

Production of 3-fucosyllactose and lactose converting α -1,3-fucosyltransferase enzymes

The present invention relates to methods for producing 3-fucosyllactose (3-FL) as well as newly identified fucosyltransferases, more specifically newly identified lactose binding α -1,3-fucosyltransferase polypeptides, and their applications. Furthermore, the present invention provides methods for producing 3-fucosyllactose (3-FL) using the newly identified lactose binding α -1,3-fucosyltransferases.

Background

Today, more than 80 compounds belonging to the family of Human Milk Oligosaccharides (HMOs), have been structurally characterized. These HMOs represent a class of complex oligosaccharides that function as prebiotics. Additionally, the structural homology of HMO to epithelial epitopes accounts for protective properties against bacterial pathogens. Within the infant gastrointestinal tract, HMOs selectively nourish the growth of selected bacterial strains and are, thus, priming the development of a unique gut microbiota in breast milk-fed infants.

Some of these Human Milk oligosaccharides require the presence of particular fucosylated structures which most likely exhibit a particular biological activity. Production of these fucosylated oligosaccharides requires the action of a fucosyltransferase. Such fucosyltransferases, which belong to enzyme family of glycosyltransferases, are widely expressed in vertebrates, invertebrates, plants, fungi, yeasts and bacteria. They catalyze the transfer of a fucose residue from a donor, generally guanosine-diphosphate fucose (GDP-fucose) to an acceptor, which include oligosaccharides, (glyco)proteins and (glyco)lipids. The thus fucosylated acceptor substrates are involved in a variety of biological and pathological processes.

Several fucosyltransferases have been identified, e.g. in the bacteria *Helicobacter pylori*, *Escherichia coli*, *Salmonella enterica*, in mammals, *Caenorhabditis elegans* and *Schistosoma mansoni*, as well as in plants.

Fucosyltransferases are classified based on the site of fucose addition into for example α -1,2, α -1,3, α -1,4 and O-fucosyltransferases.

Several α -1,3-fucosyltransferases are already described in the art. WO 1998/055630 describes a bacterial α -1,3-fucosyltransferase gene of *Helicobacter pylori* which can be used in the production of oligosaccharides such as Lewis X, Lewis Y, and sialyl Lewis X. WO 2016/040531 describes several α -1,3-fucosyltransferases for the production of fucosylated oligosaccharides. Here, α -1,3-fucosyltransferases are described with 25 to 100% sequence identity to the *Bacteroides nordii* CafC enzyme. However, in table 1 of that filing, the authors clearly show that over half (7 out of 12) of their tested enzymes, many of which with > 25% sequence identity to CafC, are unable to produce 3-fucosyllactose using lactose as the acceptor substrate. This illustrates that clearly not all hypothetical fucosyltransferases indeed have lactose

binding fucosyltransferase activity. WO2012/049083 describes some new alpha-1,3-fucosyltransferases and their use for the production of fucosylated products. Huang *et al.* 2017 did a comparison of various exogenous alpha-1,3-fucosyltransferase candidates, as well as a series of *E. coli* host strains, and demonstrated that futA from *Helicobacter pylori* using *E. coli* BL21(DE3) as the host strain yielded the highest titers of 3-fucosyllactose, one of the Human Milk Oligosaccharides.

In general, alpha-1,3-fucosyltransferases, also known as 3-fucosyltransferases or 3-fucosyltransferase enzymes are known to have low affinity for lactose. A 3-fucosyltransferase is needed for the production of the HMO 3-fucosyllactose. The low affinity has a negative effect on the productivity of 3-fucosyllactose. In order to improve conversion rates and productivity, there is need for transferases with sufficient lactose affinity, preferably higher lactose affinity.

Thus, it is an object of the present invention to provide for tools and methods by means of which 3-fucosyllactose can be produced or synthesized in an efficient, time and cost-effective way and which yields similar or higher amounts of the desired product compared to state of the art methods.

Description

Surprisingly, it has now been found that the newly identified lactose binding alpha-1,3-fucosyltransferase enzymes of the present invention provide for transferases with similar or higher lactose binding and/or transferase properties than the presently known lactose binding alpha-1,3-fucosyltransferase enzymes.

The invention therefore provides methods for producing 3-fucosyllactose (3FL) using the newly identified lactose binding alpha-1,3-fucosyltransferases. The 3FL can be obtained by reacting lactose in the presence of alpha-1,3-fucosyltransferase, capable of catalysing the formation of the 3-fucosyllactose oligosaccharides from lactose and GDP-fucose. Alternatively, it can also be obtained from a microorganism producing an alpha-1,3-fucosyltransferase according to the present invention.

Definitions

The words used in this specification to describe the invention and its various embodiments are to be understood not only in the sense of their commonly defined meanings, but to include by special definition in this specification structure, material or acts beyond the scope of the commonly defined meanings. Thus, if an element can be understood in the context of this specification as including more than one meaning, then its use in a claim must be understood as being generic to all possible meanings supported by the specification and by the word itself.

The various embodiments and aspects of embodiments of the invention disclosed herein are to be understood not only in the order and context specifically described in this specification, but to

include any order and any combination thereof. Whenever the context requires, all words used in the singular number shall be deemed to include the plural and vice versa. Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization described herein are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications.

10 In the drawings and specification, there have been disclosed embodiments of the invention, and although specific terms are employed, the terms are used in a descriptive sense only and not for purposes of limitation, the scope of the invention being set forth in the following claims. It must be understood that the illustrated embodiments have been set forth only for the purposes of example and that it should not be taken as limiting the invention. It will be apparent to those skilled in the art that alterations, other embodiments, improvements, details and uses can be made consistent with the letter and spirit of the disclosure herein and within the scope of this disclosure, which is limited only by the claims, construed in accordance with the patent law, including the doctrine of equivalents. In the claims which follow, reference characters used to designate claim steps are provided for convenience of description only, and are not intended to imply any particular order for performing the steps.

20 According to the present invention, the term "polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotide(s)" include, 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. In addition, "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotide(s)" according to the present invention. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, are to be understood to be covered by the term "polynucleotides". It will be appreciated that a great variety of

modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells. The term "polynucleotide(s)" also embraces short polynucleotides often referred to as oligonucleotide(s).

"Polypeptide(s)" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" refers to both short chains, commonly referred to as peptides, oligopeptides and oligomers and to longer chains generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to the skilled person. The same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Furthermore, a given polypeptide may contain many types of modifications. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

"Isolated" means altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Similarly, a "synthetic" sequence, as the term is used herein, means any sequence that has been generated synthetically and not directly isolated from

a natural source. "Synthesized", as the term is used herein, means any synthetically generated sequence and not directly isolated from a natural source.

"Recombinant" means genetically engineered DNA prepared by transplanting or splicing genes from one species into the cells of a host organism of a different species. Such DNA becomes part of the host's genetic makeup and is replicated. "Mutant" cell or microorganism as used within the context of the present disclosure refers to a cell or microorganism which is genetically engineered or has an altered genetic make-up.

The terms "cell genetically modified for the production of 3-fucosyllactose" within the context of the present disclosure refers to a cell of a microorganism which is genetically manipulated to comprise at least one of i) a recombinant gene encoding an α 1,3 fucosyltransferase necessary for the synthesis of said 3-fucosyllactose, ii) a biosynthetic pathway to produce a GDP-fucose suitable to be transferred by said fucosyltransferase to lactose, and/or iii) a biosynthetic pathway to produce lactose or a mechanism of internalization of lactose from the culture medium into the cell where it is fucosylated to produce the 3-fucosyllactose.

The terms "nucleic acid sequence coding for an enzyme for 3-fucosyllactose synthesis" relates to nucleic acid sequences coding for enzymes necessary in the synthesis pathway to 3-fucosyllactose, e.g. an enzyme able to transfer the fucose moiety of a GDP-fucose donor substrate onto the 3 hydroxyl group of the galactose moiety of lactose and thus producing 3-fucosyllactose.

The term "endogenous," within the context of the present disclosure refers to any polynucleotide, polypeptide or protein sequence which is a natural part of a cell and is occurring at its natural location in the cell chromosome. The term "exogenous" refers to any polynucleotide, polypeptide or protein sequence which originates from outside the cell under study and not a natural part of the cell or which is not occurring at its natural location in the cell chromosome or plasmid.

The term "heterologous" when used in reference to a polynucleotide, gene, nucleic acid, polypeptide, or enzyme refers to a polynucleotide, gene, nucleic acid, polypeptide, or enzyme that is from a source or derived from a source other than the host organism species. In contrast a "homologous" polynucleotide, gene, nucleic acid, polypeptide, or enzyme is used herein to denote a polynucleotide, gene, nucleic acid, polypeptide, or enzyme that is derived from the host organism species. When referring to a gene regulatory sequence or to an auxiliary nucleic acid sequence used for maintaining or manipulating a gene sequence (e.g. a promoter, a 5' untranslated region, 3' untranslated region, poly A addition sequence, intron sequence, splice site, ribosome binding site, internal ribosome entry sequence, genome homology region, recombination site, etc.), "heterologous" means that the regulatory sequence or auxiliary sequence is not naturally associated with the gene with which the regulatory or auxiliary nucleic acid sequence is juxtaposed in a construct, genome, chromosome, or episome. Thus, a promoter operably linked to a gene to which it is not operably linked to in its natural state (i.e. in the genome

of a non-genetically engineered organism) is referred to herein as a "heterologous promoter," even though the promoter may be derived from the same species (or, in some cases, the same organism) as the gene to which it is linked.

The term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides that include a sequence encoding a polypeptide of the invention, particularly an α -1,3-fucosyltransferase having the amino acid sequence as set forth in SEQ ID NOs 2, 4, 6, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing. For sake of clarity, also the polynucleotide encoding the polypeptides of SEQ ID NO 18, 24 and 26 is a polynucleotide encompassed by the definition, but the polynucleotide of SEQ ID NO 18 is a prior art α -1,3-fucosyltransferase used as a reference and the polynucleotides of SEQ ID NO 24 and 26 are α -1,3-fucosyltransferase enzymes that are non-functional towards lactose as acceptor. 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.

"Variant(s)" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively but retains essential properties. 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. In some embodiments, the present disclosure contemplates making functional variants by modifying the structure of a membrane protein as used in the present invention. Variants can be produced by amino acid substitution, deletion, addition, or combinations thereof. For instance, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g., conservative mutations) will not have a major effect on the biological activity of the resulting

molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Whether a change in the amino acid sequence of a polypeptide of the disclosure results in a functional homolog can be readily determined by assessing the ability of the variant polypeptide to produce a response in cells in a fashion similar to the wild-type polypeptide, an in case of the present invention to provide better yield, productivity, and/or growth speed than a cell without the variant.

The term "functional homolog" as used herein describes those molecules that have sequence similarity and also share at least one functional characteristic such as a biochemical activity. Functional homologs will typically give rise to the same characteristics to a similar, but not necessarily the same, degree. Functionally homologous proteins give the same characteristics where the quantitative measurement produced by one homolog is at least 10 percent of the other; more typically, at least 20 percent, between about 30 percent and about 40 percent; for example, between about 50 percent and about 60 percent; between about 70 percent and about 80 percent; or between about 90 percent and about 95 percent; between about 98 percent and about 100 percent, or greater than 100 percent of that produced by the original molecule. Thus, where the molecule has enzymatic activity the functional homolog will have the above-recited percent enzymatic activities compared to the original enzyme. Where the molecule is a DNA-binding molecule (e.g., a polypeptide) the homolog will have the above-recited percentage of binding affinity as measured by weight of bound molecule compared to the original molecule.

A functional homolog and the reference polypeptide may be naturally occurring polypeptides, and the sequence similarity may be due to convergent or divergent evolutionary events. Functional homologs are sometimes referred to as orthologs, where "ortholog", refers to a homologous gene or protein that is the functional equivalent of the referenced gene or protein in another species. Functional homologs can be identified by analysis of nucleotide and polypeptide sequence alignments. For example, performing a query on a database of nucleotide or polypeptide sequences can identify homologs of biomass-modulating polypeptides. Sequence analysis can involve BLAST, Reciprocal BLAST, or PSI-BLAST analysis of non-redundant databases using amino acid sequence of a biomass-modulating polypeptide as the reference sequence. Amino acid sequence is, in some instances, deduced from the nucleotide sequence. Typically, those polypeptides in the database that have greater than 40 percent sequence identity are candidates for further evaluation for suitability as a biomass-modulating polypeptide. Amino acid sequence similarity allows for conservative amino acid substitutions, such as substitution of one hydrophobic residue for another or substitution of one polar residue for another. If desired, manual inspection of such candidates can be carried out in order to narrow the number of candidates to be further evaluated. Manual inspection can be performed by selecting those candidates that appear to have domains present in productivity-modulating polypeptides, e.g., conserved functional domains.

"Fragment", with respect to a polynucleotide, refers to a clone or any part of a polynucleotide molecule, particularly a part of a polynucleotide that retains a usable, functional characteristic. Useful fragments include oligonucleotides and polynucleotides that may be used in hybridization or amplification technologies or in the regulation of replication, transcription or translation. A

5 "polynucleotide fragment" refers to any subsequence of a polynucleotide, typically, of at least about 9 consecutive nucleotides, for example at least about 30 nucleotides or at least about 50 nucleotides of any of the sequences provided herein. Exemplary fragments can additionally or alternatively include fragments that comprise, consist essentially of, or consist of a region that encodes a conserved family domain of a polypeptide. Exemplary fragments can additionally or
10 alternatively include fragments that comprise a conserved domain of a polypeptide.

Fragments may additionally or alternatively include subsequences of polypeptides and protein molecules, or a subsequence of the polypeptide. In some cases, the fragment or domain is a subsequence of the polypeptide which performs at least one biological function of the intact polypeptide in substantially the same manner, or to a similar extent, as does the intact
15 polypeptide. For example, a polypeptide fragment can comprise a recognizable structural motif or functional domain such as a DNA-binding site or domain that binds to a DNA promoter region, an activation domain, or a domain for protein-protein interactions, and may initiate transcription. Fragments can vary in size from as few as 3 amino acid residues to the full length of the intact polypeptide, for example at least about 20 amino acid residues in length, for example at least
20 about 30 amino acid residues in length. Preferentially a fragment is a functional fragment that has at least one property or activity of the polypeptide from which it is derived, such as, for example, the fragment can include a functional domain or conserved domain of a polypeptide. A domain can be characterized, for example, by a Pfam or Conserved Domain Database (CDD) designation.

25 The terms "alpha-1,3-fucosyltransferase", "alpha 1,3 fucosyltransferase", "3-fucosyltransferase", "α-1,3-fucosyltransferase", "α 1,3 fucosyltransferase", "3 fucosyltransferase", "3-FT" or "3FT" as used in the present invention, are used interchangeably and refer to a glycosyltransferase that catalyses the transfer of fucose from the donor substrate GDP-L-fucose, to the acceptor molecule lactose in an alpha-1,3-linkage. A polynucleotide encoding an "alpha-1,3-fucosyltransferase" or
30 any of the above terms, refers to a polynucleotide encoding such glycosyltransferase that catalyses the transfer of fucose from the donor substrate GDP-L-fucose, to the acceptor molecule lactose in an alpha-1,3-linkage.

The terms "3-fucosyllactose", "alpha-1,3-fucosyllactose", "alpha 1,3 fucosyllactose", "α-1,3-fucosyllactose", "α 1,3 fucosyllactose", "Galβ-4(Fucα1-3)Glc", "3FL" or "3-FL" as used in the
35 present invention, are used interchangeably and refer to the product obtained by the catalysis of the alpha-1,3-fucosyltransferase transferring the fucose residue from GDP-L-fucose to lactose in an alpha-1,3-linkage.

