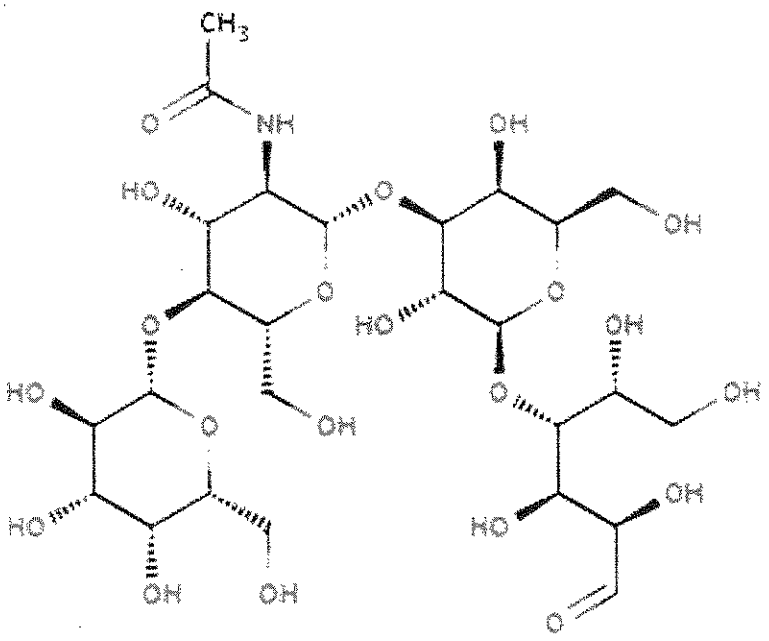
~~Biotechnological production~~Bioengineering of LNT, LNnT and ~~the~~their fucosylated derivatives ~~thereof~~

The present invention relates to genetically modified microorganisms for in vivo synthesis of lacto-N-tetrose (LNT) and lacto-N-neotetrose (LNnT), and their fucosylated derivatives, and to uses of such microorganisms in methods of producing lacto-N-tetrose and lacto-N-neotetrose, and their fucosylated derivatives.

Human breast milk is considered to have an important role in healthy infant development. The oligosaccharides present therein (human milk oligosaccharides (HMO)) are one of the major constituent components of breast milk, and their core structure has a lactose unit at the reducing end and is continued with N-acetyllactosamine units in a branched or chain-like manner. Structural variability is additionally expanded by fucosyl or sialyl modifications at the terminal positions.



With regard to health and development promoting action, the biological function of HMOs has been the subject of numerous studies, although this requires the recovery of pure compounds in sufficient quantities. Currently, the most commonly used method is the rather complicated extraction from breast milk. Biotechnological methods of producing HMOs have been described (see Han et al., *Biotechnol. Adv.* **2012**, *30*, 1268-1278), but lacto-N-tetrose, for example, as one of the most common HMOs, is currently not available for research at a reasonable price. Both chemical and enzymatic syntheses for LNT are known from the literature (see Aly et al., *Carbohydr. Res.* **1999**, *316*, 121-132; Murata et al., *Glycoconj. J.* **1999**, *16*, 189-195). However, since chemical synthesis requires multiple steps of protection and deprotection of reactive groups and enzymatic synthesis suffers from unfavorable equilibrium product distributions and regioselectivities, these methods do not produce any satisfactory results.

It was therefore a primary object of the ~~present invention~~embodiments disclosed herein to specify a system, preferably microorganisms, which is/are capable of producing high yields of LNT and LNnT and of their fucosylated derivatives.

Another object of the ~~present invention~~embodiments disclosed herein was to provide a corresponding method enabling LNT and LNnT and their fucosylated derivatives to be biotechnologically produced in an efficient and inexpensive manner.

~~The primary object is achieved according to the invention by~~In an aspect, there is provided a genetically modified microorganism for *in vivo* synthesis of lacto-N-tetrose ~~or lacto-N-neotetrose~~, said microorganism comprising

(i) a first transgene coding for β1,3-*N*-acetylglucosaminyltransferase,

and

(ii) a second transgene coding for β1,3-galactosyltransferase ~~(in the case of the synthesis of lacto-N-tetrose) or for β1,4-galactosyltransferase (in the case of the synthesis of lacto-N-neotetrose).~~

In another aspect, there is provided a genetically modified microorganism for *in vivo* synthesis of lacto-N-neotetrose, said microorganism comprising

(i) a first transgene coding for β1,3-*N*-acetylglucosaminyltransferase,

and

(ii) a second transgene coding for β1,4-galactosyltransferase.

In another aspect, there is provided the use of a genetically modified microorganism as defined above for *in vivo* synthesis of lacto-N-tetrose or lacto-N-neotetrose or a fucosylated derivative of lacto-N-tetrose or lacto-N-neotetrose.

In another aspect, there is provided a method of preparing lacto-N-tetrose or lacto-N-neotetrose or a fucosylated derivative of lacto-N-tetrose or lacto-N-neotetrose, comprising the following steps:

(a) providing a genetically modified microorganism as defined above,

(b) culturing said genetically modified microorganism under conditions that permit synthesis of lacto-N-tetrose and lacto-N-neotetrose,

(c) optionally adding fucose,

(d) optionally isolating the synthesized lacto-N-tetrose or lacto-N-neotetrose or fucosylated derivative of lacto-N-tetrose or lacto-N-neotetrose.

In another aspect, there is provided the genetically modified microorganism as defined above or the use as defined above or the method as defined above, wherein the genetically modified microorganism is selected from the group consisting of bacteria, fungi, and plants, preferably microorganisms of the genera *Corynebacterium, Bevibacterium, Bacillus, Saccharomyces* and *Escherichia*.

