Claims

1. A metabolically engineered cell producing a mixture of at least three different oligosaccharides, wherein said cell:
   - is metabolically engineered for the production of said mixture,
   - is capable to express, preferably expresses at least two glycosyltransferases, and
   - is capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferase.
2. Cell according to claim 1, wherein said mixture comprises at least four, preferably at least five, more preferably at least six, even more preferably at least seven, even more preferably at least eight, even more preferably at least nine, most preferably at least ten, different oligosaccharides.
3. Cell according to claim 1 or 2, wherein the relative abundance of each oligosaccharide in the mixture is at least 3%, preferably at least 5%, more preferably at least 10%.
4. Cell according to any one of claims 1 to 3, wherein at least one, preferably more than one, of said oligosaccharides is a mammalian milk oligosaccharide (MMO).
5. Cell according to any one of claims 1 to 4, wherein said cell is capable to express, preferably expresses, at least three, preferably at least four, more preferably at least five, most preferably at least six, glycosyltransferases.
6. Cell according to any one of claims 1 to 5, wherein the cell is modified in the expression or activity of at least one of said glycosyltransferases.
7. A cell according to any one of claims 1 to 6, wherein:
   - one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid; and/or
   - one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-fucose; and/or
   - one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine; and/or
   - one of said glycosyltransferases is a galactosyltransferase and one of said donor nucleotide-sugars is UDP-galactose; and/or
   - one of said glycosyltransferases is an N-acetylgalactosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylgalactosamine; and/or
   - one of said glycosyltransferases is an N-acetylmannosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylmannosamine.
8. Cell according to any one of claims 1 to 7, wherein said oligosaccharides are produced intracellularly.

9. Cell according to any one of claims 1 to 8, wherein said mixture is composed of charged and neutral oligosaccharides.
10. Cell according to any one of claims 1 to 8, wherein said mixture is composed essentially of at least three, preferably at least four, more preferably at least five, even more preferably at least six, most preferably at least seven, neutral oligosaccharides and no charged oligosaccharides.
11. Cell according to claim 10, wherein said neutral oligosaccharides comprises at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, different neutral non-fucosylated oligosaccharides.
12. Cell according to claim 10 or 11, wherein said neutral oligosaccharides comprises at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, different neutral fucosylated oligosaccharides.
13. Cell according to any one of claims 1 to 8, wherein said mixture comprises at least three, preferably at least four, more preferably at least five, even more preferably at least six, most preferably at least seven, charged oligosaccharides and no neutral oligosaccharides.
14. Cell according to any one of claims 1 to 9 and 13, wherein said mixture comprises at least one sialylated oligosaccharide.
15. Cell according to claim 9, 13 or 14, wherein all said charged oligosaccharides are sialylated oligosaccharides.
16. Cell according to any one of claims 9, 13, 14 and 15, wherein the relative abundance of said charged oligosaccharides in said mixture is at least 5%, preferably at least 7%, more preferably at least 10%.
17. Cell according to any one of claims 9 and 13 to 16, wherein the relative abundance of said charged oligosaccharides in said mixture is less than 20%, preferably less than 15%.
18. Cell according to any one of claims 1 to 17, wherein the relative abundance of fucosylated oligosaccharides in said mixture is at least 10%, preferably at least 20%, more preferably at least 30%, even more preferably at least 35%, even more preferably at least 40%, most preferably at least 50%
19. Cell according to any one of claims 1 to 18, wherein the relative abundance of fucosylated oligosaccharides in said mixture is less than 90%, preferably less than 80%, more preferably less than 70%, even more preferably less than 60%, most preferably less than 55%.

20. Cell according to any claim 9, wherein the relative abundance of fucosylated oligosaccharides in the neutral oligosaccharides fraction of said mixture is 10-60%, preferably 20-60%, more preferably 30-60%, even more preferably 30-50%, most preferably 35-50%.
21. Cell according to any one of claims 1 to 20, wherein said cell is a bacterium, fungus, yeast, a plant cell, an animal cell, or a protozoan cell, preferably a bacterium, more preferably an *Escherichia coli* strain.

~~2. Cell according to claim 1, wherein said mixture comprises, consists of or consists essentially of charged and neutral fucosylated and non-fucosylated oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.~~

~~3. Cell according to claim 1, wherein said mixture comprises, consists of or consists essentially of neutral fucosylated and non-fucosylated oligosaccharides and no charged oligosaccharides.~~

~~4. Cell according to claim 1, wherein said mixture comprises, consists of or consists essentially of neutral fucosylated oligosaccharides and no neutral non-fucosylated or charged oligosaccharides.~~

~~5. Cell according to claim 1, wherein said mixture comprises, consists of or consists essentially of neutral non-fucosylated oligosaccharides and no neutral fucosylated or charged oligosaccharides.~~

~~6. Cell according to claim 1, wherein said mixture comprises, consists of or consists essentially of charged oligosaccharides and no neutral oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.~~

~~7. Cell according to any one of claim 1 to 6, wherein said cell is modified with gene expression modules, characterized in that the expression from any of said expression modules is either constitutive or is created by a natural inducer.~~