"Oligosaccharide" as the term is used herein and as generally understood in the state of the art, refers to a saccharide polymer containing a small number, typically three to ten, of simple sugars, i.e. monosaccharides.

The term "purified" refers to material that is substantially or essentially free from components which interfere with the activity of the biological molecule. For cells, saccharides, nucleic acids, and polypeptides, the term "purified" refers to material that is substantially or essentially free from components which normally accompany the material as found in its native state. Typically, purified saccharides, oligosaccharides, proteins or nucleic acids of the invention are at least about 50 %, 55 %, 60 %, 65 %, 70 %, 75 %, 80 % or 85 % pure, usually at least about 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, or 99 % pure as measured by band intensity on a silver stained gel or other method for determining purity. Purity or homogeneity can be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein or nucleic acid sample, followed by visualization upon staining. For certain purposes high resolution will be needed and HPLC or a similar means for purification utilized. For oligosaccharides, e.g., 3-fucosyllactose, purity can be determined using methods such as but not limited to thin layer chromatography, gas chromatography, NMR, HPLC, capillary electrophoresis or mass spectroscopy.

The terms "identical" or percent "identity" or % "identity" in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using sequence comparison algorithms or by visual inspection. For sequence comparison, one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are inputted into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. Percent identity can be determined using BLAST and PSI-BLAST (Altschul *et al.*, 1990, J Mol Biol 215:3, 403- 410; Altschul *et al.*, 1997, Nucleic Acids Res 25: 17, 3389-402). For the purposes of this invention, percent identity is determined using MatGAT2.01 (Campanella *et al.*, 2003, BMC Bioinformatics 4:29). MatGAT utilizes a Myers and Miller global alignment algorithm for conducting pairwise alignments. The following default parameters for protein are employed: (1) Gap cost Existence: 12 and Extension: 2; (2) The Matrix employed was BLOSUM50.

The term "control sequences" refers to sequences recognized by the host cells transcriptional and translational systems, allowing transcription and translation of a polynucleotide sequence to a polypeptide. Such DNA sequences are thus necessary for the expression of an operably linked coding sequence in a particular host cell or organism. Such control sequences can be, but are

not limited to, promoter sequences, ribosome binding sequences, Shine Dalgarno sequences, Kozak sequences, transcription terminator sequences. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers. DNA for a presequence or secretory leader may be operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Said control sequences can furthermore be controlled with external chemicals, such as, but not limited to, IPTG, arabinose, lactose, allo-lactose, rhamnose or fucose via an inducible promoter or via a genetic circuit that either induces or represses the transcription or translation of said polynucleotide to a polypeptide.

The term "end of fermentation" as used in the present invention refers to the time at which a fermentation is harvested for product purification.

Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

Detailed description of the invention

According to a first embodiment, the present invention provides a method for producing α -1,3-fucosyllactose. The method comprising the steps of:

- a) providing a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate wherein said polypeptide comprises
 - i) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);
 - ii) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), and
 - iii) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of ii) equals DM[A/S]VSF (SEQ ID NO 36);wherein X can be any distinct amino acid; and wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain;
- b) contacting the polypeptide with α -1,3-fucosyltransferase activity of step a) with a mixture comprising GDP-fucose as donor substrate, and lactose as acceptor substrate, under conditions where the polypeptide catalyses the transfer of a fucose residue from the donor substrate to the acceptor substrate, thereby producing α -1,3-fucosyllactose.

These newly identified polypeptides comprising both (or all of SEQ ID NO 33 to 36, as the case may be) of the above domains provide for an alternative α -1,3-fucosyltransferase having the ability to use lactose as acceptor substrate over the presently known α -1,3-fucosyltransferases. Polypeptides comprising both (or all of SEQ ID NO 33 to 36, as the case may be) of the above domains provide for transferases with similar or higher lactose binding and/or similar or higher transferase properties than presently known α -1,3-fucosyltransferases.

In a first preferred embodiment of the present invention, a polypeptide useful in the present invention comprises both (or all of SEQ ID NO 33 to 36, as the case may be) of the domains with SEQ ID NO 33 to 34 or 36 and wherein said SEQ ID NO 33 is a conserved domain with amino acid sequence YXTEK (SEQ ID NO: 37), wherein X can be any distinct amino acid.

In a second preferred embodiment of the present invention, a polypeptide useful in the present invention comprises both (or all of SEQ ID NO 33 to 36, as the case may be) of the domains with SEQ ID NO 33 to 34 or 36 and wherein said SEQ ID NO 34 is a conserved domain with amino acid sequence [K/D]LX[I/L/M]G[F/Y] (SEQ ID NO: 38), [K/D][L/K]xL[S/G][F/Y] (SEQ ID NO: 39), or [K/D]LXLG[F/Y] (SEQ ID NO: 40), wherein X can be any distinct amino acid.

A further advantage of using some of the polypeptides newly identified to have the ability to use lactose as acceptor substrate and having α -1,3-fucosyltransferase activity and with the newly identified domains resides in the fact that 3-fucosyllactose is produced with a higher purity, than the purity obtained with a reference prior art polypeptide with SEQ ID NO 18, at the end of reaction or fermentation due to a better conversion ability of the newly identified 3-fucosyltransferases to use lactose for 3FL production. More specifically, the lactose concentration to 3-fucosyllactose concentration ratio is smaller than 1:5, preferably smaller than 1:10, more preferably smaller than 1:20, optimally smaller than 1:40. In another preferred embodiment, the 3-fucosyllactose purity is 80% or higher at the end of fermentation.

According to the invention, the method for producing α -1,3-fucosyllactose may be performed in a cell-free system or in a system containing cells. The substrates GDP-fucose and lactose are allowed to react with the α -1,3-fucosyltransferase polypeptide for a sufficient time and under sufficient conditions to allow formation of the enzymatic product. These conditions will vary depending upon the amounts and purity of the substrate and enzyme, and whether the system is a cell-free or cellular based system. These variables will be easily adjusted by those skilled in the art.

In cell-free systems, the polypeptide according to the invention, the acceptor substrate(s), donor substrate(s) and, as the case may be, other reaction mixture ingredients, including other glycosyltransferases and accessory enzymes are combined by admixture in an aqueous reaction medium for performing the enzymatic reaction. The enzymes can be utilized free in solution, or they can be bound or immobilized to a support such as a polymer and the substrates may be added to the support. The support may be, e.g., packed in a column.

Cell containing systems or cellular based systems for the synthesis of 3-fucosyllactose as described herein may include genetically modified host cells. According to one aspect of the invention the polypeptide with α -1,3-fucosyltransferase activity is produced by a cell producing the polypeptide, e.g. a host cell as described herein. According to another aspect of the invention, the GDP-fucose and/or lactose is provided by a cell producing said GDP-fucose and/or lactose. The cell can be the host cell which is also producing the α -1,3-fucosyltransferase. Alternatively, the cell can be another cell than the host cell producing the α -1,3-fucosyltransferase, in which case the skilled person would talk about cell coupling. Such cell producing GDP-fucose can express an enzyme converting, e.g., fucose, which is to be added to the host cell, to GDP-fucose. This enzyme may be, e.g., a bifunctional fucose kinase/fucose-1-phosphate guanylyltransferase, like Fkp from *Bacteroides fragilis*, or the combination of one separate fucose kinase together with one separate fucose-1-phosphate guanylyltransferase like they are known from several species including *Homo sapiens*, *Sus scrofa* and *Rattus norvegicus*.

In another embodiment, the invention relates to a method for producing α -1,3- fucosyllactose, comprising the following steps:

i) providing a cell genetically modified for the production of α -1,3-fucosyllactose, said cell comprising at least one nucleic acid sequence coding for an enzyme for α -1,3-fucosyllactose synthesis, said cell comprising the expression of a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate, wherein said polypeptide comprises:

a) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);

b) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), and

c) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of b) equals DM[A/S]VSF (SEQ ID NO 36);

wherein X can be any distinct amino acid; and

wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain, and

ii) cultivating the cell in a medium under conditions permissive for the production of α -1,3-fucosyllactose, thereby producing 3-FL.

In a further embodiment, the invention relates to a method for producing α -1,3-fucosyllactose the method comprising the steps of:

a) providing a host cell expressing said polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate, as defined herein;

b) growing, under suitable nutrient conditions permissive for the production of the α -1,3-fucosyllactose, and permissive for the expression of said polypeptide with α -1,3-fucosyltransferase activity, said host cell;

c) providing simultaneously or subsequently to step b) a donor substrate GDP-fucose and the acceptor substrate lactose, in order for the α -1,3-fucosyltransferase polypeptide to catalyse the transfer of a fucose residue from GDP-fucose to lactose, thereby producing α -1,3-fucosyllactose. Optionally the produced 3FL is then separated from the host cell and/or the medium of its growth. According to yet another embodiment, the production of said 3-fucosyllactose in the methods as described herein is performed by means of a heterologous or homologous (over)expression of the polynucleotide encoding the α -1,3-fucosyltransferase by the cell.

In the methods of the invention as described herein the host cell can be transformed or transfected to express an exogenous polypeptide as described herein and with α -1,3-fucosyltransferase activity and with the ability to use lactose as an acceptor substrate. As such, the invention relates to a method for producing α -1,3-fucosyllactose using a host cell, comprising the following steps:

a) growing, a host cell transformed or transfected to express an exogenous polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as an acceptor substrate, wherein the polypeptide is set forth herein; and
b) providing, simultaneously or subsequently to step a), a donor substrate GDP-fucose and an acceptor substrate lactose, wherein the α -1,3-fucosyltransferase polypeptide catalyzes the transfer of a fucose residue from the donor substrate to the acceptor substrate, thereby producing α -1,3-fucosyllactose.

Preferably the exogenous polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as an acceptor substrate as used herein, produces 3FL with a lactose concentration to 3FL concentration ratio at the end of fermentation smaller than 1:5.

The ratio concentration lactose to concentration 3FL can be less than 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, 1:25, 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70, 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, 1:110, 1:120, 1:130, 1:140, 1:150, 1:160, 1:170, 1:180, 1:190, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1000.

In a preferred embodiment the ratio lactose concentration on 3FL concentration of lower than 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, 1:25, 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70, 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, 1:110, 1:120, 1:130, 1:140, 1:150, 1:160, 1:170, 1:180, 1:190, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1000 is obtained within a production process resulting in a final lactose concentration of lower than 25

g/L, 20 g/L, 15 g/L, 10g/L, 9g/L, 8g/L, 7 g/L, 6 g/L, 5 g/L, 4 g/L, 3 g/L, 2 g/L, 1 g/L, 0.5 g/L, 0.25 g/L, 0.1 g/L or 0 g/L.

In another embodiment the ratio lactose concentration on 3FL concentration of lower than 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, 1:25, 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70, 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, 1:110, 1:120, 1:130, 1:140, 1:150, 1:160, 1:170, 1:180; 1:190, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1000 is obtained within a production process wherein the lactose concentration is fed at substrate limiting conditions, wherein the substrate limitation is defined as the concentration in the bioreactor that determines the rate of conversion of the substrate.

In another embodiment the ratio lactose concentration on 3FL concentration of lower than 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, 1:20, 1:21, 1:22, 1:23, 1:24, 1:25, 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:45, 1:50, 1:55, 1:60, 1:65, 1:70, 1:75, 1:80, 1:85, 1:90, 1:95, 1:100, 1:110, 1:120, 1:130, 1:140, 1:150, 1:160, 1:170, 1:180; 1:190, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1000 is obtained within a production process wherein the lactose is formed in the cell at rate limiting conditions.

In another embodiment, the 3-fucosyllactose purity in the broth is higher than about 80%, such as 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 95,5%, 96%, 96,5%, 97%, 97,5%, 98%, 98,5%, 99%, 99,5%, 99,6%, 99,7%, 99,8%, 99,9% on sum of (lactose and 3FL) in broth. As used herein, the 3-fucosyllactose purity is defined as the ratio of the 3FL concentration to the sum of the 3FL concentration and the lactose concentration ($[\text{3FL}] / ([\text{3FL}] + [\text{lactose}])$).

According to the invention, the GDP-fucose and/or lactose can be fed to the host cell in the fermentation medium or aqueous culture medium. Alternatively, the GDP-fucose and/or lactose can be provided by an enzyme simultaneously expressed in the host cell or by the metabolism of the host cell. Accordingly, the host cell will also produce the α -1,3-fucosyltransferase next to the GDP-fucose and/or lactose. In another embodiment, the GDP-fucose and/or lactose can be produced by a cell which is another cell than the host cell producing the α -1,3-fucosyltransferase, in which case the skilled person would talk about cell coupling. Such cell producing GDP-fucose can express an enzyme converting, e.g., fucose, which is to be added to the host cell, to GDP-fucose. This enzyme may be, e.g., a bifunctional fucose kinase/fucose-1-phosphate guanylyltransferase, like Fkp from *Bacteroides fragilis*, or the combination of one separate fucose kinase together with one separate fucose-1-phosphate guanylyltransferase like they are known from several species including *Homo sapiens*, *Sus scrofa* and *Rattus norvegicus*.

According to yet another embodiment, the production of said α -1,3-fucosyllactose is performed by means a host cell as described herein comprising a heterologous or homologous (over)expression of the polynucleotide encoding the α -1,3-fucosyltransferase.

In a further aspect, the present invention provides for a method for producing α -1,3- fucosyllactose
5 as described herein, wherein the method further comprises a step of separating the α -1,3-fucosyllactose from the host cell or the medium of its growth.

As used herein, the term "separating" means harvesting, collecting or retrieving from the reaction mixture and/or from the cell producing the α -1,3-fucosyltransferase, the α -1,3-fucosyllactose produced by the α -1,3-fucosyltransferase according to the invention.

10 In case α -1,3-fucosyllactose is made by use of cells or fermentation, the 3-FL can be separated in a conventional manner from the aqueous culture medium, in which the mixture was made. In case the alpha-1,3-fucosyllactose is still present in the cells producing the α -1,3-fucosyllactose, conventional manners to free or to extract the α -1,3-fucosyllactose out of the cells can be used, such as cell destruction using high pH, heat shock, sonication, French press, homogenisation,
15 enzymatic hydrolysis, chemical hydrolysis, solvent hydrolysis, detergent, hydrolysis,... The culture medium, reaction mixture and/or cell extract, together and separately called 3-FL containing mixture, can then be further used for separating the 3-FL. This preferably involves clarifying the 3-FL containing mixtures to remove suspended particulates and contaminants, particularly cells, cell components, insoluble metabolites and debris produced by culturing the
20 genetically modified cell and/or performing the enzymatic reaction. In this step, the 3-FL containing mixture can be clarified in a conventional manner. Preferably, the 3-FL containing mixture is clarified by centrifugation, flocculation, decantation and/or filtration. A second step of separating the 3-FL from the 3-FL containing mixture preferably involves removing substantially all the proteins, as well as peptides, amino acids, RNA and DNA and any endotoxins and
25 glycolipids that could interfere with the subsequent separation step, from the 3-FL containing mixture, preferably after it has been clarified. In this step, proteins and related impurities can be removed from the 3-FL containing mixture in a conventional manner. Preferably, proteins, salts, byproducts, colour and other related impurities are removed from the 3-FL containing mixture by ultrafiltration, nanofiltration, reverse osmosis, microfiltration, activated charcoal or carbon
30 treatment, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography (such as but not limited to cation exchange, anion exchange, mixed bed ion exchange), hydrophobic interaction chromatography and/or gel filtration (i.e., size exclusion chromatography), particularly by chromatography, more particularly by ion exchange chromatography or hydrophobic interaction chromatography or ligand exchange
35 chromatography. With the exception of size exclusion chromatography, proteins and related impurities are retained by a chromatography medium or a selected membrane, while 3-FL remains in the 3-FL containing mixture.

