280) found in *E. coli* K-12.

The term “overexpression” or “overexpressed” as used herein refers to a level of enzyme or polypeptide expression that is greater than what is measured in a wild-type progenitor cell, i.e. a cell of the same species as the genetically engineered microbial cell and which cell that has not been genetically engineered.

Additionally and/or alternatively, intracellular UDP-galactose supply can be achieved or improved by feeding galactose to the microbial cells via the culture medium said microbial cell is cultivated in. The exogenously supplied galactose is taken up by the microbial cell and subsequently phosphorylated to galactose-1-phosphate which is then converted to UDP-galactose. In this GDP-galactose biosynthesis pathway, the genes encoding the enzymes possessing the required enzymatic activities are known in the literature (Groissoird *et al.*, “Characterization, Expression, and Mutation of the *Lactococcus lactis* galPMKTE Genes, Involved in Galactose Utilization via the Leloir Pathway (2003) *J. Bacteriol.* 185(3) 870-878). The *galP* gene (acc. no. NP_417418) encodes a galactose-proton symporter. The *galM* gene (acc. no. NP_415277) encodes an aldolase 1-epimerase, the *galK* gene (acc. no. NP_415278) a galactokinase, the *galT* gene (acc. no. NP_415279) a galactose-1-phosphate uridylyltransferase, and the *galE* gene an UDP-galactose-4-epimerase.

Hence, UDP-galactose biosynthesis can also be supplied by expression of genes encoding a galactose-proton symporter, a galactose kinase, and a galactose-1-phosphate uridylyltransferase in the microbial cell, or improved by overexpression of the genes encoding a galactose-proton symporter, a galactose kinase, and a galactose-1-phosphate uridylyltransferase in the microbial cell.

In another embodiment, the genetically engineered microbial cell possesses an GDP-fucose biosynthesis pathway for intracellular formation of GDP-L-fucose (GDP-Fuc). GDP-Fuc is a substrate of fucosyltransferases, and the enzymatic activity of

fucosyltransferases may lead to the formation of fucosylated disaccharides or fucosylated oligosaccharides.

In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses a GDP-L-fucose biosynthesis pathway comprising a mannose-6-phosphate isomerase, a phosphomannomutase, a mannose-1-phosphate-guanylyltransferase, a GDP-mannose-4,6-dehydratase and a GDP-L-fucose synthase.

The intracellular supply of GDP-L-fucose can be improved by genetic engineering in that one or more genes encoding a mannose-6-phosphate isomerase, a phosphomannomutase, a mannose-1-phosphate-guanylyltransferase, a GDP-mannose-4,6-dehydratase or a GDP-L-fucose synthase are overexpressed and/or additional copies of one or more of the genes encoding a mannose-6-phosphate isomerase, a phosphomannomutase, a mannose-1-phosphate-guanylyltransferase, a GDP-mannose-4,6-dehydratase and a GDP-L-fucose synthase, or functional variants thereof are expressed in the microbial cell. An example of a gene encoding a mannose-6-phosphate isomerase is the *E. coli manA* gene (acc. no. NP_416130) found in *E. coli* K-12, an example for a gene encoding a phosphomannomutase is the *E. coli manB* gene (acc. no. NP_416552) found in *E. coli* K-12, an example for a gene encoding a mannose-1-phosphate-guanylyltransferase is the *E. coli manC* gene (acc. no. NP_416553) found in *E. coli* K-12, an example for a gene encoding a GDP-mannose-4,6-dehydratase is the *E. coli gmd* gene (acc. no. NP_416557) found in *E. coli* K-12, and an example of a gene encoding a GDP-L-fucose synthase is the *E. coli wcaG* gene (acc. no. NP_416556) found in *E. coli* K-12.

Additionally and/or alternatively, GDP-L-fucose supply can be achieved or improved by feeding L-fucose to the microbial cells via the culture medium said microbial cell is cultivated in. The exogenously supplied L-fucose is taken up by the microbial cell and first phosphorylated to fucose-1-phosphate by the enzyme fucose kinase. Fucose-1-phosphate is subsequently converted to GDP-L-fucose by the enzymatic activity of the enzyme fucose-1-phosphate guanylyltransferase. Genes encoding the enzymes possessing the required enzymatic activities are known to the skilled artisan. Exemplarily, the *fkp* gene (acc. no. WP_010993080), encoding the

bifunctional L-fucokinase/L-fucose 1-phosphat guanylyltransferase of *Bacteroides fragilis*, may be overexpressed within the genetically engineered host cell.

In another embodiment, the genetically engineered microbial cell possesses an UDP-*N*-acetylglucosamine biosynthesis pathway for intracellular formation of UDP-*N*-acetylglucosamine (UDP-GlcNAc), required for *N*-acetylglucosaminyltransferase reactions leading e.g. to the formation of *N*-acetylglucosaminylated di- or oligosaccharides.

In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses a UDP-*N*-acetylglucosamine biosynthesis pathway, e.g. comprising a L-glutamine:D-fucose-6-phosphate aminotransferase, a phosphoglucosamine mutase and a *N*-acetylglucosamine-1-phosphate uridyltransferase/ glucosamine-1-phosphate acetyltransferase.

The intracellular supply of UDP-*N*-acetylglucosamine can be improved by genetic modifications such as an expression or overexpression of one or more of the genes encoding polypeptides exhibiting L-glutamine:D-fucose-6-phosphate aminotransferase activity (e.g. the *E. coli* K-12 *glmS* gene (acc. no. NP_418185)), phosphoglucosamine mutase activity (e.g. the *E. coli* K-12 *glmM* gene (acc. no. NP_417643)) and *N*-acetylglucosamine-1-phosphate uridyltransferase/ glucosamine-1-phosphate acetyltransferase activity (e.g. the *E. coli* K-12 *glmU* gene (acc. no. NP_418186)), or variants thereof.

In another embodiment, the genetically engineered microbial cell possesses an CMP-*N*-acetylneuraminic acid/CMP-sialic acid biosynthesis pathway for intracellular formation of CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac), required for sialyltransferase reactions leading e.g. to the formation of sialylated di- or oligosaccharides.

In an additional and/or alternative embodiment, the genetically engineered microbial cell possesses a CMP-*N*-acetylneuraminic acid biosynthesis pathway, e.g. comprising a L-glutamine:D-fucose-6-phosphate aminotransferase, a phosphoglucosamine mutase, a *N*-acetylglucosamine-1-phosphate uridyltransferase/ glucosamine-1-phosphate acetyltransferase, UDP-*N*-acetylglucosamine-2-epimerase, a glucosamine-6-phosphate acetyltransferase, a *N*-acetylglucosamine-6-

phosphate phosphatase (preferably a HAD-like sugar phosphatase), a *N*-acetylglucosamine 2-epimerase, a sialic acid synthase and a CMP-sialic acid synthetase.

The intracellular supply of CMP-*N*-acetylneuraminic acid can be improved by genetic modifications such as an expression or overexpression of one or more of the genes encoding polypeptides exhibiting L-glutamine:D-fucose-6-phosphate aminotransferase activity (e.g. the *E. coli* K-12 *glmS* gene), phosphoglucosamine mutase activity (e.g. the *E. coli* K-12 *glmM* gene), *N*-acetylglucosamine-1-phosphate uridylyltransferase/ glucosamine-1-phosphate acetyltransferase activity (e.g. the *E. coli* K-12 *glmU* gene), UDP-*N*-acetylglucosamine-2-epimerase activity (e.g. the *Campylobacter jejuni neuC* gene (acc. no. AF305571)), a glucosamine-6-phosphate acetyltransferase activity (e.g. the *Saccharomyces cerevisiae gna1* gene (acc. no. NP_116637)), a *N*-acetylglucosamine-6-phosphate phosphatase activity (e.g. the *E. coli* K-12 *yihX* gene (acc. no. NP_418321)), a *N*-acetylglucosamine 2-epimerase activity (e.g. the *Synechocystis sp.* PCC6803 *slr1975* gene (acc. no. BAL35720)), a sialic acid synthase activity (e.g. the *Campylobacter jejuni neuB* gene (acc. no. AF305571)) and a CMP-sialic acid synthetase activity (e.g. the *Campylobacter jejuni neuA* gene (acc. no. AF305571)), or variants thereof.

The genetically engineered microbial cell may further comprise a glycosyltransferase. In a preferred embodiment, at least one glycosyltransferase is a fucosyltransferase, a sialyltransferase, a glucosaminyltransferase or a galactosyltransferase, more preferably, the at least one glycosyltransferase exhibits β -1,3-galactosyltransferase activity, β -1,4-galactosyltransferase activity, β -1,6-galactosyltransferase activity, β -1,3-*N*-acetylglucosaminyltransferase activity, α -2,3-sialyltransferase activity, α -2,6-sialyltransferase activity, α -1,2-fucosyltransferase activity, α -1,3-fucosyltransferase activity, α -1,4-fucosyltransferase activity. Suitable glycosyltransferases are known to the skilled artisan or can be found in the literature.

Generally, and throughout the present disclosure, the term "glycosyltransferase activity" or "glycosyltransferase" designates and encompasses enzymes that are responsible for the biosynthesis of disaccharides, oligosaccharides and polysaccharides, and they catalyze the transfer of monosaccharide moieties from an activated nucleotide monosaccharide/sugar (e.g. UDP-Glc, UDP-Gal, GDP-Fuc,

UDP-GlcNAc, CMP-Neu5Ac) to a glycosyl acceptor molecule (e.g. mono-, di- or oligosaccharides).