~~8. Cell according to any one of claim 1 to 7, wherein said cell comprises multiple copies of the same coding DNA sequence encoding for one protein.~~

~~9. Cell according to any one of claims 1 to 8, wherein said oligosaccharide mixture comprises at least three different oligosaccharides differing in degree of polymerization.~~

~~10. Cell according to any one of claims 1 to 9, wherein said cell produces four or more different oligosaccharides.~~

~~11. Cell according to any one of claims 1 to 10, wherein any one of said glycosyltransferase is chosen from the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases, glucosyltransferases, mannosyltransferases, N-acetylglucosaminyltransferases, N-acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases, N-acetylrhamnosyltransferases, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases, UDP-*N*-acetylglucosamine**

enolpyruvyl transferases and fucosaminyltransferases,

- preferably, said fucosyltransferase is chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase,
- preferably, said sialyltransferase is chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase,
- preferably, said galactosyltransferase is chosen from the list comprising beta-1,3-galactosyltransferase, N-acetylglucosamine beta-1,3-galactosyltransferase, beta-1,4-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase and alpha-1,4-galactosyltransferase,
- preferably, said glucosyltransferase is chosen from the list comprising alpha-glucosyltransferase, beta-1,2-glucosyltransferase, beta-1,3-glucosyltransferase and beta-1,4-glucosyltransferase,
- preferably, said mannosyltransferase is chosen from the list comprising alpha-1,2-mannosyltransferase, alpha-1,3-mannosyltransferase and alpha-1,6-mannosyltransferase,
- preferably, said N-acetylglucosaminyltransferase is chosen from the list comprising galactoside beta-1,3-N-acetylglucosaminyltransferase and beta-1,6-N-acetylglucosaminyltransferase,
- preferably, said N-acetylgalactosaminyltransferase is chosen from the list comprising alpha-1,3-N-acetylgalactosaminyltransferase and beta-1,3-N-acetylgalactosaminyltransferase.

12. Cell according to any one of claims 1 to 11, wherein said cell is capable to express, preferably expresses, at least three, more preferably at least four, even more preferably at least five, most preferably at least six glycosyltransferases.
13. Cell according to any one of claims 1 to 12, wherein said cell is modified in the expression or activity of at least one of said glycosyltransferases.
14. Cell according to any one of claims 1 to 4, 7 to 13, wherein one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-Fucose (GDP-Fuc).
15. Cell according to any one of claims 1, 2, 6 to 14, wherein one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid (CMP-Neu5Ac).
16. Cell according to any one of claims 1 to 15, wherein one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine (UDP-GlcNAc).
17. Cell according to any one of claims 1 to 16, wherein one of said glycosyltransferases is a

galactosyltransferase and one of said donor nucleotide-sugars is UDP-galactose (UDP-Gal).

18. Cell according to any one of claim 1 to 17, wherein said glycosyltransferase is an N-acetylgalactosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylgalactosamine (UDP-GalNAc).

19. Cell according to any one of claim 1 to 18, wherein said glycosyltransferase is an N-acetylmannosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylmannosamine (UDP-ManNAc).

20. Cell according to any one of claims 1 to 19, wherein any one of said nucleotide-sugars is chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), UDP-2-acetamido-2,6-dideoxy-L-arabino-4-hexulose, UDP-2-acetamido-2,6-dideoxy-L-lyxo-4-hexulose, UDP-N-acetyl-L-rhamnosamine (UDP-L-RhaNAc or UDP-2-acetamido-2,6-dideoxy-L-mannose), dTDP-N-acetylfucosamine, UDP-N-acetylfucosamine (UDP-L-FucNAc or UDP-2-acetamido-2,6-dideoxy-L-galactose), UDP-N-acetyl-L-pneumosamine (UDP-L-PneNAC or UDP-2-acetamido-2,6-dideoxy-L-talose), UDP-N-acetylmuramic acid, UDP-N-acetyl-L-quinovosamine (UDP-L-QuiNAc or UDP-2-acetamido-2,6-dideoxy-L-glucose), GDP-L-quinovose, CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), CMP-Neu4Ac, CMP-Neu5Ac9N_3, CMP-Neu4,5Ac_2, CMP-Neu5,7Ac_2, CMP-Neu5,9Ac_2, CMP-Neu5,7(8,9)Ac_2, UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose.

21. Cell according to any one of claims 1 to 20, wherein said cell expresses one or more polypeptides chosen from the list comprising mannose-6-phosphate isomerase, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase, GDP-L-fucose synthase, fucose permease, fucose kinase, GDP-fucose pyrophosphorylase, fucose-1-phosphate guanylyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acylglucosamine 2-epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylmannosamine-6-phosphate 2-epimerase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, N-acetylneuraminate synthase, N-acetylneuraminate lyase, N-acylneuraminate-9-phosphate synthase, N-acylneuraminate-9-phosphate phosphatase, N-acylneuraminate cytidylyltransferase, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate uridylyltransferase, phosphoglucomutase, *UDP-*

N-acetylglucosamine 4-epimerase, N-acetylgalactosamine kinase and UDP-N-acetylgalactosamine pyrophosphorylase, preferably wherein said cell is modified in the expression or activity of any one of said polypeptides.