3-FL is further separated from the reaction mixture and/or culture medium and/or cell with or without further purification steps by evaporation, lyophilization, crystallization, precipitation, and/or drying, spray drying.

In an even further aspect, the present invention also provides for a further purification of the α -1,3-fucosyllactose. A further purification of said α -1,3-fucosyllactose may be accomplished, for example, by use of (activated) charcoal or carbon, nanofiltration, ultrafiltration or ion exchange to remove any remaining DNA, protein, LPS, endotoxins, or other impurity. Alcohols, such as ethanol, and aqueous alcohol mixtures can also be used. Another purification step is accomplished by crystallization, evaporation or precipitation of the product. Another purification step is to dry, spray dry or lyophilize α -1,3-fucosyllactose.

The separated and preferably also purified 3-FL can be used as a supplement in infant formulas and for treating various diseases in newborn infants.

Another aspect of the invention provides for a method wherein the polypeptide and preferably also the 3-FL is produced in and/or by a fungal, yeast, bacterial, insect, animal and plant expression system or cell as described herein. The expression system or cell is chosen from the list comprising a bacterium, a yeast, or a fungus, or, refers to a plant or animal cell. The latter bacterium preferably belongs to the phylum of the Proteobacteria or the phylum of the Firmicutes or the phylum of the Cyanobacteria or the phylum Deinococcus-Thermus. The latter bacterium belonging to the phylum Proteobacteria belongs preferably to the family *Enterobacteriaceae*, preferably to the species *Escherichia coli*. The latter bacterium preferably relates to any strain belonging to the species *Escherichia coli* such as but not limited to *Escherichia coli* B, *Escherichia coli* C, *Escherichia coli* W, *Escherichia coli* K12, *Escherichia coli* Nissle. More specifically, the latter term relates to cultivated *Escherichia coli* strains - designated as *E. coli* K12 strains - which are well-adapted to the laboratory environment, and, unlike wild type strains, have lost their ability to thrive in the intestine. Well-known examples of the *E. coli* K12 strains are K12 Wild type, W3110, MG1655, M182, MC1000, MC1060, MC1061, MC4100, JM101, NZN111 and AA200. Hence, the present invention specifically relates to a mutated and/or transformed *Escherichia coli* host cell or strain as indicated above wherein said *E. coli* strain is a K12 strain. More preferably, the *Escherichia coli* K12 strain is *E. coli* MG1655. The latter bacterium belonging to the phylum Firmicutes belongs preferably to the Bacilli, preferably Lactobacilliales, with members such as *Lactobacillus lactis*, *Leuconostoc mesenteroides*, or Bacillales with members such as from the genus *Bacillus*, such as *Bacillus subtilis* or, *B. amyloliquefaciens*. The latter Bacterium belonging to the phylum Actinobacteria, preferably belonging to the family of the Corynebacteriaceae, with members *Corynebacterium glutamicum* or *C. afermentans*, or belonging to the family of the Streptomycetaceae with members *Streptomyces griseus* or *S. fradiae*. The latter yeast preferably belongs to the phylum of the Ascomycota or the phylum of the Basidiomycota or the phylum of the Deuteromycota or the phylum of the Zygomycetes. The latter yeast belongs preferably to the

genus *Saccharomyces*, *Pichia*, *Komagataella*, *Hansenula*, *Kluyveromyces*, *Yarrowia* or *Starmerella*. The latter fungus belongs preferably to the genus *Rhizopus*, *Dictyostelium*, *Penicillium*, *Mucor* or *Aspergillus*.

According to a further aspect of the invention, the polynucleotide encoding the polypeptide with
 5 alpha-1,3-fucosyltransferase activity is adapted to the codon usage of the respective cell or expression system.

In a further preferred embodiment, the method of the invention uses a culture medium for growth of the host cell or microorganism comprising the alpha-1,3-fucosyltransferase of the invention, wherein the lactose concentration in the culture medium ranges from 50 to 150 g/L. Such lactose
 10 concentration in the culture medium can be 50 g/L, 55g/L, 60 g/L, 65 g/L, 70 g/L, 75 g/L, 80 g/L, 85 g/L, 90 g/L, 95 g/L, 100 g/L, 105 g/L, 110 g/L, 115 g/L, 120 g/L, 125 g/L, 130 g/L, 135 g/L, 140 g/L, 145 g/L, or 150 g/L.

In a further preferred embodiment, the method of the invention produces a final concentration of 3-fucosyllactose ranges between 70 g/L to 200 g/L. Such 3-FL concentration being 70 g/L, 75 g/L,
 15 80 g/L, 85 g/L, 90 g/L, 95 g/L, 100 g/L, 105 g/L, 110 g/L, 115 g/L, 120 g/L, 125 g/L, 130 g/L, 135 g/L, 140 g/L, 145 g/L, 150 g/L, 155 g/L, 160 g/L, 165 g/L, 170 g/L, 175 g/L, 180 g/L, 185 g/L, 190 g/L, 195 g/L, or 200 g/L. Higher lactose concentrations in the culture medium can provide even higher 3-FL final concentrations obtained in the production method.

In a further preferred embodiment, the method of the invention produces a final concentration of
 20 3FL ranging between 70 g/L to 200 g/L as explained above, and wherein the 3FL purity in the broth is 80% or more. The 3FL purity according to the invention is at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 95,5%, 96%, 96,5%, 97%, 97,5%, 98%, 98,5%, 99%, 99,5%, 99,6%, 99,7%, 99,8%, 99,9%.

25 In the methods of the invention as described herein the polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate comprises:

a) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);
 b) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34),
 30 and

c) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of b) equals DM[A/S]VSF (SEQ ID NO 36);

wherein X can be any distinct amino acid; and

wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting
 35 from the first amino acid of the GDP-fucose binding domain.

Within the scope of the present invention, such polypeptide proved to have lactose binding α -1,3-fucosyltransferase activity and preferably has better lactose conversion efficiency compared to the presently known α -1,3-fucosyltransferase enzymes.

- 5 In a preferred embodiment of the invention said polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate comprises an amino acid sequence selected from the group consisting of:
- i) any one of SEQ ID NO 6, 2, 4, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing;
 - 10 ii) an amino acid sequence having 87% or more sequence identity to the full length amino acid sequence of SEQ ID NO 2, 20 or 22;
 - iii) an amino acid sequence having 80% or more sequence identity to the full length amino acid sequence of any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32;
 - iv) a fragment of an amino acid sequence shown in SEQ ID NO 2, 20 or 22, wherein said fragment
 - 15 comprises at least 45 contiguous amino acids thereof;
 - v) a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof and has lactose binding α -1,3-fucosyltransferase activity.
- Optionally said polypeptide is further modified by an N-terminal and/or C-terminal amino acid
- 20 stretch.

The amino acid sequence of the polypeptide used herein can be a sequence chosen from SEQ ID NO 6, 2, 4, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing. The amino acid sequence can also be an amino acid sequence that has greater than about 87% sequence

25 identity, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 95,5%, 96%, 96,5%, 97%, 97,5%, 98%, 98,5%, 99%, 99,5%, 99,6%, 99,7%, 99,8%, 99,9% sequence identity to the full length amino acid sequence of any one of SEQ NO 2, 20 or 22. The amino acid sequence can also be an amino acid sequence that has greater than about 80% sequence identity, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 95,5%, 96%, 96,5%, 97%, 97,5%,

30 98%, 98,5%, 99%, 99,5%, 99,6%, 99,7%, 99,8%, 99,9% sequence identity to the full length amino acid sequence of any one of SEQ NO 6, 8, 10, 12, 14, 16, 28, 30 or 32.

Furthermore, within the scope of the present invention, the amino acid sequence can be a fragment of an amino acid sequence shown in any one of SEQ ID NO 2, 20 or 22, wherein said fragment comprises at least 45 contiguous amino acids thereof; alternatively the amino acid

35 sequence can be a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof and has lactose binding α -1,3-fucosyltransferase activity.

Further included in the scope of the invention, is an α -1,3-fucosyltransferase polypeptide as described herein which is optionally further modified by an N-terminal and/or C-terminal amino acid stretch. Such amino acid stretch is to be understood as an addition of polypeptide sequences at the N-terminus and/or C-terminus of the polypeptide. For example, polypeptide sequences may be fused to the α -1,3-fucosyltransferase polypeptide in order to effectuate additional enzymatic activity. Such amino acid stretch can be a specific tag and/or HQ-tag; an extension of up to 20 amino acids, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids; such extension can also be 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 or more amino acids long. The optional N-terminal and/or C-terminal amino acid stretch can also be a tag for purification, a tag for increasing the solubility of the polypeptide, a tag or amino acid stretch for metabolon formation, a tag for protein metabolomics, a tag for substrate binding, another polypeptide with the same or a different function in a gene fusion, such as but not limited to a polypeptide coding for GDP-fucose synthase, galactosyltransferase, fucosyltransferase, bifunctional fucose kinase/fucose-1-phosphate guanylyltransferase or fucose-1-phosphate guanylyltransferase, wherein said other polypeptide is optionally fused to the α -1,3-fucosyltransferase polypeptide via a peptide linker. For example, the α -1,3-fucosyltransferase polypeptide as described herein optionally includes one or more exogenous affinity tags, e.g., purification or substrate binding tags, such as a 6 His tag sequence, a GST tag, a HQ tag, an HA tag sequence, a plurality of 6 His tag sequences, a plurality of GST tags, a plurality of HA tag sequences, a SNAP-tag, a SUMOstar tag. Other examples include proteolytic cleavage sites, retention sites, cleavage sites, polyhistidine tags, biotin, avidin, BiTag sequences, S tags, enterokinase sites, thrombin sites, antibodies or antibody domains, antibody fragments, antigens, receptors, receptor domains, receptor fragments, ligands, dyes, acceptors, quenchers, or combinations thereof.

In addition, α -1,3-fucosyltransferase polypeptides may include proteins or polypeptides that represent functionally equivalent polypeptides. Such an equivalent α -1,3-fucosyltransferase polypeptide may contain deletions, additions or substitutions of amino acid residues within the amino acid sequence encoded by the α -1,3-fucosyltransferase polynucleotides described herein, but which results in a silent change, thus producing a functionally equivalent α -1,3-fucosyltransferase. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; planar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Within the context of this invention, "functionally equivalent", as used herein, refers to a polypeptide capable of exhibiting a substantially similar in

vivo activity as the lactose binding α -1,3-fucosyltransferase polypeptides of the present invention as judged by any of a number of criteria, including but not limited to enzymatic activity.

Included within the scope of the invention are alpha-1,3-fucosyltransferase proteins, polypeptides, and derivatives (including fragments) which are differentially modified during or after translation.

5 Furthermore, non-classical amino acids or chemical amino acid analogues can be introduced as a substitution or addition into the alpha-1,3-fucosyltransferase polypeptide sequence.

The α -1,3-fucosyltransferase polypeptide may be produced by expression by polynucleotides produced via recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing
10 α -1,3-fucosyltransferase coding sequences and appropriate transcriptional and/or translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. See, for example, the techniques described in Sambrook et al. (2001) Molecular Cloning: a laboratory manual, 3rd Edition, Cold Spring Harbor Laboratory Press, CSH, New York or to Current Protocols in Molecular Biology,
15 John Wiley and Sons, N.Y. (1989 and yearly updates). Alternatively, the α -1,3-fucosyltransferase polypeptide may be produced by direct synthesis, by extraction of the cell which produces the polypeptide in nature or within a cell free and/or in vitro system.

The suitability of the newly identified alpha-1,3-fucosyltransferases having the ability to bind lactose to be used for producing 3-fucosyllactose, and preferably producing such 3FL with a purity
20 of 80% or more, is highly surprising, and, thus, their use represents an excellent tool to easily, efficiently and cost-effectively produce 3-fucosyllactose.

The polynucleotide encoding the α -1,3-fucosyltransferase polypeptide may be produced via recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing α -1,3-
25 fucosyltransferase coding sequences and appropriate transcriptional and/or translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. See, for example, the techniques described in Sambrook et al. (2001) Molecular Cloning: a laboratory manual, 3rd Edition, Cold Spring Harbor Laboratory Press, CSH, New York or to Current Protocols in Molecular Biology, John Wiley and
30 Sons, N.Y. (1989 and yearly updates).

According to another aspect of the invention, a vector is provided, containing a polynucleotide encoding a polypeptide with alpha-1,3-fucosyltransferase activity as described herein, wherein the polynucleotide is operably linked to control sequences recognized by a host cell transformed with the vector. In a particularly preferred embodiment, the vector is an expression vector, and,
35 according to another aspect of the invention, the vector can be present in the form of a plasmid, cosmid, phage, liposome, or virus.

Thus, the polynucleotide according to the invention, may, e.g., be comprised in a vector which is to be stably transformed/transfected into host cells. In the vector, the polynucleotide of the invention is under control of a promoter. The promoter can be e.g. an inducible promoter, so that the expression of the gene/polynucleotide can be specifically targeted, and, if desired, the gene
5 may be overexpressed in that way. The promoter can also be a constitutive promoter.

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
10 derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. These vectors may contain selection markers such as but not limited to antibiotic markers, auxotrophic markers, toxin-antitoxin markers, RNA sense/antisense markers. The expression system constructs may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain,
15 propagate or express polynucleotides and/or to express 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., see above.

For recombinant production, host cells can be genetically engineered to incorporate expression
20 systems or portions thereof or polynucleotides of the invention. Introduction of a polynucleotide into the host cell can be effected by methods described in many standard laboratory manuals, such as Davis et al., Basic Methods in Molecular Biology, (1986), and Sambrook et al., 1989, supra.

According to another aspect of the invention, a host cell is provided containing the vector as
25 described above.

According to a further aspect, the invention provides a host cell genetically modified for the production of α -1,3-fucosyllactose, wherein the host cell comprises at least one nucleic acid sequence coding for an enzyme for 3-fucosyllactose synthesis and wherein said cell comprises the expression of a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use
30 lactose as acceptor substrate. Said polypeptide being as described herein.

As used herein, the term "host cell" is presently defined as a cell which has been transformed or transfected or is capable of transformation or transfection by an exogenous polynucleotide sequence, thus containing at least one sequence not naturally occurring in said host cell.

A variety of host-expression vector systems may be utilized to express the alpha-1,3-fucosyltransferase polynucleotides of the invention. Such host-expression systems represent
35 vehicles by which the coding sequences of interest may be produced and subsequently purified,

but also represent cells which, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the alpha-1,3-fucosyltransferase gene product of the invention in situ. According to another aspect of the invention, a host cell for the production of 3-fucosyllactose is provided wherein the host cell contains a sequence consisting of a polynucleotide encoding a polypeptide with lactose binding alpha-1,3-fucosyltransferase activity as described herein, wherein the sequence is a sequence foreign to the host cell and wherein the sequence is integrated in the genome of the host cell. The polynucleotide is operably linked to control sequences recognised by the host cell.

According to an alternative aspect of the invention, a host cell for the production of 3-fucosyllactose is provided wherein the host cell contains a vector comprising said polynucleotide described herein, wherein the polynucleotide being operably linked to control sequences recognized by a host cell transformed with the vector.