In an additional and/or alternative embodiment, the genetically engineered microbial cell synthesizes more phosphoenolpyruvate (PEP) than the wildtype of the cell. In an additional and/or alternative embodiment, the genetically engineered microbial cell has been genetically engineered to possess an enhanced PEP biosynthesis pathway. For example, the genetically engineered microbial cell has been genetically engineered to possess an increased phosphoenolpyruvate carboxykinase activity, e.g. by overexpressing the *E. coli* K-12 *pckA* gene (acc. no. NP_417862) or a functional variant thereof. Preferably, the genetically engineered host cell has been genetically engineered to possess an increased phosphoenolpyruvate synthase activity, for example in that the *E. coli* K-12 *ppsA* gene (acc. no. NP_416217) encoding phosphoenolpyruvate synthase is overexpressed and/or in that the nonnaturally-occurring microorganism contains at least one additional copy of a nucleotide sequence allowing the expression of a phosphoenolpyruvate synthase or a functional variant thereof. Overexpression of *pckA* or *ppsA* enhances intracellular PEP synthesis such that more PEP is available, e.g. for the production of sialic acid.

In an additional and/or alternative embodiment, the intracellular PEP-availability of the genetically engineered host cell can be improved by decreasing and/or diminishing the activity of a phosphoenolpyruvate:sugar phosphotransferase dependent importer required for the transfer of a certain sugar (e.g. glucose), used as carbon- and energy source, from the culture medium into the cell. Consequently, in order to maintain the capability of the genetically engineered host cell to use said certain sugar as carbon- and energy source, a phosphoenolpyruvate:sugar phosphotransferase independent importer needs to be expressed or overexpressed. In an additional and/or alternative embodiment, the expression or overexpression of a kinase, capable to phosphorylate said certain sugar, is required or decreased and/or diminished, in order to realize metabolism of said sugar or its accumulation in an unphosphorylated form within the cell, respectively.

Phosphoenolpyruvate:sugar phosphotransferase dependent importer or components thereof, whose expression and/or activity might be decreased and/or

diminished in the genetically engineered microbial cell (e.g. *E. coli* K-12) comprise at least one selected from the group consisting of the glucose PEP-PTS genes *ptsG* (acc. no. NP_415619), *malX* (acc. no. NP_416138), *crr* (acc. no. NP_416912), *bglF* (acc. no. NP_418178) and/or the fructose PEP-PTS genes *fruA* (acc. no. NP_416672), *fruB* (acc. no. NP_416674) and/or the mannose PEP-PTS genes *manX* (acc. no. NP_416331), *many* (acc. no. NP_416332), *manZ* (acc. no. NP_416333) and or the *N*-acetylglucosamine PEP-PTS gene *nagE* (acc. no. NP_415205).

Suitable non-PEP-PTS transporter or variants thereof, capable of transferring monosaccharides (glucose and/or fructose and/or galactose and/or *N*-acetylglucosamine and/or *N*-acetylneuraminic acid and/or fucose) into a microbial cell, are known to the skilled artisan. Non-limiting examples of genes encoding said non-PEP-PTS transporter are *galP* of *Escherichia coli* K-12 (SEQ ID NO. 1), *glf* of *Zymomonas mobilis* (SEQ ID NO. 2), *cscB* of *Escherichia coli* W (SEQ ID NO. 3), *fupL* of *Leuconostoc pseudomesenteroides* (SEQ ID NO. 4), *lacY* of *Escherichia coli* K-12 (SEQ ID NO. 5), *fucP* of *Escherichia coli* K-12 (SEQ ID NO. 6), *nanT* of *Escherichia coli* K-12 (SEQ ID NO. 7), *nagP* of *Xanthomonas campestris* (SEQ ID NO. 8), *glcP* of *Bifidobacterium longum* NCC2705 (SEQ ID NO. 9), *glcP* of *Bacillus subtilis* (SEQ ID NO. 10), *sglS* of *Vibrio parahaemolyticus* (SEQ ID NO. 11), *xylE* of *Escherichia coli* K-12 (SEQ ID NO. 12), *araE* of *Bacillus subtilis* 168 (SEQ ID NO. 13). Thus, in an additional and/or alternative embodiment, the genetically engineered host cell comprises and expresses at least one gene comprising the protein coding region of the aforementioned genes or functional variants thereof.

In a preferred embodiment, suitable non-PEP-PTS-glucose transporter are a sugar facilitated diffusion protein and/or a glucose translocation permease. A suitable glucose facilitated fusion protein is encoded by the *glf* gene of *Zymomonas mobilis*. A suitable glucose translocation permease is encoded by the *E. coli* K-12 *galP* gene. The glucose translocation permease is also known as galactose-proton symporter or galactose permease, but also imports glucose across the cell membrane.

In another preferred embodiment, suitable non-PEP-PTS-fructose transporter are a sugar facilitated diffusion protein and/or a fructose carrier protein. A suitable fructose facilitated fusion protein is encoded by the *glf* gene of *Zymomonas mobilis*. A

suitable fructose carrier protein is encoded by the *Leuconostoc pseudomesenteroides fupL* gene.

Generally, and throughout the present disclosure the terms “increased intracellular availability of a sugar phosphate” or “increased/improved supply of a sugar phosphate” refer to an increased capability of the genetically modified microbial cell to generate elevated intracellular amounts of said sugar phosphate, compared to an unmodified microbial cell, enabling an elevated carbon flux through the biosynthesis pathway of a desired carbohydrate. Consequently, an elevated production of said desired carbohydrate by said genetically modified microbial cell is a direct measure of an elevated intracellular amount of said sugar phosphate. Besides, methods targeting the intracellular metabolite quantification and/or the carbon flux of cells can be found in the literature known to the skilled artisan (e.g. Lämmerhofer and Weckwerth, “Metabolomics in Practice: Successful Strategies to Generate and Analyze Metabolic Data”, Wiley-VCH, Weinheim, Germany (2013)).

In an additional and/or alternative embodiment, the expression and/or activity of genes and/or proteins, respectively, competing with the biosynthesis of the desired carbohydrate may be decreased and/or diminished within the genetically engineered host cell. Non-limiting examples of such counteracting proteins/protein activities comprise at least one selected from the group consisting of β -galactosidase (e.g. *E. coli* K-12 LacZ (acc. no. NP_414878)), UDP-glucose:undecaprenylphosphate glucose-1-phosphate transferase (e.g. *E. coli* K-12 WcaJ (acc. no. NP_416551)), L-fucose isomerase (e.g. *E. coli* K-12 FucI), fuculokinase (e.g. *E. coli* K-12 FucK (acc. no. NP_417282)), *N*-acetylglucosamine-6-phosphate deacetylase (e.g. *E. coli* K-12 NagA (acc. no. NP_415203)), glucosamine-6-phosphate deaminase (e.g. *E. coli* K-12 NagB (acc. no. NP_415204)), *N*-acetylmannosamine kinase (e.g. *E. coli* K-12 NanK (acc. no. NP_417689)), *N*-acetylmannosamine-6-phosphate epimerase (e.g. *E. coli* K-12 NanE (acc. no. NP_417690)), *N*-acetylneuraminic acid aldolase (e.g. *E. coli* K-12 NanA (acc. no. NP_417692)), sialic acid permease (e.g. *E. coli* K-12 NanT (acc. no. NP_417691)).

In an additional and/or alternative embodiment, the genetically engineered microbial cell is capable to metabolize sucrose, thus, comprising one or more genes encoding

(i) a heterologous PTS-dependent sucrose utilization transport system (e.g. the *Klebsiella pneumoniae* *scrYAB* genes (SEQ ID NO. 14) or the *Salmonella typhimurium* *scrYAB* genes (SEQ ID NO. 15) consisting of a sucrose porin (*scrY*), a PTS sucrose-specific transporter subunit II (*scrA*) and a sucrose-6-phosphate hydrolase (*scrB*) and/or (ii) a heterologous PTS-dependent sucrose transport system (e.g. the *Klebsiella pneumoniae* *scrYA* genes or the *Salmonella typhimurium* *scrYA* genes) in combination with a sucrose phosphate synthase gene (e.g. the *Anabaena* sp. PCC7120 *spsA* gene (acc. no. AJ302071)) and/or (iii) a heterologous PTS-independent sucrose utilization system (e.g. the *E. coli* W *cscBKA* genes (SEQ ID NO. 16) consisting of a fructokinase (*cscK*), a sucrose hydrolase (*cscA*) and a sucrose permease (*cscB*) and/or (iv) a heterologous sucrose utilization system consisting of a sucrose permease gene (e.g. *E. coli* W *cscB*) in combination with a sucrose phosphorylase gene (e.g. the *Bifidobacterium adolescentis* *basP* gene (acc. no. WP_011742626)) or a sucrose synthase gene (e.g. the *Anabaena* sp. *susA* gene (acc. no. CAA09297)).

In an additional and/or alternative embodiment, the microbial cell comprises an exporter protein or a permease exporting the carbohydrate of interest from the cell, preferably a sugar efflux transporter.

In an additional and/or alternative embodiment, the host cell is a microbial cell, preferably a bacterial cell selected from the group consisting of bacteria of the genera *Escherichia*, *Lactobacillus*, *Corynebacterium*, *Bacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus* and *Clostridium*, preferably a bacterial cell that is selected from the group of bacterial species consisting of *Escherichia coli*, *Corynebacterium glutamicum*, *Clostridium cellulolyticum*, *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Clostridium acetobutylicum*, *Bacillus subtilis*, *Bacillus megaterium*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, and *Lactococcus lactis*. In another embodiment, the microbial cell is an *Escherichia coli*. A person skilled in the art will be aware of further bacterial strains when reading the present disclosure.