22. Cell according to any one of claims 1 to 21, wherein said cell is capable to synthesize at least two nucleotide-sugars, preferably at least three nucleotide-sugars, more preferably at least four nucleotide-sugars, even more preferably at least five nucleotide-sugars.

23. Cell according to any one of claims 1 to 22, wherein at least one of said oligosaccharides is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated, contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylgalactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.

24. Cell according to any one of claims 1 to 4, 7 to 23, wherein said oligosaccharide mixture comprises at least one fucosylated oligosaccharide.

25. Cell according to any one of claims 1 to 2, 6 to 24, wherein said oligosaccharide mixture comprises at least one sialylated oligosaccharide.

26. Cell according to any one of claims 1 to 25, wherein said oligosaccharide mixture comprises at least one oligosaccharide that comprises an N-acetylglucosamine monosaccharide unit.

27. Cell according to any one of claims 1 to 26, wherein said oligosaccharide mixture comprises at least one galactosylated oligosaccharide.

28. Cell according to any one of claims 1 to 27, wherein said cell uses at least one precursor for the production of any one or more of said oligosaccharides, preferably said cell uses two or more precursors for the production of any one or more of said oligosaccharides, said precursor(*s*) being fed to the cell from the cultivation medium.

29. Cell according to any one of claims 1 to 28, wherein said cell is producing at least one precursor for the production of any one of said oligosaccharides.

30. Cell according to any one of claims 1 to 29, wherein said at least one precursor for the production of any one of said oligosaccharides is completely converted into any one of said oligosaccharides.

31. Cell according to any one of claims 1 to 30, wherein said cell produces said oligosaccharides intracellularly and wherein a fraction or substantially all of said produced oligosaccharides remains intracellularly and/or is excreted outside said cell via passive or active transport.

32. Cell according to any one of claims 1 to 31, wherein said cell is further genetically modified for

i) modified expression of an endogenous membrane protein, and/or

i) modified activity of an endogenous membrane protein, and/or

ii) expression of a homologous membrane protein, and/or

iii) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the secretion of any one of said oligosaccharides from said mixture outside said cell, preferably wherein said membrane protein is involved in the secretion of all of said oligosaccharides from said mixture from said cell.

33. Cell according to any one of claims 1 to 32, wherein said cell is further genetically modified for

i) modified expression of an endogenous membrane protein, and/or

i) modified activity of an endogenous membrane protein, and/or

ii) expression of a homologous membrane protein, and/or

iii) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the uptake of a precursor and/or an acceptor for the synthesis of any one of said oligosaccharides of said mixture, preferably wherein said membrane protein is involved in the uptake of all of the required precursors, more preferably wherein said membrane protein is involved in the uptake of all of said acceptors.

34. Cell according to any one of claim 32 or 33, wherein said membrane protein is chosen from the list comprising porters, P-P-bond-hydrolysis-driven transporters, β-barrel porins, auxiliary transport proteins, putative transport proteins and phosphotransfer-driven group translocators, preferably, said porters comprise MFS transporters, sugar efflux transporters and siderophore exporters,

preferably, said P-P-bond-hydrolysis-driven transporters comprise ABC transporters and siderophore exporters.

35. Cell according to any one of claim 32 to 34, wherein said membrane protein provides improved production and/or enabled and/or enhanced efflux of any one of said oligosaccharides.

36. Cell according to any one of claim 1 to 35, wherein said cell resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with one or more other carbon source(s).

37. Cell according to any one of claim 1 to 36, wherein said cell comprises a modification for reduced production of acetate compared to a non-modified progenitor.

38. Cell according to claim 37, wherein said cell comprises a lower or reduced expression and/or abolished, impaired, reduced or delayed activity of any one or more of the proteins comprising beta-galactosidase, galactoside O-acetyltransferase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, N-acetylglucosamine repressor, ribonucleotide monophosphatase, EIICBA-Nag, UDP-glucose:undecaprenyl phosphate glucose-1-phosphate transferase, L-fuculokinase, L-fucose isomerase, N-acetylneuraminate lyase, N-acetylmannosamine kinase, N-acetylmannosamine-6-phosphate 2-epimerase, EIIAB-Man, EIIC-

~~Man, EIID-Man, ushA, galactose-1-phosphate uridylyltransferase, glucose-1-phosphate adenylyltransferase, glucose-1-phosphatase, ATP-dependent 6-phosphofructokinase isozyme 1, ATP-dependent 6-phosphofructokinase isozyme 2, glucose-6-phosphate isomerase, aerobic respiration control protein, transcriptional repressor IclR, lon protease, glucose-specific translocating phosphotransferase enzyme IIBC component ptsG, glucose-specific translocating phosphotransferase (PTS) enzyme IIBC component malX, enzyme IIA^{Glc}, beta-glucoside-specific PTS enzyme II, fructose-specific PTS multiphosphoryl transfer protein FruA and FruB, ethanol dehydrogenase aldehyde dehydrogenase, pyruvate-formate lyase, acetate kinase, phosphoacyltransferase, phosphate acetyltransferase, pyruvate decarboxylase compared to a non-modified progenitor.~~