In a further aspect, the present invention also provides for a method for the production of α -1,3-fucosyllactose, comprising the steps of: a) providing a cell as described herein, and b) cultivating the cell in a medium under conditions permissive for the production of α -1,3-fucosyltransferase. Preferably, said α -1,3-fucosyltransferase is separated from the cultivation as described herein. Preferably, also a purification can be done as described herein.

In another further aspect, the invention provides for use of the cell as described herein for the production of 3-fucosyllactose.

According to a further aspect of the invention, a microorganism is provided expressing the alpha-1,3-fucosyltransferase as described herein and preferably encoded by the polynucleotide as described herein.

The term micro-organism or organism or cell or host cell as used herein refers to a microorganism chosen from the list comprising a bacterium, a yeast, or a fungus, or, refers to a plant or animal cell. The latter bacterium preferably belongs to the phylum of the Proteobacteria or the phylum of the Firmicutes or the phylum of the Cyanobacteria or the phylum Deinococcus-Thermus. The latter bacterium belonging to the phylum Proteobacteria belongs preferably to the family *Enterobacteriaceae*, preferably to the species *Escherichia coli*. The latter bacterium preferably relates to any strain belonging to the species *Escherichia coli* such as but not limited to *Escherichia coli* B, *Escherichia coli* C, *Escherichia coli* W, *Escherichia coli* K12, *Escherichia coli* Nissle. More specifically, the latter term relates to cultivated *Escherichia coli* strains - designated as *E. coli* K12 strains - which are well-adapted to the laboratory environment, and, unlike wild type strains, have lost their ability to thrive in the intestine. Well-known examples of the *E. coli* K12 strains are K12 Wild type, W3110, MG1655, M182, MC1000, MC1060, MC1061, MC4100, JM101, NZN111 and AA200. Hence, the present invention specifically relates to a mutated and/or transformed *Escherichia coli* host cell or strain as indicated above wherein said *E. coli* strain is a K12 strain. More preferably, the *Escherichia coli* K12 strain is *E. coli* MG1655. The latter

bacterium belonging to the phylum Firmicutes belongs preferably to the Bacilli, preferably Lactobacilliales, with members such as *Lactobacillus lactis*, *Leuconostoc mesenteroides*, or Bacillales with members such as from the genus *Bacillus* such as *Bacillus subtilis* or *B. amyloliquefaciens*. The latter Bacterium belonging to the phylum Actinobacteria, preferably
 5 belonging to the family of the Corynebacteriaceae, with members *Corynebacterium glutamicum* or *C. afermentans*, or belonging to the family of the Streptomycetaceae with members *Streptomyces griseus* or *S. fradiae*. The latter yeast preferably belongs to the phylum of the Ascomycota or the phylum of the Basidiomycota or the phylum of the Deuteromycota or the phylum of the Zygomycetes. The latter yeast belongs preferably to the genus *Saccharomyces*,
 10 *Pichia*, *Komagataella*, *Hansenula*, *Kluyveromyces*, *Yarrowia* or *Starmerella*. The latter fungus belongs preferably to the genus *Rhizopus*, *Dictyostelium*, *Penicillium*, *Mucor* or *Aspergillus*.

According to another aspect of the invention, the polynucleotide encoding the polypeptide with lactose binding alpha-1,3-fucosyltransferase activity is adapted to the codon usage of the respective host cell.

15 A further aspect of the invention provides for the use of a polypeptide as described herein for the production of alpha-1,3-fucosyllactose. A further aspect of the invention provides for the use of a polynucleotide as described herein or of the vector as described herein, for the production of alpha-1,3-fucosyllactose.

According to one other embodiment, there is provided hitherto unknown lactose binding alpha-
 20 1,3-fucosyltransferases. The invention provides an isolated and/or synthesised polypeptide with a lactose binding alpha-1,3-fucosyltransferase activity wherein said polypeptide comprises:

- an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33) and a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), where additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35)
 25 is present at the N-terminal region if this domain equals DM[A/S]VSF (SEQ ID NO 36), wherein X can be any distinct amino acid, and the C-terminus of said amino acid sequence having less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain.

Preferably said polypeptide is selected from the group consisting of:

- 30 i) SEQ ID Nos 2, 4, 6, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing;
 ii) an amino acid sequence having 87% or more sequence identity to the full length of SEQ ID NO 2, 20 or 22;
 iii) an amino acid sequence comprising at least 80% sequence identity to the full-length amino acid sequence of any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32;
 35 iv) a fragment of an amino acid sequence shown in any one of SEQ ID NO 2, 20 or 22, wherein said fragment comprises at least 45 contiguous amino acids thereof;

v) a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof.

Optionally said polypeptide is further modified by an N-terminal and/or C-terminal amino acid stretch.

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Within the scope of the present invention, the isolated and/or synthesised polypeptide has lactose binding alpha-1,3-fucosyltransferase activity. Such polypeptide comprises an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33) and a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), where additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if this domain equals DM[A/S]VSF (SEQ ID NO 36), wherein X can be any distinct amino acid, and the C-terminus of said amino acid sequence having less than or equal to 100 amino acids, such as 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40 amino acids, starting from the first amino acid of the above defined conserved GDP-fucose binding domain.

Further included in the scope of the invention is an alpha 1,3-fucosyltransferase polypeptide as described herein which is optionally further modified by an N-terminal and/or C-terminal amino acid stretch.

The newly identified lactose binding alpha-1,3-fucosyltransferases were surprisingly found to be useable to perform reactions which are not naturally occurring. Furthermore, it has been found that the above identified alpha-1,3-fucosyltransferases are able to use lactose as substrate with similar or higher lactose binding properties than the presently known alpha-1,3-fucosyltransferase enzymes and are able to produce 3-fucosyllactose.

Up to the present day, the newly identified fucosyltransferases of the invention were not described to have lactose binding alpha-1,3-fucosyltransferase activity, as can be seen in table 1.

Table 1:

SEQ ID NO	DNA Identifier	NCBI_name	Organism
SEQ ID 1 - 2	SMF69967.1	Glycosyltransferase family 10 (fucosyltransferase) C-term [Azospirillum oryzae]	<i>Azospirillum oryzae</i> A2P
SEQ ID 3 - 4	WP_042442472.1	putative glycosyltransferase [Azospirillum lipoferum 4B]	<i>Azospirillum lipoferum</i>
SEQ ID 5 - 6	AIL32582.1	hypothetical protein IX83_03990 [Basilea psittacipulmonis DSM 24701]	<i>Basilea psittacipulmonis</i>

SEQ ID 7 - 8	ADG66884.1	putative LPS biosynthesis related glycosyltransferase [<i>Planctopirus limnophila</i> DSM 3776]	<i>Planctopirus limnophila</i> (strain ATCC 43296 / DSM 3776 / IFAM 1008 / 290) (<i>Planctomyces limnophilus</i>)
SEQ ID 9 - 10	KHJ37904.1	glycosyltransferase family 10 (fucosyltransferase) [<i>Pedobacter glucosidilyticus</i>]	<i>Pedobacter glucosidilyticus</i>
SEQ ID 11 - 12	WP_081748371.1	hypothetical protein [<i>Porphyromonas catoniae</i>]	<i>Porphyromonas catoniae</i> (WGS, in genbank: JDFF01000001 till JDFF010000025)
SEQ ID 13 - 14	WP_052080772.1	hypothetical protein [<i>Porphyromonas</i> sp. COT-239 OH1446]	<i>Porphyromonas</i> sp. COT-239 OH1446 (contig_18; NZ_JRAO01000018.1)
SEQ ID 15 - 16	EHG19535.1	hypothetical protein HMPREF9334_01850 [<i>Selenomonas infelix</i> ATCC 43532]	<i>Selenomonas infelix</i> ATCC 43532
SEQ ID 19 - 20	WP_109445332	alpha-1,3-fucosyltransferase [<i>Azospirillum</i> sp. TSH64]	<i>Azospirillum</i> sp. TSH64
SEQ ID 21 - 22	QCG87584.1	alpha-1,3-fucosyltransferase [<i>Azospirillum</i> sp. TSH100]	<i>Azospirillum</i> sp. TSH100
SEQ ID 27 - 28	SEQ36152.1	Glycosyltransferase family 10 (fucosyltransferase) C-term [<i>Butyrivibrio</i> sp. TB]	<i>Butyrivibrio</i> sp. TB
SEQ ID 29 - 30	EKX99948.1	hypothetical protein HMPREF9134_01857 [<i>Porphyromonas catoniae</i> F0037]	<i>Porphyromonas catoniae</i> F0037
SEQ ID 31 - 32	SHI32494.1	Glycosyltransferase family 10 (fucosyltransferase) C-term [<i>Butyrivibrio fibrisolvens</i>]	<i>Butyrivibrio fibrisolvens</i>

As shown in Table 2, it was also found that the newly identified α -1,3-fucosyltransferases having the ability to bind lactose to be used for producing 3-fucosyllactose, all shared the same special feature of having an amino acid sequence comprising a conserved GDP-fucose binding domain

5 [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33) wherein x can be any distinct amino acid and wherein the C-terminus of said amino acid sequence has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain. This in contrast with the known lactose binding 3-fucosyltransferases as described in e.g. WO2012/049083 having a

C-terminus which is longer than 100 amino acids starting from the first amino acid of the above defined GDP-fucose binding domain.

Table 2:

SEQ ID NO	DNA Identifier	Organism	C-terminus length from GDP-fucose binding domain
SEQ ID 1 - 2	SMF69967.1	<i>Azospirillum oryzae</i> A2P	89
SEQ ID 3 - 4	WP_042442472.1	<i>Azospirillum lipoferum</i> B510	89
SEQ ID 5 - 6	AIL32582.1	<i>Basilea psittacipulmonis</i>	97
SEQ ID 7 - 8	ADG66884.1	<i>Planctopirus limnophila</i>	96
SEQ ID 9 - 10	KHJ37904.1	<i>Pedobacter glucosidilyticus</i>	94
SEQ ID 11 - 12	WP_081748371.1	<i>Porphyromonas catoniae</i>	93
SEQ ID 13 - 14	WP_052080772.1	<i>Porphyromonas</i> sp. COT-239 OH1446	94
SEQ ID 15 - 16	EHG19535.1	<i>Selenomonas infelix</i>	96
SEQ ID 19 - 20	WP_109445332	<i>Azospirillum</i> sp. TSH64	89
SEQ ID 21 - 22	QCG87584.1	<i>Azospirillum</i> sp. TSH100	89
SEQ ID 27 - 28	SEQ36152.1	<i>Butyrivibrio</i> sp. TB	93
SEQ ID 29 - 30	EKX99948.1	<i>Porphyromonas catoniae</i> F0037	93
SEQ ID 31 - 32	SHI32494.1	<i>Butyrivibrio fibrisolvens</i> DSM 3071	93

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Furthermore, it was also found that the polypeptide sequences of SEQ ID NO 6, SEQ ID NO 10, SEQ ID NO 12, SEQ ID NO 14 and SEQ ID NO 16, share the domain PENXXXXXXXXTEK (SEQ ID NO 37), wherein X can be any distinct amino acid, as shown in figure 1 wherein the domain is put in a box. All alignments were done with MAFFT v7.307, visualisation was made with Jalview 2.10.

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Furthermore, it was also found that, in addition to the conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33), the newly identified polypeptides all share the common conserved motif [K/D][L/K/M]XXX[F/Y] (SEQ ID NO 34) wherein X can be any distinct amino acid, as well as the conserved amino acid region [FW]W which is important for lactose binding, as shown in figure 11 wherein the domains are put in a box.

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In addition, we noticed that when this common feature is DM[A/S]VSF (SEQ ID NO 36), additionally the conserved [N/H]XDPAXLD (SEQ ID NO 35) motif, wherein X can be any distinct amino acid, is required in the N-terminal domain of the protein for the enzyme to have α -1,3-fucosyltransferase activity on lactose as the acceptor substrate, as shown in the alignment of figure 12, wherein the domains are put in a box. As exemplified in example 14, the polypeptides

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with SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 20 and SEQ ID NO 22 contain both consensus motifs and appear to have alpha-1,3-fucosyltransferase activity on lactose as the acceptor substrate, while the polypeptides with SEQ ID NO 24 and SEQ ID NO 26 does not contain the N-terminal [NH]XDPAXLD motif, wherein X can be any distinct amino acid (SEQ ID NO 35) and do
5 show this activity.

Furthermore, it was also found that the polypeptide sequences of SEQ ID NO 6, SEQ ID NO 12, SEQ ID NO 14, SEQ ID NO 16, SEQ ID NO 28, SEQ ID NO 30 and SEQ ID NO 32, share the domains K[IV]F[FL]XGEN (SEQ ID NO 41) and RFPLW (SEQ ID NO 42), wherein x can be any distinct amino acid, as shown in the alignment of figure 13 wherein the domain is put in a box.

10 In a second embodiment, the present invention also relates to an isolated and/or synthesised polynucleotide encoding a polypeptide with lactose binding alpha-1,3-fucosyltransferase activity as described above.

Within the scope of the present invention, polynucleotide can be an allelic variant of a polynucleotide encoding any one of the amino acid sequences shown in SEQ ID NO 2, 6, 8, 10,
15 12, 14, 16, 20, 22, 28, 30, 32.

Accordingly, the present invention also relates to an isolated and/or synthesised polynucleotide which encodes a polypeptide with α -1,3-fucosyltransferase activity and which comprises a sequence selected from the group consisting of: a) SEQ ID NO 1, 5, 7, 9, 11, 13, 15, 19, 21, 27, 29, 31 of the attached sequence listing; b) a nucleic acid sequence complementary to SEQ ID
20 NO 1, 5, 7, 9, 11, 13, 15, 19, 21, 27, 29, 31; c) a nucleic acid sequence having 80% or more sequence identity to SEQ ID NO 1, 5, 7, 9, 11, 13, 15, 19, 21, 27, 29, 31.

Accordingly, the invention also relates to the 3-fucosyllactose obtained by the methods according to the invention, as well as to the use of a polynucleotide, the vector, host cells, microorganisms or the polypeptide as described above for the production of 3-fucosyllactose. The alpha-1,3-
25 fucosyllactose may be used as food additive, prebiotic, symbiotic, for the supplementation of baby food, adult food or feed, or as either therapeutically or pharmaceutically active compound. With the novel methods, alpha-1,3- fucosyllactose can easily and effectively be provided, without the need for complicated, time and cost consuming synthetic processes.

Unless defined otherwise, all technical and scientific terms used herein generally have the same
30 meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry described above and below are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, enzymatic reactions and purification steps are performed
35 according to the manufacturer's specifications.

Further advantages follow from the specific embodiments, the examples and the attached drawings.

It goes without saying that the abovementioned features and the features which are still to be explained below can be used not only in the respectively specified combinations, but also in other combinations or on their own, without departing from the scope of the present invention.

5 The present invention relates to the following specific embodiments:

1. A method for producing α -1,3-fucosyllactose, the method comprising the steps of:

a) providing a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate wherein said polypeptide comprises

10 i) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);

ii) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), and

15 iii) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of ii) equals DM[A/S]VSF (SEQ ID NO 36);

wherein X can be any distinct amino acid; and

wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain;

20 b) contacting the polypeptide with α -1,3-fucosyltransferase activity of step a) with a mixture comprising GDP-fucose as donor substrate, and lactose as acceptor substrate, under conditions where the polypeptide catalyses the transfer of a fucose residue from the donor substrate to the acceptor substrate,

thereby producing α -1,3-fucosyllactose

c) optionally separating said α -1,3-fucosyllactose.

25 2. Method according to embodiment 1, wherein said polypeptide is provided in a cell free system.