According to the second aspect, provided is the use of a genetically engineered host cell as described herein for the production of a desired carbohydrate, wherein said host cell is cultivated in the presence of a monosaccharide mixture consisting of

glucose and at least a second monosaccharide of the group of fructose and galactose. Said cell is genetically engineered to exhibit an increased intracellular availability of at least one sugar phosphate relevant for the production of the desired carbohydrate. Said sugar phosphate may be selected from the group consisting of phosphoenolpyruvate (PEP), dihydroxyacetone phosphate, fructose-6-phosphate, fructose-1-phosphate, fructose-1,6-bisphosphate, glucose-6-phosphate, glucose-1-phosphate, galactose-1-phosphate, mannose-1-phosphate, mannose-6-phosphate, glucosamine-6-phosphate, glucosamine-1-phosphate, *N*-acetylglucosamine-6-phosphate, *N*-acetylglucosamine-1-phosphate, *N*-acetylmannosamine-6-phosphate, UDP-glucose, UDP-galactose, CMP-*N*-acetylneuraminic acid, GDP-mannose, GDP-fucose and UDP-*N*-acetylglucosamine.

According to the third aspect, provided is a method for the fermentative production of a desired carbohydrate comprising the steps of:

- a) providing a genetically engineered microorganism capable of producing a desired carbohydrate, wherein said microorganism is exhibiting an increased intracellular availability of at least one sugar phosphate due to a decreased and/or diminished expression and/or activity of at least one protein leading to a consumption of said intracellular sugar phosphate within said engineered microorganism;
- b) cultivating said genetically engineered microorganism in a culture medium permissive for the production of said desired carbohydrate, wherein the main carbon source is a monosaccharide mixture consisting of glucose and at least a second monosaccharide of the group of fructose and galactose;
- c) recovering said desired saccharide from the culture broth.

In an additional and/or alternative embodiment, the carbohydrate of interest is a human milk oligosaccharide or a building block thereof. A list of carbohydrates of interest that can be produced by using a genetically modified microbial cell and/or a method as described herein is disclosed in table 1. Preferably, the desired carbohydrate is selected from the group consisting of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3-fucosyl-3'-sialyllactose,

lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N*-difucosylhexose I, lacto-*N*-difucosylhexaose II, lacto-*N*-sialylpentaose LSTa, LSTb, LSTc.

Preferably, the mixture of glucose and at least one additional monosaccharide is a mixed feedstock of glucose and fructose, preferably obtained by hydrolyzation of sucrose.

In an additional and/or alternative embodiment, sucrose hydrolyzation is incomplete, such that substantial amounts of sucrose remain in the feedstock. Thus, the sucrose amount in the hydrolyzed and/or heat-sterilized feedstock is greater than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65 %, 70%, 75% or greater than 80%.

In an additional and/or alternative embodiment, the microbial cell is cultivated without exogenous supply of an acceptor substrate, e.g. *N*-acetylglucosamine or lactose, in particular when cultivated for the production of the oligosaccharide of interest.

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

It is to be noticed that the term "comprising", used in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps. It is thus to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more other features, integers, steps or components, or groups thereof. Thus, the scope of the expression "a device

comprising means A and B” should not be limited to devices consisting only of components A and B. It means that with respect to the present invention, the only relevant components of the device are A and B.

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

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

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

Furthermore, some of the embodiments are described herein as a method or combination of elements of a method that can be implemented by a processor of a computer system or by other means of carrying out the function. Thus, a processor

with the necessary instructions for carrying out such a method or element of a method forms a means for carrying out the method or element of a method. Furthermore, an element described herein of an apparatus embodiment is an example of a means for carrying out the function performed by the element for the purpose of carrying out the invention.

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

Name	Abbrev.	structure
Mannose	Man	Man
Fucose	Fuc	Fuc
<i>N</i> -acetylglucosamine	GlcNAc	GlcNAc
<i>N</i> -acetylmannosamine	ManNAc	ManNAc
<i>N</i> -acetylneuraminic acid	Neu5Ac	Neu5Ac
Lacto- <i>N</i> -biose	LNB	Gal(β1-3)GlcNAc
<i>N</i> -acetyllactosamine	LacNAc	Gal(β1-4)GlcNAc
3-Fucosylglucose	Fuc-Glc	Glu Fuc(α1-3)
4'-Fucosyl- <i>N</i> -acetylglucosamine	Fuc-GlcNAc	GlcNAc Fuc(α1-4)
6'-Sialyl- <i>N</i> -acetylglucosamine	Neu5Ac-GlcNAc	GlcNAc Neu5Ac(α2-6)
2'-Fucosyllactose	2'-FL	Fuc(α1-2)Gal(β1-4)Glu
3-Fucosyllactose	3-FL	Gal(β1-4)Glu Fuc(α1-3)
2',3-Difucosyllactose	DF-L	Fuc(α1-2)Gal(β1-4)Glu Fuc(α1-3)
Blood group H antigen type I	HA-T I	Fuc(α1-2)Gal(β1-3)GlcNAc
Blood group H antigen type II	HA-T II	Fuc(α1-2)Gal(β1-4)GlcNAc
Blood group antigen	Le ^a	Gal(β1-3)GlcNAc

Name	Abbrev.	structure
Lewis ^a		Fuc(α1-4)
Blood group antigen Lewis ^b	Le ^b	Fuc(α1-2)Gal(β1-3)GlcNAc Fuc(α1-4)
Blood group antigen Lewis ^x	Le ^x	Gal(β1-4)GlcNAc Fuc(α1-3)
Blood group antigen Lewis ^y	Le ^y	Fuc(α1-2)Gal(β1-4)GlcNAc Fuc(α1-3)
Blood group antigen Sialyl-Lewis ^x	SLe ^x	Neu5Ac(α2-3)Gal(β1-4)GlcNAc Fuc(α1-3)
Lacto- <i>N</i> -triose II	LNT II	GlcNAc(β1-3)Gal(β1-4)Glu
Lacto- <i>N</i> -tetraose	LNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glu
Lacto- <i>N</i> -neotetraose	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glu
Lacto- <i>N</i> -fucopentaose I	LNFP I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glu
Lacto- <i>N</i> -neofucopentaose I	LNnFP I	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glu
Lacto- <i>N</i> -fucopentaose II	LNFP II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glu Fuc(α1-4)
Lacto- <i>N</i> -fucopentaose III	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glu Fuc(α1-3)
Lacto- <i>N</i> -fucopentaose V	LNFP V	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glu Fuc(α1-3)
Lacto- <i>N</i> -neofucopentaose V	LNnFP V	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glu Fuc(α1-3)
Lacto- <i>N</i> -difucohexaose I	LNDH I	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glu Fuc(α1-2) Fuc(α1-4)
Lacto- <i>N</i> -difucohexaose II	LND	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glu Fuc(α1-4) Fuc(α1-3)
6'-Galactosyllactose	6'-GL	Gal(β1-6)Gal(β1-4)Glu
3'-Galactosyllactose	3'-GL	Gal(β1-3)Gal(β1-4)Glu
Lacto- <i>N</i> -hexaose	LNH	Gal(β1-4)GlcNAc(β1-6)Gal(β1-4)Glu Gal(β1-3)GlcNAc(β1-3)
Lacto- <i>N</i> -neohexaose	LNnH	Gal(β1-4)GlcNAc(β1-6)Gal(β1-4)Glu Gal(β1-4)GlcNAc(β1-3)

Name	Abbrev.	structure
<i>para</i> -Lacto- <i>N</i> -hexaose	paraLNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glu
<i>para</i> -Lacto- <i>N</i> -neohexaose	paraLNnH	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glu
Difucosyl-lacto- <i>N</i> -neohexaose	DF-LNnH	$ \begin{array}{c} \text{Fuc}(\alpha 1-3) \\ \\ \text{Gal}(\beta 1-4)\text{GlcNAc}(\beta 1-6)\text{Gal}(\beta 1-4)\text{Glu} \\ \\ \text{Gal}(\beta 1-4)\text{GlcNAc}(\beta 1-3) \\ \\ \text{Fuc}(\alpha 1-3) \end{array} $
3'-Sialyllactose	3'-SL	Neu5Ac(α2-3)Gal(β1-4)Glu
6'-Sialyllactose	6'-SL	Neu5Ac(α2-6)Gal(β1-4)Glu
3'-Sialyl-lacto- <i>N</i> -biose	3'-SLNB	Neu5Ac(α2-3)Gal(β1-3)GlcNAc
6'-Sialyl-lacto- <i>N</i> -biose	6'-SLNB	Neu5Ac(α2-6)Gal(β1-3)GlcNAc
3'-Sialyl- <i>N</i> -acetyllactosamine	3'-SLN	Neu5Ac(α2-3)Gal(β1-4)GlcNAc
6'-Sialyl- <i>N</i> -acetyllactosamine	6'-SLN	Neu5Ac(α2-6)Gal(β1-4)GlcNAc
Lacto- <i>N</i> -sialylpentaose a	LST-a	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glu
Lacto- <i>N</i> -sialylpentaose b	LST-b	$ \begin{array}{c} \text{Gal}(\beta 1-3)\text{GlcNAc}(\beta 1-3)\text{Gal}(\beta 1-4)\text{Glu} \\ \\ \text{Neu5Ac}(\alpha 2-6) \end{array} $
Lacto- <i>N</i> -sialylpentaose c	LST-c	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glu
Fucosyl-lacto- <i>N</i> -sialylpentaose a	F-LST-a	$ \begin{array}{c} \text{Neu5Ac}(\alpha 2-3)\text{Gal}(\beta 1-3)\text{GlcNAc}(\beta 1-3)\text{Gal}(\beta 1-4)\text{Glu} \\ \\ \text{Fuc}(\alpha 1-4) \end{array} $
Fucosyl-lacto- <i>N</i> -sialylpentaose b	F-LST-b	$ \begin{array}{c} \text{Fuc}(\alpha 1-2)\text{Gal}(\beta 1-3)\text{GlcNAc}(\beta 1-3)\text{Gal}(\beta 1-4)\text{Glu} \\ \\ \text{Neu5Ac}(\alpha 2-6) \end{array} $
Fucosyl-lacto- <i>N</i> -sialylpentaose c	F-LST-c	$ \begin{array}{c} \text{Neu5Ac}(\alpha 2-3)\text{Gal}(\beta 1-3)\text{GlcNAc}(\beta 1-3)\text{Gal}(\beta 1-4)\text{Glu} \\ \\ \text{Fuc}(\alpha 1-3) \end{array} $
Disialyl-lacto- <i>N</i> -tetraose	DS-LNT	$ \begin{array}{c} \text{Neu5Ac}(\alpha 2-3)\text{Gal}(\beta 1-4)\text{GlcNAc}(\beta 1-3)\text{Gal}(\beta 1-4)\text{Glu} \\ \\ \text{Neu5Ac}(\alpha 2-6) \end{array} $
Disialyl-lacto- <i>N</i> -fucopentaose	DS-LNFP V	$ \begin{array}{c} \text{Neu5Ac}(\alpha 2-3)\text{Gal}(\beta 1-4)\text{GlcNAc}(\beta 1-3)\text{Gal}(\beta 1-4)\text{Glu} \\ \qquad \qquad \qquad \\ \text{Neu5Ac}(\alpha 2-6) \qquad \text{Fuc}(\alpha 1-3) \end{array} $
3-Fucosyl-3'-sialyllactose	3F-3'-SL	$ \begin{array}{c} \text{Neu5Ac}(\alpha 2-3)\text{Gal}(\beta 1-4)\text{Glu} \\ \\ \text{Fuc}(\alpha 1-3) \end{array} $
3-Fucosyl-6'-sialyllactose	3F-6'-SL	$ \begin{array}{c} \text{Neu5Ac}(\alpha 2-6)\text{Gal}(\beta 1-4)\text{Glu} \\ \\ \text{Fuc}(\alpha 1-3) \end{array} $

