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

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

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

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.

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 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:
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 claim~~s~~ 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<u>s</u> 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; <u>or</u>
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 alpha-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 <u>α-1,3-fucosyllactose</u> ~~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 α-1,3-fucosyllactose ~~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 alpha-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; or

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 alpha-1,3-fucosyltransferase activity;~~

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

15. Method according to any one of the claims 3 to 11 or cell according to any one of claim~~s~~ 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 claim<u>s</u> 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<u>s</u> 12 to 15, characterized in that the host cell is a yeast cell.

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,

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 claims 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 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);

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.

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 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; or

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 alpha-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 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 said alpha-1,3-fucosyllactose from the host cell or the medium of its growth.

26. The method of any one of claim<u>s</u> 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 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.

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.

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 <u>α-1,3-fucosyllactose</u> ~~3-fucosyllactose~~ ranges between 70 g/L to 200g/L.

34. A method of any one of clams 1 to 11, 15, 19, 25 to 33 wherein said production results in a lactose concentration to <u>α-1,3-fucosyllactose</u> ~~3-fucosyllactose~~ concentration ratio of less than 1:5 at the end of <u>step b) of claim 1</u>~~fermentation~~.

35. A method of any one of claims 1 to 11, 15, 19, 25 to 34 wherein said production results in a α-1,3-fucosyllactose ~~3-fucosyllactose~~ purity of 80% or more at the end of step b) of claim 1~~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.~~