3. Method according to embodiment 1, wherein said polypeptide is produced by a cell comprising a polynucleotide encoding said polypeptide.

4. Method according to any one of embodiment 1 or 3, wherein said GDP-fucose and/or lactose is provided by a cell producing said GDP-fucose and/or lactose.

30 5. A method according to any one of embodiments 1, 3 or 4, the method comprising the steps of: i) providing a cell genetically modified for the production of α -1,3-fucosyllactose, said cell comprising at least one nucleic acid sequence coding for an enzyme for α -1,3-fucosyllactose synthesis,

said cell comprising the expression of said polypeptide with α -1,3-fucosyltransferase activity and
35 with the ability to use lactose as acceptor substrate

ii) cultivating the cell in a medium under conditions permissive for the production of α -1,3-fucosyllactose,

- iii) preferably separating the α -1,3-fucosyllactose from the cultivation.
6. Method according to embodiment 3, the method comprising the steps of:
- a) providing a host cell expressing said polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate;
- 5 b) growing, under suitable nutrient conditions permissive for the production of the α -1,3-fucosyllactose, and permissive for the expression of said polypeptide with α -1,3-fucosyltransferase activity, said host cell;
- c) providing simultaneously or subsequently to step b) a donor substrate GDP-fucose and the acceptor substrate lactose, in order for the α -1,3-fucosyltransferase polypeptide to catalyse the
- 10 transfer of a fucose residue from GDP-fucose to lactose, thereby producing α -1,3-fucosyllactose;
- d) optionally separating said α -1,3-fucosyllactose from the host cell or the medium of its growth.
7. A method according to any one of embodiments 5 or 6 wherein the host cell is transformed or transfected to express an exogenous polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as an acceptor substrate.
- 15 8. Method according to any one of embodiments 3 to 7 characterized in that the GDP-fucose and/or lactose is provided by an enzyme simultaneously expressed in the host cell or by the metabolism of the host cell.
9. The method of any one of embodiments 1 to 8, further comprising purification of α -1,3-fucosyllactose.
- 20 10. Method according to any one of the preceding embodiments, wherein said polypeptide is selected from the group consisting of:
- i) any one of SEQ ID NO 6, 2, 4, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing;
- ii) an amino acid sequence having 87% or more sequence identity to the full length amino acid
- 25 sequence of SEQ ID NO 2, 20 or 22;
- iii) an amino acid sequence having 80% or more sequence identity to the full length amino acid sequence of any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32;
- iv) a fragment of an amino acid sequence shown in SEQ ID NO 2, 20 or 22, wherein said fragment comprises at least 45 contiguous amino acids thereof;
- 30 v) a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof and has lactose binding α -1,3-fucosyltransferase activity;
- optionally said polypeptide is further modified by an N-terminal and/or C-terminal amino acid stretch.
- 35 11. Method for the production of 3-fucosyllactose according to any one of the preceding embodiments, the method further comprising at least one of the following steps:

- i) adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per initial reactor volume, preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said lactose feed;
- ii) adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;
- iii) adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably, 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L; and wherein preferably the pH of said solution is set between 3 and 7 and wherein preferably the temperature of said feed solution is kept between 20°C and 80°C;
- iv) said method resulting in a 3-fucosyllactose concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final volume of said culture medium.
12. Host cell genetically modified for the production of α -1,3-fucosyllactose, wherein the host cell comprises at least one nucleic acid sequence coding for an enzyme involved in α -1,3-fucosyllactose synthesis; said cell comprising the expression of a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate, wherein said polypeptide comprises:
- i) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);
- ii) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34),
- and
- iii) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of ii) equals DM[A/S]VSF (SEQ ID NO 36); wherein X can be any distinct amino acid; and wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain.
13. Cell according to embodiment 12, the host cell comprising i) a sequence comprising a polynucleotide encoding said polypeptide with lactose binding α -1,3-fucosyltransferase

activity, wherein the sequence is a sequence foreign to the host cell and wherein the sequence is integrated in the genome of the host cell, or ii) containing a vector comprising a polynucleotide encoding said polypeptide, wherein the polynucleotide being operably linked to control sequences recognized by a host cell transformed with the vector.

5 14. Cell according to any one of embodiment 12 or 13, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of:

i) any one of SEQ ID NO 6, 2, 4, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing;

10 ii) an amino acid sequence having 87% or more sequence identity to the full length amino acid sequence of SEQ ID NO 2, 20 or 22;

iii) an amino acid sequence having 80% or more sequence identity to the full length amino acid sequence of any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32;

iv) a fragment of an amino acid sequence shown in SEQ ID NO 2, 20 or 22, wherein said fragment comprises at least 45 contiguous amino acids thereof;

15 v) a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof and has lactose binding alpha-1,3-fucosyltransferase activity;

Optionally said polypeptide is further modified by an N-terminal and/or C-terminal amino acid stretch.

20 15. Method according to any one of the embodiments 3 to 11 or cell according to any one of embodiments 12, 13 or 14, wherein said cell is selected from the group consisting of microorganism, plant, or animal cells, preferably said microorganism is a bacterium, fungus or a yeast, preferably said plant is a rice, cotton, rapeseed, soy, maize or corn plant, preferably said animal is an insect, fish, bird or non-human mammal; preferably the cell is an *Escherichia coli* cell.

16. Host cell according to any one of embodiments 12 to 15, characterized in that the host cell is a cell of a bacterium, preferably of an *Escherichia coli* strain, more preferably of an *Escherichia coli* strain which is a K12 strain, even more preferably the *Escherichia coli* K12 strain is *Escherichia coli* MG1655.

30 17. Host cell according to any one of embodiments 12 to 15, characterized in that the host cell is a yeast cell.

18. Host cell according to any one of embodiments 12 to 17, characterized in that the polynucleotide encoding the polypeptide with lactose binding alpha-1,2-fucosyltransferase activity is adapted to the codon usage of the respective host cell.

35 19. Method for the production of α -1,3-fucosyllactose, comprising the steps of:

a) providing a cell according to any one of embodiments 12 to 18,

- b) cultivating the cell in a medium under conditions permissive for the production of α -1,3-fucosyltransferase,
- c) preferably, separating said α -1,3-fucosyltransferase from the cultivation.
20. Use of a host cell according to any one of embodiments 12 to 18 for the production of α -1,3-fucosyllactose.
21. Use of a polypeptide as described in the method of any one of embodiment 1 or 11 for the production of α -1,3-fucosyllactose.
22. A microorganism heterologously expressing a lactose binding α -1,3-fucosyltransferase polypeptide wherein said polypeptide comprises:
- 10 i) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);
- ii) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), and
- iii) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of ii) equals DM[A/S]VSF (SEQ ID NO 36);
- 15 wherein X can be any distinct amino acid; and
- wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain.
23. Microorganism according to embodiment 22, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of:
- 20 i) any one of SEQ ID NO 6, 2, 4, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing;
- ii) an amino acid sequence having 87% or more sequence identity to the full length amino acid sequence of SEQ ID NO 2, 20 or 22;
- 25 iii) an amino acid sequence having 80% or more sequence identity to the full length amino acid sequence of any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32;
- iv) a fragment of an amino acid sequence shown in SEQ ID NO 2, 20 or 22, wherein said fragment comprises at least 45 contiguous amino acids thereof;
- v) a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16,
- 30 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof and has lactose binding α -1,3-fucosyltransferase activity;
- Optionally said polypeptide is further modified by an N-terminal and/or C-terminal amino acid stretch.
24. Use of a microorganism according to embodiment 22 or 23 for the production of α -1,3-fucosyllactose.
- 35 25. The method of any one of embodiments 1 to 11, 15, or 19, further comprising a step of separating said α -1,3-fucosyllactose from the host cell or the medium of its growth.

26. The method of any one of embodiments 1 to 11, 15, 19 or 25, wherein said separation comprises at least one of the following steps: clarification, ultrafiltration, nanofiltration, reverse osmosis, microfiltration, activated charcoal or carbon treatment, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and/or gel filtration, ligand exchange chromatography.
27. The method of any one of embodiments 1 to 11, 15, 19, 25 or 26, further comprising purification of alpha-1,3-fucosyllactose.
28. The method of embodiment 27, wherein said purification of said alpha-1,3-fucosyllactose comprises at least one of the following steps: use of activated charcoal or carbon, use of charcoal, nanofiltration, ultrafiltration or ion exchange, use of alcohols, use of aqueous alcohol mixtures, crystallization, evaporation, precipitation, drying, spray drying or lyophilization.
29. The method of any one of embodiments 1 to 11, 15, 19, 25 to 28, wherein the polypeptide is produced in a fungal, yeast, bacterial, insect, animal and plant expression system.
30. The method of embodiment 29, wherein the host cell is a cell of a bacterium, preferably of an *Escherichia coli* strain, more preferably of an *Escherichia coli* strain which is a K12 strain, even more preferably the *Escherichia coli* K12 strain is *Escherichia coli* MG1655.
31. The method of embodiment 29, wherein the host cell is a yeast cell.
32. The method of any one of embodiments 1 to 11, 15, 19, 25 to 31, wherein the lactose concentration in the culture medium ranges from 50 to 150 g/L.
33. The method of any one of embodiments 1 to 11, 15, 19, 25 to 32 wherein the final concentration of 3-fucosyllactose ranges between 70 g/L to 200g/L.
34. A method of any one of embodiments 1 to 11, 15, 19, 25 to 33 wherein said production results in a lactose concentration to 3-fucosyllactose concentration ratio of less than 1:5 at the end of fermentation.
35. A method of any one of embodiments 1 to 11, 15, 19, 25 to 34 wherein said production results in a 3-fucosyllactose purity of 80% or more at the end of fermentation.
36. A method for the production of α -1,3-fucosyllactose, the method comprising the steps of:
- a) providing a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate
 - b) contacting the polypeptide with α -1,3-fucosyltransferase activity of step a) with a mixture comprising GDP-fucose as donor substrate, and lactose as acceptor substrate, under conditions where the polypeptide catalyses the transfer of a fucose residue from the donor substrate to the acceptor substrate, thereby producing α -1,3-fucosyllactose,
 - c) wherein said catalysis results in a lactose concentration to 3-fucosyllactose concentration ratio of less than 1:5 at the end of fermentation
 - d) optionally separating said α -1,3-fucosyllactose.

37. A method for the production of α -1,3-fucosyllactose, the method comprising the steps of:

a) providing a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate

b) contacting the polypeptide with α -1,3-fucosyltransferase activity of step a) with a mixture comprising GDP-fucose as donor substrate, and lactose as acceptor substrate, under conditions where the polypeptide catalyses the transfer of a fucose residue from the donor substrate to the acceptor substrate,

thereby producing α -1,3-fucosyllactose,

c) wherein said catalysis results in a 3-fucosyllactose purity of 80% or more at the end of fermentation

d) optionally separating said α -1,3-fucosyllactose.

38. Method for the production of 3-fucosyllactose comprising at least one of the following steps:

i) Adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per initial reactor volume, preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said lactose feed;

ii) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;

iii) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L; and wherein preferably the pH of said solution is set between 3 and 7 and wherein preferably the temperature of said feed solution is kept between 20°C and 80°C;

Said method resulting in a 3-fucosyllactose concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final volume of said culture medium and preferably a lactose concentration to 3FL concentration ratio lower than 1:5, more preferably 1:10, even more preferably 1:20, most preferably 1:40 in the final volume of said culture.

39. Method for the production of 3-fucosyllactose comprising at least one of the following steps:

- i) Adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per initial reactor volume, preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said lactose feed;
- ii) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;
- iii) Adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably, 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L; and wherein preferably the pH of said solution is set between 3 and 7 and wherein preferably the temperature of said feed solution is kept between 20°C and 80°C;
- Said method resulting in a 3-fucosyllactose concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final volume of said culture medium and preferably with a 3FL purity of 80% or more in the final volume of said culture.
- The following drawings and examples will serve as further illustration and clarification of the present invention and are not intended to be limiting.

Brief description of the drawings

- FIG. 1 shows an alignment of the polypeptide sequences of SEQ ID NO 6, SEQ ID NO 10, SEQ ID NO 12, SEQ ID NO 14 and SEQ ID NO 16.
- FIG. 2 shows normalised production of 3-fucosyllactose in a growth experiment.
- FIG. 3 shows normalised production of 3-fucosyllactose in a growth experiment with low to high amounts of lactose in the medium.
- FIG. 4 shows normalised production of 3-fucosyllactose in a growth experiment with low amounts of lactose in the medium.
- FIG. 5 shows the percentage of lactose that is converted to 3-FL of one of the identified lactose binding α -1,3-fucosyltransferases.

FIG. 6 shows the percentage of lactose that is converted to 3-FL of different of the identified lactose binding alpha-1,3-fucosyltransferases driven by different promoters.

FIG. 7 shows the normalised production of 3-fucosyllactose of a further experiment.

FIG. 8 shows the normalised production of 3-fucosyllactose of a subset of the identified lactose binding alpha-1,3-fucosyltransferases driven by different promoters.

FIG. 9 shows the normalised production of 3-fucosyllactose of strains expressing *H. pylori* fucT (SEQ ID 18) from 2 different promoters

FIG. 10 shows the normalised production of 3-fucosyllactose of strains expressing polypeptides with the DM[AS]VSF consensus motif

FIG. 11 shows an alignment of the polypeptide sequences of SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8, SEQ ID NO 10, SEQ ID NO 12, SEQ ID NO 14, SEQ ID NO 16, SEQ ID NO 20, SEQ ID NO 22, SEQ ID NO 28, SEQ ID NO 30 and SEQ ID NO 32. The consensus motifs [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R], [K/D][L/K/M]XXX[F/Y] and [FW]W, wherein X can be any distinct amino acid, are marked with a box.

FIG. 12 shows an alignment of the polypeptide sequences of SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 20, SEQ ID NO 22, SEQ ID NO 24 and SEQ ID NO 26. The consensus motifs DM[A/S]VSF and [N/H]XDPAXLD, wherein X can be any distinct amino acid (and unrelated motifs) are marked with a box.

FIG. 13 shows an alignment of the polypeptide sequences of SEQ ID NO 6, SEQ ID NO 12, SEQ ID NO 14, SEQ ID NO 16, SEQ ID NO 28, SEQ ID NO 30 and SEQ ID NO 32. The consensus motifs K[I/V]F[F/L]XGEN (SEQ ID NO 41) and RFPLW (SEQ ID NO 42), wherein X can be any distinct amino acid, are marked with a box.

Examples

Example 1: Materials and methods *Escherichia coli*

Media

The Luria Broth (LB) medium consisted of 1% tryptone peptone (Difco, Erembodegem, Belgium), 0.5% yeast extract (Difco) and 0.5% sodium chloride (VWR, Leuven, Belgium). The medium for the shake flasks experiments contained 2.00 g/L NH₄Cl, 5.00 g/L (NH₄)₂SO₄, 2.993 g/L KH₂PO₄, 7.315 g/L K₂HPO₄, 8.372 g/L MOPS, 0.5 g/L NaCl, 0.5 g/L MgSO₄·7H₂O, 14.26 g/L sucrose or another carbon source when specified in the examples, 1 ml/L vitamin solution, 100 µl/L molybdate solution, and 1 mL/L selenium solution. The medium was set to a pH of 7 with 1M KOH. Vitamin solution consisted of 3.6 g/L FeCl₂·4H₂O, 5 g/L CaCl₂·2H₂O, 1.3 g/L MnCl₂·2H₂O, 0.38 g/L CuCl₂·2H₂O, 0.5 g/L CoCl₂·6H₂O, 0.94 g/L ZnCl₂, 0.0311 g/L H₃BO₄, 0.4 g/L Na₂EDTA·2H₂O and 1.01 g/L thiamine.HCl. The molybdate solution contained 0.967 g/L NaMoO₄·2H₂O. The selenium solution contained 42 g/L SeO₂.