Name	Abbrev.	structure
Lacto- <i>N</i> -neodifucohexaose I	LNnDFH I	$ \begin{array}{c} \text{Gal}(\beta 1-4)\text{GalNAc}(\beta 1-3)\text{Gal}(\beta 1-4)\text{Glu} \\ \qquad \qquad \qquad \\ \text{Fuc}(\alpha 1-3) \qquad \qquad \text{Fuc}(\alpha 1-3) \end{array} $

Table 1: Non-limiting list of carbohydrates of interest that can be produced by using a genetically modified microbial cell and/or a method as described herein.

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

In an embodiment an *E. coli* strain possessing the genotype *nagABE*⁻, *manXYZ*⁻ is metabolically engineered as an efficient producer of *N*-acetylglucosamine (GlcNAc) using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source. Therefore, the heterologous expression of a gene encoding for a glucosamine-6-phosphate acetyltransferase, capable to transfer acetate from acetyl-CoA to glucosamine-6-phosphate, thus generating *N*-acetylglucosamine-6-phosphate, is necessary. In a preferred embodiment, this production strain is further genetically engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate dehydrogenase gene *zwf*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (fructose-6-phosphate) for *N*-acetylglucosamine production. In an additional embodiment a gene encoding for a glutamine-fructose-6-phosphate aminotransferase (e.g. *E. coli* GlmS) and/or a gene encoding for a HAD-like sugar phosphatase (e.g. *E. coli* YihX, *E. coli* YqaB), capable to dephosphorylate *N*-acetylglucosamine-6-phosphate to *N*-acetylglucosamine, is expressed/ overexpressed in order to foster the synthesis of GlcNAc.

In another embodiment, an *E. coli* strain possessing the genotype *lacY*⁺, *lacZ*⁻, *fucIK*⁻, *wcaJ*⁻ is metabolically engineered as an efficient producer of L-fucose using a

mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source as well as lactose as acceptor substrate. Therefore, the overexpression of at least one of the *E. coli* genes *manA*, *manC*, *manB*, *gmd* and *wcaG* as well as the expression of a heterologous α -1,2-fucosyltransferase, capable to transfer fucose from GDP-fucose to lactose, thus generating 2'-fucosyllactose, and an α -1,2-fucosidase, capable to release free L-fucose from 2'-fucosyllactose, is necessary. In a preferred embodiment, this production strain is further engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate dehydrogenase gene *zwf*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (fructose-6-phosphate) for L-fucose production.

In another embodiment an *E. coli* strain possessing the genotype *nanKETA*⁻, *nagAB*⁻ is metabolically engineered as an efficient producer of *N*-acetylneuraminic acid (Neu5Ac) using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source. Therefore, the overexpression of at least one of the genes encoding for either (i) a glucosamine-6-phosphate acetyltransferase and a *N*-acetylglucosamine-2 epimerase and a *N*-acetylneuraminic acid synthetase, or (ii) a UDP-*N*-acetylglucosamine 2-epimerase and a *N*-acetylneuraminic acid synthetase, is necessary. Since the *N*-acetylneuraminic acid synthesis is a phosphoenolpyruvate (PEP)-dependent process, competing reactions leading to PEP consumption are preferably avoided. Thus, in a preferred embodiment, this production strain is further engineered by decreasing and/or diminishing the import of said carbon- and energy source(s) through a phosphoenolpyruvate:sugar phosphotransferase dependent mechanism, e.g. by decreasing and/or diminishing the expression of the glucose PEP permease gene *ptsG* and/or the fructose PEP permease gene *fruA* and/or the mannose PEP permease genes *manXYZ*. Along with the expression/overexpression of at least one gene encoding for a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter, enabling monosaccharide(s) transfer into the engineered cell, as well as a fructose kinase (e.g. the *E. coli* W *cscK* gene) and a glucose kinase (e.g. the *E. coli* K-12 *glk* gene), capable to activate fructose to fructose-6-phosphate and

glucose to glucose-6-phosphate, respectively, this further genetic modification allows the cultivation of the engineered production strain on a mixed-mono-saccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (fructose-6-phosphate, phosphoenolpyruvate) for *N*-acetylneuraminic acid production.

In another embodiment an *E. coli* strain possessing the genotype *nagABE*, *manXYZ*, *lacZ* is metabolically engineered as an efficient producer of *N*-acetylglucosamine (LacNAc) using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source as well as GlcNAc as acceptor substrate. Therefore, the expression/overexpression of genes encoding for a glucosamine-6-phosphate acetyltransferase, a phosphoenolpyruvate:sugar phosphotransferase independent transporter, capable to transfer *N*-acetylglucosamine into the cell, and a β -1,4-galactosyltransferase, capable to transfer galactose from UDP-Gal to free *N*-acetylglucosamine, thus generating *N*-acetylglucosamine, is necessary. In a preferred embodiment, this production strain is further genetically engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate isomerase gene *pgi* and/or the glucose-6-phosphate dehydrogenase gene *zwf*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (glucose-6-phosphate) for *N*-acetylglucosamine production. In an additional embodiment at least one of the *E. coli* genes *pgm*, *galU* and *galE* is overexpressed in order to foster the synthesis of UDP-Gal.

In another embodiment an *E. coli* strain possessing the genotype *nagAB* is metabolically engineered as an efficient producer of lacto-*N*-biose (LNB) by means of total fermentation using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source. Therefore, the expression/ overexpression of genes encoding for a glucosamine-6-phosphate acetyltransferase, capable to transfer acetate from acetyl-CoA to glucosamine-6-phosphate, thus generating *N*-acetylglucosamine-6-phosphate, a HAD-like sugar phosphatase,

capable to dephosphorylate *N*-acetylglucosamine-6-phosphate, thus generating *N*-acetylglucosamine, and a β -1,3-galactosyltransferase, capable to transfer galactose from UDP-Gal to free *N*-acetylglucosamine, thus generating lacto-*N*-biose, is necessary. In a preferred embodiment, this production strain is further genetically engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate dehydrogenase gene *zwf*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (fructose-6-phosphate and glucose-6-phosphate) for lacto-*N*-biose production. In an additional embodiment at least one of the *E. coli* genes *glmS*, *pgm*, *galU*, *galE* is overexpressed in order to foster the synthesis of GlcNAc and/or UDP-Gal.

In another embodiment an *E. coli* strain possessing the genotype *lacY*⁺, *lacZ*⁻, *nanKETA*⁻, *nagAB*⁻ is metabolically engineered as an efficient producer of 3'-sialyllactose using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source as well as lactose as acceptor substrate. Therefore, an *N*-acetylneuraminic acid production strain, as described, is genetically engineered by the expression of a heterologous CMP-*N*-acetylneuraminic acid synthetase, a *N*-acetylneuraminic acid synthase and an α -2,3-sialyltransferase, capable to transfer *N*-acetylneuraminic acid from CMP-Neu5Ac to lactose, thus generating 3'-sialyllactose. As for the synthesis of *N*-acetylneuraminic acid, 3'-SL production is a phosphoenolpyruvate (PEP)-dependent process. Thus, in a preferred embodiment, said 3'-SL production strain is further engineered by decreasing and/or diminishing the import of said carbon- and energy source(s) through a phosphoenolpyruvate:sugar phosphotransferase dependent mechanism, e.g. by decreasing and/or diminishing the expression of the glucose PEP permease gene *ptsG* and/or the fructose PEP permease gene *fruA* and/or the mannose PEP permease genes *manXYZ*. Along with the expression/overexpression of at least one gene encoding for a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter, enabling monosaccharide(s) transfer into the engineered cell, as well as a fructose kinase (e.g. the *E. coli* W *cscK* gene) and a glucose kinase (e.g. the *E. coli* K-12 *glk* gene), capable to activate fructose to

fructose-6-phosphate and glucose to glucose-6-phosphate, respectively, this further genetic modification allows the cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (fructose-6-phosphate, phosphoenolpyruvate) for 3'-sialyllactose production.