~~39. Cell according to any one of claim 1 to 38, wherein the cell is capable to produce phosphoenolpyruvate (PEP).~~

~~40. Cell according to any one of claim 1 to 39, wherein said cell is modified for enhanced production and/or supply of phosphoenolpyruvate (PEP) compared to a non-modified progenitor.~~

~~41. Cell according to any one of claims 1 to 40, wherein any one of said oligosaccharides is a mammalian milk oligosaccharide.~~

~~42. Cell according to any one of claims 1 to 41, wherein all said oligosaccharides are mammalian milk oligosaccharides.~~

~~43. Cell according to any one of claims 1 to 4, 7 to 41, wherein any one of said oligosaccharides is an antigen of the human ABO blood group system.~~

~~44. Cell according to any of claims 1, 3, 4, 7 to 40, 41, wherein all said oligosaccharides are antigens of the human ABO blood group system.~~

~~45.~~22. A method to produce a mixture of at least three different oligosaccharides by a cell, preferably a single cell, the method comprising the steps of:

i) providing a cell which is capable to express, preferably expressing at least two glycosyltransferases and capable to synthesize one or more nucleotide-sugar(s), wherein said nucleotide-sugar(s) is/are donor(s) for said glycosyltransferases, and

ii) cultivating said cell under conditions permissive to express said glycosyltransferases and to synthesize said nucleotide-sugar(s),

iii) preferably, separating at least one of said oligosaccharides from said cultivation,

optionally further comprising purification of any one of said oligosaccharides from said cultivation.

23. Method according to claim 22, wherein said mixture is composed of charged and neutral oligosaccharides.

24. Method according to claim 22, wherein said mixture is composed essentially of at least three, preferably at least four, more preferably at least five, even more preferably at least six, most preferably at least seven, neutral oligosaccharides and no charged oligosaccharides.

25. Method according to claim 24, wherein said neutral oligosaccharides comprises at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, different neutral non-fucosylated oligosaccharides.

26. Method according to claim 24 or 25, wherein said neutral oligosaccharides comprises at least one, preferably at least two, more preferably at least three, even more preferably at least four, even more preferably at least five, even more preferably at least six, most preferably at least seven, different neutral fucosylated oligosaccharides.

27. Method according to claim 22, wherein said mixture comprises at least three, preferably at least four, more preferably at least five, even more preferably at least six, most preferably at least seven, charged oligosaccharides and no neutral oligosaccharides.

~~46. Method according to claim 45, wherein said mixture comprises, consists of or consists essentially of charged and neutral fucosylated and non-fucosylated oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.~~

~~47. Method according to claim 45, wherein said mixture comprises, consists of or consists essentially of neutral fucosylated and non-fucosylated oligosaccharides and no charged oligosaccharides.~~

~~48. Method according to claim 45, wherein said mixture comprises, consists of or consists essentially of neutral fucosylated oligosaccharides and no neutral non-fucosylated or charged oligosaccharides.~~

~~49. Method according to claim 45, wherein said mixture comprises, consists of or consists essentially of neutral non-fucosylated oligosaccharides and no neutral fucosylated or charged oligosaccharides.~~

~~50. Method according to claim 45, wherein said mixture comprises, consists of or consists essentially of charged oligosaccharides and no neutral oligosaccharides, preferably at least one of said charged oligosaccharides is a sialylated oligosaccharide.~~

~~51.~~28. Method according to any one of claims 22~~45~~ to 27~~50~~, wherein said cell is a metabolically engineered cell according to any one of claims~~embodiments~~ 1 to 21~~44~~.

~~52. Method according to claim 51, wherein said cell is modified with gene expression modules, characterized in that the expression from any of said expression modules is either constitutive or is created by a natural inducer.~~

~~53. Method according to any one of claim 51 or 52, wherein said cell comprises multiple copies of the~~

~~same coding DNA sequence encoding for one protein.~~

~~54. Method according to any one of claims 45 to 53, wherein said oligosaccharide mixture comprises at least three different oligosaccharides differing in degree of polymerization.~~

~~55. Method according to any one of claims 45 to 54, wherein said cell produces four or more different oligosaccharides.~~

~~56. Method according to any one of claims 45 to 55, wherein any one of said glycosyltransferases is chosen from the list comprising fucosyltransferases, sialyltransferases, galactosyltransferases, glucosyltransferases, mannosyltransferases, N-acetylglucosaminyltransferases, N-acetylgalactosaminyltransferases, N-acetylmannosaminyltransferases, xylosyltransferases, glucuronyltransferases, galacturonyltransferases, glucosaminyltransferases, N-glycolylneuraminyltransferases, rhamnosyltransferases, N-acetylrhamnosyltransferases, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminases, UDP-*N*-acetylglucosamine enolpyruvyl transferases and fucosaminyltransferases,~~