The minimal medium for fermentations contained 6.75 g/L NH_4Cl , 1.25 g/L $(\text{NH}_4)_2\text{SO}_4$, 2.93 g/L KH_2PO_4 and 7.31 g/L KH_2PO_4 , 0.5 g/L NaCl , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 14.26 g/L sucrose, 1 mL/L vitamin solution, 100 $\mu\text{L/L}$ molybdate solution, and 1 mL/L selenium solution with the same composition as described above.

- 5 Complex medium was sterilized by autoclaving (121°C., 21') and minimal medium by filtration (0.22 μm Sartorius). When necessary, the medium was made selective by adding an antibiotic (e.g. chloramphenicol (20 mg/L), carbenicillin (100mg/L), spectinomycin (40mg/L) and/or kanamycin (50mg/L)).

10 Plasmids

pKD46 (Red helper plasmid, Ampicillin resistance), pKD3 (contains an FRT-flanked chloramphenicol resistance (cat) gene), pKD4 (contains an FRT-flanked kanamycin resistance (kan) gene), and pCP20 (expresses FLP recombinase activity) plasmids were obtained from Prof. R. Cunin (Vrije Universiteit Brussel, Belgium in 2007).

- 15 Plasmids for alpha-1,3-fucosyltransferase expression were constructed in a pMB1 ori vector using Golden Gate assembly. The genes were expressed using promoters apFAB305 ("PROM0012"), apFAB146 ("PROM0032") (both as described by Mutalik *et al.* (Nat. Methods 2013, No. 10, 354-360)), and p14 ("PROM0016" in combination with "UTR0019") (as described by De Mey *et al.* (BMC Biotechnology 2007)) and UTRs Gene10-LeuAB-BCD2 ("UTR0002") (as described by
- 20 Mutalik *et al.* (Nat. Methods 2013, No. 10, 354-360)).

Plasmids were maintained in the host *E. coli* DH5alpha (F^- , $\text{phi80d}/\text{lacZ}/\text{deltaM15}$, $\text{delta}(\text{lacZYA-argF})$ U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk^- , mk^+), *phoA*, *supE44*, lambda^- , *thi-1*, *gyrA96*, *relA1*) bought from Invitrogen.

25 Strains and mutations

Escherichia coli K12 MG1655 [lambda^- , F^- , *rph-1*] was obtained from the Coli Genetic Stock Center (US), CGSC Strain#: 7740, in March 2007. Gene disruptions as well as gene introductions were performed using the technique published by Datsenko and Wanner (PNAS 97 (2000), 6640-6645). This technique is based on antibiotic selection after homologous recombination performed

- 30 by lambda Red recombinase. Subsequent catalysis of a flippase recombinase ensures removal of the antibiotic selection cassette in the final production strain.

Transformants carrying a Red helper plasmid pKD46 were grown in 10 ml LB media with ampicillin, (100 mg/L) and L-arabinose (10 mM) at 30 °C to an OD_{600nm} of 0.6. The cells were made electrocompetent by washing them with 50 ml of ice-cold water, a first time, and with 1ml

- 35 ice cold water, a second time. Then, the cells were resuspended in 50 μl of ice-cold water. Electroporation was done with 50 μl of cells and 10-100 ng of linear double-stranded-DNA product by using a Gene Pulser™ (BioRad) (600 Ω , 25 μFD , and 250 volts).

After electroporation, cells were added to 1 ml LB media incubated 1 h at 37 °C, and finally spread onto LB-agar containing 25 mg/L of chloramphenicol or 50 mg/L of kanamycin to select antibiotic resistant transformants. The selected mutants were verified by PCR with primers upstream and downstream of the modified region and were grown in LB-agar at 42 °C for the loss of the helper plasmid. The mutants were tested for ampicillin sensitivity.

The linear ds-DNA amplicons were obtained by PCR using pKD3, pKD4 and their derivatives as template. The primers used had a part of the sequence complementary to the template and another part complementary to the side on the chromosomal DNA where the recombination must take place. For the genomic knock-out, the region of homology was designed 50-nt upstream and 50-nt downstream of the start and stop codon of the gene of interest. For the genomic knock-in, the transcriptional starting point (+1) had to be respected. PCR products were PCR-purified, digested with DpnI, repurified from an agarose gel, and suspended in elution buffer (5 mM Tris, pH 8.0).

The selected mutants (chloramphenicol or kanamycin resistant) were transformed with pCP20 plasmid, which is an ampicillin and chloramphenicol resistant plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis. The ampicillin-resistant transformants were selected at 30 °C, after which a few were colony purified in LB at 42 °C and then tested for loss of all antibiotic resistance and of the FLP helper plasmid. The gene knock outs and knock ins are checked with control primers (Fw/Rv-gene-out).

A mutant strain derived from *E. coli* K12 MG1655 was created by knocking out the genes *lacZ*, *lacY*, *lacA*, *glgC*, *agp*, *pfkA*, *pfkB*, *pgi*, *arcA*, *iclR*, *wcaJ*, *pgi*, *lon* and *thyA*. Additionally, the *E. coli* *lacY* gene, a fructose kinase gene (*frk*) originating from *Zymomonas mobilis* and a sucrose phosphorylase (SP) originating from *Bifidobacterium adolescentis* were knocked in into the genome and expressed constitutively. The constitutive promoters originate from the promoter library described by De Mey *et al.* (BMC Biotechnology, 2007). These genetic modifications are also described in WO2016075243 and WO2012007481.

All constructed plasmids with the hypothetical alpha-1,3-fucosyltransferase genes were evaluated in this mutant strain derived from *E. coli* K12 MG1655. All strains are stored in cryovials at -80°C (overnight LB culture mixed in a 1:1 ratio with 70% glycerol). A list of all successful lactose binding alpha-1,3-fucosyltransferases (SEQ ID NOs 1 to 16, 19 to 22 and 27 to 32) together with a prior art alpha-1,3-fucosyltransferase (SEQ ID NO 17-18) and two non-functional alpha-1,3-fucosyltransferases (SEQ ID NO 23 to 26) is provided in Table 3.

Table 3:

SEQ ID	Organism	Country origin
SEQ ID 1 - 2	<i>Azospirillum oryzae</i> A2P	Japan
SEQ ID 3 - 4	<i>Azospirillum lipoferum</i> B510	Japan

SEQ ID 5 - 6	<i>Basilea psittacipulmonis</i>	Switzerland
SEQ ID 7 - 8	<i>Planctopirus limnophila</i> (strain ATCC 43296 / DSM 3776 / IFAM 1008 / 290) (<i>Planctomyces limnophilus</i>)	Germany
SEQ ID 9 - 10	<i>Pedobacter glucosidilyticus</i>	Germany
SEQ ID 11 - 12	<i>Porphyromonas catoniae</i> (WGS, in genbank: JDFF01000001 till JDFF010000025)	United States
SEQ ID 13 - 14	<i>Porphyromonas</i> sp. COT-239 OH1446 (contig_18; NZ_JRAO01000018.1)	United Kingdom
SEQ ID 15 - 16	<i>Selenomonas infelix</i> ATCC 43532	Unknown
SEQ ID 17 - 18	<i>Helicobacter pylori</i>	Australia
SEQ ID 19 - 20	<i>Azospirillum</i> sp. TSH64	Japan
SEQ ID 21 - 22	<i>Azospirillum</i> sp. TSH100	Japan
SEQ ID 23 - 24	<i>Azospirillum brasiliense</i>	Unknown
SEQ ID 25 - 26	<i>Azospirillum</i> sp. B510	Japan
SEQ ID 27 - 28	<i>Butyrivibrio</i> sp. TB	Unknown
SEQ ID 29 - 30	<i>Porphyromonas catoniae</i> F0037	Unknown
SEQ ID 31 - 32	<i>Butyrivibrio fibrisolvens</i> DSM 3071	Unknown

Heterologous and homologous expression

All potential alpha-1,3-fucosyltransferase genes that needed to be expressed, be it for a plasmid or for the genomic insertion, were synthetically synthesized at Twist Biosciences (San Francisco, USA). Expression could be further facilitated by optimizing the codon usage to the codon usage of the expression host. Genes were optimized using the tools of the supplier.

Cultivation conditions

A preculture of 96well microtiter plate experiments was started from a cryovial, in 150 µL LB and was incubated overnight at 37 °C on an orbital shaker at 800 rpm. This culture was used as inoculum for a 96well square microtiter plate, with 400 µL MMs medium by diluting 400x. These final 96-well culture plates were then incubated at 37°C on an orbital shaker at 800 rpm for 72h, or shorter, or longer. At the end of the cultivation experiment samples were taken from each well to measure sugar concentrations in the broth supernatant (extracellular sugar concentrations, after spinning down the cells), or by boiling the culture broth for 15 min at 90°C before spinning down the cells (= whole broth measurements, average of intra- and extracellular sugar concentrations).

A preculture for the bioreactor was started from an entire 1 mL cryovial of a certain strain, inoculated in 250 mL or 500 mL of MMs medium in a 1 L or 2.5 L shake flask and incubated for 24 h at 37°C on an orbital shaker at 200 rpm. A 5 L bioreactor was then inoculated (250 mL

inoculum in 2 L batch medium); the process was controlled by MFCS control software (Sartorius Stedim Biotech, Melsungen, Germany). Culturing condition were set to 37 °C, and maximal stirring; pressure gas flow rates were dependent on the strain and bioreactor. The pH was controlled at 6.8 using 0.5 M H₂SO₄ and 20% NH₄OH. The exhaust gas was cooled. 10% solution
5 of silicone antifoaming agent was added when foaming raised during the fermentation.

Optical density

Cell density of the cultures was frequently monitored by measuring optical density at 600 nm (Implen Nanophotometer NP80, Westburg, Belgium or with a Spark 10M microplate reader,
10 Tecan, Switzerland).

Liquid chromatography

Standards for 3-fucosyllactose were synthesized in house. Other standards such as but not limited to lactose, sucrose, glucose, fructose were purchased from Sigma.
Carbohydrates were analyzed via a HPLC-RI (Waters, USA) method, whereby RI (Refractive
15 Index) detects the change in the refraction index of a mobile phase when containing a sample. The sugars were separated in an isocratic flow using an X-Bridge column (Waters X-bridge HPLC column, USA) and a mobile phase containing 75 ml acetonitrile and 25 ml Ultrapure water and 0.15 ml triethylamine. The column size was 4.6 x 150mm with 3.5 µm particle size. The temperature of the column was set at 35°C and the pump flow rate was 1 mL/min.

20

Example 2: Evaluation of different lactose binding alpha-1,3-fucosyltransferase enzymes incorporated in Escherichia coli

An experiment was set up to evaluate a number of genes coding for potential alpha-1,3-fucosyltransferase enzymes that are able to produce 3-fucosyllactose (3-FL) from GDP-fucose
25 and lactose. A growth experiment was performed according to the cultivation conditions provided in Example 1.

Figure 2 shows the normalised production of 3-fucosyllactose obtained in a growth experiment of the strains successfully expressing various lactose binding alpha-1,3-fucosyltransferases using two different promoters (PROM0012 and PROM0016) with 20 g/L lactose in the production
30 medium. Each datapoint corresponds to data from one well. The dashed horizontal line indicates the setpoint to which all datapoints were normalized.

The experiment identified the following polypeptides with lactose binding 3-fucosyltransferase activity: SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8, SEQ ID NO 10, SEQ ID NO 12 and SEQ ID NO 14 having similar to better lactose binding α-1,3-fucosyltransferase activity
35 compared to a strain containing SEQ ID NO 18 with previously confirmed lactose binding α-1,3-fucosyltransferase activity. The polypeptide of SEQ ID NO 4 has 90,8% global sequence identity

to SEQ ID NO 2, herewith showing that also sequences which have 87% or more sequence identity to SEQ ID NO 2 have lactose binding α -1,3-fucosyltransferase activity.

Example 3: Evaluation of a lactose binding α -1,3-fucosyltransferase enzyme incorporated in *Escherichia coli* for its ability to produce 3-FL with low to high lactose concentrations in minimal media

A gene coding for SEQ ID NO 6 (and combined with PROM0016) is evaluated on its ability to produce 3-FL in minimal media with various concentrations of lactose. A growth experiment was performed according to the cultivation conditions provided in Example 1. Strains with SEQ ID NO 6 and SEQ ID NO 18 (driven by PROM0016) were grown in multiple wells of an 96-well plate as described above. SEQ ID NO 18 has previously confirmed α -1,3-fucosyltransferase activity on lactose.

Figure 3 shows the normalised production of 3-fucosyllactose with 6 different concentrations of lactose as a precursor for 3-FL (90 g/L and a 1:2 dilution series thereof, until 2.8 g/L, as indicated in the figure). Each datapoint corresponds to data from one well. The dashed horizontal line indicates the setpoint to which all datapoints were normalized.

The experiment identified the polypeptide of SEQ ID NO 6 to have better lactose binding α -1,3-fucosyltransferase activity at all lactose concentrations compared to a strain expressing SEQ ID NO 18, a polypeptide with previously confirmed lactose binding α -1,3-fucosyltransferase activity.

Example 4: Evaluation of various lactose binding α -1,3-fucosyltransferase enzymes incorporated in *Escherichia coli* for their ability to produce 3-FL at low concentrations of lactose in minimal media

Several of the above identified strains with genes coding for SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 12 and SEQ ID NO 14 were evaluated on their ability to produce 3-fucosyllactose from GDP-fucose and lactose in a growth experiment at low concentrations of lactose. A growth experiment was performed according to the cultivation conditions provided in Example 1.

Figure 4 shows the normalised production of 3-fucosyllactose with strains expressing various α -1,3-fucosyltransferases (using two different promoters PROM0012 and PROM0016) and grown in a medium with low amounts of lactose (2.8 g/L lactose). Each datapoint corresponds to data from one well. The dashed horizontal line indicates the setpoint to which all datapoints were normalized.

The experiment identified the following polypeptides with SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 12 and SEQ ID NO 14 to having similar to better lactose binding α -1,3-fucosyltransferase activity when provided with low concentrations of lactose compared to a strain

containing SEQ ID NO 18 with previously confirmed lactose binding alpha-1,3-fucosyltransferase activity.

Example 5: evaluation of enzyme activity of the polypeptide of SEQ ID NO 6 incorporated in *Escherichia coli* on two low concentrations of lactose

A gene coding for SEQ ID NO 6 (and combined with PROM0016) was evaluated for its ability to convert lactose into 3-fucosyllactose in a strain producing GDP-fucose in a growth experiment providing 2.8 g/L or 5.62 g/L of lactose and sucrose at 30 g/L. A growth experiment was performed according to the cultivation conditions provided in Example 1.

Figure 5 shows the % of lactose that is converted to 3-FL, calculated by dividing the measured amount of 3-FL by the amount that could theoretically be obtained based on the input concentration of lactose. Theoretically, if all lactose is converted, a value of 100% is obtained. Each datapoint corresponds to data from one well.

The strain expressing polypeptide as shown in SEQ ID NO 6 is compared to a strain expressing the polypeptide as shown in SEQ ID NO 18 (driven by PROM0016) which is previously confirmed to have alpha-1,3-fucosyltransferase activity on lactose. At both concentrations of lactose the strain expressing the polypeptide as shown in SEQ ID NO 6 is able to convert much more lactose to 3-FL than the strain expressing the polypeptide as shown in SEQ ID NO 18 for a given amount of carbon source (30 g/L of sucrose).