In another embodiment an *E. coli* strain possessing the genotype *lacY*⁺, *lacZ*⁻, *fucIK*⁻, *wcaJ*⁻ is metabolically engineered as an efficient producer of 3-fucosyllactose using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source as well as lactose as acceptor substrate. Therefore, the overexpression of at least one of the *E. coli* genes *manA*, *manC*, *manB*, *gmd* and *wcaG* as well as the expression of a heterologous α -1,3-fucosyltransferase, capable of transferring fucose from GDP-fucose to lactose, thus generating 3-fucosyllactose, is necessary. In a preferred embodiment, this production strain is further genetically engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate dehydrogenase gene *zwf*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (fructose-6-phosphate) for 3-fucosyllactose production.

In one embodiment an *E. coli* strain possessing the genotype *lacY*⁺, *lacZ*⁻, *nagB*⁻, *wcaJ*⁻ is metabolically engineered to efficiently produce lacto-*N*-triose II (LNT-II) using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source as well as lactose as acceptor substrate. Therefore, the expression of a heterologous β -1,3-*N*-acetylglucosaminyltransferase, capable to transfer *N*-acetylglucosamine from UDP-GlcNAc to lactose, thus generating lacto-*N*-triose II, is necessary. In a preferred embodiment, this production strain is further genetically engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate dehydrogenase gene *zwf*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g.

hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (fructose-6-phosphate) for lacto-*N*-triose II production. In an additional embodiment at least one of the *E. coli* genes *glmS*, *glmU* and *glmM* is overexpressed in order to foster UDP-GlcNAc synthesis.

In another embodiment an *E. coli* strain possessing the genotype *lacY*⁺, *lacZ*⁻, *nagB*⁻, *wcaJ*⁻ is metabolically engineered as an efficient producer of lacto-*N*-tetraose (LNT) using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source as well as lactose as acceptor substrate. Therefore, the expression of a heterologous β -1,3-*N*-acetylglucosaminyltransferase, capable to transfer *N*-acetylglucosamine from UDP-GlcNAc to lactose, and a β -1,3-galactosyltransferase, capable to transfer galactose from UDP-galactose to lacto-*N*-triose II, thus generating lacto-*N*-tetraose, is necessary. In a preferred embodiment, this production strain is further genetically engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate dehydrogenase gene *zwf* and/or the glucose-6-phosphate isomerase gene *pgi*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (fructose-6-phosphate and glucose-6-phosphate) for lacto-*N*-tetraose production. In an additional embodiment at least one of the *E. coli* genes *glmS*, *glmU*, *glmM*, *pgm*, *galU* and *galE* is overexpressed in order to foster the synthesis of UDP-GlcNAc and/or UDP-Gal.

In another embodiment an *E. coli* strain possessing the genotype *lacY*⁺, *lacZ*⁻, *nagB*⁻, *wcaJ*⁻ is metabolically engineered to efficiently produce lacto-*N*-neotetraose (LNnT) by means of total fermentation using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source. Therefore, the LNnT production strain is genetically engineered by decreasing and/or diminishing the import of glucose through a phosphoenolpyruvate:sugar phosphotransferase dependent mechanism, e.g. by decreasing and/or diminishing the expression of the glucose PEP permease gene *ptsG*, while expressing at least one gene encoding for

a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter, enabling glucose transfer into the engineered cell. Furthermore, the expression of the glucokinase gene *glk* and/or the glucose dehydrogenase gene *gcd* is decreased and/or abolished. Essentially, the heterologous expression of a β -1,4-galactosyltransferase, capable to transfer galactose from UDP-galactose on glucose, thus generating lactose, and a β -1,3-*N*-acetylglucosaminyltransferase, capable of transferring *N*-acetylglucosamine from UDP-GlcNAc to lactose, thus generating lacto-*N*-triose II, and a β -1,4-galactosyltransferase, capable to transfer galactose from UDP-galactose to lacto-*N*-triose II, thus generating lacto-*N*-neotetraose, is necessary. In a preferred embodiment, this production strain is further engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate dehydrogenase gene *zwf* and/or the glucose-6-phosphate isomerase gene *pgi*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (glucose and fructose-6-phosphate and glucose-6-phosphate) for lacto-*N*-neotetraose production by total fermentation. In an additional embodiment at least one of the *E. coli* genes *glmS*, *glmU*, *glmM*, *pgm*, *galU* and *galE* is overexpressed in order to foster the synthesis of UDP-GlcNAc and/or UDP-Gal.

In another embodiment, an *E. coli* strain possessing the genotype *lacY*⁻, *lacZ*⁻, *fucIK*⁻, *wcaJ*⁻ is metabolically engineered to efficiently produce 2'-fucosyllactose by means of total fermentation using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source. Therefore, the expression of the glucokinase gene *glk* and/or the glucose dehydrogenase gene *gcd* as well as the activity of a phosphoenolpyruvate:glucose phosphotransferase dependent mechanism is decreased and/or abolished. In addition, phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter gene is expressed or overexpressed in said *E. coli* strain. Furthermore, at least one of the *E. coli* genes *manA*, *manC*, *manB*, *gmd*, *wcaG*, *pgm*, *galU* and *galE* as well as a heterologous β -1,4-galactosyltransferase, capable to transfer galactose from UDP-galactose on glucose, thus generating lactose, and a α -1,2-fucosyltransferase, capable to transfer fucose from GDP-fucose to lactose, thus generating 2'-fucosyllactose, are

expressed / overexpressed. In a preferred embodiment, this production strain is further engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB* and/or the glucose-6-phosphate dehydrogenase gene *zwf* and/or the glucose-6-phosphate isomerase gene *pgi*. This further genetic modification allows cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (glucose and fructose-6-phosphate and glucose-6-phosphate) for 2'-fucosyllactose production by total fermentation.

In another embodiment an *E. coli* strain possessing the genotype *lacY*⁺, *lacZ*⁻, *fucIK*⁻, *wcaJ*⁻, *manA*⁻ is metabolically engineered as an efficient producer of 2'3-difucosyllactose using a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source as well as lactose as acceptor substrate. Therefore, the expression of the genes exhibiting fructokinase activity (e.g. the *E. coli* K-12 *mak* gene) and the activity of a phosphoenolpyruvate:fructose phosphotransferase dependent mechanism is decreased and/or abolished. This cell is further genetically engineered by the expression/overexpression of at least one gene encoding for a phosphoenolpyruvate:fructose phosphotransferase independent (non-PEP-PTS) transporter, enabling fructose transfer into the engineered cell, a mannose isomerase, capable of transforming fructose to mannose (e.g. *E. coli* BL21 *yihS*, *Agrobacterium radiobacter* M-1 *manI*), a mannokinase (e.g. *Prevotella bryantii* B₁₄ *manK*, *Arthrobacter* sp. Strain KM *manK*), capable of activating mannose to mannose-6-phosphate, as well as for at least one heterologous fucosyltransferase exhibiting an α -1,2- and/or an α -1,3-fucosyltransferase activity, capable of transferring fucose from GDP-fucose to lactose and/or 2'-fucosyllactose and/or 3-fucosyllactose, thus generating 2'3-difucosyllactose. These further genetic modifications allow cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. hydrolyzed sucrose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but increasing precursor supply (mannose/mannose-6-phosphate) for 2'3-difucosyllactose production. In an additional embodiment the overexpression of at least one of the *E. coli* genes *manC*, *manB*, *gmd* and *wcaG* is overexpressed in order to foster the synthesis of GDP-Fuc.

Non-limiting examples for suitable proteins exhibiting mannose isomerase (ManI) activity or mannokinase (ManK) activity can be found in the literature (Hirose et al., Biosci Biotechnol Biochem. 2001 Mar;65(3):658-61.; Hirose et al., Biotechnol Lett. 2003 Feb;25(4):349-52.; Patel et al., Appl Environ Microbiol. 2011 May;77(10):3343-50.; Hu et al., Int J Biol Macromol. 2016 Aug;89:328-35.; Huang et al., Appl Microbiol Biotechnol. 2018 Mar;102(5):2051-2062.; Mukai et al., Appl Environ Microbiol. 2003 Jul;69(7):3849-57.; Fields and Russel, Microbiology. 2001 Apr;147(Pt 4):1035-43.; Kroschewski et al., Mol Biochem Parasitol. 2000 Jan 5;105(1):71-80.).