- ~~preferably, said fucosyltransferase is chosen from the list comprising alpha-1,2-fucosyltransferase, alpha-1,3-fucosyltransferase, alpha-1,4-fucosyltransferase and alpha-1,6-fucosyltransferase,~~
- ~~preferably, said sialyltransferase is chosen from the list comprising alpha-2,3-sialyltransferase, alpha-2,6-sialyltransferase and alpha-2,8-sialyltransferase,~~
- ~~preferably, said galactosyltransferase is chosen from the list comprising beta-1,3-galactosyltransferase, N-acetylglucosamine beta-1,3-galactosyltransferase, beta-1,4-galactosyltransferase, N-acetylglucosamine beta-1,4-galactosyltransferase, alpha-1,3-galactosyltransferase and alpha-1,4-galactosyltransferase,~~
- ~~preferably, said glucosyltransferase is chosen from the list comprising alpha-glucosyltransferase, beta-1,2-glucosyltransferase, beta-1,3-glucosyltransferase and beta-1,4-glucosyltransferase,~~
- ~~preferably, said mannosyltransferase is chosen from the list comprising alpha-1,2-mannosyltransferase, alpha-1,3-mannosyltransferase and alpha-1,6-mannosyltransferase,~~
- ~~preferably, said N-acetylglucosaminyltransferase is chosen from the list comprising galactoside beta-1,3-N-acetylglucosaminyltransferase and beta-1,6-N-acetylglucosaminyltransferase,~~
- ~~preferably, said N-acetylgalactosaminyltransferase is chosen from the list comprising alpha-1,3 N-acetylgalactosaminyltransferase and beta-1,3 N-acetylgalactosaminyltransferase,~~
- ~~preferably, said cell is modified in the expression or activity of at least one of said glycosyltransferases.~~

57. Method according to any one of claims 45 to 56, wherein said cell is capable to express, preferably expresses, at least three, more preferably at least four, even more preferably at least five, most preferably at least six glycosyltransferases.

58. Method according to any one of claims 45 to 48, 51 to 57, wherein one of said glycosyltransferases is a fucosyltransferase and one of said donor nucleotide-sugars is GDP-Fucose (GDP-Fuc).

59. Method according to any one of claims 45, 46, 50 to 58, wherein one of said glycosyltransferases is a sialyltransferase and one of said donor nucleotide-sugars is CMP-N-acetylneuraminic acid (CMP-Neu5Ac).

60. Method according to any one of claims 45 to 59, wherein one of said glycosyltransferases is an N-acetylglucosaminyltransferase and one of said donor nucleotide-sugars is UDP-N-acetylglucosamine (UDP-GlcNAc).

61. Method according to any one of claims 45 to 60 wherein one of said glycosyltransferases is a galactosyltransferase and one of said donor nucleotide-sugars is UDP-galactose (UDP-Gal).

62. Method according to any one of claim 45 to 61, wherein said glycosyltransferase is an N-acetylgalactosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylgalactosamine (UDP-GalNAc).

63. Method according to any one of claim 45 to 62, wherein said glycosyltransferase is an N-acetylmannosaminyltransferase and said donor nucleotide-sugar is UDP-N-acetylmannosamine (UDP-ManNAc).

64. Method according to any one of claims 45 to 63, wherein any one of said nucleotide-sugars is chosen from the list comprising GDP-Fuc, CMP-Neu5Ac, UDP-GlcNAc, UDP-Gal, UDP-N-acetylgalactosamine (UDP-GalNAc), UDP-N-acetylmannosamine (UDP-ManNAc), GDP-mannose (GDP-Man), UDP-glucose (UDP-Glc), UDP-2-acetamido-2,6-dideoxy-L-arabino-4-hexulose, UDP-2-acetamido-2,6-dideoxy-L-lyxo-4-hexulose, UDP-N-acetyl-L-rhamnosamine (UDP-L-RhaNAc or UDP-2-acetamido-2,6-dideoxy-L-mannose), dTDP-N-acetylfucosamine, UDP-N-acetylfucosamine (UDP-L-FucNAc or UDP-2-acetamido-2,6-dideoxy-L-galactose), UDP-N-acetyl-L-pneumosamine (UDP-L-PneNAC or UDP-2-acetamido-2,6-dideoxy-L-talose), UDP-N-acetylmuramic acid, UDP-N-acetyl-L-quinovosamine (UDP-L-QuiNAc or UDP-2-acetamido-2,6-dideoxy-L-glucose), GDP-L-quinovose, CMP-N-glycolylneuraminic acid (CMP-Neu5Gc), CMP-Neu4Ac, CMP-Neu5Ac9N_3, CMP-Neu4,5Ac_2, CMP-Neu5,7Ac_2, CMP-Neu5,9Ac_2, CMP-Neu5,7(8,9)Ac_2, UDP-glucuronate, UDP-galacturonate, GDP-rhamnose, UDP-xylose.