Example 6: evaluation of enzyme activity of various lactose binding alpha-1,3-fucosyltransferase enzymes incorporated in *Escherichia coli* at limited concentrations of lactose and sucrose

Genes coding for the above identified polypeptides SEQ ID NO 2, SEQ ID NO 6, SEQ ID NO 12 and SEQ ID NO 14 were evaluated on their ability to convert lactose into 3-fucosyllactose in a strain producing GDP-fucose in a growth experiment at low concentrations of lactose (2 g/L) and sucrose (7.5 g/L). A growth experiment was performed according to the cultivation conditions provided in Example 1.

Figure 6 shows the % of lactose that is converted to 3-FL, calculated by dividing the measured amount of 3-FL by the amount that could theoretically be obtained based on the input concentration of lactose. Each datapoint corresponds to data from one well.

The strains expressing the polypeptides with SEQ ID NO 2, SEQ ID NO 6, SEQ ID NO 12 or SEQ ID NO 14 are able to convert more lactose to 3-FL than the strain expressing the polypeptide with SEQ ID NO 18 for a given amount of carbon source (7.5 g/L sucrose).

Example 7: evaluation of *Escherichia coli* strains expressing various lactose binding alpha-1,3-fucosyltransferase enzymes in a batch fermentation

Batch fermentations at bioreactor scale were performed to evaluate strains, derived from the mutant *E. coli* K12 MG1655 strain background as described in example 1, expressing various
5 alpha-1,3-fucosyltransferase enzymes with SEQ ID NO 2, SEQ ID NO 6, SEQ ID NO 12 and SEQ ID NO 14. The bioreactor runs were performed as described in Example 1. In these examples, sucrose was used as a carbon source. Lactose was added in the batch medium at 90 g/L as a precursor for 3-FL formation.

Figure 7 shows the normalised production of 3-fucosyllactose obtained in batch fermentations
10 with strains successfully expressing various lactose binding alpha-1,3-fucosyltransferases with lactose in the production medium as a precursor. Each datapoint corresponds to data from one fermentation run. The dashed horizontal line indicates the setpoint to which all datapoints were normalized.

The experiment shows that mutant *E. coli* strains expressing the lactose binding alpha-1,3-fucosyltransferase genes with SEQ ID NO 2, SEQ ID NO 6, SEQ ID NO 12 or SEQ ID NO 14
15 produce higher amounts of 3-FL compared to the strain expressing the polypeptide with SEQ ID NO 18.

Example 8: Evaluation of different lactose binding alpha-1,3-fucosyltransferase enzymes incorporated in *Escherichia coli*

A further experiment was set up with strains expressing the enzymes with SEQ ID NO 2, SEQ ID NO 6, SEQ ID NO 12, SEQ ID NO 14 and SEQ ID NO 16 and evaluated whether these are able to produce 3-fucosyllactose from lactose in a strain producing GDP-fucose. A growth experiment was performed according to the cultivation conditions provided in Example 1.

Figure 8 shows normalized production of 3-fucosyllactose with strains successfully expressing various lactose binding alpha-1,3-fucosyltransferases (using three different promoters PROM0012, PROM0016 and PROM0026) with 20 g/L lactose in the production medium. Each datapoint corresponds to data from one well. The dashed horizontal line indicates the setpoint to which all datapoints were normalized.

The experiment confirmed the results from example 2 for the strains expressing polypeptides with SEQ ID NO 12, SEQ ID NO 6, SEQ ID NO 12 and SEQ ID NO 14, and identified the polypeptide with SEQ ID NO 16 to also have better lactose binding alpha-1,3-fucosyltransferase activity compared to a strain containing SEQ ID 18 with previously confirmed lactose binding alpha-1,3-fucosyltransferase activity.

Example 9: Material and methods *Saccharomyces cerevisiae*Media

Strains are grown on Synthetic Defined yeast medium with Complete Supplement Mixture (SD CSM) or CSM drop-out (SD CSM-Ura) containing 6.7 g/L Yeast Nitrogen Base without amino acids (YNB w/o AA, Difco), 20 g/L agar (Difco) (solid cultures), 22 g/L glucose monohydrate or 20 g/L lactose and 0.79 g/L CSM or 0.77 g/L CSM-Ura (MP Biomedicals).

Strains

Saccharomyces cerevisiae BY4742 created by Bachmann *et al.* (Yeast (1998) 14:115-32) was used available in the Euroscarf culture collection. All mutant strains were created by homologous recombination or plasmid transformation using the method of Gietz (Yeast 11:355-360, 1995). *Kluyveromyces marxianus lactis* is available at the LMG culture collection (Ghent, Belgium).

Plasmids

Yeast expression plasmid p2a_2μ_sia_GFA1 (Chan 2013 (Plasmid 70 (2013) 2-17)) was used for expression of foreign genes in *Saccharomyces cerevisiae*. This plasmid contains an ampicillin resistance gene and a bacterial origin of replication to allow for selection and maintenance in *E. coli*. The plasmid further contains the 2μ yeast ori and the Ura3 selection marker for selection and maintenance in yeast. Next, this plasmid can be modified to p2a_2μ_fl to contain a lactose permease (for example LAC12 from *Kluyveromyces lactis*), a GDP-mannose 4,6-dehydratase (such as *Gmd* from *E. coli*) and a GDP-L-fucose synthase (such as *fcl* from *E. coli*).

Yeast expression plasmids p2a_2μ_fl_3ft is based on p2a_2μ_ft but modified in a way that also SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8, SEQ ID NO 10, SEQ ID NO 12, SEQ ID NO 14, SEQ ID NO 16 or SEQ ID NO 18 are expressed. Preferably but not necessarily, the fucosyltransferase proteins are N-terminally fused to a SUMOstar tag (e.g. obtained from pYSUMOstar, Life Sensors, Malvern, PA) to enhance the solubility of the fucosyltransferase enzymes.

Plasmids were maintained in the host *E. coli* DH5alpha (F⁻, phi80d/lacZdeltaM15, delta(lacZYA-argF)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk⁻, mk⁺), *phoA*, *supE44*, lambda⁻, *thi-1*, *gyrA96*, *relA1*) bought from Invitrogen.

Gene expression promoters

Genes are expressed using synthetic constitutive promoters, as described in by Blazeck (Biotechnology and Bioengineering, Vol. 109, No. 11, 2012).

Heterologous and homologous expression

Genes that needed to be expressed, be it from a plasmid or from the genome were synthetically synthesized with one of the following companies: DNA2.0, Gen9 or IDT.

- 5 Expression could be further facilitated by optimizing the codon usage to the codon usage of the expression host. Gene were optimized using the tools of the supplier.

Cultivations conditions

- 10 In general, yeast strains were initially grown on SD CSM plates to obtain single colonies. These plates were grown for 2-3 days at 30 °C.

Starting from a single colony, a preculture was grown over night in 5 mL at 30 °C, shaking at 200rpm. Subsequent 125 mL shake flask experiments were inoculated with 2% of this preculture, in 25 mL media. These shake flasks were incubated at 30 °C with an orbital shaking of 200 rpm. The use of an inducer is not required as all genes are constitutively expressed.

15

Example 10: production of 3-fucosyllactose in *Saccharomyces cerevisiae* using various lactose binding alpha-1,3-fucosyltransferase enzymes

- Another example provides use of an eukaryotic organism, in the form of *Saccharomyces cerevisiae*, for the invention. Using the strains, plasmids and methods as described in example 20 9, strains are created that express SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8, SEQ ID NO 10, SEQ ID NO 12, SEQ ID NO 14, SEQ ID NO 16 or SEQ ID NO 18.

- On top of that, further modifications are made in order to produce 3-fucosyllactose. These modifications comprise the addition of a lactose permease, a GDP-mannose 4,6-dehydratase and a GDP-L-fucose synthase. The preferred lactose permease is the *KILAC12* gene from 25 *Kluyveromyces lactis* (WO 2016/075243). The preferred GDP-mannose 4,6-dehydratase and the GDP-L-fucose synthase are respectively *gmd* and *fcl* from *Escherichia coli*.

- These strains are capable of growing on glucose or glycerol as carbon source, converting the carbon source into GDP-L-fucose, taking up lactose, and producing 3-fucosyllactose using GDP-L-fucose and lactose as substrates for the enzymes represented by SEQ ID NO 2, SEQ ID NO 30 4, SEQ ID NO 6, SEQ ID NO 8, SEQ ID NO 10, SEQ ID NO 12, SEQ ID NO 14, SEQ ID NO 16 or SEQ ID NO 18, with SEQ ID NO 18 as reference.

- Preculture of said strains are made in 5mL of the synthetic defined medium SD-CSM containing 22 g/L glucose and grown at 30°C as described in example 9. These precultures are inoculated in 25 mL medium in a shake flask with 10g/L sucrose as sole carbon source and grown at 30°C. 35 Regular samples are taken and the production of 3-fucosyllactose is measured as described in example 1.

Example 11: enzymatic production of 3-fucosyllactose

Another example provides the use of an enzyme with SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8, SEQ ID NO 10, SEQ ID NO 12, SEQ ID NO 14 or SEQ ID NO 16 of the present invention. These enzymes are produced in a cell-free expression system such as but not limited to the PURExpress system (NEB), or in a host organism such as but not limited to *Escherichia coli* or *Saccharomyces cerevisiae*, after which the above listed enzymes can be isolated and optionally further purified.

Each of the above enzyme extracts or purified enzymes are added to a reaction mixture together with GDP-fucose, lactose and a buffering component such as Tris-HCl or HEPES. Said reaction mixtures is then incubated at a certain temperature (for example 37°C) for a certain amount of time (for example 24 hours), during which the lactose will be converted to 3-fucosyllactose by the enzyme using GDP-fucose. The 3-fucosyllactose is then separated from the reaction mixture by methods known in the art. Further purification of the 3-FL can be performed if preferred. At the end of the reaction or after separation and/or purification, the production of 3-fucosyllactose is measured as described in example 1.

Example 12: 3-fucosyllactose production with different lactose concentrations

A fermentation process as described in example 1 and example 7, wherein the lactose concentration in the culture medium ranges from 50 to 150 g/L. Said lactose is converted during the process into 3-fucosyllactose until minor amounts of lactose is left. The final ratio lactose to 3-fucosyllactose may be manipulated during this process by stopping the process earlier (higher lactose to 3-fucosyllactose ratio) or later (lower lactose to 3-fucosyllactose ratio) The lactose concentration may be increased in the vessel by feeding high concentrations of lactose solution with or without another carbon source to the bioreactor. Said lactose feed contains lactose concentrations between 100 and 700g/L and is kept at a temperature so that the lactose is kept soluble at a pH below or equal to 6 to avoid lactulose formation during the process, a standard method used in the dairy industry. The final concentrations of 3-fucosyllactose reached in such a production process ranges between 70 g/L when lower lactose concentrations are used and 200 g/L or higher when high lactose concentrations are used in the process as described above.

Example 13: Evaluation of the *Helicobacter pylori* alpha-1,3-fucosyltransferase fucT (SEQ ID 18) expressed from various promoters

The gene coding for the *H. pylori* alpha-1,3-fucosyltransferase fucT (SEQ ID NO 18) was cloned in an expression vector under control of promoters PROM0012 or PROM0016, and the resulting plasmids were transformed to the *E. coli* mutant strain as described in Example 1. These strains were then evaluated in a growth experiment for their ability to produce 3-FL. Both strains were grown in multiple wells of an 96-well plate.

Figure 9 shows the normalized production of 3-fucosyllactose produced by the strains. Each datapoint corresponds to data from one well. The dashed horizontal line indicates the setpoint to which all datapoints were normalized.

The experiment shows that the 3-FL production in a strain expressing *H. pylori* FucT using promoter PROM0012 drops to $\pm 30\%$ of the levels observed for a similar strain expressing the fucosyltransferase from promoter PROM0016.

By extrapolation of the data provided in Examples 2, 4 and 8, we can conclude that all strains containing any of the SEQ ID NOs 2 – 16 show a significantly higher production compared to the control strain with α 1,3-fucosyltransferase fucT (SEQ ID NO 18) when the fucosyltransferase is expressed from the same promoter (PROM0012 OR PROM0016), except for the strain with SEQ ID NO 10 which shows a similar production as the control strain.

Example 14: Evaluation of strains expressing polypeptides with the DM[AS]VSF consensus motif for the production of 3-fucosyllactose

Mutant *E. coli* strains containing an expression construct for either SEQ ID NO 4, SEQ ID NO 20, SEQ ID NO 22, SEQ ID NO 24 and SEQ ID NO 26 were evaluated for their 3-FL production in a growth experiment as described in Example 1. As indicated in figure 12, all polypeptide sequences contain the consensus domain DM[AS]VSF (SEQ ID NO 36), but only SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 20 and SEQ ID NO 22 additionally contain the consensus motif [NH]xDPAXLD (SEQ ID NO 35) in the N-terminal region of the protein. The strain containing the *H. pylori* α 1,3-fucosyltransferase fucT (SEQ ID NO 18) was taken along as a positive control. All strains were grown in multiple wells of an 96-well plate and tested in standard medium with 30 g/L sucrose and 20 g/L lactose.

Figure 10 shows the normalized production of 3-fucosyllactose produced by the strains. Each datapoint corresponds to data from one well. The dashed horizontal line indicates the setpoint to which all datapoints were normalized.

The experiment shows that only the strains containing polypeptides with both consensus motifs [NH]xDPAXLD and DM[AS]VSF: i.e. SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 20 or SEQ ID NO 22, are able to produce 3-FL, while the strains with polypeptides with DM[AS]VSF but lacking [NH]xDPAXLD: i.e. SEQ ID NO 24 and SEQ ID NO 26, do not produce any 3-FL. Based on this data, we can conclude that the presence of the [NH]xDPAXLD (SEQ ID NO 35) consensus motif at the N-terminal region of polypeptides with the DM[AS]VSF (SEQ ID NO 36) domain is crucial for the enzyme to have lactose binding α 1,3-fucosyltransferase activity.

Moreover, the polypeptide of SEQ ID NO 22 has 92% global sequence identity to SEQ ID NO 2, herewith showing that also sequences which have 87% or more sequence identity to SEQ ID NO 2 have lactose binding α 1,3-fucosyltransferase activity.

Example 15: Evaluation of strains expressing polypeptides with SEQ ID NO 28, SEQ ID NO 30 or SEQ ID NO 32

Mutant *E. coli* strains containing an expression construct for either SEQ ID NO 28, SEQ ID NO 30 or SEQ ID NO 32 can be evaluated for their 3-FL production in a growth experiment as described in Example 1. At the end of the growth experiment, the production of 3-fucosyllactose can be observed in the culture broth.

10 Example 16: Evaluation of the 3FL purity at the end of a fed-batch fermentation

Fed-batch fermentations at bioreactor scale were performed to evaluate strains, derived from the mutant *E. coli* K12 MG1655 strain background as described in example 1, expressing various alpha-1,3-fucosyltransferase enzymes with SEQ ID NO 2, SEQ ID NO 6 and SEQ ID NO 18. The bioreactor runs were performed as described in Example 1. In these examples, sucrose was used as a carbon source. Lactose was added in the batch medium at 90 g/L as a precursor for 3-FL formation, and a concentrated sucrose solution was fed during the fed-batch. For each strain, three independent fermentations were performed.