In another embodiment an *E. coli* strain possessing the genotype *nagAB*, *fucIK*, *wcaJ* is metabolically engineered as an efficient producer of H antigen type I (HA-T I) by means of total fermentation using a mixed-monosaccharide feedstock (e.g. a mixture of hydrolyzed sucrose and hydrolyzed lactose) as main carbon- and energy source. Therefore, the production strain is genetically engineered by decreasing and/or diminishing the import of glucose through a phosphoenolpyruvate:sugar phosphotransferase dependent mechanism, while expressing/overexpressing at least one gene encoding for a non-PEP-PTS transporter as well as a monosaccharide kinase, enabling glucose and/or galactose transfer into the engineered cell as well as activating these monosaccharides into a phosphorylated form, respectively. Furthermore, at least one gene encoding for a glucosamine-6-phosphate acetyltransferase, capable to transfer acetate from acetyl-CoA to glucosamine-6-phosphate, thus generating *N*-acetylglucosamine-6-phosphate, a HAD-like sugar phosphatase, capable to dephosphorylate *N*-acetylglucosamine-6-phosphate, thus generating *N*-acetylglucosamine, a heterologous β -1,3-galactosyltransferase, capable to transfer galactose from UDP-galactose on *N*-acetylglucosamine, thus generating lacto-*N*-biose, and an α -1,2-fucosyltransferase, capable to transfer fucose from GDP-fucose to lacto-*N*-biose, thus generating H antigen type I, are expressed/overexpressed. In a preferred embodiment, this production strain is further genetically engineered by decreasing and/or diminishing the expression of the phosphofructokinase genes *pfkA* and/or *pfkB*. These genetic modifications allow cultivation of the engineered production strain on a mixed-monosaccharide feedstock (e.g. a mixture of hydrolyzed sucrose and hydrolyzed lactose) as main carbon- and energy source, while preventing to hamper the strain's metabolism but

increasing precursor supply (*N*-acetylglucosamine, fructose-6-phosphate and galactose-1-phosphate) for H antigen type I production. In an additional embodiment at least one of the *E. coli* genes *glmS*, *galT*, *galE*, *manC*, *manB*, *gmd* and *wcaG* is overexpressed in order to foster the synthesis of GlcNAc and/or UDP-Gal and/or GDP-Fuc.

Referring to Fig. 1, an exemplary natural microbial cell is schematically shown. Said microbial cell is capable of consuming a mixed feedstock consisting of glucose and fructose, leading to a minute intracellular pool of glucose-6-phosphate and fructose-6-phosphate. The microbial cell expresses polynucleotides encoding phosphoenolpyruvate:sugar phosphotransferase systems for glucose and fructose (PTS) import into the cell. The reaction products primarily enter the glycolysis.

Fig. 2 displays schematically an exemplary microbial cell of the invention being capable of supplying glucose-6-phosphate to a high degree when cultivated on a mixed feedstock consisting of glucose and fructose. As expression of the glucose-6-phosphate isomerase Pgi and/or the glucose-6-phosphate dehydrogenase Zwf have been decreased or diminished by deletion, functional inactivation or silencing of the *pgi* and/or *zwf* gene(s), any glucose-6-phosphate generated by glucose import into the cell may be utilized by the microbial cell for generating UDP-Glc and/or UDP-Gal. In a variant of the microbial cell (Fig. 3), the cell's phosphoenolpyruvate:sugar phosphotransferase system(s) for fructose has been disabled, e.g. by deletion of the *fruA* gene. Instead, fructose enters the cell via a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter getting activated by a fructose kinase (e.g. *E. coli* W CscK) to fructose-6-phosphate. In a variant of the microbial cell (Fig. 4), the cell's phosphoenolpyruvate:sugar phosphotransferase system(s) for glucose has also been disabled, e.g. by deletion of the *ptsG* gene. Instead, glucose enters the cell via a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter getting activated by a glucose kinase (e.g. *E. coli* K-12 Glk) to glucose-6-phosphate.

Fig. 5 displays schematically an exemplary microbial cell of the invention being capable of supplying glucose-6-phosphate and fructose-6-phosphate to a higher degree when cultivated on a mixed feedstock consisting of glucose and fructose. The cell's phosphoenolpyruvate:sugar phosphotransferase system(s) for fructose

has been disabled, e.g. by deletion of the *fruA* gene. Instead, fructose enters the cell via a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter getting activated by a fructose kinase (e.g. *E. coli* W CscK) to fructose-6-phosphate. In a variant of the microbial cell (Fig. 6), the expression of the phosphofructokinase(s) Pfk and/or the glucose-6-phosphate dehydrogenase Zwf have been decreased or diminished by deletion, functional inactivation or silencing of the *pfkA* and/or *pfkB* and/or *zwf* gene(s), yielding increased availability of glucose-6-phosphate and fructose-6-phosphate, which may be utilized by the microbial cell for generating UDP-Gal and/or GDP-Fuc and/or UDP-GlcNAc and/or CMP-Neu5Ac. In a variant of the microbial cell (Fig. 7), the cell expresses polynucleotides encoding phosphoenolpyruvate:sugar phosphotransferase systems for glucose and fructose (PTS) import into the cell. The expression of the phosphofructokinase(s) Pfk and/or the glucose-6-phosphate dehydrogenase Zwf have been decreased or diminished by deletion, functional inactivation or silencing of the *pfkA* and/or *pfkB* and/or *zwf* gene(s), yielding increased availability of glucose-6-phosphate and fructose-6-phosphate.

Fig. 8 displays schematically an exemplary microbial cell of the invention being capable of supplying glucose-6-phosphate and fructose-6-phosphate to a higher degree when cultivated on a mixed feedstock consisting of glucose and fructose. The cell expresses polynucleotides encoding phosphoenolpyruvate:sugar phosphotransferase systems for glucose and fructose (PTS) import into the cell. The expression of the phosphofructokinase(s) Pfk and/or the glucose-6-phosphate isomerase Pgi and/or the glucose-6-phosphate dehydrogenase Zwf have been decreased or diminished by deletion, functional inactivation or silencing of the *pfkA* and/or *pfkB* and/or *pgi* and/or *zwf* gene(s). Along with an overexpression of a fructose-1,6-bisphosphatase (e.g. *glpX*, *fbp*) an increased availability of glucose-6-phosphate and fructose-6-phosphate is generated and may be utilized by the microbial cell for generating UDP-Gal and/or GDP-Fuc and/or UDP-GlcNAc and/or CMP-Neu5Ac.

Fig. 9 displays schematically an exemplary microbial cell of the invention being capable of generating high availability of glucose-6-phosphate and fructose-6-phosphate and phosphoenolpyruvate when cultivated on a mixed feedstock

consisting of glucose and fructose. The cell's phosphoenolpyruvate:sugar phosphotransferase systems for glucose and fructose have been disabled, e.g. by deletion of the *ptsG* and *fruA* genes, respectively. Instead, fructose and glucose enter the cell via a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter getting activated by a fructose kinase (e.g. *E. coli* W CscK) and glucose kinase (e.g. *E. coli* K-12 Glk), to fructose-6-phosphate and glucose-6-phosphate, respectively. Moreover, PEP consumption by the phosphoenolpyruvate:sugar phosphotransferase systems is circumvented. Along with the decreased and/or diminished expression of the phosphofructokinase(s) Pfk and/or the glucose-6-phosphate dehydrogenase Zwf, realized by deletion, functional inactivation or silencing of the *pfkA* and/or *pfkB* and/or *zwf* gene(s), increased availability of glucose-6-phosphate and fructose-6-phosphate and phosphoenolpyruvate is achieved. This may be utilized by the microbial cell for generating UDP-Gal and/or GDP-Fuc and/or UDP-GlcNAc and/or CMP-Neu5Ac.

Fig. 10 displays schematically another exemplary microbial cell of the invention capable of generating increased availability of free glucose and glucose-6-phosphate and fructose-6-phosphate when cultivated on a mixed feedstock consisting of glucose and fructose. The cell's phosphoenolpyruvate:sugar phosphotransferase systems for glucose and fructose have been disabled, e.g. by deletion of the *ptsG* and *fruA* genes, respectively. Instead, fructose and glucose enter the cell via a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter. Along with the deletion of the *glk* gene, the microbial cell has been genetically engineered such that free glucose monomer becomes available in the cell. Instead, fructose gets activated by a fructose kinase (e.g. *E. coli* W CscK) to fructose-6-phosphate. The expression of the phosphofructokinase(s) Pfk and/or the glucose-6-phosphate dehydrogenase Zwf have been decreased or diminished by deletion, functional inactivation or silencing of the *pfkA* and/or *pfkB* and/or *zwf* gene(s). Overall, this is yielding an improved intracellular supply of free glucose and glucose-6-phosphate and fructose-6-phosphate. Glucose becomes available as substrate for various glycosylation reactions, whereas glucose-6-phosphate and fructose-6-phosphate may be utilized by the microbial cell for generating UDP-Gal and/or GDP-Fuc and/or UDP-GlcNAc and/or CMP-Neu5Ac.

Additionally, PEP consumption by the phosphoenolpyruvate:sugar phosphotransferase systems is circumvented.

Fig. 11 displays schematically another exemplary microbial cell of the invention capable of achieving an increased intracellular availability of free mannose and/or mannose-6-phosphate when cultivated on a mixed feedstock consisting of glucose and fructose. The cell's phosphoenolpyruvate:sugar phosphotransferase systems for fructose have been disabled, e.g. by deletion of the *fruA* gene and/or the *manXYZ* genes. Instead, fructose enters the cell via a sugar permease and/or channel. Along with the deletion of genes exhibiting fructose kinase activity (e.g. *mak*) and/or mannose-6-phosphate isomerase activity (e.g. *manA*) as well as the expression/overexpression of a suitable mannose isomerase (ManI) and mannokinase (ManK), the microbial cell has been genetically engineered such that mannose-6-phosphate becomes available in the cell. This may be utilized by the microbial cell for generating GDP-Man and/or GDP-Fuc.

Fig. 12 displays schematically another exemplary microbial cell of the invention capable of generating a higher intracellular availability of free fructose-6-phosphate and/or galactose-1-phosphate when cultivated on a mixed feedstock consisting of glucose and fructose and galactose. The cell's phosphoenolpyruvate:sugar phosphotransferase systems for glucose has been disabled, e.g. by deletion of the *ptsG* gene. Instead, glucose enters the cell via a phosphoenolpyruvate:sugar phosphotransferase independent (non-PEP-PTS) transporter. The expression of the phosphofructokinase(s) Pfk have been decreased or diminished by deletion, functional inactivation or silencing of the *pfkA* and/or *pfkB* gene(s). Along with the expression/overexpression of a suitable non-PEP-PTS transporter as well as of a monosaccharide kinase, enabling galactose transfer into the engineered cell as well as its activation to galactose-1-phosphate, respectively, the microbial cell has been genetically engineered such that fructose-6-phosphate and galactose-1-phosphate become available in the cell. These may be utilized by the microbial cell for generating GlcNAc and/or GDP-Fuc and/or UDP-Gal and/or CMP-Neu5Ac.