65. Method according to any one of claims 45 to 64, wherein said cell expresses one or more polypeptides chosen from the list comprising mannose-6-phosphate isomerase, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-

dehydratase, GDP-L-fucose synthase, fucose permease, fucose kinase, GDP-fucose pyrophosphorylase, fucose-1-phosphate guanylyltransferase, L-glutamine—D-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate deaminase, phosphoglucosamine mutase, N-acetylglucosamine-6-phosphate deacetylase, N-acylglucosamine 2-epimerase, UDP-N-acetylglucosamine 2-epimerase, N-acetylmannosamine-6-phosphate 2-epimerase, glucosamine 6-phosphate N-acetyltransferase, N-acetylglucosamine-6-phosphate phosphatase, N-acetylmannosamine-6-phosphate phosphatase, N-acetylmannosamine kinase, phosphoacetylglucosamine mutase, N-acetylglucosamine-1-phosphate uridylyltransferase, glucosamine-1-phosphate acetyltransferase, N-acetylneuraminate synthase, N-acetylneuraminate lyase, N-acylneuraminate-9-phosphate synthase, N-acylneuraminate-9-phosphate phosphatase, N-acylneuraminate cytidylyltransferase, galactose-1-epimerase, galactokinase, glucokinase, galactose-1-phosphate uridylyltransferase, UDP-glucose 4-epimerase, glucose-1-phosphate uridylyltransferase, phosphoglucomutase, *UDP-N-acetylglucosamine 4-epimerase, N-acetylgalactosamine kinase and* UDP-N-acetylgalactosamine pyrophosphorylase, preferably wherein said cell is modified in the expression or activity of any one of said polypeptides.

66. Method according to any one of claims 45 to 65, wherein said cell is capable to synthesize at least two nucleotide-sugars, preferably at least three nucleotide-sugars, more preferably at least four nucleotide-sugars, even more preferably at least five nucleotide-sugars.

67. Method according to any one of claims 45 to 48, 51 to 66, wherein said oligosaccharide mixture comprises at least one fucosylated oligosaccharide.

68. Method according to any one of claims 45, 46, 50 to 67, wherein said oligosaccharide mixture comprises at least one sialylated oligosaccharide.

69. Method according to any one of claims 45 to 68, wherein said oligosaccharide mixture comprises at least one oligosaccharide that comprises an N-acetylglucosamine monosaccharide unit.

70. Method according to any one of claims 45 to 69, wherein said oligosaccharide mixture comprises at least one galactosylated oligosaccharide.

71. Method according to any one of claims 45 to 70, wherein said oligosaccharide mixture comprises at least one oligosaccharide that is fucosylated, sialylated, galactosylated, glucosylated, xylosylated, mannosylated, contains an N-acetylglucosamine, contains an N-acetylneuraminate, contains an N-glycolylneuraminate, contains an N-acetylgalactosamine, contains a rhamnose, contains a glucuronate, contains a galacturonate, and/or contains an N-acetylmannosamine.

72. Method according to any one of claims 45 to 71, wherein said cell uses at least one precursor for the production of any one or more of said oligosaccharides, preferably said cell uses two or more

precursors for the production of any one or more of said oligosaccharides, said precursor(s) being fed to the cell from the cultivation medium.

73. Method according to any one of claims 45 to 72, wherein said cell is producing at least one precursor for the production of any one of said oligosaccharides.

74. Method according to any one of claims 45 to 73, wherein said at least one precursor for the production of any one of said oligosaccharides is completely converted into any one of said oligosaccharides.

75. Method according to any one of claims 45 to 74, wherein said cell produces said oligosaccharides intracellularly and wherein a fraction or substantially all of said produced oligosaccharides remains intracellularly and/or is excreted outside said cell via passive or active transport.

76. Method according to any one of claims 45 to 75, wherein said cell is further genetically modified for

i) modified expression of an endogenous membrane protein, and/or

i) modified activity of an endogenous membrane protein, and/or

ii) expression of a homologous membrane protein, and/or

iii) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the secretion of any one of said oligosaccharides from said mixture outside said cell, preferably wherein said membrane protein is involved in the secretion of all of said oligosaccharides from said mixture from said cell.

77. Method according to any one of claims 45 to 76, wherein said cell is further genetically modified for

i) modified expression of an endogenous membrane protein, and/or

i) modified activity of an endogenous membrane protein, and/or

ii) expression of a homologous membrane protein, and/or

iii) expression of a heterologous membrane protein,

wherein said membrane protein is involved in the uptake of a precursor and/or an acceptor for the synthesis of any one of said oligosaccharides of said mixture, preferably wherein said membrane protein is involved in the uptake of all of the required precursors, more preferably wherein said membrane protein is involved in the uptake of all of said acceptors.

78. Method according to any one of claim 76 or 77, wherein said membrane protein is chosen from the list comprising porters, P-P-bond-hydrolysis-driven transporters, β-barrel porins, auxiliary transport proteins, putative transport proteins and phosphotransfer-driven group translocators, preferably, said porters comprise MFS transporters, sugar efflux transporters and siderophore exporters,

preferably, said P-P-bond-hydrolysis-driven transporters comprise ABC transporters and siderophore exporters.

79. Method according to any one of claim 76 to 78, wherein said membrane protein provides improved production and/or enabled and/or enhanced efflux of any one of said oligosaccharides. Method according to any one of claim 1 to 34, wherein said cell resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with one or more other carbon source(s).