At the end of the fermentation, the broth was analyzed for the presence of lactose and 3-FL and the 3-FL purity was calculated using the formula $3FL\ (g/L) / (3FL\ (g/L) + lactose\ (g/L))$. For strains containing SEQ ID NO 18, an average purity of 85% was obtained, while for strains containing SEQ ID NO 2 or 6 an average purity of over 98% and over 99% was obtained respectively.

The experiment shows that mutant *E. coli* strains expressing the lactose binding alpha-1,3-fucosyltransferase genes with SEQ ID NO 2 or SEQ ID NO 6 produce, in fed-batch fermentations at bioreactor scale, a broth with a higher 3-FL purity than similar strains containing SEQ ID NO 18.

Claims

1. A method for producing α -1,3-fucosyllactose, the method comprising the steps of:
 - a) providing a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate wherein said polypeptide comprises
 - 5 i) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);
 - ii) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), and
 - iii) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of ii) equals DM[A/S]VSF (SEQ ID NO 36);
 - 10 wherein X can be any distinct amino acid; and
 - wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain;
 - b) contacting the polypeptide with α -1,3-fucosyltransferase activity of step a) with a mixture
 - 15 comprising GDP-fucose as donor substrate, and lactose as acceptor substrate, under conditions where the polypeptide catalyses the transfer of a fucose residue from the donor substrate to the acceptor substrate,
 - thereby producing α -1,3-fucosyllactose
 - c) optionally separating said α -1,3-fucosyllactose.
- 20 2. Method according to claim 1, wherein said polypeptide is provided in a cell free system.
3. Method according to claim 1, wherein said polypeptide is produced by a cell comprising a polynucleotide encoding said polypeptide.
4. Method according to any one of claim 1 or 3, wherein said GDP-fucose and/or lactose is provided by a cell producing said GDP-fucose and/or lactose.
- 25 5. A method according to any one of claim 1, 3 or 4, the method comprising the steps of:
 - i) providing a cell genetically modified for the production of α -1,3-fucosyllactose, said cell comprising at least one nucleic acid sequence coding for an enzyme for α -1,3-fucosyllactose synthesis,
 - said cell comprising the expression of said polypeptide with α -1,3-fucosyltransferase activity and
 - 30 with the ability to use lactose as acceptor substrate
 - ii) cultivating the cell in a medium under conditions permissive for the production of α -1,3-fucosyllactose,
 - iii) preferably separating the α -1,3-fucosyllactose from the cultivation.
6. Method according to claim 3, the method comprising the steps of:
 - 35 a) providing a host cell expressing said polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate;

- b) growing, under suitable nutrient conditions permissive for the production of the α -1,3-fucosyllactose, and permissive for the expression of said polypeptide with α -1,3-fucosyltransferase activity, said host cell;
- c) providing simultaneously or subsequently to step b) a donor substrate GDP-fucose and the acceptor substrate lactose, in order for the α -1,3-fucosyltransferase polypeptide to catalyse the transfer of a fucose residue from GDP-fucose to lactose, thereby producing α -1,3-fucosyllactose;
- d) optionally separating said α -1,3-fucosyllactose from the host cell or the medium of its growth.
7. A method according to any one of claims 5 or 6 wherein the host cell is transformed or transfected to express an exogenous polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as an acceptor substrate.
8. Method according to any one of claim 3 to 7 characterized in that the GDP-fucose and/or lactose is provided by an enzyme simultaneously expressed in the host cell or by the metabolism of the host cell.
9. The method of any one of claims 1 to 8, further comprising purification of α -1,3-fucosyllactose.
10. Method according to any one of the preceding claims, wherein said polypeptide is selected from the group consisting of:
- i) any one of SEQ ID NO 6, 2, 4, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing;
 - ii) an amino acid sequence having 87% or more sequence identity to the full length amino acid sequence of SEQ ID NO 2, 20 or 22;
 - iii) an amino acid sequence having 80% or more sequence identity to the full length amino acid sequence of any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32;
 - iv) a fragment of an amino acid sequence shown in SEQ ID NO 2, 20 or 22, wherein said fragment comprises at least 45 contiguous amino acids thereof;
 - v) a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof and has lactose binding α -1,3-fucosyltransferase activity;
- optionally said polypeptide is further modified by an N-terminal and/or C-terminal amino acid stretch.
11. Method for the production of 3-fucosyllactose according to any one of the preceding claims, the method further comprising at least one of the following steps:
- i) adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per initial reactor volume, preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said lactose feed;

- ii) adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;
- iii) adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably, 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L; and wherein preferably the pH of said solution is set between 3 and 7 and wherein preferably the temperature of said feed solution is kept between 20°C and 80°C;
- iv) said method resulting in a 3-fucosyllactose concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final volume of said culture medium.
12. Host cell genetically modified for the production of α -1,3-fucosyllactose, wherein the host cell comprises at least one nucleic acid sequence coding for an enzyme involved in α -1,3-fucosyllactose synthesis; said cell comprising the expression of a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate, wherein said polypeptide comprises:
- i) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);
- ii) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), and
- iii) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of ii) equals DM[A/S]VSF (SEQ ID NO 36); wherein X can be any distinct amino acid; and wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain.
13. Cell according to claim 12, the host cell comprising i) a sequence comprising a polynucleotide encoding said polypeptide with lactose binding α -1,3-fucosyltransferase activity, wherein the sequence is a sequence foreign to the host cell and wherein the sequence is integrated in the genome of the host cell, or ii) containing a vector comprising a polynucleotide encoding said polypeptide, wherein the polynucleotide being operably linked to control sequences recognized by a host cell transformed with the vector.
14. Cell according to any one of claim 12 or 13, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of:

- i) any one of SEQ ID NO 6, 2, 4, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence listing;
- ii) an amino acid sequence having 87% or more sequence identity to the full length amino acid sequence of SEQ ID NO 2, 20 or 22;
- 5 iii) an amino acid sequence having 80% or more sequence identity to the full length amino acid sequence of any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32;
- iv) a fragment of an amino acid sequence shown in SEQ ID NO 2, 20 or 22, wherein said fragment comprises at least 45 contiguous amino acids thereof;
- v) a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16,
- 10 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof and has lactose binding alpha-1,3-fucosyltransferase activity;
- optionally said polypeptide is further modified by an N-terminal and/or C-terminal amino acid stretch.
- 15 15. Method according to any one of the claims 3 to 11 or cell according to any one of claims 12, 13 or 14, wherein said cell is selected from the group consisting of microorganism, plant, or animal cells, preferably said microorganism is a bacterium, fungus or a yeast, preferably said plant is a rice, cotton, rapeseed, soy, maize or corn plant, preferably said animal is an insect, fish, bird or non-human mammal; preferably the cell is an *Escherichia coli* cell.
- 20 16. Host cell according to any one of claim 12 to 15, characterized in that the host cell is a cell of a bacterium, preferably of an *Escherichia coli* strain, more preferably of an *Escherichia coli* strain which is a K12 strain, even more preferably the *Escherichia coli* K12 strain is *Escherichia coli* MG1655.
17. Host cell according to any one of claim 12 to 15, characterized in that the host cell is a yeast cell.
- 25 18. Host cell according to any one of claims 12 to 17, characterized in that the polynucleotide encoding the polypeptide with lactose binding alpha-1,2-fucosyltransferase activity is adapted to the codon usage of the respective host cell.
19. Method for the production of α -1,3-fucosyllactose, comprising the steps of:
- a) providing a cell according to any one of claims 12 to 18,
- 30 b) cultivating the cell in a medium under conditions permissive for the production of α -1,3-fucosyltransferase,
- c) preferably, separating said α -1,3-fucosyltransferase from the cultivation.
20. Use of a host cell according to any one of claim 12 to 18 for the production of α -1,3-fucosyllactose.
- 35 21. Use of a polypeptide as described in the method of any one of claim 1 or 11 for the production of α -1,3-fucosyllactose.

22. A microorganism heterologously expressing a lactose binding alpha-1,3-fucosyltransferase polypeptide wherein said polypeptide comprises:
- i) an amino acid sequence encoding a conserved GDP-fucose binding domain [Y/W/L/H/F/M]X[T/S/C][E/Q/D/A][K/R] (SEQ ID NO 33);
 - 5 ii) an amino acid sequence encoding a conserved [K/D][L/K/M]XXX[F/Y] domain (SEQ ID NO 34), and
 - iii) wherein additionally the conserved motif [N/H]XDPAXLD (SEQ ID NO 35) is present at the N-terminal region if the domain of ii) equals DM[A/S]VSF (SEQ ID NO 36); wherein X can be any distinct amino acid; and
- 10 wherein the C-terminus of said polypeptide has less than or equal to 100 amino acids starting from the first amino acid of the GDP-fucose binding domain.
23. Microorganism according to claim 22, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of:
- i) any one of SEQ ID NO 6, 2, 4, 8, 10, 12, 14, 16, 20, 22, 28, 30 or 32 of the attached sequence
 - 15 listing;
 - ii) an amino acid sequence having 87% or more sequence identity to the full length amino acid sequence of SEQ ID NO 2, 20 or 22;
 - iii) an amino acid sequence having 80% or more sequence identity to the full length amino acid sequence of any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32;
 - 20 iv) a fragment of an amino acid sequence shown in SEQ ID NO 2, 20 or 22, wherein said fragment comprises at least 45 contiguous amino acids thereof;
 - v) a fragment of an amino acid sequence shown in any one of SEQ ID NO 6, 8, 10, 12, 14, 16, 28, 30 or 32, wherein said fragment comprises at least 10 contiguous amino acids thereof and has lactose binding alpha-1,3-fucosyltransferase activity;
 - 25 optionally said polypeptide is further modified by an N-terminal and/or C-terminal amino acid stretch.
24. Use of a microorganism according to claim 22 or 23 for the production of alpha-1,3-fucosyllactose.
25. The method of any one of claims 1 to 11, 15, or 19, further comprising a step of separating
- 30 said alpha-1,3-fucosyllactose from the host cell or the medium of its growth.
26. The method of any one of claim 1 to 11, 15, 19 or 25, wherein said separation comprises at least one of the following steps: clarification, ultrafiltration, nanofiltration, reverse osmosis, microfiltration, activated charcoal or carbon treatment, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography,
- 35 hydrophobic interaction chromatography and/or gel filtration, ligand exchange chromatography.
27. The method of any one of claims 1 to 11, 15, 19, 25 or 26, further comprising purification of alpha-1,3-fucosyllactose.

28. The method of claim 27, wherein said purification of said alpha-1,3-fucosyllactose comprises at least one of the following steps: use of activated charcoal or carbon, use of charcoal, nanofiltration, ultrafiltration or ion exchange, use of alcohols, use of aqueous alcohol mixtures, crystallization, evaporation, precipitation, drying, spray drying or lyophilization.
- 5 29. The method of any one of claims 1 to 11, 15, 19, 25 to 28, wherein the polypeptide is produced in a fungal, yeast, bacterial, insect, animal and plant expression system.
30. The method of claim 29, wherein the host cell is a cell of a bacterium, preferably of an *Escherichia coli* strain, more preferably of an *Escherichia coli* strain which is a K12 strain, even more preferably the *Escherichia coli* K12 strain is *Escherichia coli* MG1655.
- 10 31. The method of claim 29, wherein the host cell is a yeast cell.
32. The method of any one of claims 1 to 11, 15, 19, 25 to 31, wherein the lactose concentration in the culture medium ranges from 50 to 150 g/L.
33. The method of any one of claims 1 to 11, 15, 19, 25 to 32 wherein the final concentration of 3-fucosyllactose ranges between 70 g/L to 200g/L.
- 15 34. A method of any one of claims 1 to 11, 15, 19, 25 to 33 wherein said production results in a lactose concentration to 3-fucosyllactose concentration ratio of less than 1:5 at the end of fermentation.
35. A method of any one of claims 1 to 11, 15, 19, 25 to 34 wherein said production results in a 3-fucosyllactose purity of 80% or more at the end of fermentation.
- 20 36. A method for the production of α -1,3-fucosyllactose, the method comprising the steps of:
- a) providing a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate
- b) contacting the polypeptide with α -1,3-fucosyltransferase activity of step a) with a mixture comprising GDP-fucose as donor substrate, and lactose as acceptor substrate, under conditions
- 25 where the polypeptide catalyses the transfer of a fucose residue from the donor substrate to the acceptor substrate,
- thereby producing α -1,3-fucosyllactose,
- c) wherein said catalysis results in a lactose concentration to 3-fucosyllactose concentration ratio of less than 1:5 at the end of fermentation
- 30 d) optionally separating said α -1,3-fucosyllactose.
37. A method for the production of α -1,3-fucosyllactose, the method comprising the steps of:
- a) providing a polypeptide with α -1,3-fucosyltransferase activity and with the ability to use lactose as acceptor substrate
- b) contacting the polypeptide with α -1,3-fucosyltransferase activity of step a) with a mixture
- 35 comprising GDP-fucose as donor substrate, and lactose as acceptor substrate, under conditions where the polypeptide catalyses the transfer of a fucose residue from the donor substrate to the acceptor substrate,

thereby producing α -1,3-fucosyllactose,

c) wherein said catalysis results in a 3-fucosyllactose purity of 80% or more at the end of fermentation

d) optionally separating said α -1,3-fucosyllactose.

5 38. Method for the production of 3-fucosyllactose comprising at least one of the following steps:

i) adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per initial reactor volume, preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said lactose feed;

ii) adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;

iii) adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably, 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L; and wherein preferably the pH of said solution is set between 3 and 7 and wherein preferably the temperature of said feed solution is kept between 20°C and 80°C;

Said method resulting in a 3-fucosyllactose concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final volume of said culture medium and preferably a lactose concentration to 3FL concentration ratio lower than 1:5, more preferably 1:10, even more preferably 1:20, most preferably 1:40 in the final volume of said culture.

39. Method for the production of 3-fucosyllactose comprising at least one of the following steps:

30 i) adding to the culture medium a lactose feed comprising at least 50, more preferably at least 75, more preferably at least 100, more preferably at least 120, more preferably at least 150 gram of lactose per initial reactor volume, preferably in a continuous manner, and preferably so that the final volume of the culture medium is not more than three-fold, preferably not more than two-fold, more preferably less than 2-fold of the volume of the culture medium before the addition of said lactose feed;

35 ii) adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution;

- iii) adding a lactose feed in a continuous manner to the culture medium over the course of 1 day, 2 days, 3 days, 4 days, 5 days by means of a feeding solution and wherein the concentration of said lactose feeding solution is 50 g/L, preferably 75 g/L, more preferably 100 g/L, more preferably 125 g/L, more preferably 150 g/L, more preferably 175 g/L, more preferably 200 g/L, more preferably 225 g/L, more preferably 250 g/L, more preferably 275 g/L, more preferably 300 g/L, more preferably 325 g/L, more preferably 350 g/L, more preferably 375 g/L, more preferably, 400 g/L, more preferably 450 g/L, more preferably 500 g/L, even more preferably, 550 g/L, most preferably 600 g/L; and wherein preferably the pH of said solution is set between 3 and 7 and wherein preferably the temperature of said feed solution is kept between 20°C and 80°C;
- 10 said method resulting in a 3-fucosyllactose concentration of at least 50 g/L, preferably at least 75 g/L, more preferably at least 90 g/L, more preferably at least 100 g/L, more preferably at least 125 g/L, more preferably at least 150 g/L, more preferably at least 175 g/L, more preferably at least 200 g/L in the final volume of said culture medium and preferably with a 3FL purity of 80% or more in the final volume of said culture.