According to the fourth aspect, provided is the use of the desired carbohydrates in a pharmaceutical and/or nutritional composition, wherein the carbohydrate of interest

is preferably produced by a method or genetically engineered host cell according to the invention.

Examples

Example 1 - Preparation of a mixed monosaccharide feedstock

A 50 % (w/v) sucrose solution was prepared by dissolving 500 g of sucrose in water. The final volume of the solution was 1 litre. At a temperature of 30°C to 35°C the pH was adjusted by using 96 % (v/v) sulfuric acid. Afterwards, the solution was sterilized in a vertical autoclave (Systec VX-65, Linden, Germany) at 121°C for 45 minutes. Samples were taken before and after heat sterilization and kept frozen prior to analysis by high performance liquid chromatography (HPLC). HPLC was carried out using a RID-10A refractive index detector (Shimadzu, Germany) and a Waters XBridge Amide Column 3.5 µm (250 x 4.6 mm) (Eschborn, Germany) connected to a Shimadzu HPLC system. Isocratic elution was carried out with 30% solvent A (50% (v/v) acetonitrile in double distilled water, 0.1% (v/v) NH₄OH) and 70% solvent B (80% (v/v) acetonitrile in double distilled water, 0.1% (v/v) NH₄OH) at 35°C and at a flow rate of 1.4 mL min⁻¹. Samples were cleared by solid phase extraction on an ion exchange matrix (Strata ABW, Phenomenex). Ten microliters of the sample (dilution of 1:5) was applied to the column. Finally, the relative amount of detected sugars was determined. As depicted in table 2, the sucrose conversion into the monosaccharides glucose and fructose increased with decreasing pH values of the solutions prior to heat treatment. Full sucrose cleavage could be observed at pH values ≤ 3.50 when acidification was carried out with sulfuric acid.

pH	Relative composition [%]		
	Sucrose	Glucose	Fructose
before heat sterilization			
3.50 - 7.10	100	-	-
after heat sterilization			
7.10 (not acidified)	100	-	-
pH 5.50	87.55	6.30	6.15
pH 5.05	68.25	16.62	15.13
pH 3.87	13.90	45.05	41.06
pH 3.50	-	51.68	48.32

Table 2: Relative amount of sugars detected in pH adjusted 50 % (w/v) sucrose solution before and after heat sterilization. The pH adjustment was carried out using 96 % (v/v) sulfuric acid. Depicted is the percental amount of sugars (area under the curve; AUC) detected by HPLC.

Example 2 - Feedstock-dependent growth of various gene deletion strains

The growth behavior of an *E. coli* BL21(DE3) strain (wild type) as well as the mutated strains *E. coli pfkA*⁻ ($\Delta pfkA$), *E. coli pfkB*⁻ ($\Delta pfkB$), *E. coli pfkA*⁻ *pfkB*⁻ ($\Delta pfkA \Delta pfkB$) was compared. Genomic deletions were performed according to the method of Datsenko and Wanner (Proc. Natl. Acad. Sci. USA 97:6640-6645 (2000)). All strains were cultivated at 30°C in 100 mL-shake flasks with 20 mL mineral salts medium, containing 7 g·L⁻¹ NH₄H₂PO₄, 7 g·L⁻¹ K₂HPO₄, 2 g·L⁻¹ KOH, 0.3 g·L⁻¹ citric acid, 2 g·L⁻¹ MgSO₄ × 7·H₂O and 0.015 g·L⁻¹ CaCl₂ × 6·H₂O, supplemented with 1 mL·L⁻¹ trace element solution (54.4 g·L⁻¹ ammonium ferric citrate, 9.8 g·L⁻¹ MnCl₂ × 4·H₂O, 1.6 g·L⁻¹ CoCl₂ × 6·H₂O, 1 g·L⁻¹ CuCl₂ × 2·H₂O, 1.9 g·L⁻¹ H₃BO₃, 9 g·L⁻¹ ZnSO₄ × 7·H₂O, 1.1 g·L⁻¹ Na₂MoO₄ × 2·H₂O, 1.5 g·L⁻¹ Na₂SeO₃, 1.5 g·L⁻¹ NiSO₄ × 6·H₂O) and containing either 2 % (m/v) glucose (A) or 1 % (w/v) glucose/1 % (w/v) fructose (B) as carbon source. Cultures were inoculated to OD 0.1 and growth development was monitored over 26 hours by OD₆₀₀ measurement. As shown in Fig. 2, *E. coli pfkA*⁻ *pfkB*⁻ hardly showed growth when glucose was provided as sole carbon- and energy source, whereas its growth was indistinguishable from the wild type strain as well as the single deletion mutants when the mixed monosaccharide feedstock was available.

Example 3 - Production of 2'-fucosyllactose by an engineered *E. coli* strain

An *E. coli* BL21 (DE3) strain exhibiting the genotype *lacY*⁺, *lacZ*⁻, *fucIK*⁻, *wcaJ*⁻, *pfkA*⁻, was further genetically engineered by overexpressing enzymes for the de novo synthesis of GDP-Fucose (ManB, ManC, Gmd, WcaG), the 2'-fucosyltransferase gene *wbgL* from *E. coli*:O126, the sugar efflux transporter yberc0001_9420 from *Yersinia bercovieri* ATCC 43970 and the *csc*-gene cluster of *E. coli* W (acc. no. CP002185.1) comprising the genes for sucrose permease, fructokinase, sucrose hydrolase as well as a transcriptional repressor (genes *cscB*, *cscK*, *cscA*, and *cscR*, respectively), enabling the strain to grow on sucrose as sole carbon source. Genomic deletions were performed according to the method of Datsenko and Wanner (Proc. Natl. Acad. Sci. USA 97:6640-6645 (2000)). Genomic integration of heterologous genes was performed by transposition. Either the EZ-Tn5TM transposase (Epicentre, USA) was used to integrate linear DNA-fragments or the hyperactive C9-mutant of the mariner transposase Himar1 (Proc. Natl. Acad. Sci. 1999, USA 96:11428-11433) was employed for transposition. The genes were codon-optimized for expression in *E. coli* and prepared synthetically by GenScript cooperation. The resulting strain was cultivated at 30°C in 100 mL-shake flasks with 20 mL mineral salts medium, containing 3 g/L KH₂PO₄, 12 g/L K₂HPO₄, 5 g/L (NH₄)₂SO₄, 0.3 g/L citric acid, 2 g/L MgSO₄ × 7H₂O, 0.1 g/L NaCl and 0.015 g/L CaCl₂ × 6H₂O with 1 mL/L trace element solution (54.4 g/L ammonium ferric citrate, 9.8 g/L MnCl₂ × 4H₂O, 1.6 g/L CoCl₂ × 6H₂O, 1 g/L CuCl₂ × 2H₂O, 1.9 g/L H₃BO₃, 9 g/L ZnSO₄ × 7H₂O, 1.1 g/L Na₂MoO₄ 2H₂O, 1.5 g/L Na₂SeO₃, 1.5 g/L NiSO₄ × 6H₂O), and containing 2 % (v/v) glycerol, 2 % (m/v) sterile-filtered sucrose or 2 % sucrose hydrolysate as carbon source. Additionally, 15 mM lactose were added, yielding as acceptor substrate for 2'-fucosyllactose production. Cultures were inoculated to OD 0.1 and cultivation was discontinued after 26 hours. For quantifying 2'-FL in the culture broth, HPLC analyses were performed using a refractive index detector (RID-10A) (Shimadzu, Germany) and a Waters XBridge Amide Column 3.5µm (250 x 4.6mm) (Eschborn, Germany) connected to an HPLC system (Shimadzu, Germany). Elution was performed isocratically with 30% A: 50% (v/v) ACN in ddH₂O, 0.1% (v/v) NH₄OH and 70% B: 80% (v/v) ACN in ddH₂O, 0.1% (v/v) NH₄OH (v/v) as eluent at 35°C and at a flow rate of 1.4 ml·min⁻¹. The culture supernatant was sterile filtered (0.22 µm pore size) and cleared by solid phase

extraction on an ion exchange matrix (Strata ABW, Phenomenex). 10 µl of the samples were applied to the column, and the 2'-fucosyllactose concentration was calculated according to a standard curve. The productivity of the engineered strain during growth on glycerol was set to 100 %. As depicted in table 3, 2'-FL production was highest when sucrose hydrolysate was provided as carbon- and energy source.

	Glycerol	Sucrose	Sucrose hydrolysate
Relative 2'-FL production	100 %	199 %	482 %

Table 3: Relative 2'-FL production of the *E. coli* strain described in example 3 during cultivation on glycerol, sucrose or sucrose hydrolysate as carbon- and energy source. The productivity on glycerol was set to 100 %.