80. Method according to any one of claim 45 to 79, wherein said cell comprises a modification for reduced production of acetate compared to a non-modified progenitor.

81. Method according to claim 80, wherein said cell comprises a lower or reduced expression and/or abolished, impaired, reduced or delayed activity of any one or more of the proteins comprising beta-galactosidase, galactoside O-acetyltransferase, N-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase, N-acetylglucosamine repressor, ribonucleotide monophosphatase, EIICBA-Nag, UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase, L-fuculokinase, L-fucose isomerase, N-acetylneuraminate lyase, N-acetylmannosamine kinase, N-acetylmannosamine-6-phosphate 2-epimerase, EIIAB-Man, EIIC-Man, EIID-Man, ushA, galactose-1-phosphate uridylyltransferase, glucose-1-phosphate adenylyltransferase, glucose-1-phosphatase, ATP-dependent 6-phosphofructokinase isozyme 1, ATP-dependent 6-phosphofructokinase isozyme 2, glucose-6-phosphate isomerase, aerobic respiration control protein, transcriptional repressor IclR, lon protease, glucose-specific translocating phosphotransferase enzyme IIBC component ptsG, glucose-specific translocating phosphotransferase (PTS) enzyme IIBC component malX, enzyme IIA^{Glc}, beta-glucoside specific PTS enzyme II, fructose-specific PTS multiphosphoryl transfer protein FruA and FruB, ethanol dehydrogenase aldehyde dehydrogenase, pyruvate-formate lyase, acetate kinase, phosphoacyltransferase, phosphate acetyltransferase, pyruvate decarboxylase compared to a non-modified progenitor.

82. Method according to any one of claim 45 to 81, wherein the cell is capable to produce phosphoenolpyruvate (PEP).

83. Method according to any one of claim 45 to 82, wherein said cell is modified for enhanced production and/or supply of phosphoenolpyruvate (PEP) compared to a non-modified progenitor.

84. Method according to any one of claims 45 to 83, wherein any one of said oligosaccharides is a mammalian milk oligosaccharide.

85. Method according to any one of claims 45 to 84, wherein all said oligosaccharides are mammalian milk oligosaccharides.

86. Method according to any one of claims 45 to 49, 51 to 84, wherein any one of said oligosaccharide is an antigen of the human ABO blood group system.

87. Method according to any of claims 45 to 49, 51 to 83 and 86, wherein all said oligosaccharides are antigens of the human ABO blood group system.

88. Method according to any one of claim 45 to 87, wherein said conditions comprise:

- use of a culture medium comprising at least one precursor and/or acceptor for the production of any one of said oligosaccharides, and/or
- adding to the culture medium at least one precursor and/or acceptor feed for the production of any one of said oligosaccharides.

89. Method according to any one of claim 45 to 88, the method comprising at least one of the following steps:

i) Use of a culture medium comprising at least one precursor and/or acceptor;

i) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m^3 (cubic meter), 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 two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed;

ii) Adding to the culture medium in a reactor at least one precursor and/or acceptor feed wherein the total reactor volume ranges from 250 mL (millilitre) to 10.000 m^3 (cubic meter), 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 two-fold of the volume of the culture medium before the addition of said precursor and/or acceptor feed and wherein preferably, the pH of said precursor and/or acceptor feed is set between 3 and 7 and wherein preferably, the temperature of said precursor and/or acceptor feed is kept between 20°C and 80°C;

iii) Adding at least one precursor and/or acceptor 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;

iv) Adding at least one precursor and/or acceptor 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 preferably, the pH of said feeding solution is set between 3 and 7 and wherein preferably, the temperature of said feeding solution is kept between 20°C and 80°C;

said method resulting in any one of said oligosaccharides with a 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 cultivation.

90. Method according to any one of claim 45 to 88, the method comprising at least one of the following steps:

i) Use of a culture medium 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 litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m^3 (cubic meter);

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 litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m^3 (cubic meter), 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 two-fold of the volume of the culture medium before the addition of said lactose feed;

ii) 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 litre of initial reactor volume wherein the reactor volume ranges from 250 mL to 10.000 m^3 (cubic meter), 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 two-fold of the volume of the culture medium before the addition of said lactose feed and wherein preferably the pH of said lactose feed is set between 3 and 7 and wherein preferably the temperature of said lactose feed is kept between 20°C and 80°C;

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;

iv) 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 feeding solution is set between 3 and 7 and wherein preferably the temperature of said feeding solution is kept between 20°C and 80°C;

said method resulting in any one of said oligosaccharides with a 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 cultivation.

91. Method according to claim 90, wherein the lactose feed is accomplished by adding lactose from the beginning of the cultivation in a concentration of at least 5 mM, preferably in a concentration of 30, 40, 50, 60, 70, 80, 90, 100, 150 mM, more preferably in a concentration > 300 mM.

92. Method according to any one of claim 90 or 91, wherein said lactose feed is accomplished by adding lactose to the cultivation in a concentration, such, that throughout the production phase of the cultivation a lactose concentration of at least 5 mM, preferably 10 mM or 30 mM is obtained.