A genetically modified microorganism in the present context means a microorganism in which individual genes have been switched off and/or endogenous or exogenous genes have been incorporated (transgenes) in a specific manner using biotechnological methods. A transgene in accordance with the present invention may be a gene imported from a different organism or else a gene which is naturally present in the microorganism concerned that has been integrated by genetic engineering at a different site in the genome and as a result is expressed, for example, under a promoter different from the natural promoter.

Surprisingly, it was found in the course of the present invention that micro-organisms routinely employed in genetic engineering, which transgenically express β1,3-*N*-acetylglucosaminyltransferase and a β1,3-galactosyltransferase or β1,4-galactosyltransferase, can successfully be employed in the synthesis of LNT and LNnT, respectively. It is possible to use here, for example, Leloir glycosyltransferases (LgtA or LgtB) which firstly react lactose as substrate for glycosylation to give lacto-N-triose II (LNT II) as intermediate and then, in a step dependent on nucleotide-activated sugars, elongate it to give LNT (see fig. 1 and Frey et al. *FASEB J.* **1996**, *10,* 461–70). Examples of transgenes with proven

Claims

1. A genetically modified microorganism for *in vivo* synthesis of lacto-N-tetrose, said microorganism comprising

(i) a first transgene coding for β1,3-*N*-acetylglucosaminyltransferase,

and

(ii) a second transgene coding for β1,3-galactosyltransferase.

2. A genetically modified microorganism for *in vivo* synthesis of lacto-N-neotetrose, said microorganism comprising

(i) a first transgene coding for β1,3-*N*-acetylglucosaminyltransferase,

and

(ii) a second transgene coding for β1,4-galactosyltransferase.

~~2~~3. The genetically modified microorganism according to claim 1 or 2, said microorganism in addition being genetically modified so as to suppress expression of LacZ and LacA.

~~3~~4. The genetically modified microorganism according to claim ~~2~~3, wherein the (i) first transgene has been integrated into the LacZYA locus and the microorganism comprises a further transgene coding for LacY.

~~4~~5. The genetically modified microorganism according to claim ~~3~~4, wherein the transgene coding for LacY has been integrated into the FucIK locus.

~~5~~6. The genetically modified microorganism according to any of claims 1 to ~~4~~5, said microorganism comprising a further transgene coding for UDP-sugar pyrophosphorylase.

~~6~~7. The genetically modified microorganism according to any of claims 1 to ~~5~~6, said microorganism in addition being genetically modified so as to suppress expression of UDP-glucose 4-epimerase.

~~7~~8. The genetically modified microorganism according to any of claims 1 to ~~6~~7, wherein one or both, or one, multiple or all, transgenes is/are chromosomally integrated.

~~8~~9. The genetically modified microorganism according to any of claims 1 to ~~7~~8, said microorganism comprising a further transgene coding for a

bifunctional enzyme having L-fucokinase activity and L-fucose-1-phosphate guanylyl-transferase activity, and at least one transgene coding for an enzyme capable of alpha1,2-fucosylation, alpha1,3-fucosylation or alpha1,4-fucosylation.

910. The genetically modified microorganism according to claim 89, wherein the transgene coding for said bifunctional enzyme having L-fucokinase activity and L-fucose-1-phosphate guanylyltransferase activity is chromosomally integrated, and the at least one transgene coding for an enzyme capable of alpha1,2-fucosylation, alpha1,3-fucosylation or alpha1,4-fucosylation is expressed on a plasmid vector.

~~10~~11. The genetically modified microorganism according to claim 89, wherein both the transgene coding for said bifunctional enzyme having L-fucokinase activity and L-fucose-1-phosphate guanylyltransferase activity and the at least one transgene coding for an enzyme capable of alpha1,2-fucosylation, alpha1,3-fucosylation or alpha1,4-fucosylation are chromosomally integrated.

~~11~~12. The use of a genetically modified microorganism according to any of the preceding claims for *in vivo* synthesis of lacto-N-tetrose or lacto-N-neotetrose or a fucosylated derivative of lacto-N-tetrose or lacto-N-neotetrose.

~~12~~13. A method of preparing lacto-N-tetrose or lacto-N-neotetrose or a fucosylated derivative of lacto-N-tetrose or lacto-N-neotetrose, comprising the following steps:

(a) providing a genetically modified microorganism according to any of claims 1 to ~~10~~11,

(b) culturing said genetically modified microorganism under conditions that permit synthesis of lacto-N-tetrose and lacto-N-neotetrose,

(c) optionally adding fucose,

(d) optionally isolating the synthesized lacto-N-tetrose or lacto-N-neotetrose or fucosylated derivative of lacto-N-tetrose or lacto-N-neotetrose.

~~13~~14. The method according to claim ~~12~~13, wherein step (b) comprises

- using galactose as carbon source for said microorganism,

or

- using glycerol and galactose as carbon source for said microorganism.

~~14~~15. The method according to either of claims ~~12~~13 and ~~13~~14, wherein step (b) comprises adding one or more carbon source(s), preferably selected from the group consisting of lactose, glucose, glycerol, galactose, and any mixtures thereof, continuously or in batches.

~~15~~16. The method according to any of claims ~~12~~13 to ~~14~~15, said method being carried out by way of a fed batch process with a batch volume in the range from 2 to 30 L, preferably from 3 to 20 L, particularly preferably from 5 to 15 L.

~~16~~17. The genetically modified microorganism according to any of claims 1 to ~~10~~11 or the use according to claim ~~11~~12 or the method according to any of claims ~~12~~13 to ~~15~~16, wherein the genetically modified microorganism is selected from the group consisting of bacteria, fungi, and plants, preferably microorganisms of the genera *Corynebacterium, Bevibacterium, Bacillus, Saccharomyces* and *Escherichia*.