Example 4 - Total fermentation of 2'-fucosyllactose by an engineered *E. coli* strain during growth on a mixed-monosaccharide feedstock

An *E. coli* BL21 (DE3) strain exhibiting the genotype *pfkA*⁻, *lacZ*⁻, *fucIK*⁻, *wcaJ*⁻, *glk*⁻, *gcd*⁻, *ptsG*⁻ was further genetically engineered by overexpressing enzymes for the de novo synthesis of GDP-Fucose (ManB, ManC, Gmd, WcaG), the 2'-fucosyltransferase gene *wbgL* from *E. coli*:O126, the sugar efflux transporter gene *yberc0001_9420* from *Yersinia bercovieri* ATCC 43970, the glucose facilitator gene *glf* from *Zymomonas mobilis*, the β-1,4-galactosyltransferase gene *galTpm1141* from *Pasteurella multocida* (GenBank: AEC04686) as well as the *E. coli* genes *galE* and *pgm*, encoding a UDP-glucose 4-epimerase and a phosphoglucomutase, respectively. Genomic deletions were performed according to the method of Datsenko and Wanner (Proc. Natl. Acad. Sci. USA 97:6640-6645 (2000)). Genomic integration of heterologous genes was performed by transposition. Either the EZ-Tn5TM transposase (Epicentre, USA) was used to integrate linear DNA-fragments or the hyperactive C9-mutant of the mariner transposase Himar1 (Proc. Natl. Acad. Sci. 1999, USA 96:11428-11433) was employed for transposition. The genes were codon-optimized for expression in *E. coli* and prepared synthetically by GenScript cooperation.

The resulting *E. coli* strain was cultivated at 30°C in a 3 L fermenter (New Brunswick, Edison, USA) starting with 1000 mL mineral salts medium containing 7 g·L⁻¹

$\text{NH}_4\text{H}_2\text{PO}_4$, $7 \text{ g} \cdot \text{L}^{-1}$ K_2HPO_4 , $2 \text{ g} \cdot \text{L}^{-1}$ KOH , $0.3 \text{ g} \cdot \text{L}^{-1}$ citric acid, $2 \text{ g} \cdot \text{L}^{-1}$ $\text{MgSO}_4 \times 7 \cdot \text{H}_2\text{O}$ and $0.015 \text{ g} \cdot \text{L}^{-1}$ $\text{CaCl}_2 \times 6 \cdot \text{H}_2\text{O}$, supplemented with $1 \text{ mL} \cdot \text{L}^{-1}$ trace element solution ($54.4 \text{ g} \cdot \text{L}^{-1}$ ammonium ferric citrate, $9.8 \text{ g} \cdot \text{L}^{-1}$ $\text{MnCl}_2 \times 4 \cdot \text{H}_2\text{O}$, $1.6 \text{ g} \cdot \text{L}^{-1}$ $\text{CoCl}_2 \times 6 \cdot \text{H}_2\text{O}$, $1 \text{ g} \cdot \text{L}^{-1}$ $\text{CuCl}_2 \times 2 \cdot \text{H}_2\text{O}$, $1.9 \text{ g} \cdot \text{L}^{-1}$ H_3BO_3 , $9 \text{ g} \cdot \text{L}^{-1}$ $\text{ZnSO}_4 \times 7 \cdot \text{H}_2\text{O}$, $1.1 \text{ g} \cdot \text{L}^{-1}$ $\text{Na}_2\text{MoO}_4 \times 2 \cdot \text{H}_2\text{O}$, $1.5 \text{ g} \cdot \text{L}^{-1}$ Na_2SeO_3 , $1.5 \text{ g} \cdot \text{L}^{-1}$ $\text{NiSO}_4 \times 6 \cdot \text{H}_2\text{O}$) and containing 2 % (m/v) hydrolyzed sucrose as carbon source. Cultivation was started with the addition of a 2.5 % (v/v) inoculum from a pre-culture grown in the same medium. The end of the batch phase was characterized by a rise in the dissolved oxygen level. A carbon feed consisting of fully hydrolyzed sucrose, supplemented with $2 \text{ g} \cdot \text{L}^{-1}$ $\text{MgSO}_4 \times 7 \cdot \text{H}_2\text{O}$, $0.015 \text{ g} \cdot \text{L}^{-1}$ $\text{CaCl}_2 \times 6 \cdot \text{H}_2\text{O}$ and $1 \text{ mL} \cdot \text{L}^{-1}$ trace element solution, was applied instantaneously after leaving the batch phase. A feeding rate of $12.0 - 15.0 \text{ mL} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ was applied, referring to the starting volume. Aeration was maintained at $3 \text{ L} \cdot \text{min}^{-1}$. Dissolved oxygen was maintained at 20–30 % saturation by controlling the rate of agitation. The pH was maintained at 6.7 by adding 25 % ammonia solution. Cultivation lasted for 86 hours and yielded substantial amounts of 2'-FL in the culture supernatant.

CLAIMS

1. A genetically engineered microbial cell for the production of a carbohydrate of interest, wherein the microbial cell possesses an increased intracellular availability of at least one sugar phosphate as compared to the wild-type cell, and produces the carbohydrate of interest when being cultivated in a culture medium comprising a mixed monosaccharide feedstock as main carbon and energy source, wherein said mixed monosaccharide feedstock consists of glucose and at least one additional monosaccharide selected from the group consisting of fructose and galactose.
2. The genetically engineered microbial cell according to claim 1, wherein the genetically engineered microbial cell further comprises an UDP-galactose biosynthesis pathway for intracellular formation of UDP-galactose.
3. The genetically engineered microbial cell according to claim 1 or 2, wherein the genetically engineered microbial cell further comprises a GDP-fucose biosynthesis pathway for intracellular formation of GDP-L-fucose.
4. The genetically engineered microbial cell according to any one of claims 1 to 3, wherein the genetically engineered microbial cell further comprises an UDP-N-acetylglucosamine biosynthesis pathway for intracellular formation of UDP-N-acetylglucosamine.
5. The genetically engineered microbial cell according to any one of claims 1 to 4, wherein the genetically engineered microbial cell further comprises an CMP-N-acetylneuraminic acid/CMP-sialic acid biosynthesis pathway for intracellular formation of CMP-N-acetylneuraminic acid.
6. The genetically engineered microbial cell according to any one of claims 1 to 5, wherein the genetically engineered microbial cell further comprises at least one glycosyltransferase.

7. The genetically engineered microbial cell according to any one of claims 1 to 6, wherein the genetically engineered microbial cell further comprises an enhanced synthesis of phosphoenolpyruvate.
8. The genetically engineered microbial cell according to any one of claims 1 to 7, wherein the genetically engineered microbial cell further comprises at least one monosaccharide transporter for translocating a monosaccharide from the medium into the cytoplasm of the microbial cell.
9. The genetically engineered microbial cell according to any one of claims 1 to 8, wherein the genetically engineered microbial cell further comprises a decreased or diminished expression of at least one gene encoding for a protein competing with the biosynthesis of the desired carbohydrate and/or decreased or diminished activity of at least one protein competing with the biosynthesis of the carbohydrate of interest.
10. The genetically engineered microbial cell according to any one of claims 1 to 9, wherein the protein leading to a consumption of said intracellular sugar phosphate within said engineered microorganism is selected from the group consisting of phosphofructokinase, glucose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase, components of a phosphoenolpyruvate:sugar phosphotransferase system.
11. The genetically engineered microbial cell according to any one of claims 1 to 10, wherein the genetically engineered microbial cell further comprises at least one exporter protein or permease exporting the carbohydrate of interest from the cell.
12. Use of a genetically engineered microbial cell according to any one of claims 1 to 11 for the production of a carbohydrate of interest.

13. A method for the fermentative production of a carbohydrate of interest, the method comprising:
 - a) providing a genetically engineered microorganism capable of producing a desired carbohydrate, wherein said microorganism is exhibiting an increased intracellular availability of at least one sugar phosphate due to a decreased and/or diminished expression and/or activity of at least one protein leading to a consumption of said intracellular sugar phosphate within said engineered microorganism;
 - b) cultivating said genetically engineered microorganism in a culture medium permissive for the production of said desired carbohydrate, wherein the main carbon source is a monosaccharide mixture consisting of glucose and at least a second monosaccharide of the group of fructose and galactose; and
 - c) recovering said carbohydrate of interest.

14. The method according to claim 13, wherein the carbohydrate of interest is selected from the group consisting of mannose, fucose, *N*-acetylglucosamine, *N*-acetylmannosamine, *N*-acetylneuraminic acid, lacto-*N*-biose, *N*-acetyl-lactosamine, 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, blood group H antigen type I, blood group H antigen type II, blood group antigen Lewis^a, blood group antigen Lewis^b, blood group antigen Lewis^x, blood group antigen Lewis^y, blood group antigen Sialyl-Lewis^x, lacto-*N*-triose II, lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-fucopentaose I, lacto-*N*-neofucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N*-neofucopentaose V, lacto-*N*-difucohexaose I, lacto-*N*-difucohexaose II, 6'-galactosyllactose, 3'-galactosyllactose, lacto-*N*-hexaose, lacto-*N*-neohexaose, *para*-lacto-*N*-hexaose, *para*-Lacto-*N*-neohexaose, difucosyl-lacto-*N*-neohexaose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-*N*-acetyllactosamine, 6'-sialyl-*N*-acetyllactosamine, lacto-*N*-sialylpentaose a, lacto-*N*-sialylpentaose b, lacto-*N*-sialylpentaose c, fucosyl-lacto-*N*-sialylpentaose a, fucosyl-lacto-*N*-sialylpentaose b, fucosyl-lacto-*N*-sialylpentaose c, disialyl-lacto-*N*-tetraose, disialyl-lacto-*N*-fucopentaose, 3-fucosyl-3'-sialyllactose, 3-fucosyl-6'-sialyllactose, lacto-*N*-neodifucohexaose I.

15. Use of a carbohydrate of interest being produced by a microbial cell according to any one of claims 1 to 11 and/or a method according to any one of claims 13 to 14 for the manufacturing of a pharmaceutical and/or nutritional composition.