93. Method according to any one of claim 45 to 92, wherein the host cells are cultivated for at least about 60, 80, 100, or about 120 hours or in a continuous manner.

94. Method according to any one of claim 45 to 93, wherein said cell is cultivated in a culture medium comprising a carbon source comprising a monosaccharide, disaccharide, oligosaccharide, polysaccharide, polyol, glycerol, a complex medium including molasses, corn steep liquor, peptone, tryptone or yeast extract; preferably, wherein said carbon source is chosen from the list comprising glucose, glycerol, fructose, sucrose, maltose, lactose, arabinose, malto-oligosaccharides, maltotriose, sorbitol, xylose, rhamnose, galactose, mannose, methanol, ethanol, trehalose, starch, cellulose, hemi-cellulose, molasses, corn-steep liquor, high-fructose syrup, acetate, citrate, lactate and pyruvate.

95. Method according to any one of claim 45 to 94, wherein the culture medium contains at least one precursor selected from the group comprising lactose, galactose, fucose, sialic acid, GlcNAc, GalNAc, lacto-N-biose (LNB), N-acetyllactosamine (LacNAc).

96. Method according to any one of claim 45 to 95, wherein a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium before the precursor, preferably lactose, is added to the culture medium in a second phase.

97. Method according to any one of claim 45 to 96, wherein a first phase of exponential cell growth is provided by adding a carbon-based substrate, preferably glucose or sucrose, to the culture medium comprising a precursor, preferably lactose, followed by a second phase wherein only a

carbon-based substrate, preferably glucose or sucrose, is added to the culture medium.

98. Method according to any one of claim 45 to 97, wherein a first phase of exponential cell growth is provided by adding a carbon based substrate, preferably glucose or sucrose, to the culture medium comprising a precursor, preferably lactose, followed by a second phase wherein a carbon-based substrate, preferably glucose or sucrose, and a precursor, preferably lactose, are added to the culture medium.

99. Method according to any one of claims 45 to 98, wherein said separation comprises at least one of the following steps: clarification, ultrafiltration, nanofiltration, two-phase partitioning, reverse osmosis, microfiltration, activated charcoal or carbon treatment, treatment with non-ionic surfactants, enzymatic digestion, tangential flow high-performance filtration, tangential flow ultrafiltration, affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and/or gel filtration, ligand exchange chromatography.

100. Method according to any one of claims 45 to 99, further comprising purification of any one of said oligosaccharides from said cell.

101. Method according to claim 100, wherein said purification comprises at least one of the following steps: use of activated charcoal or carbon, use of charcoal, nanofiltration, ultrafiltration, electrophoresis, enzymatic treatment or ion exchange, use of alcohols, use of aqueous alcohol mixtures, crystallization, evaporation, precipitation, drying, spray drying, lyophilization, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying or vacuum roller drying.

102. Cell according to any one of claims 1 to 44 or method according to any one of embodiments 45 to 101, wherein said cell is a bacterium, fungus, yeast, a plant cell, an animal cell, or a protozoan cell,

- preferably said bacterium is an *Escherichia coli* strain, more preferably an *Escherichia coli* strain which is a K-12 strain, even more preferably the *Escherichia coli* K-12 strain is *E. coli* MG1655,
- preferably said fungus belongs to a genus chosen from the group comprising *Rhizopus, Dictyostelium, Penicillium, Mucor* or *Aspergillus,*
- preferably said yeast belongs to a genus chosen from the group comprising *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Komagataella*, *Hansenula*, *Yarrowia, Starmerella*, *Kluyveromyces* or *Debaromyces,*
- preferably said plant cell is an algal cell or is derived from tobacco, alfalfa, rice, tomato, cotton, rapeseed, soy, maize, or corn plant,
- preferably said animal cell is derived from non-human mammals, birds, fish, invertebrates, reptiles, amphibians or insects or is a genetically modified cell line derived from human cells

~~excluding embryonic stem cells, more preferably said human and non-human mammalian cell is an epithelial cell, an embryonic kidney cell, a fibroblast cell, a COS cell, a Chinese hamster ovary (CHO) cell, a murine myeloma cell, an NIH 3T3 cell, a non-mammary adult stem cell or derivatives thereof, more preferably said insect cell is derived from *Spodoptera frugiperda*, *Bombyx mori*, *Mamestra brassicae*, *Trichoplusia ni* or *Drosophila melanogaster*,~~

~~preferably said protozoan cell is a *Leishmania tarentolae* cell.~~

~~103. Cell according to claim 102, or method according to claim 102, wherein said cell is a viable Gram-negative bacterium that comprises a reduced or abolished synthesis of poly-N-acetyl-glucosamine (PNAG), Enterobacterial Common Antigen (ECA), cellulose, colanic acid, core oligosaccharides, Osmoregulated Periplasmic Glucans (OPG), Glucosylglycerol, glycan, and/or trehalose compared to a non-modified progenitor.~~

~~104.~~29. Use of a cell according to any one of claims 1 to 21~~44, 102, 103, or method according to any one of claim 45 to 103~~ for the production of a mixture of at least three different oligosaccharides.